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IMPEDIMETRIC BIOSENSING OF TRIGLYCERIDES USING CONDUCTIVE ACRYLIC MICROSPHERE–MODIFIED ELECTRODE

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Triglycerides are clinically important biomarkers associated with metabolic and cardiovascular disorders, and their detection requires rapid and reliable methods. While conventional analytical techniques are accurate, they are often time-consuming, require complex instrumentation, and are not suitable for point-of-care applications. Here, we report a label-free impedimetric triglyceride biosensor based on novel conductive acrylic microspheres (ACM) that function simultaneously as an enzyme immobilization matrix and conductive sensing interface. ACM were synthesized via microemulsion and photopolymerization, followed by succinimide functionalization for covalent immobilization of lipase. Incorporation of polyacetylene (PA) imparted intrinsic conductivity, enabling ACM to function simultaneously as an enzyme immobilization matrix and conductive sensing interface. Electrochemical impedance spectroscopy confirmed efficient electron transfer, indicating that ACM can serve as a lower-cost alternative to gold nanoparticles, which typically require additional fabrication steps. The biosensor exhibited a linear response from 1–1000 mg dL^{-1} (R^2 = 0.992) with a limit of detection of 0.0087 mg dL^{-1} and good repeatability and reproducibility with relative standard deviation of 4.41 % and 2.86 % (n = 8). Validation using tripalmitin-spiked pork fat demonstrated the practical feasibility of the proposed biosensor, supported by comparison with the standard titration method. The simplified design eliminates the need for gold nanoparticles, redox mediators, and multi-enzyme systems, offering a cost-effective and practical approach for triglyceride monitoring.

Keywords: conductive acrylic microsphere; triglyceride; triacylglycerol; impedimetric biosensor; electrochemical biosensor

ИМПЕДИМЕТРИСКО БИОСЕНЗОРСКО ДЕТЕКТИРАЊЕ НА ТРИГЛИЦЕРИДИ СО УПОТРЕБА НА ЕЛЕКТРОДА МОДИФИЦИРАНА СО ПРОВОДЛИВИ АКРИЛНИ МИКРОСФЕРИ

Триглицеридите се клинички важен индикатор поврзан со метаболички и кардиоваскуларни нарушувања, што бара брзи и сигурни методи на детекција. Конвенционалните аналитички техники, иако се точни, честопати одземаат многу време, бараат сложена инструментација и не се погодни за апликации на лице место. Овде, ние известуваме за импедиметриски триглицериден биосензор без етикета, базиран на нова акрилна спроводлива микросфера (АСМ) што функционира истовремено како матрица за имобилизација на ензими и интерфејс за спроводливо мерење. АСМ беше синтетизиран преку микроемулзија и фотополимеризација, проследено со сукцинимидна функционализација за ковалентна имобилизација на липазата. Вклучувањето на полиацетилен даде внатрешна спроводливост, овозможувајќи му на АСМ да функционира истовремено како матрица

за имобилизација на ензими и интерфејс за спроводливо мерење. Електрохемиската импедансна спектроскопија потврди ефикасен пренос на електрони, што укажува дека ACM може да послужи како пониска алтернатива на златните наночестички, кои обично бараат дополнителни чекори на производство. Биосензорот покажа линеарен одговор од 1–1000 mg/dL (R^2 = 0,992) со граница на детекција од 0,0087 mg/dL и добра повторување и репродуктивност со релативна стандардна грешка од 4,41% и 2,86% (n = 8). Валидацијата на реални примероци со употреба на свинска маст збогатена со трипалмитин ја покажа практичната изводливост на предложениот биосензор, поткрепена со споредба со стандардниот метод на титрација. Поедноставениот дизајн ја елиминира потребата од златни наночестички, редокс медијатори и мултиензимски системи, нудејќи економичен и практичен пристап за следење на триглицеридите.

Клучни зборови: спроводлива акрилна микросфера; триглицерид; триацилглицерол; импедиметриски биосензор; електрохемиски биосензор

1. INTRODUCTION

Triglycerides (triacylglycerols) are a major class of circulating blood lipids that serve as the body's primary energy reserve and play a critical role in metabolic regulation.[1] Abnormal triglyceride levels are strongly associated with a range of pathological conditions, including cardiovascular disease, diabetes, pancreatitis, and hepatic dysfunction, making them essential clinical biomarkers for routine diagnostics. Normal blood triglyceride levels are below 150 mg dL^{-1} (1.6 mmol L^{-1}), whereas levels from 150 to 199 mg dL^{-1} (1.6–2.1 mmol L^{-1}) indicate hyperlipoproteinemia, levels above 500 mg dL^{-1} (5.2 mmol L^{-1}) correspond to hyperlipidemia, and levels exceeding 1000 or 5000 mg dL^{-1} are associated with eruptive xanthoma and enlargement of the liver and spleen, respectively.[2,3] High triglyceride levels are an important risk factor for many diseases, making them valuable clinical biomarkers. Despite their importance, triglyceride analysis is still predominantly performed in centralized laboratories, limiting rapid screening and point-of-care applications, particularly in resource-limited settings.

Conventional analytical techniques such as colorimetric, chromatographic, and fluorometric methods provide reliable detection; however, they often require multiple enzymes, complex sample preparation, or sophisticated instrumentation. These limitations hinder their applicability for rapid, cost-effective, and on-site monitoring. Consequently, there is a growing demand for alternative sensing platforms that enable simplified, sensitive, and real-time triglyceride detection. Biosensors, particularly electrochemical platforms, address these limitations by enabling rapid, sensitive, and user-friendly detection with minimal sample preparation.[1–5] Electrochemical biosensors are advanced electroanalytical tools that use nanoscale surface modifications.[6,7] The field of electrochemical diagnostics utilizes various electroanalytical techniques, which are generally categorized into four primary operational modalities: amperometric, potentiometric, voltammetric, and impedimetric biosensors. Each approach employs a distinct electrical measurement mechanism to convert biochemical interactions into interpretable signals. Among electrochemical methods, amperometric biosensors dominate the literature, whereas impedimetric biosensors remain underexplored. Even with the advantages of impedimetric approaches, their application in biosensing remains limited due to poor reproducibility, unstable immobilization matrices, and reliance on complex signal interpretation, hindering its practical deployment.[8]

Electrochemical impedance spectroscopy (EIS) enables label-free detection by monitoring changes in charge transfer resistance (R_{ct}) at the electrode–electrolyte interface. Its performance, however, critically depends on the immobilization matrix, which affects enzyme stability, electron transfer efficiency, and analytical reliability.[9,10] Conventional acrylic microspheres are effective for enzyme immobilization but lack intrinsic conductivity, requiring the addition of gold nanoparticles to enhance electron transfer.[11–13] While gold nanoparticles provide excellent conductivity, their high cost and additional fabrication steps increase complexity and reduce the feasibility of low-cost, scalable biosensor fabrication. Multi-enzyme or mediator-based systems further increase cost and limit device lifetime. A critical challenge in impedimetric biosensor design lies in developing a single material that can simultaneously ensure efficient electron transfer and stable enzyme immobilization without requiring additional conductive nanomaterials.

To address these limitations, this study presents a label-free, mediator-free impedimetric triglyceride biosensor based on newly synthesized conductive acrylic microspheres (ACM) incorporating polyacetylene (PA). Unlike conventional systems

that require additional conductive nanomaterials, ACM function as a dual-purpose platform, providing both intrinsic conductivity and an effective matrix for enzyme immobilization. *Candida antarctica* lipase A is employed as a single biorecognition element to catalyze triglyceride hydrolysis, inducing measurable changes in (R_{ct}). This simplified design eliminates the need for gold nanoparticles, redox mediators, and multi-enzyme systems, thereby reducing cost and fabrication complexity for triglyceride monitoring. At the same time, this work highlights the potential of conductive polymer-based microspheres as a new class of multifunctional materials for next-generation electrochemical biosensors.

2. EXPERIMENTAL

2.1. *Chemicals*

2,2-Dimethoxy-2-phenylacetophenone (DMPP), 1,6-hexanediol diacrylate (HDDA), sodium dodecyl sulfate (SDS), lipase (EC 3.1.1.3 from *Candida antarctica*), tripalmitin, myristic acid, oleic acid, linoleic acid, dodecanoic acid, stearic acid, and phenylvinylsulfoxide (PVS) were purchased from Sigma-Aldrich (USA). Palmitic acid was purchased from Fluka (Switzerland). Sodium chloride (NaCl), *n*-acryloxysuccinimide (NAS), and ethanol were obtained from Systerm (Malaysia). Sodium dihydrogen phosphate (NaH_2PO_4) and *n*-butyl acrylate (nBA) were procured from Merck (German). Disodium hydrogen phosphate (Na_2HPO_4) was purchased from BDH (United Kingdom). All chemicals were used without purification. Deionized water was used in all experiments.

2.2. *Instrumentation*

Impedance spectra were recorded across a frequency range of 100 Hz to 1000 kHz using an Autolab PGSTAT 302N (Metrohm AG, Switzerland) with a 10 Mv sine wave potential amplitude. The resulting real (Z') and imaginary (Z'') impedance components were analyzed using frequency response analysis software. The electrochemical measurements employed a three-electrode configuration consisting of screen-printed carbon paste electrodes (SPCPE) modified with ACM, platinum electrodes, and a silver/silver chloride (Ag/AgCl) reference electrode. All EIS measurements were conducted in 0.05 M sodium phosphate buffer (Na-Pbs) at pH 7.

2.3. *Synthesis and characterization of ACM*

ACM were synthesized using microemulsion and photopolymerization method adapted with slight modifications from Ulianas.[11] Briefly, 2 mg of DMPP, 20 µL of HDDA, 40 µL of PVS, 1 mg of NAS, 20 µL of nBA, 1 mg of SDS, and 100 µL of deionized water were combined and sonicated for 10 minutes until micelle droplets formed, as evidenced by the solution turning milky white. Photopolymerization was then initiated by irradiating the micelle suspension with ultraviolet light under a nitrogen gas flow (< 0.5 bar) for 10 minutes. The resulting milky suspension was centrifuged at 10,000 rpm for 10 minutes, and the white precipitate obtained was washed with ethanol and air-dried at room temperature. Heat treatment at 250 °C under a continuous nitrogen atmosphere was subsequently performed to generate conductive polymer segments. The ACM were stored at 4 °C until further use. The size and morphology of the ACM were determined by field emission scanning electron microscopy (FESEM). The impedance technique was used to analyze conductivity. Approximately 5 mg of ACM was dispersed in 500 µL of 80 % ethanol for further use in enzymatic biosensor fabrication.

2.4. *Fabrication of the enzymatic biosensor*

ACM were dispersed in a solution containing 30 % ethanol and 70 % deionized water. After dropping ACM onto the surface of the SPCPE, the electrode was air-dried at room temperature (27 °C) for 2 hours to promote uniform and stable adhesion of ACM. A lipase solution (5 mg μL^{-1}) was placed on the surface of the ACM/SPCPE and allowed to react overnight. Then, the resulting enzymatic biosensor was carefully rinsed with 0.05 M Na-Pbs (pH 7) to remove any unbound lipase enzyme. The modified electrode was immersed in a reaction solution containing 0.1 mM Na-Pbs (pH 7), 0.9 % sodium chloride (NaCl), triglyceride solution (1–250 mg dL^{-1}), and surfactant, at 35 °C for 10 seconds. EIS was used to examine the biosensor response.

Figure 1 presents a schematic of the developed biosensor. ACM contain phenyl vinyl sulfoxide, which exhibits electrical conductivity primarily due to its π-conjugated structure and molecular polarization. The conjugation between

the phenyl ring and vinyl group enables partial electron delocalization, facilitating charge transport through π–π interactions, particularly in polymerized or closely packed systems. In addition, the polar sulfoxide (S=O) group enhances charge separation and stabilization of charge carriers, contributing to improved charge mobility. When incorporated into a polymeric network, these combined effects can result in measurable conductivity. ACM also contain succinimide functional groups, which can form covalent bonds with primary amine groups of lipase, leading to stable enzyme immobilization through the formation of amide linkages.

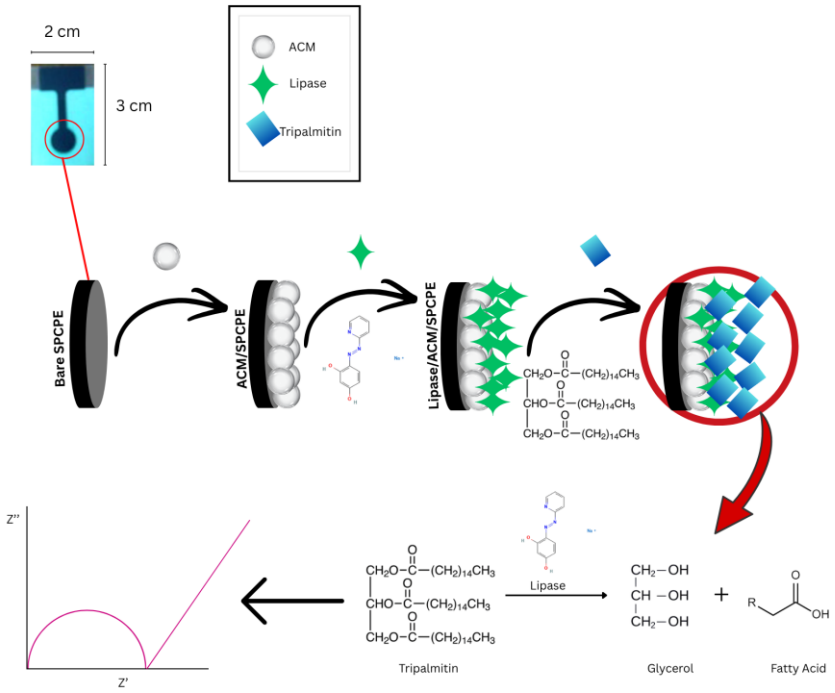


Fig. 1. The fabrication of label-free triglyceride biosensor, developed from immobilized lipase onto conductive acrylic microspheres (ACM)

2.5 *Optimization of the enzymatic biosensor for triglyceride detection*

The amount of lipase immobilized onto ACM was optimized by keeping the amount of ACM constant at 6 µg and varying the amount of by lipase varied from 5 to 35 µg. Lipase was immobilized for 20–240 minutes. The incubation time between lipase and triglyceride was determined by exposing the fabricated biosensor to triglyceride solution for 20–200 minutes. To investigate the effect of Triton-X-100, the surfactant was added to the entire triglyceride solution at a concentration ranging from 0.2 % to 0.8 %. The calibration curve of the developed biosensor was established with a series of triglyceride concentrations (1–1000 mg dL^{-1}). The results are expressed as mean ± standard deviation (n = 3).

The interference study was performed by mixing tripalmitin with different fatty acids at a ratio of 1:1. The relative response (%) was used to evaluate the interference effect of each fatty acid on the biosensor response toward triglycerides. The repeatability and reproducibility of the biosensor were evaluated based on eight replicates. Repeatability was assessed using the same bioelectrode, whereas reproducibility was determined by using independently prepared bioelectrodes under identical conditions. The results are expressed as relative standard deviation (RSD) to assess measurement precision and consistency. At the same time, stability experiments were conducted over a 60-day period.

2.6 *Sample testing and confirmation*

The impedimetric triacylglycerol biosensor was validated with a real sample of lard obtained from the Halal Institute, Universiti Putra Malaysia (UPM), and compared with standard analysis based on the method that reported by Seriburi and Akoh.[14] The lard sample was diluted to several concentrations. Lard contains a high amount of palmitic acid (16:0), the content of which was

determined by titrating the sample with 0.1 M sodium hydroxide (NaOH) using 30 mL of titrant per 0.5 g of sample, with 3–4 drops of phenolphthalein used as an indicator. Titration continued until a stable pink color appeared and remained for 2–5 seconds. The percentage of fatty acids, expressed as palmitic acid, was calculated according to the standard AOCS method Ca 5a-40:

$$\%\, FFA\ 16{:}0 = V_{NaOH} \times N \times \frac{28.2}{m}$$

where V_{NaOH} is the volume of NaOH (mL) required to reach the titration endpoint, and m is the weight of the lard (g).

3. RESULTS AND DISCUSSION

3.1. *Morphology and characterization of ACM and electrochemical response of the modified SPCPE*

Figure 2a presents a field emission scanning electron micrograph showing the morphology of ACM. They have a spherical morphology with an average particle size ranging from approximately 0.3 to 1.25 µm. Emulsification is a method aimed at producing smaller and more uniform spherical sizes,[15] while photopolymerization can accelerate polymer formation and enhance the conductivity of ACM.[16] SDS acts as surfactant and doping agent to control particle size and improve the polymers conductive properties. ACM were heated at 250 °C to remove benzene sulfenic acid from PPVS, the precursor of PA, thereby forming conductive segments within the polymer.[17]

Fourier-transform infrared spectroscopy (FTIR) was used to characterize the features of ACM before and after heat treatment (Figure 2b). Heat-treated ACM lacks the hydroxyl (–OH) band at 3440 cm^{-1}, attributed to adsorbed water in untreated ACM. Only untreated ACM presents bands at 3068 and 1445 cm^{-1}, corresponding to aromatic benzene rings and sulfone groups, respectively. Both untreated and heat-treated ACM exhibit C–H stretching at 2936 and 2852 cm^{-1}, while C=O (ester), C=O (amide), and S=O (sulfoxide) vibrations appear at 1750, 1636, and 1062–1052 cm^{-1}, originating from nBA, NAS, and PVS monomers, respectively. There are slight shifts in the peaks, likely due to dopant incorporation during polymerization, which may alter electron localisation.[18]

Both heat-treated and untreated samples present bands related to CH_3 bending (1451 cm^{-1}) and CH stretching (1395 cm^{-1}). A band near 1000 cm^{-1} indicates out-of-plane C–H deformation, confirming the formation of trans-PA.[19] Heat-treated ACM shows an increase in %T, consistent with Shirakawa,[20] who reported a direct correlation between trans-PA absorption and elevated heating in Ziegler–Natta-catalyzed systems. The reduced peak intensity at 748 cm^{-1} in heat-treated ACM suggests conversion of cis-PA to trans-PA, driven by the thermodynamic instability of cis-PA, in agreement with Ito et al.,[21] who noted irreversible isomerization above 145 °C.

Figure 2c shows the impedance analysis of ACM compared with bare SPCPE, acrylic microsphere (AM)/SPCPE, and gold colloid (GC)/AM/SPCPE over the frequency range of 10–100 kHz. EIS measurements were performed using a working electrode with a geometric surface area of 2.094 cm^2 and a diameter of 0.67 cm. Accordingly, all impedance values in this study are reported as raw data in ohms based on this fixed geometric area. EIS is a powerful tool for evaluating electrode–electrolyte interfacial resistance (R_{ct}), surface coverage (θ), and kinetic parameters of modified electrodes.

The Nyquist plots reveal that ACM/SPCPE exhibits a markedly lower R_{ct} relative to bare SPCPE and AM/SPCPE, with values approaching that of GC/AM/SPCPE. This is due to highly conductive materials that shunt the electrode and inhibit electric field penetration into the solution. This reduction demonstrates that ACM effectively enhances the conductivity of SPCPE by leveraging its intrinsic conductive properties, thereby minimizing interfacial resistance. Equivalent circuit fitting yields an R_{ct} of 450 Ω for ACM/SPCPE, 910 Ω for bare SPCPE, and 776 Ω for AM/SPCPE. The larger semicircles observed for bare SPCPE and AM/SPCPE indicate electrochemical insulating behavior, which restricts the adsorption of redox probe molecules. The close agreement between ACM/SPCPE (450 Ω) and GC/AM/SPCPE (447 Ω) highlights the potential of ACM as a viable alternative to gold nanoparticles, which are conventionally used to enhance electrochemical signals.

Using the relation $\sigma = \frac{L}{R \cdot A}$, the conductivity of ACM was quantified as $1.01 \cdot 10^{-4}$ S cm^{-1}. This level of conductivity is comparable to a previous report by Le and Yoon,[22] confirming its suitability for effective electrochemical performance. The conductive behavior of ACM has previously been attributed to the presence of PA networks and PVS-derived conductive pathways within the polymer matrix. These structural features have been proposed to facilitate charge transport and improve

electron-transfer processes. Although the conductivity mechanism of ACM was not directly investigated in the present study, the observed reduction in R_{ct} is consistent with its reported conductive characteristics and supports its potential use as a conductive platform for electrochemical biosensing. This reduction suggests enhanced electron-transfer kinetics at the electrode–electrolyte interface. This improved electrochemical behavior is consistent with the reported conductive characteristics of ACM and is expected to enhance the sensitivity of the impedimetric response by enabling more efficient transduction of interfacial changes arising from enzyme immobilization and substrate hydrolysis.

Figure 2d shows impedance measurements of ACM/SPCPE with lipase immobilized on its surface. Adsorption of lipase on the ACM provokes significant impedance changes. The R_{ct} of lipase/ACM/SPCPE increases, indicating that lipase was successfully immobilized within ACM, which led to a slight hindrance in the electron transfer and diffusion processes.[9,23] This suggests that there is an interaction between lipase and ACM, affecting the electrical properties of the system.[23] The increase in resistance can be attributed to the functional groups of lipase (–NH_2 and –CHO) present on the electrode surface, as well as the intrinsic insulating nature of the enzyme. The efficiency of enzyme immobilization is also influenced by the inherent properties of the supporting matrix, which can affect enzyme conformation during the process. Accordingly, ACM/SPCPE, with its hydrophobic surface, provides a favorable environment that enhances the catalytic activity of the immobilized enzyme. As further highlighted by Califano et al.,[24] this effect is associated with conformational rearrangements of the enzyme, enabling the formation of an open active site accessible to the substrate. Such conformational adaptation facilitates substrate recognition similar to the enzyme's natural environment, thereby promoting interfacial activation during immobilization.

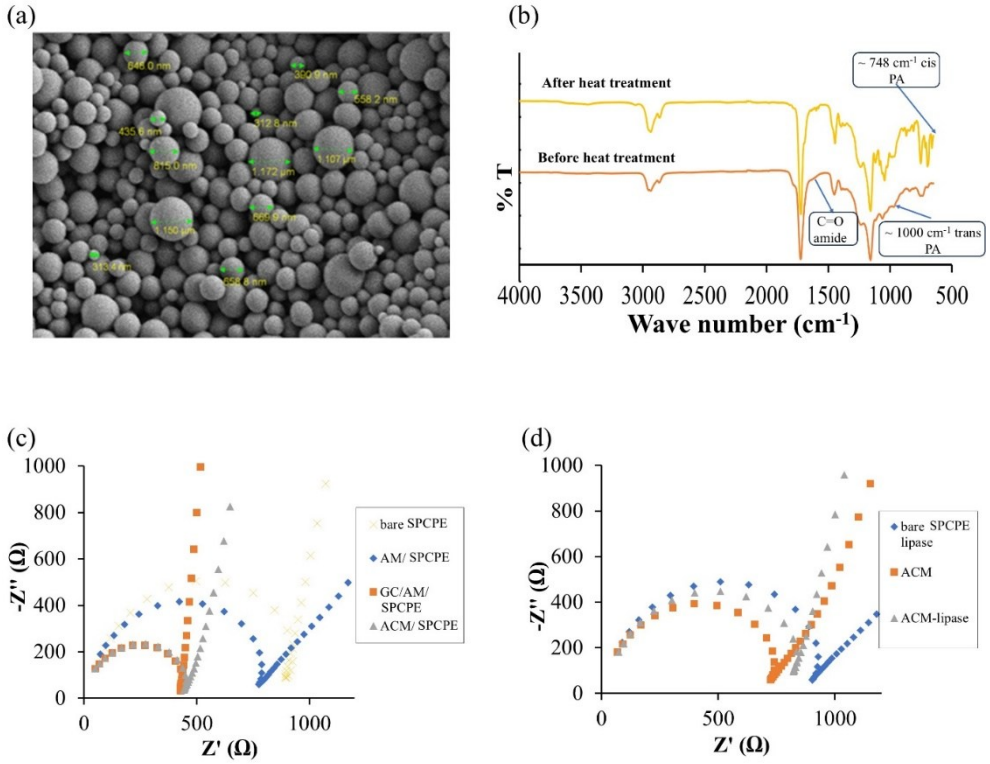


Fig. 2. (a) Field emission scanning electron micrograph of conductive acrylic microspheres (ACM) at a magnification of 10,000×. (b) Superimposed Fourier-transform infrared spectra of ACM, before and after heat treatment. (c) Conductivity of ACM in 5 mM $[Fe(CN)_6]^{3-/4-}$. (d) Electrochemical impedance spectra showing the effect of lipase immobilization in 10 mM Na-Pbs. The impedance data are presented as raw values (in ohms), corresponding to a working electrode geometric surface area of 2.094 cm^2

To quantitatively evaluate the electrochemical changes occurring at the electrode interface during triglyceride hydrolysis, EIS data were fitted using a modified Randles equivalent circuit model consisting of solution resistance (R_s), a constant phase element (CPE) representing the double-layer capacitance, and polarization/charge-transfer resistance (R_p, equivalent to R_{ct}) (Figure 3). The choice of a CPE instead of an ideal capacitor account for electrode surface roughness and nonuniform charge

distribution, yielding an exponent value $N = 0.983$ that is close to unity.

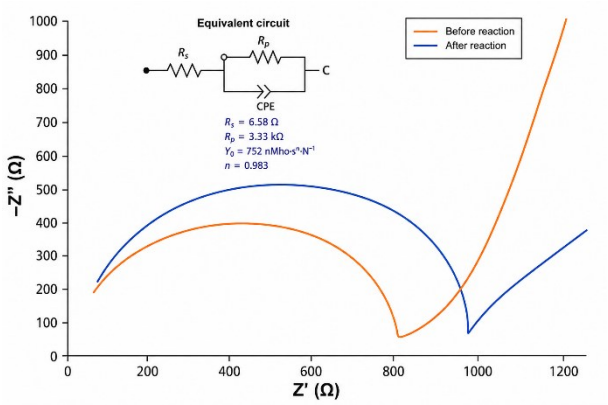


Fig. 3. Equivalent Randles circuit model representing the electrochemical response of the triglyceride biosensor constructed with conductive acrylic microspheres

R_s reflects the bulk properties of the electrolyte and remains virtually unaffected by bio-recognition events at the electrode surface. In contrast, R_{ct}, represented by the diameter of the semicircle in the Nyquist plot at high-to-medium frequencies, serves as the dominant sensing parameter because interfacial modifications directly impede electron transfer.[9]

Upon the initiation of triglyceride hydrolysis, R_{ct} increased in a concentration-dependent manner relative to baseline, indicating that electron transfer at the electrode–electrolyte interface is the rate-limiting process. Mechanistically, lipase-catalyzed cleavage of triglycerides yields glycerol and long-chain free fatty acids, while simultaneously generating three protons (H^+) per triglyceride molecule. The progressive accumulation and adsorption of these liberated, poorly conductive fatty acid chains at the electrode interface create a physical barrier. This hydrophobic layer passivates the electrode surface, restricting mass transport and electron transfer of the redox probe to the underlying conductive substrate. Furthermore, the concomitant release of H^+ decreases the local pH within the enzyme-immobilization matrix. These local ionic shifts modulate the interfacial charge distribution within the electrical double layer, directly correlating with the observed variations in the CPE parameters. This comprehensive equivalent circuit analysis confirms that the impedimetric sensing mechanism is primarily governed by surface-blocking effects and local double-layer alterations driven by the enzymatic reaction.

3.2. *Optimization of the impedimetric triglyceride biosensor response*

Several parameters were optimized for the detection of triglycerides, including the amount of lipase, the immobilization time, pH, the surfactant concentration, and the incubation time. All experiments were conducted in 10 mM Na-Pbs (pH 7) containing 0.9 % NaCl and 50 mg dL^{-1} tripalmitin as the analyte. Tripalmitin, a representative triglyceride, was utilized as a model compound throughout the experimental procedure.

Varying amounts of lipase (5–35 µg) were immobilized by drop-casting onto the surface of ACM/SPCPE (Figure 4a). In general, higher lipase concentrations increased the rate of triglyceride hydrolysis into fatty acids and glycerol, which accelerated the decrease in the pH of the solution. This pH reduction increased the concentration H^+ in Na-Pbs, inducing electrostatic repulsion from the positively charged electrode surface, thereby increasing the interfacial resistance, as reflected in the optimum R_{ct}. Moreover, higher enzyme loading introduced additional active sites within the same area, enhancing the probability of substrate binding. However, beyond a certain threshold, crowding effects in the bulk phase reduced enzyme flexibility, diminishing its catalytic efficiency in triglyceride hydrolysis.[25] The results indicated that R_{ct} increased with the lipase concentration up to an optimum at 25 µg, after which further enzyme addition decreased R_{ct}.

The effect of immobilization time (20–240 minutes) was evaluated by drying at room temperature. This approach aimed to balance enzyme usage costs while maintaining sufficient sensor performance. As shown in Figure 4b, R_{ct} increased as the immobilization time increased up to 180 minutes, after which there a decline in R_{ct}. Thus, the optimal immobilization time (180–240 minutes) provided a sufficient duration for covalent bonding between lipase and the ACM/SPCPE surface, thereby enhancing catalytic activity toward triglyceride hydrolysis.

pH plays a crucial role in regulating the catalytic activity of enzymes for triglyceride detection. In general, changes in pH affect the ionization state of both free substrate and enzyme. According to Goswani et al.,[26] extreme pH conditions can denature lipase and degrade the substrate, thereby reducing the reaction rate. The buffer pH may also influence charge-transfer properties at the immobilized bioelectrode interface. Accordingly, the effect of buffer pH in the range of 6.0–8.0 was evaluated (Figure 4c). The

observed increase in the imaginary component of impedance as pH increases is attributed to a reduction in the H^+ concentration at the electrode surface, leading to an increase in the thickness of the electrical double layer. This behavior arises from a lower ionic charge density, which simultaneously decreases the double-layer capacitance. Consequently, pH 7 was identified as the optimum.

Amino acids within enzymes exhibit amphoteric behavior, enabling them to donate or accept protons and thereby confer partial resistance to variations in pH. Nevertheless, the overall structural conformation of enzymes remains highly sensitive to pH changes. At the optimum pH, the active site is stabilized by hydrogen bonding and ionic interactions involving the NH_2 and COOH groups of polypeptides, which ensures the proper configuration for catalysis. Even slight deviations from this optimum can disrupt the geometry of the active site, ultimately leading to enzyme denaturation and loss of substrate specificity.

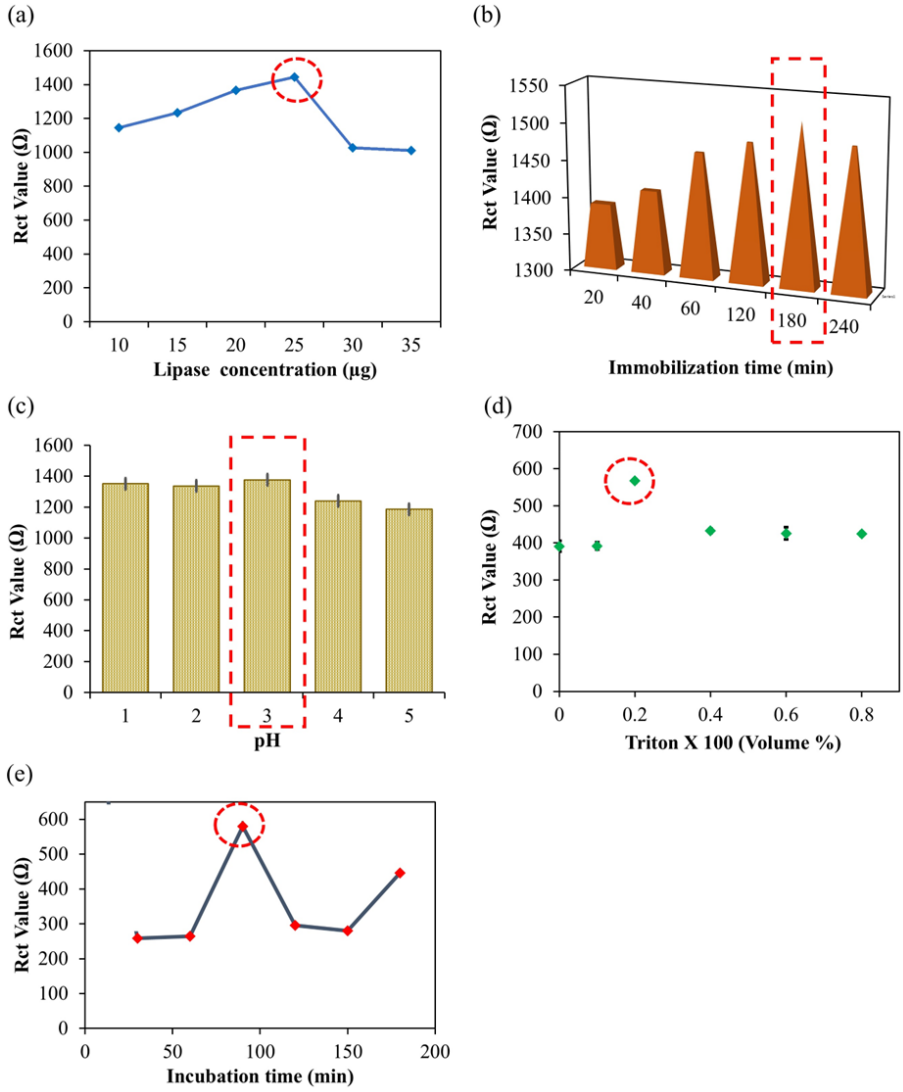


Fig. 4. The effects of (a) the amount of lipase, (b) the lipase immobilization time, (c) pH, (d) surfactant loading, and (e) the incubation time on the triglyceride biosensor response based on charge transfer resistance (R_{ct})

During triglyceride hydrolysis, the fatty acids generated contain carboxylic acid groups that are inherently acidic. In aqueous media, these groups donate H^+ to water, forming hydronium ions (H_3O^+). The accumulation of carboxylic acids consequently increases the H^+ concentration in the reaction medium. As a result, the progressive hydrolysis of triglycerides into fatty acids and glycerol leads to a decrease in the overall pH of the solution.

Surfactants have been reported to enhance solubility and increase interfacial area. The incorporation of surfactants results in more stable emulsions with triacylglycerol, thereby improving solubility and providing a larger reactive interface for enzymatic catalysis. However, excessive

surfactant concentrations may lead to the formation of larger and more complex emulsion structures, which reduce the effective interfacial area. In this study, the effect of Triton X-100 as a surfactant was examined by incorporating it into the substrate solution (5 mL PBS and 1 mL tripalmitin) at concentrations ranging from 0.2 % to 0.8 % (Figure 4d). The optimum response was obtained at a concentration of 0.2 %.

Figure 4e illustrates the effect of incubation time on the impedance response of the triglyceride biosensor. Increasing the incubation time allowed triglyceride hydrolysis to proceed to completion. A comparison between the non-incubated and incubated conditions showed that incubation reduced electrode resistance by enhancing electron transfer between the enzyme and the electrode. The optimum incubation time was determined to be 90 minutes; at this time, increased fatty acid production intensified electrostatic repulsion between the positively charged electrode surface and the hydrolysis products, thereby amplifying the impedance response.

3.3. *Analytical performance of the impedimetric triglyceride biosensor*

The performance of the triglyceride biosensor was evaluated in 0.1 mM Na-Pbs (pH 7) containing 0.9 % NaCl and a tripalmitin solution. Measurements were carried out using an impedimetric triglyceride biosensing technique to obtain a linear calibration curve (Figure 5a). Electrode resistance increased as the tripalmitin concentration increased from 1 to 1000 mg dL^{-1}. The fabricated biosensor exhibited a wide linear response over the concentration range of 1–1000 mg dL^{-1} (R^2 = 0.992). This broad linear range is attributed to the three-dimensional structure of ACM, which provides a larger surface area for enzyme immobilization compared to conventional two-dimensional membrane structures. The limit of detection (LOD) was calculated as three times the standard deviation of the biosensor response within the linear range divided by the slope of the corresponding calibration curve. Accordingly, the LOD of the impedimetric triglyceride biosensor was 0.0087 mg dL^{-1}. This is due to improved electron transfer and large active surface area provided by the ACM-modified electrode interface.

A reproducibility test was performed using eight different electrodes prepared over the same period under identical optimum conditions and tested simultaneously at the same tripalmitin concentration (Figure 5b). The average reproducibility of the triglyceride biosensor, expressed as the RSD, was 2.56 % (n = 8). These results indicate high sensor reproducibility, demonstrating its ability to deliver stable and consistent responses based on the proposed fabrication and detection strategy. Repeatability was assessed by testing the same bioelectrode eight times under identical conditions to evaluate the impedance response of the triglyceride biosensor. The measurements demonstrated highly consistent resistance values, with an RSD of 4.41 % (n = 8).

Figure 5d illustrates the interference effects on the impedance response of the triglyceride biosensor. The bar chart presents mixtures of triglyceride and potential interferents at a 1:1 ratio. The relative response percentages for fatty acid interferents ranged from 0.27 % to 13.5 %. Only oleic acid and linoleic acid caused significant interference in the triglyceride biosensor response.

The stability of the developed impedance-based triglyceride biosensor was assessed over a period of 60 days at 4 °C (Figure 5e). Within the first 5 days, there was a significant 31.7 % increase in resistance, attributed to enzyme deactivation on the bioelectrode surface. Stability continued to decline gradually, with a 34.4 % increase in resistance at 30 days and a nearly 50 % increase in resistance after 40 days.

The overall performance of the ACM-based triglyceride biosensor is summarized in Table 1. The observed decline in biosensor response during storage represents a limitation of the present sensing platform, particularly for applications requiring extended shelf life. The gradual loss of signal is likely associated with reduced lipase activity and changes in the stability of the enzyme–ACM interface during storage.

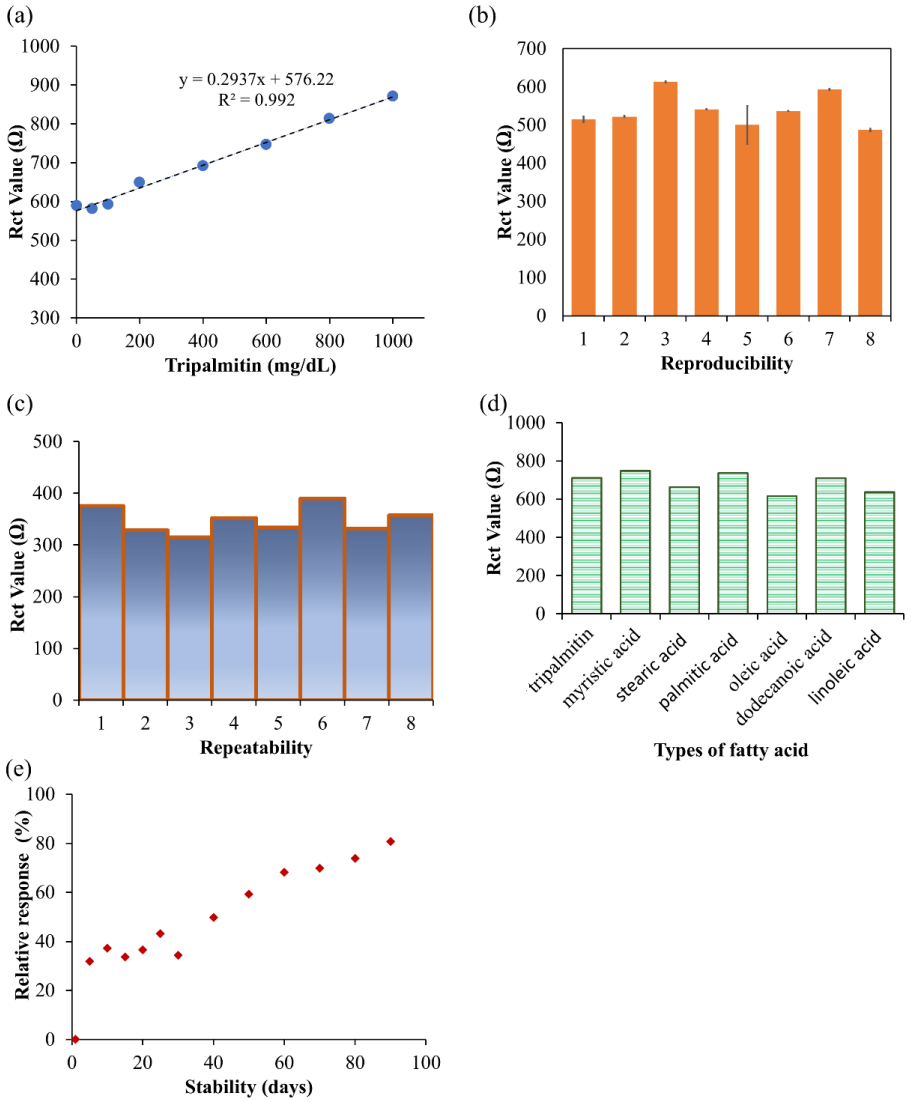


Fig. 5. The (a) linear range, (b) reproducibility, (c) repeatability, (d) interference effect at a 1:1 ratio of triglyceride to interferent, and (e) stability of the impedimetric triglyceride biosensor

Table 1. Summary of the performance of the impedimetric triglyceride biosensor

Linear range	1–1000 mg dL^{-1}
Limit of detection	0.0087 mg dL^{-1}
Reproducibility	Relative standard deviation = 2.56 % (n = 8)
Repeatability	Relative standard deviation = 4.41% (n = 8)
Interference effect	0.27 % to 13.5 %
Stability	31.8 % was retained after 60 days

3.4. *Validation using real samples and assessment of triglyceride recovery*

The impedimetric triglyceride biosensor was evaluated using spiked pork fat samples, and its performance was compared with that of the standard titration method (Table 2). Pork fat samples were spiked with tripalmitin at different concentrations, and the percentage recovery was determined using both methods. The results were compared with the standard titration method. The biosensor produced recoveries ranging from 61.9 % to 140 %, compared with 89 % to 116 % for the standard method. The substantially wider recovery range obtained with the biosensor indicates greater variability in its analytical response under the conditions tested. This variability may be related to the complex pork-fat matrix and to the sensitivity of impedance measurements to factors such as sample preparation and measurement conditions. In addition, the validation was based only on spiked pork-fat samples, so the results cannot be assumed to apply to other food or biological matrices. Further optimization of the sensing procedure and control of experimental variables are therefore required to improve the accuracy, reproducibility, and robustness of the biosensor. Additional validation

using a wider range of samples and concentrations would also be needed to establish its analytical performance.

Table 2. Comparison of the recovery ranges using the impedimetric triglyceride biosensor and the standard titration method

Actual concentration of pork fat (mg ml^{-1})	Determination of pork fat using developed biosensor	Percentage recovery (%)	Determination of pork fat using titration technique	Percentage recovery (%)
2	2.19	109.5	1.97	98.5
1	0.62	61.9	0.89	89
0.5	0.48	96.2	0.54	108
0.1	0.14	140	0.09	90

3.5. *Comparison of the analytical performance of the developed impedimetric biosensor with other reported triglyceride biosensors*

The performance of the impedimetric biosensor developed using ACM was compared with another reported triglyceride biosensor designs (Table 3). This comparison is important for validating the developed biosensor. Triglyceride biosensors use diverse immobilization strategies, primarily involving enzyme entrapment within polymeric matrices or direct surface adsorption, each of which impacts the resulting linearity and detection capabilities.[4,27] This enzymatic approach typically exploits irreversible electron flow without the need for an electron mediator, thereby enhancing the efficiency and stability of the biosensing platform.[3]

Another biosensor in which lipase was co-immobilized with glycerol-3-phosphate oxidase and glycerol kinase on a zinc oxide and chitosan nanofilm exhibited a detection range of 50–650 mg dL^{-1} and a LOD of 20 mg dL^{-1}.[4] Furthermore, biosensors that utilize graphene oxide nanoparticles on pencil graphite electrodes have been developed to enhance analytical performance, demonstrating a linear range of 0.01–60 mM for triolein and a LOD of 0.2 nM.[3] Another notable advancement is the development of amperometric biosensors incorporating multi-enzymatic systems on modified glassy carbon electrodes, which offer enhanced selectivity and sensitivity, particularly in lyophilized serum samples.[5] Moreover, the incorporation of sol–gel-derived silica and zirconia matrices modified with gold nanoparticles has shown promise for lipase immobilization, resulting in a tributyrin biosensor with a low LOD (1.86 μmol L^{-1}) and high sensitivity (5.37 μΩ μmol L^{-1}).[28]

To date, only a few impedimetric biosensors have been reported for the determination of triglycerides. The newly developed biosensor reported in the present study is a valuable addition to the tools available for triglyceride detection. Using ACM as an immobilization matrix has improved the wide linear range and LOD of the constructed biosensors because of the three-dimensional structure of ACM that provide a wider surface area for enzyme immobilization. ACM show strong potential as an alternative to conventional gold-based electrodes commonly used in biosensor development as they can enhance current response attributed to its intrinsic conductive properties.

Table 3. Comparison of the performance of the newly developed triglyceride biosensor and biosensors previously reported in the literature

Immobilisation matrix	Detection technique	Linear range (mg/dL)	Limit of Detection (LOD) (mg/dL)	Reference
ACM/SPCPE	Impedimetric	1-1000	0.0087	This work
ENPs/GrONPs/PGE	Amperometric	0.89-5310	$1.77 \cdot 10^{-5}$	Pundir et al.[3]
GCE/GO/MWCNTPEI/TiO_2	Amperometric	100-500	nA	Thakkar et al.[4]
NAF-LIP/MWCNTs/NAF-GO/MWCNTs/PABS/GCE	Amperometric	1-20	0.1	Jalalvand[5]
Gelatine membrane	Amperometric	26.6-115.1	nA	Manoj et al.[27]

4. CONCLUSION

In this study, a mediator-free impedimetric triglyceride biosensor based on ACM was developed and evaluated. ACM serve as a conductive platform for enzyme immobilization and facilitate electron transfer without the use of an external redox mediator. The fabricated ACM/SPCPE exhibited a LOD of 0.0087 mg dL^{-1} and a linear response over the concentration range of 1–1000 mg dL^{-1}, demonstrating its capability for triglyceride detection across a broad range of concentrations. The observed analytical performance may be attributed to the conductive properties of ACM and their suitability as an enzyme-supporting matrix.

These findings suggest that ACM have potential as a functional material for electrochemical biosensing applications while offering a relatively simple sensor configuration. Nevertheless, the recovery study revealed variability in analytical performance under the investigated conditions, indicating that further optimization and broader validation studies are required. Future work should focus on evaluating the sensor across more diverse sample matrices and benchmarking its performance against established analytical methods to further assess its practical applicability for triglyceride monitoring.

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