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Development of an electrochemical immunosensor using gold nanoparticles (AuNPs) for the detection of Apolipoprotein AI (ApoAI) in chicken blood plasma

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ABSTRACT

The research aims to develop an electrochemical immunosensing assay using gold nanoparticles (AuNPs) on screen printed carbon electrode (SPCE) for the specific detection of Apolipoprotein A1 (ApoA1) proteins in chicken blood plasma (CBP). The biotin anti-ApoAI antibody was first immobilized on magnetic beads (MBs), while antibody ApoA1 was then conjugated with AuNPs. After a series of sandwich-type immunoreaction complexes was performed on the screen-printed carbon electrode, AuNPs were quantified by subjecting the immunocomplex to a peroxidation process of high potential at 1.2 V for 330 s and immediately reduced and scanned by differential pulse voltammetry (DPASV). This study obtained a linear relationship between reduction peak current signals and ApoA1 concentration from 0 to 100% (correlation coefficient of 0.996 with a detection limit of 5% of chicken blood plasma (CBP). The efficacy of our immunosensor is predicated on the seamless integration of antibodies with MBs to the target analyte, which provide ample surface area, biocompatibility, and user-friendliness. This breakthrough improves preliminary food fraud detection's sensitivity, reliability, and usability. It could improve diagnostic food safety, leading to a worldwide health breakthrough.

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1. INTRODUCTION

Surimi, a widely enjoyed seafood-based product, is created by processing fish meat and transforming it into a paste-like consistency [1]. In recent years, surimi's popularity has grown due to its accessibility, high protein and low lipid content, and delicious flavor, which has been incorporated into well-known products such as fish cakes, crab flesh imitation, and fish balls, all of which originated in Japan. The production of surimi commonly makes use of marine fishes as raw materials. Some examples of marine fishes that are employed include Pacific whiting (*Merluccius productus)*, Alaska pollock (*Gadus chalcogrammus*), threadfin bream (*Nemipterus virgatus*), and various species of freshwater fish. One of the crucial aspects in the production of surimi is protein gelation, as it plays a significant role in achieving the desired texture. However, this process is frequently impaired in some species. Therefore, to enhance the gelation, texture, and water-holding capacity of surimi, a variety of additives have been added during the production process using various treatments consisting of high-frequency defrosting, superheated steam, and quick-freezing to improve the texture and quality of surimi [2]. Furthermore, others have explored incorporating plant-based proteins in surimi products for binding, which later enhances the gelation and texture [3],[4].

One of the excellent binder used in surimi products is chicken blood plasma (CBP), which is a by-product of poultry processing containing valuable proteins, minerals, and other constituents that hold the potential to provide nutritional benefits and functional properties. The high protein content in CBP consists of a common proteins such as albumin and prealbumin, α1-acid glycoprotein, transferrin, lipoproteins (HDL, LDL, and VLDL), immunoglobulins, complement proteins, and coagulation proteins (thrombin, plasminogen, and fibrinogen) [5]. Previous studies have highlighted that the addition of CBP as a food additive has the potential to enhance surimi texture and elasticity [6], emulsification properties [7], gelling characteristics, and binding capacities [8]. A recent study showed optimal conditions were achieved with a CBP concentration of 0.79% and sorbitol concentration of 4.68%, resulting in predicted solubility of 49.09 mg/ml, cohesiveness of 0.654, and whiteness of 75.55, making it more similar to real seafood [9]. One of the many proteins that may be found in the CBP is called Apolipoprotein A1 (ApoA1), and it is the prominent structural and functional protein component of high-density lipoprotein (HDL) in blood plasma. [10]. ApoA1 makes up over 70% of HDL and is important in transporting cholesterol in the opposite direction and maintaining cholesterol balance within cells [10],[11]. In addition to its role in cholesterol transport, ApoA1 has been found to have multifunctional roles in immunity, inflammation [12], apoptosis, and combating viral and bacterial infections. It also inhibits tumour-associated neo-angiogenesis and matrix degradation, making it a possible cancer treatment [10]. The unique structural and functional characteristics of ApoAI make it an intriguing target for further investigation and utilisation in various applications [13] especially markers of the risk of cardiovascular disease. The statement indicates the gap in markers specific to ApoA1 detection in foods. Developing a reliable and efficient detection method for ApoA1 as a biomarker in surimi products is crucial to ensure product quality and safety. The approaches to detect adulterants in foods by biochemical and molecular techniques are chromatography-based, spectroscopy-based, electrophoresis-based, immunology-based, and DNA-based [14]. DNA-based techniques such as Polymerase Chain Reaction (PCR) and its variants, the Real-Time PCR and Multiplex PCR, often to amplify and detect specific DNA sequences in food samples. While mass spectrometry (MS) under chromatography-based methods has proven useful for analysing complex and processed adulterated meat products [15],[16]. The establishment of detection in various food samples using PCR and MS-based techniques offers valuable tools for detecting food adulteration, however, they each possess destructive drawbacks, require trained personnel, limited sensitivity, are expensive, and are time-consuming. Addressing these limitations requires advancements in assay design, sample preparation methods, instrumentation, and data analysis algorithms to enhance the accuracy, reliability, and practicality of these analytical approaches in food safety and quality assurance applications [17].

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To address these issues, there is a need to create an enhanced detection platform utilizing antibody-antigen interactions for protein identification in CBP, focusing on ApoA1 detection with the aid of gold nanoparticles (AuNP) as labels in the sensor. The rationale of using AuNP in this study is driven by its high surface-to-volume ratio, quick electron transport, and biocompatibility. The direct binding of antibodies to their surface, enables the creation of electrochemical immunosensors that have excellent sensitivity and a low limit of detection (LOD) for a wide range of substances, including clinically significant biomolecules such as protein biomarkers for diseases [18]. AuNPs added to conductive substrates like carbon paste or screen-printed carbon electrodes (SPCEs) can increase electrode roughness, enhance antibody adsorption on the exposed AuNPs surface, and lower the detection limit. [18]. In addition, gold nanoparticles (AuNPs) can enhance the conductivity and surface area of electrodes to engage thiolated linkers, which can then covalently bind capture antibodies (CAbs) [18]. AuNPs' high surface area and conductivity improve electrochemical immunosensor sensitivity, which makes them ideal for point-of-care (POC) applications. [18][19].

Hence, the research objective was to develop a platform in electrochemical immunosensing assay based on biorecognition using AuNPs for the detection of ApoA1 from CBP as a model. AuNPs were successfully synthesised using the Turkevich-Frens method, involving the reduction of chloroauric acid with trisodium citrate. The biotin anti-ApoAI antibody was subsequently immobilized on magnetic beads (MBs), while antibody ApoA1 was then conjugated with the synthesised AuNPs. After a series of sandwich-type immunoreaction complexes was performed on the SPCEs, AuNPs were quantified by subjecting the immunocomplex to a peroxidation process of high potential at 1.2 V for 330 s and immediately reduced and scanned by differential pulse voltammetry (DPV). Electrodeposition of gold during the reduction stage of the redox reaction was determined by cyclic voltammetry (CV) that showed a linear relationship with the different ApoAI concentrations with no cross-reactivity with fish paste.

2. MATERIALS AND METHODS

2.1 Chemicals

Monoclonal anti-apolipoprotein AI antibody (ApoA1) and Biotin anti-apolipoprotein AI antibody (ab48329) were obtained from ABCAM (Boston, MA, USA). Streptavidin beads are 2.8-µm were obtained from Thero Fisher Scientific (USA). Gold (III) chloride trihydrate (HAuCl4•3H2O), trisodium citrate (Na3C6H5O7), bovine serum albumin (BSA), chicken albumin (CA), and Br2 were purchased from Sigma Aldrich Corporation (St. Louis, MO, USA). HBr, sodium chloride (NaCl), trisodium chloride trihydrate (C6H5Na3O7•2H2O), potassium carbonate (K2CO3), potassium dihydrogen phosphate (KH2PO4), di-potassium hydrogen phosphate (K2HPO4), acetone (C3H6O), APTES 99.5%, and GA 25% in aqueous solution was purchased from Merck KGaA (Darmstadt, Germany). The buffer solutions used in this study included phosphate buffer (PB; pH 7.4; 0.2 M KH2PO4, 0.2 M K2HPO4, and 0.9 M NaOH), and phosphate-buffered saline (PBS; pH 7.4; 0.2 M KH2PO4, 0.2 M K2HPO4, 3.0 M NaCl, and 0.9 M NaOH). The HBr/Br2 dissolution medium was composed of a mixture of 1.0 M HBr and 0.15 mM Br2. All aqueous solutions were produced using deionized water (deH2O) obtained from an Arium® Pro Ultrapure Water System (Sartorius AG, Göttingen, Germany).

2.2 Instrumentations

Differential Pulse Voltammetry (DPASV) were performed using an Autolab PGSTAT204 (Metrohm, Utrecht, The Netherlands), working together with its Nova 2.1.6 software. The screen-printed carbon electrodes (SPCEs) were purchased from Quasense (Thailand) and consisted of a carbon working electrode, a carbon counter-electrode, and a silver/silver chloride reference electrode. All measurements were made at room temperature (21 Å} 1℃).

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2.3 Preparation of fresh chicken blood plasma (CBP)

The chicken (*Gallus gallus*) blood sample was obtained from the local market in Dengkil, Selangor. 100 chickens have been certified healthy by the local Department of Veterinary Selangor before being slaughtered. The fresh blood was collected directly into a vacuum blood collection tube containing 3.2% sodium citrate (Vacutube, Algeria) at a volume ratio of 9:1 blood to anticoagulant. The mixture was shaken vigorously to prevent clotting. The tubes were maintained at a temperature of 4 °C throughout the collection and transportation process in an icebox. Subsequently, the blood was centrifuged using Rotofix 32A Benchtop Centrifuge (Kubota, Japan) for 20 min at 2,300 g. Two layers were formed, the upper was blood plasma with a yellow colour, while the lowest was red blood cells with a red colour. The upper layer was taken and stored at −40 °C until further usage [9].

2.4 Preparation of fish paste

The fish paste was prepared in the laboratory to eliminate contaminants and food additives. Minced fish were obtained from QL Food Sdn. Bhd. Teluk Intan. After deboning, the minced fish were washed three times with distilled water (meat: water ratio, 1:3 w/w) at a temperature below 10 ^{0}C. The next step was dewatering until the moisture content of the fish paste reached around 78-80%. The paste was then mixed with the cryoprotectants of sucrose (4%) and sorbitol (4%). The prepared fish paste was covered with polyethylene bags and frozen at -18 °C using an air-blast freezer for 24 hours. It was stored at -18 ^{0}C until further use [20].

2.5 Synthesis of Gold Nanoparticles (AuNP) using Turkevich-Frens method

The gold nanoparticles (AuNPs) were prepared by reducing Chloroauric acid ($HAuCl_4$) with trisodium citrate ($Na_3C_6H_5O_7$), which acts as a reducer and stabilizer. The colour of the solution eventually changed from translucent to black, purple, and deep red accordingly within 2 mins. The solution continuously stirs while boiling for another 15 mins. The temperature of the solution should drop naturally to room temperature. The cool-down solution was stored at 4 ˚C until further use.

2.6 Characterization of AuNP using TEM

The characterization involves the use of transmission electron microscopy (TEM) and was performed to investigate the size, morphology, and particle-size distribution of the synthesised AuNPs.

2.7 Bioconjugation of Biotin anti-ApoAI antibody to Magnetic Bead-Streptavidin (MBS)

This study used streptavidin-coupled magnetic beads (MBs) as the solid-phase support for immunoreactions with biotin anti-ApoAI antibody. This platform was prepared by immobilizing biotinylated anti-ApoAI capturing antibody (α-bAPoAI/CAP) onto MBs. Briefly, 25 µg of MBs were incubated under tilting mixing with 5 µL of 0.1 mg/mL α-bAPoAI/CAP solution in PBS buffer containing 0.1% v/v Tween20 (PB/T20) for 30 mins. This step was followed by washing with the same buffer for magnetic separation using a magnetic separator. The separated α-bAPoAI/CAP-MB conjugate was stored at 4 °C for further use.

2.8 Bioconjugation of AuNPs with anti-ApoAI antibody detection antibodies

AuNPs were conjugated with anti-ApoAI antibody detection antibody (α-APoAI /DET) for specific labelling of target ApoAI. The bioconjugation of AuNPs with the α-APoAI /DET process was performed using a conventional adsorption technique. Briefly, 100 µL of AuNP, pH 8.0 were mixed with 3 µL of 0.1 mg/mL α-APoAI /DET solution in PB buffer under tilting mixing for 30 mins. This α-APoAI /DET-AuNP conjugate was blocked with 10% (w/v) bovine serum albumin (BSA) for 40 mins in PB buffer. All the unconjugated α-APoAI /DET and free BSA were washed off with PB buffer containing 10% BSA

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(PB/BSA) by centrifugation. The soft sediment obtained was resuspended in 0.5 mL of PB/BSA and was stored at 4 °C for further use.

2.9 Electrochemical magneto-sensing of ApoAI from CBP

The α-ApoAI/CAP-MB conjugate was resuspended in 100µL of variable concentrations of CBP in PB/T20 buffer and was incubated at ambient temperature for the formation of immunocomplexes to occur. The unbound components were removed from the MB surface by washing with PB/T20 via magnetic separation. Next, the washed ApoAI-bound MB complexes were resuspended in 10 µL of α-ApoAI/DET-AuNP suspension. The labelling procedure will then proceed at ambient temperature for 30 mins, followed by washing with PB/T20 to remove any excess unreacted immunocomplexes. Lastly, the AuNP probed ApoAI-bound MB complexes were resuspended in 100 µL of detection medium (1 M HBr/0.15 mMBr_2) to chemically dissolve the AuNP probes as aqueous Au3+ ions. Electrochemical-quantitative analyses of resulting Au3+ ions were performed on SPCE the differential pulse anodic stripping voltammetry (DPASV) method (Fig 1). The aqueous Au3+ ions were subjected to electrochemical analysis by depositing the entire detection medium onto the SPE sensing area. This was followed by DPASV analysis using the specified parameters: The process involves applying an electrode at a voltage of -0.55 V for 390 seconds, followed by scanning the potential from 0.5 V to 1.0 V. The step potential is set at 6.0 mV, the modulation amplitude is 5 mV, the modulation period is 50 ms, and the scan rate is 50 mV/s. The current value at a potential +0.68 V was recorded for data analysis with the measured current signal expected to be quantitatively proportional to the amount of target protein biomarkers present in the reaction mixture. All experiments were performed in triplicate for each condition.

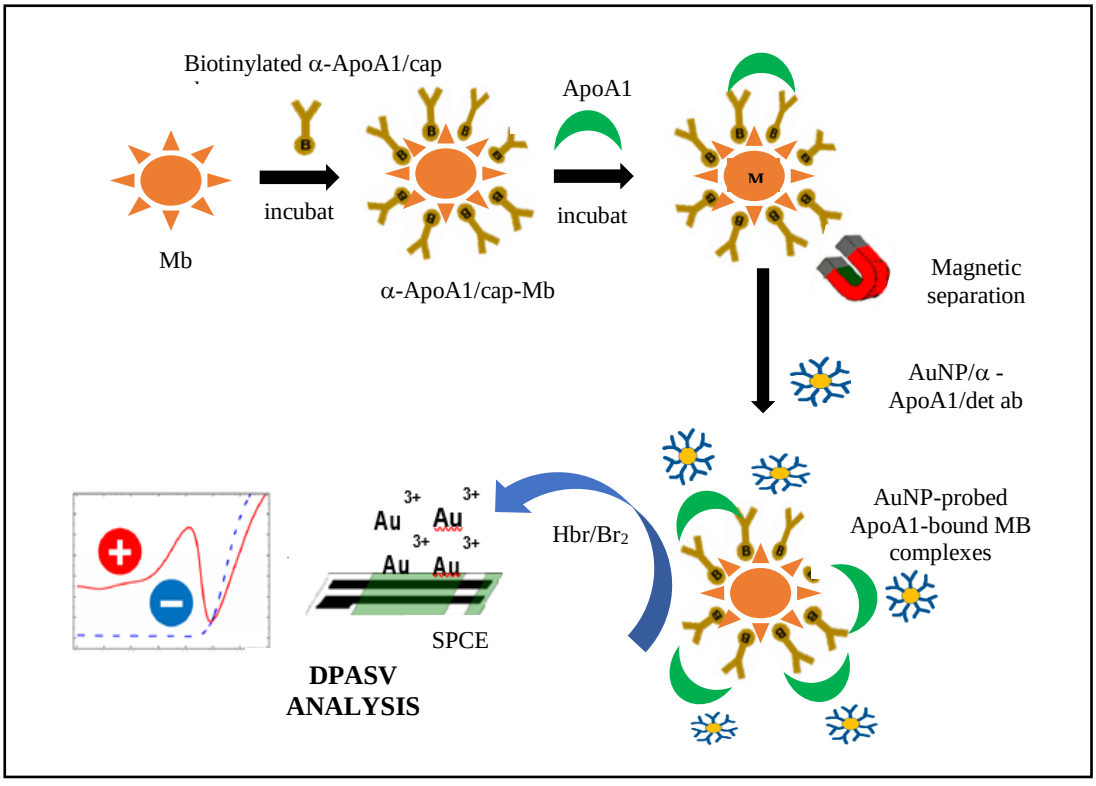

Fig. 1. Schematic representation showing the development of the proposed AuNP-based electrochemical immunosensor for detection of ApoA1.

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3. RESULT AND DISCUSSION

3.1 The synthesis of AuNP

The method adopted for producing AuNPs in this study was based on reducing a $HAuCl_4$ solution using sodium citrate under boiling conditions. As the reaction progressed and sodium citrate was introduced to the $HAuCl_4$ solution, the reaction medium gradually transitioned from a pale-yellow hue to a colourless in 10 mins, and ultimately a wine-red shade (Fig. 2(a)). The appearance of the wine-red colour solution signified the formation of colloidal AuNPs. Particle aggregation affects the molecules' colour. Zhang and colleagues [21] proposed an alteration in the hue of gold nanoparticles through the utilization of surface plasmon resonance (SPR). The colour change was observed to be accompanied by an increase in particle size, resulting in a shift from dark red to dim purple. The SPR exhibited by these particles in the visible range of the electromagnetic spectrum was found to be the primary cause of this variation [21].

3.2 TEM for AuNP and conjugated α-bAPoAI/CAP-MB- α-ApoAI/DET-AuNP

The morphological characteristics of chemically synthesised colloidal gold nanoparticles (AuNPs) were investigated using transmission electron microscopy (TEM). As depicted in Figure 2(b) and (d), the resulting AuNPs displayed spherical uniformity in size, with an average diameter of 15.20 ± 1.38 nm (n=100), with a calculated RSD value of 9.12%. Such RSD value which is less than 10%, signifies the high monodispersity of the synthesised AuNPs. AuNPs of varying sizes have distinct mechanical properties, with smaller particles exhibiting greater mechanical strength. The enhancement of mechanical strength is attributed to the reduction in interparticle gaps, resulting in the narrowing of the valence and conduction bands. Enhanced surface area is a significant characteristic in smaller AuNPs [22].

Additionally, TEM was utilized to capture an image of the α-APoAI/CAP-MB immunocomplex, which had been conjugated with the α-ApoAI/DET-AuNP. This biorecognition event is portrayed in Figure 2 (c), confirming the successful interaction between the and the platform.

3.3 Detection performance of ApoAI using the electrochemical immunosensor from CBP.

The present study evaluated the detection sensitivity of the Apo AI electrochemical immunosensor by subjecting it to a known percentage of serially diluted CBP in 0-100% (v/v) PB/T20. Electrochemical immunosensors' functioning starts with the synergistic integration of nanomaterials, in this study by using AuNPs and antibodies (α-ApoAI) specifically designed for targeting ApoA1 proteins. Upon introducing the CBP sample to the sensor, the ApoA1 proteins present in the CBP initiate a binding process with the antibodies immobilized on the sensor surface. This binding event generates a detectable electrochemical signal, facilitating the quantitative detection and characterisation of ApoA1 proteins within. The DPASV responses were then compared with the mean current signals observed for each percentage of the CBP sample. The study's findings suggest that there was a direct relationship between the electric current and the increasing percentage of CBP, with the highest value observed when CBP reached 100% (Fig. 3). This escalation in CBP percentage led to the formation of th1e Apo AI-bound MBS immunocomplex, which, in turn, resulted in the labelling of an increased number of α-Apo AI/AuNP signalling probes, thus leading to amplified current signals. A calibration curve was plotted to establish a linear relationship between the current signal and the CBP percentage within the range of 0-100% with a coefficient of determination (R^2) of 0.996, indicating the voltammetric responses directly to the binding of ApoAI available in CBP with different percentages to the bioconjugated MBs through biospecific antigen-antibody interactions. The experiment was performed in triplicate, and the relative standard deviation (RSD) was calculated to be 1.50% - 10.00% (n = 3) (Fig. 3). The current signals obtained by evaluating the detection sensitivity were used to calculate a threshold value for the determination of positivity and negativity.

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In this study, the calculated threshold value was determined as 0.58 µA (M + 3SD = 0.55 µA + [3 × 0.01]). The threshold point for a positive result was determined as a current signal greater than or equal to the mean (M) plus three times the standard deviation (SD) of the current signals of the blank samples containing no ApoAI. Consequently, samples with current signals ≥ 0.58µA were considered positive, while those falling below this threshold were classified as negative. Using this threshold, the limit of detection (LoD) was established with a negative current value presented by the fish paste (0.079µA), indicating no ApoAI was present in fish paste (FP).

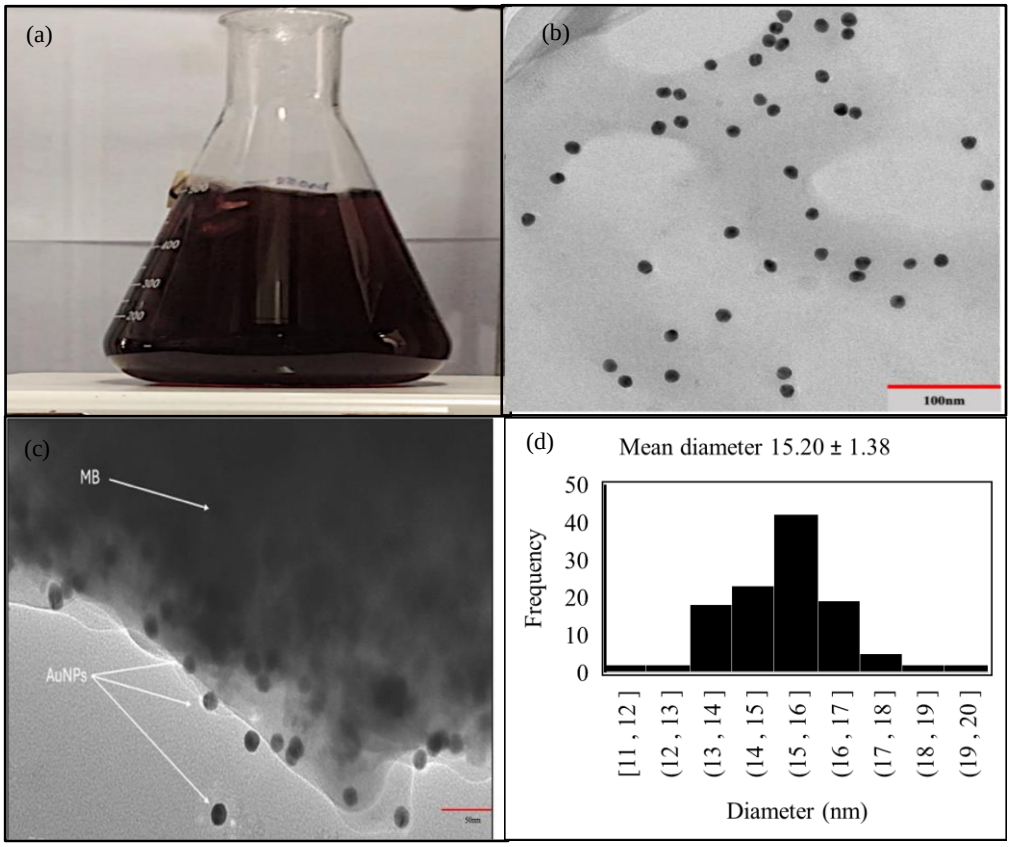

Fig. 2. shows the (a) AuNPs wine-red shade colour ; (b) TEM image of AuNPs synthesisd at 63000× magnification; (c) TEM image showing the α-APoAI/CAP-MB conjugated α-ApoAI/DET-AuNP at 20000× magnification and (d) particle size distribution histogram of the colloidal AuNP

Generally, the types of proteins include various categories based on their structure and function such as enzymes, and storage proteins (casein and ovalbumin) are essential in growth and development. Other than that, contractile proteins (actin and myosin) aid in muscle contractions and movement and transport protein (blood components, haemoglobin) which facilitate the transport of molecules across cell membranes or within the bloodstream. Apo A1 is a protein with a calculated molecular weight of 30,662 Da and 264 amino acid residues. It is a major protein part of high-density lipoprotein (HDL) that regulates cholesterol transport and metabolism [23]. It is present in the plasma of 95% of individuals [24] and is composed of stable α-helices that are associated with HDL. Subsequently, fish muscle is rich in various proteins, including myosin, actin, tropomyosin, collagen, and sarcoplasmic proteins. Myosin is the primary contractile protein, responsible for muscle contraction and movement. Actin forms thin filaments and plays a key role in muscle movement. Tropomyosin regulates the interaction between actin and myosin during

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muscle contraction. Collagen provides support and structure to fish muscle tissue. Sarcoplasmic proteins, including enzymes, structural proteins, and regulatory proteins, play diverse roles in muscle function and metabolism [25]. Hence, it is further confirmed that there were no cross-reactivity or false positives of the FP or blood from the FP itself due to the different anatomy of the proteins presented accordingly.

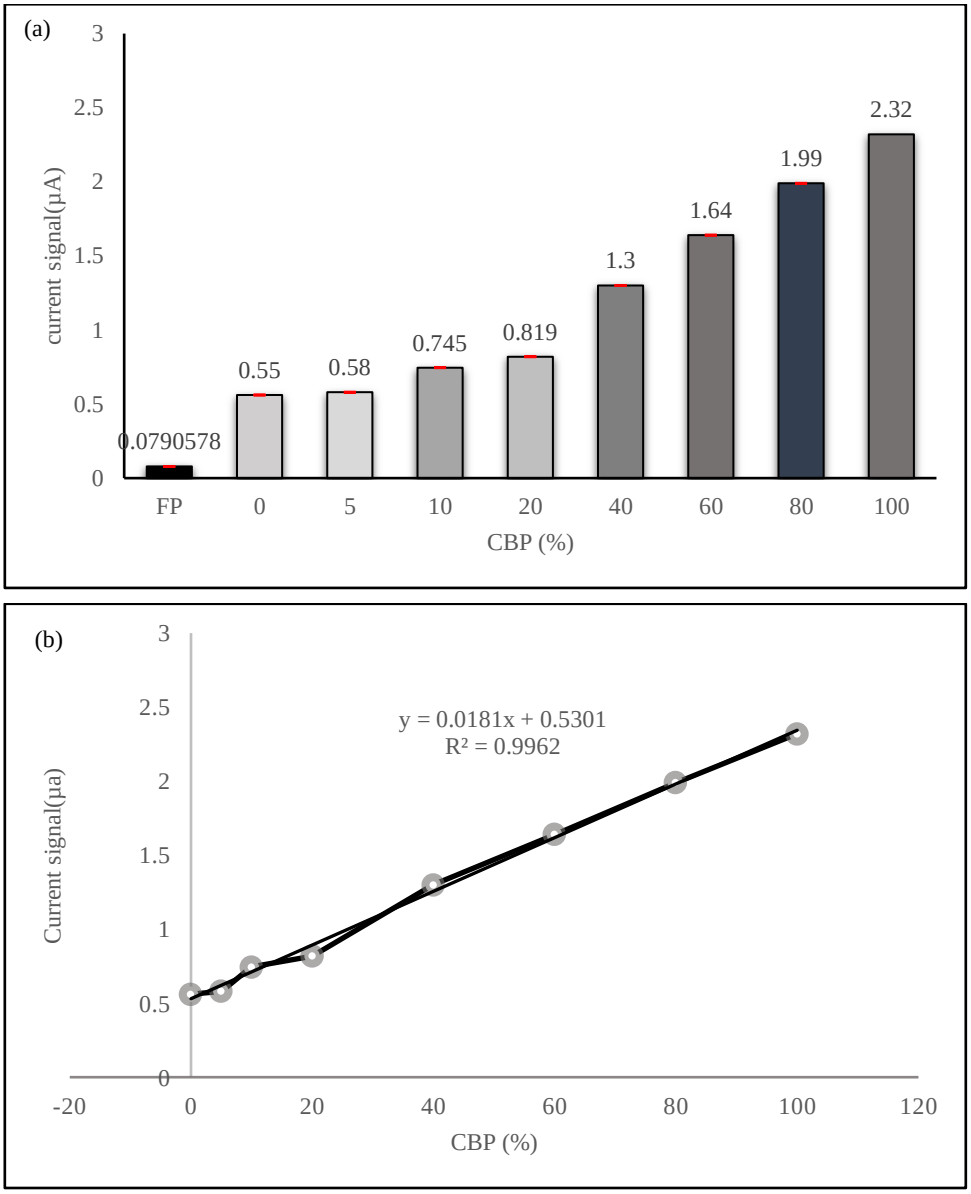

Fig. 3. (a) Detection sensitivity of the ApoA1-MBs-AuNPs immunocomplex in electrochemical immunosensor using serially diluted concentrations from extracted CBP and b) the corresponding linear range is depicted in the calibration plot. The error bars indicate the standard deviation of triplicate assays (n = 3).

Other literature has reported on the detection of ApoA1 using different platforms in other samples. A recent study demonstrated the utilisation of electrochemical aptasensor in human serum using gold nanorod

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(AuNR) and gold nanowire (AuNW) nanocomposites (AuNR/AuNW/CS) as electrode modifiers on a glassy carbon electrode (GCE) [26]. It was found that the detection limit of 0.04 pg mL1 and a wide linear range of 0.1 to 1000 pg mL1 for the detection of ApoA1 was due to the uses of purified ApoA1 purchased in human serum. Similar findings have been observed in [27] with complex employment of dialdehyde cellulose nanocrystal (DCNC) as a reducing and stabilizing agent, to propose an efficient method for generating stable silver nanoparticles (AgNPs). The immunosensor that was developed demonstrated enhanced sensitivity towards APO-A1, with a linear range spanning from 0.01 pg/mL to 1000 pg/mL and a limit of detection (LOD) of 2.20 fg/mL.Therefore, as both techniques use fabrication of the electrode modifiers, the similarity on the excellent sensitivity is reasonable with the focus on medical sections.

The research paper underscores the basic utilization of the AuNP developing an electrochemical immunosensor tailored for the precise detection of ApoA1 from extracted CBP, addressing a notable research gap within the current scientific literature. This whole platform demonstrated great potential and established immunosensors available to date for the detection of ApoAI in foods.

4. CONCLUSION

The combined merits of the utilization of AuNPs and NBs in performing in the developed electrochemical immunosensor platforms were explored in this preliminary study. Synthesised AuNPs showed uniformity in size with an average diameter of 15.20 ± 1.38 nm (n=100), with a calculated RSD value of 9.12%. An electrochemical immunosensor was successfully developed for the detection of ApoAI protein using AuNPs as an electrochemical label on SPCEs.AuNPs were quantified by subjecting the immunocomplex to a peroxidation process of high potential at 1.2 V for 330 s and immediately reduced and scanned by differential pulse voltammetry (DPASV). A linear relationship between reduction peak current signals and ApoAI concentration was obtained, with a detection limit of 5% (0.55 μA) of CBP with threshold value of 0.58 μA. To the best of our knowledge, this is the first study that uses AuNPs and MBs on carbon SPCEs for the detection of ApoAI from CBP, which is novel in foods.

It is recommended that further study should be conducted on the different types of surimi with purified ApoAI proteins extracted from CBP. The significant results of this study will enable regulatory bodies to facilitate the prompt identification on screening of fraud in the food sector, improve their oversight of food safety, and encourage immediate preventive measures to tackle the widespread problem of food adulteration.

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6. CONFLICT OF INTEREST STATEMENT

The authors agree that this research was conducted in the absence of any self-benefits, commercial or financial conflicts and declare the absence of conflicting interests with the funders.

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7. AUTHORS' CONTRIBUTIONS

Nurhazirah Azmi: Conceptualisation, data curation, formal analysis, investigation and writing-original draft; **Nur Izzati Gati**: Conceptualisation, methodology, and formal analysis; **Abdul Hadi Mahmud**: Conceptualisation, formal analysis, and validation; **Siti Aimi Sarah Zainal Abidin**: Conceptualisation, supervision, writing- review and editing, and validation; **Low Kim Fatt**: Conceptualisation, supervision, and editing, and validation; **Saiful Anuar Karsani**: Conceptualisation, supervision, editing, and validation.

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