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ORIGINAL PAPER

Development of an optical immunoassay based on peroxidase-mimicking Prussian blue nanoparticles and a label-free electrochemical immunosensor for accurate and sensitive quantification of milk species adulteration

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Abstract

In contrast to reported enzyme-based immunoassays, an enzyme-free immunoassay (optical and electrochemical) is presented here for the first time that can be used as point-of-need detection bioplatforms of bovine IgG as goat milk adulterant. In the first format, Prussian blue nanoparticles (PBNPs) were used as antibody catalytic labels in a competitive colorimetric microplate immunoassay. Absorbance measurement was performed photometrically at 450 nm. After in-depth optimization, excellent sensitivity was achieved (0.01% cow/goat volume ratio), which is 100 times lower than the limit allowed by the European legislation (EL) (1% v/v), thanks to the high catalytic activity of PBNPs compared with natural peroxidase. Moreover, the antibody-PBNPs bioconjugates showed excellent stability over 4 weeks (> 94% of the initial response) confirming the successful anchoring of the antibodies to the surface of the PBNPs. On the other hand, a label-free voltammetric immunoassay for the detection of bovine IgG was developed. The sensing principle was based on the hindrance of charge transfer between ferri-ferrocyanide redox couple and the screen-printed gold electrodes modified with bovine IgG antibody. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were used to characterize the step-by-step modification of the electrode surface. Under optimal conditions, this single-step electrochemical analysis achieved a high sensitivity of 0.1% (cow/goat) when monitoring the ferrocyanide oxidation at + 0.092 V (vs. Ag/AgCl) using differential pulse voltammetry (DPV). The selectivity of the developed immunoassays was evaluated for different species of milk of similar composition, and both immunoassays exhibited a selective response only to bovine IgG. Unlike conventional immunoassays, the developed enzyme-free immunoassays have many attractive features for the detection of milk adulteration, whether they are used in quality control laboratories for routine milk analysis (optical immunoassay) or at on-site checkpoints (electrochemical immunoassay) using wireless electrochemical detectors. The sensors provide high sensitivity (≤ 0.1%), excellent precision (RSD < 6%), low cost (no enzyme is required) and ease of operation, including handling of milk samples.

Keywords Milk species adulteration · Enzyme-free immunoassay · Prussian blue nanoparticles · Peroxidase-like activity · Label-free detection · Electrochemical biosensor · Differential pulse voltammetry

Introduction

With the depletion of the world market's resources due to the rapid increase in the world's population, along with the ease of international trade, economically motivated adulteration has found a way to meet the increased needs of consumers and has therefore spread to all food sectors (milk, meat, oil, honey and spices) [1]. Fraudsters (producers, processors or

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retailers) are constantly looking for new and tricky ways to counterfeit products in order to reduce production costs and seek economic profit. Milk is a nutrient-rich liquid food that is widely consumed by all age groups because of its valuable proteins, fats, carbohydrates, vitamins and minerals [2]. However, adulteration of milk and related products accounted for 18% of food product adulteration issues between 1980 and 2010 [3]. Milk adulteration involves the addition of toxic chemicals such as melamine and urea, as well as the addition of reconstituted milk powder, sugar, starch and flour. Adulteration of milk also includes diluting it with water, removing beneficial fats and substituting milk of one species with milk of another species [4–9]. All of these fraudulent tactics result in less nutritious and lower quality dairy products, deceiving consumers and potentially threatening their lives [10].

A high-profile case is the adulteration of cow's milk into goat's milk thanks to its high digestibility, low lactose content and limited seasonal production [11]. To ascertain goat milk authenticity, conventional tedious methods including spectroscopy [12], chromatography [13], electrophoresis [14] and fluorescence [15] have been successfully employed. In addition, molecular biology techniques based on DNA analysis, although they are time-consuming and difficult to implement in limited source areas, have attracted great interest because of their ability to detect adulteration in heat-treated dairy products [16]. A particular emphasis is given to immunological methods, namely enzyme linked-immunosorbent assay (ELISA) [17–19], in addition to lateral flow immunoassay (LFIA) [20, 21] and immunosensor [22] due to their excellent specificity, sensitivity, reproducibility and affordability compared to the above-mentioned methods. Although these methods have shown great merit in detecting low levels of adulteration in milk, they all require the use of a detection antibody which is usually labeled with natural horseradish peroxidase (HRP). Such natural enzymes require specific physiological conditions of pH and temperature to maintain their catalytic activity, they are not very stable, and their synthesis, isolation and purification are very expensive [23, 24]. Therefore, enzyme-based immunoassays are somewhat demanding and not suitable for decentralized applications and low-cost devices.

To overcome these shortcomings, enzyme-like nanomaterials (also called nanozymes) have been developed as alternative to natural enzymes. Nanozymes with intrinsic enzyme-like activity possess obvious advantages over natural enzymes, such as simple synthesis methods, low-cost, easy large-scale production, high catalytic activity and excellent stability under various pH (3–12) and temperature (4–90 °C) conditions [25–28]. To date, various nanomaterials with inherent peroxidase catalytic properties have been evaluated as artificial peroxidase, including carbon based-materials (graphene oxide nanosheets, carbon nanotubes, and fullerene), noble metals nanoparticles (gold nanoparticles), metal oxides (CeO_2, CuO, Co_3O_4, MnO_2), metal–organic frameworks (MOF), metal sulfides (MoS_2), hemin micelles, palladium nanoparticles, magnetite nanoparticles and Prussian blue nanoparticles (PBNPs) [29]. PBNPs with chemical formula $Fe_4[Fe(CN)_6]_3$ are composed of alternating ferric and ferrous irons coordinated with cyanides [30]. The high peroxidase-like activity of these artificial enzymes is assigned to the presence of Fe^{3+} and Fe^{2+} in the core of PBNPs [31, 32]. It was reported that PBNPs were able to catalyze the oxidation of various typical peroxidase substrates such as diammonium salt of 2, 2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), diazo-aminobenzene (DAB), o-phenylenediamine (OPD), di-azo-aminobenzene (DAB) and 3,3′,5,5′-tetramethylbenzidine (TMB) [30]. Therefore, PBNPs have sufficient merit to replace natural peroxidase in immunological assays.

On the other hand, enzyme-free biosensors based on the use of redox probes can also be explored to develop rapid and inexpensive in situ monitoring devices for milk authentication. Label-free electrochemical immunosensors do not require any label, neither enzymes nor nanozymes; they are based on the study of charge transfer changes recorded at the surface of the electrode as a consequence of antibody–antigen interactions [33]. To date, only one immunosensor has been reported for the detection of adulteration in milk based on a sandwich configuration using an antibody pair (unconjugated and conjugated with horseradish peroxidase enzyme) [22]. Therefore, additional efforts are needed to implement decentralized devices that today's society requires to ensure on-site control of food authenticity.

In the present work, two enzyme-free immunoassays (optical and electrochemical) were developed for the detection of bovine IgG as a biomarker of goat milk adulteration. In the first format, a competitive microplate immunoassay based on the use of PBNPs nanozymes with inherent peroxidase-like activity was developed for the sensitive detection of goat milk adulteration. To the best of our knowledge, this is the first time that nanozymes, used as antibody labels, are combined with an immunoassay for milk quality assessment. The affinity interaction between antibodies and their specific analytes was photometrically monitored at 450 nm in the presence of TMB. In addition, a label-free electrochemical immunosensor for the detection of bovine IgG was constructed as a second inexpensive, simple, and sensitive enzyme-free immunosensing platform. In this case, the hindrance of charge transfer between the ferri-ferrocyanide redox couple and the electrode surface witnessed the successful binding of the analyte to the antibody immobilized on screen-printed gold electrodes, as confirmed by differential pulse voltammetry (DPV) measurements at + 0.092 V (vs. Ag/AgCl). Both developed immunoassays were optimized carefully and applied to the detection of bovine IgG in

standard bovine IgG solutions, cow milk samples and binary mixtures of goat and cow milk.

Experimental section

Chemicals and reagents

IgG from bovine serum (referred to as standard bovine IgG), anti-bovine IgG (whole molecule) antibody produced in rabbit, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), cysteamine, glutaraldehyde solution (25%), 3,3′,5,5′-Tetramethylbenzidine powder and 3,3′,5,5′-tetramethylbenzidine (TMB) ready-to-use supersensitive liquid chromogenic substrate were purchased from Merck, Germany. Potassium hexacyanoferrate (II) ($K_4[Fe(CN)_6]$), potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$), iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), citric acid, potassium chloride (KCl), sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), potassium phosphate monobasic (KH_2PO_4) and sucrose were bought from Sigma-Aldrich, Germany. Bovine serum albumin (BSA), 2-(N-morpholino) ethanesulfonic acid (MES) and Tween-20 were obtained from VWR Life Science AMRESCO, Ireland. Acetone, ethanol, dimethyl sulfoxide (DMSO), hydrogen peroxide (H_2O_2) and sulfuric acid (H_2SO_4) were of analytical grade and used as received without further purification. Screen-printed gold electrodes (SPGEs) were obtained from BVT Technologies, Czech Republic. They consist of gold as the working electrode (Ø = 1 mm), graphite as the counter electrode and silver chloride as the reference electrode.

Experimental instructions

Phosphate buffer (PB, pH 7.4) made of 0.05 M Na_2HPO_4 and 0.05 M NaH_2PO_4 was used as PBNPs working buffer. Phosphate-buffered saline (PBS, pH 7.4) made of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 was used for the preparation of biomolecules and glutaraldehyde solutions. PBS supplemented with 0.05% Tween-20 ($PBST_{0.05\%}$) was used as the washing buffer in all experiments. TMB stock solution (1 mg/mL) was prepared in DMSO, and the working concentrations were made by diluting TMB stock solution with acetate buffer pH 5.0. The experimental data shown in the whole manuscript are the mean of at least three replicate measurements (mean ± SD, n = 3).

Apparatus

An automated ELx800 absorbance microplate reader (BioTek Instruments, Winooski, USA) was used to measure the optical density of the developed colorimetric product at 450 nm. Data were evaluated with Gen5 software. The UV–visible absorption spectra were taken on a spectrophotometer (JENWAY Model 6850 UV–visible double beam spectrophotometer) in the wavelength range of 400–900 nm with a slight width of 2 nm. Electrochemical measurements were carried out with a PalmSens 3 potentiostat (PalmSens BV, Houten, The Netherlands) controlled by a PC running PSTrace 5.0 software. OriginPro8 was used as software for data analysis, processing, and graphing. Eppendorf high-speed micro-centrifuge DLAB (DLAB Scientific Co., Ltd., China) was used for nanoparticles separation.

Preparation of antibody-Prussian blue conjugates (Ab-PBNPs)

Prussian blue nanoparticles (PBNPs) with an average size ranging from 15 to 24 nm were synthesized according to previous work [34] with slight modifications (details are described in Supplementary Material section). Afterward, 1 mL PBNPs dispersion was washed using successive centrifuge cycles (13000 rpm/30 min) in the presence of an equal volume of acetone, followed by solubilizing the blue pellet in 0.05 M phosphate buffer (PB, pH 7.4). The principle of conjugation between antibody (Ab) and PBNPs was based on EDC/NHS coupling method. In short, 4 mg of EDC and 8 mg of NHS prepared in 0.1 M MES buffer (pH 5) were added to 1 mL of PBNPs to activate the surface of citric acid-coated PBNPs before Ab grafting. The activation step was maintained for 1 h at 37 °C. Afterward, an adequate volume of anti-bovine IgG antibody was dropped into the above solution and incubated for 2 h at 37 °C. Then, 5% BSA was used to block the remaining sites of the Prussian blue nanoparticles. After centrifuge cycles, the resulting bioconjugates (anti-bovine IgG/PBNPs) were stored in PBS containing 1% BSA and 1% sucrose and kept at 4 °C until use.

Preparation of the competitive colorimetric immunoassay

The first step in the development of the competitive colorimetric immunoassay involved the coating of polystyrene microplate wells with 100 µL of bovine serum IgG (bovine IgG). Next, the nonspecific binding sites were blocked with 5% BSA-$PBST_{0.05\%}$ (200 µL/well) for 1 h at 4 °C to avoid nonspecific adsorption. The modified microplates can be stored at 4 °C until use, by covering the wells with trehalose which allows maintaining long stability of the biomolecules, as demonstrated in our previous work [35]. Meanwhile, a mixture of bovine IgG-loaded samples and anti-bovine IgG/PBNPs bioconjugates were prepared at a ratio of 1:1 (v/v); then, 100 µL of the above mixture was introduced into the wells. The competition between bovine IgG bound in the

microplate wells and bovine IgG-loaded samples was maintained for 5 min. Subsequently, the peroxidase-like activity of PBNPs was revealed by adding 100 µL of an appropriate concentration of TMB/H_2O_2. After 20 min of incubation in the dark, 50 µL of 0.1 M sulfuric acid was added to fully oxidize the obtained colorimetric product whose optical density was measured at 450 nm using a microplate reader. After each modification step, the microplate wells were thoroughly washed five times with the washing buffer.

Preparation of the label-free electrochemical immunosensor

Prior to modification, screen-printed gold electrodes (SPGEs) were activated in a 0.5 M sulfuric acid solution by running 6 scans between -0.2 V and 1.6 V (vs. Ag/AgCl) at a scan rate of 100 $mV.s^{-1}$ until a reproducible cyclic voltammogram was obtained. Once washed, the clean bare SPGE were coated with a thiol self-assembled monolayer (SAM) by dropping 1 µL of ethanolic cysteamine solution on the surface of SPGE for 4 h at room temperature. Absolute ethanol and water were used to wash away the physically adsorbed cysteamine. For the biomodification, glutaraldehyde was used as an effective protein crosslinker to activate the terminal amino groups of cysteamine-modified gold (Cyst/SPGE) electrodes. Accordingly, 1 µL of 2.5% glutaraldehyde solution was dropped on the surface of Cyst/SPGE and left at room temperature for 15 min. After excessive washing, 1 µL of anti-bovine IgG was added to the activated surface and incubated for 1 h at 4 °C to achieve complete binding. The electrodes were thoroughly washed with $PBST_{0.05\%}$ in order to remove any possible unbounded antibody molecules and denoted as Anti-IgG/Cyst/SPGE. After antibody immobilization, the unoccupied sites on Anti-IgG/Cyst/SPGE surface were blocked using 5% BSA for 30 min incubation at 4 °C to prevent the non-specific adsorption of biomolecules. Finally, 1 µL of bovine IgG was deposited on the surface of the prepared immunosensor, and left to react for 1 h at 4 °C (denoted IgG/Anti-IgG/Cyst/SPGE). The immunosensor was washed with $PBST_{0.05\%}$ before the label-free detection of bovine IgG using DPV measurements.

Electrochemical measurements

The electrochemical characterization of the developed immunosensor was performed by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS), in 5 mM $[Fe(CN)_6]^{3-/4-}$ redox probe in the presence of 0.1 M KCl. CV measurements were carried out at a potential window of − 0.4 V and + 0.5 V and at a scan rate of 50 $mV.s^{-1}$. EIS measurements were conducted under an applied potential of + 0.2 V, with an AC voltage amplitude of 0.1 V and a voltage frequency from 5 Hz to 50 kHz.

Label-free detection of the analyte (bovine IgG) was accomplished in 5 mM $[Fe(CN)_6]^{3-/4-}$ redox probe containing 0.1 M KCl, using differential pulse voltammetry (DPV). The parameters for DPV measurements are initial potential -0.4 V, end potential + 0.6 V, step potential 0.005 V, pulse potential 0.1 V, pulse time 0.2 s and scan rate 0.01 $V.s^{-1}$.

Milk samples preparation

Casein is the dominant protein in milk, representing 80% (24–29 g/L) of total milk protein. Its isoelectric pH (pI) is between 4.6 and 4.7. In contrast, whey proteins represent only 20% of total milk proteins including β-lactoglobulin (2–4 g/L; pI~5.1), α-lactalbumin (1.2–1.5 g/L; pI~4.2), bovine serum albumin (< 1 g/L; pI~4.7) and immunoglobulins (IgG, IgA, IgM) with a pI of 6.5 to 8.3 and a concentration of below 1 g/L [36]. Therefore, acidification of milk samples (initial pH~6.1) with 2 M hydrochloric acid was conducted until reaching a pH equal to 4.6. This will ensure maximum precipitation of casein as well as further milk protein fractions, except for the immunoglobulin fraction. Finally, the precipitate was filtered using a syringe filter (Ø = 0.45 µm) which also removed the fatty phase, and the resulting filtrate was stored at 4 °C until use. The milk samples were subjected to acidification immediately after milking to ensure authenticity.

Selectivity study of the developed immunoassays

The study of possible cross-reactivity was performed by comparing the performance of the immunoassays developed against the most similar milk species, namely cow, goat and sheep milks. Milk samples from goat and cow were used immediately after milking to ensure their genuineness before being analyzed. Thus, goat's milk was authentic without any trace of cow's milk, which was itself authentic. Milk samples were handled separately to avoid any unexpected cross-reactivity. IgG was collected from each milk species as described in "Milk samples preparation" and then analyzed by the developed immunoassays.

Results and discussion

Bovine IgG as a biomarker of goat milk adulteration

Casein and β-lactoglobulin are the most detected milk adulterants due to their high abundance [19, 21, 37, 38]. However, the detection of these biomarkers is not always accurate because the type of casein fraction (α or β) and the amount of β-lactoglobulin differ among milk species and they are sensitive to proteolysis [39]. Therefore, immunoglobulins that are less sensitive to proteolysis have been used

as biomarkers of milk adulteration [17, 22, 40–42]. In the present work, immunoglobulin G (IgG), which forms 80% of total immunoglobulins [43], was detected as an adulterant in goat milk.

Herein, IgG (pI 6.5–8.3) was separated from bulk milk proteins (initial pH 6.1) by removing the maximum of casein as well as other milk proteins that may interfere with IgG detection. Ideally, all the casein in milk sample would be precipitated simply by adding enough acid to bring the pH value to 4.6 [44]. Therefore, 1.4 mL of 2 M HCl solution was added to 1 mL of milk to bring the pH of milk to 4.6. It was observed that at a pH of about 5.3, casein begins to precipitate out of solution, and at a pH of about 4.6, maximum casein precipitation occurs. In addition, when the pH of milk is lowered from 6.1 to 4.6, it passes through the pI of other milk proteins (β-lactoglobulin, bovine serum albumin) which in turn go into the insoluble state and undergo precipitation. After the acidification step, milk was filtered through a syringe filter (Ø = 0.45 µm) to get rid of the precipitated proteins and the fat fraction. The filtrate-loaded IgG was stored at 4 °C for until use. This simple pretreatment step allowed the separation of IgG from bulk milk proteins due to its isoelectric pH (pI) which is far different from that of casein. In addition, this simple method enables the removal of interfering milk proteins that can be found in diluted or non-treated milk samples. The proposed method is suitable for decentralized applications since it requires only one reagent and no sophisticated equipment is needed such as a centrifuge.

Nanozyme preparation and functionalization with antibody

PBNPs peroxidase-like activity

PBNPs were synthesized by mixing equimolar aqueous solutions of $FeCl_3$ and $K_4[Fe(CN)_6]$ in the presence of citric acid, which is the most reproducible synthesis method [45]. As shown in Fig. 1a, a maximum absorption peak was observed at 708 nm confirming the co-precipitation of ferric ions (Fe^{2+}/Fe^{3+}) to a blue colloidal suspension in the presence of citric acid. The use of citric acid as capping agent did not only mediate nucleation between $FeCl_3$ and $K_4[Fe(CN)_6]$, but also resulted in a very stable suspension of PBNPs that remain perfectly dispersed in the working solution for at least 7 months, the minimum period examined.

The peroxidase-like activity of the as prepared PBNPs was investigated by evaluating their catalytic activity toward the oxidation of TMB supersensitive liquid chromogenic substrate. TMB is the commonly used natural peroxidase substrate which shows two absorption peaks at 369 nm and 652 nm when oxidized in the presence of hydrogen peroxide [46]. Thus, the oxidation of TMB in the presence of PBNPs presents a well-controlled reaction that can be used to analyze the catalytic activity of the prepared nanozymes. As shown in Fig. 1b, when PBNPs and TMB were not mixed, no change in the absorbance values was observed during at least 10 min, suggesting the high stability of both PBNPs and TMB. In contrast, when PBNPs were mixed with TMB, a sharp and quick increase in the absorbance values at 562 nm was observed, indicating the fast catalytic oxidation of TMB in the presence of PBNPs. These results confirm that PBNPs possess an intrinsic peroxidase-like activity which is mainly ascribed to Fe^{2+}/Fe^{3+} present in the core of PBNPs. The peroxidase-like activity of PBNPs was further demonstrated by conducting kinetic assays in the presence of different substrate and nanozyme dilutions. As displayed in Fig. S1a and Fig. S1b, the catalytic behavior of PBNPs was found to be dependent on the dilution factor of TMB and PBNPs indicating that these nanozymes can behave like natural enzyme and thus can be used as artificial peroxidase.

Ab-PBNPs bioconjugates preparation

In a typical enzyme-free immunoassay, called NLISA (nanozyme-linked immunosorbent assay), PBNPs used to replace the conventional HRP, were covalently linked to the detector antibody (anti-bovine IgG) by various EDC-based immobilization strategies, as described in "Preparation of antibody-Prussian blue conjugates (Ab-PBNPs)". Generally, EDC is a zero-length carboxyl- and amine-reactive crosslinking agent that initially reacts with a carboxyl group to form an O-acylisourea intermediate [47]. Conjugation efficiency was assessed by a direct NLISA (the protocol is described in Supplementary Material section), comparing the ability of each bioconjugate (Ab-PBNPs) to distinguish between 0 µg/mL (negative control) and 10 µg/mL (positive control) of bovine IgG. Fig. S2a represents the absorbance values recorded for 0 and 10 µg/mL bovine IgG after affinity interaction with Ab-PBNPs bioconjugates obtained by various functionalization strategies, including only EDC, EDC/NHS powdered in solution of PBNPs and EDC/NHS dissolved in MES buffer before mixing with PBNPs. As can be seen in Fig. S2a (orange histogram), the absorbance values obtained with 0 and 10 µg/mL are almost the same, indicating a poor anchoring density of Ab on PBNPs using EDC alone. In addition, during the functionalization step, PBNPs underwent aggregation over time which can be explained by the release of the highly unstable isourea by-product [48]. In the second approach, NHS was added to EDC to form a semi-stable amine-reactive NHS ester, which is more stable than the isourea by-product. As shown in Fig. S2a (purple histogram), an obvious difference between 0 and 10 µg/mL was observed confirming the successful anchoring of Ab on PBNPs. However, the stability of the obtained Ab-PBNPs was not maintained during functionalization steps and the

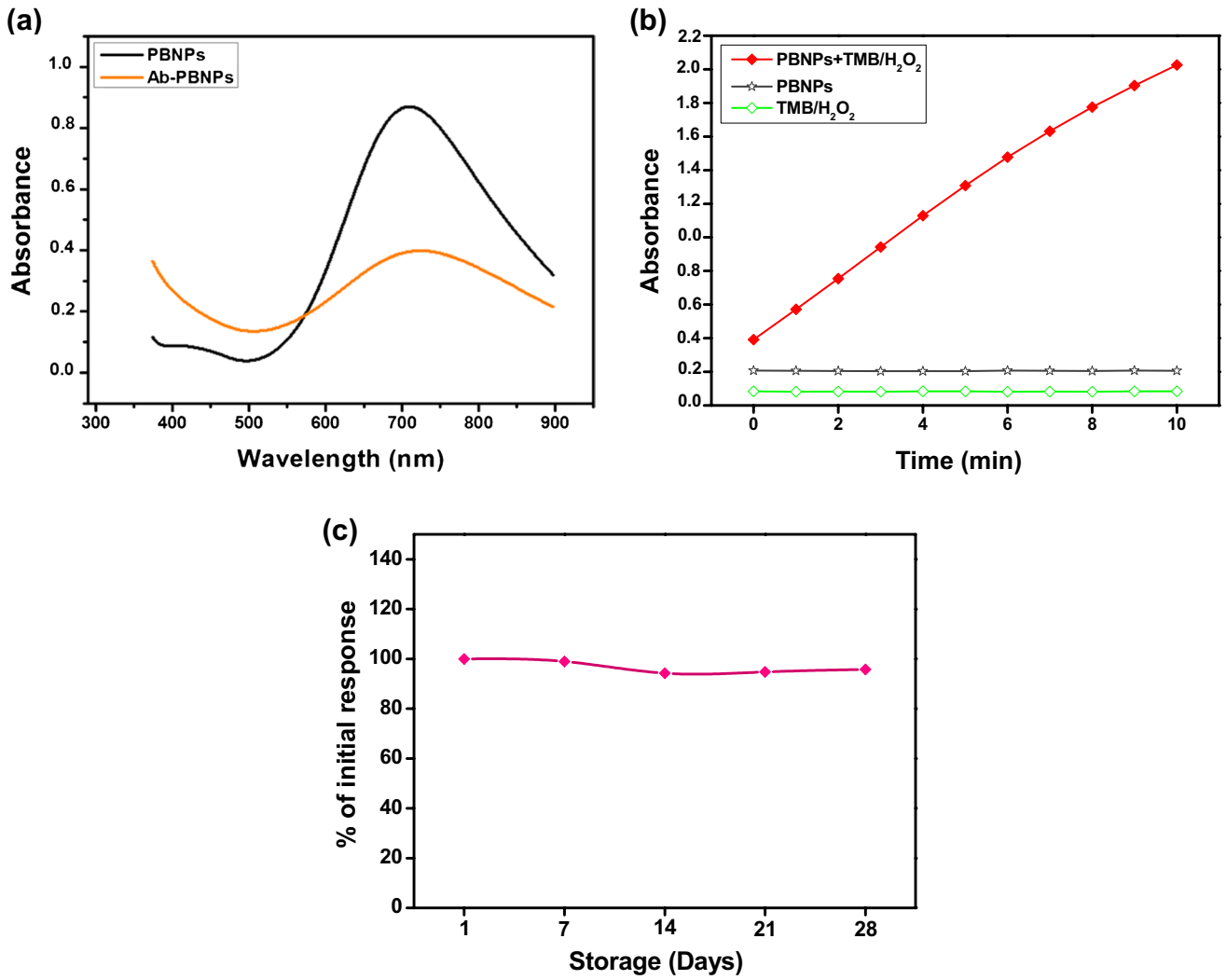
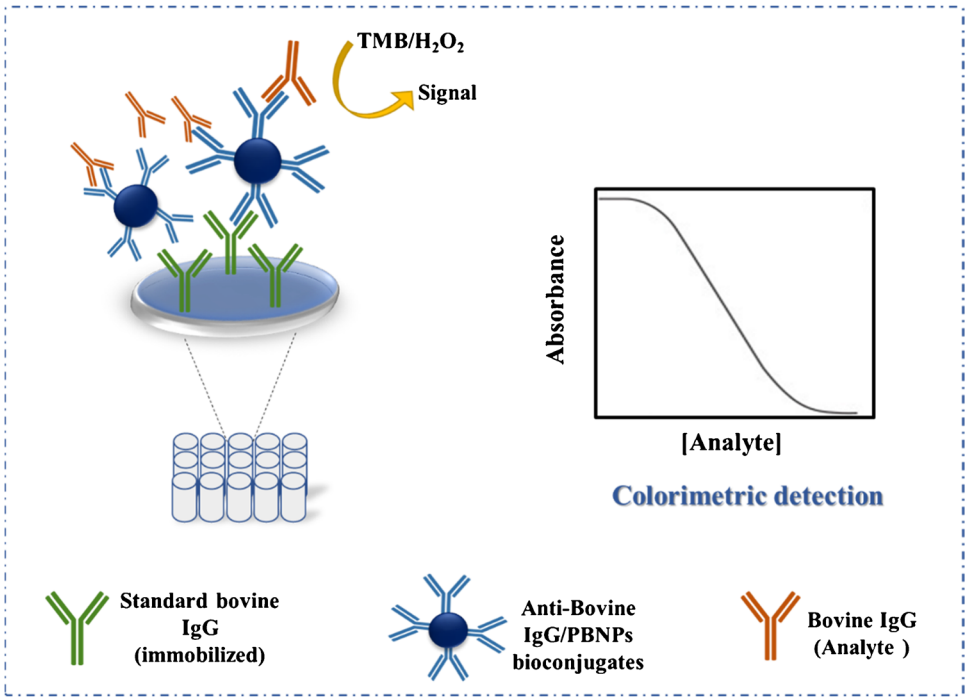


Fig. 1 **a** Typical UV–Vis absorbance spectra of the synthesized Prussian blue nanoparticles before and after conjugation with bovine IgG antibody, **b** UV–Vis absorbance–time curves of the TMB–H_2O_2 system under different conditions, **c** stability assessment of the prepared Ab-PBNPs bioconjugates when stored at 4 °C for up to 28 days. Data are mean ± SD, n = 5

Ab-PBNPs underwent aggregation at the end of functionalization, which is due to the reduced stability over time (a few hours) of the amine-reactive NHS ester at pH 7.4 [49]. Therefore, EDC and NHS were pre-dissolved in MES buffer (pH 5) before mixing with PBNPs. As can be concluded from Fig. S2a (pink histogram), a huge difference between the absorbance values were obtained, confirming the successful anchoring of more antibodies on PBNPs. Furthermore, no aggregation of Ab-PBNPs was observed throughout the assay, confirming the high stability of the bioconjugate achieved by the preparation of EDC/NHS in MES buffer, which is the optimal buffer solution for EDC/NHS chemistry, as the hydrolysis of NHS esters is much slower at low pH [50]. Thus, Ab immobilization on PBNPs through the use of EDC/NHS prepared in MES buffer was used for the next experiments. This was further corroborated by the orange spectra shown in Fig. 1a, since a slight shift in the absorption peak was observed from 708 to 717 nm, which can be attributed to the successful coating of the antibody on the surface of PBNPs.

In addition, the optimal amount of antibody to be conjugated with PBNPs along with other crucial parameters that may affect the immobilization of antibody on PBNPs were deeply studied. Optimizations results are reported in Fig. S2b and Table S1.

An important feature of a good bioconjugate is its stability for long-term storage, which ensures a long shelf life of biomolecules such as antibodies in the present study. The stability of Ab-PBNPs was investigated over 4 weeks. Bioconjugates were stored at 4 °C in PBS enriched with 1% BSA and 1% sucrose. The peroxidase-like activity of PBNPs and the ability of antibodies to recognize the antigen



Scheme 1 Schematic illustration of the developed colorimetric enzyme-free immunoassay based on the competitive detection of bovine IgG as goat milk adulteration biomarker

were assessed weekly by a NLISA. From the finding plotted in Fig. 1c, the response, as indicated by % of the initial response, tends to be almost constant, ranging from 94.2% to 95.7% over 4 weeks. This excellent stability was reached thanks to the thorough study of the conjugation of PBNPs with the antibody. From the results obtained, it can be deduced that the prepared bioconjugates can remain stable for long time up to one month.

Competitive colorimetric immunoassay for adulteration detection in milk

Principle of the competitive immunoassay and optimizations of the working variables

In the present work, an enzyme-free colorimetric immunoassay was developed for the sensitive detection of cow milk adulteration in goat milk. The developed colorimetric immunoassay is based on the competitive detection of bovine IgG by a limited amount of anti-bovine IgG antibody conjugated to Prussian blue nanoparticles (Ab-PBNPs). Following a competitive sensing strategy, increasing the analyte concentration (bovine IgG loaded samples) results in the binding of a large amount of Ab-PBNPs available for binding to immobilized antigens (bovine IgG used for coating), leading to low color development and thus low absorbance values, measured by a microplate reader. A schematic illustration of the competitive immunoassay is depicted in Scheme 1. Indeed, the concentration of the coating antigen is the most important parameter that directly affects a competitive immunoassay. Therefore, the amount of bovine IgG to be used for coating the microtiter plate wells was carefully studied by conducting different immunoassays at once in the presence of different coating IgG (1–10 µg/mL) and target IgG (0–10 µg/mL) concentrations. Moreover, different concentrations of peroxidase substrates (TMB/H_2O_2) were tested to achieve better sensitivity. According to the results summarized in Table S1, coating the microplate wells with 10 µg/mL of bovine IgG enables the competitive detection of different analyte concentrations in the presence of 0.5 mM TMB mixed with 125 mM H_2O_2.

Analytical performances

Once all parameters affecting the preparation of the competitive immunoassay were optimized, the analytical performance of the developed assay was assessed in the presence of an increasing concentration of standards bovine IgG prepared in PBS. Figure 2a displays the absorbance values obtained for different concentrations of bovine IgG. As we are conducting a competitive immunoassay, the absorbance value of the sample has a negative correlation with bovine IgG concentration confirming that the competition process was successfully carried out. The absorbance at 450 nm versus bovine IgG ($\mu g.mL^{-1}$) concentration in the range of 0.5–10 $\mu g.mL^{-1}$ was fitted by a linear regression $OD_{450nm} = -0.095 \log C + 0.373$; where C corresponds to the absorbance/$\mu g.mL^{-1}$, ($R^2 = 0.98$). The limit of detection (LOD) was found to be 0.034 $\mu g.mL^{-1}$, and the relative standard deviation (RSD) value calculated from the

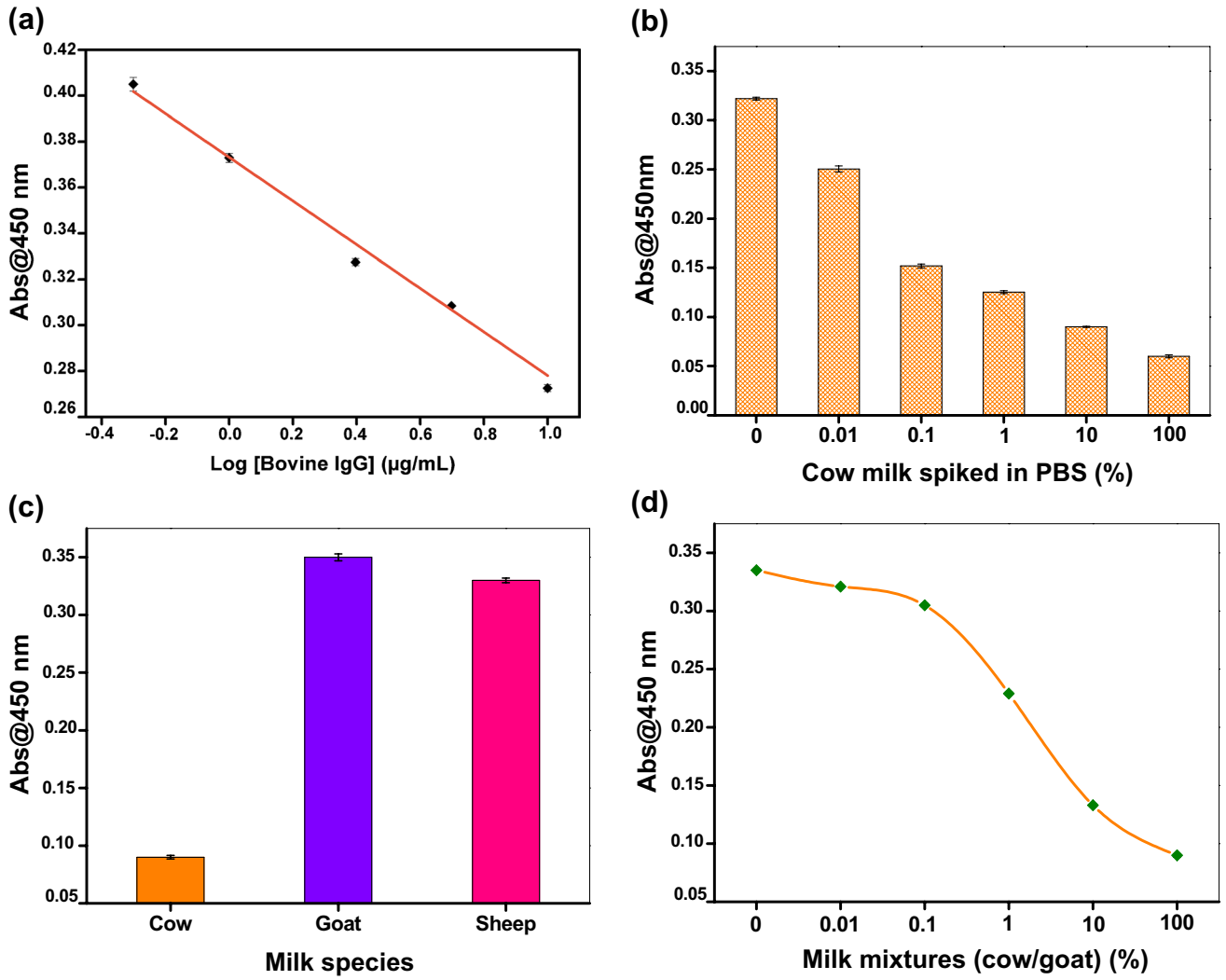


Fig. 2 **a** Colorimetric responses obtained by the developed competitive immunoassay corresponding to different concentrations of standard bovine IgG in spiked PBS solutions, **b** analysis of different amounts of undiluted cow milk spiked in PBS solutions, **c** selectivity assessment toward similar milk species (cow, goat, sheep) and **d** different levels of cow milk adulteration in goat milk ranging from 0 to 100%. Data are mean ± SD, n = 5

colorimetric responses of at least five immunoassays was equal to 1%, indicating the high reproducibility of the developed immunoassay when detecting bovine IgG in PBS spiked solutions.

Application to milk samples analysis

In view of demonstrating the potential application of the developed competitive immunoassay in the detection of bovine IgG as an adulterant in goat milk, the determination of this biomarker in a complex food matrix, namely milk, was mandatory. The ability to detect bovine IgG among other milk proteins will provide insight into the efficiency of milk sample treatment and allow the test to be applied to the analysis of milk mixtures from different species. Thus, PBS solutions were spiked with several amounts of undiluted cow milk and then analyzed by the competitive immunoassay. Figure 2b consists of the absorbance values obtained as a function of different cow milk contents in PBS solutions ranging from 0 to 100%. 0% reflects IgG-depleted PBS, which is the negative control, while 100% of cow milk was used as the positive control. The absorbance values obviously decrease when the bovine IgG content in the buffer solution rises from 0 to 100%. These findings confirm the ability of the developed competitive immunoassay to detect as low as 0.01% of bovine IgG in a complex milk matrix, with excellent reproducibility (RSD = 4.9%).

Selectivity study

To investigate the specificity of the prepared immunoassay, different milk species with similar composition were tested as complex matrices often associated with milk adulteration. Undiluted milk samples were handled separately in order to avoid any unexpected cross-reactivity. IgG from goat, cow and sheep's milks was obtained as described in "Milk samples preparation". Figure 2c shows the absorbance values obtained when analyzing different milk species. As can be seen, the absorbance value obtained with goat and sheep milk were almost the same, excluding cow milk which showed weak absorbance value, as expected, since we are dealing with a competitive assay. This negative cross-reactivity study confirms that the colorimetric immunoassay developed has great merit for application to the selective detection of bovine IgG as a biomarker of milk adulteration among other milk species.

Milk adulteration detection

To demonstrate the practical use of the developed immunoassay to detect adulteration of goat milk by cow milk, several cow/goat milk fortifications were prepared by spiking undiluted goat milk samples with undiluted cow milk from 0.01% to 10% (v/v). 0% of adulteration means that the sample is composed only of goat's milk and is authentic, while a 100% of adulteration indicates that goat's milk was completely substituted by cow's milk. Figure 2d reports the absorbance values recorded in the analytical range mentioned above. As can be seen, the absorbance values decrease competitively when the concentration of cow milk increases from 0 to 100% in the milk mixtures, which confirms that the developed immunoassay is able to successfully detect 0.01% (v/v) cow milk adulteration in goat milk with high reproducibility (RSD = 1.64%). It is noteworthy that the detected level of adulteration is 100 times lower than the level allowed by the European Community (EC), 1.0% (v/v), according to the European Community (EC) Regulation (No. 273/2008) [41, 51]. Compared to previously reported immunoassays aimed at the detection of cow milk adulteration in goat milk (Table 1), the proposed analytical method encompasses several features: (1) The single-step IgG isolation by milk acidification is not only fast and very simple, but it also removed a very important part of milk proteins that would interfere with IgG detection. Thus, this rapid and one-reagent treatment of milk samples significantly reduced the total time of the assay, (2) the use of PBNPs as an artificial peroxidase instead of natural peroxidase for the development of highly stable bioconjugates (antibody-PBNPs) provides an enzyme-free, inexpensive and robust immunoassay to check milk quality in limited-sources settings, and (3) the greatest advantage of using nanozymes is the improved sensitivity of bovine IgG detection, which resulted in a very low limit of quantification (0.01% v/v) despite the low content of IgG in whey. All these features make the proposed colorimetric immunoassay a powerful tool for milk authenticity control, as it is highly sensitive, rapid, stable and inexpensive in all its steps compared to existing immunoassays. In addition, the proposed method can be extended to the identification of other milk adulterants by changing only the antibody involved.

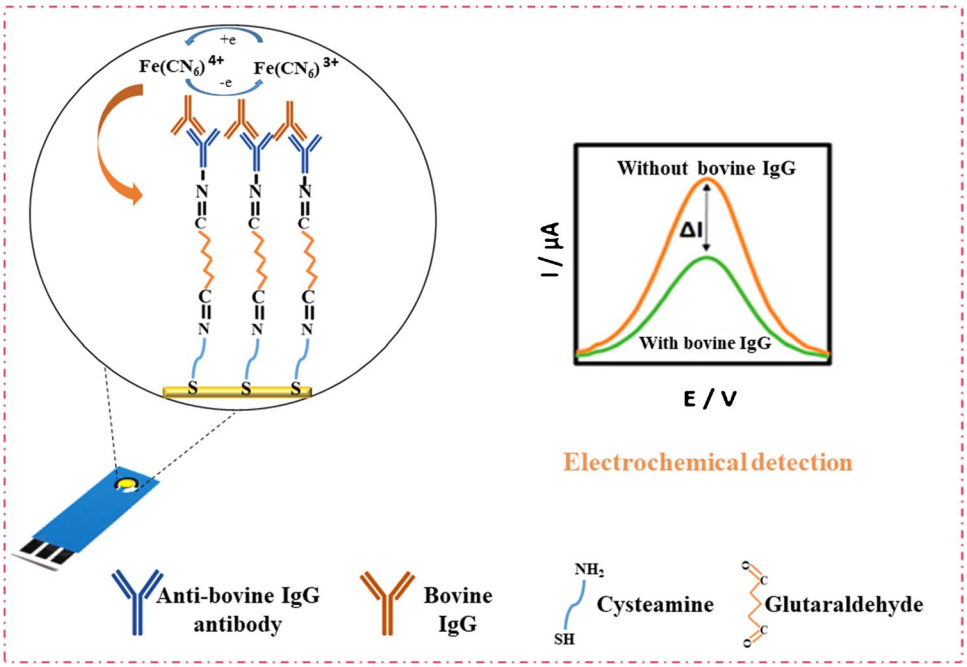
Label-free electrochemical immunoassay for adulteration detection in milk

Immunosensor construction and optimizations

In the present work, two different enzyme-free immunoassays were developed as simple, sensitive, selective, and inexpensive devices for goat milk authenticity testing. Regarding the electrochemical immunoassay, it is based on the use of a

Table 1 Comparative table of different immunoassay formats developed for the detection of cow milk adulteration in goat milk

Immunoassay	Adulterant	Antibody label	Detection limit (milk mixtures)	Signal readout	Ref
Indirect ELISA	Bovine β-casein	Peroxidase (HRP)	2% (v/v)	Colorimetry	[19]
Competitive ELISA	Bovine casein	Peroxidase (HRP)	1% (v/v)	Colorimetry	[38]
Competitive lateral flow immunoassay	Bovine casein	Colloidal gold nanoparticles	0.07% (v/v)	Colorimetry	[21]
Indirect competitive ELISA	Bovine IgG	Peroxidase (HRP)	0.1% (v/v)	Colorimetry	[40]
Sandwich immunosensor	Bovine IgG	Peroxidase (HRP)	0.1% (v/v)	Amperometry	[22]
Indirect competitive ELISA	β-lactoglobulin	Alkaline phosphatase	0.1% (v/v)	Colorimetry	[52]
Competitive NLISA	Bovine IgG	Prussian blue nanoparticles (PBNPs)	0.01% (v/v)	Colorimetry	Present work
Label-free immunosensor	Bovine IgG	Label-free	0.1% (v/v)	Differential Pulse Voltammetry (DPV)	Present work



Scheme 2 Schematic illustration of the developed electrochemical immunosensor based on the label-free detection of bovine IgG as goat milk adulterant

label-free detection antibody immobilized on screen-printed gold electrodes through cysteamine/glutaraldehyde cross-linking. The affinity interaction of the target (bovine IgG) with its specific antibody leads to a hindrance of the charge transfer between ferri-ferrocyanide redox probe and the electrode, and thus to a decrease of current value when conducting DPV measurements. The schematic illustration of the developed immunosensor is depicted in Scheme 2**.** The experimental parameters that may affect the performance of the developed immunosensor such as cysteamine concentration, antibody dilution and BSA concentration were carefully optimized, as summarized in Table S2.

Electrochemical characterization of the immunosensor

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) are important methods to reveal electrochemical interactions at the electrode surface. Screen-printed gold electrodes (SPGEs) were characterized electrochemically by CV and EIS using 5 mM ferri-ferrocyanide in 0.1 M KCl. Figure 3a shows the cyclic voltammograms of the $[Fe(CN)_6]^{3-/4-}$ redox probe recorded for bare SPGE (curve a); cysteamine-modified electrode (Cyst/SPGE) (curve b), antibody immobilized on cysteamine-modified gold electrodes using glutaraldehyde (Anti-IgG/Cyst/SPGE) (curve c), and after target IgG binding (IgG/Anti-IgG/Cyst/SPGE) (curve d). As observed, the redox couple, $[Fe(CN)_6]^{3-/4-}$, shows a well-defined reversible redox cyclic voltammogram with anodic to cathodic current peak ratio of approximately one and peak-to-peak separation of 100 mV (curve a). The cysteamine SAM formed on SPGE, induces a further significant increase in the peak current (curve b) due to electrostatic interactions between the negative charge of $[Fe(CN)_6]^{3-/4-}$ and the positively charged amino-end groups of cysteamine, as reported previously [53]. However, the current intensity decreases greatly after antibody immobilization (curve c), due to hindrance of the charge transfer mediated by the successful anchoring of antibody on the electrode surface. A further decrease in the current and an increase in peak separation were also observed after the affinity interaction between bovine IgG antigens and immobilized antibodies (curve d).

The cyclic voltammetry results were further confirmed by EIS measurements. Indeed, the electron-transfer resistance is well known as a parameter for controlling the electron-transfer kinetics of the redox couple through the electrode surface, which is indicative of the blocking behavior between the redox couple and the electrode interface. Figure 3b illustrates the Nyquist plots for bare SPGE (curve a), cysteamine modified electrode (Cyst/SPGE) (curve b), antibody immobilization (Anti-IgG/Cyst/SPGE) (curve c), and after IgG affinity binding (IgG/Anti-IgG/Cyst/SPGE) (curve d). As expected, bare SPGE (curve a) and Cyst/SPGE (curve b) showed a fast electron-transfer process confirming the excellent conductivity of the electrode surface at these stages. But, antibody immobilization on the modified electrode increased the electron-transfer resistance (curve c), indicating that the antibodies were successfully immobilized and thus reduced the accessibility of the redox probe to the electrode. The resistance was extensively enlarged (curve d) when the analyte (bovine IgG) was incubated with the modified electrode. These results are in agreement with the

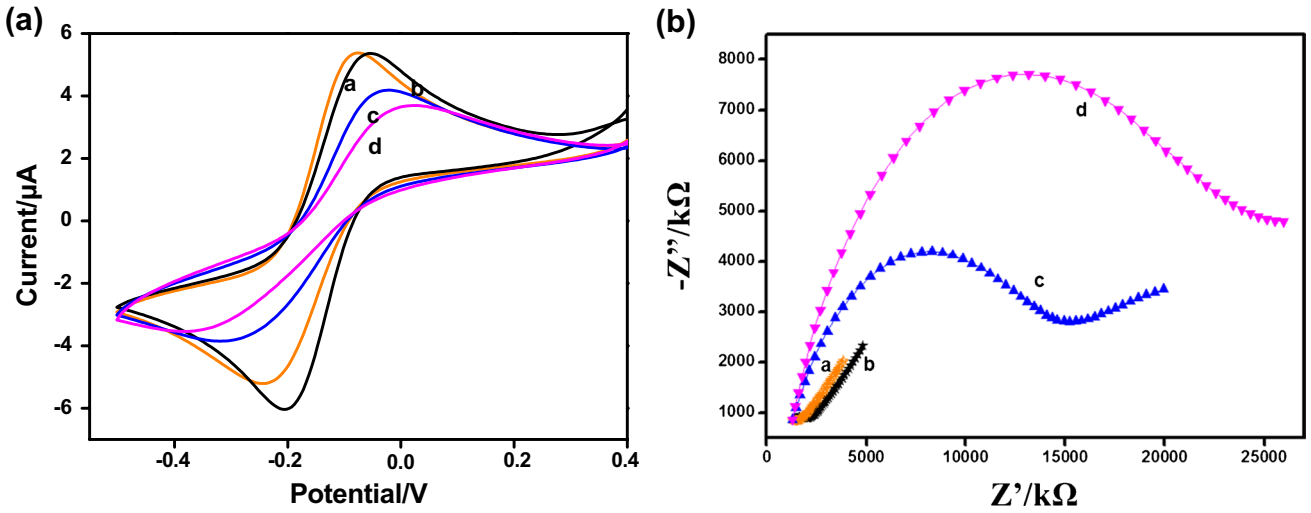


Fig. 3 **a** Cyclic voltammograms and **b** Nyquist plots of 5 mM $[Fe(CN)_6]^{3-/4-}$ redox probe prepared in 0.1 M KCl, recorded for bare SPGE (curve a); cysteamine-modified electrode (Cyst/SPGE) (curve b), antibody immobilized on cysteamine-modified gold electrodes using glutaraldehyde (Anti-IgG/Cyst/SPGE) (curve c), and after target IgG binding (IgG/Anti-IgG/Cyst/SPGE) (curve d)

changes observed in current by cyclic voltammetry (CV), both confirming the successful construction of the developed immunosensor.

Electrochemical detection of bovine IgG

Under the above-mentioned optimal conditions, the prepared immunosensor was incubated for 1 h at 4 °C with PBS solutions containing different standard bovine IgG concentrations ranging from 0 µg/mL to 10 µg/mL. The immunoaffinity interaction between antibody and the analyte was monitored by measuring the intensity of ferrocyanide oxidation at + 0.092 V (vs. Ag/AgCl) using DPV. Figure 4a displays the DPV voltammograms obtained for each bovine IgG concentration. As can be seen, the more the amount of analyte rises, the more the peak current intensities diminish in the studied analytical range. This can be explained by the strong binding between the antibody and the analyte, which constitutes a barrier resulting in a significant blockage of electron transfer between the redox probe and the electrode surface. The current intensity (I) versus bovine IgG concentration ($\mu g.mL^{-1}$) was fitted by a linear regression I (µA) = -0.82 log C + 1.24; where C corresponds to the current intensity/$\mu g.mL^{-1}$, ($R^2 = 0.99$). Accordingly, 0.054 µg.mL^{-1} of standard bovine IgG in PBS spiked solutions was successfully detected by the developed immunosensor (RSD = 5%).

Selectivity of the developed immunosensor

The selectivity of the proposed immunosensor was investigated by analyzing IgGs obtained from the most similar milk species (caprine, ovine and bovine). Figure 4b shows the DPV measurements recorded upon the separate incubation of the prepared immunosensors with different IgGs. In the case of caprine and ovine IgG, the current intensity is maximal for both analytes, whereas an obvious decrease of current intensity was found after binding with bovine IgG, indicating that there was no cross-reaction of the immunosensor with other milk species. These results demonstrated that the electron-transfer hindrance recorded is assigned to the selective interaction between the antibody and the target bovine IgG, therefore showing the ability of the developed immunosensor to detect bovine IgG adulteration in milk with excellent specificity.

Application to milk mixtures analysis

Herein, the developed immunosensor was applied to determine different cow milk adulteration levels in goat milk. Milk binary mixtures were prepared by fortifying undiluted goat milk samples with undiluted cow milk in the range of 0% to 100%. Besides, 0% adulteration (= 100% goat's milk) was tested as the negative control, while 100% adulteration (= 100% cow's milk) was tested as the positive control. As reported in Fig. 4c, the current intensities recorded by DPV decrease when the adulteration level of goat milk with cow milk increases from 0 to 100%. 0.1% cow milk adulteration in goat milk can be considered as the low level of adulteration that the proposed immunosensor was able to quantify successfully (RSD = 6%). The identified level of adulteration (0.1%) is very satisfactory as the level of cow milk accepted by the EC is 1% [51]. Moreover, compared with the previously reported immunosensor [22], the developed label-free

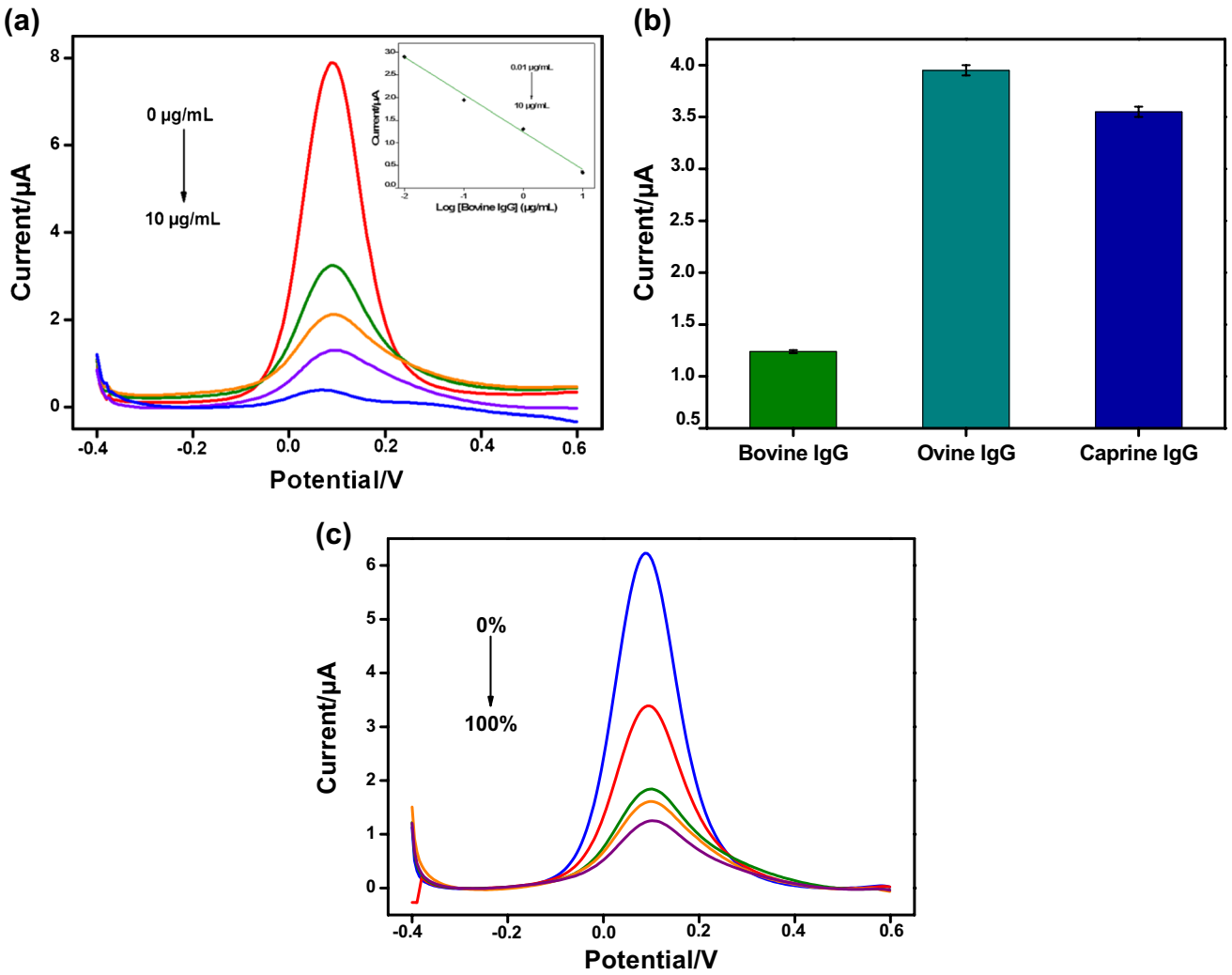


Fig. 4 **a** DPV responses obtained by the developed immunosensor for increasing concentrations of standard bovine IgG ranging from 0 to 10 µg/mL in PBS spiked solutions, **b** specificity test results of bovine IgG compared to ovine and caprine IgGs obtained from sheep and goat's milk, respectively, and **c** different levels of undiluted goat milk adulteration with undiluted cow milk ranging from 0 to 100%. Data are mean ± SD, n = 5

immunosensor is simpler, inexpensive, requires small volumes of reagent and reduced time compared to the sandwich immunosensor. Nevertheless, more attention should be devoted to biosensors as they are generally affordable, sensitive, specific, user-friendly, fast and robust, equipment-free, and deliverable to end-users (ASSURED), and thus perfectly suited for decentralized monitoring of milk adulteration.

Conclusion

Two enzyme-free immunoassays (optical and electrochemical) were constructed for centralized and decentralized detection of bovine IgG as adulterants in goat milk. The first format consists of a PBNPs nanozyme-based competitive immunoassay, while the second format deals with a label-free voltammetric immunosensor based on ferrocyanide oxidation.

The proposed biosensing platforms were extensively studied and applied to the determination of bovine IgG in three different matrices: standard IgG solutions, cow milk samples and binary mixtures of goat and cow milk. Under optimal conditions, excellent sensitivity and high specificity were achieved by the developed bioplatforms. The biosensing strategies described here are advantageous compared to already reported immunoassays in terms of sensitivity, cost, simplicity, precision, stability and possible portability. In addition, the proposed one-step treatment of milk samples allowed the removal of interfering milk proteins that can be found in diluted or untreated milk samples, and it is also suitable for decentralized applications since this single-step preparation requires only one

reagent and no sophisticated equipment such as a centrifuge is needed.

We believe that the developed enzyme-free immunoassays hold great promise for use in quality control laboratories for routine analysis of milk (optical immunoassay) or at on-site checkpoints (electrochemical immunoassay) using portable electrochemical detectors.

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Declarations

Declaration of competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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