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A novel electrochemical aptamer–antibody sandwich assay for lysozyme detection

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In this paper, we have reported a novel electrochemical aptamer–antibody based sandwich biosensor for the detection of lysozyme. In the sensing strategy, an anti-lysozyme aptamer was immobilized onto the carbon electrode surface by covalent binding *via* diazonium salt chemistry. After incubating with a target protein (lysozyme), a biotinylated antibody was used to complete the sandwich format. The subsequent additions of avidin-alkaline phosphatase as an enzyme label, and a 1-naphthyl phosphate substrate (1-NPP) allowed us to determine the concentration of lysozyme (Lys) *via* Differential Pulse Voltammetry (DPV) of the generated enzyme reaction product, 1-naphthol. Using this strategy, a wide detection range from 5 fM to 5 nM was obtained for a target lysozyme, with a detection limit of 4.3 fM. The control experiments were carried out by using bovine serum albumin (BSA), cytochrome *c* and casein. The results showed that the proposed biosensor had good specificity, stability and reproducibility for lysozyme analysis. In addition, the biosensor was applied for detecting lysozyme in spiked wine samples, and very good recovery rates were obtained in the range from 95.2 to 102.0% for lysozyme detection. This implies that the proposed sandwich biosensor is a promising analytical tool for the analysis of lysozyme in real samples.

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1. Introduction

Nowadays, use of biosensors for detection and quantification of proteins plays a vital role in research,¹ clinical applications² and food industry.³ Biosensors are analytical devices which incorporate a biomolecule to provide specific recognition for an analyte together with transduction technology to detect and quantify the binding taking place between the analyte and the detector molecule.⁴ Traditionally, enzymes, antibodies and proteins have been employed in biosensors as biorecognition species. Since their discovery in 1990,⁵ aptamers have attracted considerable attention in biosensor development.⁶ Aptamers are artificial DNA or RNA oligonucleotides selected *in vitro* which have the ability to bind to proteins, small molecules or even whole cells, with high affinity and specificity.⁷ They offer many advantages over antibodies such as relatively easy production, high affinity and specificity, easy chemical modifi-

cation and high stability.⁸ Thanks to these excellent properties, a number of aptamer-based sensors have been developed using different transducer techniques.^{9,10} Among different types of biosensors, electrochemical biosensing is of particular interest due to its remarkable sensitivity, simple instrumentation, fast response, low cost and portability.¹¹

Lysozyme (Lys) is a relatively small protein (14.3 kDa) consisting of only 129 amino acid residues, and is widely distributed in nature.¹² It has an isoelectric point of 11.0 and constitutes 3.5% of egg white protein. It is clear that lysozyme's relatively small size and simplicity makes it an excellent model analyte for novel methods in protein detection. This protein is also known as *N*-acetylmuramide glycan hydrolase due to its properties to destroy bacterial cellular membranes by catalyzing the hydrolysis of glycosidic bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine peptidoglycan residues of Gram-positive bacterial cell walls.¹³ Moreover, the monitoring of the lysozyme level is used as a marker for some health problems such as bronchopulmonary dysplasia in newborns,¹⁴ conjunctivitis, kidney problems¹⁵ and leukemia.¹⁶ In addition, Lys has been widely used as an antimicrobial agent in the production of wine,¹⁷ cheese,¹⁸ beers¹⁹ as well as to prolong the shelf-life of shrimp, surimi products and sausages.²⁰ In particular, in wine-making, Lys has been used since 1990 to prevent or mitigate heterolactic fermentation.¹⁷ The maximum permitted level of lysozyme in wine samples is

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500 mg L⁻¹ (~35 μM).²¹ Being an egg-protein, lysozyme is considered as an allergen; therefore developing new, rapid, cheap and sensitive methods for the detection of Lys is of great significance.

Presently, the available analytical methods for the detection of lysozyme include conventional methods like chromatographic or immunosensing techniques based on ELISA, which have high sensitivity, but high cost and experimental complexity. Thus, numerous sensors have been presented as alternatives to overcome these limitations based on mainly electrochemical, surface plasmon resonance and optical detection.^{22–35} In this work, we report a novel electrochemical aptamer–antibody sandwich assay for the detection of Lys. The differential pulse voltammetry (DPV) technique was used to detect Lys *via* 1-naphthol oxidation signal changes. For this purpose, the aptamer was immobilized onto the electrode surface by covalent binding *via* diazonium salt. After incubating with lysozyme, a biotinylated antibody was used to form the sandwich format. The addition of avidin modified alkaline phosphatase and the 1-NPP enzymatic substrate allowed us to detect lysozyme based on the electrochemical oxidation signals of 1-naphthol. Results showed that this novel biosensor can be used for accurate quantification of the concentration of Lys in spiked wine samples. The developed biosensor is simple, sensitive, specific and fast for the detection of Lys.

2. Experimental

2.1. Chemicals and materials

Potassium dihydrogen phosphate, sodium monophosphate, bovine serum albumin (BSA), lysozyme (Lys), avidin-labeled alkaline phosphatase (Av-ALP), casein, biotin-labeled rabbit anti-chicken Lys antibody (AbLysBio), magnesium chloride, potassium chloride, sodium chloride, ethanolamine, diethanolamine (DEA), *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N'*-ethyl-carbodiimide hydrochloride (EDC), 4-aminobenzoic acid (ABA), 1-naphthyl phosphate (1-NP) and sodium nitrite were purchased from Sigma (St. Louis, MO, USA). All reagents were of analytical grade. The aptamer used was: AptLysCOX²⁴ 5'-NH₂-GCA GCT AAG CAG GCG GCT CAC AAA ACC ATT CGC ATG CGG C-3' and was provided by Eurogenetic (France)

All solutions were prepared using MilliQ water. The buffers employed were: binding buffer (BB) (1 mM MgCl₂, 2.7 mM KCl, 140 mM NaCl, 0.1 mM Na₂HPO₄ and 1.8 mM KH₂PO₄, pH 7.4), 10% DEA buffer (pH 9.5) and 100 mM MES buffer containing 0.09% NaCl.

2.2. Equipment

The electrochemical measurements were performed using an Autolab PGSTAT100 potentiostat/galvanostat (Eco Chemie, Netherlands) controlled by General Purpose Electrochemical System software (GPES) (4.9) for voltammetry. Screen printed carbon electrodes (SPCEs) were fabricated by using a DEK 248 screen-printing system. The SPCE consists of a conventional

three electrode configuration with graphite as working (4-mm diameter disk) and counter (16 mm × 1.5 mm curved line) electrodes, and Ag/AgCl (16 mm × 1.5 mm straight line) as a pseudo reference electrode.

2.3. Experimental protocol

2.3.1. Electrochemical SPE pretreatment. SPE was subjected to electrochemical pretreatment by 10 cyclic potential scans between 1.0 and -1.5 V at a scan rate of 0.2 V s⁻¹ in 0.5 M H₂SO₄ and 0.1 M KCl. Then, the electrodes were rinsed with water.

2.3.2. Immobilization of AptLys onto the electrode surface. In detail, a diazonium cation was synthesized by *in situ* reaction of 2 μL of 1 M NaNO₂ and 1 mL of 2 mM ABA prepared in 0.5 M HCl. The mixture was left to react for 5 min at room temperature. 100 μL of this mixture and 100 μL of 0.5 M HCl were deposited onto the electrode surface and the electrochemical modification was performed by linear sweep voltammetry from 0.6 to -0.8 V. After modification, the electrode was rinsed three times with distilled water. Linear voltammetry was used for surface modification to avoid the multilayer formation, and to have a uniform monolayer on the electrode surface. As the thickness of the layer and blocking effect are related to diazonium reduction, electrodes with the same magnitude of a reduction peak for diazonium were used to immobilize the aptamer. The carboxylic groups onto the electrode surface were activated with 100 μL of 100 mM EDC and 25 mM NHS in 100 mM MES buffer for 1 hour. After rinsing three times with distilled water, 30 μL of 10 μM solution of Apt were incubated onto the electrode surface for 1 hour. After that, the electrodes were washed three times with BB to remove the unbound aptamer. In order to deactivate the remaining succinimide groups, the electrodes were incubated with 30 μL of 1 M ethanolamine solution. After washing three times with BB, the electrodes were incubated with 30 μL of 5% BSA solution for 1 h to avoid nonspecific adsorption. The modified electrodes can be used directly or stored dry at 4 °C for several days without decrease in the sensitivity.

2.3.3. Aptamer–antibody sandwich assay. The electrodes were incubated with different concentrations of Lys for 15 min. Then, the electrodes were washed three times with BB. In order to achieve the aptamer–antibody sandwich, the electrodes were incubated with 30 μL of anti-Lys antibody from a 1/1500 dilution of the stock solution in the BB buffer. The incubation took place for 1 h. This was followed by three washing steps using BB. Then, 30 μL of av-ALP, from 1/12 500 dilution from the stock solution of enzyme, were deposited on the electrodes for 1 h. After that, the electrodes were washed three times with BB.

2.3.4. Electrochemical detection. 90 μL of 10% DEA buffer and 10 μL of 1-NPP 5 mg mL⁻¹ were added on the electrode surface and incubated for 2 min at room temperature. Electrochemical detection was performed by DPV. A modulation time of 2 s, an interval time of 0.2 s, an initial potential of 0.1 V, an end potential of 0.4 V, a step potential of 0.01 V, a modulation amplitude of 0.06 V and a stand-by potential of 0 V were

applied. The height of the resulting oxidation peak was recorded and plotted against Lys concentration to give a calibration curve.

2.3.5. Wine sample preparation. Wine samples were prepared following a protocol from a previous study.²³ Briefly, 1 mL of a wine sample was spiked with 200 μ M of Lys and allowed to stand for 3 min. Next, 200 μ L of a 5 M NaCl solution containing a 5% Tween-20 surfactant were added to 200 μ L of a lysozyme–wine mixture and diluted to a final volume of 1 mL using 20 mM MES buffer, pH 6 with 1 mM MgCl_2 . This mixture was further centrifuged at 5000 rpm for 5 min and diluted using the aforesaid buffer to obtain the desired concentration of Lys.

3. Results and discussion

3.1. Principle of the aptamer–antibody sandwich assay

Fig. 1 illustrates the different steps involved in the fabrication of a biosensor for the detection of Lys based on its specific recognition by an aptamer–antibody assay. As described in section 2.3, the aptamer was covalently immobilized through EDC/NHS chemistry *via* diazonium salt on the SPE surface. This step was followed by a blocking step with BSA to avoid non-specific adsorption on the transducer surface. Then, anti-Lys antibody was incubated on the sensing platform to obtain sandwich type detection. Thereafter, a solution of avidin modified-ALP was deposited onto the electrode surface to achieve coupling to the antibody through biotin–avidin affinity.

Finally, the biosensor was immersed in DEA buffer solution of pH 9.5 containing 1-NPP as an ALP substrate, and Lys was determined by differential pulse voltammetry of the generated 1-naphthol as the enzyme reaction product.

3.2. Optimization of the working experimental conditions

In order to achieve the improved analytical characteristics of a proposed biosensor, it is of vital importance to optimize the different experimental parameters. In this context, several parameters including pH, temperature conditions, concentrations of anti-Lys antibody and avidin modified enzyme, and their incubation time with Lys were optimized prior to performing concentration dependence response of the proposed sandwich assay. The electrochemical response of the sandwich biosensor was investigated for different pHs of PBS buffer (6.8–8.2) and under varying temperature conditions. The maximum electrochemical output signal was obtained at pH 7.4 under room temperature. Fig. 2a shows the voltammetric peak response of 1-NPP in the presence of different concentrations of anti-Lys antibody (from 1/3000 to 1/500, dilutions from the stock solution of an antibody). As can be observed, the current response increased rapidly with increasing concentration of anti-Lys antibody, with a maximal electrochemical output signal at 1/1500. Similarly, Fig. 2b shows that the peak current increased with increasing concentration of an avidin modified ALP till 1/12 500 (dilution from the stock enzyme solution), which was followed by a decrease in response for the subsequent increasing concentration, indicating that the saturation point of an enzyme label is reached.

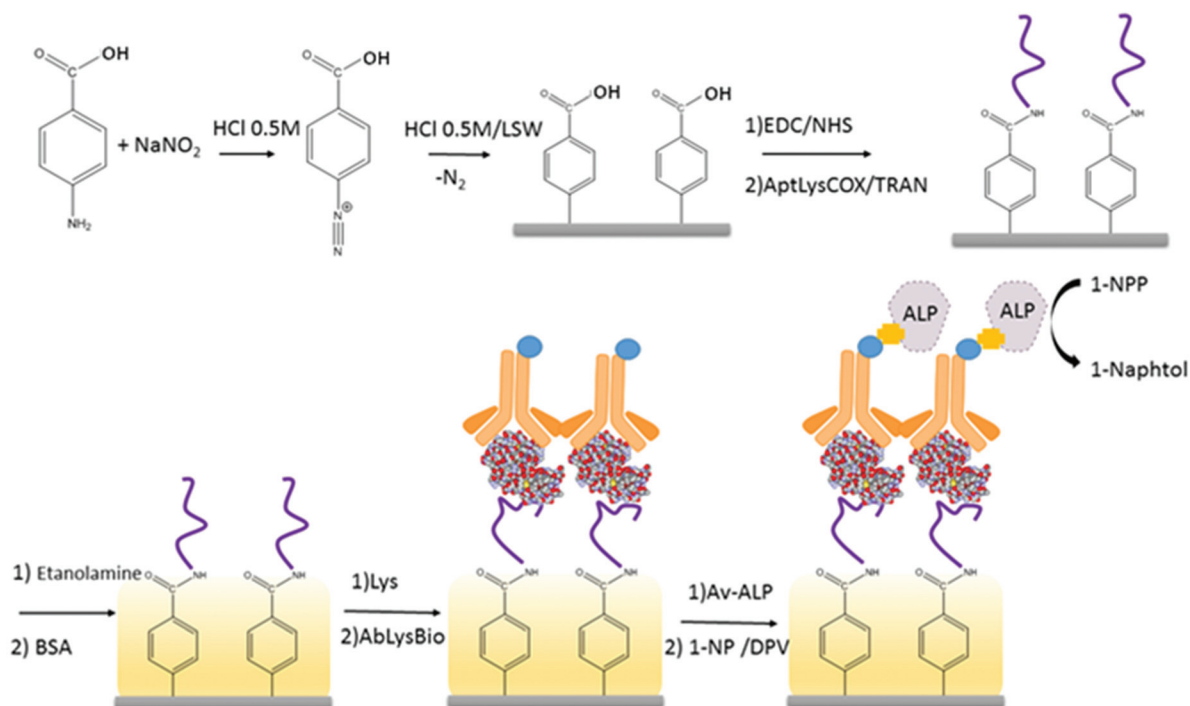


Fig. 1 Schematic representation of the developed sandwich biosensor.

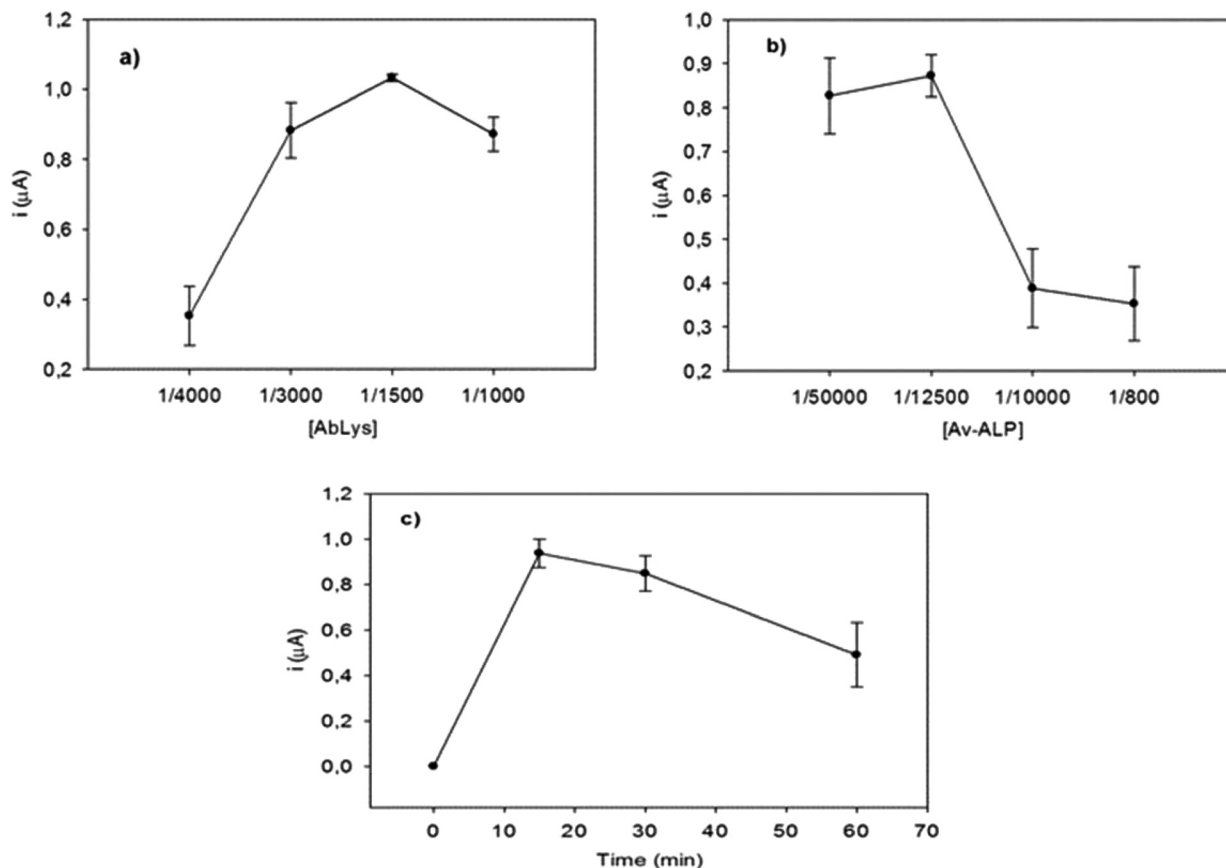


Fig. 2 (a) Optimization of the concentration of AbLys antibody. (b) Optimization of the concentration of av-ALP. (c) Optimization of incubation time with Lys. Uncertainty values corresponding to replicate experiments ($n = 3$).

Thus, 1/1500 and 1/12 500 were chosen as the optimal dilutions for anti-Lys antibody and avidin modified-ALP respectively.

The effect of the incubation time of Lys on the current response of the biosensor was also studied. As can be seen from Fig. 2c, the maximum peak current of 1-NPP was observed for an incubation period of 15 min. For longer incubation times, the peak current was decreased. Thus, according to the experimental results, an incubation time period of 15 min was selected to perform further experiments.

3.3. Analytical performance of the aptamer-antibody sandwich biosensor

In order to perform the quantitative analysis, the designed biosensor was incubated with different concentrations of Lys under the optimal conditions, and the DPV responses were recorded to draw a calibration curve. As shown in Fig. 3a, the oxidation peak current increased with the increasing concentration of Lys. The peak current was plotted against the concentration of Lys, and the calibration plots (Fig. 3b) exhibited a good linear correlation between the peak current and the logarithm of Lys concentrations in the range from 5 fM to 5 nM with a correlation coefficient of 0.998. The calculated limit of detection (LOD) for Lys was 4.3 fM. The reproducibility of the method showed a relative standard deviation (RSD) of 5.5%,

obtained from a series of 3 experiments carried out in a concentration of 5 nM of Lys. Table 1 provides a comparison of different biosensors reported in the literature for Lys detection. The data in Table 1 indicate the lowest limit of detection of our proposed sandwich biosensor as compared to the LOD of previously reported biosensors for Lys detection. In addition, the linear range was greatly improved, being much wider than for other biosensors. The improved analytical performance can be attributed to the use of an aptamer as a capturing probe for the analyte on the transducer surface. Aptamers have a unique number of characteristics including higher binding affinities, immobilization onto the solid surface in a controlled orientation and retention of their conformation upon immobilization. Based on the fact that an aptamer was used as a capturing probe in the sandwich format, the enhanced analytical performance was expected in the purposed assay as compared to the conventional antibody based sandwich format where an antibody is used as the capturing probe.

The aptamer modified transducer surface was observed to retain its electrochemical signal after a storage period of 2 weeks at 4 °C with a negligible loss of activity. This suggests that the immobilization method has resulted in the improved stability of the aptamer, and the aptamer modified electrode

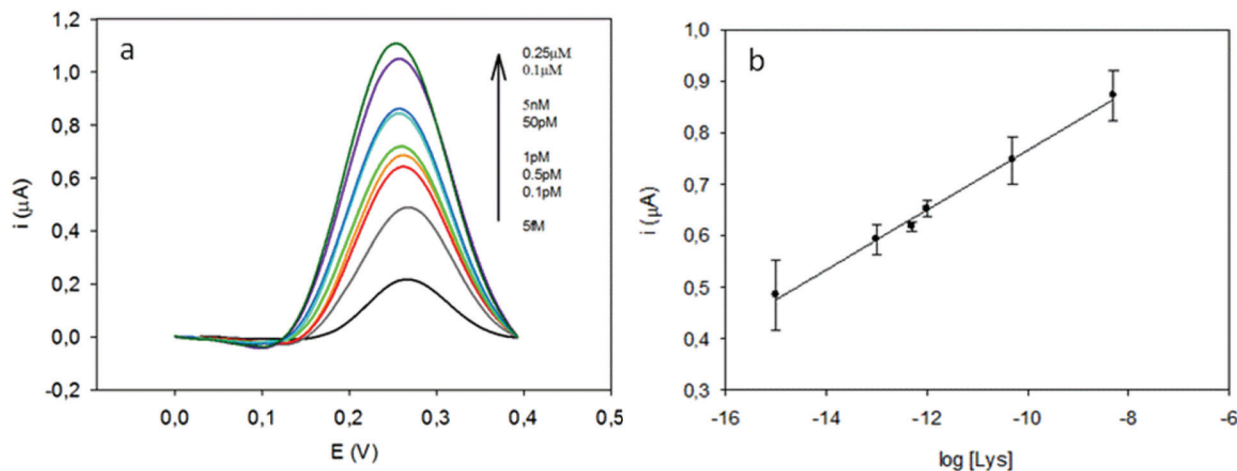


Fig. 3 (a) DPV curves for different concentrations of Lys. (b) Calibration curve and regression plot of the biosensor. Uncertainty values corresponding to replicate experiments ($n = 3$).

Table 1 Comparison of the proposed biosensor with other reported methodologies for lysozyme detection

Analytical technique	Detection limit	Linear range	Ref.
Electroluminescence	120 pM	64 pM–0.64 μM	25
Electroluminescence	0.15 ng mL ⁻¹ (~10.4 pM)	0.5 nM–9 nM	26
SPR	2.4 nM	0.5–80 μg mL ⁻¹	23
Impedance	6 fM	0.01–0.5 pM	27
Impedance	28.53 nM		28
Impedance	862 nM	0–400 μg mL ⁻¹	29
Impedance	0.07 nM	0.2 nM–4 nM	30
Square wave voltammetry	0.2 nM	0.5 nM–100 nM	31
Square wave voltammetry	38/16 nM	0–30 mg L ⁻¹	32
Square wave voltammetry	1 nM	7–30 nM	33
Square wave voltammetry	0.3 pg mL ⁻¹ (~20.8 fM)	1–50 pg mL ⁻¹	34
Cyclic voltammetry	0.1 pM	5 pM–1 nM	35
Differential pulse voltammetry	4.3 fM	5 fM–5 nM	Our work

can be employed as a ready to use tool for target analyte detection.

3.4. Selectivity of the biosensor

In order to establish the specificity and selectivity of the designed biosensors, different proteins including casein, BSA and cytochrome *c* were incubated on the biosensor surface and DPV measurements were carried out under the same experimental conditions as those described for Lys analysis. As shown in Fig. 4, the presence of these proteins exhibited a negligible response compared with that of Lys, even in the case of cytochrome *c* which is structurally similar to Lys. Therefore, the results demonstrated that the developed strategy could be used to identify Lys with high specificity.

3.5. Application of the biosensor for the detection of Lys in spiked wine samples

In order to demonstrate the analytical reliability and applicability of the method for a real sample matrix, analysis of wine samples were performed by spiking at three different concentrations of Lys (1.5 nM–5 pM). The recovery values were deter-

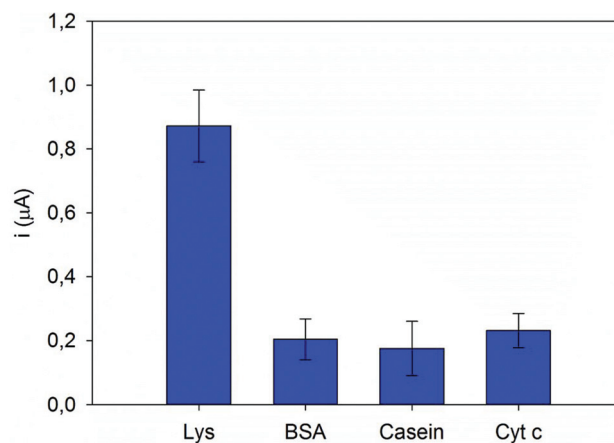


Fig. 4 Selectivity of the biosensor to casein, BSA and cytochrome Cat 5 nM, respectively. Error bars are obtained based on three independent measurements.

mined in accordance with the calibration curve performed in buffer. The obtained Lys concentrations were in consistent with the spiked values, indicating the suitability of the method

Table 2 Recovery studies performed in spiked wine samples for the applicability of the biosensor ($n = 3$)

Spiked [Lys] (M)	Found [Lys] (M)	R.S.D. %	Recovery %	Relative error %
1.5×10^{-9}	$1.45 \times 10^{-9} \pm 0.04 \times 10^{-9}$	3.1	96.67	3.33
2.5×10^{-11}	$2.38 \times 10^{-11} \pm 0.099 \times 10^{-11}$	4.2	95.2	4.8
5×10^{-12}	$5.1 \times 10^{-12} \pm 0.26 \times 10^{-12}$	5.1	102	1.96

for real sample analysis. The recovery results along with other analytical characteristics for the proposed biosensors are presented in Table 2.

4. Conclusions

In conclusion, a novel electrochemical biosensor has been successfully developed for the detection of lysozyme, a protein based on a hybrid sandwich protocol. Differential pulse voltammetry was used to detect Lys *via* the changes in 1-naphthol oxidation signals. The described biosensor showed a lower detection limit (4.3 fM) as compared to the previously reported biosensors for lysozyme detection (Table 1), a wide linear range for lysozyme detection from 5 fM to 5 nM, high sensitivity and selectivity. In addition, the biosensor was used for detecting lysozyme in spiked wine samples and very promising recovery values were obtained, indicating the great potential of the proposed methodology for detecting lysozyme in wines and possibly other food matrices.

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