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Aptasensing of chloramphenicol in the presence of its analogues: reaching the maximum residue limit

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ABSTRACT: A novel label-free folding induced aptamer-based electrochemical biosensor for the detection of chloramphenicol (CAP) in the presence of its analogues has been developed. CAP is a broad-spectrum antibiotic which has lost its favor due to its serious adverse toxic effects on human health. Aptamers are artificial nucleic acid ligands (ssDNA or RNA) able to specifically recognize a target such as CAP. In this article, the aptamers are fixed onto a gold electrode surface by a self-assembly approach. In the presence of CAP, the unfolded ssDNA on the electrode surface changes to a hairpin structure bringing the target molecules close to the

surface and trigger electron transfer. Detection limits were determined to be 1.6×10^{-9} mol L^{-1}. In addition, thiamphenicol (TAP) and florfenicol (FF), antibiotics with a similar structure to CAP, did not influence the performance of the aptasensor, suggesting a good selectivity of the CAP-aptasensor. Simplicity and lower detection limit (because of the home-selected aptamers) make that the electrochemical aptasensor is suitable for practical use in the detection of CAP in milk samples.

1. Introduction

Antibiotics are a large and natural group of pharmaceuticals used in modern medicine. They are applied for the treatment of infectious diseases in human and veterinary. However, accumulation of antibiotics in food-producing animals and foods has been implicated in the occurrence of resistant bacterial strains causing a serious threat for public health. Subsequently, their use in the farming of food-producing animals has been strongly regulated in different countries. Since the maximum residue limit (MRL) has been set for these compounds in food-safety control programs in many countries, a fast and sensitive analytical method is needed for reaching the lowest possible detection limit.[1,2]

Phenicols such as chloramphenicol (CAP), thiamphenicol (TAP) and florfenicol (FF) are broad-spectrum antibiotics. The main member of this group, CAP, is a neutral nitrobenzene derivative (Fig.1a). CAP was initially isolated from *Streptomyces venezuelae* and was widely used in the treatment of serious infections such as typhoid fever and salmonellosis.[3] Low cost and efficiency against infections made CAP a widely used antibiotic in veterinary. However, CAP lost its favor due to its side effect on the haemopoietic system.[3,4] For this reason, the European Union (EU) has set the MRL for CAP at a level of 0.3×10^{-6} g kg^{-1}.[4]

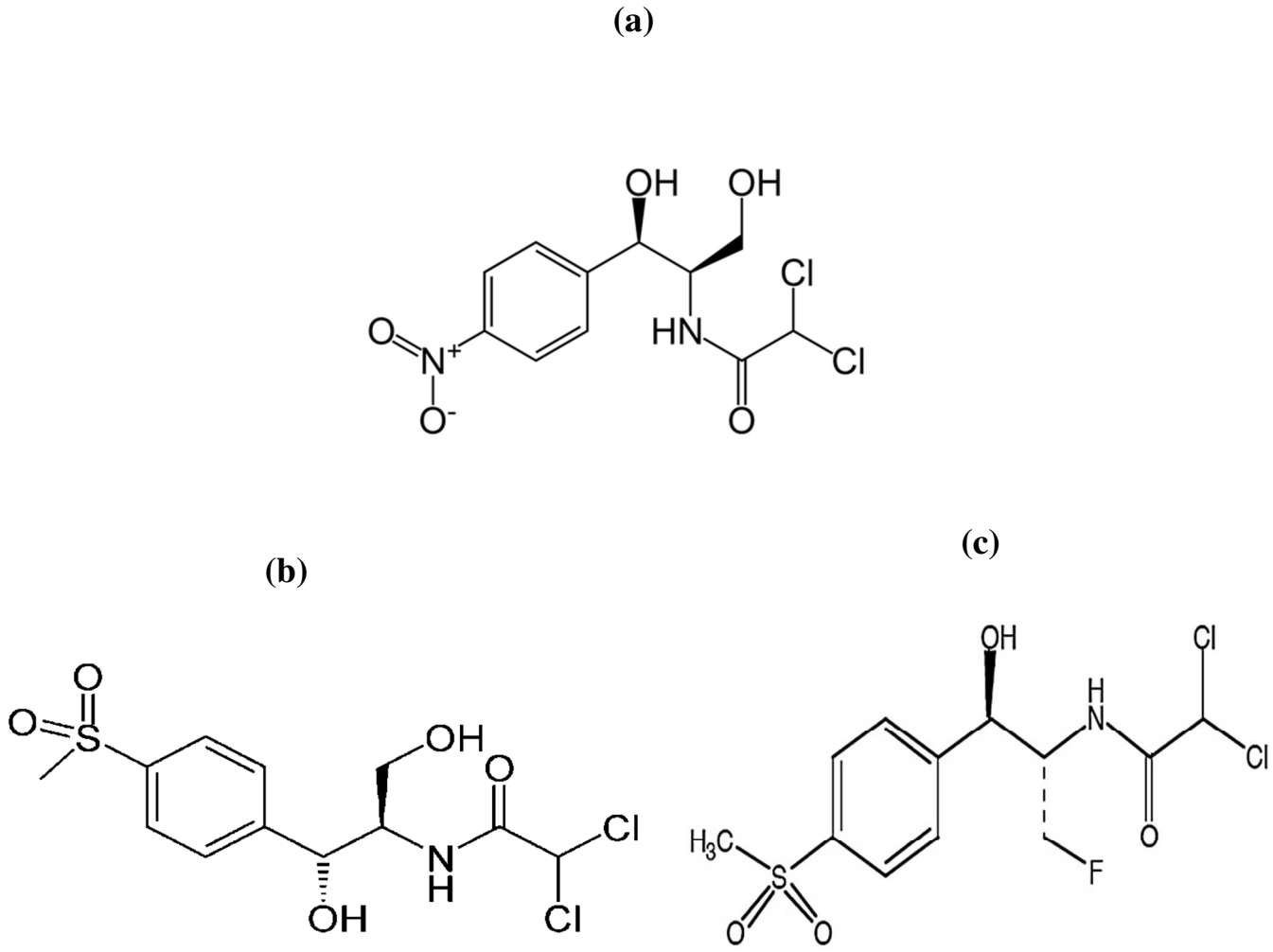


Figure 1. Chemical structures of phenicols (CAP (a), TAP (b), FF(c))

The other members of the phenicol family, TAP and FF, have a very similar structure as CAP (Figure 1b-c). The chemical structure of TAP differs from CAP in having a sulpho group instead of a nitro group. The chemical structure of florfenicol differs from CAP in the presence of a p-methyl sulphonyl group and a fluorine atom. All three compounds are mainly used in veterinary medicine as antimicrobial agents, because of their extremely broad spectrum of activity. The toxicity of TAP and FF is not as distinct as for CAP. Nevertheless, these compounds still have some side effects and the presence of them is unsafe from the standpoint of human health safety.[5] In addition, since they have a similar structure and may appear in the same matrices, cross-reactions of CAP with TAP and FF should be considered.

Several techniques have been used for the detection of CAP residue: photoinduced chemiluminescence[6], GC (gas chromatography) and HPLC (high performance liquid chromatography) with UV[7], capillary electrophoresis with amperometric detection[8]. These methods are quite sensitive and accurate but expensive and complex in operation and thus limiting their application. Providing an answer to the latter, biosensors have been developed. Biosensors are analytical devices that consist of a recognition element targeting an analyte of interest and a transduction element for converting the recognition process into a measurable signal. Since the molecular recognition element is the main part of a biosensor and affects the selectivity of the sensor, there has been growing attention on the development of new molecular recognition molecules with higher selectivity for important food-safety targets.[9]

Aptamers (ssDNA) are synthetic oligonucleic acid sequences which can bind to their targets with high affinity and specificity due to their flexibility that results in binding to their ligands via adaptive recognition involving conformational alteration. The recognition process is based on the sequence of the aptamers and the three dimensional structure of the target molecules. These

specific ligands are designed and selected in vitro by SELEX process (selection evolution of ligands by exponential enrichment)[10-11] from wide populations of random sequences. Aptamers are accurate and reproducible in production. In addition, they are stable and can be selected in complex conditions. Immunization and animal hosts are not necessary for aptamer synthesis. Moreover, these oligonucleic acids can be easily modified by attachment of functional groups without affecting their affinity.[9-11]

Biosensors based on aptamers as bio-recognition elements have been called aptasensors. Electrochemical aptasensors which use electrochemistry to monitor binding-specific conformational changes in an electrode-bound aptamer suggests promise with regard to rapid, reagent-less detection under complex conditions. The common methods for immobilizing aptamers onto electrode surfaces are covalent attachment, bio-coating, and self-assembling.[12] The latter results in the formation of a stable and reproducible aptamer layer without affecting the affinity and special shape of the aptamers. Several detection approaches have been used for aptamer based biosensors. These are divided into two categories: labeled and label-free detection systems. The former is based on a redox-active signal from a reporter molecule or a label, which changes upon the interaction with the target. Label-free electrochemical detection exploits intrinsic redox-active target molecules.[13-15] Although redox-labeled electrochemical aptasensors are selective and sensitive, some complicated labeling steps and specific reagents for fabrication are needed, which limit the use of such aptasensors. As a result, efforts have been made in the direction of using label-free and low-cost aptasensors.

Electrochemical methods for the analysis of CAP have already been published but none of them is able to specifically detect CAP in the presence of its analogues and the MRL is not reached except in one case. Agui et al.[16] developed a sensor with electrochemically activated carbon fiber microelectrodes for the detection of chloramphenicol; the detection limit was

4.7×10^{-8} mol L^{-1} and the linear range was 1.0×10^{-7}–1.0×10^{-5} mol L^{-1}. Chen et al. [17] used a novel on-off amperometric sensor incorporating a three-electrode configuration for direct detection of CAP, and the detection limit was 4.2×10^{-7} mol L^{-1}. Xiao et al [18] developed modified glassy carbon electrodes based on a single-wall carbon nanotube–gold nanoparticle–ionic liquid composite film for the detection of CAP with the linear range of 1.0×10^{-8}–6.0×10^{-6} mol L^{-1} and 5.0×10^{-9} mol L^{-1} detection limit. Most of these detection methods include the detection of CAP in the absence of its derivatives and does not cover real sample analysis. Moreover, most of the validation studies are not in conformity with the new EU requirements.[2] With the detection of CAP in the presence of its derivatives in the same matrices, it would be very desirable to be able to analysis CAP in real samples such as milk.

This work was carried out in order to evaluate the performances of the developed aptasensor for CAP detection in complex matrices which contain the CAP derivatives, i.e. TAP and FF, in food of animal origin following the EU criteria for qualitative screening methods.

The challenge was to develop a sensitive and selective method for the detection of CAP in the presence of TAP and FF on the basis of label-free and binding-induced conformational change aptamers. Changes in the interfacial properties due to the aptamer immobilization and specific binding of aptamer with CAP were characterized by cyclic voltammetry (CV) and square wave voltammetry (SWV). To our best knowledge, no method has been reported for the determination of the CAP, reaching the MRL, in the presence of its derivatives in real samples.

2. Experimental Section

2.1. Chemical and material

CAP (M_w=323.13, CAS Number=56-75-7), FF (M_w=358.21, CAS Number=73231-34-2) and TAP (M_w=356.22, CAS Number=15318-45-3) were purchased from Sigma (Belgium). Tris

buffer containing 100×10^{-3} mol L^{-1} NaCl, 20×10^{-3} mol L^{-1} Tris HCl, 2×10^{-3} mol L^{-1} $MgCl_2$, 5×10^{-3} mol L^{-1} KCl , 1×10^{-3} mol L^{-1} $CaCl_2$, (pH=7.6) was obtained from VWR (Belgium) and used as binding buffer solution. The CAP stock solution was first prepared in EtOH and deionized water (10×10^{-3} mol L^{-1}) and then diluted with Tris buffer. Potassium ferricyanide and ferrocyanide were purchased from Sigma (Belgium).

CAP binding aptamer sequence (5'-SH-$(CH_2)_6$-AGC-AGC-ACA-GAG-GTC-AGA-TGC-ACT-CGG-ACC-CCA-TTC-TCC-TTC-CAT-CCC-TCA-TCC-GTC-CACCCT-ATG-CGT-GCT-ACC-GTG-AA-3'), selected and designed at SPHERE laboratory of the University of Antwerp, was purchased from Eurogentec.[11] Before use, the stock solution of the oligonucleotides were prepared by dissolving them in the Tris buffer solution (100×10^{-6}). The aptamer and CAP solutions were stored at 4°C to prevent decomposition. All other chemicals and solvents used were of analytical grade.

2.2. Apparatus

CV and SWV were performed by using a PGSTAT20 potentiostat controlled by GPES 4.9 005 software package running (ECO Chemie, The Netherlands). Electrochemical impedance spectroscopy (EIS) measurements were performed by using an FRA (frequency response analyser) module.

Electrochemical experiments were performed in a conventional three electrode cell configuration. A gold electrode inlaid disk (□=1.2 mm) was used as working electrode. A saturated calomel electrode (SCE) and graphite were used as the reference and the auxiliary electrode, respectively. All the potentials in the text are referred to the SCE. The cell was placed in a Faraday cage to reduce electrical noise. All the experiments were conducted at room temperature (20±1°C).

FTIR spectra were obtained with a Bruker Vector 22 FT-IR spectrometer equipped with a PIKE Miracle single reflection ATR accessory. All spectra were recorded through 32 scans with 4 cm^{-1} resolution.

2.3. Fabrication of the aptasensor

Prior to surface modification, the gold electrode was mechanically polished with 1.0 and 0.05 μm alumina slurry separately followed by rinsing thoroughly with double distilled water. Then it was washed ultrasonically in double distilled water and ethanol for 15 min, respectively. The electrode was rinsed with distilled water and dried at room temperature. Subsequently, electrodes were electrochemically cleaned by potential scanning between −1.2 and 1.2 V until a reproducible cyclic voltammetric scan was obtained.

In order to immobilize the thiolated aptamers on the gold surface, 3×10^{-6} L of a 5×10^{-6} mol L^{-1} aptamer solution was dropped onto a freshly smoothed gold surface, and the solvent was then evaporated at $4^{\circ}C$ for 6 hours. The final sensing interface was ready after rinsing with buffer solution.

The concentration of CAP specific DNA aptamer immobilized on a gold electrode was optimized. A series of different aptamer concentrations was chosen to determine the effective concentration of ssDNA aptamer to be immobilized. The optimal concentration of CAP specific ssDNA aptamer was estimated to be 5×10^{-6} mol L^{-1} at which the current changes by CAP binding to aptamer was most effective in both of CV and SWV analysis (data not shown). A low concentration of aptamers can result in an inefficient current signal, while high concentrations of aptamer might block the electrode surface and leading to a decrease in the signal sensitivity.

3. Results and discussion

3.1. Sensing mechanism of the CAP-aptasensor

The aptasensor was made by covalently attaching the thiol-modified label free CAP aptamer to the gold surface. The CAP binding aptamer contains 80-base sequences and specifically binds to CAP with high affinity and specificity.[11] The aptamers have the flexibility to change their conformational structure once they bind to a CAP molecule. The CAP determination is based on the change of the current response before and after the aptamer-target binding recognition reaction (when applying a certain reduction potential). In the absence of CAP, the aptamer structure is partially unfolded. Upon CAP binding, the aptamer is folded into the stem-loop structure, and this conformational change forces the CAP molecules to be in close proximity with the electrode, this results in a significant increase in electron transfer efficiency. The detailed immobilization and detection process is illustrated in scheme 1.

The ratio between the increase of peak current upon target binding and the peak current in the absence of target was used to calculate the % current response according to equation 1.

$$\text{Current response } (\mu A) = (I - I_0)/I_0 \qquad \text{(Equation 1)}$$

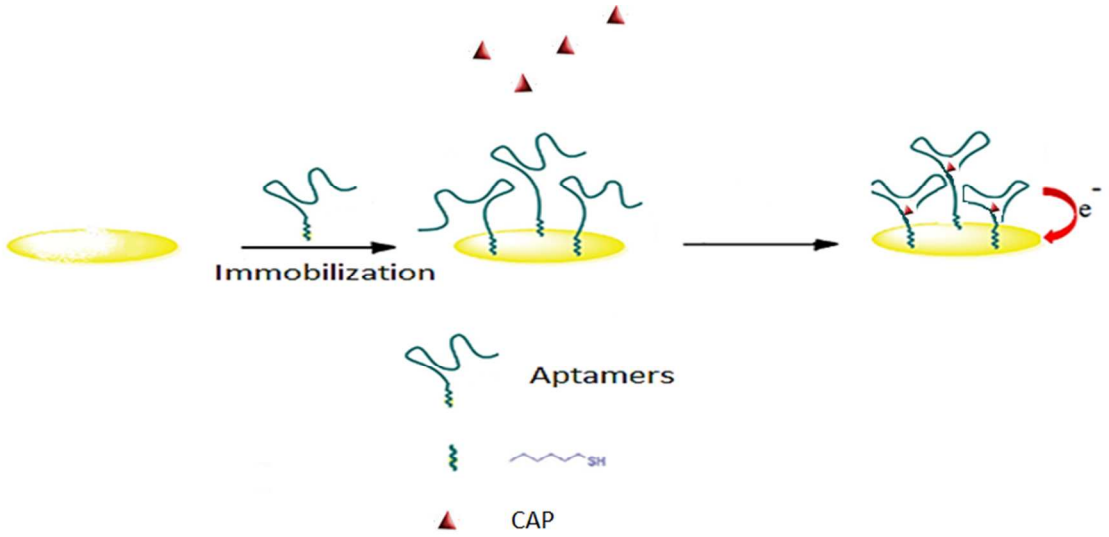
In this equation, I is the reduction peak current obtained in the presence of the target, and I_0 is the background current in the blank solution.

3.2. Characterization of the CAP-aptasensor

The modification of the gold surface with aptamers was characterized by EIS and FT-IR techniques. EIS is one of the most powerful electrochemical technologies for interfacial investigation. Therefore, EIS was applied to prove the aptamer immobilization. EIS measurements were done in the presence of 5×10^{-2} mol L^{-1} $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture and is based on interfacial electron transfer. A Nyquist plot (Z_{re} vs. Z_{im}) was drawn to

analyze the impedance results.[16] Figure 2(I) shows the Nyquist plots of EIS measurements which was conducted under a potential of 5 mV over the frequency range of 10^5 Hz -0.1 Hz.

Scheme.1. Fabrication of an electrochemical aptasensor based on label free target-induced folded aptamer.



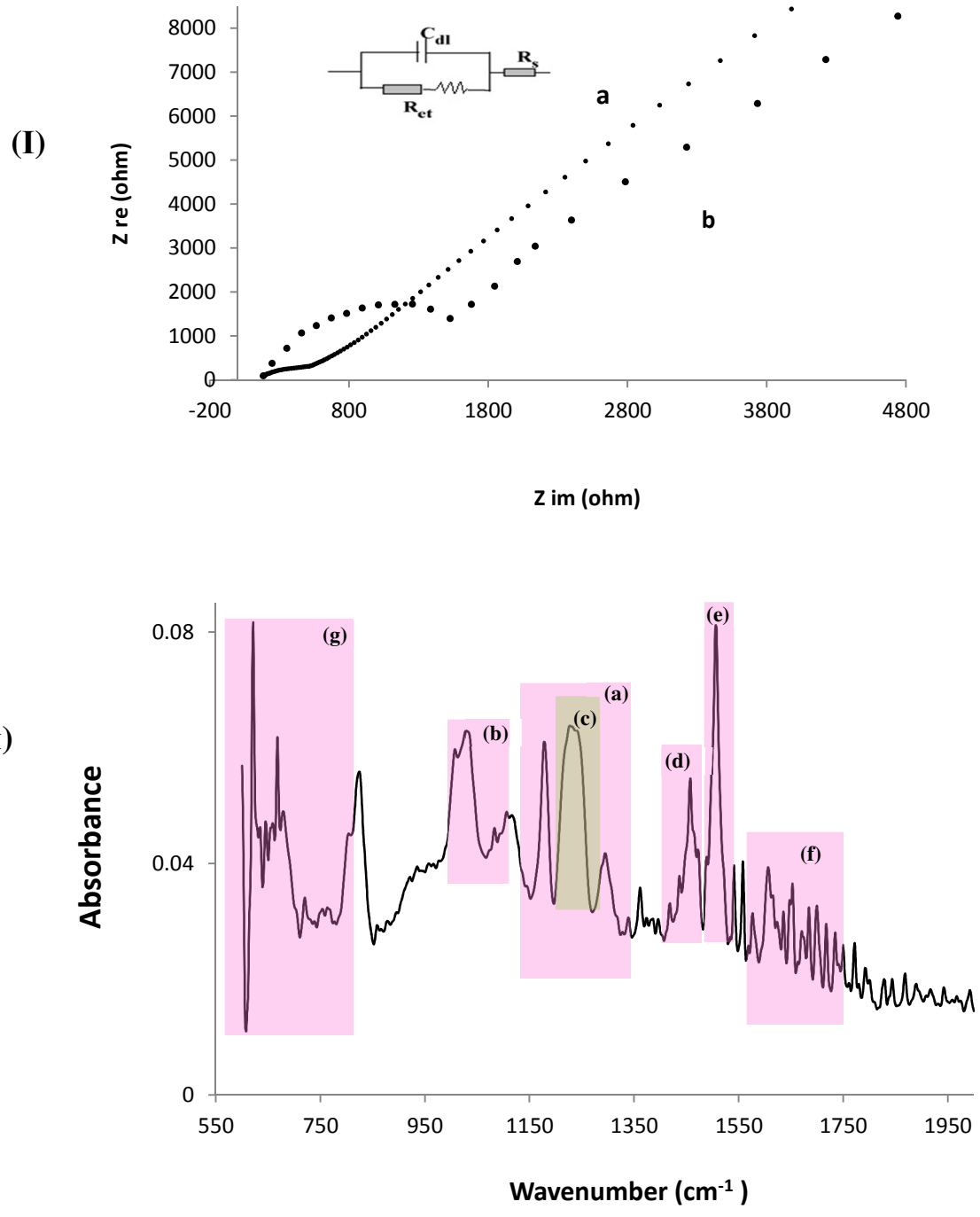


Figure 2. Nyquist plot of a bare gold (a) and ssDNA aptamer modified gold (b) electrode in the presence of 5×10^{-2} mol L^{-1} $[Fe(CN)_6]^{4-/3-}$ (frequency range 0.1 to 10^5 Hz, amplitude 5.0 mV) (I). FTIR spectrum of the thiolated aptamer modified gold electrode between 600- 2000 cm^{-1}(II).

The bare Au electrode has a very small semicircle domain (R_{et}) that is characterized by a fast electron transfer.[19] In contrast, for the aptamer immobilized electrode, the response of the equimolar $[Fe(CN)_6]^{4-/3-}$ is blocked, resulting in an increased electron-transfer resistance with, to a certain extent, a large semicircle. This is attributed to the fact that single stranded nucleic acid with negative charges on its phosphate backbone makes an electrostatic repulsive force to $[Fe(CN)_6]^{3-/4-}$ anions. The results show that the aptamer has been immobilized on the gold electrode surface successfully.[19,20] In addition, it can be concluded from the results that the developed aptasensor shows poor affinity toward non-specific target molecules due to the surface blocking effect of aptamers which results in blocking of the electron transfer pathway. In contrast, when the specific target molecules (CAP) are present in the solution, the binding induced conformational change of the aptamers results in bringing CAP molecules in close proximity of the surface, and thus enhancing the electron transfer.[19,20]

The binding of the aptamers onto a gold electrode was confirmed by FTIR spectroscopy, shown in Figure 2 (II). The occurrence of the peaks in the 1000-1140 cm^{-1} range is assigned to nucleic acid sequences and indicated that aptamer molecules were attached to the gold electrode surface (a). For instance, the modes at 1082 and 1238 cm^{-1} correspond to the symmetric and asymmetric PO^{2-} group of the DNA phosphodiester backbone (b, c). The modes at 1464 and 1514 cm^{-1} result from the purine and pyrimidine (DNA bases) ring modes (d, e), while the region from 1600 to 1750 cm^{-1} is due to carbonyl (C=O), C=N stretching, and exocyclic -NH_2 bending vibrations in the DNA bases (f).[21,22] A band between 550 and 700 cm^{-1} indicates the presence of a covalently gold-sulphur bonding (g).[23]

3.3. Performance of the CAP- aptasensor

3.3.1. CV response and calibration curves

Figure 3 shows the cyclic voltammograms of a bare gold electrode and an aptamer modified gold electrode in a buffer solution and in a solution containing 1×10^{-6} mol L^{-1} CAP.

The cyclic voltammetric behaviour of a gold electrode in a blank Tris buffer solution is represented as curve I. Within the potential window from 0.6 to -0.9 V, no considerable redox reaction appeared. When the bare gold electrode is incubated in 1×10^{-6} mol L^{-1} CAP solution, a small reduction peak appears around -0.7 V (curve II). This peak can be related to the irreversible reduction of the nitro group (NO_2) (present in CAP molecules) with formation of hydroxylamine (NHOH) described by the following electrochemical reaction (1).[24]

$$R\text{-}NO_2 + 4e^- + 4H^+ \rightarrow R\text{-}NHOH + H_2O \quad (1)$$

When the aptamer modified gold electrode is incubated in a solution containing 1×10^{-6} mol L^{-1} CAP, the NO_2-reduction peak increases markedly and the signal gain changes to 180.3% (curve III). This can be explained by a conformational change of the aptamers into G-quadruplex structures upon binding to CAP with high specificity, bringing the nitro group, present in CAP molecules, close to the electrode surface, and thus increasing the current response markedly. [25]

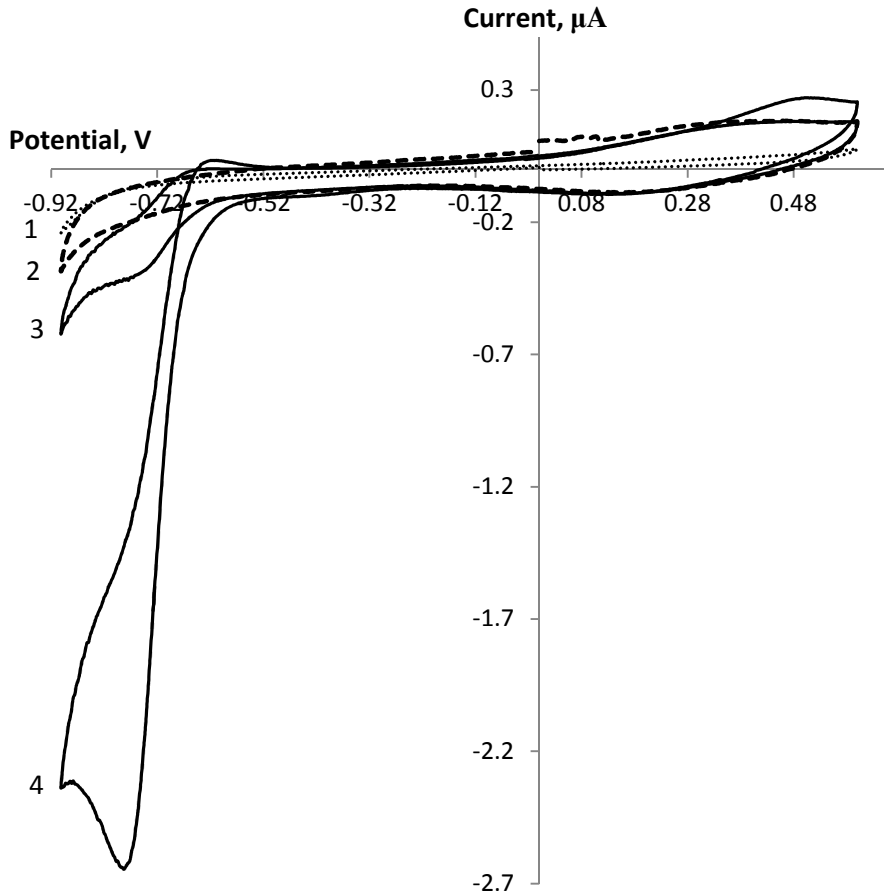


Figure 3. Cyclic voltammograms of an unmodified gold electrode in a blank buffer solution (2), and in the presence of 1×10^{-6} mol L^{-1} CAP (3). The behavior of an aptamer modified gold electrode in (1) a blank buffer solution and in the presence of 1×10^{-6} mol L^{-1} CAP is shown as curve (4).

3.3.2. Stability

The stability of successive assays with an aptamer modified gold electrode and a bare gold electrode was studied by 5 cycles of CV measurements in Tris buffer after being incubated in 1 $\times 10^{-6}$ mol L^{-1} CAP. [26]

The result shows that the peak current decreased only 3.01% for an aptamer modified gold electrode. The good stability of the sensing device can be ascribed to the covalent bonding of the aptamers onto the gold surface and the stability of aptamers. Moreover, when repeating the impedance measurements after a CAP electrochemical detection, the observed semicircle has the same value for the diameter, suggesting that the self-assembled aptamers form stable films which are not influenced when making contact with the target molecule CAP.

3.3.3. SWV response

In comparison to cyclic voltammetry, SWV has a broader dynamic range and a lower limit of detection because the current is sampled twice during each square-wave cycle, once at the end of the forward pulse and once at the end of the reverse pulse. The difference between the two measurements is plotted against the potential. The resulting peak-shaped voltammogram displays excellent sensitivity and effective discrimination against the capacitance current. Subsequently, the peak height is directly proportional to the concentration of the electroactive species.

The SWV behaviour of the sensing interface is shown in Figure 4. As can be seen, a cathodic peak at ca. -0.65 V appears upon adding CAP to the buffer solution. This peak is related to the irreversible reduction of the nitro group in CAP molecules with the formation of hydroxylamine.[24] In order to establish that this new electrochemical aptasensor can quantitatively assay the target molecule, a series of solutions containing different concentration of CAP have been prepared. The prepared aptasensor was incubated with different concentrations of CAP. A

calibration plot was obtained over the linearity range (1.6×10^{-9} to 4.2×10^{-7} mol L^{-1} CAP). The linear equation was $\Delta I = 0.238 C_{cap} + 1.62$ with a correlation coefficient of 0.9957 (N=3 and $p<0.05$) and a detection limit of 1.6×10^{-9} mol L^{-1} (calculated according to International Conference on Harmonization (ICH) guidelines). It can be seen that the linear range and the detection limit of the proposed aptasensor was greatly improved and a lower detection limit was achieved compared to the electrochemical sensing of chloramphenicol with a bare gold electrode which was done before in our group and the analytical methods described in literature.[27] This can be explained by the fact that the immobilization of aptamers on the electrode surface not only improved the amount of CAP molecules, but also decreased the distance between the CAP molecules and electrode surface upon binding of aptamers with them.

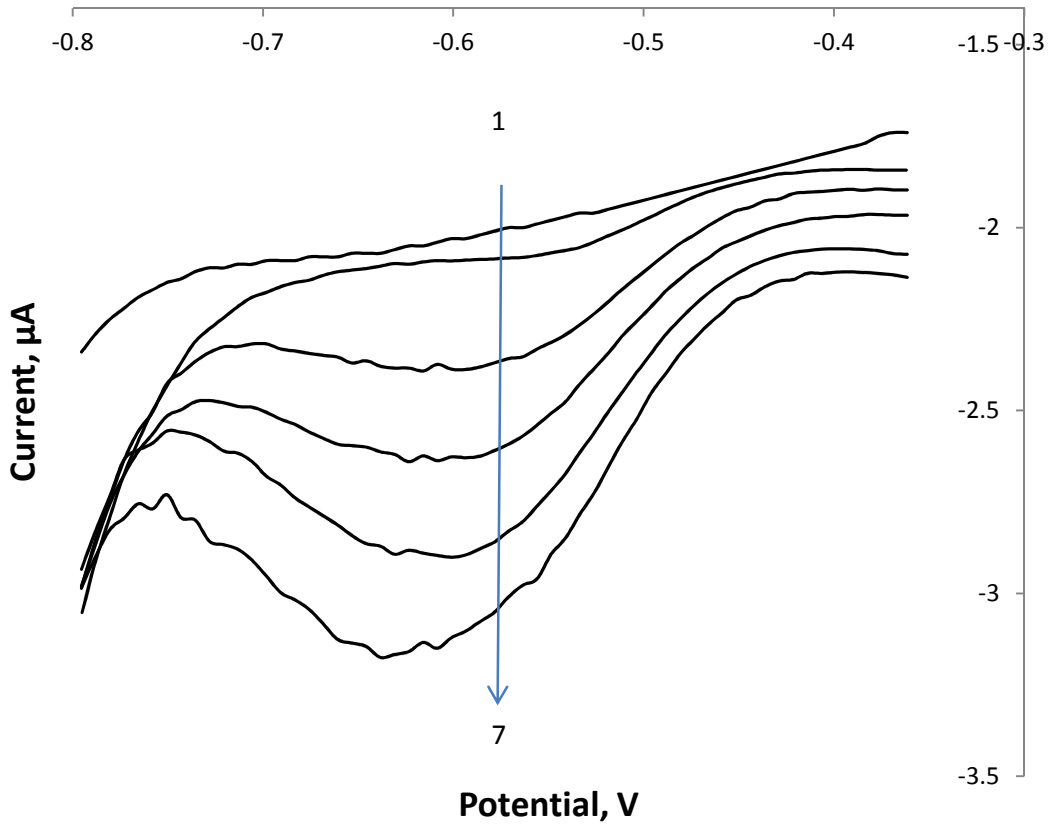


Figure 4. Square wave voltammogram of an aptamer immobilized gold electrode in a Tris buffer solution (pH 7.6) containing different concentrations of CAP: 0 (1), 1.3 (2), 2.5 (3), 3.5 (4), 5 (5), 6.2 (6) and 7.2 (7) $\times 10^{-6}$ mol L^{-1} (frequency 50 Hz, amplitude 10 mV).

3.3.4. The specificity of the aptasensor

In order to estimate the specificity of the aptasensor towards CAP, experiments were performed in the solution containing 1×10^{-6} mol L^{-1} CAP, 1×10^{-6} mol L^{-1} of TAP and 1×10^{-6} mol L^{-1} of FF, all being antibiotics from the same family which may interfere with the detection of CAP. Figure 8 compares the response of the developed electrode to CAP and the structurally similar antibiotics. It can be seen in Figure 5 that only the CAP sample showed the highest ΔI while the other two antibiotics had almost negligible responses. These results showed that the CAP-specific aptamer sequence was highly specific to CAP. Moreover, results indicated that TAP or FF have negligible influence on the CAP detection and is also able to discriminate CAP in complex matrices from its analogues.

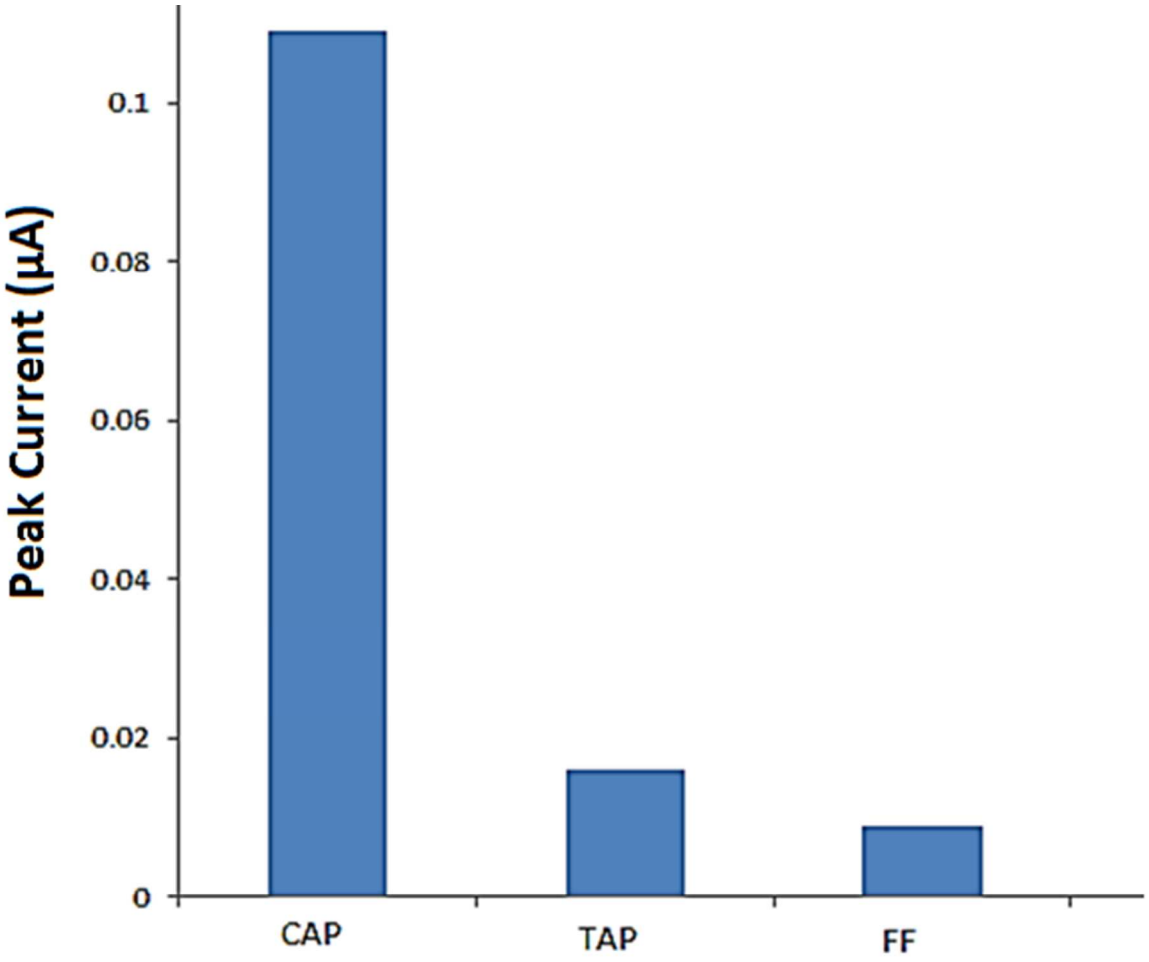


Figure 5. The selectivity of the DNA aptamer (1×10^{-6} mol L^{-1} of CAP, TAP and FF)

3.4. Determination of chloramphenicol in spiked milk samples

The feasibility and applicability of the proposed aptasensor was investigated by standard addition method of calibration (SAM). Herein, a series of samples were prepared by adding CAP of different concentrations to milk (2, 4, 6 $\times 10^{-6}$ mol L^{-1}), the recovery is in the range of (87.3, 88.0, 92.1 %). SWV voltammetry was chosen to determine the residue level of CAP in milk samples (Figure 6). The detection limit of aptamer modified gold electrode in milk samples was estimated to be 6×10^{-9} mol L^{-1}.

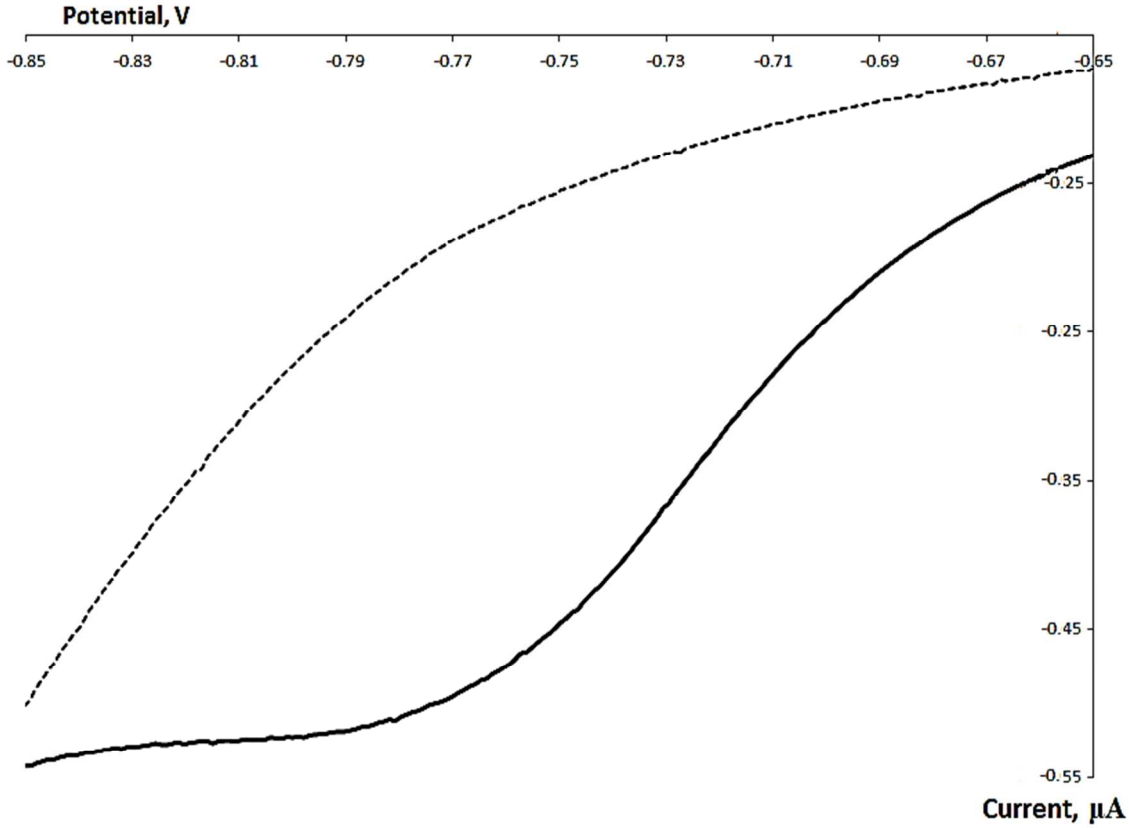


Figure 5. Square wave voltammogram of an aptamer immobilized gold electrode in a blank (dash line) and 2 $\times 10^{-9}$ CAP containing milk solution (solid line).

4. Conclusions

In this study, a novel and highly sensitive label-free aptamer based electrochemical biosensor is developed for the detection of CAP in the presence of its analogues. The principle was based on a target-induced conformational change of the aptamer. The study demonstrates the ability of label-free aptasensing to operate in complex matrixes with high sensitivity. In addition, the developed aptasensor shows a detection limit of 1.6×10^{-9} mol L^{-1}, reaching the MRL. The method could fulfill the requirement of the confirmatory criteria according to European Commission Decision 2002/657/EC with high sensitivity and selectivity. The approach can be an alternative for the construction of aptamer based biosensors for the reliable determination of CAP in contaminated milk samples, offering advantages over other analytical techniques in terms of simplicity, sensitivity and cost effectiveness.

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ABBREVIATIONS

CAP, Chloramphenicol; TAP, Thiamphenicol; FF, Florfenicol; CV, Cylic voltammetry; SWV, Square wave voltammetry; IR, Infrared Spectroscopy.

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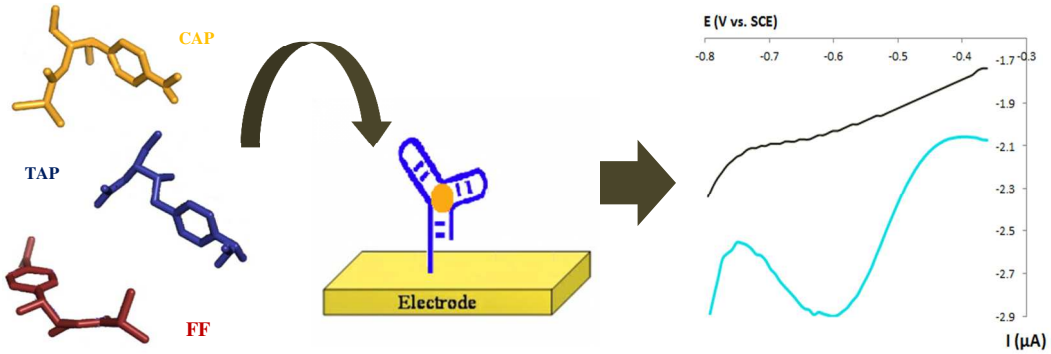

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