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Determination of E. coli in water using the enzyme free electrochemical impedimetric immunosensors

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Abstract. In this article, we have shown the results of E. coli bacteria determination in water using the enzyme free electrochemical immunosensor with covalent via click chemistry immobilized receptor layer in comparison with dropwise immobilized antibodies. Covalent immobilization was realized by creating precursors on the surface of the working electrode with the reaction of azide-alkyne copper-catalysed cycloaddition approach. The detection limit of the immunosensor with covalent and dropwise immobilization of antibodies was estimated as 6.6 CFU/ml and 11.2 CFU/ml, respectively, a linear range was \(10^3-10^6\) CFU/ml. Moreover, the sensor with “click” immobilized antibodies showed good stability for 30 days when stored in a phosphate buffer, while the sensor with dropwise immobilized receptor layer was stable for 3 days.

1. Introduction
Currently, the development of new methods and approaches for the rapid and accurate detection of infectious agents is one of the most urgent tasks. E. coli bacteria normally inhabit the lower intestines of humans and animals, but in some cases, they act as environmental and food contaminants and can cause severe infectious diseases.

For express detection of bacterial pathogens, electrochemical biosensors are widely used [1]. Electrochemical biosensors open up possibilities for miniaturization, automation and cost reduction of the device, not inferior to optical biosensors in analytical performance [2]. The most important factor in the creation of electrochemical biosensors is the method of immobilizing the receptor layer on the surface of the working electrode. When creating immunosensors, oriented covalent immobilization of antibodies can significantly improve the sensitivity and accuracy of detection [3].

Earlier, we proposed a method for covalent immobilization of antibodies based on the reaction of azide-alkyne copper-catalysed cycloaddition [4]. The main advantage of this reaction for the immobilization of antibodies is its proceeding under conditions close to physiological with a high yield of the reaction product [5, 6]. It allows a significant reduction in immobilization time (30 minutes) in comparison with similar methods (2-12 hours) [7, 8]. The acceleration of the CuAAC reaction is associated with the use as a catalyst of the products of electrooxidation of copper particles, pre-deposited in a polymer film of polyvinylbenzylazide [4].

The aim of this work is the detection of E. coli bacteria in water using the enzyme free electrochemical immunosensors with covalently linked (via click reaction) and dropwise immobilized receptor layer.
2. Methods

All chemicals were purchased from Sigma Aldrich: sodium azide, ascorbic acid, vinylbenzyl chloride, copper II hexafluoroacetylacetonate, potassium ferrocyanide (K₃Fe[CN]₆), potassium ferricyanide (K₄Fe[CN]₆), dimethylformamide (DMF), anti-Escherichia coli polyclonal antibodies (produced from a rabbit), lithium perchlorate, polyvinyl pyridine, formamide, potassium chloride, cetyltrimethylammonium bromide, and propargyl-N-hydroxysuccinimide ester. *E. coli* bacteria were provided by the department of experimental biology and biotechnology of Ural Federal University (Prof. I.S. Kiseleva).

A screen-printed electrode (SPE) served as a basis for three-stage antibody immobilization. At the first stage, vinyl benzyl azide was immobilized on the electrode surface by electro polymerization. It was carried out by 10-fold cyclic potential sweep in the range from 0 to -2.5 V from a 25% monomer solution in dimethylformamide, while copper particles were electrodeposited into a polymer film from a solution of copper hexafluoroacetylacetonate. The second stage consisted in reaction of copper-catalysed azide-alkyne cycloaddition between the azide fragment of polyvinyl benzyl azide and propargyl-N-hydroxysuccinimide ether. The oxidation products of copper particles precipitated in the polymer film by linear potential sweep from 0 to 1 V were used as a catalyst. At the third stage, the electrode was incubated in a suspension containing 0.1 mg/ml antibodies to *E. coli* for 30 minutes [4]. On the working area of the electrode, 5 μl of the suspension was dropwise applied and dried at room temperature.

After modification, the electrode was incubated in the analysed sample for 20 minutes. The analytical signal in the proposed sensor was an increase in the charge transfer resistance, which was recorded by the method of electrochemical impedance spectroscopy and calculated by the formula:

$$\Delta R = \frac{R_{E.coli} - R_{Ab}}{R_{E.coli}} \times 100\%,$$

where $R_{Ab}$ – charge transfer resistance of the modified antibodies SPE and $R_{E.coli}$ – charge transfer resistance of the modified *E. coli* SPE.

Electrochemical measurements were performed using the µAutolab Type III e. The voltammetric and impedimetric measurements were performed in the standard three-electrode cell: SPE as a working electrode, Ag/AgCl/KCl as a reference, and a carbon rod as auxiliary electrodes. The 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ was used as a redox probe. The Nyquist diagrams were registered using the electrochemical impedance spectroscopy in the potentiostatic regime. The working potential was equal to the CV oxidation peak potential with the amplitude of 0.02 V. The frequency range was $10^2$-0.1 Hz.

3. Results

The analytical characteristics of impedimetric sensors were compared depending on the method of antibody immobilization. In order to study the electrochemical response when immobilizing antibodies by different methods, EIS measurements were performed in the presence of various concentrations of *E. coli*. An increase in the concentration of *E. coli* led to an increase in charge transfer resistance, which indicates the formation of an immunocomplex between antibodies and bacteria (Fig. 1).

Figure 2a shows calibration curves plotted using $\Delta R$ values. The correlation coefficient ($R^2$) for the linear relationship in the case of covalent and non-covalent immobilization of antibodies was 0.9950 and 0.9602, respectively. Limit of Detection (LOD) was estimated as 6.6 CFU/ml and 11.2 CFU/ml, respectively, a linear range was $10^3$ - $10^6$ CFU/ml. Thus, an impedimetric immunosensor using oriented covalent immobilization of antibodies by the CuAAC reaction demonstrates a higher correlation coefficient with a lower detection limit than with immobilization through physical sorption.

In addition, the stability of the manufactured sensor during storage is an important indicator of the effectiveness of the antibody immobilization strategy. This parameter was evaluated when storing modified electrodes in phosphate buffered saline for 30 days. During physical sorption, the sensor shows satisfactory results during the first three days, after which a significant decrease in sensitivity is observed, while the sensor using covalent immobilization of antibodies shows satisfactory results during all 30 days, which indicates the effectiveness of the proposed approach (Fig. 2b).
Figure 1. The enzyme free impedimetric immunosensor with covalent immobilized (via click reaction) antibodies for *E. coli* detection.

Figure 2. Calibration plot $\Delta R = f (\log C_{E. coli})$ in model suspensions (a), the diagrams showing stability of immunosensor $n = 3$, $P = 0.95$, $C_{E. coli} = 10^3$ CFU/ml spiked in phosphate buffer solution (b).

Table 1. Real sample analysis.

| Sample      | Spiked *E. coli*, CFU/ml | Found *E. coli*, CFU/ml (Covalent immobilization) | Found *E. coli*, CFU/ml (Physical sorption) | Found *E. coli*, CFU/ml (Plate culture counting) |
|-------------|--------------------------|--------------------------------------------------|------------------------------------------|--------------------------------------|
| Drinking water | 0                        | not found                                       | not found                                | not found                            |
|              | $5 \cdot 10^3$           | $(4.93 \pm 0.01) \cdot 10^3$                     | $(5.20 \pm 0.11) \cdot 10^3$             | $4.91 \cdot 10^3$                    |
|              | $5 \cdot 10^4$           | $(5.02 \pm 0.03) \cdot 10^4$                     | $(4.62 \pm 0.19) \cdot 10^4$             | $4.91 \cdot 10^4$                    |
| Milk        | 0                        | not found                                       | not found                                | not found                            |
|              | $5 \cdot 10^3$           | $(5.03 \pm 0.02) \cdot 10^3$                     | $(4.54 \pm 0.25) \cdot 10^3$             | $5.23 \cdot 10^3$                    |
|              | $5 \cdot 10^4$           | $(5.09 \pm 0.02) \cdot 10^3$                     | $(4.74 \pm 0.15) \cdot 10^3$             | $5.21 \cdot 10^4$                    |
To evaluate the work of the proposed immunosensor in real samples, objects such as drinking water and milk were selected (Table 1). In the initial samples, no *E. coli* was detected, after which they were supplemented with *E. coli* with a final concentration of $5 \times 10^3$ CFU/ml and $5 \times 10^4$ CFU/ml in the analysed sample. The plate culture counting method was used as a method for comparing the operation of the impedimetric sensor. The *E. coli* concentration detected with impedimetric immunosensor was in close agreement (recovery is more than 90%) with the results obtained from conventional plate culture counting method. We observe an improvement in sensitivity when using an impedimetric sensor with covalently immobilized antibodies, which allows us to judge the effectiveness of the chosen strategy of electrode modification.

4. Conclusion
In the work, the efficiency of the approach of oriented covalent immobilization of antibodies based on the CuAAC reaction was evaluated in comparison with the immobilization of antibodies through physical sorption when *E. coli* bacteria were present in the water. The sensor with covalent immobilization of antibodies has a better linearity with a higher detection limit and a higher stability (30 days) when analysing real spiked water and milk samples as the model bacterial suspensions. The detection limit of the immunosensor was estimated as 6.3 CFU/ml, the linear range was $10^3$-$10^6$ CFU/ml ($R^2 = 0.9950$).

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