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APPLICATION OF AN APTAMER AND A REAGENT BASED ON GOLD NANOPARTICLES FOR DETECTION OF *Escherichia coli*

Plates functionalized with an *Escherichia coli* binding aptamer and a reagent based on gold nanoparticles were used for the detection of bacterial cells. Bacteria suspended in samples were caught by specific aptamers immobilized on a plastic plate whereas the gold nanoparticle reagent is an indicator reagent for visualization of the test result. Changes in colour of the gold nanoparticle reagent caused by gold nanoparticle agglomeration were observed with the naked eye. The test was designed for the detection of *Escherichia coli* in water suspensions.

1. INTRODUCTION

Escherichia coli is a bacterium that naturally occurs in the mammalian digestive system to help and to take a part in the synthesis of vitamins and to protect the digestive system against other pathogenic bacteria. Live bacterial cells are excreted in the faeces. Thus, presence of *E. coli* cells in food and drinking water indicates faecal contaminations [1]. All this normally harmless commensal of mammalian organisms needs to become a pathogen capable of causing a range of diseases (from gastroenteritis to extraintestinal infections of the urinary tract, bloodstream, and the central nervous system) as a mobile genetic element [2]. Worldwide reports of those diseases suggest that hundreds of millions people are affected annually. Traditional methods for detection of *E. coli* involving standard clinical microbiological assays are sensitive, inexpensive and give both qualitative and quantitative information on the number and the nature of tested microorganisms, however they are still time consuming – they are characterized by long incubation time on the order of 24–72 h [3–5]. Moreover, diag-

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nostic tests for identification of pathogens very often require additional sample cultivation and visual inspection by means of microscopy, flow cytometry or polymerase chain reaction.

Thus, biosensors, biochips and diagnostic tests are considered promising tools for rapid detection and identification of bacteria. The main concept of development of new diagnostic tools is conversion of a specific analyte recognition event into quantitative or semi-quantitative analytical information.

A well-known analytical procedure, known as enzyme-linked immunosorbent assay (ELISA), uses an antibody for specific analyte detection and enzymatic conversion (enzyme-linked antibody) of a recognition event into a quantitative analytical signal [6]. In numerous analytical techniques, short specific oligonucleotide sequences, known as an aptamer, might be used as an alternative for the antibody. That is why we decided to develop a simple procedure for the detection and identification of bacteria based on aptamers and a colorimetric reaction that occur in the presence of bacterial cells. Aptamers are a special class of nucleic acid molecules that are beginning to be investigated for clinical use. Nucleic acid aptamers are relatively short single-stranded DNA or RNA oligonucleotides which can form secondary and tertiary structures capable of specifically binding proteins or other cellular targets. Aptamers have the advantage of being highly specific, relatively small in size, and non-immunogenic. Thus, *in vitro* selection, high affinity and specificity make them an alternative specific receptor in numerous bioanalytical applications [7–10].

In the proposed solution, an *Escherichia coli* binding aptamer immobilised on a plastic surface was the specific recognition element, whereas a gold reagent composed from gold nanoparticles (AuNPs), gold ions and hydrogen peroxide was the indicator. An aptamer immobilised on a plastic surface is responsible for the specific catching of cells and their concentration in a defined area, whereas the gold substrate is an indicator for peroxidise used for colour results visualization.

2. EXPERIMENTAL

Materials. Salts, molecular biology grade (gold chloride (III), trisodium citrate dihydrate, disodium carbonate, sodium hydroxycarbonate and sodium chloride), nonfat-dried milk bovine, avidin from the egg white, and Luria Broth (LB) medium were purchased from Sigma-Aldrich (Germany). A biotinylated aptamer (selected in our laboratory, data not published) with the sequence: AGCCTCGTCTGTTCT CCCTGGGGTGGGTGGTGGGGTTGCCGGTGGTTGCCAGTGTTGTGGGAAGA CAAGCAGACGTA of the HPLC purity was purchased from Genomed (Poland). Bacterial strains of *Escherichia coli*, *Salmonella Thypimurium* and *Stapyloccocus aureus* were obtained from the Polish Collection of Microorganism, Polish Academy of Sciences, Wroclaw, Poland. 96 well bottom microtiter plates were obtained from VWR (Germany).

Functionalization of 96 well bottom microtiter plates with an aptamer. Avidin (10 μg/cm^3) in 0.1 M bicarbonate buffer (pH = 8.5) was coated on a microtiter plate at 100% humidity and 4 °C overnight. After immobilization of the avidin, the polystyrene surface was treated with 0.1 cm^3 of 2% milk solution in tris-buffered saline (TBS) (50 mM tris-base, 0.9% NaCl, pH 8.4) for 2 h at room temperature for blocking non-specific interaction. After the blocking mixture was removed, the plate was washed twice with TBS, and 0.1 cm^3 of biotinylated aptamer (1 μM) were applied per each well of the microtiter plate and incubated for 1 h. After incubation, the microtiter plate was washed twice with TBS.

Gold reagent. Gold nanoparticles (AuNPs) were prepared using the method described by Turkevich [11]. 95 cm^3 of gold chloride solution (containing 5 mg of gold) was heated on a magnetic stirrer. After boiling, 5 cm^3 of 1% sodium citrate were added. The obtained mixture was heated for the next 10 min.

Gold reagent solutions were prepared from AuNPs solution, 3% hydrogen peroxide solution, gold chloride solution, and water in adequate proportions (Table 1).

Table 1

Composition of gold reagent solutions

No.	AuNPs [μl]	0.005% Au^{3+} chloride solution [μl]	3% H_2O_2 solution [μl]	Distilled water [μl]
1	500	200	250	50
2	500	150	250	100
3	500	250	250	0

Bacterial samples. Bacterial strains of *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus* were cultured on an LB-medium overnight. Then 1 cm^3 of bacteria culture was harvested by centrifugation at 3000 rpm for 15 min. The bacteria were washed with 1 cm^3 of saline solution (0.9% NaCl) and diluted to optical density of 0.509 to obtain stock solutions. For determination of the detection limit and test specificity, a series of 2-fold serial dilutions of bacterial stock suspensions were prepared from the stock cultures and physiological solutions.

The number of bacteria in stock solutions was determined by surface plating of 0,1 cm^3 of 10-fold serial dilutions on LB agar. After 24 h incubation at 37 °C, colonies were calculated and the amounts of bacteria in 2-fold serial dilutions were estimated.

Procedure for sample analysis by means of the gold reagent test. 50 μl of prepared liquid samples were added to wells on the microtiter plate and incubated for 30 min at room temperature. After incubation, liquid was removed and the microtiter plate was

washed with 50 µl of deionized water. After washing, 50 µl of gold reagent was added to each well of the microtiter plate and incubated at room temperature for 5 min. Spectra of the reagent were analyzed by means of Nanodrop ND 1000 spectrophotometer.

3. RESULTS

A diagnostic test based on an *Escherichia coli* binding aptamer and a gold reagent was developed for detection of specific bacteria. An aptamer immobilized on a polystyrene surface by means of streptavidin-biotin complex performs the role of a specific molecular receptor which specifically recognizes bacterial cells (Fig. 1a, b), whereas the gold reagent used in the test is the colour marker strongly sensitive for the presence of bacteria (Fig. 1c).

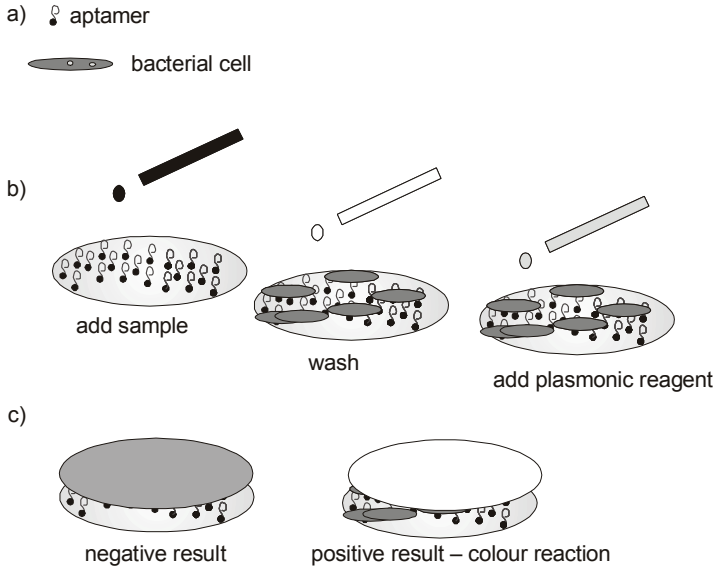

Fig. 1. Developed diagnostic test for detection of *Escherichia coli*:
a) aptamer coated plate, b) analytical procedure, c) result interpretation

For the test development purposes, the composition of the gold reagent was optimized. Optimization of the diagnostic test was performed by using two samples: one containing sterile physiological solution, and other contaminated with bacterial cells (*E. coli*) of the optical density (λ = 600 nm) approximately of 0.509. The greatest colour changes were observed for the composition of the gold reagent No. 1 (Table 1). The results are presented in Fig. 2a. Under this condition, the greatest colour changes as well as the greatest variations in the absorbance (in the range of 400–650 nm) were observed for the results presented in the figures (Figs. 2a–c, Fig. 6A–C).

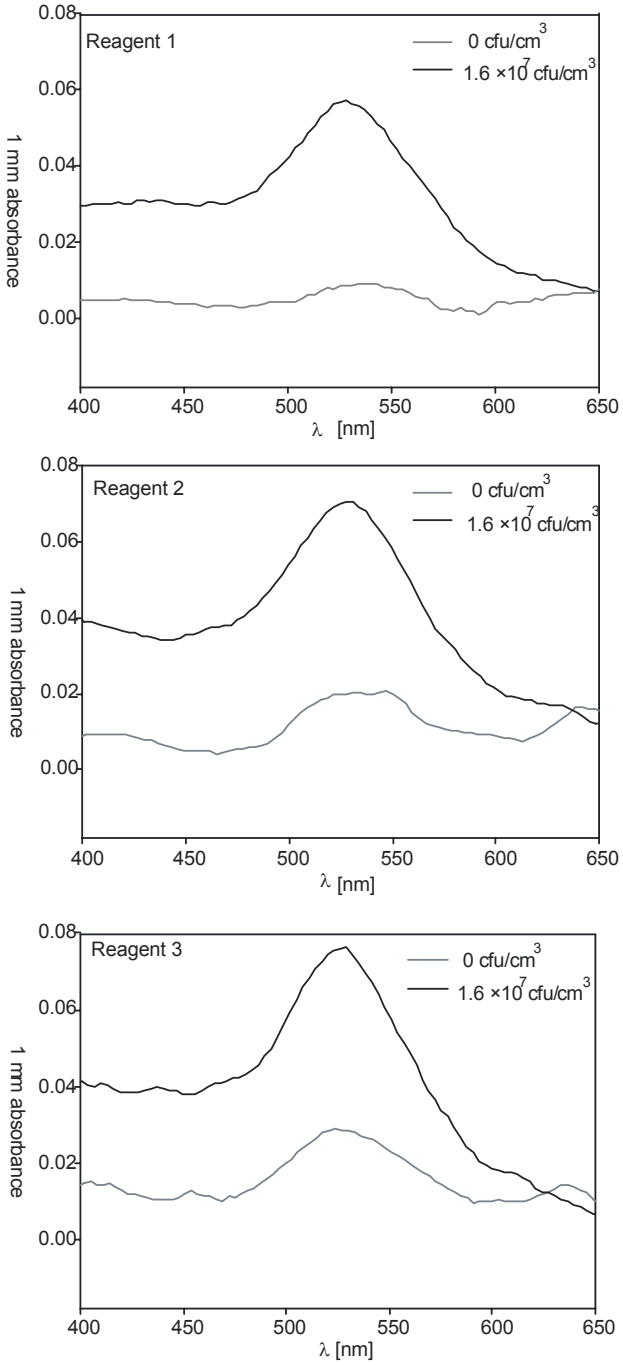

Fig. 2. Diagnostic test optimization: spectra of gold reagent
in the range of 400–650 nm and colour reaction for the tested reagent

Samples of gold reagent after reaction were observed by means of transmission electron microscopy. The obtained results demonstrated that the colour reaction during the detection of bacterial cells was caused by changes in the structure of gold nanoparticles. Gold nanoparticles present in the reagent can agglomerate and increase their diameter from approximately 20 nm to 100–150 nm (Fig. 3).

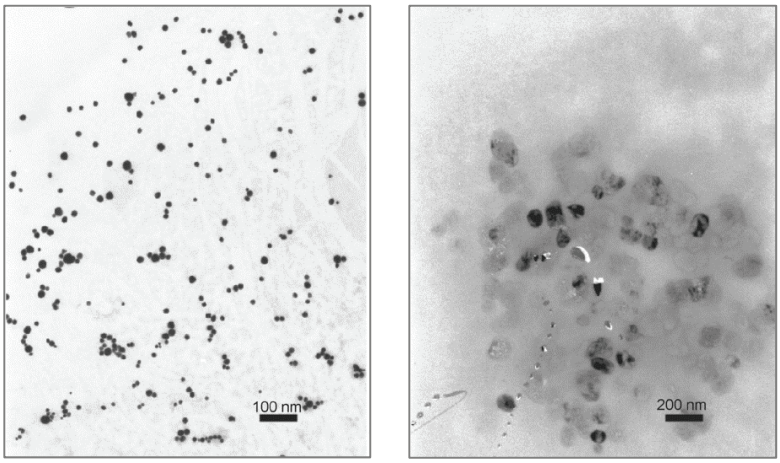

Fig. 3. Structure of gold nanoparticles in gold reagent after tests of samples (left) and sterile physiological solution (right) contaminated with bacterial cells

A diagnostic test was used for the analysis of samples contaminated with bacterial strains of *E. coli*, *Staphylococcus aureus* and *Salmonella typhimurium*.

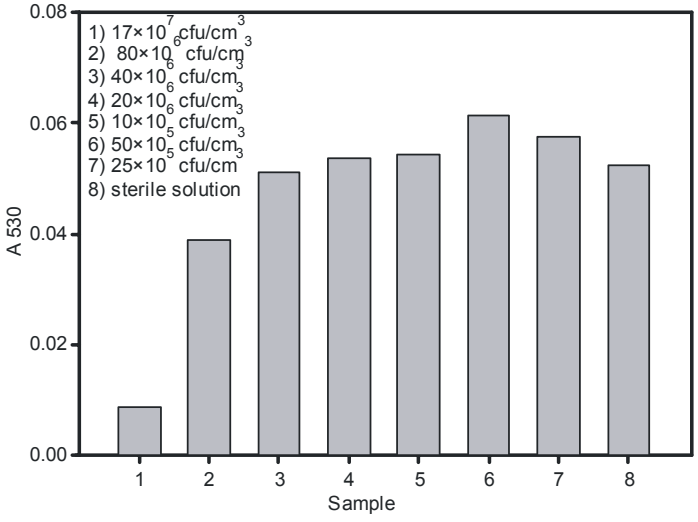

Fig. 4. Absorbance analysis for *Escherichia coli* samples at 530 nm

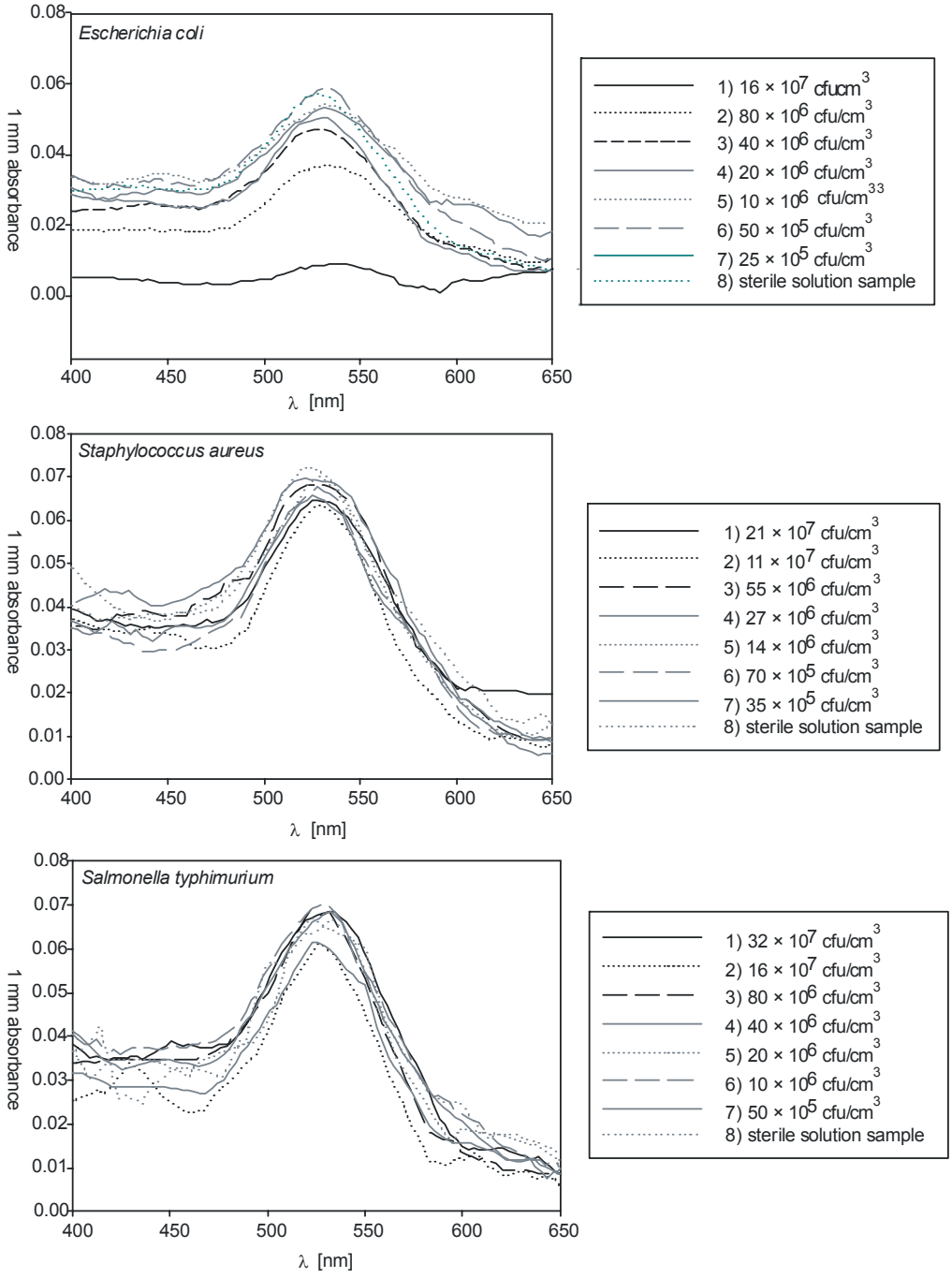

Fig. 5. Absorbance spectra in the range of 400–650 nm
and colour reaction for samples contaminated with bacteria cells

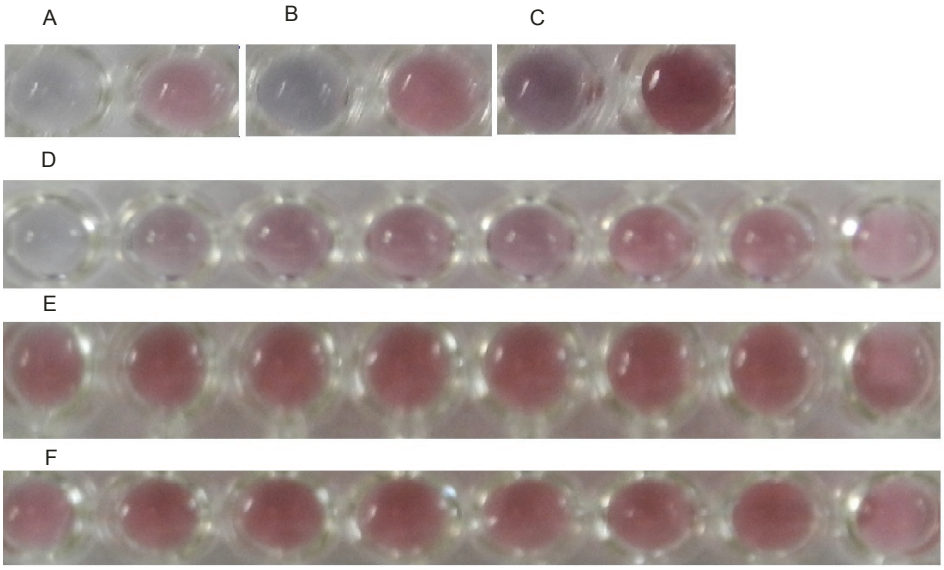

Fig. 6. Colour reaction: A, B, C – composition of the optimalization pigment (cf. Table 1), D – analysis of samples contaminated with *Escherichia coli*, E – analysis of samples contaminated with *Staphylococcus*, F – analysis of samples contaminated with *Salmonella typhimurium*

The performed analysis demonstrated an intensive colour reaction for samples contaminated with *Escherichia coli* cells. The detection limit developed by the diagnostic procedure is around 40×10^6 cfu/cm^3. For samples contaminated with *Stapylococcus aureus* and *Salmonella typhimurium* no colour reaction was observed. Specific detection was obtained by using an *Escherichia coli* binding aptamer which specifically recognizes bacterial cells on a polystyrene coated microtiter plate. According to the measured spectra, we observed strong changes in the absorbance (at 530 nm) for samples containing more than 40×10^6 cfu/cm^3 (Figs. 4–6).

4. CONCLUSION

A simple diagnostic test for bacterial cell (*Escherichia coli*) detection was developed. Its results might be analyzed with the naked eye and also by means of a spectrophotometer to measure absorbance. The obtained detection limit enables the use of this test for identification of *Escherichia coli* colonies cultured on a non-specific solid growth medium. Specificity of the test is limited by the aptamers used as specific elements recognizing bacterial cells. Thus, by changing aptamer specificity (for example, an aptamer specific for other microorganism), we can probably obtain a test for the detection other bacterial species.

The colour reaction that occurs during detection of bacterial cells might be caused by enzymatic bacterial activity that stimulates gold nanoparticle agglomeration induced by concentration changes in the detection reagent components or by releasing salts from bacterial cells during their lysis under the presence of a coloured reagent.

ACKNOWLEDGEMENTS

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