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***Highlights (for review)**

- Ultra-rapid colorimetric biosensor for the detection of *E. Coli was developed*
- The designed colorimetric biosensor was based on the detection of the amidolytic activity of *E. Coli* protease
- The detection limit of this colorimetric sensor were 12 CFU mL^{-1} in broth samples and 30-300 CFU mL^{-1} in spiked complex food matrices.

July 28th 2016

Professor A.P.F. Turner

Europe- Editor- of J. of Biosensors and Bioelectronics

Re: Submission of an original research as a Research Article to Biosensors and Bioelectronics

Dear Prof. Turner,

Please find enclosed the manuscript entitled "Paper-Based Magnetic Nanoparticle-peptide probe for Rapid and Quantitative Colorimetric Detection of *Escherichia coli* O157:H7 ", by Ghadeer A.R.Y. Suaifan, Sahar Alhogail, Mohammed Zourob, submitted as a research article to Biosensors and Bioelectronics for consideration of publication.

In this manuscript, we developed a novel, ultra-rapid colorimetric biosensor for the detection of *E. Coli*. The designed colorimetric biosensor was based on the detection of the amidolytic activity of *E. Coli* protease using a specific protease substrate which covalently interacts with black nano-magnetic beads and with a gold sensor. This interaction will mask the golden color of the senor platform and turns it into black. Upon proteolysis of the substrate-magnetic beads, a visible colorimetric readout of the sensor golden color using the naked eye is directly proportional with *E. Coli* protease concentration. The detection limits of this colorimetric sensor were12 CFU mL^{-1}in broth samples and 30-300 CFU mL^{-1} in spiked complex food matrices. In conclusion, this approach permits the use of a disposable biosensor chips that can be mass-produced at low cost and can be used not only by the food manufacturing and companies but also by regulatory agencies for better control of potential health risks associated with the consumption of contaminated foods.

To my knowledge as expert in Biosensors and lab on a chip and editor of 6 books in the area, the present work is the first report of developing simple and highly sensitive sensing platform for *E. Coli*. We believe that our findings could be of interest to the readers of J. Biosensors and Bioelectronics.

We hope that you and the editorial board will agree on the interest and significance of the present work.

Yours sincerely,

Prof. Mohammed Zourob

Biosensors BioMEMS and Bionantotechnology Lab (BBBL)

Paper-Based Magnetic Nanoparticle-peptide probe for Rapid and Quantitative Colorimetric Detection of *Escherichia coli* O157:H7

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ABSTRACT

There is a critical and urgent demand for a simple, rapid and specific qualitative and quantitative colorimetric biosensor for the detection of the food contaminant *Escherichia coli* O157:H7 (*E. coli* O157:H7) in complex food products due to the recent outbreaks of food-borne diseases. Traditional detection techniques are time-consuming, require expensive instrumentation and are labour-intensive. To overcome these limitations, a novel, ultra-rapid visual biosensor was developed based on the ability of *E. coli* O157:H7 proteases to change the optical response of a surface-modified, magnetic nanoparticle-specific (MNP-specific) peptide probe. Upon proteolysis, a gradual increase in the golden color of the sensor surface was visually observed. The intensification of color was correlated with the *E. coli* O157:H7 concentration. The color change resulting from the dissociation of the self-assembled monolayer (SAM) was detected by the naked eye and analysed using an image analysis software for the purpose of quantitative detection. This biosensor demonstrated high sensitivity and applicability, with lower limits of detection of 12 CFU mL^{-1} in broth samples and 30-300 CFU mL^{-1} in spiked complex food matrices. In conclusion, this approach permits the use of a disposable biosensor chip that can be mass-produced at low cost and can be used not only by food manufacturers but also by regulatory agencies for better control of potential health risks associated with the consumption of contaminated foods.

Keywords

Colorimetric Biosensor; *Escherichia coli* O157:H7 ; Magnetic-nano Particles; Food-borne illness.

1. Introduction

Food-borne illnesses linked to the consumption of freshly and minimally processed food cause wide discomfort and economic loss for many people each year(Beuchat 2002; Shriver-Lake et al. 2007; Tauxe et al. 1997). In general, washing fresh fruits and vegetables with cold water removes dirt lingering on the surface but does not remove pathogens that may exist inside. Fresh products might be contaminated with microbial pathogens upon contact with contaminated water or manure from the soil(Ingham et al. 2004; Song et al. 2006). A current report shows that the United States Department of Food Safety and Inspection Services (FSIS) spends over half a billion dollars annually on food inspection for bacterial contaminants. Approximately 73000 cases of food-borne infections occur every year, of which 2–7% result in a severe complication called hemolytic uremic syndrome (HUS), as reported by the Center for Disease Control and Prevention(CDC, *Escherichia coli* O157:H7, 2006, www.cdc.gov/ncidod/dbmd/diseaseinfo/*Escherichia coli* g.htm.) .

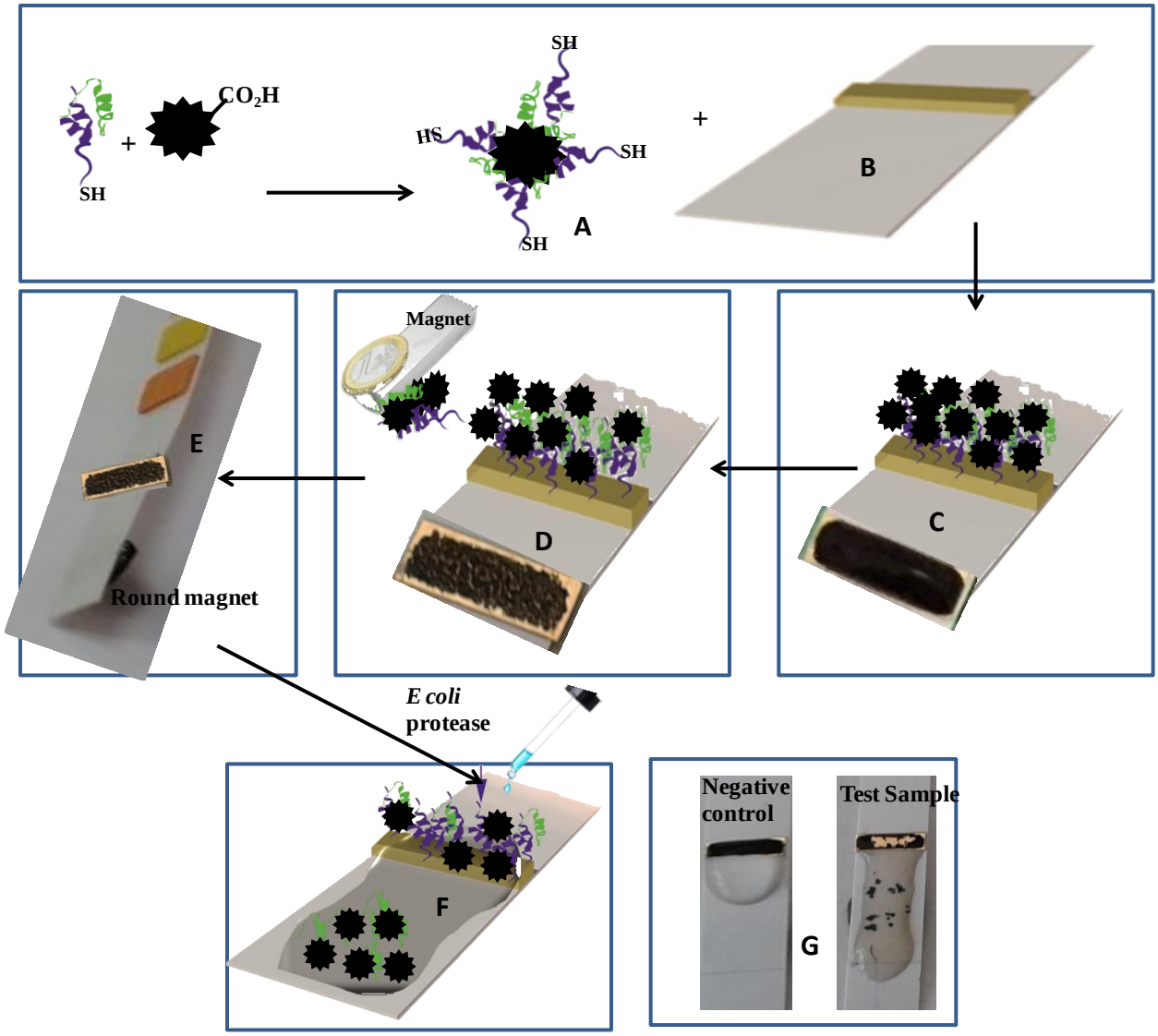
It is imperative to develop techniques for the identification of food-borne pathogens after food ingestion in order to ensure proper disease treatment. However, it is more important to prevent the occurrence of infections. One way of doing this is to identify contaminated food products prior to ingestion, preferably before they are distributed to grocery stores, restaurants and manufacturing facilities. This could be achieved through the development of rapid detection devices applicable at the retail level in order to protect the consumer.

There are several techniques that are conventionally used to detect pathogenic microbes: the culturing method is commonly used but remains problematic due to the lack of phenotypic characteristics to distinguish between generic pathogens(Gould et al.); other techniques such as DNA-based assays are currently the most specific and sensitive tests available as confirmatory

assays(Call 2005; Deisingh and Thompson 2004; Simpson and Lim 2005; Uttamchandani et al. 2009). However, these assays require a long time (up to 48 h) to yield results due to the extensive list of steps required for sample pretreatment, including enrichment and extraction. Additionally, highly trained staff are required to perform these assays and to analyse the assay results. Further, polymerase chain reaction (PCR) inhibitors such as humics are commonly present in complex food matrices and must be removed prior to analysis(Shriver-Lake et al. 2007) to avoid faulty results. Other immunoassay-based methods have been employed recently to achieve high sensitivity while avoiding many of the disadvantages of DNA-based assays. However, these methods are less specific than DNA-based assays(Shriver-Lake et al. 2007). Presently, researchers are attempting to improve the specificity of these assays through the employment of antibodies and by exploiting changes in optical properties, such as transmitted light, surface plasmon or acoustic waves resulting from antibody–antigen binding(Berkenpas et al. 2006; Deisingh and Thompson 2004; Subramanian et al. 2006). Additionally, some of these assays use fluorescent labels to provide an optical signal(DeCory et al. 2005; Ho and Hsu 2003; Nyquist-Battie et al. 2004). However, the application of these assays is limited by the inconsistency and high variability of the target DNA labelling. In addition, these assays require expensive and non-portable scanners for data acquisition and analysis(Call 2005; Kuck and Taylor 2008; Vora et al. 2008). Other alternative detection methods such as enzyme-linked immunosorbent assays (Abuknesha and Darwish 2005), spectrometric techniques(Siripatrawan and Harte 2007) and electrochemical techniques(Gehring and Tu) have also been employed for the detection of food-borne pathogens. With these methods, a detection range of 10^3 to 10^5 cells mL^{-1} was achieved without enrichment and was as low as 1 $CFUmL^{-1}$ with enrichment. Nevertheless, this low limit of detection is valid only for the detection of microorganisms that can be grown on specific

media. (Kuck et al. 2008) used a particular colorimetric assay for the detection of *E. coli*. This assay involves unstable reagents that require a temperature-controlled environment with variable development times, leading to an increase in the nonspecific background(Kuck and Taylor 2008). Notably, none of the above-mentioned detection methods fully satisfy the detection performance criteria because they are sophisticated, costly in terms of both time and money and require cumbersome preparation steps.

This study aimed at the development of simple, sensitive, specific and cost-effective strip-format colorimetric biosensor for the qualitative and quantitative detection of *E. coli* O157:H7 in food products at the retail level or even at home. This biosensor is based on the use of the proteolytic activity of *E. coli* proteases as a biomarker. Specific *E. coli* O157:H7 proteases peptide substrate was labelled with magnetic nanoparticles (MNPs) and then immobilized on a gold sensing platform to provide a hand-held peptide probe in strip format. Upon application of *E. coli* O157:H7 proteases, the MNP-peptide moiety leaves the gold sensing platform, resulting in a color change. This method is simple, and the signal change can be detected by the naked eye. Therefore, the sensor holds potential for the specific detection of *E. coli* O157:H7 (Scheme 1).



Scheme 1. Schematic and actual image of the colorimetric assay designed to specifically detect *E. coli* O157:H7. (**A)** *E. coli* O157:H7-specific peptide substrate labelled with magnetic-nanoparticles (MNPs). (**B)** Gold biosensing platform. (**C)** Immobilization of the sensing monolayer (SAM). (**D)** Sensor platform under the effect of an external magnet to remove immobilized functionalized MNPs. (**E)** Side view of the sensing platform showing the round magnet fixed on the strip back. (**F)** Schematic view of the *E. coli* O157:H7 biosensing step. (**G)** Actual application of the test *E. coli* O157:H7 proteases and a negative control on the paper-based biosensor.

2. Experimental

2.1. Materials and Reagents

Carboxyl-terminated beads (50 nm in diameter), *N*-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDC) and plastic pH indicator strips were purchased from Sigma Aldrich (Dorset, UK). Self-adhesive magnet sheets were purchased from Polarity Magnets Company (UK). The peptide for *E. coli* O157:H7, NH_2-Ahx-KVSRRRRRGGDKVDRRRRRGGD-Ahx-Cys, was synthesized by Pepmic Co., Ltd (Suzhou, China). The self-adhesive tape was purchased from Whatman (London, U.K). Nutrient agar and nutrient broth were purchased from Oxoid (Amman, Jordan). Sterile filters (0.22 µm) were purchased from Millipore (Amman, Jordan). The wash/storage buffer (10 mM Tris base, 0.15 M sodium chloride, 0.1% (w/v) bovine serum albumin, 1 mM ethylenediaminetetraacetic acid, 0.1% sodium azide, pH 7.5) and the coupling buffer (10 mM potassium phosphate, 0.15 M sodium chloride, pH 5.5) were prepared from chemicals of analytical grade.

2.2. E. coli O157:H7-specific peptide substrate labelled with MNPs

The MNP suspension (1 mL) was mixed with the peptide (1.0 mg / mL), the coupling agent EDC (0.57 mg / mL) and NHS (12 µg / mL). The mixture was shaken gently at room temperature for 24 h. The uncoupled peptides were removed by washing the beads three times with a wash buffer. Finally, the beads were stored at 4ºC in a storage buffer, as shown in Scheme 1A.

2.3. Preparation of the gold sensor platform

Self-adhesive tape was coated with a thin layer of gold at the Canfield University Engineering Department. Following this, a narrow piece (~1.5-2 X 3 mm) was cut and stacked over a plastic strip providing physical support, as shown in Scheme 1B.

2.4. Immobilization of the sensing monolayer (SAM)

The gold sensing surface was mounted with the MNP-peptide solution and allowed to stand at room temperature for 1 h. Following this, an external magnet was passed over the immobilized sensor to remove any unattached MNP-peptide moieties (Scheme 1D). Then, a round paper magnet was fixed on the back of the strip at a distance of 2-3 mm below the gold sensing platform, as shown in Scheme 1E (side view).

2.5. Bacterial strain culturing

E. coli O157:H7, *Listeria monocytogenes* ATCC 19115, *Staphylococcus aureus* (*S. aureus*) ATCC 25923 and *Pseudomonas. aeruginosa* (ATCC 15692), purchased from Sigma Aldrich UK, were grown in 5 ml of nutrient broth and incubated at 37ºC for 18 h. The primary bacterial culture (PBC) was then enumerated using the serial dilution and plate counting method. Consequently, each bacterial strain was pelted by centrifugation at 3000 × g for 10 min. The supernatant was then filtered using a sterile 0.22-µm syringe filter to obtain the crude protease solution. The bacterial protease concentration was associated with the bacterial concentration and referred to as a colony forming unit (CFU).

2.6. Food matrix spiking and protease preparation

Food samples (ground beef, turkey sausage, lettuce and milk) were obtained from a local grocery market. Ground beef and turkey sausage were homogenized with sterile water at a 1:5 (w:v) ratio in a blender for 1 min and then rocked at room temperature for 2 h. Lettuce leaves were rinsed with vegetable detergent and phosphate-buffered saline (PBS PH 7.4) and then cutted into 4-cm^2 pieces using sterile scissors to remove any potential adulterates on the leafy surface.

Next, the PBC stock of *E. coli* O157:H7 was added to food matrices to create 10-fold dilution samples. The spiked matrices were allowed to incubate at room temperature for 2 h and were then

clarified by centrifugation (5 min, 2000 rpm)(Medina 2003). The supernatant was then collected, centrifuged (10 min, 10.000 X g) to sediment bacteria, filtered through a 0.22-µm sterile filter and tested directly. The positive control was prepared by creating a 10-fold dilution from the PBC stock of *E. coli*. The negative control containing no bacteria was also prepared in a similar way and tested to check the effect of the food matrix content on the biosensor. Different *E. coli* O157:H7 concentrations in CFU/mL were determined by placing 10-fold serial dilutions of the positive control (PBC) on the agar plates. The experiments were conducted in triplicate.

2.7. Biosensing of E. coli O157:H7 proteases

The solution of *E. coli* O157:H7 proteases was down-streamed over the immobilized sensing platform. During the enzymatic cleavage reaction, the permanent round magnet stacked at the back of the sensor stripe (Scheme 1E) attracts the cleaved MNP-peptide moiety, prompting a visual observation for qualitative evaluation of the tested sample (Scheme 1F and 1G). The experiments were conducted in triplicate.

2.8. Quantitative measurement of the color change

Because the developed biosensor was intended to be used by visual observation, the color change (black to golden) as a result of the proteolytic activity of *E. coli* O157:H7 proteases was easily viewed by the naked eye. Additionally, the intensification of golden color increased with increasing the concentration of *E. coli* O157:H7 PBC (1.21×10^6 CFU/ mL, 1.21×10^5 CFU/ mL, 1.21×10^4 CFU/ mL, 1.21×10^3 CFU/ mL, 1.21×10^2 CFU/ mL, 121 CFU/ mL), as shown in Fig. 1A and 1B. The color change was quantified by a simple method using the ImageJ program (a public-domain, Java-based image processing program developed at the National Institute of Health)(Collins). ImageJ is a simple, practical and freely downloadable program that can be used on any computer with Java 5 or a virtual machine(Rajwa et al.). Images were

obtained by direct photography of the biosensor using smart phone camera after protease application and were saved in JPEG format. These images were processed through the red channel, which shows lower background levels. The black-colored peptide-MNP moieties immobilized on the gold sensing platform were highlighted by the threshold function with a red color. The intensity color of the black peptide-MNPs area on the sensor surface was highlighted by the color threshold function with a red color and then the area was measured(Fig. 2C-II). Next, the cleaved MNPs attracted by the backwards magnet were also highlighted and measured (Fig. 2C-III).

The total amount of peptide-MNPs immobilized on the biosensor surface = Black MNPs intensity colored area on the sensor surface after protease application + Cleaved MNPs intensity color area.

The percentage of MNPs cleaved = Cleaved MNPs intensity color area / Total amount of peptide-MNPs immobilized on the biosensor surface Intensity color area.

However, as the threshold adjustment process is subjective, the analysis was performed by two experimenters who followed the proposed protocol. The two experimenters measured the highlighted red-colored area. The correlation between different proteases produced by different PBC and the percentages of peptide-MNPs moieties cleavage is shown in Fig. 2C.

The selected red-colored areas were plotted with respect to different *E. coli* O157:H7 concentrations to obtain a standard curve. However, as the threshold adjustment process is subjective, the analysis was performed twice by two experimenters who followed the proposed protocol. The two experimenters measured the highlighted red-colored area. A highly accurate standard curve was obtained for the quantitative evaluation, as shown in Fig. 1C. All experiments were conducted in triplicate.

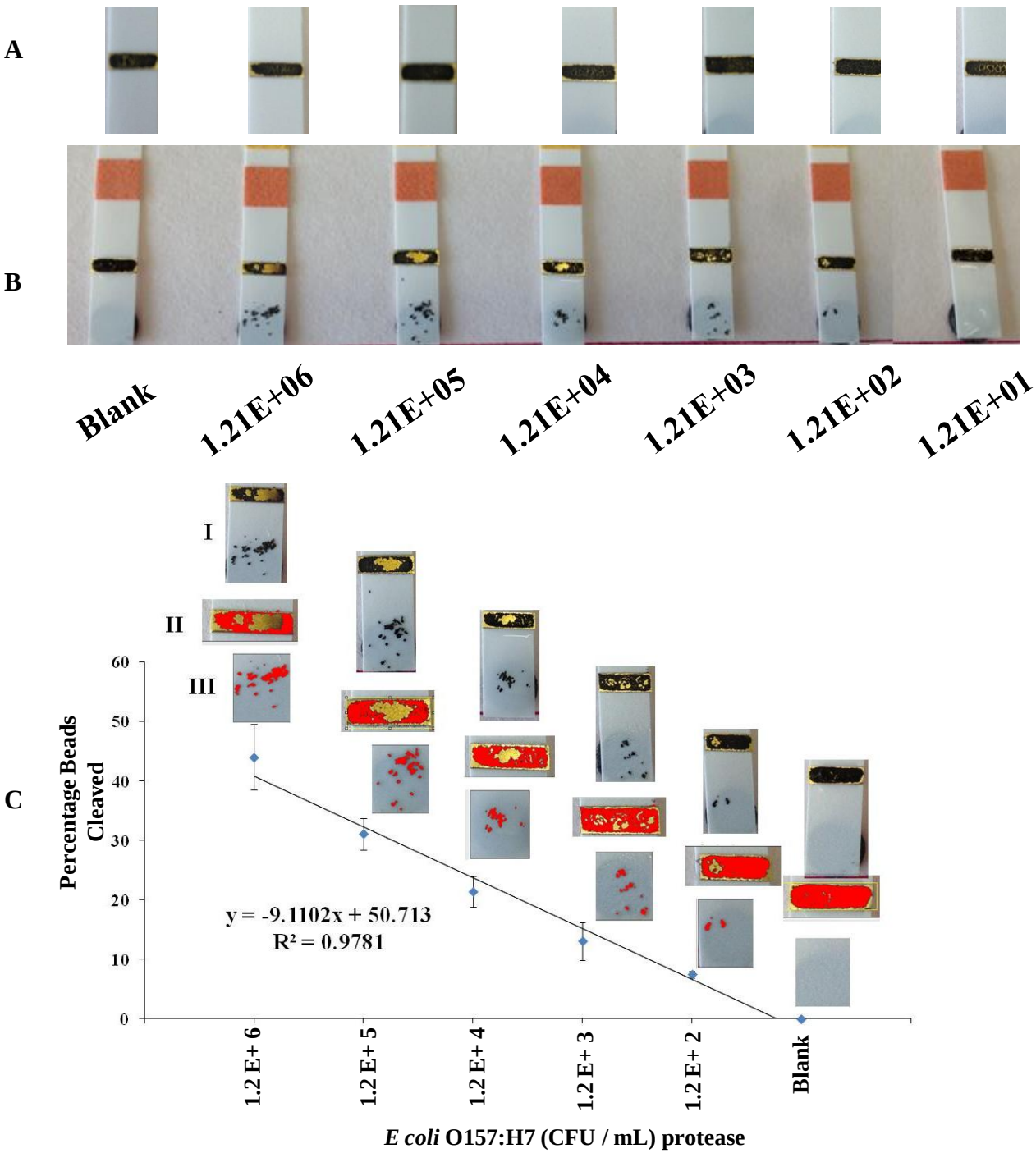


Fig. 1. Colorimetric *E. coli* sensor probe (*E. coli*-specific peptide substrate covalently bound to magnetic nanobeads). (A) Biosensor chip immobilized with magnetic bead–specific *E. coli* O157:H7 peptide substrate. (B) Immobilized biosensor under the effect of different *E. coli* O157:H7 protease concentrations. (C) Direct correlation of different *E. coli* O157:H7 proteases proteolytic activity and the percentage of peptide-magnetic beads moieties cleaved. I Biosensor JPG photo. II The intensity color of the black peptide-MNPs area on the sensor surface as prcessed by imageJ software. III Cleaved MNPs attracted by the backwards magnet as processed by imageJ software.

3. Results and discussion:

The detection and identification of the food-borne pathogen *E. coli* O157:H7 relies on the culturing methodology, evaluation of colony morphology, identification of characteristic nucleic acid sequences, biochemical markers and antigenic signatures associated with the microbe(Ivnitski et al. 1999; Marusov et al. 2012). However. these assays are complex, tedious, labour-intensive and time-consuming(Siripatrawan et al. 2006).

Recently, colorimetric assays have gained importance for clinical and environmental diagnosis because of their simplicity and low cost. These assays are convenient because of the ease of qualitative detection using the naked eye without the need for a specific apparatus. In comparison, quantitative detection of the color change is usually accomplished by using instruments such as spectrophotometers, epifluorescence microscopes and luminescence counters. Thus far, a number of colorimetric methods have been reported for the detection of *E. coli* O157:H7. However, these assays require several preparative steps as well as sophisticated instrumentation and trained personnel.

Colorimetric disposable biosensors, which identify specific *E. coli* O157:H7 proteins, such as proteases, as biomarkers (Hanumegowda et al. 2005; Zhou et al. 2006), would provide a detection tool for use in retail stores or at home to detect contamination by the food-borne pathogen *E. coli* O157:H7. Therefore, this work reports the development of a rapid, strip-format biosensor that uses MNPs, demonstrating the implementation of nanotechnology to the field of biosensors for the specific detection of *E. coli* O157:H7.

3.1. Sensor fabrication

The designed sensing platform is based on the detection of the proteolytic activity of *E. coli* O157:H7 proteases on a specific peptide substrate. This substrate is covalently attached at one end to a carboxyl-terminated MNP, while the other end possesses an S-S linkage joining it to a gold sensor surface (Scheme 1 A). A color change occurs upon proteolysis of the peptide-MNP moieties, indicating the presence of the contaminating *E. coli* O157:H7, as described in Scheme 1.

The biosensor peptide probe was prepared using a specific *E. coli* O157:H7 peptide substrate sequence (NH_2-Ahx-KVSRRRRRGGDKVDRRRRRGGD-Ahx-Cys)(Bayliff et al. 2012), with an Ahx-residue linker on either terminus of the peptide. The N-terminus of the peptide was attached to the MNP. The cysteine residue at the C-terminus allows gold-sulfur interaction for the formation of the peptide-MNP complex SAM on the gold sensing platform, as shown in Scheme 1A, B and C (Esseghaier et al. 2014; Suaifan et al. 2012, 2013). The amount of the peptide substrate required for bead functionalization was optimized in previous work to achieve optimal monolayer performance and stability.

The peptide-MNPs solution was mounted over the gold sensing platform and left intact at room temperature until dry to ensure proper immobilization of the SAM. Following this, the golden color of the biosensor turned black as shown in Scheme 1C. Next, an external magnetic field was applied over the SAM to remove any peptide-MNPs moieties that were not immobilized (Scheme 1D). After which, a round paper magnet was stacked at the back of the strip (Scheme 1E). The sensor probe was then ready for *E. coli* O157:H7 detection using the *E. coli* O157:H7 proteases as a biomarker (Scheme 1E).

3.2. Sensor testing

The proteolytic activity of the *E. coli* O157:H7 proteases was analysed by dropping the protease solution (1.21×10^6 CFU/mL) over the functionalized gold sensor (Scheme 1F and G). The cleavage of the peptide-MNP moieties from the sensor surface was accelerated by the round paper magnet stacked at the back of the plastic physical support and beneath the sensing surface revealing the golden color visible to the naked eye, as shown in Fig. 1F and G.

To gain insight into the applicability of the sensor for quantitative detection of *E. coli* O157:H7, different concentrations (1.21×10^6 CFU/ mL, 1.21×10^5 CFU/ mL, 1.21×10^4 CFU/ mL, 1.21×10^3 CFU/ mL, 1.21×10^2 CFU/ mL and 12 CFU/ mL) of crude *E. coli* O157:H7 protease solutions were dripped over the black-colored sensing platform. A gradual visible increase in the golden area was observed, correlated with the concentrations of the *E. coli* O157:H7 protease solutions. This is due to the proteolytic activity of *E. coli* O157:H7 proteases, which results in the dissociation of the peptide-MNP moieties. Additionally, a quantitative analytical approach was developed for the *E. coli* O157:H7-specific detection using ImageJ software (NIH, Bethesda, Maryland, USA; http://rsb. info.nih.gov/ij). A standard curve (R^2= 0.9557) was obtained as a tool for the quantitative detection of *E. coli*.

3.2.1 Response time

The test response time (using a digital stopwatch with naked eye inspection) was defined as the time needed for the *E. coli* O157:H7 protease solution to dissociate the peptide-MNP SAM, followed by attraction of these moieties to the magnet stacked at the back of the biosensor. The response time was found to be 30 sec, which is quite a rapid response. Sensor reproducibility was examined by performing tests under the same experimental conditions in triplicate.

3.2.2 Sensor Stability

To evaluate the stability of the biosensor under storage, the ready-to-use immobilized sensing strips were stored in empty petri dishes at 4ºC. Every week, three strips were used to detect a known stock of *E. coli* O157:H7 protease solution. In every experiment, a negative control (no *E. coli* O157:H7 protease) was tested to ensure that the SAM dissociation could be attributed to the *E. coli* O157:H7 proteolytic activity. The designed biosensor showed adequate long-term stability for up to six months.

3.3. Specificity Testing

To assess the specificity of the biosensor, proteases of different food-contaminating pathogens, including *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, were tested over the designed *E. coli* O157:H7 biosensor. No disruption of the SAM layer and no read-out for color change was observed by the naked eye, showing sufficient detection specificity, as shown in Fig. 2. The experiments were conducted in triplicate.

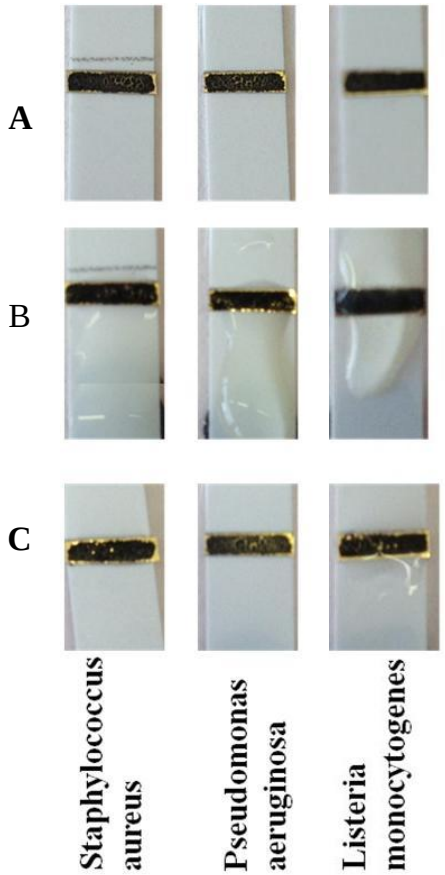


Fig. 2. Colorimetric *E. coli* protease sensor probe under the effect of other proteases. (A), Biosensor chip functionalized with magnetic bead–specific *E. coli* peptide substrate. (B) Functionalized biosensor incubated with different proteases. (C) Functionalized biosensor after incubation with different proteases.

3.4 Biosensor detection of E. coli-spiked food matrices

Because our developed colorimetric biosensor was able to detect *E. coli* O157:H7, we then determined its applicability for the detection of *E. coli* O157:H7 in contaminated food products. Thus, fresh PBC of *E. coli* O157:H7 was spiked into different food matrices, including ground beef, turkey sausage, lettuce and milk. First, 9 mL of various samples of different food products was spiked with 1.0 mL of fresh PBC of *E. coli* O157:H7 at eight bacterial concentrations ranging from 2.9×10^8 CFU / mL to 2.9×10^1 CFU / mL. A positive control was also prepared by spiking 9 mL

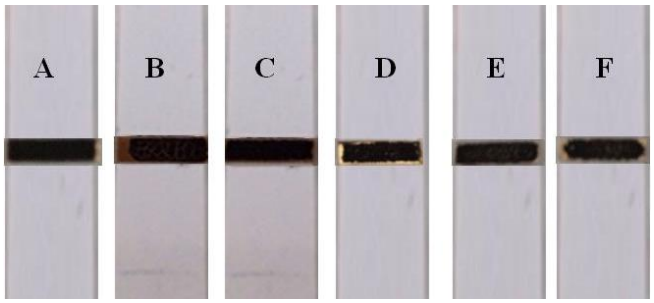
of water with 1 mL of *E. coli* PBC. A negative control, containing no bacteria, was prepared and tested to check the effect of the content of food matrices on the sensor.

The positive control and the spiked samples were allowed to incubate at room temperature for 2 h, after which *E. coli* O157:H7 was enumerated. The spiked samples were then centrifuged, and the supernatant was examined on the developed biosensor. A clear and steady increase was observed in the gold-colored area of the sensing platform in relation to the bacterial concentration. The developed biosensor displayed the ability to detect *E. coli* O157:H7 spiked in different food matrices, with a lower detection limit of 30 CFU/ mL in ground beef, turkey sausage and lettuce and a lower detection limit of 300 CFU /mL in milk, as shown in Fig. 3(A-D). Thus, the developed biosensor is suitable for the detection of *E. coli* O157:H7 food contamination at the retail level and even at home.

In view of our results, the developed sensor was able to exhibit high sensitivity without the use of any instrument and with a short detection time of 30 sec. It must be noted that although different colorimetric sensors have been developed previously, these methods do not fully satisfy the detection performance criteria because they are sophisticated and involve cumbersome preparative steps. For example, Beatriz Quinõnes *et al.*(Quinones et al. 2011) designed a colorimetric detection method for *E. coli* O157:H7 based on the use of DNA microarrays in combination with photopolymerization. The detection limit range was 100–1000 CFU/mL. Additionally, Patricio *et al.*(Villalobos et al. 2012) reported the use of polymerized lipid vesicles as a colorimetric biosensor for detecting the presence of *E. coli* O157:H7 in water, with a detection limit of over 10^8 CFU. The detection sensitivity was improved by the use of a cytochrome C peroxidase-encoding bacteriophage (limit 10^7 CFU/ mL)(Hoang et al. 2014) or by the application of an aptamer-based technique (aptasensor) (limit 10^4-10^8 CFU/mL)(Wu et al.

2012). However, to the best of our knowledge and accessibility to literature resources, the lowest limit of detection for *E. coli* O157:H7 is 7 CFU/mL in a method that involves the measurement of absorbance at 652 nm. This low sensitivity was achieved using functionalized Au@Pt nanoparticles with a detection time of approximately 40 min(Su et al. 2013). In comparison, the sensor reported in this work was able to specifically detect *E. coli* O157:H7 with a lower limit of detection of 12 CFU/mL without the use of any instrument and in 30 sec.

(1)



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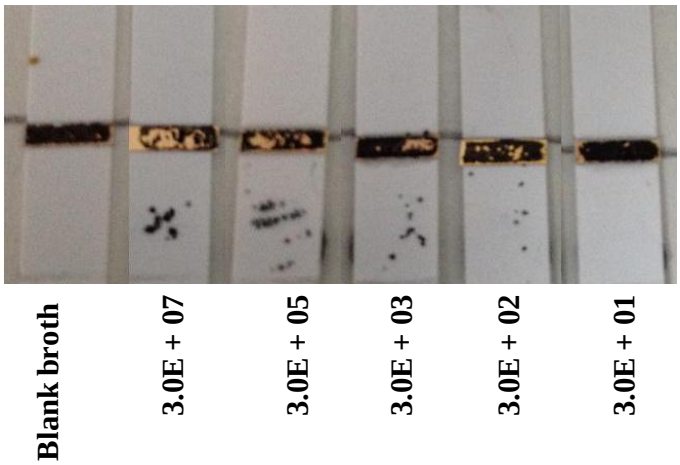
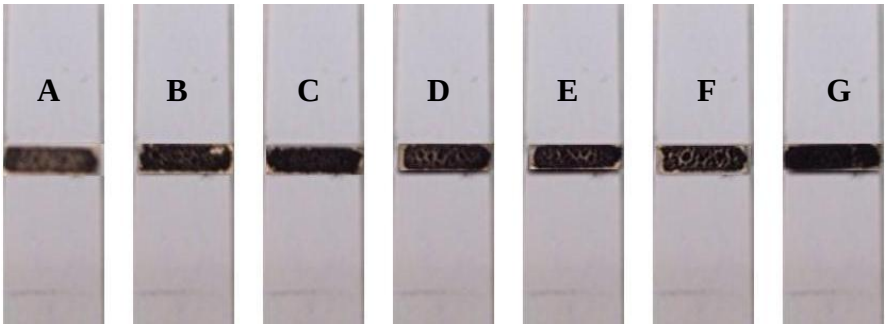


Fig. 3A. Application of the colorimetric ***E coli*** O157:H7 **sensor probe** to spiked milk samples (specific *E coli* substrate peptide covalently bound to a magnetic bead). (1), Biosensor chip functionalized with magnetic nanobead–specific *E coli* O157:H7 peptide substrate. (2) Immobilized biosensor under the effect of different *E coli* O157:H7 protease concentrations spiked in milk.

(1)



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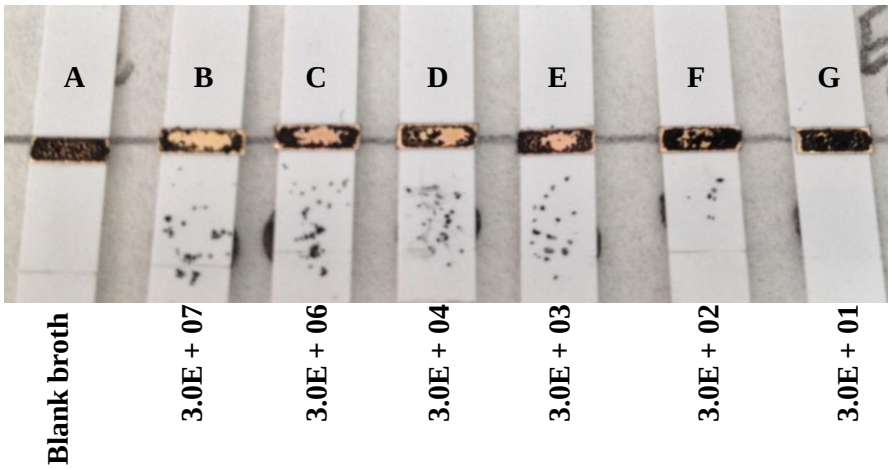
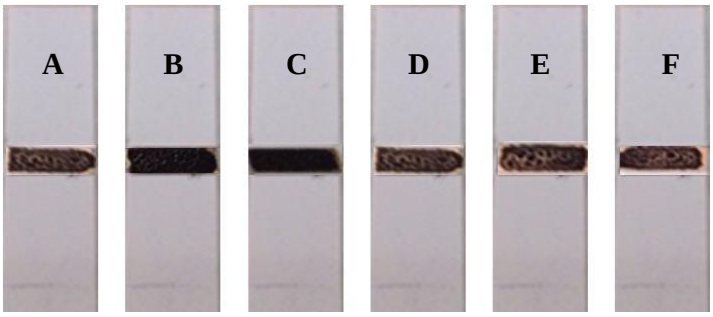


Fig. 3B. Application of the colorimetric *E coli* sensor to a spiked turkey sausage samples (specific *E coli* substrate peptide covalently bound to a magnetic bead). (1), Biosensor chip functionalized with magnetic bead–specific *E coli* peptide substrate. (A2, B2, C2, D2) Functionalized biosensor under the effect of different *E coli* protease concentrations spiked in food products.

(1)



(2)

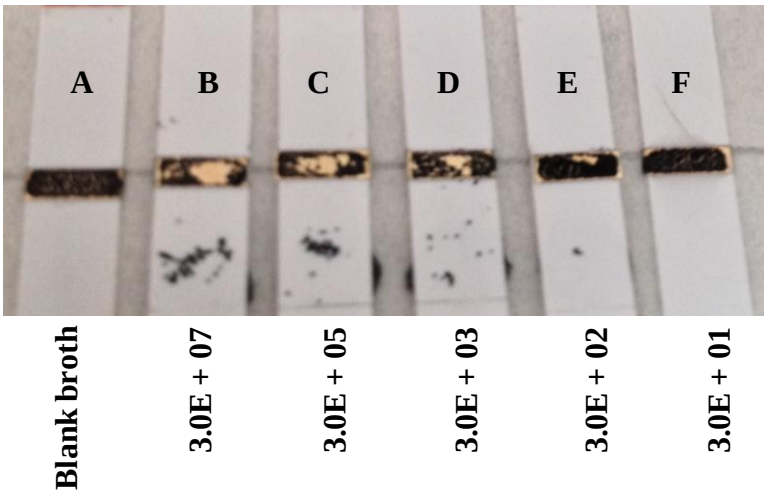
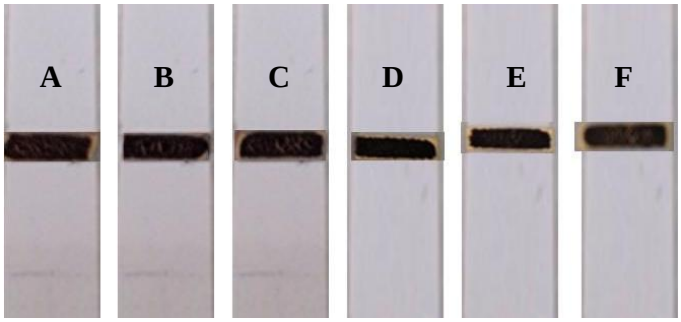


Fig. 3D. Application of colorimetric *E coli* sensor probe to spiked ground meat samples (specific *E coli* substrate peptide covalently bound to a magnetic bead). (1), Biosensor chip functionalized with magnetic bead–specific *E coli* peptide substrate. (A2, B2, C2, D2) Functionalized biosensor under the effect of different *E coli* protease concentrations spiked in food products.

(1)



(2)

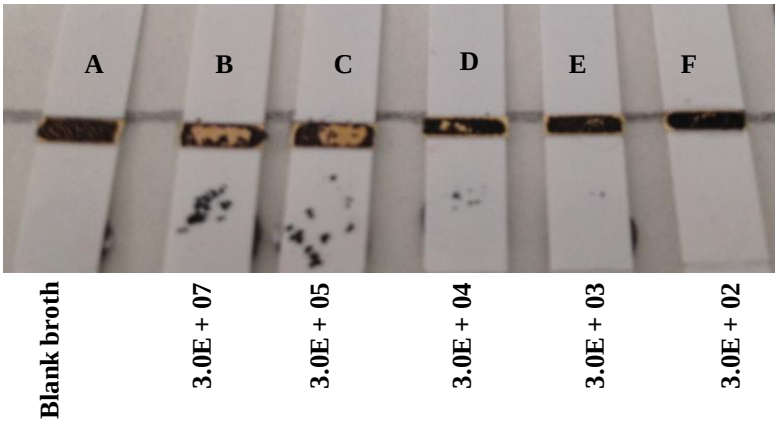


Fig. 3E. Application of the colorimetric *E coli* O157:H7 sensor probe to spiked milk samples (specific *E coli* substrate peptide covalently bound to a magnetic bead). (1), Biosensor chip functionalized with magnetic nanobead–specific *E coli* O157:H7 peptide substrate. (2) Immobilized biosensor under the effect of different *E coli* O157:H7 protease concentrations spiked in milk.

4. Conclusions

This work demonstrated the ability of the developed biosensor to specifically and simultaneously detect *E. coli* O157:H7 proteases in complex food matrices. The limits of detection were 12 CFU/mL in broth and 30-300 CFU/mL in food matrices. The assay was simple to perform, was rapid, required no sample pretreatment or pre-concentration and could be performed at the retail level to protect the consumer and at home. The detection mechanism does not require any labelling or amplification schemes and can be applied without requirement for any sophisticated and/or expensive instrumentation. This novel, low-cost colorimetric method used covalently attached specific substrate-magnetic nanobead complexes. This biosensing configuration is amenable to the qualitative and quantitative detection of *E. coli* O157:H7 proteases. The main advantage of the developed biosensor over existing technology is its ability to detect *E. coli* O157:H7 food contamination in less than 30 sec and with a very low limit of detection. This study provides the first step towards establishing a *proof-of-concept* biosensor for the detection of other microbial pathogens in contaminated food. Thus, this biosensor presents a valuable tool not only for the produce industry but also for agencies to better control potential risks associated with the consumption of contaminated foods.

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