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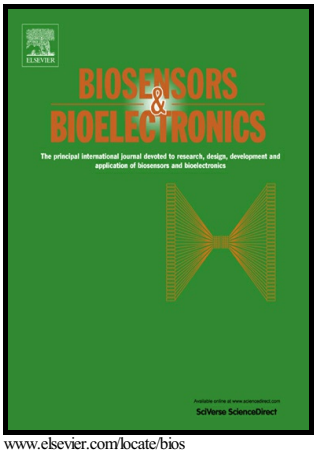

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Rapid and low-cost biosensor for the detection of *Staphylococcus aureus*

Ghadeer A.R.Y. Suaifan[a], Sahar Alhogail[b], Mohammed Zourob[b,c*]

[a] Department of Pharmaceutical Sciences, Faculty of Pharmacy, The University of Jordan, Amman11942 Jordan.

[b] Department of Chemistry, Alfaisal University, Al Zahrawi Street, Al Maather, AlTakhassusi Rd, Riyadh 11533, Saudi Arabia.

[c] King Faisal Specialist Hospital and Research Center, Zahrawi Street, Al Maather, Riyadh 12713, Saudi Arabia.

[*]**Corresponding author: E-mail: mzourob@alfaisal.edu**

Abstract

Staphylococcus aureus (S. aureus) is one of the most common etiological agents in hospital-acquired infections and food-borne illness. *S. aureus* toxins and virulence proteases often circulate in host blood vessels leading to life-threatening diseases. Standard identification approaches include bacterial culturing method, which takes several days. Other nucleic acid-based methods were expensive and required trained personnel. To surmount these limitations, a paper-based biosensor was developed. The sensing mechanism was based on the proteolytic activity of *S. aureus* proteases on a specific peptide substrate, sandwiched between magnetic nanobeads and gold surface on top of a paper support. An external magnet was fixed on the back of the sensor to accelerate the cleavage of the magnetic nanobeads-peptide moieties away from the sensor surface upon test sample dropping. The colour change resulting from the dissociation of the magnetic nanobeads moieties was detected by the naked eye and analysed using ImageJ analysis software for the purpose of quantitative

measurement. Experimental results showed detection limits as low as 7, 40 and 100 CFU/mL for *S. aureus* in pure broth culture, and inoculated in food produces and environmental samples, respectively upon visual observation. The specificity of the sensor was examined by blind testing a panel of food-contaminating pathogens (*Listeria monocytogenesis* 19115 and *E. coli O157:H7*), clinical isolates (methicillin-resistant *S. aureus* (*MRSA*) and *Candida albicans*) and standard (*Pseudomonas aeruginosa* 15692) pathogens. Negative read-out was observed by the naked eye for all tested isolates except for MRSA. Moreover, this sensing tool requires one minute's time to obtain the results. In conclusion, this sensing platform is a powerful tool for the detection of *S. aureus* as a potential point-of-care diagnostic platform in hospitals and for use by regulatory agencies for better control of health-risks associated with contaminated food consumption.

Keywords: *Methicillin-Resistant Staphyloccocus aureus*, Hospital acquired infections, Colorimetric detection assay, Magnetic nanobeads, Biosensors.

1. Introduction

Staphylococcus aureus (*S. aureus*) is a facultative anaerobic, gram-positive bacterium discovered by Dr. Alexander Ogston (Ogston, 1984). Since 1980, *S. aureus* bacterium received a lot of attention, being allied with health care-associated infections (HAIs) (http://www.cdc.gov/HAI/organisms/staph.html). These infections were correlated with a number of risk factors, including long-time hospitalization, hospital-costs and mortality. On the other hand, *S. aureus* bacterium was among the top five pathogens associated with food-borne illnesses (http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html; Wang et al., 2011).

S aureus pathogens usually grow in open wounds and urinary tracts, leading to numerous ailments, from minor skin infections to life-threatening diseases such as abscesses (Kapral et al., 1980), pneumonia (Robertson et al., 1958), meningitis (Gordon et al., 1985), endocarditis (Fowler et al., 2007) and septicaemia (Cross et al., 1983). The National Institute of Health and Center for Disease Control and Prevention reported 94,000 life-threatening antibiotic-resistant infection cases out of which 500,000 people were infected with *S. aureus* pathogen in the United States of America annually (Klein et al., 2007).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common *S aureus* resistant strains in hospitals (Wang et al., 2011). MRSA infections always cause serious antibiotic resistant illnesses and high mortality (Cosgrove et al., 2003). Thus, to combat the spread of MRSA infections and to minimize the waste of public resources, new strategies for the development of a specific and feasible diagnostic biosensor for the detection of *S. aureus* is urgently needed.

It is worth mentioning that the *Staphylococcus* species is usually present in the nostrils and on the skin of warm-blooded animals, and thus can contaminate food

products derived from animals such as meat, milk and eggs (Yang et al., 2011). Also, poor hygienic conditions by food handlers during manufacturing processes can contaminate foods (Goto et al., 2007). *S. aureus* bacterium can also live in harsh environments, and so, under temperature-abused conditions, it grows and produces virulence proteases in addition to other endotoxins, leading to staphylococcal food poisoning (SFP) (Doyle MP et al., 2007), which is marked by severe gastrointestinal symptoms such as emesis, diarrhea, and/or abdominal pain (Doyle MP et al., 2007). Recently, the Center for Disease Control and Prevention (CDC) estimated 240,000 illnesses with 1,000 hospitalizations and 6 deaths associated with SFP annually (Scallan et al., 2011).

Clearly, in case of food poisoning, specific *S. aureus* detection is important for proper treatment, but it is more important to prevent the occurrence of the disease. One way of doing so is to spot contaminated products and sites. For example, it is preferred to detect contaminated food products before reaching consumers. Conspicuously, this could be achieved through the development of cheap, stripe formate biosensors applicable at the retail level in order to protect the consumer.

Conventionally, *S. aureus* detection was based on bacterial culture methodology (Bocher et al., 2008). However, this process is time-consuming, labor-intensive and takes several days. This delays bacterium identification, which is unacceptable in emergency and critical illnesses such as sepsis, thus limiting its practical application for rapid diagnosis (Gilbert, 2002). Other ultra-sensitive detection methods were reported based on nucleic acid amplification, such as ligase chain reaction (LCR) (Moore and Curry, 1998), strand displacement amplification (SDA) (Edman et al., 2000) and polymerase chain reaction (PCR) (Cheng et al., 2006). Fortunately, these technologies were capable of detecting low numbers of bacterial cells, but took

several hours. Moreover, these technologies were expensive and required prior bacterial DNA isolation, preparation of enzyme reaction mixtures and expensive instruments for nucleic acid amplification. Alternative methods such as antibody-based immunoassays and immuno-PCR assays were well-established and used extensively (Huang and Chang, 2004; Zhu et al., 2014). However, antibody-DNA conjugation and purification processes are tedious, in addition to the high costs of the instruments, which prevents them from being procured quickly. Chang et al. (2013) reported the development of a non-PCR–based method, which combines aptamer-conjugated gold nanoparticles and a resonance light-scattering detection system. This method successfully detects a single *S. aureus* cell within 1.5 hours. However, this method needs sophisticated instruments. At present, nanotechnology's application in the development of biosensors-in particular, those capable of identifying protease activity as a disease biomarker, were very attractive due to their high specificity and sensitivity (Esseghaier et al., 2014; Suaifan et al., 2013; Wignarajah et al., 2015; Suaifan et al., 2016; Alhogail et al., 2016). However, expensive instruments are still required for these real-time detection methods. Therefore, there is a need for a ''sample-to-answer'', ''hand-held'', specific and sensitive diagnostic biosensor capable of detecting *S. aureus* proteases, as a biomarker for the presence/contamination of *S. aureus* in a short time.

Accordingly, this study aimed at the development of a magnetic nanobeads-peptide probe, which would be cleaved specifically by *S. aureus* virulence proteases. This probe would then be integrated with a gold sensing platform via Au-S linkage to provide specific and cost-effective strip-format diagnostic biosensor for the qualitative and quantitative detection of *S. aureus*. This biosensor would be label-free, cost-effective, quick, simple and can be read by the naked eye.

2. Experimental Section

2.1 Materials and Method.

Carboxyl-terminated magnetic nanobeads (50 nm in diameter) were supplied by Turbo beads (Switzerland). *N*-Hydroxysuccinimide (NHS), 1-(3-Dimethylaminopropyl)-3-Ethyl-Carbodiimide (EDC), pH indicator strips were purchased from Sigma Aldrich (Dorset, UK). Self-adhesive Magnet sheets were purchased from Polarity Magnets Company (UK). The peptide sequence NH_2-Ahx-ETKVEENEAIQK-Ahx-Cys was synthesised by Pepmic Co., Ltd. (Suzhou, China). Self-adhesive tape was purchased from Whatman (London, UK). Brain Heart Infusion broth and agar were purchased from SDA Oxoid, Ltd. (Basingstoke, UK). Sterile filter 0.22 µm was from Millipore (UK). 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. Bacterial Strain Culturing and protease preparation.

S aureus (ATCC 25923), *Listeria* monocytogenes (ATCC 19115) and *Pseudomonas* aeruginosa (*P. aeruginosa*, ATCC 15692), purchased from Sigma Aldrich (UK) were streaked on BHI agar plate and incubated at 37.0 °C for 24 h. One colony was then isolated, inoculated into BHI broth and incubated overnight at 37 °C. The primary bacterial culture (PBC) was serially diluted to get the bacterial colony forming unit (CFU/ml) value of the original stocks by using viable count spread dilution method. Then, the bacteria were pelted by centrifugation at 3000Xg for 10 minutes. The supernatant was then filtered to obtain the crude protease solution of each bacterial

concentration. The protease's proteolytic activity was measured by the universal, non-specific casien assay and was determined as the amount in micromoles of tyrosine equivalents released from casien per minute (Cupp-Enyard, 2008). An increase in *S. aureus* protease's proteolytic activity strength was correlated to bacterial culture concentration (colony forming unit (CFU/mL)).

2.3 Conjugation of S. aureus peptide substrates with magnetic-nanobeads.

Magnetic-nanobeads suspension (1 ml) was mixed with the peptide substrate NH_2-Ahx-ETKVEENEAIQK-Ahx-Cys (1.0 mg / mL), coupling agent EDC (0.57 mg / mL) and NHS (12 µg / mL). The mixture was tremor gently at room temperature for 24 hours. The uncoupled peptides were removed by washing the magnetic-nanobeads three times with a wash buffer. Finally, the beads were stored at 4ºC in a storage buffer.(Esseghaier et al., 2014; Suaifan et al., 2012a; Suaifan et al., 2013)

2.4 Fabrication of the gold sensing platform.

Schematic 1 shows the detailed steps for the preparation of the sensing platform. Self-adhesive tape was coated with a thin layer (30 nm) of gold which was sputtered using RF magnetron sputtering (Nordiko Ltd). Conditions 5mT pressure Argon gas. Power 100W, 5mins at School of Engineering at Cranfield University. Following this, a rectangular piece (~1.5-2 X 3 mm) was stacked over the front face of the plastic physical support (Scheme 1A).

2.5 Immobilization of the sensing monolayer (SAM).

Magnetic nanobeads-peptide solution was placed over the gold sensing platform and allowed to dry at room temperature (Scheme 1B). Upon completion of the immobilization step, the sensor platform golden color turns into black as observed by

the naked eye. Following this, an external magnet (12.5 X 12.5 X 5 mm) with field strength of 573 gauss at a distance of 10 mm was passed over the sensor platform to remove any unattached magnetic nanobeads as shown in (Scheme 1C). Then, a round paper magnet was fixed on the back of the strip at around 5 mm distance beneath the gold sensor platform as shown in (Scheme 1D).

2.6 Biosensing of S. aureus proteases.

The current diagnostic sensing platform is based on the measurement of *S. aureus proteases* proteolytic activity using a specific magnetic nanobeads-peptide gold sensing probe. Accordingly, *S. aureus* proteases solution (30 µl) was dropped over the black color functionalized sensing platform. During the enzymatic cleavage reaction, the round permanent magnets stacked at the back of the strip will attract the cleaved magnetic nanobeads-peptide moiety, which in turn prompting a visual observation of the sensor golden colour for a qualitative evaluation of the tested sample (Scheme 1).

2.7 Quantitative measurements of biosensor colour change.

The designed biosensor was intended to be used by visual observation of the colour change (black to golden) via naked eye. Additionally, the area of the golden colour increased with increasing the concentration of *S. aureus* protease prepared from different PBC (7.5 X 10^6 CFU/mL, 7.5 X 10^5 CFU/mL, 5 X 10^4 CFU/mL, 5 X 10^3 CFU/mL, 5 X 10^2 CFU/mL, 75 CFU/mL, 7.5 CFU/mL) as shown in Figure 2A. The black colour magnetic nanobeads quantity was quantified by using the ImageJ program developed at National Institute of Health (Collins, 2007; Rajwa et al., 2004). Images were obtained by direct photography of the biosensor after protease application by a smartphone and were saved in JPEG format. Then, images were split

into red, green and blue channels using the RGB stack command. Next, the images were performed through red channel following the discard of the blue and green channels since this channel showed less background level. Threshold was adjusted manually until only the black beads were highlighted in red and then Set Measurements dialogue was checked for ‘‘Area’’. Afterwhich, the area of the magnetic nanobeads on the sensor surface after protease application and the area of the cleaved magnetic nanobeads-peptide moieties were selected and measured separately as shown in Fig. 2BII

Total magnetic nanobeads-peptide SAM area = Black magnetic nanobeads area on the sensor surface after protease application + Cleaved magnetic nanobeads moieties area. The percentage of cleavage = Cleaved magnetic nanobeads moieties area / Total magnetic nanobeads SAM 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. Different *S. aureus* protease concentration obtained from different *S. aureus* pure culture (CFU/mL) were plotted against the percent cleavage of the magnetic nanobeads-peptide moieties as shown in Fig. 2B I and II.

2.8. Bacterial Spiking and proteases preparation.

2.8.1. Environmental samples.

Dust samples (Wiped from different places, including Jordan University Hospital Intensive Care Unit, Jordan University Hospital stairs and The University of Jordan courtyard corners) were suspended in sterile water. Afterward, different *S. aureus* PBC dilution was spiked into the dust samples and incubated at room temperature for

about 2 hours. The samples were then clarified by centrifugation at 3000 x g for 10 min and the supernatant was filtered through a 0.22 µm sterile syringe filter. The filtrates were either used immediately or stored at -20°C for later use. Unspiked dust samples were used as a negative control.

2.8.2.Food products.

Food products (ground beef, turkey sausage, lettuce and milk) were purchased from local markets. Ground beef and turkey sausages were homogenized with sterile water at 1:5 (w:v) ratio in a blender for 1 min, and then were rocked at room temperature for 2 hours. Lettuce leaves were rinsed with vegetable detergent and phosphate buffered saline (PBS, pH 7.4), and then cut into 4-cm^2 pieces using sterile scissors, to remove any potential adulterates which may exist on the leafy surface.

Afterward, the PBC stock of *S. aureus* was added to food products 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, 3000 X *g*) to pellet bacteria and then filtered through a 0.22 µm sterile filter. The filtrate containing crude bacterial proteases was used immediately. Positive control was prepared by creating 10-fold serial dilution from the *S aureus* PBC stock. Different *S aureus* concentrations in CFU/mL were determined by placing 10-fold serial dilutions of the positive control (PBC) on the BHI agar plates. The plates were then incubated overnight at 37°C and then enumerated. Negative blank samples containing no bacteria were also prepared in a similar way and tested to check the effect of food matrices content on the sensor.

2.8.3.Clinical isolates.

Clinical isolates (methicillin-resistant *S. aureus*, *P. aeruginosa* brown and green), *E. coli* and *Candida albicans* were obtained from The University of Jordan Hospital. These isolates were identified and characterized by the Mycological investigation laboratory at the hospital and provided as an *inocula* in the proper broth with 5% glycerol and then was stored at -20 °C for later use.

3. Results and discussion

Dramatic progress has been made in the field of nanomaterials-based biosensors specifically in biomedical and bioanalytical themes(Guo et al., 2013). Of particular interest, magnetic nanobeads which has a large surface/volume ratio (Citartan et al., 2012), easily separate or enrich specific analyte from complex matrices, under the effect of an external magnetic field and provide a direct readout by naked eye (Huang et al., 2010). Based on these unique magnetic nanobeads properties, a novel diagnostic sensing probe for the specific detection of *S. aureus* by naked eye has been constructed.

3.1. Sensor fabrication and sensing mechanism.

The biosensor peptide probe was prepared by using *S. aureus* specific substrate (ETKVEENEAIQK) to detect the proteolytic activity of *S. aureus* proteases (Sanders et al., 2005) This substrate was elongated with Ahx-residue linker on both ends of the peptide (NH_2-Ahx-ETKVEENEAIQK-Ahx-Cys). The Ahx-residue spacer endorses protease's accessibility to their specific peptide cleavage site. The N-terminal of the peptide was coupled with carboxyl-terminated magnetic nanobeads. The cysteine residue at the C-terminal allows gold-sulfur interaction to construct the magnetic nanobeads-peptide complex SAM on the surface of the gold sensor (Scheme 1A, B

and C). The amount of the peptide substrate required for MNPs functionalization was optimized in a previous work to get the optimal monolayer performance (Esseghaier et al., 2014; Suaifan et al., 2013). The detailed protocol steps in sensor preparation are shown in Scheme 1A-D. The sensing mechanism is based on the proteolytic activity of *S. aureus* proteases on a specific peptide substrate, sandwiched between magnetic nanobeads and gold surface on top of a paper substrate. An external magnet fixed at the back of the sensor accelerates the cleavage of the peptide-magnetic nanobeads moieties. This dissociation process reveals the golden color of the sensor surface visible to the naked eyes as shown in (Scheme 1E and 1F).

3.2. Sensor testing.

The proteolytic activity of the *S. aureus* proteases was detected by placing 30µL of the protease solution (7.5 X 10^6 CFU/mL) over the functionalized gold sensor surface. The optimum read out time was chosen to be after one minute of protease application as it provides about 85% of the sensor response. To gain insight into the applicability of the sensor for quantitative detection of *S. aureus*, a number of biosensors were prepared and exposed to different *S aureus* proteases solutions prepared from different bacterial pure broth culture concentrations (7.5 X 10^6 CFU/mL, 7.5 X 10^5 CFU/mL, 5 X 10^4 CFU/mL, 5 X 10^3 CFU/mL, 5 X 10^2 CFU/mL, 75 CFU/mL, 7.5 CFU/mL). Figure 2 clearly showed a proportional increase in the visible sensor golden colored area with the protease solution concentrations. This is due to the proteolytic activity of the protease, which resulted in the dissociation of the peptide-magnetic nanobeads complex. Blank samples showed no reaction since the sensor demonstrated no disruption of the SAM layer. A comparable proteolytic activity for *S. aureus* protease was established upon the measurement of the proteolytic activity by the universal,

non-specific casien assay (Fig. 1) and the developed biosensor (Fig. 2I and II). The results approved the powerful use of *S aureus* proteases as a biomarker. As a ten-fold decrease in bacterial concentration showed an effect on *S. aureus* proteases proteolytic activity and the consequent biosensor cleavage. As it has been observed, ten fold dilution in bacterial proteases concentration resulted in a drop in the proteolytic activity by approximately 6-10% of magnetic nanobeads cleavage. For example, 10^5 resulted of 36% of magnetic nanobeads cleavage, whereas 10^4 resulted in 28 % and 10^3 resulted in 22%. This difference can explains the adequate sensitivity of the developed biosensor.

Additionally, a quantitative approach was developed for *S. aureus*-specific detection using ImageJ software. The image software measures the amount of cleaved area (appearance of golden colour) to the total black area (before cleavage). Thus, we have adjusted the colour threshold function, as shown in Fig. 2BII. Photos analysis was performed three times. The correlation between the percentage cleavage of magnetic nanobeads-peptide moieties and different *S. aureus* protease prepared from different PBC showed a consistent correlation (Fig. 2B).

Detection limit determination was either based on visual evaluation of the lowest protease concentration incapable of cleaving peptide-magnetic beads, covalently attached to the golden sensor surface i.e. the sensor golden surface area is invisible to the naked eye due to intact SAM layer (Fig. 2A), or based on the standard deviation of the response and the slope: LOD = 3.3 standard deviation of low concentration(σ) / slope of the calibration line (Fig. 2BI). Interestingly, the developed sensor revealed low limits of detection without the use of any instrumentation and with one minute of incubation time.

3.3. Biosensor detection of S. aureus in spiked food matrices, environmental samples and clinical isolates

The developed biosensor proved its potential ability to detect *S. aureus* in PBS. The next step was to evaluate its feasibility to detect *S. aureus* in contaminated food products and environmental samples. To contaminate food produces, 1.0 mL of different *S. aureus* concentrations (range from 4.14 X 10^5 CFU/mL to 4.14 CFU/mL) was spiked into 9 mL of ground beef, turkey sausage, lettuce and milk. The results are shown in Fig. 2. A negative control was prepared by spiking 1 mL of BHI broth with 9 mL of food samples. On the other hand, to contaminate environmental samples, dust samples collected from different sites were suspended in sterile water, and spiked with six different concentrations ranging from 1.12 X 10^7 CFU/mL to 11.2 CFU/mL. The results are shown in Fig. 3. Positive and negative controls were also prepared. Both controls and spiked food products and environmental samples were allowed to incubate at room temperature for 2 hours prior to testing. The samples were then centrifuged, filtered and exposed to the developed biosensor. It is clearly observed by the naked eye that there is a directly proportional relationship between the sensor golden area and bacteria concentrations. The results in Fig. 3 and Fig. 4 clearly demonstrate the ability to detect *S. aureus* contamination. The detection limits were as low as 40 CFU/mL and 100 CFU/mL, respectively, in one minute.

3.4. Sensor stability.

To evaluate the stability of the biosensor over time, the ready-to-use immobilized strip sensors were stored in empty petri dishes at 4ºC. Every week, three sensors were used to detect the proteolytic activity of known *S. aureus* protease solution. In every experiment, a negative control (no *S. aureus* protease) was tested to ensure that the

magnetic nanobeads-peptide moieties dissociation was attributed to *S. aureus* proteolytic activity. The designed biosensor showed adequate long-term stability for up to six months at room temperature. Figure 5A shows the comparion of the sneosr response for less than 6 month and more than month. It can be observed that the application of similar proteases concentrations revealed higher percentage of cleavage on sensor probes stored for more than 6 months in comparison with those stored for less than six months.

3.5. Sensor Specificity.

A significant improvement in disease control could be made if the target pathogen, in the clinical bio-samples, and contaminated food produces were specifically and directly detected by a low-cost and sensitive method. The developed biosensor was exposed to 30 µL of a panel of blinded clinical isolates samples (MRSA, *P. aeraginosa* brown and green and *Candida albicans*) and standard pathogen (*P. aeraginosa* ATCC2785). The results are shown in Fig. 5B. Positive read-out by the naked eye was only observed for MRSA clinical isolate. Furthermore, the biosensor was exposed to a panel of food contaminating pathogens such as *Listeria monocytogenesis and E. coli* 157 as shown in Fig. 5. It was observed clearly that there was no disruption in the SAM layer of the tested pathogens and no significant change in the sensor surface golden color, except for *S. aureus*, showing again a high specificity for the *S. aureus*.

3.6. Comparison with literature

The developed biosensor is simple and highly sensitive, which is comparable with PCR techniques without the need for bacteria preconcentration It needs only the

supernatant of the sample, which can be easily prepared by untrained personnel. On the other hand, PCR and culture conventional methods require trained personnel and/or expensive instrumentation use. The ease of on-site use of this colorimetric biosensor by untrained personal and food makers stands behind its simplicity. Currently, a number of colorimetric detection methods were developed (dos Santos Pires et al., 2011; Sung et al., 2013; Tote et al., 2008). However, these methods do not fully satisfy the optimal detection performance criteria for field applications. For comparison purposes, Sung et al. (2013) reported a detection limit as low as $1.5X10^3$ and $1.5X10^5$ CFU for *S. aureus* in PBS and milk samples, respectively, in 40 minutes using antibody/gold, nanoparticle/magnetic nanoparticles. Pires et al. (2011) reported the use of 10,12-pentacosadiynoic acid (PCDA) -N-[(2-tetradecanamide)-ethyl]-ribonamide (TDER) vesicles, to determine the colorimetric response induced by *S. aureus* in a culture medium and in apple juice. Other methods used antibody-labeled gold nanoparticles (AuNPs) as an immunological reporter for an easy visual sensing of the biomolecules (Jian and Huang, 2011; Kim et al., 2012; Li et al., 2009). However, tested samples had to be purified and concentrated prior to the immunological reaction to achieve a specific and sensitive response with low noise (Li et al., 2009).

Conclusions

This study has demonstrated the ability of the developed biosensor to detect *S. aureus* pathogen qualitatively by naked eye and quantitatively by using imaging software. Additionally, the biosensor detected *S. aureus* specifically among different clinical isolates, contaminated food matrices and environmental samples. The limits of detection were as low as 7, 40 and 100 CFU/mL for *S. aureus* in pure broth culture,

and *S. aureus* inoculated in food produces and environmental samples respectively, in one minute's time. This assay is simple to perform, cheap, can be performed at the retail to prevent the consumer from being susceptible to food-borne diseases, or at the hospital to protect patients from hospital-acquired infections. The detection mechanism does not require any amplification steps.

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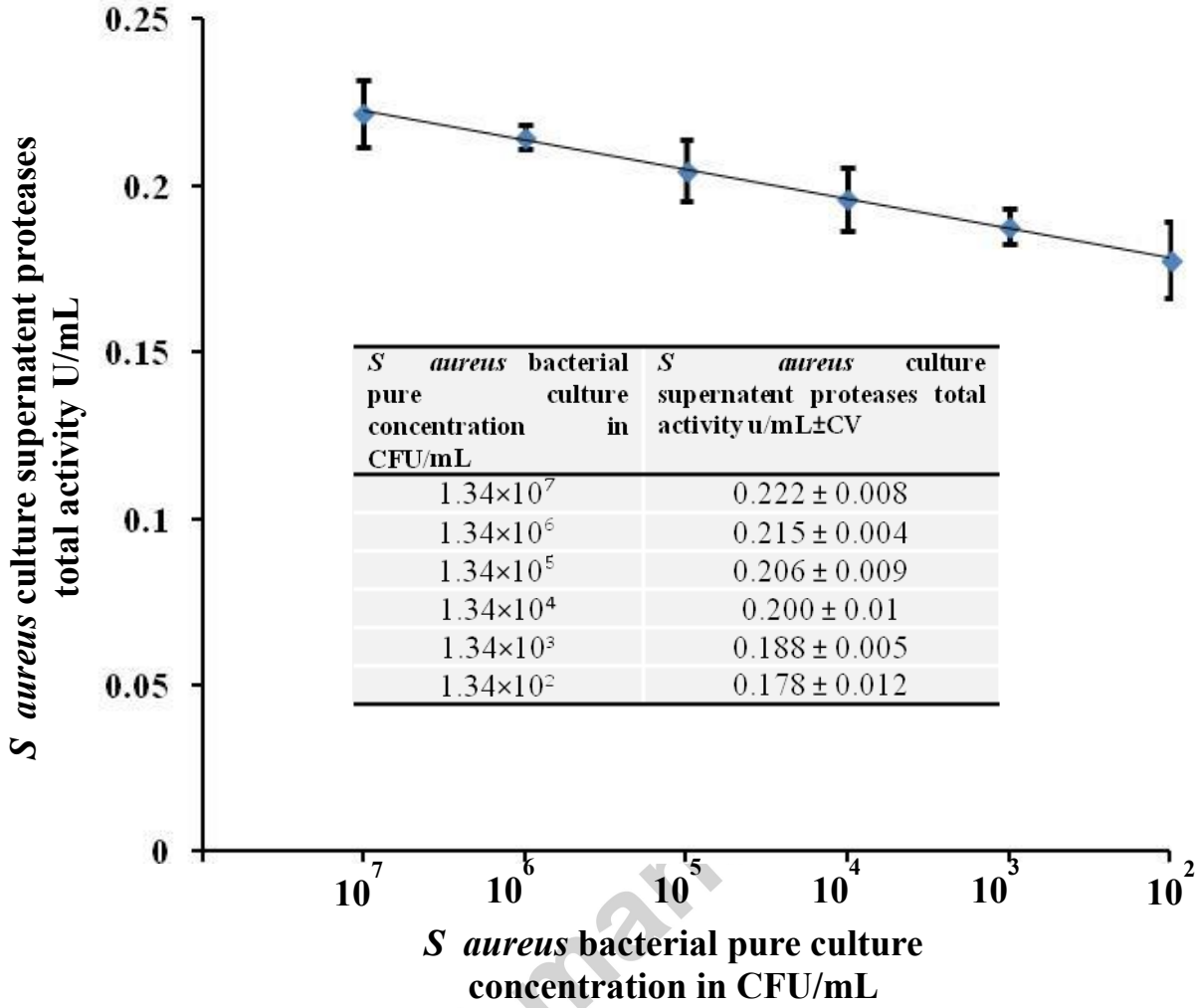


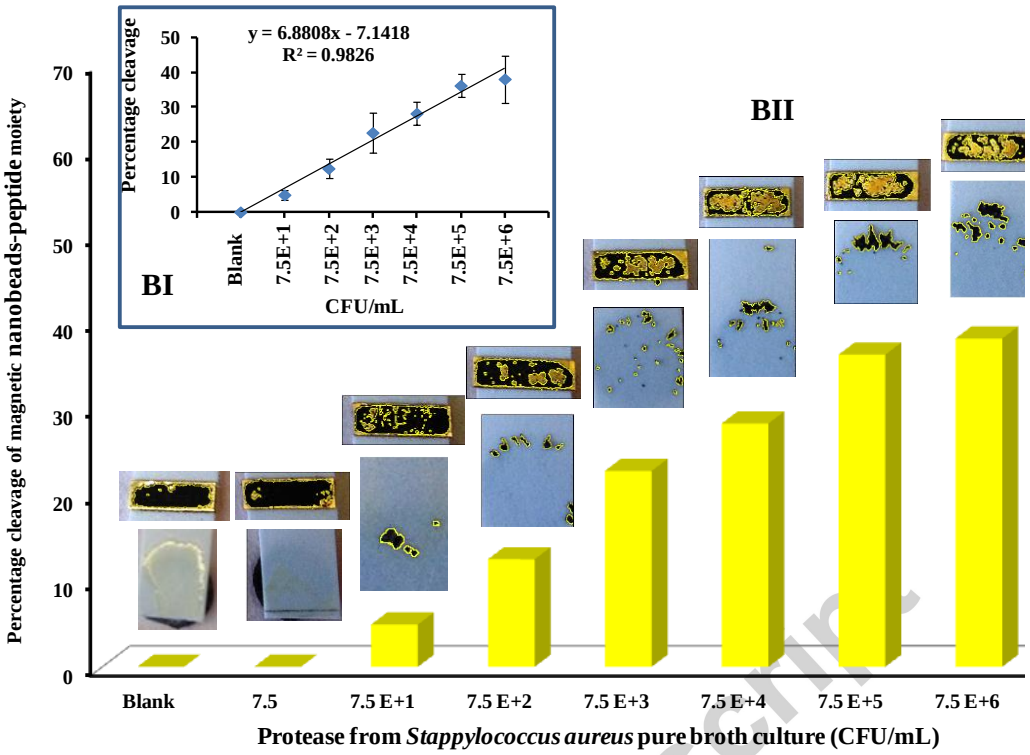
S aureus bacterial pure culture concentration in CFU/mL	*S aureus* culture supernatent proteases total activity u/mL±CV
1.34×10^{7}	0.222 ± 0.008
1.34×10^{6}	0.215 ± 0.004
1.34×10^{5}	0.206 ± 0.009
1.34×10^{4}	0.200 ± 0.01
1.34×10^{3}	0.188 ± 0.005
1.34×10^{2}	0.178 ± 0.012

Fig. 1. Proteolytic activity of different *S aureus* culture supernatent measured by Sigma's Non-specific Protease Activity Assay. Proteases activity is determined in terms of Units, which is the amount in micromoles of tyrosine equivalents released from casein per minute.

Fig. 2. (A) Colorimetric *S aureus* sensor probe (*S aureus* -specific peptide substrate covalently bound to magnetic nanobeads). (AI) Biosensor chip immobilized with magnetic nanobeads–specific *S aureus* peptide substrate. (AII) Immobilized biosensor under the effect of different *S aureus* protease concentrations.**(B) Quantitative measurement of biosensor color changes using ImageJ software. (**BI) The response curve with the respective standard deviation. (BII) The intensity color of the magnetic nanobeads on the sensor surface after protease application and the cleaved magnetic nanobeads moieties attracted by the backwards magnet as processed by imageJ software.

A

B



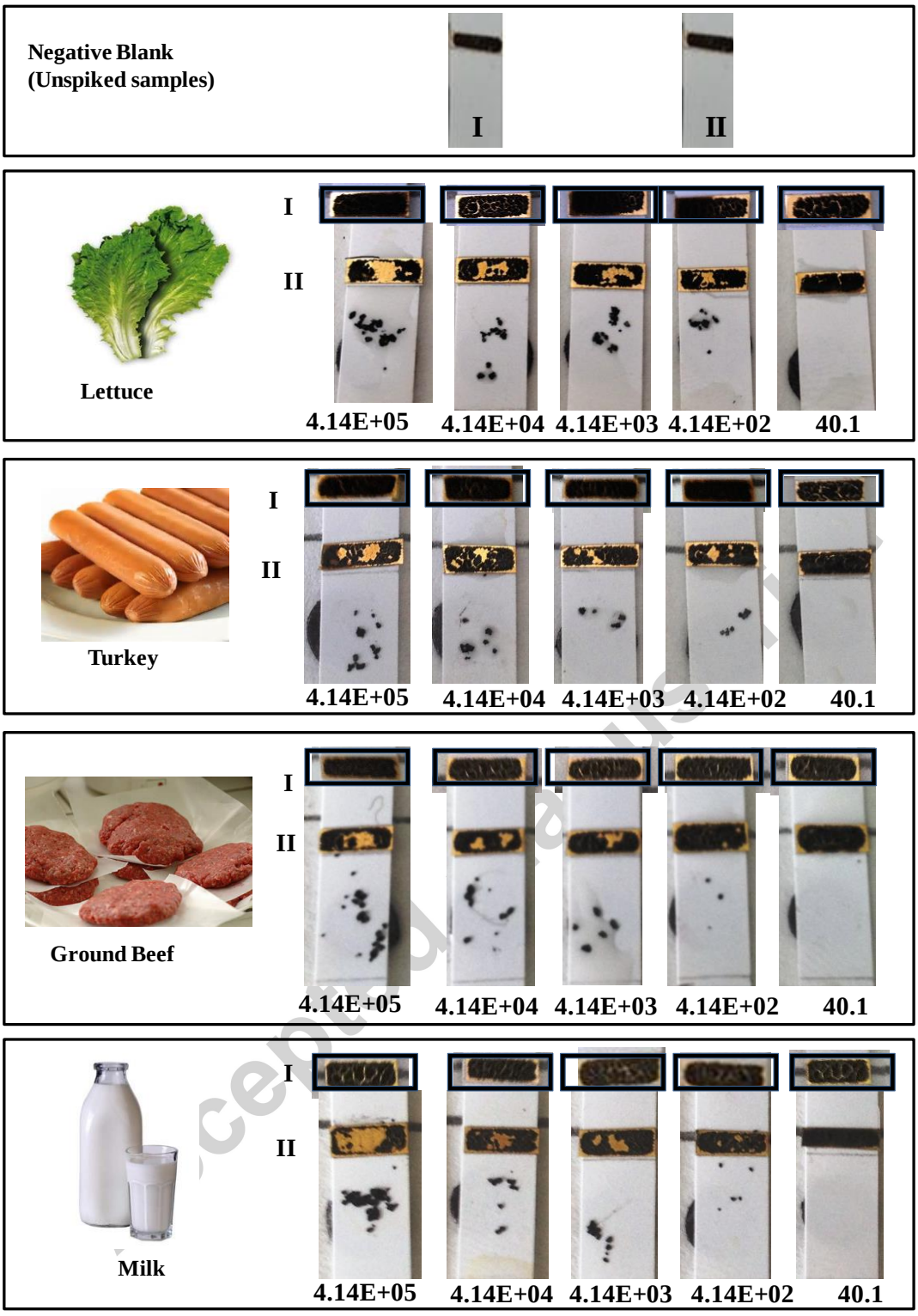


Fig. 3: Colorimetric *S. aureus* sensor probe application on spiked food produces (specific *S. aureus* substrate peptide covalently bound to a magnetic nanobeads). (I), Biosensor chip functionalized with magnetic nanobeads–specific *S. aureus* peptide substrate. (II) Functionalized biosensor under the effect of different *S. aureus* protease concentrations spiked in food produces.

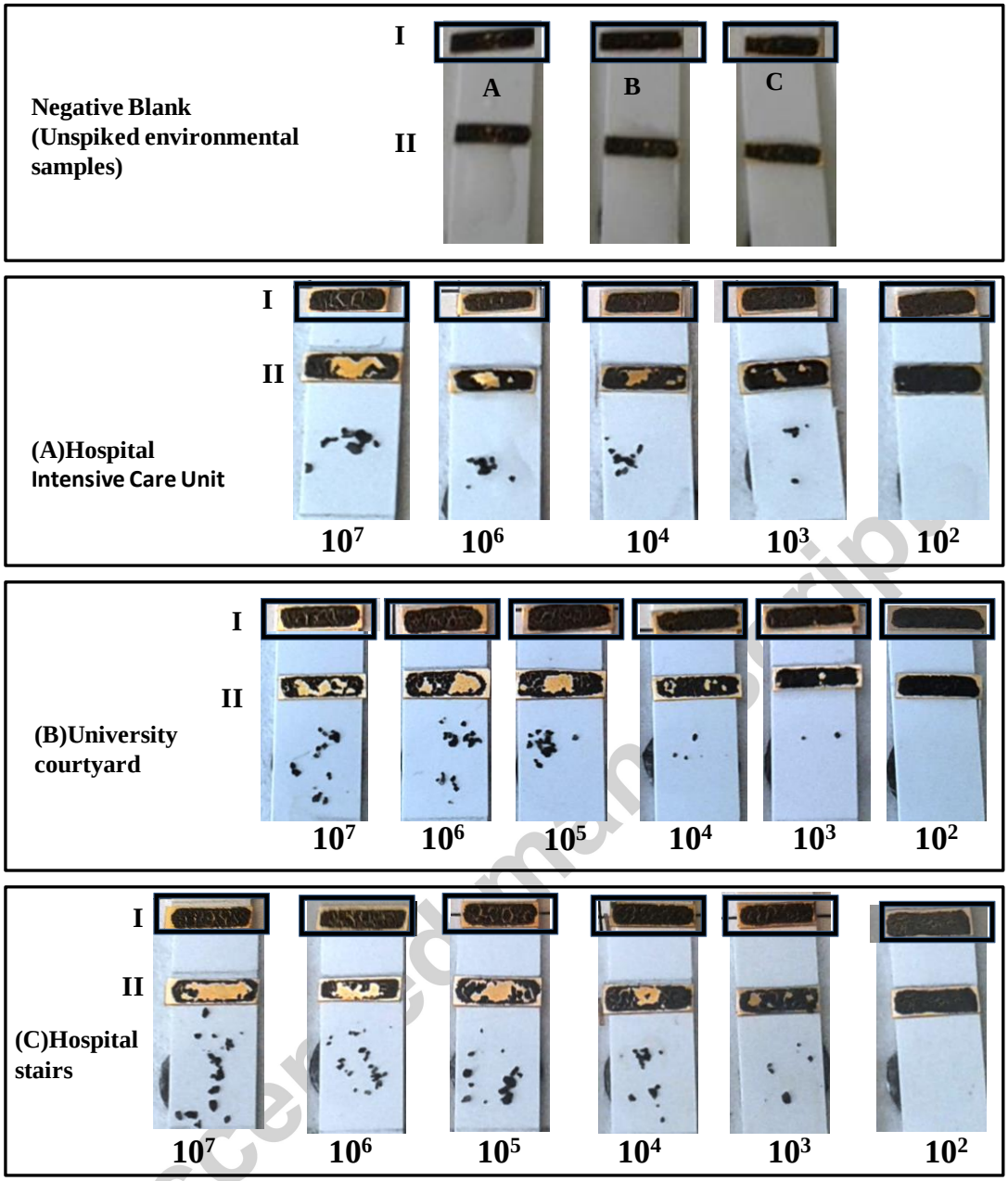


Fig. 4. Testing the colorimetric *S. aureus* sensor probe on spiked environmental samples (specific *S. aureus* substrate peptide covalently bound to a magnetic bead). **(I)** Biosensor chip functionalized with magnetic nanobeads–specific *S. aureus* peptide substrate (before exposing to the contaminated samples); **(II)** Functionalized biosensor exposed to different concentrations of *S. aureus* proteases spiked in environmental samples.

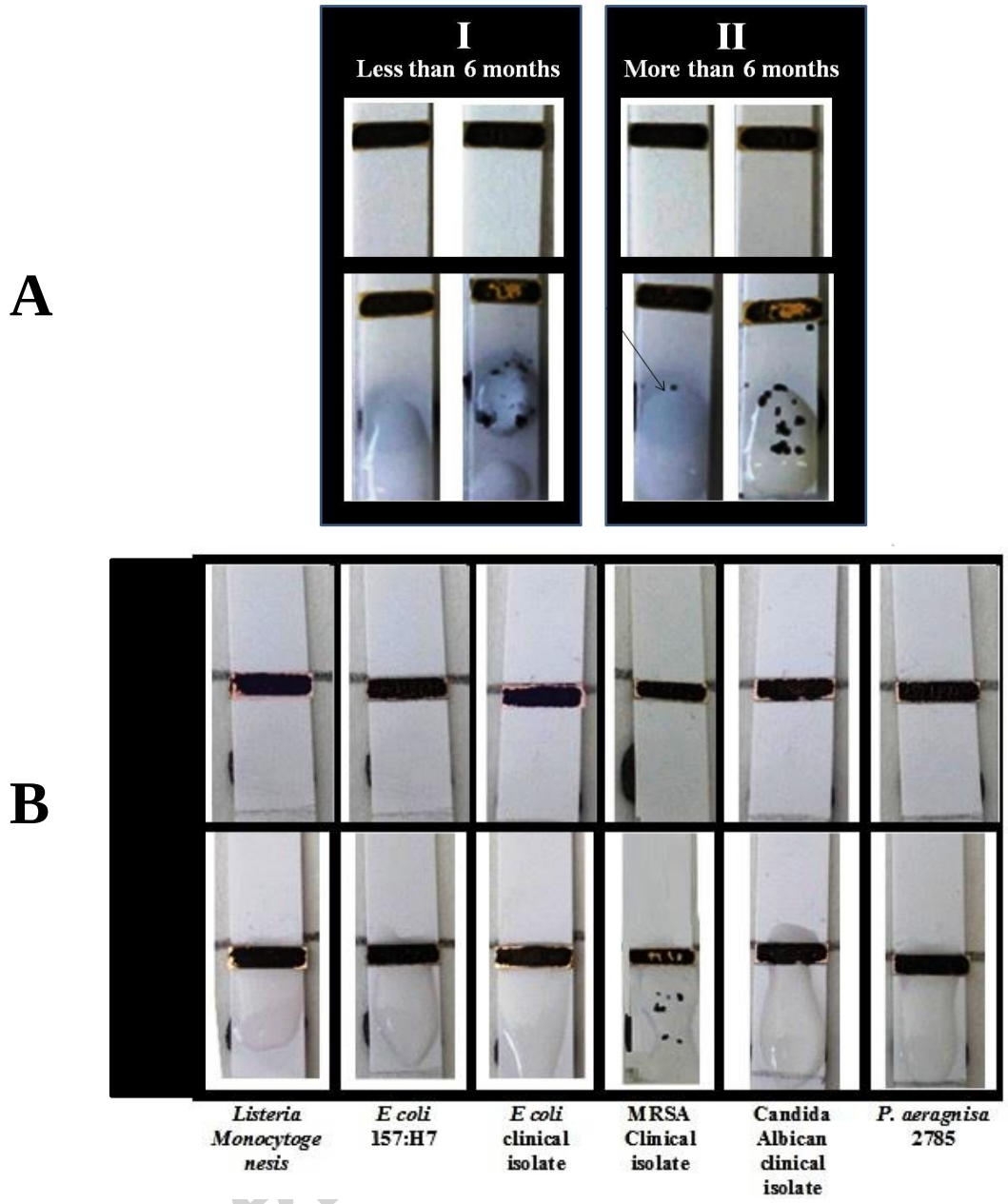
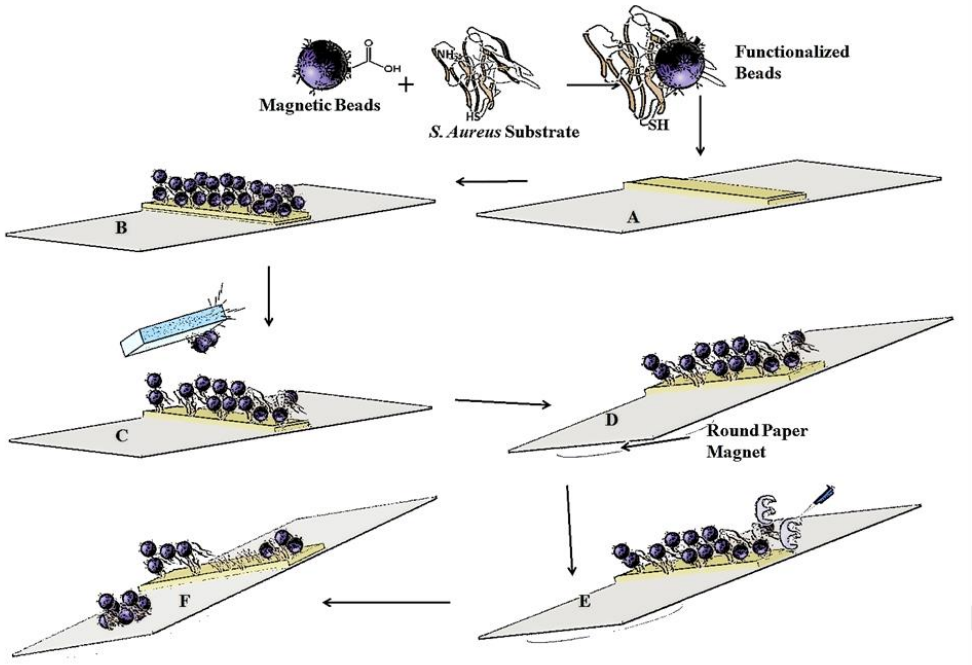


Fig. 5. (A) Stability study of *S. aureus* sensor probe . (AI) Application of negative control (broth only) and known protease concentration over biosensor probe stored for less than 6 months. (AII) Application of negative control (broth only) and the same protease concentration over biosensor probe stored for more than 6 months. **(B) Specificty of the colorimetric *S. aureus* sensor.** (BI) Biosensor chip functionalized with magnetic nanobeads-specific *S. aureus* peptide substrate. (BII) Sensor exposed to various food-borne pathogens (*L. Monocytogenesis*, *E. coli* 157:H7), clinical isolates (*E. coli*, *MRSA* and *C. albican*) and standard pathogens (*P. aeruginosa* 2785).



Scheme 1. Fabrication and mechanism of *S aureus* protease detection using colorimetric paper-based biosensor. (A) Preparation of gold sensor platform; (**B)** Gold biosensor covered *with S aureus* protease specific substrate peptide-magnetic nanobeads; **(C)** External magnet to attract unattached nanobeads; **(D)** Round paper magnet stacked at the back of the strip; **(E)** Sensing process upon application of *S aureus* proteases**; (F)** Dissociation of the peptide-magnetic nanobeads complex from the sensor surface upon dripping *S aureus* protease solution.

Highlights

- Novel, ultra-rapid colorimetric biosensor for the detection and identification of *S. aureus*.
- The designed colorimetric biosensor detect rapidly the amidolytic activity of *S. aureus* protease.
- The detection limits of this colorimetric sensor were 4 and 11 CFU/mL in less than one minute in food produces and spiked environmental samples.
- The platform presented herein is considered to be simple, fast and low-cost, thus opening new opportunities for point of care devices for other diseases.