Analytical Biochemistry 523 (2017) 58–64

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Development of magnetic nanoparticle based calorimetric assay for the detection of bovine mastitis in cow milk

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ARTICLE INFO

Article history:
Received 18 December 2016
Received in revised form
13 February 2017
Accepted 15 February 2017
Available online 17 February 2017

Keywords:
Mastitis
Colorimetric biosensor
Plasmin

ABSTRACT

Mastitis in dairy cattle is an inflammatory reaction of the udder tissue. Mastitis increases plasmin levels, leading to an increased proteolysis of milk proteins such as casein, resulting in a significant decrease in milk quality and related dairy products. Due to its key-role in mastitis, we used plasmin proteolytic activity as a biomarker for the detection of mastitis in bovine mastitic milk. Inspired by earlier studies on protease activity using mastitic milk samples, we developed a simple colorimetric assay to distinguish mastitic milk from milk derived from healthy animals. The plasmin substrate coupled to magnetic nanoparticles form a black self-assembled monolayer on a gold sensor surface. In the presence of increased levels of plasmin, the substrate is cleaved and the peptide fragment attached to the magnetic beads, will be attracted by the magnet which is present under the sensor strips revealing the golden surface. We found the area of the golden color surface proportional to plasmin activity. The sensitivity of this method was determined to be 1 ng/ml of plasmin *in vitro*. Next, we tested the biosensor using mastitis positive milk of which infection is confirmed by bacterial cultures. This newly developed colorimetric biosensor has high potential in applications for the diagnosis of mastitis with potential spin offs to health, food and environmental sectors.

1. Introduction

Bovine mastitis leads to huge economic losses in the dairy industry worldwide [1]. These losses are associated with the reduction in the milk production [2,3], treatment of contaminated milk, and costs of measures to prevent spread of mastitis within a herd. A variety of Gram positive and Gram negative bacteria, as well as algae, fungi and viruses [4] are associated with infections of the mammary glands. Mastitis alters the composition of the milk; the change in the composition depends on the pathogenicity of the microorganisms involved and extend of the mammary gland infections. For example, mastitis may lead to increased levels of ions, proteins and phagocyting cells resulting from leakage or migration, respectively. Clinical mastitis is characterised by swelling of the udder, redness, pain and reduced milk secretion from the affected quarters. The abnormal appearance of the milk can be observed by the eye and is characterised by the presence of clots or flakes [3]. Diagnosis of sub-clinical mastitis is more difficult and challenging. In general, visible signs of the udder or milk are lacking, and although impaired yields of milk are characterised by increased levels of Somatic Cell Counts (SCC), diagnosis by the naked eye is practically impossible.

Somatic cell counts (SCC) is used for measuring the quality of the milk in many methods [5,6]. For example, the California mastitis test, leukocyte number has been indirectly measures by aggregation of nucleic acids released by the leukocytes in the milk samples. Alternatively, an esterase-catalyzed enzymatic reaction based Portacheck assay indirectly determines the presence of SCC. On the other hand, the change in the fluorescence intensity of DNA

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http://dx.doi.org/10.1016/j.ab.2017.02.009
0003-2697/

of the cells upon binding with ethidium bromide were applied in the Fossomatic FC and the Delaval cell counters to detect SCC. Whereas, the other methods, electrical conductivity (EC) test and pH test do not consider SCC counts. These tests measure the conductivity of the milk due to the elevation in levels of ions such as sodium, potassium, calcium, magnesium and chloride, and changes in pH during inflammation, respectively. The cow-side tests (CMT, Portacheck, EC, pH) are quick, relatively cheap and easy to use, but have moderate test characteristics. SCC measurement with the Fossomatic performs better, but requires an expensive device that is complex to use and therefore cannot be used cow-side.

The Delaval cell counter reliably estimates the SCC and can be used cow-side, but is relatively expensive [6]. Culture techniques to selectively identify microorganisms involved in mastitis may reveal the cause of mastitis. Although this technique has little demands on equipment, it requires skilled personnel and certified laboratories. Furthermore, culturing is time-consuming, taking minimally 24 h before results are delivered. Also various protease-based methods have been developed and validated [7,8,9–12]. But, so far, as easy read-out systems are still lacking, none of them has been proved to be directly applicable for on-site purposes, Therefore a rapid, sensitive, and specific assay for the onsite detection of mastitis would be desirable [13,14].

Various host proteases can be considered as candidate molecules for their use as biomarkers for mastitis including N-acetyl-d-glucosaminidase (NAGase) [15,16] serum amyloid A (SAA) haptoglobin [17], other acute phase protein (APP) [15,16,18,19]. Alternatively proteases from bacterial origin may be interesting candidates [20,21] such as from *Escherichia coli*, *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis* [22] and *Listeria monocytogenes* [23,24], which are typical mastitogenic bacteria. Though of all proteases involved, the host derived plasmin appears to be one of the most active protease; its expression seemingly being independent of the identity of the infectious agent [14,25,26].

Plasmin is a heat stable endogenous serine protease which specifically cleave the peptide bonds with arginine (R) and lysine (-K) at P1 position [7,27]. Recently, we have used plasmin as a diagnostic biomarker for the diagnosis mastitis milk using the cleavage of plasmin specific fluorigenic substrates [7]. Inspired from this work, we now developed a proof concept of a simple colorimetric assay to distinguish mastitic milk from milk from healthy animals.

2. Materials and methods

Carboxyl-terminated magnetic nano-beads with less than 50 nm diameter were purchased from Turbobeads (Zurich, Switzerland). Molecular biology grade dimethyl sulfoxide (DMSO), bovine plasmin, *N*-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDC) and plastic pH indicator strips were purchased from Sigma-Aldrich (Saint-Louise, MO USA). Self-adhesive magnetic sheets were purchased from Polarity Magnets Company, Wickford, UK. A plasmin sensitive peptide substrate H_2N-Ahx-VLK-Ahx-Cys-COOH, was synthesised from Pepmic Co., Ltd (Suzhou, China). HPLC analysis confirmed purity of the peptide of more than 98%. The self-adhesive tape was purchased from Whatman (London, U.K). Analytical grade 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris base), sodium chloride, bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), sodium azide, potassium phosphate and sodium chloride from Sigma -Aldrich (Saint-Louise, MO, USA) were used for these experiments. Self-adhesive sheet was purchased from Whatman (London, U.K) and gold plated at the Electrical Engineering department of the King Abdullah University for Science and Technology (KAUST). The free magnetic beads, peptide linked and the plasmin treated magnetic beads were characterised by FT-IR spectroscopy done in the Thermo Scientific Nicolet Si10.

2.1. Bio-conjugation of magnetic beads

Carboxylic acid functionalised magnetic nano-beads (150 mg) were washed extensively with distilled water. The washed beads were sonicated for 5 min with a frequency of 35 Hz at 37 °C using a VWR Symphony Ultrasonic cleaner and washed extensively with coupling buffer until the supernatant was clear. Next, 500 μl of washed magnetic beads suspension in coupling buffer (10 mM potassium phosphate, 150 mM sodium chloride, pH 5.5), 500 μl of 1 mg/ml of plasmin specific peptide substrate in DMSO and 500 μl of coupling agent (20 mM EDC and 5 mM NHS in coupling buffer) was gently mixed for 4 h at room temperature. After completion of the reaction, the unreacted peptides were removed by washing the beads with washing buffer (10 mM Tris base, 150 mM sodium chloride, 0.1% (w/v) bovine serum albumin (BSA), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium azide, pH 7.5) and stored in washing buffer at 4 °C until use. The active free carboxylic acid groups on the magnetic nanobeads were blocked by the free amines containing Tris base and BSA in the washing buffer. The gold plated adhesive sheets were cut into small narrow stripes (1.5 mm wide) and stacked over a plastic strip approximately two cm from the bottom of the pH strip. The strip was used as a physical support for the gold sensor platform. A week round magnetic sheet was stacked under the strip with 1 cm distance from the sensor platform.

2.2. Characterisation of magnetic beads

The free carboxy functional magnetic beads, peptide coupled magnetic beads and the cleavage after plasmin digestion have been characterised by FT-IR spectroscopy. As the magnetic beads are composed of many functional groups, we focused on the change in the IR frequency of C=O functional group of COOH before and after coupling. The peak at 1725 cm^{-1} is assigned to C=O in COOH in the magnetic beads. After treating the magnetic beads with NH_2 of peptide, there is an additional peak appears at 1650 cm^{-1} due to amide C=O bond, confirming the amide formation. Plasmin treatment with peptide coupled magnetic beads show strong peak at 1730 cm^{-1} and 1650 cm^{-1}due to the hydrolysis of substrate amide bond to carboxylic acid and the existing amide bonds respectively.

2.3. Preparation of self-assembled monolayer (SAM) sensor platform

The cysteine moiety in the C-terminal peptide substrate was used for the formation of the self-assembled monolayer on the gold surface by irreversible Au-S bond. About 5 μl magnetic particle conjugated peptide was pipetted out on the gold surface, and kept in a closed petri dish with wet tissue paper at room temperature for one hour to avoid fast evaporation. A magnet with a field strength of 3360 G and 573 G at 10 mm and 1 mm respectively were passed over the monolayer to remove the unbound magnetic particles. A round permanent paper magnet was fixed under the strip, with 2–3 mm distance from the gold sensor platform (Fig. 1).

2.4. Plasmin biosensing

We used a plasmin sensitive peptide sequence for the detection of plasmin proteolytic activity. It is known that plasmin specifically cleaves peptide sequences with a lysine at the P1 position, we therefore designed a valine-leucine-lysine (VLK) peptide substrate

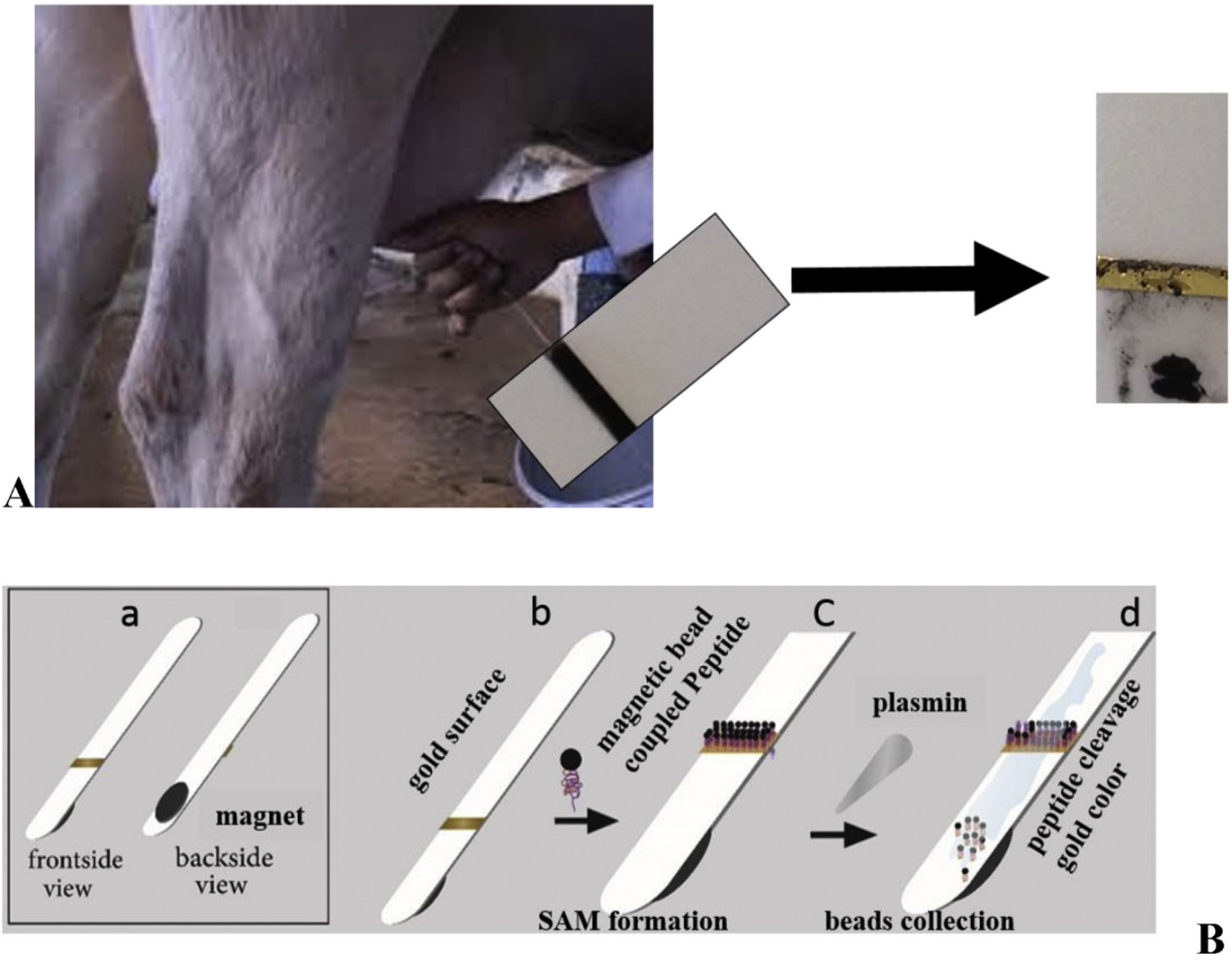


Fig. 1. (A) Application of a magnetic nanoparticle based biosensor for the on-site diagnosis of mastitis using milk. The milk from mastitis cows cleaves the biosensor self-assembled monolayer and turns the black biosensor surface gold. (B) Description of biosensor principle. The platform consists of a golden sheet stacked onto a plastic strip (front side) with a circular permanent magnet attached at the bottom of the sensor strip (backside) (a–b). The biosensor is formed by immobilization of a plasmin specific peptide coupled to magnetic nanobeads (c). Upon addition of plasmin, the plasmin specific peptide bond is cleaved and detach the magnetic beads from the gold surface and accumulate on the top of permanent magnet surface (d). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with an aminohexanoic linker (Ahx) on both sides of the sequence (H_2N-Ahx-VLK-Ahx-Cys-COOH) [28,29]. The plasmin specific substrate D-valyl-leucyl-lysine-p-nitroanilide that was developed by Rollema and co-workers using spectrophotometric assay of plasmin and plasminogen in bovine-milk [9]. The amino group in the N-terminal was bio-conjugated with carboxylic acid terminated magnetic nanoparticles. The C-terminal with cysteine was used for the formation of the gold-thiol bond SAM as the results, gold surface turned to black. Addition of 10–20 μl of plasmin on the sensor surface, the proteolytic activity of the plasmin on the specific recognition site of the peptide substrate cleave the amide bond *at* lysine (K) position within two minutes leads to free peptide fragments coupled to magnetic beads. These cleaved peptide fragments are collected by the permanent magnet fixed under the plastic strip. As a results, the gold colored sensor surface appears which can be clearly observed by the naked eye (Fig. 2). The detection limit of the plasmin biosensor was determined using serial dilution of plasmin (0.1–1000 ng/ml) in PBS buffer.

The biosensor can be used for qualitative as well as quantitative measurements. Qualitative can be used visual observation of the color change (black to golden) via naked eye. Quantitative measurements was achieved by quantifying the disappearance of black magnetic nanobeads and appearance of golden color. This was done by feeding the images to ImageJ program developed at National Institute of Health [30,31].

2.5. Milk sample collection and bacterial culture test

Milk samples from ten cows suffering from mastitis and three samples from healthy cows were collected. To avoid external contamination, the first squirts of milk were discarded and the teat ends were cleaned with alcohol soaked cotton balls. About 10 ml of each sample was collected in sterile plastic vials. Milk samples from 3 clinically healthy cows were used as a control samples. The milk from 3 cows that were enrolled were healthy and no evidence for clinical or subclinical mastitis was present. All milk samples were collected aseptically as described previously [7]. After discarding the first squirts of milk, the teat ends were scrubbed with a cotton

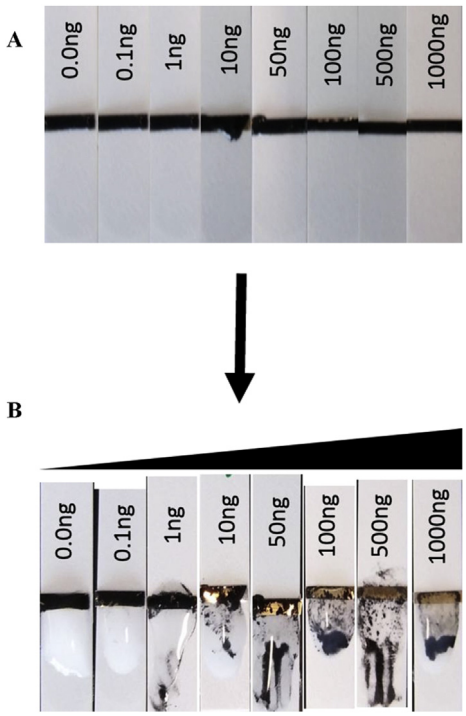


Fig. 2. Limit of detection of the plasmin biosensor (A) Plasmin sensing peptide, H_2N-Ahx-VLK-Ahx-Cys-COOH, formed a self-assembled monolayer on the sensor by using the NH_2 group for magnetic beads coupling and the -SH group of cysteine for S-Au irreversible bond on the gold surface. (B) An increase in magnetic nanoparticles cleavage by the addition of an increasing amount of plasmin protease is observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

balls soaked in alcohol, before the samples were collected. Per sample, about 10 ml of milk was collected in a sterile plastic vial. Milk samples were stored at −20 °C until analysis at the laboratory. Bacteriological culture of the milk samples were performed at the laboratory of the University for Farm Animal Practice according to the National Mastitis Council guidelines [32]. The tests confirmed the presence of pathogenic bacteria including *Enterobacter*, *S. uberis* and mixture of other bacteria. The collected milk was centrifuged at 10,000 × g for 20 min at 4 °C and the plasmin containing supernatant was filtered through 0.22 μm syringe filter. The plasmin extracts were stored at −20 °C until use.

We used ten milk samples collected from cows suffering infectious mastitis. In six cases, the presence of mastitis causing bacteria was confirmed by culture. The plasmin containing milk extract was used to study the applicability of the developed biosensor in the diagnosis of mastitis. The milk extracts were applied onto the black color plasmin specific SAM on the gold sensor surface. The peptide substrates coupled SAM was cleaved by the plasmin present in the milk extracts, and the resulting magnetic bead coupled peptide fragments were collected at the bottom of the sensor surface resulting in appearance of the golden color surface.

3. Results and discussion

Mastitis is a complex disease and its diagnosis is a big challenge. Milk from mastitis infected cows contains a variety of active enzymes [33], which can be used as potential diagnostic biomarker [4,15]. In the present colorimetric biosensor assay, we used a plasmin sensitive substrate coupled to magnetic nanoparticles to detect plasmin's proteolytic activity *in vitro* and in mastitic milk (Fig. 1). Plasmin selectively cleaves at the carboxyl side of L-lysine or L-arginine amino acids, with a preference for L-lysine [34]. Here, a tripeptide, containing a lysine residue, was employed with an aminohexanoic (Ahx) linker on both the N- and C-terminal of the sequence (H_2N-Ahx-VLK-Ahx-Cys-COOH). The Ahx-linker, containing a six carbon chain, increases the flexibility of the peptide substrate and facilitates its accessibility to the protease. As the substrate consists of only three amino acids, the linker enhances the accessibility of the peptide substrate for the plasmin protease.

Detection of plasmin proteolytic activity using the developed biosensor was studied by applying different concentrations of plasmin onto the SAM sensor surface. The applicability of such a protease-based biosensor in the diagnosis of (bacterial) infections was confirmed in a previous study wherein periodontitis was diagnosed using an elevated levels of Human Neutrophil Elastase (HNE) and Cathepsin-G proteolytic activity as a diagnostic biomarker [25]. The magnetic sheet in the bottom of the sensor surface collects the cleaved magnetic nanoparticles and as a result the color of the sensor surface changes from black to gold. The revealed golden surface area was proportional to the amount of plasmin present as determined by the eye [35] (Fig. 2). No change in SAM of the biosensor was observed using mastitis negative milk (Table 1).

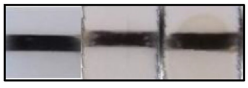
3.1. Limit of detection of the plasmin biosensor

In order to determine the detection limit of the plasmin biosensor, plasmin activity on the sensor surface was tested with different plasmin concentrations between 0.01 ng/ml and 1000 ng/ml. A visible and apparent gradual increase in gold surface area appearance with increasing plasmin concentration was observed (Fig. 2). There is no variation in the negative control sensor by the addition of PBS buffer, indicating the SAM was stable and is not altered due to PBS buffer (Fig. 2). As the sensor responses significantly with a small variation in plasmin quantity, the assay has potential for use in the semi-quantitative estimation of plasmin in milk or dairy products without any pre-concentration steps prior to analysis. It was found that the lower detection limit of plasmin *in vitro*, observable by naked eye is 1 ng/ml (Fig. 2). These results are more sensitive than the recently developed electrochemical and calorimetric biosensor for the detection of plasmin [36]. They employed ferrocene-modified peptide specific to plasmin and they detect the decrease in current measured by cyclic voltammetry. The detection limit for electrochemical and Uv–Vis spectroscopy were 45.3 ng/ml and 298 ng/ml respectively. Hiss et al, have used haptoglobin (Hp) used as s biomarker for the diagnosis of mastitis in cow milk. In this method, intracisternal injection of lipopolysaccharide (LPS) into one quarter and the milk samples were collect from the untreated quarters. The different in the Hp mRNA expression in parenchymal tissue, the tissues around the cisternal milk ducts and teat tissue from treated and control quarters evaluated by real time RT-PCR. The limit of detection of Hp from this method was 70 ng/ml. Compare to the above methods, our method is sensitive for the diagnosis of mastitis [37]. Moreover the developed method is simple, cheap, easy to use by non-skilled personnel and requires no instrumentation for readout. Although there are a large number of microorganisms involved in mastitis infection, plasmin protease content in the milk is considered as a common host-derived biomarker independent of source of the mastitis causing microbes [14].

3.2. Application of the plasmin biosensor for mastitis diagnosis

To examine whether the plasmin biosensor can be used in the diagnosis of mastitis, the sensor was validated using a limited set of

Table 1
Results of the milk samples tested in this study from healthy cows and clinically diseased animals. The change in the SAM before and after treatment.

Sample. No	Pathogen	Sensor surface before	Sensor surface after
1	No Culture		
2	*Streptococcus uberis*		
3	*Streptococcus uberis*		
4	*Streptococcus uberis*		
5	*Enterobacter spp.*		
6	No culture		
7	No culture		
8	Mixed culture		
9	No culture		
10	*Streptococcus uberis*		
C1, C2 and C3	Control (no mastitis)		

milk samples drawn from cows suffering from clinical mastitis. After centrifugation of the milk, the supernatant was applied on the self-assembled monolayer of the plasmin detection sensor platform. Bacteriological culture tests were done to identify the causative agent. Table 1 shows the results of the milk samples tested in this study from healthy cows and clinically diseased animals. Samples number 1, 2, 3, 4, 5, 8 and 10 cleaved the peptide monolayer and turned the sensor surface from black to gold color. Seven of them are confirmed to have bacterial infections. The samples 6, 7and 9 with had no effect on the monolayer, presumably no plasmin or due to the presence of lower plasmin levels than the sensor detection limit (1 ng/ml). The bacterial culture results showed that the presence of mastitis causing pathogenic bacteria *S. uberis, Enterobacter* combination with mixture of other coagulase pathogens (Table 1). We used milk from clinically healthy cows as a control to ensure that the cleavage of the SAM monolayer is only due to mastitis. There was no change in the biosensor surface by the addition of mastitis negative milk from the healthy cows (C1, C2 and C3) indicates the sensor can be applied for the diagnosis of mastitis in the dairy milk.

A quantitative measurements were achieved using ImageJ software. The software measures the amount of cleaved area (appearance of golden color) to the total black area (before cleavage). Fig. 3 shows the results for the appearance of golden color and as a result of plasmin protease on magnetic nanobeads peptide conjugate. Fig. 3 clearly showed a proportional increase in the visible sensor golden colored area with the plasmin concentrations. Blank samples showed no reaction since the sensor demonstrated no cleavage on magnetic nanobeads peptide conjugate.

4. Conclusions

In this paper, we have reported a sensitive biosensor designed for the diagnosis of mastitis using the plasmin protease as a biomarker. Proteolytic activity of plasmin was monitored using a plasmin specific substrate, coupled to a magnetic nanoparticle based calorimetric biosensor platform. In the presence of plasmin, the specific peptide bonds in the substrate monolayer were cleaved and the peptide fragments coupled to the magnetic particles were collected with a permanent magnet. The area of gold surface visualized due to the cleavage of magnetic particles is proportional to the concentration of plasmin in the sample. The detection limit of

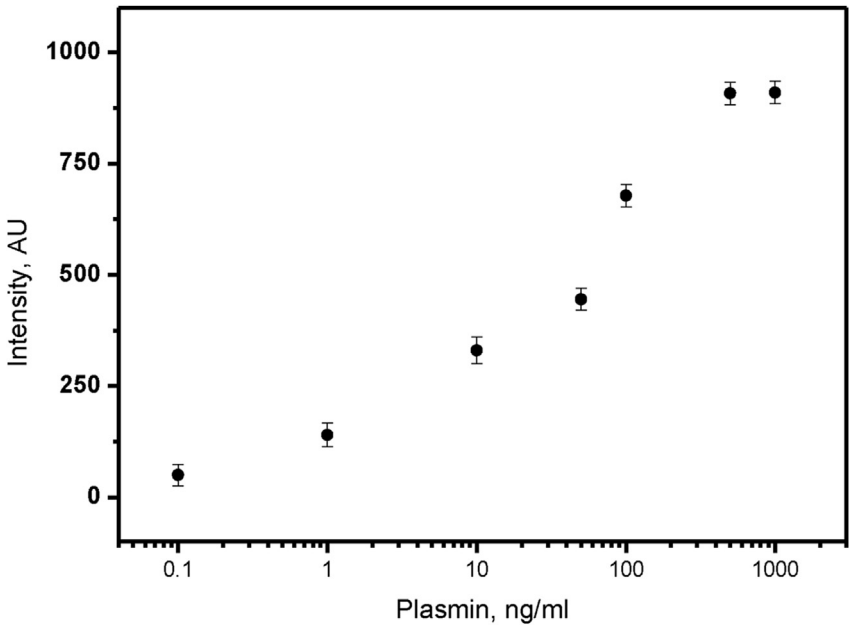


Fig. 3. Quantitative measurement of biosensor color changes using ImageJ software. The plot of gold surface intensity with increasing concentration of plasmin. The plasmin cleaves more substrate with increasing concentration (ng/ml) of plasmin in PBS buffer. The intensity of gold surface is proportional to the concentration of plasmin.

our biosensor for the detection of plasmin concentration was as low as 1 ng/ml. The proof of concept of the new sensor has been tested by the milk derived from cows suffering clinical mastitis. As our mastitis biosensor is rapid, simple, sensitive and specific, it can be applied for the cow-site detection of mastitis in milk and dairy products and might be suitable for applications in health and the environmental fields.

Acknowledgements

R.C would like to acknowledge the fund from the ORG office at Alfaisal University under grant Number 16415.

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