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Ultra-rapid colorimetric assay for protease detection using magnetic nanoparticle-based biosensors

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Sensitive protease detection methods often require time-consuming techniques and expensive instrumentation. To overcome this limitation, a novel, simple, sensitive and selective colorimetric detection approach was developed. This biosensing configuration was validated by the use of prostate specific antigen (PSA) protease as a model target. In this method, proteolytically active PSA capable of cleaving PSA substrates caused the release of black-colored magnetic carrier complexes, exposing the gold color sensor surface visible to the naked eye. The assay showed excellent sensitivity as well as specificity, capable of discriminating between different types of protease targets. The biosensor was able to quantitatively detect different PSA concentrations with a detection limit as low as 10 ng mL^{-1}. The sensor offers the possibility of developing a wash-less and cost-effective point-of-care device due to the simplicity of the probe immobilization process and the elimination of labeling and reporter molecules during the biosensing step.

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1 Introduction

Proteases are enzymes that cleave other proteins at specific peptide sequences. Proteases are estimated to comprise 2% of the human genome[1] and control a diverse array of biological processes in living organisms.[2–6] Therefore, it is not surprising that perturbations of protease activity underlie multiple pathological conditions such as cancer, neurodegenerative disorders, inflammation and cardiovascular diseases. In addition, proteases are required by many infectious microorganisms during replication. Protease activity measurements therefore have broad applications in drug screening, diagnosis, disease staging and biotechnology. For example, the detection of the prostate cancer biomarker, prostate specific antigen (PSA), increased the specificity of prostate cancer (PCa) diagnosis.[7,8] Likewise, the use of proteases as virulence factors by infectious microorganisms has facilitated the development of protease-targeted therapies for diseases of great relevance to human life such as acquired immunodeficiency syndrome (AIDS).[9]

Efforts to increase the utility and applicability of protease detection methods are usually frustrated by the limitation of assay safety, sensitivity, specificity, analysis time and ease of on-site analysis. In the past decades, the radioactive method was the method of choice for protease detection due to the availability and low cost of radioisotope-labeled antibodies. Its major drawback is the radiation exposure, environmental concern, disposal, short half-life, conjugate radiolysis and legislative bias.[10–13] For this, colorimetric detection methods were developed as a non-radioactive alternative.[14,15] However, these methods were considered to be of medium sensitivity compared to radioactive assay. Additionally, the usefulness of these methods was based on the measurement of a change in light absorbance of specific reporter groups in the targeted substrate, which is not universal. The development of chromogenic substrates was able to circumvent this shortcoming to a certain degree. Nevertheless specialized instruments such as spectrophotometer and plate reader were required which greatly reduce the potential for on-site analysis.

Currently, immunoassay detection methods including fluorescence,[16,17] absorption[18] and electrochemical approaches[19,20] are commonly used. On the other hand, a new approach in disease diagnosis has emerged as a result of recent advances in biosensor developments. Biosensors are miniaturized devices used to detect biological analytes such as proteins (antigen, antibody and enzyme), nucleic acids and metabolites. Moreover, these chip-scale devices are useful in the evaluation of the effectiveness of several medications.[21] In fact, augmented protease activities were indicative of cancer prognosis and therefore prompted their use as a cancer biomarker.[22,23] As a result, an urgent demand for simple, low-cost, highly sensitive and portable biosensors capable of detecting protease activity drives our effort toward the development of a colorimetric type biosensor. This sensor would be devoted to the detection of various biomarkers as a result of its easy-to-interpret results. Moreover, such biosensors will be highly desirable and will have

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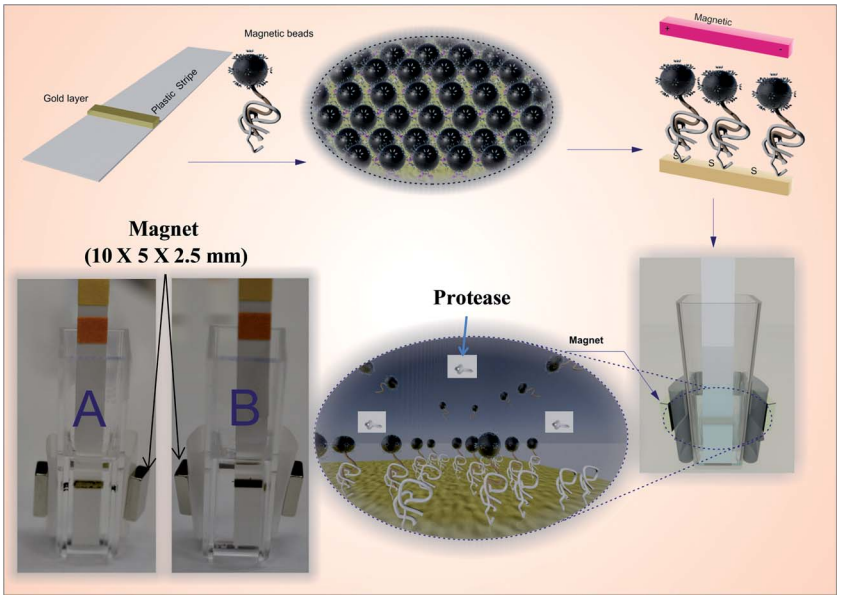

Fig. 1 Mechanism of PSA detection on a gold sensor surface. PSA-induced dissociation of the magnetic bead complex exposed the gold color of the bare sensor surface. (A) Biosensor chip functionalized with PSA substrate peptide–magnetic beads immersed in 100 ng mL^{-1} PSA solution. (B) Biosensor chip functionalized with PSA substrate peptide–magnetic beads immersed in 100 pg mL^{-1} PSA solution.

high potential in reducing the therapeutic turn-around time (TAT), healthcare costs, as well as facilitating patient compliance and clinical outcome.

This study describes the development of a novel, simple and inexpensive colorimetric sensor without any chromophoric labeling for the prostate cancer biomarker PSA. In this method, the probe consists of a specific PSA substrate peptide covalently bound to a magnetic bead through its N-terminus and linked to a gold sensor surface at the C-terminus. This construct results in a layer of magnetic beads adsorbed on the sensor surface masking its golden color. Upon cleavage of the substrate peptide by PSA, the peptide linkage between the magnetic beads and the gold sensor surface will be abolished. An external magnet will then collect the cleaved magnetic beads from the gold sensor surface as a result of protease's proteolytic activity. This will in turn reveal the golden color of the sensor surface (Fig. 1). This setup offers a highly specific and sensitive detection approach suitable for lab-on-a-chip (LOC) biosensor elaboration and will lead to an efficient and reliable device for protease detection.[24]

2 Experimental

2.1 Materials and reagents

Carboxyl-terminated beads of 30 nm diameter were provided by TurboBeads (Switzerland) suspended in a storage buffer (10 mM Tris, 0.15 M NaCl, 0.1% (w/v) bovine serum albumin (BSA), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium azide, pH 7.5). Neodymium bar magnets were purchased from Indigo Instruments Company. The recombinant prostate-specific antigen was purchased from Sigma-Aldrich (Ontario, Canada). The peptide sequences (specific peptide 1: GSGS GSGSEHSSKLQLAKGSGSGSGSC; control peptide: GGGSGSGSA RVLAEAGGGSGSGSC) were synthesized at the Sheldon Biotechnology Center at McGill University (Montreal, Canada). Coupling agents (1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS)) and the pH test indicator strips were purchased from Sigma (Ontario, Canada). Coupling buffer (10 mM potassium phosphate, 0.15 M NaCl, pH 5.5) and wash/storage buffer were prepared from chemicals of analytical grade.

2.2 Conjugation of the prostate-specific antigen substrate peptide to magnetic beads

A magnetic bead suspension (1 mL) was mixed with each peptide (1.0 mg mL^{-1}), coupling agent EDC (0.57 mg mL^{-1}) and NHS (12 μg mL^{-1}). The mixture was shaken gently at room temperature for 24 hours. The uncoupled peptides were removed by washing the beads 3 times with wash buffer. Finally, the beads were stored at 4 °C in storage buffer.

2.3 Gold sensor platform preparation

Clear adhesive tape was plated with a thin layer of gold. After which, a narrow piece was cut and stacked over the pH indicator plastic strip. This small strip was used as a physical support for the whole biofunctionalization process as well as the enzyme detection and quantification sensor.

2.4 Sensing monolayer immobilization

The gold sensing surface was covered with the magnetic bead–peptide solution and left at room temperature for one hour. Subsequently, an external magnetic field was applied using a permanent magnet (12.5 × 12.5 × 5 mm) with a field strength

of 3360 gauss and 573 gauss at 1 mm and 10 mm distance, respectively. This magnet was passed over the functionalized strip from a distance of 3 to 5 mm to remove any unattached magnetic beads.

2.5 Biosensing of PSA

In our sensor design, the detection mechanism of PSA is based on the proteolytic cleavage of a substrate peptide.[25] Therefore, we designed a peptide probe using a specific PSA substrate sequence EHSSKLQLAK,[26] with an 8-residue linker on either terminal of the peptide. The N-terminal of the peptide was attached to the magnetic bead. A cysteine residue was added at the C-terminal, allowing a gold–sulfur interaction[27] for the establishment of a self-assembled monolayer (SAM) of peptide and magnetic bead on the surface of the layer. On the other hand, two external permanent magnets were fixed on the outer side of the PSA solution reservoir (*i.e.* 2–3 mm distance from the strip according to the type of the reservoir used, *i.e.*, micro-centrifuge tube or disposable cuvette). After which, the functionalized PSA sensor platform was immersed into the solution and incubated for 5 minutes. During the enzymatic reaction, the permanent magnets would attract the released magnetic beads prompting a visual observation for a qualitative evaluation of the tested PSA samples. Moreover, a quantitative evaluation was possible by using different concentrations of PSA solution (100 ng mL^{-1}, 10 ng mL^{-1}, 1 ng mL^{-1} and 0.1 ng mL^{-1}) as shown in Fig. 2 and 3.

2.6 Statistical analysis of results

The activity of PSA was used as a quantitative indicator by measuring the ratio of black color (magnetic beads) to gold color. Fisher's exact test was conducted to assess the reproducibility between repeated assessments (*i.e.* qualitative determination of PSA present in the sample).[28] The assessment of PSA solutions of different concentrations (100 ng mL^{-1}, 10 ng mL^{-1}, 1 ng mL^{-1} and 0.1 ng mL^{-1}) was conducted four times.

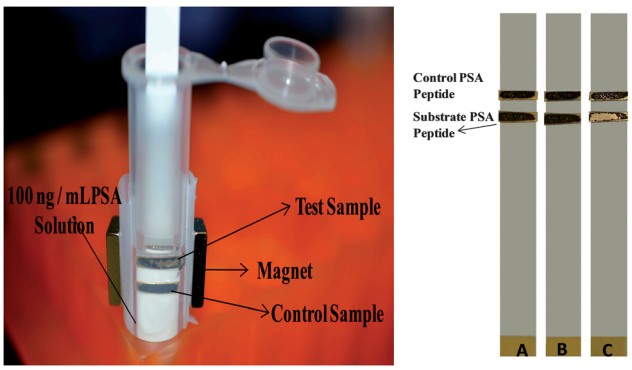

Fig. 2 Application of the colorimetric detection methods. (A) Biosensor chip functionalized with magnetic bead-specific and negative control peptides. (B) Functionalized biosensor after passing a magnet over the surface. (C) Functionalized biosensor after immersing in 100 ng mL^{-1} PSA solution.

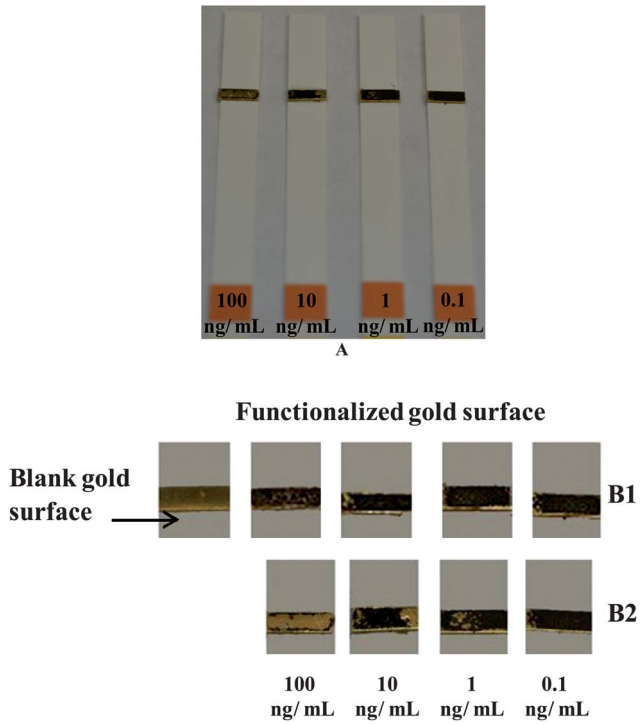

Fig. 3 (A) Colorimetric PSA sensor probe (a specific PSA substrate peptide covalently bound to a magnetic bead) under the effect of different PSA concentrations. (B) Visual comparison of the golden color of the exposed probe before (B1) and after (B2) PSA activity.

3 Results and discussion

Recent advances in nanotechnology in the field of biosensors have resulted in a broad range of biosensing applications. In particular, colorimetric biosensors that are capable of sensitive protease detection continue to develop towards accurate diagnostic and monitoring techniques for many serious disease such as cancer. PSA, a serine protease, has an elevated serum level in the presence of prostate cancers and was chosen as a model protease.[29,30] In our development of a low-cost PSA colorimetric biosensor, a gold sensor surface was completely covered with the magnetic bead–peptide complex after an optimum time of 1 hour reaction at room temperature. As a result, the golden color of the sensor chip was completely masked by the black magnetic beads. Subsequently, an external permanent magnet was passed over the functionalized gold sensor surface to remove any non-immobilized magnetic bead. The sensor was then ready for PSA detection.

The proteolytic activity of PSA was analyzed by immersing the functionalized gold sensor in 100 ng mL^{-1} PSA solution. The PSA-induced release of the peptide–magnetic bead moiety from the sensor surface was accelerated by an external magnet. The dissociation of the peptide–magnetic bead moiety from the gold sensor surface caused the destabilization of the blocking organic/metallic monolayer, revealing the golden color of the sensor surface visible to the naked eye (Fig. 2).

This biosensing method is also amenable to the quantitative determination of the amount of PSA present in solution. After

immersing the functionalized gold sensor in PSA solutions of different concentrations (100 ng mL^{-1}, 10 ng mL^{-1}, 1 ng mL^{-1} and 0.1 ng mL^{-1}) for 2 minutes under the effect of an external permanent magnetic field, we observed a gradual increase of the visible bare gold area in a comparative way with different enzyme concentrations and the blank (no enzyme) strips. This was due to the proteolytic activity of PSA which caused the dissociation of the peptide–magnetic bead complex from the sensor surface (Fig. 3). The activity of the enzymatic reaction was used as a quantitative indicator for PSA by measuring the ratio of cleaved magnetic beads of black color to gold. Moreover, Fisher's exact test indicated that there was no statistical difference ($p > 0.05$) in the proportions of samples assessed as PSA positive among the trials.

Longer incubation time (5 min) improved the sensibility of the biosensing, allowing the detection of PSA down to 1 ng mL^{-1}. This detection limit is markedly better than the recently reported detection limits of either the gold particle-based or aptamer-functionalized gold nanoparticle-based colorimetric method for other proteases such as thrombin.[31,32]

It was important to assess the specificity of the biosensor in the present detection mechanism. For this, we constructed a similar sensor monolayer with a different peptide substrate (a HIV-1 protease substrate peptide with no similarity to PSA cleavage site) and tested it with the previous ranges of PSA concentrations using the same methodology. The sensor showed no disruption of the SAM layer and no significant change in the golden color of the sensor surface, showing sufficient detection specificity.

4 Conclusions

In this report, a novel and low-cost colorimetric method using covalently attached PSA substrate–magnetic nano-carrier complexes is developed for the detection of protease activity. This biosensing configuration is amenable to an implementation for the qualitative and quantitative detection of proteases that can be performed by anybody, without the requirement of any sophisticated instrumentation. Our approach is based on a one-step biosensing monolayer preparation. The detection is also a single-step and wash-less process, based on the analyte protease-induced dissociation of the monolayer. The detection mechanism does not require any labeling or amplification schemes. In fact, the protease detection signal is a simple change of the substrate color that is visible to the naked eye. As prostate cancer diagnostics, this miniature sensor strip is capable of quantitative PSA detection, achieving a detection limit as low as 1 ng mL^{-1} with good specificity and reproducibility. This extremely simple PSA biosensing mechanism can be further optimized and developed into a cost-effective lab-on-a-chip device suitable for point-of-care usage. Moreover, the developed sensor strip has a rapid response (~5 minutes analysis time) which would lead to an ultra-rapid diagnostic tool extremely useful for TAT minimization, patient satisfaction and clinical outcome improvement. This simple and low-cost biosensor has practical applications and we believe that significant research and development efforts will be devoted to this category of biosensor.

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