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Short communication

A novel Au-nanoparticle biosensor for the rapid and simple detection of PSA using a sequence-specific peptide cleavage reaction ☆

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ABSTRACT

PSA (prostate-specific antigen) is one of the most widely used proteins for the diagnosis of breast and prostate cancer. Of note, PSA displays enzymatic activity for the specific peptide sequence HSSKLQ, which it recognizes and cleaves. In this study, we developed a site-specific enzymatic-cleavage-reaction-based biosensor for the detection of PSA using fluorescein isothiocyanate (FITC)/peptide-conjugated gold (Au) nanoparticle complexes (FPANs). The FPANs do not initially fluoresce in the spectral region associated with the fluorophore, due to the quenching effect of the Au nanoparticles. When PSA was added to a solution containing the FPANs, PSA recognized and cleaved the specific sequence of the peptides attached to the Au nanoparticles. As a result, FITCs were separated from the Au nanoparticles and emitted strong fluorescence in their spectral region. Using this detection method, PSA was successfully detected as a function of concentration (10 pM—100 nM). This approach is superior to the immunoassay with respect to the performance of sensor, which is very rapid, simple, and one-step method for the detection of PSA and other protein markers can be measured for the early detection of several diseases.

1. Introduction

Prostate-specific antigen (PSA) is the best serum marker currently available for the detection of prostate and breast cancer (Healy et al., 2007). Most current assays for PSA detection are processed by large analyzers at dedicated testing site, a procedure that requires samples to be sent away for testing. Conventional immunoassay methods, including the enzyme-linked immunosorbent assay (ELISA) (Voller et al., 1978; Yates et al., 1999), radioimmunoassay (Goldsmith, 1975; Teppo and Maury, 1987), chemiluminescence assay (Matsuya et al., 2003; Cesaro-Tadic et al., 2004), and electrochemical immunoassay (Du et al., 2010; Ahirwal and Mitra, 2010), all allow for reliable detection. These immunoassay methods can be sensitive and selective for the detection of PSA but there are some drawbacks to these approaches including time-commitment, sustained stability, exposure of active sites for antigen binding, and the fact that antibodies, as proteins, can easily denature with temperature changes. In the case of ELISA, a sandwich structure must be used to measure the concentration of a disease marker, so at least two antibodies must bind at each specific site, and repeated washing steps may wash away antibodies and reduce detection rates. Moreover, advanced skills on the part of the user are required due to several complicated steps of ELISA and the use of diverse analysis equipment. Thus, there is a need to develop PSA-sensing systems that are more stable, rapid, and simple than the immunoassay methods in order to develop a point-of-care testing (POCT) system.

Recently, biosensors that use a sequence-specific peptide decomposition reaction has been suggested as an alternative to existing immunoassay-based biosensors. This method has been used to detect some specific proteins — i.e., proteases — that enzymatically cleave specific peptide bonds. Proteases have been of particular interest as disease-relevant biomarkers because they are involved in several important human diseases, including cancer. Typical examples of proteases include matrix metalloproteinases (MMPs), caspases and thrombin (Overall and Kleifeld, 2006; Concha and Abdel-Meguid, 2002). For example, a biosensor for the detection of MMP-2 has been developed using an induced peptide-cleavage reaction through the enzymatic property of MMP-2. In addition, the development of MMP assays using nanoparticles and unique peptide sequences has been examined due to the cleavage ability of the peptides and their potential for use as cancer prognostic markers (Kim et al., 2008; Xia et al., 2008). For that reason, the development of novel protease assays with high selectivity and sensitivity is of great significance for the

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diagnosis of protease-relevant diseases and development of therapeutic agents.

In particular, the biological function of PSA involves the enzymatic cleavage of a specific peptide sequence in serum fluid, so this activity can be exploited to develop a novel assay for PSA detection that could be more simple and stable than conventional immunoassay systems—the novel assay need not be concerned with the stability of or complications arising from antigen—antibody binding. So far, aside from the aforementioned MMP-sensing systems, little has been done in this direction for PSA and other biomarker. Therefore, we developed a novel PSA biosensor that consists of Au nanoparticles and a specific peptide sequence (HSSKLQ) that is recognized and cleaved by PSA (Denmeade et al., 1997). An enhanced sensitivity for PSA detection was achieved using this detection method and the process was much simpler than available immunoassay systems.

2. Experimental methods

2.1. Materials and reagents

Au nanoparticles with a diameter of 60 nm were purchased from BBInternational (Cardiff, UK) and PSA was purchased from Sigma-Aldrich (St Louis, MO. USA). The specific peptide sequence with an attached fluorescein moiety (N′-CCCCCCGLAibAAGGHS-SLKQGK-FITC-C′) was synthesized by Peptron Inc. (Daejeon, Korea). Phosphate buffered saline (PBS; pH 7.4, 15 mM) solution was purchased from Sigma-Aldrich (St. Louis, MO, USA). Each of these solutions had a pH of 7.4 to mimic physiological pH. Deionized (DI) water, obtained from a Millipore water system, was used throughout the experiments. All other chemicals were analytical grade reagents.

2.2. Au-nanoparticle complex preparation

To prepare the Au nanoparticles (AuNPs), 1 ml of an aqueous suspension of colloidal AuNPs (diameter 60 nm, 2.6×10^{10} per ml) was centrifuged for 5 min at 10,000 rpm, and the supernatant was removed. The particles were resuspended in a 0.15 M PBS solution at pH 7.4, and C-terminal fluorescein isothiocyanate (FITC)-functionalized polypeptides (N′-CCCCCCGLAibAAGGHSSLKQGK-FITC-C′, 82.32 μmol/ml) were added to the solution. The solution was incubated on a rocking shaker for 3 h to allow the polypeptide to bind with the AuNP surface through an Au-thiol interaction.

2.3. Confirmation of peptide attachment to AuNPs

The AuNP complexes were attached to the FITC-tailed peptides to generate our novel PSA biosensor, the FITC/peptide-conjugated Au-nanoparticle (FPAN) complexes. To confirm binding, the absorbance value at 562 nm of the complex was measured and compared to the value of the peptide in the supernatant using the BCA (bicinchoninic acid) assay (BCA assay kit, Pierce) and a UV—vis spectrophotometer (Jasco, V530) after centrifugation for 10 min at 13,200 rpm. In addition, the UV spectra of the AuNP, FITC, and FPAN in the solution were measured to assess peptide binding to the AuNPs based on the red-shift of the absorbance peak compared with the original peak of the AuNPs alone.

2.4. Measurement of PSA concentration using an enzyme cleavage reaction

The FPANs do not fluoresce in the spectral region associated with the FITC due to the quenching effect of the AuNPs. When PSA is added to a solution containing the FPANs, PSA will recognize and cleave the specific sequence of the peptides attached to the AuNP surface. As a result, FITC would be separated from the AuNPs and a strong fluorescent signal proportional to the PSA concentration would be observed in the appropriate spectral region. To cause this reaction, constant concentration of PSA was added to the FPANs solution, and allowed to react for 60 min. After the enzyme reaction was complete, AuNPs were spun down and the separated fluorophores present in the supernatant were analyzed using a fluorescence spectrophotometer (High Performance Fluorescence Spectrophotometer Hitachi Model F-7000) at 521 nm.

3. Results and discussion

3.1. Characterization of the peptide-modified AuNP complex

A nanoscale sensor system for PSA detection was developed using 60 nm colloid AuNP complexes containing small peptide sequence-fluorophore (FITC) fragments. As a first step, the conformation of the FPAN was assessed using the UV absorbance value at 562 nm. The BCA assay, which is a colorimetric assay, was used to measure the peptide concentration (Fig. 1(a)). In the BCA method, proteins induce the reduction of Cu (II) cations to Cu (I), and then bicinchoninic acid is added and the resultant colorimetric change was assessed. The peptide concentration in the AuNP solution (2.6×10^{10} per ml) was 100 μmol/ml, and its absorbance value at 562 nm was ~0.32. The peptide concentration in the supernatant after attachment to the AuNP surface was lower (~0.13) than before the reaction due to the attachment to the AuNP surface. The number of peptides attached to the AuNPs can be inferred as proportional to the difference (~0.19) between pre- and post-attachment absorbance values. These results clearly indicate successful peptide attachment to the AuNPs.

In addition, it is possible to confirm the conjunction of peptide-AuNP complex directly. Fig. 1(b) shows the UV/vis absorbance spectrum for each nanomaterial (AuNPs, FITC, and FPANs), which can also be used to evaluate binding to the AuNPs. AuNPs alone had a singular absorbance peak at ~530 nm, which was shifted to the right after the biomaterials were attached to the surface of the nanomaterials due to the LSPR (localized surface plasmon resonance) effect (Delfino and Cannistraro, 2009; Li et al., 2010). The red-shift of the absorption spectra for the peptide-AuNP complex depended on nanoparticle size, particle aggregation, and local dielectric environments, which affect the surface plasmon resonance phenomena. This absorbance method was deemed suitable for directly identifying binding events between the peptide and nanoparticles. As shown in Fig. 1(b), the absorbance peak for the AuNPs alone was at 535 nm, while the peak for the AuNP complex was shifted to the right by ~10 nm. Therefore, conjunction of the peptide to the AuNPs was independently demonstrated by indirect and direct methods.

3.2. Determination of an efficient amount of peptide for binding to the AuNP surface

For the purpose of an efficient PSA sensor, it is essential to compare the fluorescence signals of FPANs in the presence or absence of PSA in order to determine a suitable peptide concentration on the AuNP surface. As shown in Fig. 1(c), the lowest and highest peptide concentrations tested (10.4 and 164.64 μmol/ml, respectively) in AuNP solution both resulted in a low positive-to-negative ratio (100 nm PSA/non-PSA), corresponding to the attachment of too few peptides for PSA cleavage to efficiently occur, or too many peptides, which may cause steric hindrance, respectively. More specifically, when the amount of peptide on the AuNP surface is too low, enzymatic cleavage by PSA is low due to the

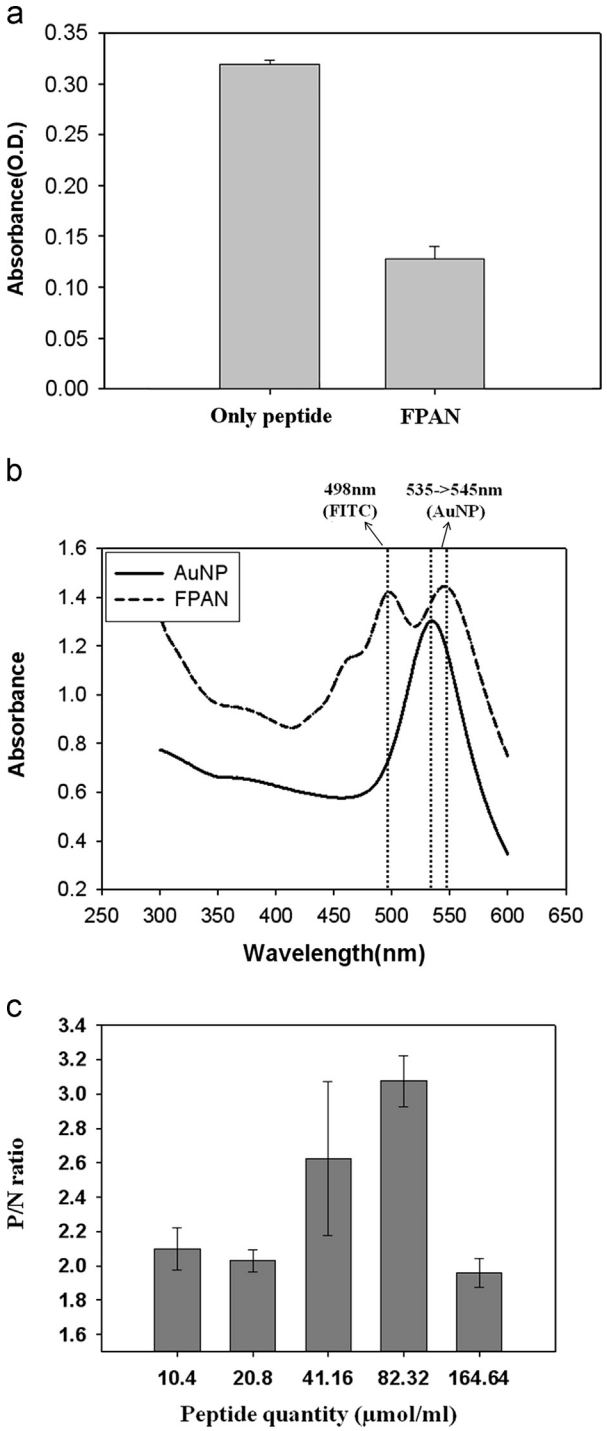

Fig. 1. Confirmation of successful conjugation of the peptides to the AuNPs. (a) UV absorbance change before and after constructing the FPANs. The first graph is the peptide absorbance value at 562 nm measured using the BCA protein assay. After their attachment to the AuNPs, the absorbance value of the peptides in the supernatant decreased due to their conjugation to the AuNPs. (b) UV—vis spectrum of AuNPs and FPANs. AuNPs had a sharp absorbance peak at 535 nm, and the absorbance peak of the FPAN after the reaction was red-shifted. (c) Assessment of the FPAN probe at different peptide-FITC concentrations. The positive-to-negative (P/N) ratio was determined from the activity in the presence and absence of PSA (100 nM each). The peptide-FITC produced the best signal ratio at a concentration of 82.32 μmol.

presence of insufficient levels of cleavable peptides on AuNPs. On the other hand, when large quantities of peptide are used, the space for the peptide-PSA reaction is insufficient because the peptide-FITC molecules are densely packed on the AuNP surface, i.e. steric hindrance. Therefore, an appropriate peptide quantity proportional to AuNP concentration is needed to develop the most effective sensing system. Through these experiments, we obtained an effective peptide concentration (82.32 μmol/ml) for efficient PSA detection.

3.3. PSA sensor using an enzyme cleavage reaction

Detection of PSA through enzymatic cleavage was accomplished using FPANs consisting of an AuNP and attached FITC-tailed polypeptides. This approach was very simple and required only reacting the AuNPs (60 nm) with the polypeptide complexes (N′-CCCCCCGLAibAAGGHSSLKQGK-FITC-C′) in a 1.5-ml tube (Fig. 2). The peptide sequence was selected to play four separate roles in the sensing system (inset). First, the six-cysteine group was used to attach the polypeptide to the AuNP surface so that the quenching effect of the AuNP would prevent the fluorescent signal from FITC from being emitted. AuNPs are known to have a superior quenching efficiency over a broad range of wavelengths compared with other organic quenchers. Second, Aib (α-isobutyric acid) maintained the rigidity of the peptide, which was important for the third role: the enzymatic cleavage reaction of the peptide sequence (HSSKLQ) by PSA. Forth, the FITC fluorophore was used to emit a fluorescent signal following the loss of quenching after peptide cleavage.

The simple process used to construct the FPAN probe in this study is superior to other sensing systems because it reduces the possibility for probe defects in the sensing process and it can be easily scaled-up for commercialization. The FITC molecules that are attached to the AuNPs through the polypeptide are quenched by the AuNPs, so that intact FPAN does not emit a fluorescence signal. When PSA was added to these suspended FPANs, they recognized and cleaved the peptide sequence (HSSKLQ), resulting in the release of the FITC molecules from the AuNPs, which produces an increase in green fluorescent light. In other words, the PSA protein un-quenches the fluorescent signal of the FITC molecules due to the enzymatic activity of PSA.

Through these experiments, we observed a linear relationship between fluorescence intensity and PSA concentration. The linear relationship with concentration of PSA and fluorescence signal intensity is very important in order to find out the concentration of PSA through the fluorescence signal in unknown sample by using the sensor. In details, if fluorescence signal is proportional to the concentration, it is possible to make the calibration curve through its relationship. The created calibration curve is available to find PSA concentration in random samples conversely. By using our sensing system, it is possible to diagnose prostate cancer through the measurement of fluorescence signal.

The advantage of the peptide-based PSA assay as compared with conventional methods, such as immune-based sensing systems, is that this system is not liable to cause protein denaturation that would result in a loss of the capture function of the target molecules. In addition, this system is less difficult than the heterogeneous microarray approach, where an expensive capture antibody is fixed at very high concentrations. This biosensor-based method requires only one step for PSA sensing, whereas sandwich ELISA requires several steps: i.e., antigen isolation, antibody fixation, antigen—antibody reaction, detection probe immobilization, etc. Moreover, particle separation is simple and fast, and offers a degree of detection simplicity even when only a few targets can be identified in a test sample. Finally, although FITC was used as the optical probe, other types of probes could be applied to this system, thus allowing for detection methods other than fluorescence. These include quantum dots, radioactive groups, catalytic groups, electrically generated groups, Raman-active groups, and redox-active groups. In principle, the detectable

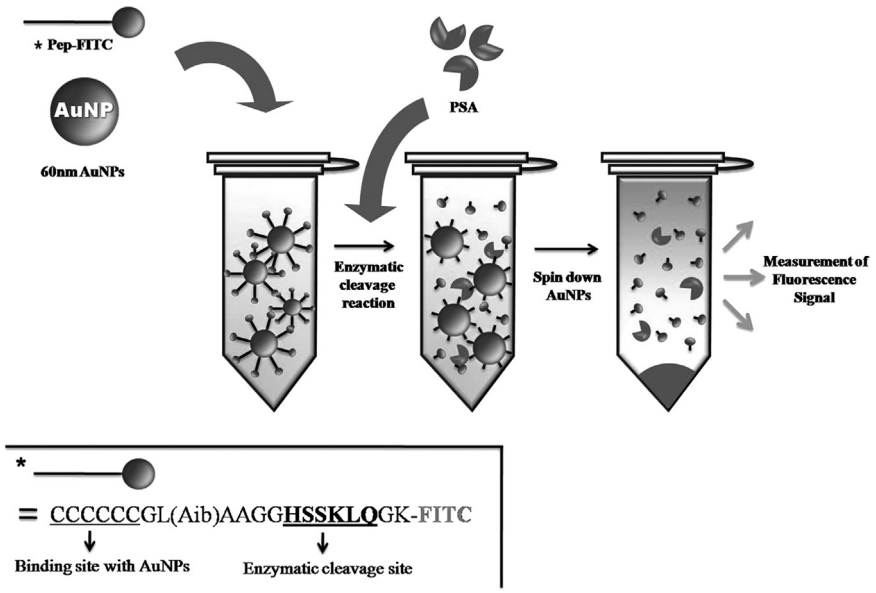

Fig. 2. Schematic illustration of the novel PSA sensor system, which utilized FPANs containing a specific peptide with FITC as a signal generator. The peptide contained six cysteine residue that was used to conjugate the peptide to the AuNP surface, an Aib (α-isobutyric acid) residue that maintained peptide rigidity, and a PSA cleavage site. PSA cleaved the specific peptide sequence to release it from the AuNP surface, which resulted in the generation of a fluorescence signal at 521 nm.

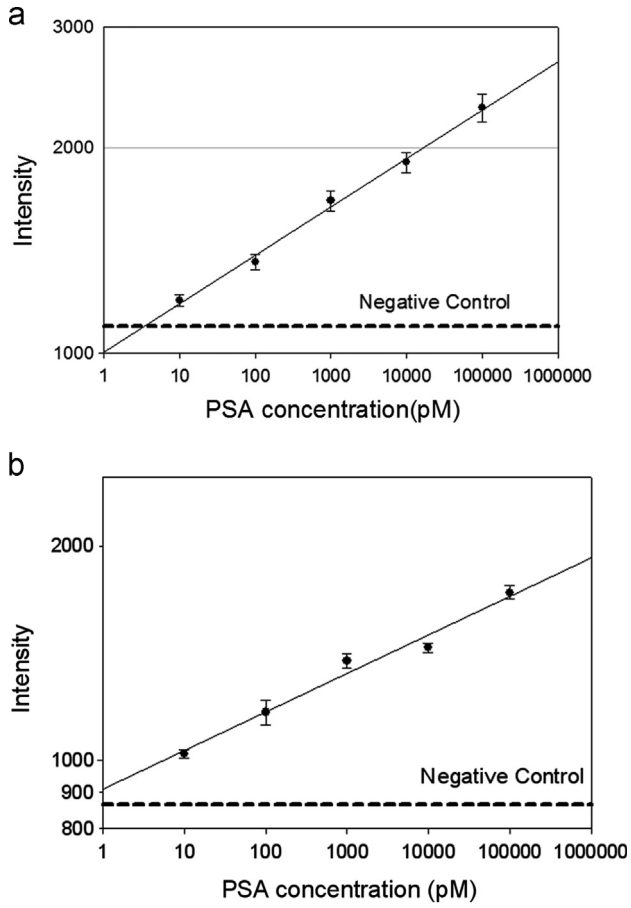

Fig. 3. Fluorescence intensity of the novel PSA sensor system in PBS (a) and in human serum (b). The excitation and emission wavelengths were 490 nm and 521 nm, respectively. Based on this data, the detection limit was found to be 10 pM of peptide. PSA cleaved the specific peptide sequence, resulting in an increase in the emitted fluorescence signal at 521 nm.

probe can be changed to anything that produces a distinct and measurable chemical or physical signature. The limit of detection and the dynamic range of our sensing system are shown in Fig. 3 for PSA in PBS and human serum. This nanoprobe-based assay system produces a proportional curve according to PSA concentration from 10 pM to 100 nM. In details, it can be measured from pico-molar to nano-molar concentration of PSA, five magnitude range with linear relationship and its correlation coefficient is about 0.98 (R^2). Fig. 3 shows a linear relationship between the fluorescence intensity and the PSA concentration under PBS and human serum conditions. As exhibited in Fig. 2, upon increasing the concentration of PSA, the value of the fluorescence intensity increased. Importantly, this sensing system has several advantages for diagnostic applications compared with other PSA sensors including: its dynamic range, which includes the clinical range of PSA (about 4—10 ng/ml, corresponding to 122—637 pM) (Kupelian et al., 1996); higher stability than immunosensors, due to the increased stability of the polypeptide chain relative to proteins or antibodies; and, simple and rapid detection, which can be achieved within 1 h, a significant reduction over conventional immunoassays without loss of sensitivity and selectivity.

4. Conclusion

In summary, PSA, which is a diagnostic marker of prostate cancer, was rapidly detected using a novel nanocomplex composed of AuNPs and polypeptides within an hour. This approach provides the framework for the development of new sensors that are based on detecting proteins that have enzymatic cleavage activities. It is superior to other PSA sensors with respect to rapid and simple detection, which originates from the enzymatic reactivity of the target protein. In addition, this system can measure over a dynamic concentration range (10 pM—100 nM), which was within the range required for clinical detection. We propose that this kind of approach is simpler, faster, and more cost-effective than conventional

immunoassay approaches, and that it can be modified for the detection of any target that displays enzymatic cleavage activity.

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