Determining the Ratio of Two Types of Prostate Specific Antigens with Biochips and Gold Nanoparticles for Accurate Prostate Cancer Diagnosis

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Prostate-specific antigen (PSA) is a well-known biomarker for prostate-cancer diagnosis. However, the serum PSA measurement alone is insufficient for accurate diagnoses because the correlation with cancer is weak within the gray zone—the biomarker level range wherein a clear-cut diagnosis is impossible. As such, accurate prostate cancer diagnosis has been supplemented by measurements of the ratio of two types of PSA: free PSA (fPSA) and complexed PSA (cPSA; α-1-antichymotrypsin-bound PSA). Herein, we describe a new method for measuring the ratio of these two types of PSA by using gold nanoparticles (AuNPs) and biochips. Both types of PSA in a sample are captured by the antibody immobilized on a biochip based on self-assembled monolayers on gold. fPSA and cPSA on the biochip are then distinguished by AuNPs that present antibodies against fPSA and cPSA, respectively. The presence of PSAs in a sample is detected with laser desorption/ionization time-of-flight mass spectrometry by observing reporter molecules, called amplification tags (Am-tags), on the AuNPs. One of the reporter molecules is an Am-tag without isotope labeling, and the other is a deuterium-labeled Am-tag (dAm-tag). These tags amplify mass signals so as to enhance the sensitivity of the method. A comparison of the mass intensities between the Am-tag and dAm-tag signals allows the determination of the ratio between fPSA and cPSA. We validated the selective measurement of fPSA and cPSA at different ratios in 50, 75, and 100 pM of total PSA (fPSA + cPSA) solutions corresponding to the gray zone in prostate-cancer diagnosis (4 - 10 ng/mL). Finally, the two types of PSA were spiked in fetal bovine serum at various ratios, and our strategy greatly afforded their accurate ratios as spiked based on a constructed calibration curve. These results clearly indicate that the strategy is applicable to human serum as a diagnostic and prognostic assay for prostate cancer.

Keywords Biochips, cancer diagnosis, gold nanoparticles, laser desorption/ionization time of flight mass spectrometry, multiplexing, prostate specific antigen, self-assembled monolayers

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Introduction

Prostate-specific antigen (PSA), a 33-kDa glycoprotein enzyme serine protease, is produced by the prostate gland in the male reproduction system, and is present in small quantities in the serum of healthy men. It is used as a biomarker for prostate cancer because increased PSA levels—more than 4 ng/mL in blood—are strongly correlated with the disease. However, the use of PSA testing for prostate cancer diagnosis is controversial because PSA levels can increase by prostatitis, benign prostatic hyperplasia, and age, thereby resulting in a false positive. Therefore, PSA levels between 4 and 10 ng/mL cannot provide clear-cut diagnostic information because the correlation with disease is weak in this range, which is called the gray zone.

Serum PSA exists in two forms: a complexed form (cPSA) bound to the serum protein α-1-antichymotrypsin (ACT) and intact PSA (free PSA; fPSA). In prostate cancer patients, the ratio of fPSA to total PSA (fPSA; fPSA + cPSA) is decreased and the risk of cancer increases if the fPSA-to-tPSA ratio is less than 25%. In addition, the probability of prostate cancer increases rapidly as the ratio decreases. Thus, quantitative information about fPSA and cPSA improves the reliability of PSA testing in men with PSA levels between 4 and 10 ng/mL, whereas total PSA levels in this range fail to provide accurate diagnostic results.

Practically and clinically, common enzyme-linked immunosorbent assays—in which two antibodies recognize the two types of serum PSA—have been widely used to measure PSA. However, this strategy is most frequently coupled with optical probes, such as chemiluminescent or fluorescent labels for reporting analyte recognition and lacks sufficient sensitivity or accuracy for prostate-cancer diagnosis, particularly in men whose PSA levels are within the gray zone (4 - 10 ng/mL). Therefore, alternative methods to accurately detect fPSA and cPSA and to measure their ratio in the gray zone are in demand to improve diagnostic precision. Until recently, very few studies have reported methods that meet this need. As a typical example, Chiriacò et al. developed a microfluidic platform based on electrochemical impedance spectroscopy that allows

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the simultaneous detection of the two types of serum PSA. As such, we propose a simple and straightforward method for multiplexed detection of fPSA and cPSA in the gray zone as well as the quantitative capability to determine their ratio to increase the diagnostic and prognostic accuracy of PSA testing in prostate cancer.

Recently, we reported an ultrasensitive PSA detection method as a diagnostic and prognostic tool for breast cancer. This mass signal amplification strategy uses organic-matrix-free laser desorption/ionization time-of-flight mass spectrometry (LDI-TOF MS) and gold nanoparticles (AuNPs).\textsuperscript{12–15} In the present study, we extended this method with a multiplexing capability to detect the two types of serum PSA and quantify their ratio in complex samples with tPSA levels between 4 and 10 ng/mL. Our strategy combines two antibodies that recognize the PSA epitopes with two amplification tags (Am-tags), one of which is deuterium labeled. Scheme 1 delineates our strategy and the structural formulas for the multiplexed detection of fPSA and cPSA and determination of their ratio in a complex sample by using biochips and AuNPs with LDI-TOF MS signal amplification. A PSA-containing sample is applied to the gold chip that presents a PSA-capturing antibody. Subsequently, fPSA-capturing AuNPs loaded with Am-tag reporter molecules and cPSA-capturing AuNPs loaded with deuterium-labeled Am-tag (dAm-tag) reporter molecules are applied to the chip. After rinsing, LDI-TOF MS analysis allows not only the multiplexed detection of the two types of PSA, but also the determination of their ratio in the sample through a comparison of their mass signal intensities. As shown in Scheme 1b, the structure of the dAm-tag is essentially identical to that of the Am-tag, but the molecular weight differs owing to the replacement of four hydrogen atoms with deuterium at two carbons. Thus, a comparison of the mass intensities between Am-tag and dAm-tag provides quantitative information about the ratio of fPSA and cPSA in samples.

**Experimental**

**Reagents and chemicals**

AuNPs (40 nm diameter) were prepared by the method previously reported by Schwartzberg \textit{et al.}\textsuperscript{16} Deuterated tri(ethylene glycol)-terminated alkanethiol (for the dAm-tag) was also prepared as previously reported.\textsuperscript{17} Gold chips were prepared via the vacuum deposition of titanium (10 nm), followed by gold (50 nm) onto cover slips. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), dimethyl sulfoxide, and PSA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS) and fetal bovine serum (FBS) were obtained from WelGENE Inc. (Seoul, Korea), and ethyl alcohol (EtOH) was purchased from Merck (Darmstadt, Germany). Antibodies (5A6, 8A6, and TMF1#4B5) were obtained from Hytest Ltd. (Turku, Finland).

**Preparation of (d)Am-tag-coated AuNPs and antibody-presenting biochips**

The self-assembled monolayers on AuNPs and gold chips to prepare (d)Am-tag-coated AuNPs and antibody-presenting biochips was performed by using a previously reported protocol.\textsuperscript{11} Briefly, AuNPs (2 mL, 1.1 nM) were incubated in a mixed solution (100 mM in EtOH) of tri(ethylene glycol)-terminated alkanethiol or deuterated tri(ethylene glycol)-terminated alkanethiol (for the Am-tag or dAm-tag, respectively) and carboxylic acid penta(ethylene glycol)-terminated alkanethiol in a ratio of 95:5. A carboxylic acid-presenting gold
Mass analysis was performed with an Autoflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam® N2 laser with the wavelength of 337 nm as an ionization source. The mass spectra of the positive ions were acquired in the reflector mode under the following parameters: accelerating voltage of –19 kV, 100 Hz repetition rate, and an average of ~500 shots without the use of a matrix.

**LDI-TOF MS analysis**

Mass analysis was performed with an Autoflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam® N2 laser with the wavelength of 337 nm as an ionization source. The mass spectra of the positive ions were acquired in the reflector mode under the following parameters: accelerating voltage of –19 kV, 100 Hz repetition rate, and an average of ~500 shots without the use of a matrix.

**Results and Discussion**

**Characterization of the Am-tag-coated AuNPs and the biochips with antibodies**

We first verified the surface modifications of the AuNPs and gold chips by using matrix-assisted LDI-TOF MS.18–20 AuNPs were modified so as to have carboxylic acid functionality among Am-tag or dAm-tag molecules in a ratio of 95:5. LDI-TOF MS of these carboxylic acid-presenting AuNPs loaded with Am-tag or dAm-tag gave peaks at m/z 552.9 [M+Na]+ and 560.9 [M+Na]+ corresponding to the Am-tag or dAm-tag disulfides, respectively, and at m/z 699.0 [M+Na]+ and 703.0 [M+Na]+ corresponding to the carboxylic acid-containing disulfides, respectively (Figs. S1a and S1c).

After conjugation of the antibodies to the AuNPs, the peaks of the carboxylic acid-containing disulfides disappeared, which implied that the antibodies had been immobilized to the AuNPs via an amide coupling reaction (Figs. S1b and S1d). Similarly, MS analysis of the carboxylic acid-presenting chips before and after antibody immobilization indicated that amide bonds had formed with carboxylic acid groups on the chips (Figs. S1e and S1f). Note that the peaks at m/z 568.9, 576.9, and 709.4 corresponded to the potassium adducts of the tri(ethylene glycol)-containing disulfide ([M+K]+).

**Multiplexed detection of the two types of serum PSA**

Mixtures of fPSA and cPSA in ratios varying from 0:10 to 10:0 at concentrations of 50, 75, and 100 pM were prepared in PBS. A sample of the solutions (10 mL) was applied to the antibody-presenting chip for 30 min, and the chip was then rinsed with PBS on a shaker for 30 min and dried under a weak stream of nitrogen. Subsequently, antibody-presenting AuNPs (5 mL each of Am-tag-coated and dAm-tag-coated AuNPs) were applied to the chip for another 30 min. The chip was then gently washed with water on a shaker for 30 min to remove nonspecifically bound AuNPs, and dried under a weak stream of nitrogen. The chip was analyzed directly with LDI-TOF MS without the use of a matrix.

**Quantification of fPSA and cPSA spiked in FBS**

The two types of serum PSA in a ratio of 1:9 and 2:8 (fPSA to cPSA) were spiked in FBS (diluted with PBS ranging from 1/10 to 10% [v/v]) at a concentration of 50 pM PSA. The remainder of the protocol was identical to that described above.

**Selective detection of the two types of serum PSA in mixed samples**

Next, we evaluated the selectivity of our strategy for the discrimination of the two types of serum PSA. We prepared PSA-spiked FBS solutions containing fPSA only, cPSA only, or a 1:1 mixture of fPSA and cPSA at a concentration of 50 pM PSA, which is within the gray zone for prostate cancer diagnosis. The anti-PSA-presenting chips were then treated with these solutions, followed by a mixed solution of Am-tag-coated AuNPs with anti-fPSA (for fPSA capture) and dAm-tag-coated AuNPs with anti-ACT (for cPSA capture). After a rinse to remove nonspecifically bound proteins and AuNPs by changing the salt concentration, the chips were analyzed by using LDI-TOF MS without a matrix. The chip treated with fPSA-containing FBS afforded one clear major peak at m/z 553 [M+Na]+ corresponding to Am-tag, whereas one major peak at m/z 561 [M+Na]+ corresponding to dAm-tag was clearly observed in the chip treated with cPSA-containing FBS (Figs. 1a and 1b). In the tPSA sample (fPSA + cPSA), peaks for both Am-tag and dAm-tag were observed with the same intensities, which reflected the quantitative aspect of our method (Fig. 1c). This result implies that our method can selectively differentiate between the two types of serum PSA by using the two antibodies on the chip and AuNPs. In addition, all mass spectra afforded the expected major peaks with very high signal-to-noise ratios, which indicated that our mass signal amplification strategy with Am-tag and dAm-tag is readily applicable to the ultra-sensitive detection of PSA in complex samples.

**Multiplexed detection of the two types of serum PSA in different ratios and the construction of calibration curve**

The quantitative aspect of our method was assessed by evaluating the capacity for the multiplexed detection of the two types of serum PSA in different ratios. We prepared mixtures of fPSA and cPSA in different ratios from 0:10 to 10:0 at concentrations of 50, 75, and 100 pM, which correspond to the gray zone in prostate-cancer diagnosis (4 – 10 ng/mL).
A sample of the solutions was applied to the antibody-presenting chip for 30 min. After rinsing, antibody-presenting Am-tag-coated and dAm-tag-coated AuNPs were applied to the chip for another 30 min. The chip was then gently washed with water on a shaker to remove nonspecifically bound AuNPs, and LDI-TOF MS analysis without a matrix was performed directly.

As shown in Fig. 2 (50 pM) and Fig. S2 (75 and 100 pM), two major peaks—at m/z 553 [M+Na]+ corresponding to Am-tag and at m/z 561 [M+Na]+ corresponding to dAm-tag—were clearly observed. Furthermore, the ratio of the mass intensities of Am-tag and dAm-tag was in close accordance with the ratio between the two types of serum PSA, which implied that the dAm-tag behaved identically to the Am-tag in the MS analysis process, and thus, the intensity ratio of Am-tag and dAm-tag directly reflected the ratio of the two types of serum PSA in the samples.

Next, we constructed calibration curves via linear regression of the ratio of signal intensities between Am-tag and dAm-tag in Fig. 2 and Fig. S2 against the fPSA/tPSA. As mentioned above, the purpose of this study was to determine the ratio of fPSA and cPSA in samples regardless of the tPSA concentration, particularly in samples within the gray zone. Therefore, the calibration curves constructed at any tPSA concentration in the gray zone were expected to have the same slope to provide the same quantitative information regarding the ratio of fPSA and cPSA. Indeed, the calibration curves constructed at the three concentrations show ed linearity with almost identical slopes of ca. 1.31, particularly over small fPSA fractions ranging from 0.1 to 0.4 (Fig. 3). This range is known to be clinically meaningful because the sera samples of prostate cancer patients have small fPSA factions. In general, the probability of prostate cancer significantly increases when the fPSA-to-tPSA ratio is less than 0.25.

**Determining the ratios of fPSA and cPSA in complex samples**

To evaluate the practical applicability of our method, we measured the ratio of fPSA and cPSA spiked in FBS. The two types of serum PSA in a ratio of 1:9 and 2:8 (fPSA to cPSA) were spiked into FBS (diluted with PBS ranging from 1 to 10% [v/v]) at a concentration of 50 pM PSA. LDI-TOF MS analysis afforded highly precise ratios of two types of PSA as spiked (Table 1). This result clearly indicated the practicability of our strategy as a quantitative assay method for determining the ratio of fPSA and cPSA in complex samples, such as serum, for improved precision in prostate cancer diagnosis.

**Conclusions**

We describe herein a strategy for the multiplexed detection of the two types of serum PSA and the determination of their ratio...
in complex samples at total PSA levels of 4 - 10 ng/mL for improved diagnostic accuracy in prostate cancer. We used an LDI-TOF mass signal-amplification strategy with two small reporter molecules on AuNPs—Am-tag without isotope labeling and dAm-tag with deuterium labeling. Comparing the mass intensities between Am-tag and dAm-tag signals allowed the determination of the ratio between fPSA and cPSA. We validated the selective measurement of fPSA and cPSA at various ratios and the determination of the ratio of these two types of PSA spiked in FBS by using our method. Our results clearly indicate the applicability of our strategy to human serum as a diagnostic and prognostic assay in prostate cancer.

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Supporting Information

Additional information as noted in the text. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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