Talanta 188 (2018) 238–244

Contents lists available at ScienceDirect

Talanta

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

Classification analyses for prostate cancer, benign prostate hyperplasia and healthy subjects by SERS-based immunoassay of multiple tumour markers

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ARTICLE INFO

Keywords:
Prostate cancer
Surface-enhanced Raman scattering
Immunoassay
Tumour marker
Serum level

ABSTRACT

Prostate cancer (PCa) is a leading cause of cancer-related death among males globally. To date, prostate-specific antigen (PSA), as a typical tumour marker, has been widely used in the early diagnosis of PCa. However, in practical clinical tests, high serum levels of PSA show a high probability for false-positive results, leading to misdiagnoses. In this study, we developed a new classification system for PCa, benign prostate hyperplasia (BPH) and healthy subjects by using a surface-enhanced Raman scattering (SERS)-based immunoassay of multiple tumour markers along with a support vector machine (SVM) algorithm. Silver nanoparticles (AgNPs) as immune probes and SiC@Ag@Ag-NPs SERS as immune substrates were constructed into a sandwich structure to serve as an ultrasensitive SERS-based immunoassay platform of tumour markers. With this assay, the limits of detection for PSA, prostate-specific membrane antigen (PSMA) and human kallikrein 2 (hK2) were as low as 0.46 fg mL^{-1}, 1.05 fg mL^{-1} and 0.67 fg mL^{-1}, respectively. Furthermore, the serum levels of PSA, PSMA and hK2 in clinical samples were successfully detected using the SERS-based immunoassay platform, and correct classifications of PCa, BPH and healthy subjects were feasible with help of the linear SVM algorithm. These results demonstrate the potential for improving the diagnostic accuracy of PCa. Overall, the linear SVM classification model with multiple tumour markers exhibited good classifications of PCa, BPH and healthy subjects, with a PCa diagnostic accuracy of 70% that was significantly superior to that of the linear SVM classification model based only on the serum level of PSA (50%). Therefore, combining the SERS-based immunoassay with pattern recognition technology can allow for comprehensive analyses of the serum levels of multiple tumour markers to effectively improve the diagnostic accuracy of cancer with potential applications in point-of-care testing.

1. Introduction

Prostate cancer (PCa) is one of the most common types of malignancies in men worldwide [1,2]. Based on clinical trials, prostate-specific antigen (PSA) has become the most widely used directional tumour marker of PCa, both for early diagnosis and therapeutic evaluations [3,4]. Free prostate-specific antigen (fPSA) is another common prostate tumour marker, and the ratio of the serum levels of fPSA and PSA (fPSA/PSA) is also frequently used as a clinical indicator for diagnosing PCa [5]. However, an increased serum level of PSA can also be caused by benign prostate hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN), acute or chronic prostatitis, and other non-malignant diseases of the prostate [6–8]. This lack of specificity may lead to a false-positive finding in up to 65% of patients if only the serum level of PSA or fPSA/PSA is used to discriminate between PCa and benign diseases [7]. To date, in practical clinical tests, the prostate biopsy method is a necessary but painful approach for accurate diagnosis. Therefore, the development of a non-invasive comprehensive analysis method for classifying patients with various prostate conditions would be greatly beneficial.

Besides PSA, prostate epithelial cells secrete other biomarkers such as prostate-specific membrane antigen (PSMA), human kallikrein 2 (hK2), and prostate stem cell antigen (PSCA) [9]. Researchers have tried to determine which of these biomarkers might be superior for the

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https://doi.org/10.1016/j.talanta.2018.05.070
Received 18 January 2018; Received in revised form 12 May 2018; Accepted 20 May 2018
Available online 23 May 2018

diagnosis of PCa. For example, PSMA as a transmembrane protein has emerged as one of the most promising tumour markers in the diagnosis and treatment of PCa [10–12]. Although PSMA is expressed at high levels in the serum of prostate carcinoma patients, this high expression is also present in BPH patients [11,13]. As another potential tumour marker, the serine protease hK2 shows 80% amino acid sequence homology to PSA and has also been reported to be expressed at higher levels in the serum of PCa than in the serum of non-PCa patients [6,14,15]. Therefore, it is reasonable to assume that the combined use of multiple tumour markers through a comprehensive analysis of the serum levels of PSA, PSMA, and hK2 would help to improve the diagnostic accuracy of PCa. However, the serum levels of PSMA and hK2 are usually 50- to 100-fold lower than those of PSA [16,17]. Thus, an ultrasensitive quantitative immunoassay should be developed to facilitate the detection of the relatively low serum levels of PSMA and hK2 simultaneously with the higher PSA levels for clinical utility.

The immunoassay has become the dominant test method of tumour markers in body fluids such as human serum, mainly owing to the highly specific molecular recognition of antibodies and epitopes of an antigen. The most common types of immunoassays include radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), fluoroimmunoassay, and chemiluminescent immunoassay (CLIA) [18–21]. These detection techniques have many advantages (e.g., sensitivity, precision, and selectivity), and thus play important roles in the clinical quantitative detection of tumour markers; however, they also suffer from some deficiencies (e.g., radiation hazards, excessive cost, requirements of sophisticated instrumentation and complex operation) [22,23], especially for screening cancer at an early stage. Therefore, it is necessary to develop new immunoassay techniques to meet the requirements of a high sensitivity, high-throughput, multi-analyte, and non-polluting immunoassay of tumour markers. From this perspective, surface-enhanced Raman scattering (SERS) spectroscopy demonstrates excellent characteristics with high sensitivity, a unique fingerprint, and non-destructive data acquisition [24–27]. Thus, a SERS-based immunoassay has become an alternative rapid analysis technique and is rapidly gaining momentum as an effective tool for detecting tumour markers in clinical serum samples.

Here, we report a SERS-based immunoassay platform that was constructed from silver nanoparticles (Ag NPs) as immune probes and SiC@Ag@Ag-NPs as a SERS immune substrate to achieve the detection of multiple tumour markers. We further developed support vector machine (SVM) algorithms to classify healthy subjects, BPH, and PCa patients based on the immunoassay data. The constructed immunoassay platform exhibits excellent performance for detecting of PSA, PSMA and hK2, such as low limits of detection (LODs) and broad dynamic detection ranges. Moreover, the specificity of the immunoassay was verified using non-specific antigens and female serum samples in control experiments. Furthermore, the contents of PSA, PSMA, and hK2 in the serum samples from patients were successfully detected with the proposed immunoassay protocol and were consistent with the test data obtained from the CLIA method. The results showed that based on the linear SVM classification model [28], the use of multiple tumour markers (PSA, PSMA, and hK2) could improve the diagnostic accuracy of PCa from 50% to 70%, also has a better classification for BPH and healthy subjects, comparing with the results of using only PSA.

2. Experimental

2.1. Chemicals

Silver nitrate ($AgNO_3$, 99.5%), phosphate buffer saline (PBS, pH 7.0), Tris-buffered saline (TBS)/0.05% Tween 20 buffer solution (0.05 M Tris, 0.138 M NaCl, 0.0027 M KCl, 0.05% Tween 20, pH 8), and 4-mercaptobenzoic acid (4MBA) were purchased from Sigma-Aldrich. Trisodium citrate ($Na_3C_6H_5O_7{\cdot}2H_2O$) was purchased from Aladdin. PSA and anti-PSA antibodies (detection and capture) were obtained from Beijing Key-Bio Biotech Co., Ltd. PSMA, anti-PSMA antibodies (detection and capture), hK2 and anti-hK2 antibodies (detection and capture) were acquired from Abcam. Bovine serum albumin (BSA) was purchased from Nanjing Sunshine Biotechnology Co., Ltd. The human blood samples were obtained from the Affiliated Hospital of School of Medicine of Ningbo University. Milli-Q water (resistivity of 18.2 $M\Omega\,cm^{-1}$) was used to prepare all solutions.

2.2. Preparation of the Ag NPs immune probes

The Ag NPs immune probes were prepared as follows. First, bare Ag NPs were prepared using a facile method according to previous work [29]. Two millilitres (1%) of sodium citrate aqueous solution was added immediately to 100 mL of 10^{-4} g mL^{-1} $AgNO_3$ boiling solution under vigorous stirring, and then the mixture was stirred vigorously and boiled for 2 h. Second, 8 mL of Ag colloid was washed by centrifugation at 8000 rpm for 20 min, and the Ag NPs sediment was re-suspended in 5 mL of deionized water. Next, 20 μL of 4MBA (1 mM) was added to the as-prepared Ag colloid under vigorous stirring and incubated at room temperature for 12 h. The unbound 4MBA molecules were removed by centrifugation and the Ag NPs sediment was re-suspended in 3 mL of PBS under ultrasonic oscillation. Third, the antibodies were immobilized on the 4MBA-labelled Ag NPs aggregates via static and hydrophobic interactions [25,30]. Taking PSA as an example, 20 μL of the anti-PSA antibody–PBS solution (0.2 mg mL^{-1}) was added to 3 mL of the 4MBA-labelled Ag NP aggregate solution and incubated at 4 °C for 1.5 h. After centrifugation, the blocking solution (3% BSA in PBS) was added to the above mixed solution to shield the bare sites on the surfaces of Ag NPs and the mixture was washed twice by centrifugation to remove the excess BSA after incubation at room temperature for 1 h. The PSA immune Ag NP probes were finally obtained by dissolving the sediment in 3 mL of PBS solution under ultrasonic oscillation. Similarly, the PSMA and hK2 immune probes were also prepared using the above procedure.

2.3. Preparation of the Ag NPs immune substrate

First, SiC sandpapers with different mesh sizes (240, 400, 800, 1200, 2000, 3000, and 5000 mesh) were used as templates for the vacuum deposition of 200-nm-thick Ag films, and 20 μL of Ag NPs colloids were dropped on the Si@Ag sandpapers with different mesh sizes, respectively. After drying, the SiC@Ag@Ag-NPs SERS substrates were obtained. Subsequently, the optimal SiC@Ag@Ag-NPs SERS substrate (1200-mesh) was selected for immobilization of the captured antibody as an immune substrate [25]. Taking PSA as an example, 20 μL (0.2 mg mL^{-1}) of the captured anti-PSA antibody in PBS was dropped onto the substrate and subsequently immobilized at 4 °C over a period of 12 h. After washing with TBS/0.05% Tween 20 buffer solution, PBS, and deionized water, the non-specific adsorption sites on the surface of the substrate were blocked with a blocking solution (3% BSA in PBS), and then washed to remove the excess BSA after incubation at room temperature for 3 h. Finally, the PSA immune substrate was prepared and stored at 4 °C for further analyses. Similarly, the PSMA and hk2 immune substrates were prepared by the above procedure.

2.4. Sandwich immunoassay

The immunoassay was performed by imitating the typical ELISA sandwich protocol, as shown in Fig. 1. First, different concentrations of the antigens were dripped onto the as-prepared SERS immune substrates and then incubated at 37 °C for 2 h. Careful attention was paid to ensure that the amount of dripped antigen was less than the amount of the captured antibody on the substrate, i.e. the antibody conjugate ratio was less than one. After rinsing with the TBS/0.05% Tween 20 buffer solution, PBS, and deionized water, 20 μL of the immune Ag NPs probes was dropped to form the sandwich immune structures and incubated at