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Discrimination of Breast Cancer by Measuring Prostate-Specific Antigen Levels in Women's Serum

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Supporting Information

ABSTRACT: Prostate-specific antigen (PSA) has been reported to be a potential biomarker of breast cancer. Serum PSA of normal women is around 1 pg/mL, which is usually undetectable by current assay methods; thus an ultrasensitive measurement of PSA expression in women's serum is necessary to distinguish normal from malignant breast diseases. To enhance the sensitivity of conventional immunoassay technology for the detection of PSA in sera, we adopted a localized surface plasmon coupled fluorescence fiber-optic biosensor, which combines a sandwich immunoassay with the localized surface plasmon technique. The concentration of total PSA (t-PSA) (from 0.1 to 1000 pg/mL) in phosphate-buffered saline solution and the normalized fluorescence signal exhibit a linear relationship where the correlation coefficient is 0.9574. In addition, the concentration of additional t-PSA in 10-fold-diluted healthly women's serum across a similar range was measured. The correlation coefficient for this measurement is 0.9142. In clinical serum samples, moreover, the experimental results of t-PSA detection show that both the mean value and median of normalized fluorescence signals in the breast cancer group (155.2 and 145.7, respectively) are higher than those in the noncancer group (46.6 and 37.1, respectively). We also examined the receiver operating characteristic curve for t-PSA, and the area under the curve (AUC) is estimated to be 0.9063, the AUC being used to measure the performance of a test to correctly identify diseased and nondiseased subjects.

Currently, breast cancer is the most common women's cancer worldwide. Development of early detection techniques for breast cancer is important for improving survival rates.[1] The conventional diagnostic methods (clinical examination and mammography) have well-described limitations;[2] for instance, mammography may only detect 70−90% of cases of breast cancer, with a false-negative rate of up to 31%.[2−4] Another possible technique is the use of serum biomarkers; however, there are currently no sensitive and specific serum biomarkers to sufficiently detect breast cancer early or to monitor minimal disease recurrence.[5] Carcinoembryonic antigen (CEA), cancer antigen (CA) 15-3 or CA27-29 are commonly used for breast cancer diagnostics and as predictors of recurrence risk, but these biomarkers are only increased in <50% of patients with metastatic disease and not recommended for surveillance purposes.[5] Thus, extensive research is under way at present to discover novel breast cancer biomarkers for improving overall sensitivity.

Prostate-specific antigen (PSA), a serine protease with chymotrypsin-like activity, has two predominant forms in human serum: either free PSA (f-PSA) or PSA complexed to α-1-antichymotrypsin (PSA−ACT). Total PSA (t-PSA) refers to the sum of these two forms.[6] The name of PSA reflects the initial general belief that the protein was exclusively expressed by the prostate gland. In fact, it has been found to be synthesized and secreted by various organs, including the breast, ovary, liver, kidney, pancreas, lung, and adrenal and parotid glands, in addition to the prostate gland.[7] It has also been demonstrated that both the molecular weight and the mRNA sequence of breast PSA are identical to those of seminal PSA.[7−9] Several lines of evidence have demonstrated that PSA is a potential biomarker for breast disease.[7,10−17] For instance, PSAs are coexpressed in nipple aspirate fluid (NAF) and in breast tumor tissue. PSA in NAF was inversely related to tumor size, nodal status, disease stage, and distant metastases and provided useful prognostic information for cancer treatment.[15] PSA expressions in human breast cancer were directly correlated with the expression of androgen receptor and progesterone receptor and inversely correlated with human epidermal growth factor receptor 2 (HER2/neu) overexpression.[18] Higher expression of PSA is usually found in the breast tumor sites and is potentially associated with the genetic polymorphism in the PSA gene promoter.[19,20] On the other hand, the low frequency (9%) of PSA-positive breast cancer tissue was also reported to disagree with the prognostic role of PSA for the hospital-based immunohistochemical method.[21]

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A suitable PSA immunoassay for analysis of women's sera must therefore have a very low detection limit (~1 pg/mL),[16,17] which is difficult to achieve in established assays, thus limiting the use of PSA for breast cancer diagnosis. Various techniques have been used to decrease the detection limit of biosensing equipment. The introduction of gold nanoparticles (GNPs) into the biosensing area is one of the most efficient ways to achieve extremely high sensitivity because of its particular optical properties known as localized surface plasmons (LSPs),[22−24] which result in selective wavelength absorption with extremely large molar extinction coefficients and significant enhancement of the localized electromagnetic field within 50−60 nm of the GNP's surface.[25−28] On the basis of the optical property, we have previously developed a fiber-optic biosensor combined with LSPs with fluorescence and a sandwich immunoassay, called the localized surface plasmon coupled fluorescence fiber-optic biosensor (LSPCF-FOB), as a tool to study protein−protein interactions at ultralow concentration reaching to the subpicogram per milliliter level.[29−32] Furthermore, the mechanism of fluorescence enhancement using metallic nanoparticles in the LSPCF-FOB was studied using scattering theory.[33] The low detection limit of the biosensing platform is attributed to the efficient enhancement of fluorescence by the localized electromagnetic field from LSPs near the GNP's surface. Then the multifluorophores of each LSPCF probe are excited simultaneously to amplify fluorescence. Finally, in the LSPCF-FOB, the fluorescence detected beside the optical fiber causes a significant decrease in the background noise compared with the conventional setup.

In this study, t-PSA at 0.4 pg/mL in phosphate-buffered saline (PBS) solution and t-PSA at 1.8 pg/mL in 10-fold-diluted healthly women's serum were measured and demonstrated. Furthermore, to compare the results from measuring the clinical serum samples, including the breast cancer group and noncancer group, both the mean value and median of normalized fluorescence signals in the breast cancer group are significantly higher than those in the noncancer group. On the basis of the ability of the LSPCF-FOB to detect t-PSA concentration at a few picograms per milliliter in women's serum, it may be helpful to differentiate healthy and breast-cancer-bearing women.

EXPERIMENTAL SECTION

Reagents and Materials. Serum samples were prepared at Taipei Veterans General Hospital (Taipei, Taiwan). In this experiment, GNP conjugate protein A (Au-PA; GNP diameter 25 nm) was purchased from Aurion (Wageningen, The Netherlands). Monoclonal and polyclonal antibodies to t-PSA, used as the capture and detection antibodies, respectively, were purchased from Meridian Life Science, Inc. (Saco, ME). Human t-PSA purified protein, used as the target antigen, was purchased from Chemicon (Billerica, MA). The fluorescent labeling kit (Lightning-Link Atto633; the fluorescence spectra can be found at the Web site http://tinyurl.com/3scjdp6) was purchased from Innova Biosciences (Cambridge, U.K.), and the multimode plastic optical fiber [material poly(methyl methacrylate) (PMMA), diameter 1 mm] was purchased from Mitsubishi Rayon Co., Ltd. (Tokyo, Japan).

Surface Modification of the PMMA Fiber. The plastic optical fibers were stripped off their cladding by immersion in ethyl acetate, and then the stripped portion was washed with 2-propanol to clean the stripped surface. Chemical adsorption was carried out using covalent binding forces to immobilize the capture antibody onto the stripped surface of the optical fiber. The protocol for chemical adsorption has been described previously.[29]

Fabrication of Sandwich Immunocomplexes. The modified stripped portion of each optical fiber was first coated with 200 μL of a 5 μg/mL concentration of the monoclonal t-PSA antibody as the capture antibody at room temperature for 2 h. After blocking with 10 mg/mL bovine serum albumin (BSA) in PBS solution for 1 h, 250 μL aliquots of (i) t-PSA serially diluted in PBS solution, (ii) t-PSA serially diluted in 10-fold-diluted healthly women's serum, and (iii) 10-fold-diluted clinical serum samples, including breast cancer and noncancer groups, were added to each individual reaction chamber and incubated at room temperature for 2 h to form the capture antibody/target antigen immunocomplex on the modified portion of the fiber surface (Figure S1, Supporting Information). The fibers were washed five times with PBS solution in each step. Finally, the LSPCF probe solution was added to each reaction chamber to form the capture antibody/target antigen/LSPCF probe sandwich immunocomplex, and the fluorescence was then measured at the same time.

Preparation of the LSPCF Probe. The LSPCF probe is composed of fluorophores labeled with detection antibodies connecting to protein A which adsorbed to colloidal GNPs (Figure S1, Supporting Information). The detection antibody was fluorescently labeled with Lightning-Link Atto633 using a commercial labeling kit. To produce the LSPCF probe, a 167 ng/mL concetration of fluorophore-labeled detection antibody was mixed with Au-PA at a concentration of 3.76×10^9 particles/mL and incubated for 10 h at 4 °C in the dark.

Experimental Measurements. A 632.8 nm He−Ne laser was used as the excitation light source, and a microscope objective (10×, numerical aperture 0.27) was used to achieve the best coupling efficiency. The fluorophore (Atto633), which has a strong absorption at 629 nm and high fluorescence emission at 657 nm (extinction coefficient 1.3×10^5 cm^{-1} M^{-1}), was excited by the enhanced localized electromagnetic field close to the GNP surface during measurement. A long-pass filter (cutoff wavelength 640 nm) was introduced into this setup to allow fluorescence detection via a photomultiplier tube. While making measurements, two lock-in amplifiers were used to synchronously detect the modulated fluorescence, and in addition, the use of the amplitude ratio algorithm was adopted to significantly improve the sensitivity of the detected signal experimentally.

RESULTS AND DISCUSSION

Detection of t-PSA in PBS Solution. To develop a highly sensitive assay for breast cancer discrimination, we used the established LSPCF-FOB platform for analyzing the detection sensitivity of t-PSA in PBS solution to calibrate the LSPCF-FOB. Different dilutions of t-PSA in PBS solution (0, 0.1, 1, 10, 100, and 1000 pg/mL) were injected into the reaction chamber to interact with the capture monoclonal t-PSA antibodies separately. Figure 1 shows the linear relationship between the normalized fluorescence signals and the t-PSA concentrations over the range 1−1000 pg/mL. The correlation coefficient (R^2) was found to be 0.9574, and the error bars indicate 1 standard deviation from multiple measurements. The fluorescence signal was calculated by subtracting the background level from the

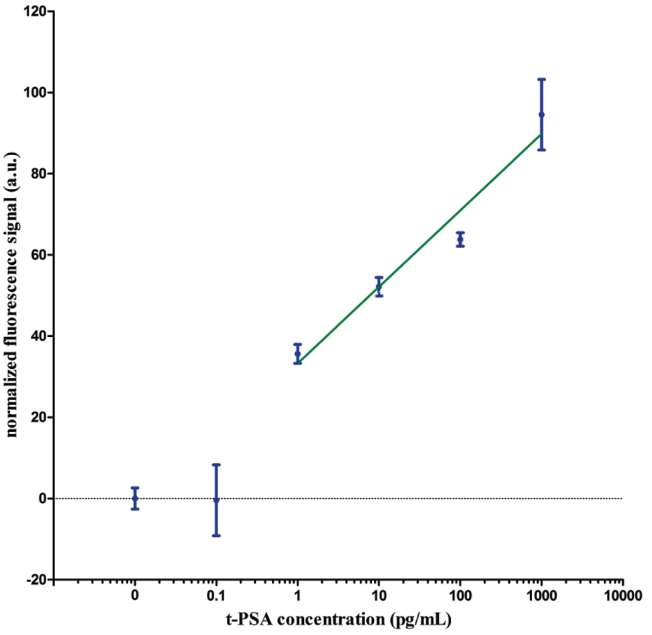

Figure 1. Linear relationship ($y = 18.84 \log x + 33.26$, $R^2 = 0.9574$) between the t-PSA concentration in PBS versus the normalized fluorescence signal. The error bars are equivalent to 1 standard deviation.

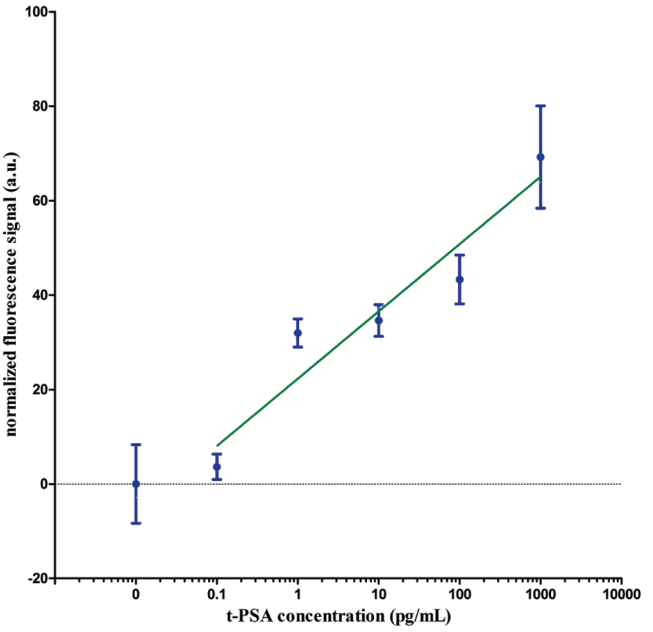

Figure 2. Linear relationship ($y = 20.30 \log x + 14.25$, $R^2 = 0.9142$) between the t-PSA concentration in 10-fold-diluted healthy women's serum versus the normalized fluorescence signal. The error bars are equivalent to 1 standard deviation.

steady fluorescence intensity in each measurement. The fluorescence intensity was obtained as the measured fluorescence normalized by the laser beam intensity to reduce the fluctuation of the laser intensity. For the purpose of decreasing the effect of misalignment in the system, the normalized fluorescence signal can be written as

$$\text{normalized fluorescence signal} = \frac{\text{fluorescence signal}}{\text{background level of fluorescence intensity}}$$

The unit of the normalized fluorescence signals is an arbitrary unit. In accordance with the International Union of Pure and Applied Chemistry (IUPAC) definition, the detection limit and analytical sensitivity of the LSPCF-FOB in the experiment are estimated at 0.4 and 1.4 pg/mL, respectively.

Detection of t-PSA in 10-Fold-Diluted Serum from Healthy Women. To demonstrate that the LSPCF-FOB can be used for clinical specimens, we prepared t-PSA in 10-fold-diluted serum from healthy women. Figure 2 shows the normalized fluorescence signals of the biomolecular interaction between the LSPCF probes and t-PSA at different concentrations in 10-fold-diluted healthly women's serum. Again, a clear linear relationship between the normalized fluorescence signal and the different concentrations of t-PSA in the 10-fold-diluted healthly women's serum over the range from 0 to 1000 pg/mL was found (Figure 2). The correlation coefficient (R^2) was 0.9142, and the error bars in the figure indicate 1 standard deviation from multiple measurements. Using the IUPAC definition, the detection limit and analytical sensitivity of the LSPCF-FOB are estimated at 1.8 and 2.3 pg/mL, respectively. From the data, the detection limit and analytical sensitivity for t-PSA in real serum samples should be around 18 and 23 pg/mL, respectively; that is, the LSPCF-FOB platform can distinguish between the signal from a sample containing 2.3 pg/mL target protein and the noise generated by the other serum proteins present in 10-fold-diluted serum.

Analysis of the Expression Levels of t-PSA in Clinical Serum Samples. Furthermore, we analyzed the expression levels of t-PSA in clinical serum samples from eight healthy women and eight women with breast cancer using the LSPCF-FOB. The experiments for noncancer groups showed a lower level of normalized fluorescence signals than the experiments for breast cancer (Figure 3). Both the mean value and median of normalized fluorescence signals in the breast cancer group (155.2 and 145.7 arbitrary units, respectively) are higher than those in the noncancer group (46.6 and 37.1 arbitrary units, respectively) as reported in Table 1. To search for criteria providing optimal discrimination, we used the fitting program (kindly provided by John Eng, ROC Analysis: Web-Based Calculator for ROC Curve, Johns Hopkins University, Baltimore, MD; http://www.jrocfit.org) for calculating the receiver operating characteristic (ROC) curve on the basis of the maximum likelihood fit of a binomial model (Figure 4). For the empirical ROC curve and the fitted ROC curve, the areas under the curve (AUC) were 0.9063 and 0.9166, the AUC being a factor for measuring the performance of a test. The larger the AUC, the better the performance of the medical test in correctly identifying diseased and nondiseased subjects.

The ROC curve is a graphical plot of the true positive (sensitivity) versus false positive rate (1 − specificity) for a binary classification system as its discrimination threshold is varied. Furthermore, each point on the ROC curve corresponds to a unique pair of values for sensitivity and specificity.[5] In this research, on the basis of the empirical ROC curve, we calculated the sensitivity and specificity for different normalized fluorescence signal cutoff levels. At a cutoff level of 107 arbitrary units, the sensitivity is 75% and the specificity is 100%; at 93.91 arbitrary units, the sensitivity is 87.5% and the specificity is 75%, and at 34.81 arbitrary units, the sensitivity reaches 100%

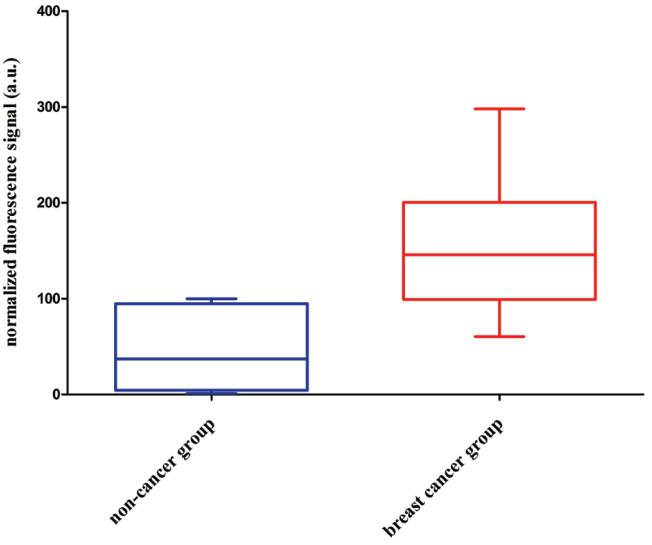

Figure 3. Box and whisker plots of normalized fluorescence signal levels from the noncancer group and breast cancer group. The median (middle line), 25th and 75th percentiles (lower and upper boundaries of the box, respectively), and lowest and highest data within the lower and upper hatch lines are illustrated.

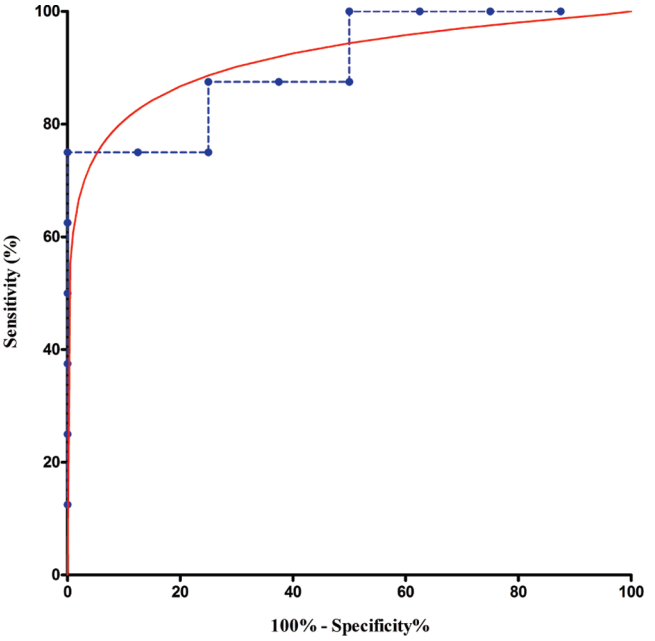

Figure 4. Empirical (blue line) and fitted (red line) ROC curves for t-PSA as described in the text.

Table 1. Mean and Median t-PSA Expression Based on the Breast Cancer and Noncancer Groups

		t-PSA levels [normalized fluorescence signal (arbitrary units)]	
	number of samples	mean	median
breast cancer group	8	155.2	145.7
noncancer group	8	46.6	37.1

but the specificity decreases to 50%. The experimental results indicate the possible existence of a cutoff level at approximately 107 arbitrary units for the discrimination of breast cancer when using the LSPCF-FOB to measure t-PSA levels in women's serum. In addition, the fitting ROC curve calculates theoretical values of sensitivity and specificity as 82.43% and 87.67%, respectively. In contrast to the results of previous research on breast cancer diagnostic assays using other potential biomarkers, such as mammaglobin, basic fibroblast growth factor, and antimalignin antibody, our method exhibits higher sensitivity and specificity whether they are experimental or theoretical values.[5,34,35] Both the sensitivity and specificity from experiment and theoretics are comparable with those using PSA as a biomarker for prostate cancer, which has been reported to have a sensitivity of 73.2% and specificity of 85.4%.[5,36,37] Furthermore, these values may compare favorably with the sensitivity and specificity of the current screening methods for breast cancer, such as clinical examination (54% sensitivity and 94% specificity) and mammogram (75% sensitivity and 92% specificity).[34,38]

Few studies discussed serum PSA associated with breast cancer, and the results were variable. These different conclusions obtained from individual research groups may be due to the selected subgroups of breast cancer and statistical methods. For example, Borchert et al. showed the PSA concentration decreased in sera after menopause;[39] however, in postmenopausal women, PSA levels in NAF and sera are correlated.[14] These suggest that a more sensitive and faster analytic method is necessary to identify the meticulous change of serum PSA in women with breast cancer. Biosensors for detection of PSA at very low concentrations are expected to provide a valuable tool for early identification and diagnosis of breast cancer in women. However, diagnostic use of PSA for breast cancer has been limited to date because of its very low diagnostic sensitivity (~20%).[17] The concentration of PSA in the sera of normal women is around 1 pg/mL, which is undetectable by current methods, except menstruating nonpregnant women with fibrocystic change produce and release more PSA in serum.[16,17,39] In the current study, we estimated the detection limits of the LSPCF-FOB for t-PSA in PBS and 10-fold-diluted normal women's serum to be 0.4 and 1.8 pg/mL, respectively, as measured experimentally via the LSPCF probe and t-PSA interaction. In the meantime, t-PSA levels in breast cancer patients' serum were measured nicely, and a significant difference between the healthy and breast cancer groups is obviously seen. Our analysis is focused on breast cancer discrimination; whether routine use is effective for prognosis determination, postoperative surveillance, and therapy monitoring remains an open question.

CONCLUSIONS

In this study, we set up a novel breast cancer diagnostic platform, the LSPCF-FOB, using monoclonal capture and polyclonal detection antibodies against t-PSA in women's serum. Linear responses between the normalized fluorescence signal and the concentration of t-PSA in PBS and in 10-fold-diluted healthy women's serum were obtained experimentally in the range of 1−1000 pg/mL. Moreover, our results demonstrate that higher mean and median normalized fluorescence signals are exhibited in the breast cancer group (155.2 and 145.7 arbitrary units) than in the noncancer group (46.6 and 37.1 arbitrary units). In

addition, the AUC for t-PSA detection is calculated to be 0.9063. In this study, the experimental results indicate that the LSPCF-FOB is capable of detecting t-PSA concentration at a few picograms per milliliter in diluted women's serum successfully. Potentially, this is helpful for breast cancer diagnosis in the near future.

■ ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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