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Research Article

A Novel Label-Free Biosensor for Detection of HE4 in Urine Based on Localized Surface Plasmon Resonance and Protein G Directional Fixed

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A non-invasive and more sensitive method for detection of HE4 is very important for the early screening and detection of ovarian carcinoma. In this study, we improved our previous localized surface plasmon resonance (LSPR) biosensor for detection of HE4 in urine to overcome disadvantages of conventional methods. Protein G directional fixed method was firstly used for LSPR biosensor to improved sensitivity, and standard HE4 and clinical samples were detected separately using this new biosensor. Compared to our previous LSPR biosensor, this new sensor was more sensitive, with other advantages as before. Under optimum conditions, this new biosensor could display a detection limit of 1 pM and wide dynamic range of 1 pM to 10,000 pM. This new biosensor was effective for detection of HE4 in urine of early ovarian cancer patients, without label and purification. To the best of our knowledge, this is first work to investigate LSPR biosensor for detection of tumor marker in urine, with great advantages and clinical application potentials.

1. Introduction

Ovarian cancer is one of the most common malignant diseases and represents the primary mortality for gynecological cancers, despite adopting more sophisticated diagnostic methods and novel methods of treatment [1–4].This is caused by the fact that most cases are not discovered until an advanced stage [2–4]. Therefore, it is crucial to find a non-invasive, sensitive and specific assay for early screening and detection of ovarian carcinoma. The human epididymis secretory protein 4 (HE4), has been widely studied and considered as a promising tumor marker for early diagnosis of ovarian cancer [4–10]. Reportedly, HE4 is highly sensitive to early ovarian cancer and can be used as a better method for diagnosis in ovarian cancer [4–6, 9].

Current methods available for detection of HE4 such as enzyme-linked immunosorbent assay (ELISA) and chemiluminescent immunoassay (CLIA), still have obvious shortcomings [10–12]. To overcome disadvantages of conventional tools, our previous study developed a localized surface plasmon resonance (LSPR) biosensor for detection HE4 in patients' blood serum, with advantages as convenient, low-cost, and label-free, and sensitivity [10]. But, for extensive general women, serological detection is invasive and difficult to accept. Therefore, it is important to develop an alternative method for clinical screening and early diagnosis of ovarian cancer. Recently, some study indicated that HE4 protein can be detected in urine since it has a molecular weight around 25kD [9]. Compared to serological detection, measuring HE4 in urine can provide a non-invasive and more convenient approach to aid early detection of ovarian cancer.

Previously, we have once achieved the detection of albumin in urine samples from mild preeclampsia patients by this LSPR biosensor [13]. However, HE4, as a tumor marker, is smaller than albumin, with concentration in urine

inferior to serum and unstable. Therefore, it is very challenging for measuring HE4 of urine in clinical patients. Up to date, LSPR biosensors have not been studied for detection of HE4 and other tumor marker in urine. To explore clinical application of LSPR biosensor, we firstly attempted to improve the sensitivity of this biosensor by introducing protein G directional fixed method, and successfully used this new build biosensor to detect HE4 in urine of early ovarian cancer patients. Compared to our previous LSPR biosensor [10, 12, 13], this new sensor was more sensitive. To the best of our knowledge, this was the first work to investigate the LSPR biosensor for the detection of tumor marker in urine. This novel LSPR biosensor provided a more sensitive and label-free method for detection HE4 in patients' urine using simple and convenient instrumentation, with great potential for applications in clinical screening of ovarian cancer and other tumors.

2. Materials DND Methods

2.1. Materials. 11-Mercaptoundecanoic acid (MUA), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were acquired from Sigma-Aldrich (Missouri, the United States of America). Protein G was obtained from BioVision (California, the United States of America). Mouse monoclonal anti-HE4 antibody and standard HE4 were purchased from Abnova (Taiwan, China). Quartz glass substrates from Juke (Chengdu, China) and Ag wire from Jubo (Beijing, China). Ultrapure water (18.3 MV/cm) used for the preparation of all solutions was from Millipore (Massachusetts, the United States of America). All reagents used were of analytical reagent grade.

2.2. Build of LSPR Biosensor. The integrated LSPR biosensor was built on-site, as our previous study reported in detail [10, 12]. Briefly, the sensor chip of silver nanoparticles was fabricated as triangular-shaped array by using nanosphere lithography (NSL) technology was used. The peak wavelength of LSPR absorption spectrum (λmax) excited by the silver nanoparticles was detected and recorded by a Ultraviolet Rays-visible spectrometer (Model 9055; Sciencetech, Ottawa, Canada). The incident light was provided by white light emerging from the optical fiber bundle. Coupled with the optical detection probe, the noble LSPR absorption spectra could be excited and directly obtained by the spectroscope at room temperature, ranging from 400 nm to 800 nm, and timely displayed on the computer screen.

All the absorption spectra were recorded and analyzed through Spectra Suite software (Ocean Optics, Florida, the United States of America). The relative LSPR wavelength shift ($\Delta\lambda$max) was used to monitor the binding of target analytes. A shift towards the longer LSPR wavelength region was referred to as a red-shift and denoted as (+); whereas, a shift towards the shorter LSPR wavelength region was defined as a blue-shift and indicated as (–). The resolution of our system archived 3 nm and indicated that our biosensor could analyze the analyte on the nanoparticles-solution interface when $\Delta\lambda$max was over +3 nm.

2.3. Further Functionalization of the LSPR Biosensor. A multistep process was made to prepare the sensor chip for biodetection events (Figure 1). During every experimental process, peak wavelength of LSPR absorption spectrums were excited, observed and used to improve the experimental conditions. The experimental conditions of each step were obtained after repeated explorations and attempts. Finally, optimal conditions were achieved as followed. First, the silver nanochip was incubated in 1 mM 11-MUA solution (in ethanol) for 10 hours at room temperature to form a self-assembled monolayer on the slice surface more effectively, then washed thoroughly with pure ethanol to remove nonspecific binding and dried at room temperature. Next, the nanochip was incubated in 75 mM EDC-HCl/15 mM NHS (volume/volume =1) solution for another hour at room temperature to preferably activate the carboxyl group of 11-MUA, which would effectively react with amino groups of protein G to form amides. Subsequently, the sensor chip was incubated with 100ug/ml protein G solution (in PBS, pH 5.5) for thirty minutes at room temperature to betterly couple the amine groups of protein G to the carboxyl groups on MUA. Next, in order to immobilization the antibody onto the chip by coupling with protein G, 50 μL of anti-HE4 solution (10 μg/mL) was spotted onto nanochip surface and overnight incubation at 4°C followed. After above steps, functional nanochip was immersed in ethanolamine solution (pH 8.5) for 30 minutes to deactivate coupled esters, then the surface was washed by phosphate-buffered solution (PBS) (pH 7.4) to remove nonspecific binding. At last, a new functionalization of HE4 LSPR biosensor was finished, as demonstrated in Figure 1. Meanwhile, the silver nanoparticles characterization using SEM (scanning electron microscope) were, respectively, observed before and after modification, as shown in Figure 2.

2.4. Clinical Samples. All detection samples in this study were from leftover urine samples in routine clinical urine tests collected from West China Second University Hospital (Chengdu, China), with no use and no harm to patients and institution. Urines of healthy women, benign ovarian cyst patients and early (I/II) ovarian cancer patients were, respectively, obtained. The disease diagnosis and staging involved were acquired from surgical and pathological results of gynecological oncologist and gynecological pathology experts. No personal privacy and no ethical harm to patients were involved in this study, owing to other medical records such as name, age, and registration number, etc were not needed. So, Research Ethics Approval from Ethics Institutional Board and written informed consents from patients were not needed in this study.

2.5. Detection of HE4 in Urine. In detection stage, at first, the different concentrations of commercial HE4 were, respectively, incubated on new functionalized LSPR chips at room temperature for 30 minutes. The biosensor chips were then dried after thorough rinsing with PBS (0.05% Tween-20) and subsequently by ultrapure water to dissociate the nonspecific binding. At last, the change in LSPR wavelength caused by antibody-antigen combination was observed and recorded. As above method, all urine samples were detected,

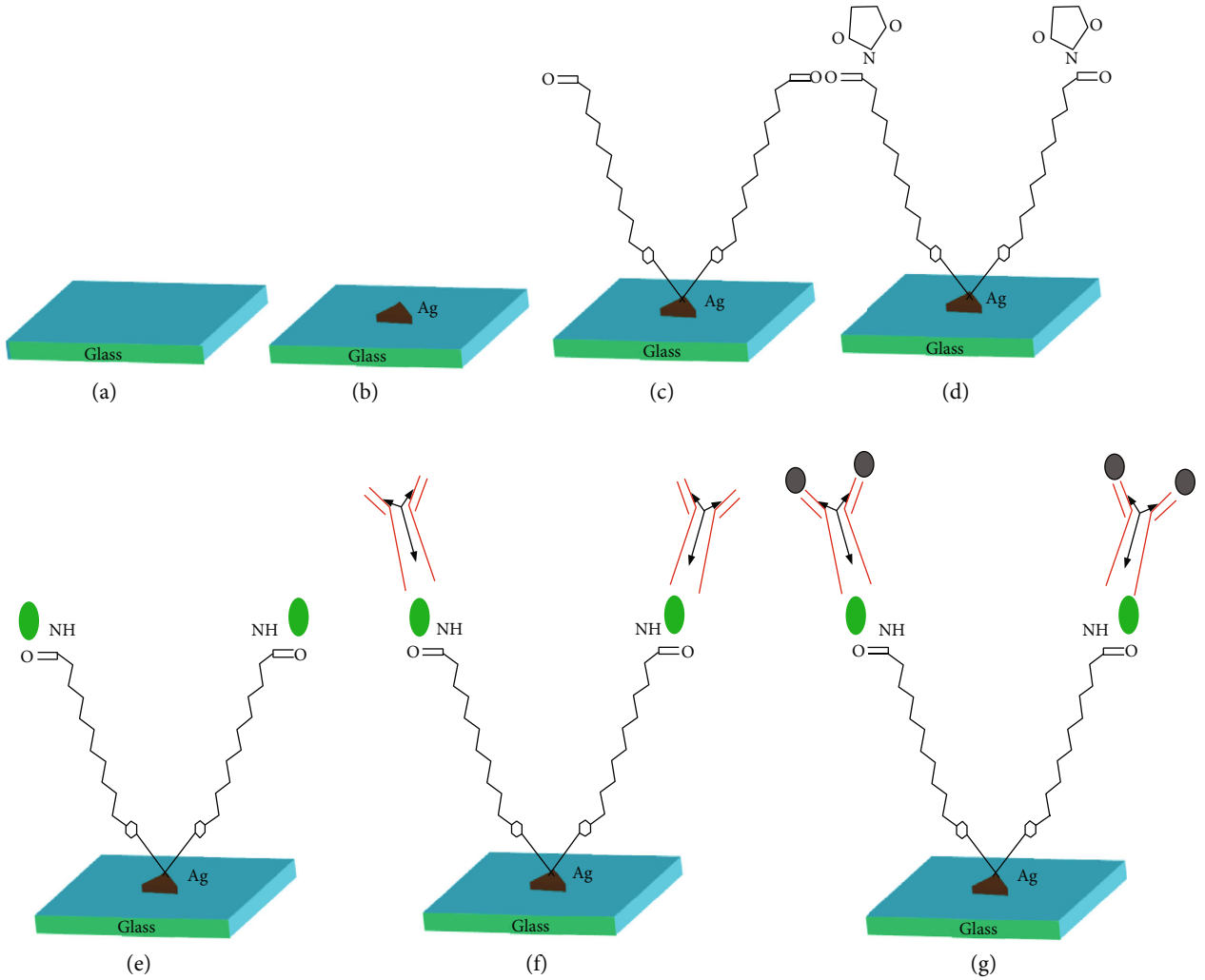

FIGURE 1: Design of the novel LSPR biosensor for detection of HE4. (a). Glass substrate. (b). Triangle-shaped silver nanoparticles array synthesized on glass substrate. (c). Incubation of 1 mM MUA. (d). Incubated in 75 mM EDC-HCl/15 mM NHS. (e). Incubated with 100ug/ml protein G. (f). Immobilization of anti-HE4 monoclonal antibodies (10 μg/mL). (g). Immunoassay of HE4 in buffer or urine samples.

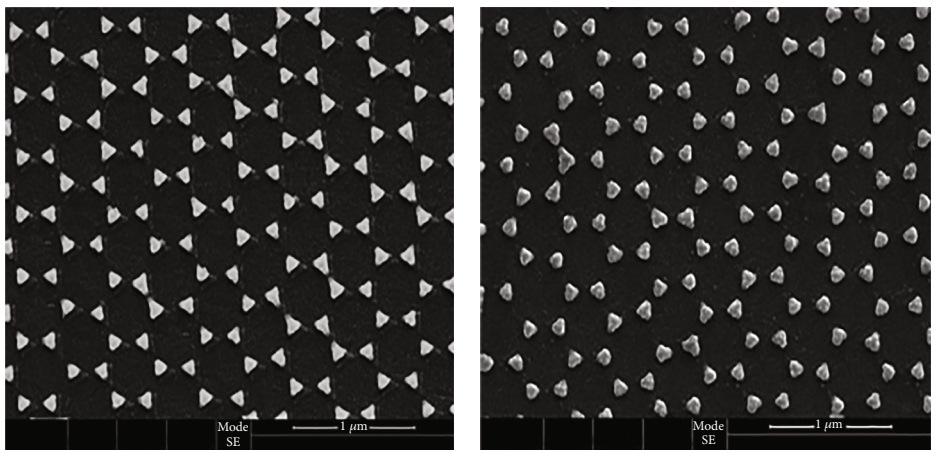

FIGURE 2: the silver nanoparticles characterization with SEM before and after modification. (a) characterization with SEM before modification. (b) characterization with SEM after modification (triangular silver nanosurfaces became obviously blunt with material binding).

respectively, using previous and new LSPR biosensors. Meanwhile, same clinical samples were confirmed using the CLIA (Chemiluminescence Immunoassay) method according to the manufacturer's instructions [14]. The detection results were collected and analyzed.

2.6. Statistical Analyses. Descriptive statistics were made by means with standard deviations and medians. Different study groups were compared using non-parametric tests. All p-values were two-sided, and the $P<0.05$ was considered statistically significantly. Statistical analyses were performed using SPSS version 19.0 (IBM, Armonk, NY, USA).

3. Results and Discussion

3.1. Spectroscopy Observation of Each Process. The LSPR responses to the new functionalization process and detection of HE4 were shown such as Figure 3. It is well known that an increase in the local refractive index around nanoparticles could be induced by the molecular binding to the nanochip surface, and result in LSPR spectrum peak red-shift indicated as the $\Delta\lambda$max [10, 12, 15, 16]. Thus, $\Delta\lambda$max could be a reliable indicator of the analytes bound to nanoparticles. The LSPR wavelength (λmax) of bare silver nanochip before modification was 606.16 nm as measured in Figure 3(a). When MUA bound onto the nanochip, the LSPR λmax shifted to 619.12 nm (Figure 3(b)). After modification of 100ug/ml protein G onto the silver surface, the LSPR λmax shifted to 624.79 nm with a corresponding $\Delta\lambda$max of +5.67 nm (Figure 3(c)). At last, an additional red-shift (+8.69 nm) of LSPR λmax was observed when immobilization of 10 μg/mL anti-HE4 monoclonal antibodies onto the nanochip (Figure 3(d)). Importantly, the LSPR λmax shifted to +5.87 nm as showing a λmax of 639.35 nm (Figure 3(e)) when incubation in 1pM HE4, and indicated that new biosensor could successfully detect the target analyte on the nanoparticles-solution interface.

3.2. Detection of Different Concentrations of Standard HE4 with New LSPR Biosensor. The new biosensors were incubated with different concentrations of standard HE4 solution ranging from 0.1 pM to 100,000 pM under optimal conditions, and LSPR absorption spectra were measured with peak shifts recorded. Each concentration measurement was repeated three times, and mean $\Delta\lambda$max were calculated, as shown in Table 1. It can be seen from the results that the LSPR $\Delta\lambda$max value increased gradually with increasing HE4 concentrations, and indicated that the biosensor had a broader detection range. However, No significant changes or peak shift were observed in the spectra, when the concentration of standard HE4 solution was decreased to 1 pM or increased to 10,000 pM. Compared to previous sensor, this study showed that the new biosensor was more sensitive. The detection limit of HE4 only could accomplish 4 pM by using our previous LSPR biosensor [10]. This work was the first explore of LSPR biosensor by using Protein G directional fixed method to increase sensitivity, which could display a detection limit of 1 pM, with wide dynamic range of 1 pM to 10,000 pM. Due to the limited number of new chips, the relation between HE4 concentrations and wavelength shift ($\Delta\lambda$max), limit of quantification, and inter/intraday variation would only be done in further study, which need more detections using a lot of new LSPR biosensors.

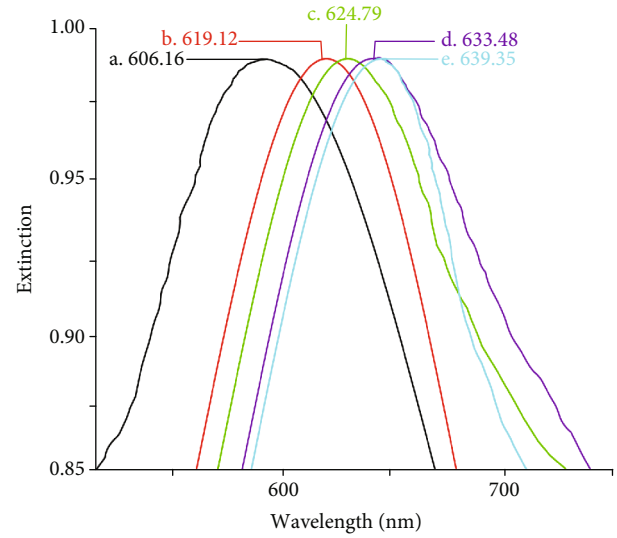

Figure 3: LSPR spectra for biosensor design and detection of 1 pM HE4. (a) Bare silver nanochip, λmax =606.16 nm. (b) Modification of 1 mM MUA, λmax =619.12 nm. (c) Incubation with 100ug/ml protein G, λmax =624.79 nm. (d) Immobilization of 10 μg/mL anti-HE4 monoclonal antibodies, λmax =633.48 nm. (e) Detection of 1 pM HE4, λmax =639.35 nm. Note: All spectra were collected at room temperature in air.

Table 1: Mean $\Delta\lambda$max of different concentrations of standard HE4 detection with new LSPR biosensor (based on localized surface plasmon resonance and protein G directional fixed [10, 17]).

HE4 concentrations	0.1 pM	1 pM	10 pM	100 pM	1000 pM	10000 pM	100000 pM
Mean $\Delta\lambda$max	0.71	5.43	6.85	8.42	8.83	9.17	0.35

3.3. Selectivity Test with New LSPR Biosensor. To the biosensor, selectivity is very important and should acquire. In this study, control experiments were designed to ensure that the results were not interfered by nonspecific bindings. To achieve specific detection, PBS and ultrapure water were used as surface washing agents after sufficient immobilization of antibodies. As another tumor marker, squamous cell carcinoma (SCC) and albumin, one protein component often present in urine were implemented as interferences. During the experiment, 10 pM SCC or albumin was, respectively, incubated onto the functionalized biosensor for same time at room temperature, and all control experiments were executed in triplicate. Results indicated that there was hardly any shift about the LSPR peak after PBS rinsing. But, under the same condition, 10 pM HE4 was introduced and LSPR peak was found shift to +6.85 nm. Above experiment confirmed superior selectivity of the detection property based on the new biological-sensitive layer.

Table 2: $\Delta\lambda$max of HE4 detection in urine of different study groups with previous LSPR biosensor (based on localized surface plasmon resonance [10]).

Study groups	LSPR wavelength shift ($\Delta\lambda$max)					$\bar{X}$±SD
	Case 1	Case 2	Case 3	Case 4	Cases 5	(n =5, nm)
Healthy women	0.32	0.16	0.25	0.14	0.39	0.25 ± 0.11
Benign ovarian cyst patients	0.33	0.41	0.27	0.23	0.40	0.33 ± 0.08
Early ovarian cancer patients	0.42	0.12	0.58	0.29	0.33	0.35 ± 0.17

Table 3: $\Delta\lambda$max of HE4 detection in urine of different study groups with new LSPR biosensor (based on localized surface plasmon resonance and protein G directional fixed [10, 17]).

Study groups	LSPR wavelength shift ($\Delta\lambda$max)					$\bar{X}$±SD
	Case 1	Case 2	Case 3	Case 4	Cases 5	(n =5, nm)
Healthy women	0.49	0.05	0.85	0.43	0.06	0.38 ± 0.33
Benign ovarian cyst patients	0.91	0.38	0.50	0.12	0.63	0.51 ± 0.29
Early ovarian cancer patients	4.31	5.39	8.66	4.19	7.24	5.96 ± 1.94

Table 4: HE4 detection in urine of different study groups with CLIA method (based on Chemiluminescence Immunoassay method [14]).

Study groups	Testing HE4 in urine (concentration)					$\bar{X}$±SD
	Case 1	Case 2	Case 3	Case 4	Cases 5	(n =5, pmol/L)
Healthy women	0.38	0.45	0.33	0.41	0.36	0.39 ± 0.05
Benign ovarian cyst patients	0.59	0.48	0.62	0.47	0.51	0.53 ± 0.07
Early ovarian cancer patients	2.01	2.79	1.96	2.82	2.33	2.38 ± 0.41

3.4. Urine Sample Detection with Previous and New LSPR Biosensor. Under optimal conditions, the clinical urine samples from healthy women, benign ovarian cyst patients, and early (I/II) ovarian cancer patients were, respectively, detected by previous and new LSPR biosensors. LSPR absorption spectra were accordingly measured with peak shifts recorded, with each sample measurement repeated three times. The results were shown as in Table 2 and Table 3. There were no significant LSPR spectra peak shift and difference among all study groups when using our previous LSPR biosensor. But, significant LSPR spectra peak red shifts were observed in early ovarian cancer patients by using new LSPR biosensor, without obvious LSPR $\Delta\lambda$max found in healthy women or benign ovarian cyst patients. Based on statistical analyses, differences in response of HE4 detection between early ovarian cancer groups and the control groups (healthy women and benign ovarian cyst patients) were statistically significant by using new LSPR biosensor ($P<0.05$). Meanwhile, in order to verify our experimental results, same clinical urine samples were also calculated using CLIA method, according to the manufacturer's instructions. The consistent results were confirmed as showed in Table 4. There were also significant differences in HE4 testing concentration between early ovarian cancer groups and the control groups (healthy women and benign ovarian cyst patients) by using CLIA. In this study, although the CLIA was also sensitive, it needed to be marked and the operation steps were tedious.

Thus it could be seen that the new LSPR biosensor was able to specifically distinguish between early ovarian cancer and the negative controls without need for labeling and purification of the clinical samples, and just by the HE4 detection of women urine. Moreover, this new LSPR biosensor had good specificity that could not be interfered by other proteins or components in urine. Contrast to previous LSPR biosensor, this work firstly explored to enhance the detection sensitivity by using protein G directional fixed method. As a cell wall protein isolated from group G Streptococcus, protein G could combine directionally with Fc crystallizable segments of most mammalian immunoglobulin G and make the antibody antigen binding fragment pointing to the outer surface of nanostructure uniformly [17, 18]. Therefore, the antigen binding end of the immunoglobulin antibody could be more advantageous to the binding of target antigen and provided with high sensitivity detection for the LSPR biosensor.

The LSPR spectra of this new biosensor was measured during every processing and the results confirmed that the new fabrication procedures for biological-sensitive layer construction could be feasible and effective, as shown in Figures 1 and 3. After modification, the characterization with SEM also indicated triangular silver nanosurfaces became obviously blunt, owning to material binding on surface of nanoparticles, as shown in Figure 2. This improvement was very important for the LSPR biosensor to identify precisely low concentrations of biological substances in urine and could be potentially used in cancer screening and early diagnosis. Using this detection method, urine HE4 could be measured in real time within 40 minutes, without label or purification of sample, which was an important issue in wide clinical application for tumor screening.

4. Conclusion

To the best of our knowledge, this was the first use of LSPR method for detection of tumor marker in urine of cancer patients. As an important tumor marker of ovarian cancer, HE4 was reported that can be measured in urine, and perform similar sensitivity and specificity to that by assaying serum [9, 19–22]. Compared with traditional immunoassay approaches, our previous-built LSPR detection system had outstanding advantages, such as label-free, miniaturization, portability, rapid test time and low cost [10, 12, 13]. In this study, we firstly improved the sensitivity of LSPR biosensor by using protein G directional fixed method, and successfully used this new build biosensor to detect HE4 in urine. The new built biosensor in this study demonstrated that: 1) the self-assembled protein G onto silver nanochips via amino coupling could make anti-HE4 pointing to the outer surface uniformly, which improved the efficiency of antibody and sensitivity of detection system. 2) the new functionalized LSPR sensor was simple to prepare, could provide better sensitivity, had a lower detection limit, and had good selectivity for testing HE4 in urine, which could provide a non-invasive and more convenient approach in clinical ovarian cancer screening and early diagnosis. 3) the new-built LSPR system could be available for analysis of HE4 in human urine with advantages such as rapid test time, label-free, and purification-free process. Moreover, a HE4 urine calibration curve would be done in further study, which need a large, randomized, case-controlled clinical study to further evaluate applicability of this new biosensor in clinical tumor screening and medical diagnosis. In conclusion, the new biosensor was anticipated to be a promising platform for cancer biomarker detection in urine and expand clinical application of LSPR sensor.

Data Availability

Answer: Yes. Comment: The dataset supporting the conclusions of this article is included within the article.

Ethical Approval

This study was approved by the Institutional Review Board of West China Second University Hospital. Research Ethics Approval from Ethics Institutional Board and written informed consents from patients were not needed in this study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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