Quartz crystal microbalance immunoassay for carcinoma antigen 125 based on gold nanowire-functionalized biomimetic interface†

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Gold nanowires with designed length on a solid substrate have been proven as an efficiently immobilized affinity support for the detection of carcinoma antigen 125 (CA 125) in this study. The presence of gold nanowires provides a well-defined three-dimensional structure, and greatly amplifies the coverage of the anti-CA 125 protein on the probe surface. Moreover, the amount of anti-CA 125 varied with the change of the morphology of the probe, and achieved an optimal quartz crystal microbalance (QCM) response towards anti-CA 125 adsorption at the number of gold nanolayers of 5. The formed immune-probe exhibits good QCM responses for the detection of CA 125, and allows the detection of CA 125 at concentrations as low as 0.5 U ml⁻¹. The QCM immunosensor exhibited good precision, high sensitivity, acceptable stability, accuracy and reproducibility. The as-prepared immunosensors were used to analyze CA 125 in human serum specimens. Analytical results suggest that the developed immunoassay method is a promising alternative approach for detecting CA 125 in the clinical diagnosis. Compared with conventional ELISA, the proposed immunoassay system was simple and rapid without multiple labeling and separation steps. Importantly, the route provides an alternative approach to incorporate multiple gold nanolayers onto the solid matrix for biosensing applications.

Introduction

Tumor markers play an essential role in modern medicine. This includes classical diagnostic applications such as detection, diagnosis, prognosis and monitoring of diseases.¹ Recently, great efforts have been made worldwide to develop and improve immunoassays for the detection of tumor markers with the aim of making portable and affordable devices.²,³ Despite many advances in this field, it is still a challenge to exploit new approaches that can improve the simplicity, selectivity, and sensitivity of clinical immunoassays, to meet the requirements of modern medical diagnostics and biomedical research applications.⁴ The quartz crystal microbalance (QCM) immunosensor, which offers some advantages including high sensitivity, low cost, real-time output, and label- or radiation-free entities, has been the active subject of investigations of biomolecular interactions and clinical bioassays.⁵–⁸

The QCM immunosensors measure the resonant frequency (f) using the standard oscillator technique, and the frequency change (Δf) is usually explained by Sauerbrey equation, which states that the decrease in f is linearly proportional to the increase in surface mass (m) loading of the QCM.⁹–¹¹ This Sauerbrey equation, however, holds only for the case of rigid coated material. The qualitative aspects of the resulting data and the magnitude of the observed effect are shown to be independent of film thickness for values as low as 115 Å. When an overlayer is thick, the relationship between Δf, and Δm is no longer linear and corrections are necessary. In QCM immunoassays, the detection sensitivity can be improved by increasing the coverage of biomolecules and by decreasing the steric-hindrance effect on the sensor surface.¹² When antibodies are immobilized on a solid-phase surface, their binding activity is usually less than that of soluble antibodies.¹³ One of the main reasons for this reduction of binding activity is due to steric hindrance of the molecules in the solid phase compared with that in solution.¹⁴,¹⁵ Another possible reason is attributed to the random orientation of biomolecules on the solid-phase surface.¹⁶ In these regards, noble metal nanowires have paved a versatile path for the immobilization of biomolecules with acceptable stability and good activity retention capacity.¹⁷,¹⁸

Gold nanowires could provide a large well-defined surface area and the capacity for modification of proteins on the surface of the gold nanowires, which would adopt an more flexible orientation and result in a high amount of proteins. Another issue is to fabricate gold nanowires of a desired length because it is one of the important parameters that affect their sensitivity in analytical applications. In this contribution, we introduced a general route to fabricate gold nanowires by using the self-assembly technique and chemical reduction method, and assessed their application in the clinical immunoassays. A direct and real-time immunoassay was first performed by immobilizing anti-CA 125 antibodies onto the crystal surface. The CA 125 was then detected directly by the frequency change observed as it binds to the specific antibodies. The change in frequency
was proportional to the concentration of the binding antigen. The present immunoassay system exhibited good analytical performances such as sensitivity, precision, accuracy, analytical time, stability and reproducibility towards the quantification of CA 125.

**Experimental**

**Materials**

Cancer antigen 125 (CA 125, 0–500 U ml\(^{-1}\)) and CA 125 monoclonal antibody (anti-CA 125) were purchased from Biocell Biochem. Co. (Zhengzhou, China). 2-Aminoethanethiol (AET), bovine serum albumin (BSA) and sodium borohydride (NaBH\(_4\)) were purchased from Sigma (USA). Phosphate buffered saline (PBS) solutions of various pHs were prepared by mixing 100 mM K\(_2\)HPO\(_4\), 137 mM NaCl and 2.7 mM KCl purchased from Sigma (USA). Hydrogen tetrachloroaurate(III) hydrate (HAuCl\(_4\)), phosphate buffered saline (PBS) and sodium borohydride (NaBH\(_4\)) were purchased from Biocell Biochem. Co. (Zhengzhou, China). 2-Aminoethanethiol (AET), clonal antibody (anti-CA 125) were purchased from Biocell Biochem. Co. (Zhengzhou, China). 2-Aminoethanethiol (AET), clonal antibody (anti-CA 125) were purchased from Biocell Biochem. Co. (Zhengzhou, China).

**Preparation of gold nanowires**

Prior to the bottom-up layer formation process, the gold electrodes (substrates) were cleaned by immersing them in piranha solution for 5 min. Preparation of layer-by-layer assembly of gold nanowires with AET as the monolayer spacer was carried out as described below. A cleaned gold substrate was initially immersed into AET acidic solution (200 \(\mu\)M, pH 4.5) for 2 h at 4 °C, and then exposed into 100 \(\mu\)M HAuCl\(_4\) solution in the dark with slight stirring for 2 h after washing with ethanol. During this process, [AuCl\(_4\)]\(^-\) ions were complexed with the amino or amide groups of the immobilized AET. Afterwards, this probe was again dipped into AET and HAuCl\(_4\) solution. The procedure is alternated and repeated for the assembly of AET and [AuCl\(_4\)]\(^-\) ions (Scheme 1). Following that, the formed precursor of gold nanowires was immersed into 2 ml of 0.1 M NaBH\(_4\) (0.5 mM) containing 0.3 M NaOH solution with slight stirring. During this procedure, the Au(III) in every layer was reduced to the zero-valent gold nanoparticles, and the gold nanowires were formed. The surface of the probe changed from yellow to deep red.

**Fabrication of the immunosensor**

After a washing step with distilled water, the gold nanowire-modified probes were transferred into anti-CA 125 solution for 6 h at 4 °C. The films were carefully washed with water and then dried with \(\text{N}_2\) gas. Finally, the modified probes with gold nanowires were incubated in 0.25 wt% BSA for 60 min at 37 °C to eliminate any non-specific binding effect and block the remaining active groups. The finished immunosensors were stored at 4 °C when not in use (Scheme 1).

**Measurement methods**

Electrochemical measurements were performed with an Autolab (Eco Chemie, The Netherlands) system. A three-electrode system [a prepared working electrode, a platinum wire as auxiliary electrode and a saturated calomel electrode (SCE) as reference] was employed for all electrochemical experiments. All gravimetric measurements were performed using a quartz crystal microbalance with AT-Cut, 10 MHz quartz crystals and a gold-plated electrode (diameter 13.7 mm, 1000 Å thickness) on both sides mounted in a HC6/U holder (QCM, Pico Balance, Italy). The principle of the QCM sensors is based on changes \(\Delta f/\), in the fundamental oscillation frequency upon the antigen–antibody interaction. To a first approximation, the frequency change \(\Delta f/\), results from an increase in the oscillating mass \(\Delta m\) according to the Sauerbrey equation:

\[
\Delta f/ = -2.3 \times 10^{-9} f_0 \Delta m/A \tag{1}
\]

where \(\Delta f/\) is the resonant frequency difference (Hz); \(f_0\) is the basic resonant frequency of the crystal (MHz); \(\Delta m\) is the mass accumulation on the crystal surface (g); and \(A\) is the electrode area (1.47 cm\(^2\)). The frequency shift, \(\Delta f/\), (Hz), was defined as the absolute value of the frequency difference \(\Delta f/\).

The prepared QCM probe was first mounted on one side of the detection vessel containing an assay buffer solution (PBS, pH 7.4). Each of the samples to be analyzed was then introduced into the detection vessel after stabilization of the resonance frequency (a shift of <1 Hz min\(^{-1}\)). To avoid the possible error resulting from different additions of samples and to deduce the response induced by non-specific adsorption, the frequency changes were recorded as the immunoreaction proceeded from 30 s (after the addition of samples) until equilibrium. The control tests were performed accordingly. The frequency changes in all experiments were referred to the average responses of the immunoreaction with corresponding standard deviations (\(\Delta f/\pm\) SD) of triplicate measurements, unless otherwise indicated. All measurements were conducted at room temperature.

**Results and discussion**

**Construction of gold nanowires**

In this experiment the AET molecule is bifunctional, with the \(-\text{SH}\) group at one end and an \(-\text{NH}_2\) group at the other. At the first layer, AET molecules were assembled on the surface of the gold substrate, which provided an immobilized matrix for complexing [AuCl\(_4\)]\(^-\) with the \(-\text{NH}_2\) of the AET according to the literature. The complexing [AuCl\(_4\)]\(^-\) ions were used for self-assembling the second layer of AET molecules. When [AuCl\(_4\)]\(^-\)
Table 1  Element analysis and complexing ratio of the [AuCl4]−–AET ligands with various layers

<table>
<thead>
<tr>
<th>Layer number</th>
<th>C (%)</th>
<th>H (%)</th>
<th>N (%)</th>
<th>Sum (%)</th>
<th>Complexing ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.38</td>
<td>0.09</td>
<td>0.23</td>
<td>0.70</td>
<td>0.007</td>
</tr>
<tr>
<td>2</td>
<td>0.53</td>
<td>0.15</td>
<td>0.31</td>
<td>0.99</td>
<td>0.010</td>
</tr>
<tr>
<td>3</td>
<td>0.75</td>
<td>0.19</td>
<td>0.44</td>
<td>1.38</td>
<td>0.014</td>
</tr>
<tr>
<td>4</td>
<td>0.96</td>
<td>0.24</td>
<td>0.56</td>
<td>1.76</td>
<td>0.018</td>
</tr>
<tr>
<td>5</td>
<td>1.20</td>
<td>0.30</td>
<td>0.70</td>
<td>2.20</td>
<td>0.023</td>
</tr>
</tbody>
</table>

ions were immobilized to the –NH2 groups of the first AET layer, the –SH group of the second layer AET could complex to the [AuCl4]− ion. Although –NH2 and –SH could complex to the Au(III) on the AET, the bond force of Au(III) complexes with sulfur-containing ligands is stronger than that with nitrogen-containing ligands from the viewpoints of the literature. Thus, the [AuCl4]− ions were firstly complexed with the –SH group of the AET molecules, and then complexed with the –NH2 group of the AET. Thus, the AET molecules and [AuCl4]− ions were assembled alternately in a layer-by-layer fashion on the gold substrate. With the growth of the [AuCl4]−–AET composite on the solid substrate, the probe surface acquired a distinct yellowish coloration, similar to that of HAuCl4 solution. After the reduction of the [AuCl4]− ions in NaBH4 aqueous solution, the probe surface achieved a typical red-purple coloration of the colloidal gold particles since NaBH4 is a strong reducing agent.

To further verify that the [AuCl4]− ion could be complexed onto the –NH2 group of AET after the AET-modified gold electrode was immersed into the HAuCl4 solution, we used atomic absorption spectroscopy (AAS, Hitachi 180-80, Zeeman, Japan) to qualitatively investigate the aurum element. The measurement method was as follows: the [AuCl4]− and AET-modified electrode was initially dipped into the distilled water and continuously sonicated for 10 min, and then the resulting solution was assayed by AAS. The complexing ratios of the AET–[AuCl4]−–AET ligands are shown in Table 1, calculated from the EA data. Successful growth of the [AuCl4]−–AET on the gold electrode was confirmed by the EA data. On the basis of the AET and EA results, we might qualitatively make a conclusion that the AET–[AuCl4]−–AET complex could be formed on the base surface. However, it is still a challenge for elucidating the exact mechanism of the interaction between [AuCl4]− ions and AET molecules. Herein, we only thought that the [AuCl4]− ions were complexed onto the –SH and –NH2 groups of the AET molecules.

Characteristics of gold nanowires

To investigate the topology characteristics of differently modified surfaces, atomic force microscopy (AFM) was used to observe the various {AuCl4−/AET}, layer-functionalized gold surfaces obtained by using the chemical reduction method (Fig. 1). For each cycle of the reduction process, the templates were thoroughly rinsed and dried, and AFM images were recorded to evaluate the growth of gold nanowires in the templates. As shown in Fig. 1a, spherical gold nanoparticles with...
a homogeneous dispersion were observed on the base surface at the first layer \( (n = 1) \). The reason is that the prepared procedure of first gold nanowires was similar to the self-assembly process of gold nanoparticles. With the increment of layer number of gold nanoparticles, however, the shape of the gold nanowires was changed from sphere-like to wire-like (Fig. 1b–d). Moreover, there were some interspaces between the gold nanowires. To further verify the formation of gold nanowires, scanning electron microscopy (SEM) and UV-vis absorption spectra were also used to investigate the surface. The SEM images exhibited the same results as the AFM images (Fig. 1e–h). Furthermore, the series of UV-vis absorption spectra clearly exhibits the evolution of optical absorbance of the gold nanoparticles with the increment of gold nanolayer number (figure not shown). In terms of the plasmon resonance band of the gold nanoparticles, we can see a progressive enhancement in its intensity, which indicated the increased content of gold in the template that yields the high absorbance features.\(^{26}\) On the basis of AFM, SEM and UV-vis results, we might make a conclusion that the gold nanowires could be formed on the substrate surface via a self-assembly technique and chemical reduction method.

**Electroactive surface area comparison of different electrodes**

In this paper, we constructed pure 24 nm gold nanoparticles and gold nanowires with five \{AuCl\(^{-}/AET\}\( n = 5 \)\)-functionalized electrodes, respectively. Cyclic voltammograms (CVs) of various electrodes were also investigated in 10 mM Fe(CN)\(_6\)\(^{3-}/\)PBS (pH 7.4) from \(-200\) to \(600\) mV at \(50\) mV s\(^{-1}\). A pair of well-defined redox peaks of Fe(CN)\(_6\)\(^{3-}/\)\(^{4+}\) was observed at \(+90\) and \(+200\) mV at the gold nanowire-modified electrode, which are similar to those at the pure 24 nm gold nanoparticle-modified electrode. According to the Randles–Sevcik equation:

\[
i_p = 2.69 \times 10^5 AD^{1/2}n^{3/2}C^{1/2}
\]

where \( D \), \( n \), \( \gamma \) and \( C \) are constants, and the electroactive surface area \( (A) \) is linear to the peak current of the redox couple; the electroactive surface area of the gold nanowire-functionalized electrode is about 53.7 mm\(^2\) while that of the gold nanoparticle-functionalized probe is about 27.4 mm\(^2\). Moreover, the background current at the gold nanowire-functionalized probe could increase about 2.5 times compared with that at the gold nanoparticle-functionalized probe. The reason might be the fact that the formed gold nanoparticles and cross-linkage AET could act as a promoter, which is facile to the diffusion of Fe(CN)\(_6\)\(^{3-}/\)\(^{4+}\) towards the electrode surface. Meanwhile, the high electroactive surface area of the gold nanowire-modified electrode could provide more room for the subsequent immobilization of biomolecules. However, the signal-to-background ratio at the gold nanowire-modified electrode might not be better due to the high background current. Thus, we did not choose cyclic voltammetry for the detection of analyte in this study.

**Comparison of different probes**

In QCM immunoassay systems, the key step is the immobilization of sensing biomolecules, which should be simple, fast and lead to robust materials with stable and highly active immobilized reagents that do not leach from the substrate.\(^{27}\) Another important issue is to enhance the coverage of the biomolecules on the transducer surface leading to directly influencing the sensitivity of the immunosensors.\(^{28}\) To examine the effect of gold nanoparticles and gold nanowires with \{AuCl\(^{-}/AET\}\( n \) layers on the immobilization of anti-CA 125, the QCM technique was also used to investigate the abilities of different probe surfaces for the immobilization of anti-CA 125. One can find that the probe with gold nanowires shows a much greater frequency change \((84.2 \pm 1.5 \text{ Hz})\) than the probe with pure gold nanoparticles \((51.7 \pm 1.9 \text{ Hz})\). According to the Sauerbrey equation [eqn (1)], the frequency shift corresponding to a mass increase of 279.5 ng cm\(^{-2}\) was less optimistic for the anti-CA 125 adsorption on the gold nanowires than that for the gold nanoparticle conjugation \((171.6 \text{ ng cm}^{-2})\). Moreover, the probe with gold nanowires exhibited a more rapid QCM response than the probe with gold nanoparticles in terms of the frequency response rate. The result reveals that the gold nanowire-modified probe could provide more binding sites and room for protein adsorption.

In addition, we used the QCM technique to investigate the influence of gold nanowires with various \{AuCl\(^{-}/AET\}\( n \) layers on the surface coverage of anti-CA 125 on the substrate (Fig. 2). As seen from the inset of Fig. 2, the surface coverage of anti-CA 125 was increased with the increment of the layer number of \{AuCl\(^{-}/AET\}\( n \), and leveled off at \( n = 5 \), which might be ascribed to the enlarged surface area of the gold nanowires. Another reason may be ascribed to the steric hindrance of the protein. The steric structure of the gold nanowires greatly prevented the access of anti-CA 125 to the inner gold nanowires. Thus, we chose the prepared gold nanowires with \{AuCl\(^{-}/AET\}\( n \) layers \( (n = 5) \) as the immobilized affinity support for the detection of CA 125 in the following experiments.

![QCM responses to CA 125](image)

**QCM responses to CA 125**

The binding capacity of the proposed immune-probe was examined for various concentrations of CA 125 antigen based...
on QCM measurements. Fig. 3 displays the typical frequency responses monitored in situ for CA 125. The linear regression equation is 
\[ \Delta f = 1.89 + 0.32c_{CA\text{125}} (U\text{ ml}^{-1}) \] 
\( (R^2 = 0.997) \) at the dynamic range of 1.5–180 U ml\(^{-1}\) with a detection limit of 0.5 U ml\(^{-1}\) (it was estimated according to IUPAC Recommendations 1994). The saturation of dose response at the higher concentration of CA 125 is mainly due to the limitation of the CA 125 antibodies’ binding site. Commonly, the normal level of CA 125 is less than 25 U ml\(^{-1}\) in human serum. Thus, the linear relationship is suitable for an ‘order of magnitude’ test of CA 125. When the CA 125 concentration was more than 150 U ml\(^{-1}\), the coefficient of determination was 7.7%, 15.8%, 28.4%, and 29.6% at the CA 125 concentrations of 5, 50, 100 and 150 U ml\(^{-1}\), respectively, while the inter-assay variation coefficient with this method was 4.8, 7.7, 8.9, and 9.3% at 100 U ml\(^{-1}\). The intra-assay precision of the proposed immune-probe was acceptable. In addition, the stability of the as-prepared immune-probes used independently was 9.3% at 100 U ml\(^{-1}\), an appropriate dilution of sample was necessary in the incubation solution.

**Reproducibility, precision and stability**

The intra-assay precision of the proposed immune-probe was evaluated via assaying the CA 125 levels of four sera for five replicate measurements in the same run. The variation coefficients of the intra-assay with this method were 4.8, 7.7, 2.6 and 5.4 at the CA 125 concentrations of 5, 50, 100 and 150 U ml\(^{-1}\), respectively, while the inter-assay variation coefficient on six CA 125 immune-probes used independently was 9.3% at 100 U ml\(^{-1}\). Thus, the precision and reproducibility of the proposed immune-probe were acceptable. In addition, the stability of the as-prepared immunosensor was examined. When the proposed immunosensor was stored in pH 7.4 PBS at 4 °C, it retained 89.3% of its initial response after a storage period of 11 days. The slow decrease of response seemed to be related to the gradual deactivation of the immobilized anti-CA 125 incorporated in the composite membrane.

**Specificity and preliminary application**

To investigate the selectivity of the developed immunosensor, the as-prepared immune-probes were incubated into the incubation solution containing different concentrations of CA 125 and various concentrations of interfering agents, such as carcinoembryonic antigen (CEA), cancer antigen 199 (CA 199), α-fetoprotein and hepatitis B surface antigen. Table 2 represents the selectivity of the immunosensor relative to various concentrations of CA 199 as an example. The obtained QCM responses were compared with the results measured in pure CA 125 solution. Experimental results suggest that, below some tolerance limit in normal human serum, these interfering agents do not interfere remarkably with the QCM response of the immunosensor.

To monitor the feasibility of the newly developed immunoassay, the spiked serum samples were prepared by injection of CA 125 standard into normal serum to make CA 125 concentrations of 0–180 U ml\(^{-1}\). These samples were then assayed by using the developed immunoassay. Fig. 4 shows the analytical results of the spiked serum sample and the recoveries in this method. It can be seen that these data are tightly scattered about the calibration lines. The mean recovery was 103.4%, and the

**Table 2** Selectivity of gold nanowires with the five [AuCl\(_4\)]\(^{-}\) layer-conjugating immunosensor

| \( C_{CA\text{125}}/U\text{ ml}^{-1} \) | Frequency shift/Hz\(^{*}\) for various \( C_{CA\text{199}}/U\text{ ml}^{-1} \) |
|-------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|       | 0               | 5               | 20              | 60              | 140             |
| 5     | -3.5 ± 0.3      | -3.9 ± 0.5      | -4.1 ± 0.7      | -3.8 ± 0.4      | -4.3 ± 0.3      |
| 20    | -8.3 ± 1.1      | -8.6 ± 0.7      | -8.9 ± 1.2      | -7.9 ± 2.1      | -9.3 ± 1.2      |
| 40    | -14.7 ± 1.3     | -15.3 ± 1.7     | -14.3 ± 2.4     | -15.8 ± 2.6     | -16.4 ± 1.3     |
| 80    | -27.5 ± 1.7     | -28.6 ± 1.9     | -29.1 ± 2.3     | -28.4 ± 2.3     | -29.6 ± 2.4     |
| 160   | -53.1 ± 2.3     | -54.7 ± 2.5     | -56.7 ± 2.2     | -57.3 ± 5.1     | -54.9 ± 3.7     |

\(^{*}\) All frequency responses (Hz) are presented as the mean ± SD (standard deviation) of triplicate measurements for each sample.
The regression equation (linear) for these data is as follows: \( y = 4.81 (\pm 3.15) + 0.967 (\pm 0.105)x \) \((R^2 = 0.952)\) (x-axis, the standard concentration; y-axis, the concentration obtained by the developed immunoassay). These data show that this method was comparable and acceptable for CA 125 detection; that is, the developed immunoassay may provide a promising alternative tool for determining CA 125 in human serum in a clinical laboratory. Importantly, the consumed time of the developed CA 125 detection method is shorter than that of the commercial ELISA (>4 h).

**Conclusions**

This manuscript has introduced a general route for the fabrication of multiple gold nanowires by using a self-assembly technique and chemical reduction method, and the application as an immobilized affinity support for the detection of CA 125 in clinical immunoassays. The immobilization shows a quite large increase in adsorption capacity, although still in random orientation. Then the sensitivity of the resulting immunosensor has been improved clearly by the QCM measurements. In addition, it has been demonstrated that the adsorption amount of biomolecules could be controlled by focusing on the control of the layer number on the substrate surface.

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