Detecting the golgi protein 73 of liver cancer with micro cantilever

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Received 16 October 2014
Accepted for publication 24 October 2014
Published 27 November 2014

Abstract
Golgi protein 73 (GP73) is a potential serum biomarker used in diagnosing human hepatocellular carcinoma (HCC). Compared to alpha-fetoprotein, detection of GP73 is expected to give better sensitivity and specificity and thus offers a better method for diagnosis of HCC at an early stage.

In this paper, silicon nitride microcantilever was used to detect GP73. The cantilever was modified through many steps to contain antibody of GP73. The result shows that the cantilever can be used as a label-free sensor to detect this kind of biomarker.

Keywords: GP73, AFP, microcantilever, biomarker

Mathematics Subject Classification: 6.09

1. Introduction

Golgi protein (GP73) is a type II membrane protein and has just been known in the diagnosis of liver cancer. Its function is still unclear in liver cells in patients suffered from giant cell hepatitis [1]. In healthy liver, the expression level of GP73 is very low and mainly in biliary epithelial cells. Expression level of GP73 in infected liver increases significantly and is independent of infection agents, whereas the expression level in the biliary epithelial cells is virtually unchanged [2].

GP73 biomarker is considered to be more valuable than alpha-fetoprotein (AFP) which has been used widely in the diagnosis of liver cancer. According to Mao et al human hepatocellular carcinoma (HCC) diagnosis cancer diagnosis with GP73 has sensitivity at 76.9%, higher than AFP 48.6%, and the specificity for HCC is 92.9% compared with only 75% for AFP [2]. Therefore, GP73 is expected to be a new biomarker for early diagnosis of HCC.

Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) are two of many methods used to detect the biomarker based on antibody–antigen interaction, in which the target molecule will be detected by the probe molecules labeled with radioactive substance or enzyme (such as horseradish peroxidase). The probe molecule is immobilized on surfaces such as glass, nitrocellulose membranes, particles or in the form of a solution. However, the downside of these methods includes danger to humans and the fact that they are time-consuming, because of additional steps of labeling with radioactive or enzyme substances [3].

In this paper we study the microcantilever, a new device for biological diagnosis. Cantilever-based biosensor is a micro/nano device capable of reading chemical reaction on its surface via the changes of its physical properties such as deflection or frequency [4]. It has been used as a promising device with various applications, especially in environment monitoring and for detecting biomarkers and early diagnosis in medicine. The advantage of this kind of sensor is that it is label-free [5].

Moreover, we present the modification of microcantilever chips to functionalize them into biosensor. Fluorescence technique was used to investigate the modification efficiency. Finally, the cantilevers were used to detect GP73, a promising biomarker for HCC as mentioned above.

2. Experimental

The cantilever chips were washed with acetone, ethanol and deionized water. After that, the chips were immersed in solutions of 3-aminopropyltriethoxysilane (APTES) (Sigma Aldrich) in ethanol solvent to form self-assembled monolayer due to the bonding reaction between silane groups and silanol groups on the sample surface. This reaction happened through many steps (hydrolysis, silanol condensation, and coating on surface [6]) as shown in figure 1. Aqueous 2% glutaraldehyde
(Sigma Aldrich) was prepared. Samples were immersed in this solution for 1 h in order for the APTES amine group to react with aldehyde group. After that samples were washed with PBS solution.

In order to evaluate the density of APTES and glutaraldehyde on the samples, we use fluorescence method. Lens culinaris agglutinin conjugated with fluorescein isothiocyanate (F-LCA) was immobilized on the sample. Amine groups in F-LCA are attached to the free ends of aldehyde group of glutaraldehyde. The samples were then observed under a fluorescence microscope BX41 (Olympus) using excitation wavelength 490 nm. F-LCA emits the strongest fluorescent light at wavelength 520 nm (green color). The fluorescence microscope is equipped with a filter to eliminate the excitation wavelength from the output fluorescence light.

In our experiment we also immobilized horseradish peroxidase (HRP) enzyme in phosphate buffer (Sigma Aldrich) on the samples. The samples were immersed overnight. After washing, the samples were put into solutions of H2O2 and o-dianisidine. Changes in color of the solutions help identify whether the HRP enzyme was immobilized on the sample surface or not.

3. Results and discussion

3.1. Verification of surface modification with fluorescence images

Figure 2 shows the fluorescence image of a modified cantilever. The image was taken on the bottom side of the cantilever, because the cantilever top side was coated with Cr and Au, which did not undergo reaction with APTES. The bright green color from fluorescein isothiocyanate (FITC) conjugated with lens culinaris agglutinin (LCA) proved that the silicon nitride surface was successfully activated with APTES and GAD and capable of reacting with molecules containing amine groups. The uniformity of APTES and GAD attached on the cantilever was quite high as shown in the uniformity of green color from F-LCA.

3.2. Effect of APTES concentration on the surface modification efficiency

The duration of forming APTES layer was investigated. We immersed the samples in 2% APTES solution over various
durations: 60, 90, 120, 150 and 180 min. Figure 3 shows no clear trend. The sample immersed in APTES for only 60 min had RFU even stronger than the samples immersed for 2 h and 3 h.

The fact that the sample immersed in APTES for 60 min had quite strong relative fluorescence intensity (or relative fluorescence unit, RFU) suggested to us that the reaction between APTES and silanol groups was quick. After 60 min, it is very likely that most of the silanol groups had reacted with APTES and longer immersion could be unnecessary.

3.3. Effect of APTES concentration on the surface modification efficiency

In the step of creating an APTES layer on silicon nitride surface, the concentration of APTES was varied from 0.01% to 2%. The RFU of F-LCA immobilized on the surface is shown in figure 4. We can see that even at small concentration 0.01%, which is 200 times smaller than the concentration 2%, the RFU was only slightly smaller. The RFU can be related directly to the density of F-LCA molecules on the surface. Therefore, we suggested that the velocity of reaction between APTES silane groups and the silanol groups on silicon nitride surface was very high, thus using APTES solution with high concentration may be not necessary.

There was a maximum at the concentration 1%. The fact that RFU at 2% APTES was lower than that at 1% APTES was quite surprising. Perhaps too much APTES was not good for the self-assembled monolayer (SAM) formation; the APTES could have formed a multilayer and then detached in PBS in subsequent steps of modification.

3.4. Test with horseradish peroxidase enzyme

The test was conducted on silicon nitride wafer (5 × 5 mm²) and also on silicon nitride cantilever. The absorption spectra of the solutions are shown in figure 5. The color of the H₂O₂: o-dianisidine turned gradually from clear to red during the reaction. The solution immersed with the control sample, not conjugated with horseradish peroxidase (HRP), showed no change in color. The absorption peaks at 540 nm indicate that HRP existed on the surface of the chips and catalyzed the reaction between H₂O₂ and o-dianisidine.

3.5. Detecting golgi protein 73 with modified cantilever

The procedure was used to modify silicon nitride cantilever. The cantilever chips included silicon nitride cantilever with thickness 1 μm, length 500 μm and width 100 μm. The top side of the cantilever was Au with 20 nm thickness. After modification with APTES and glutaraldehyde (GAD), the chips were immersed in a solution of anti-GP73 antibody in phosphate buffer saline overnight. After that, the chips were immersed in bovine serum albumin solution (2%) to passivate residual aldehyde group of GAD. Then the chips were immersed into GP73 solution at different concentration from 0 to 400 ng mL⁻¹. The deflection of each cantilever was scanned with the laser in the system Scala (Mecwins, Spain). This system utilizes a diode laser, a micropositioning system to move the laser and a position-sensitive detector (PSD). The diode laser shines upon the gold-coated cantilever and the reflected beam is collected by the PSD. The bending of the
cantilever, upwards or downwards, induces a movement of the reflected laser spot on the PSD. A computer is connected with the PSD and calculates the bending of the cantilever tip from the signal sent back from the PSD.

From the graph, one can see that the cantilevers bent up after immersion in GP73 solution. This could be due to the tensile stress created by the interaction between the GP73 protein molecules captured on the lower surface (the silicon nitride surface of the cantilever). The deflection increased with higher concentration of GP73, which can be explained by the higher surface density of GP73 captured on the cantilevers. Therefore, we suggest that the silicon nitride cantilever after modified with APTES and GAD can be used to detect golgi protein 73, a promising biomarker in diagnosis of HCC.

4. Conclusion

In this paper we investigated the effect of concentration and time in modifying the silicon nitride surface with APTES and GAD on the modification efficiency. The fluorescence images proved that the modification was most successful with moderate concentration of APTES (1%). On the other hand, the effect of time on the efficiency was vague. The success was also proved by enzyme HRP immobilized on the chips and the solution of o-dianisidine and H2O2. The chips were used to detect GP73 and proved to be capable of detecting GP73 with the concentration up to 400 ng mL⁻¹.

Acknowledgments

This study comes from the project KC.04.07/11-15 performed at the Laboratory for Nanotechnology, Vietnam National University in Ho Chi Minh City.

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