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A lable-free SPR biosensor based on one peptide sequence with three recognition sites for O-GlcNAc transferase detection

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ABSTRACT

Abnormal O-linked N-acetylglucosamine (O-GlcNAc) concentrations have been associated with many diseases, but the lack of accurate detection method limited O-GlcNAc to be used as a biomarker in clinical diagnosis. Then O-GlcNAc transferase (OGT) has drawn researchers' attention as it closed related to the level of O-GlcNAc and be considered to be a promising new target for diseases diagnosis. Nevertheless, the existing OGT detection methods are either need labeling or the sensitity can not meet the needs of clinic testing. Herein, a label-free and sensitive SPR biosensor was developed for accurate detection of OGT based on a multi-functional peptide. The designed peptide contains three recognition sites, one is the cleavage site of protease K, one is the O-GlcNAcylated site by OGT, and another is six histidine which be used as the signal report probe to recognize Ni^{2+}. The immobilized peptide would be cleavaged by proteinase K, then the His-tag residue part will leave the surface of Au film, resulting less His-tag could bind to Ni^{2+} and a small SPR signal would be record. If the peptide is O-GlcNAcylated by OGT, the cleaving reaction would be limited due to the adjacent site of O-GlcNAcylation. Then more His-tag can be left on the Au film and a bigger SPR signal could be record, this signal is associated with the concentration of OGT. Utilizing the change of the peptide configuration as a signal report probe for OGT detection not only avoids labeling of peptide, but also makes the method more sensitive. The determination linear range of OGT is from 2.00×10^{-13} to 5.00×10^{-8} M with a detection limit of 1.19×10^{-13} M, and the separation of two enzyme reactions ensured the high selectivity of the method. Finally, the sensing system was successfully used for OGT detection in blood samples with satisfied recovery. In summary, the label-free SPR platform for accurate detection of OGT in real samples is helpful to promote OGT serve as a biomarker for early clinical diagnosis of O-GlcNAc related diseases.

1. Introduction

O-linked-β-N-acetylglucosamine glycosylation (O-GlcNAcylation) is a universally protein post-translational modification in intracellular, which was discovered by Torres and Hart in 1984 [1,2]. O-GlcNAcylation has an essential role in sustaining vital biological processes, and aberrant O-GlcNAc modification be considered to be related to some serious diseases, like cancer, Alzheimer, cardiovascular disease, etc [3–6].

O-GlcNAcylated proteins are obtained via the hexosamine biosynthetic pathway (HBP), and UDP-GlcNAc is used as the sugar donor [7] during the modification process. In normal conditions, most of the glucose involved in glycolysis, aerobicoxidation and other processes, only 2–3% of glucose is shunted into the HBP. In the case of canceration, the proliferation of cancer cell would altere glucose metabolism, then normal glycolysis is insufficient to cater to the continuously increasing requirement of nutrients [8,9] for the proliferation. Therefore, the flux through the HBP would increased, generating higher amount of the sugar donor of UDP-GlcNAc [10], leading to an over-expression of O-GlcNAc transferase (OGT). While OGT can transfer the GlcNAc of the sugar donor (UDP-GlcNAc) to the serine and threonine residues of biomolecules, as a result, more biomolecules would be O-GlcNAcylated. In normal cells, these oncogenic factors can be degraded by ubiquitination and proteosomal degradation. While in malignant cells, the hyper-O-GlcNAcylation is helpful to the stability of oncogenic factors against the natural degradation pathway [11]. Hence, as a result of over-expression of OGT and UDP-GlcNAc, hyper-O-GlcNAcylation of biomoleculars are considered as an indicator of tumorigenicity and has

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been reported in many kinds of cancers [5,9,11,12]. Thus, the detection of O-GlcNAc become important for the diagnosis or research of cancer. Unfortunately, it is still difficult to realized the accurate detection of O-GlcNAc until now, due to some questions of low specifity, low sensitivity and can not used for the unkown O-GlcNAcylated protein detection or the detection result can not be verified, though many methods have been reported on the detection of O-GlcNAc [13–17]. Then, OGT detection has attracted the attention of researchers, because of its concentration is positively and closely correlated with the O-GlcNAcylation of biomoleculars. OGT is believed can be used as a new biomarker of cancer, which would contribute to promote early prevention and detection of O-GlcNAc related diseases.

In recent years, numerous efforts have been devoted to the detection of OGT based on the transfer reaction of GlcNAc by OGT to form O-GlcNAc [18–23]. In early research, the radiolabeled GlcNAc from the sugar donor of UDP-[3H]GlcNAc was transferred to the protein substrate by OGT [18], then the OGT could be detected specificly by recording the radio signal. However, this method is rarely used because of the production of radiochemical waste. Subsequently, Leavy et al. [19] have introduced ELISA method to study the transfer efficiency of O-GlcNAcylation reaction by OGT. Phosphine-FLAG labeled GlcNAz of UDP-GlcNAz was trasfered to peptide by OGT, HRP was introduced to the system by a HRP-labeled FLAG antibody interact with FLAG modified biomoleculars. Then the OGT transfer efficiency of O-GlcNAcylation could be evaluated by colorimetric signal produce by the reaction of HRP enzymatic. Though the method avoids using radioactive substance, the experiment procedure is too complicated and the relative lower sensitivity of ELISA method make it difficult for this method to be used in early disease diagnosis [24]. In order to establish more applicable and sensitive methods, fluorescence methods have been used for OGT sensitive detection based on the Fluorescence Resonance Energy Transfer (FRET) [21,22]. One fluorescence probe and one fluorescence quenching probe are labeled at two end of a peptide chain, respectively, and the peptide contains a glycosylation site which is adjacent to a protease site. Then the degree of glycosylation controlled by OGT would affect the protease reaction and then the distance of FRET pair, leading to a difference in fluorescence signal. So the fluorescence signal that depended on the distance of the FRET pair was indirectly related to the concentration of OGT. The FRET method [22] was very sensitive with a detect limit of 3.47×10^{-13} M, and was successfully used for OGT detection in HEK-293 cells. However, either ELISA or FRET method for OGT detection mentioned above both need to label the peptide. While labeling for biological molecular may affect the interaction with its targets [25,26], it also prolongs the detection time due to the complicat procedures [27]. Then an label-free electrochemical biosensor was developed for the OGT detection [23] based on the catalyst reaction of the part of the peptide to $Os(bpy)_3^{2+}$, then label free of the peptide makes this method simpler than other methods, but the LOD of this method was only 10 nM, about 4 orders higher than that of FRET biosensors, which may not enough be used for the real samples detection. In additional, it could also be concluded that the OGT detection methods used at present eigher need labeling or with unsatisfied sensitivity, so it is necessary to develop a label-free and sensitive method for the detection of OGT.

Surface plasmon resonance (SPR) is a label-free technology, it can sensitively receive refractive index changes on the surface of Au film, and be widely used in the analysis of biological samples [28]. Here, we present a label-free SPR biosensor for accurate detection of OGT utilizing one peptide chain with multiple recognition sites. When the peptide was O-GlcNAcylated by OGT-catalyzed, some His-tag residues would be left on the Au film and combined with Ni^{2+}. Otherwise, the His-tag residue could leave from the Au film and a smaller SPR signal could be recorded when Ni^{2+} was introduced. The resulting SPR signal of His-tag binding to Ni^{2+} was measured, which was associated with the concentration of OGT. The influence of different peptide immobilized methods was explored. With the best experiment conditions, the linear response and detection limit of the method for the detection of OGT were investigated. The selectivity of this method have also been evaluated. At last, the method was successfully used to detect OGT in blood samples. This label-free SPR biosensor shows potential for applying in the clinical diagnosis of O-GlcNAc related to diseases.

2. Experimental section

2.1. Materials and instruments

The peptide with the sequence of STPVSRANMKHHHHHH was synthesized by Sangon Biotech (Shanghai) Co.,Ltd. O-GlcNAc transferase (OGT) was purchased from R&D Systems Inc. Uridine 5′-diphospho-N-acetylglucosamine sodium salt (UDP-GlcNAc), alkaline phosphatase, albumin bovine (BSA), hemoglobin (Hb), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 11-mercaptoundecanoic acid (MUA), Tween-20, K_2HPO_4, KH_2PO_4 and (Hydroxymethyl)aminomethane (Tris) were obtained from Sigma Aldrich Co. Proteinase K was purchased from Aladdin Co.,Ltd. $NiCl_2.6H_2O$ and alloxan was obtained from Macklin Co.,Ltd. Bovine gland mucin (BSM) was obtained from Shanghai kayon Biological Technology Co.,Ltd. Nonidet P 40 (NP-40) was obtained from Beyotime Biotechnology Co.,Ltd. $CaCl_2$, $MgCl_2$ were purchased from Sinopharm Chemical Reagent Co.,Ltd. All reagents were analytical reagent grade. Millipore Milli-Q (18.2 MΩ cm) water was used in all experiments. The home-built FI-SPR instrument has been reported many times elsewhere [29–31], which was builted according the reported [32–34].

2.2. Preparation of samples

OGT, alkaline phosphatase, UDP-GlcNAc and the interferents protein (BSA、BSM、Hb、HRP) were prepared in 25 mM Tris-HCl (pH 7.4) containing 10 mM $CaCl_2$, 0.05% Tween-20 and 0.05% NP-40. OGT and UDP-GlcNAc were stored at −20 °C and others were stored at 4 °C. The peptide and proteinase K were dissolved in a PBS buffer (50 mM, pH 7.4) and were stored at −20 °C. $NiCl_2{\cdot}6H_2O$ was dissolved in deionized water and was stored at room temperature.

2.3. Preparation of the SPR sensor surface

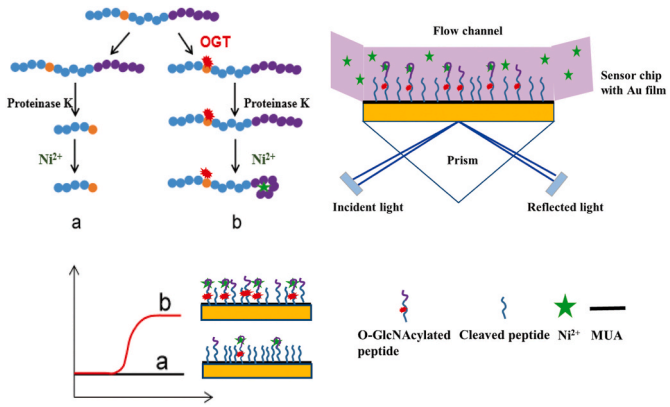
The BK7 glass slides (Fisher, 1.8 cm × 1.8 cm) were treated in piranha solution (30% H_2O_2 and 70% concentrated H_2SO_4) at 80 °C for 30 min (Note: This is a dangerous cleaning solution, care must be taken when handling the solution) and cooled to room temperature. After that, the glass slides were rinsed thoroughly with deionized water and dried by N_2. Then, Au films of a 50-nm thickness and a 2-nm Cr underlayer were deposited onto the dried glass slides using a sputtering coater (Model 108, Kert J. Lester Inc., Clairton, PA).

The Au film was rinsed with ultrapure water to reduce surface contamination. Next, 0.1 ml of 0.01 M MUA was dropped onto the Au film and incubated at 4 °C overnight. After that, the modified film was entirely rinsed with ethyl alcohol and ultrapure water to remove the weakly adsorbed MUA, and then rinsed entirely and dried with N_2, then the MUA modified Au film was prepared for the detection of OGT.

2.4. Two methods of peptide immobilization for OGT detection

There have two methods for the immobilization of peptide, the pre-immobilized method and the post-immobilized method. In the pre-immobilized method, the peptide was immobilized on the Au film firstly, and then O-GlcNAcylated and cleavage reaction was carried out, respectively. For the post-immobilized method, the two enzyme reaction was finished both in solusion, then the peptide was immobilized. Follows are the details of these two methods.

The pre-immobilized method: First, 80 μL mixture solution containing 0.3 mg/ml peptide, 0.4 M EDC solution and 0.1 M NHS solution



Scheme 1. The principle of label-free SPR biosensor for the detection of OGT.

was dropped onto the MUA-modified Au film and kept for 1 h at room temperature. After washing the Au film, the reaction mixture solution containing 20 μL 5U alkaline phosphatase, 20 μL 0.5 mM UDP-GlcNAc and 20 μL various concentration of OGT in Tris-Hcl buffer (25 mM, pH = 7.4, 10 mM $CaCl_2$, 0.05% Tween-20, 0.05% NP-40) was dropped onto the peptide modified Au film and incubated in a humidified container at 37 °C for 1 h to make the O-GlcNAcylated reaction completely. After thorough rinsing, the enzyme cleavage reaction was carried by adding 60 μL 0.3 mg/ml proteinase K onto the surface of the Au film in a humidified container at 37 °C for 2 h. After that step, the Au film was rinsed entirely and installed in the flow channel. At last, 80 mM Ni^{2+} solutions were introduced into the carrier solution by a 50-ml sample loop on a six-port valve and delivered to the flow cell at a constant speed of 1.0 ml/h by a syringe pump (KDS100, KD Scientific Inc., Holliston, MA, USA) and the SPR signal was recorded.

The post-immobilized method: First, the reaction mixture solution containing 20 μL 0.5 mM UDP-GlcNAc, 5 U alkaline phosphatase and 20 μL various concentration of OGT in Tris-Hcl buffer (25 mM, pH = 7.4, 10 mM $CaCl_2$, 0.05% Tween-20, 0.05% NP-40) was added into 60 μL 0.3 mg/ml peptide solution and incubated at 37 °C for 1 h. After the O-GlcNAcylated reaction, 60 μL 0.3 mg/ml proteinase K was added into the mixture solution in a humidified container at 37 °C for 2 h for the cleavage reaction. After that step, 40 μL 0.4 M EDC solution and 0.1 M NHS solution and 40 μL reacted peptide mixture solution was dropped onto the MUA-modified Au film and allowed to react for 1 h. The modified Au film was thoroughly rinsed and installed in the flow channel. Finally, 80 mM Ni^{2+} solutions were introduced into the Au film.

2.5. Preparation of blood samples

The blood samples (four cancer blood samples (C1–C4) and two healthy blood samples (S1 and S2) were treated as follows. First, 1 ml blood samples were centrifuged at 3600 rpm for 10 min, the serum was discarded and the remaining haematocytes was placed at −20 °C overnight to break the cells. Then 50 mM PBS (pH 7.4) was added into haematocytes to 1 ml and the haematocytes was sonicated for 30 min at 0 °C to further break the cells. At last, 1 ml haematocytes lysates were centrifuged at 3600 rpm for 10 min and the supernatant was stored at 4 °C for OGT activity assay.

3. Results and discussion

3.1. The principle of OGT detection

The principle of this SPR biosensor for OGT detection based on one peptide chain with three different recognize sites is illustrated in Scheme 1 The peptide was immobilized on MUA modified Au films via a cross linking reaction between the amino group at end of peptide and carboxylic acid groups of MUA. The peptide including an O-GlcNAcylation reaction site, a unique proteinase cleavage site adjacent to the glycosylation site and a section of affinty peptide of six histidine (His-tag) at the other end of peptide that could bind to Ni^{2+}. Especially, the strong affinity interaction between His-tag and Ni^{2+} would result in a big conformational change of peptide [35] with a great change in refractive index on the Au film, then a large SPR dip shift change could be recorded. The peptide could be easily cleavaged into two pieces by proteinase K, and the His-tag fragments would leave from the Au film. Therefore, none or less His-tag would be left on the Au film to combine with Ni^{2+}, then no or a quite samll SPR signal (Scheme 1 curve a) be recorded. However, if the GlcNAc of UDP-GlcNAc was transferred to the serine residue of the peptide by OGT, the peptide would be O-GlcNAcylated. The O-GlcNAcylated modified site is close to the recognition site of proteinase K, so that O-GlcNAcylation of the peptide could block the peptide chain from being cleavaged by proteinase K [21]. Thus more His-tag would be left and a larger change in SPR signal (Scheme 1 curve b) could be observed, when Ni^{2+} was introduced into the flow cell. In short, OGT could be detected indirectly through the SPR signal, which was related to the degree of peptide glycosylation. The higher concentration of OGT, the more O-GlcNAcylated peptide could be produced and the less peptide could be cut by proteinase K, the bigger SPR signal could be recorded. Thus, the detection of OGT could be realized by just one peptide chain with different recognition site, especially, the recognization reaction between His-tag part of the peptide and Ni^{2+} be used as a signal report probe, these designs avoid the complex procedure of labeling and greatly simplify the experimental process.

Overall, the concentration of OGT was detected by the following steps: (1) OGT transferred the GlcNAc of UDP-GlcNAc to forms the O-GlcNAcylated peptide; (2) proteinase K could cleave the peptide but not the O-GlcNAcylated peptide; (3) His-tag residues of peptide reacted with Ni^{2+} of peptide chain to produce a SPR signal.

3.2. Quantification of OGT

Experimental conditions for OGT detection were screened firstly, at last, 80 mM Ni^{2+} concentration, 0.3 mg/ml proteinase K concentration, 37 °C proteinase K reaction temperature and 60 min O-GlcNAcylated reaction time were selected (details show in Fig.S1) and used for the experiment. Then the effect of different OGT concentrations on O-GlcNAcylated reaction of the peptide were also investigated with the pre-immobilized method (Fig. 1A). It shows that almost no SPR signal was observed when Ni^{2+} was introduced to the proteinase K treated peptide modified Au film (curve a), which suggests that the complete cleavage of unglycosylated peptide by proteinase K. The result also proves that the peptide was immobilized to the Au film by the crosslinking reaction between the amino group at the end of peptide and carboxylic acid groups of MUA, the amino group of lysine could not combine with the carboxylic groups of MUA on the Au film, which maybe due to the amino group of lysine is located in the middle of peptide, the steric hindrance is not benefit to the crosslinking reaction. An obvious SPR signal was observed after the peptide modified Au film was interact with 2.00×10^{-13} M OGT, indicating that some of His-tag fragments still remained on the Au film because O-GlcNAcylation of peptide could affect the reaction of proteinase K. The left His-tag can bind with Ni^{2+} and form a stable three dimentional structure from a linear molecular, thus making an obviously configuration change of peptide on the Au film. The change is close to the Au surface and can result a big refractive index change on the surface of Au film, thus an obvious SPR signal could be recorded even with lower OGT concerntration. It is also show in Fig. 1B that the SPR signal increased gradually when the concentration of OGT increased from 2.00×10^{-13} to 5.00×10^{-8} M, the more OGT was added, the more O-GlcNAcylated peptides was formed and less His-tag fragments would be left, inducing a bigger SPR signal. The linear relationship between the SPR signal and

the logarithmic value of OGT concentration is $\Delta\theta = 0.0244\log c + 0.3228$ ($R^2 = 0.987$) with a limit of detection (LOD) of 1.19×10^{-13} M calculated by 3σ, where σ is the standard deviation of blank.

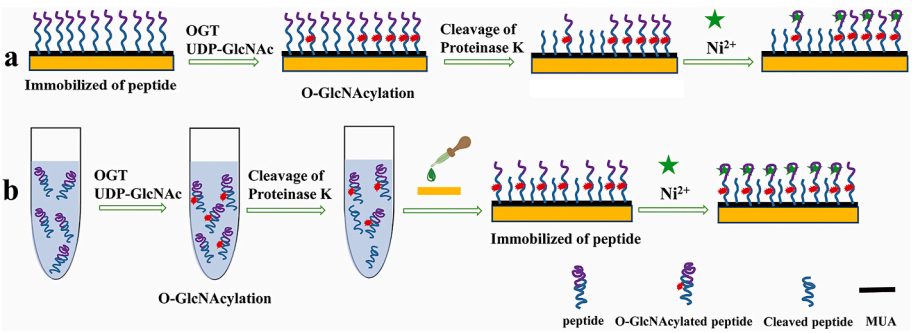
Comparing with other methods for OGT detection, the determination range of the proposed method is approximately two orders of magnitude wider than that of FRET method [22] (4.00×10^{-13} to 2.50×10^{-10} M). And a wider linear range is beneficial to the OGT detection, because the content of OGT is fluctuating greatly in real samples [36]. In addition, the detection limit of this method is far lower than that of electrochemical method (10 nM) [23] and approximately 3 times lower than that of the fluorescence method [22]. This highly sensitivity of the method may be related to the reaction mode of peptide on the Au film: The SPR signal originates from the affinity reaction between His-tag and Ni^{2+}. When interact with Ni^{2+}, the peptide would change its configuration from sigle chain to fold state and get closed to the surface of the Au film. This big configuration changes of peptide are very closed to the surface of the Au film, thus would result a big refractive index change [37]. As a result, a small amount of Ni^{2+} binding to peptide can also lead to the obviously SPR signal changed. Besides that, such a low detection level may be also due to the high sensitivity of the home-built SPR instrument which use a bi-cell photodiode detector and make the enstrument have much-improved angular resolution [29,31,33]. The developed biosensing system combine three recognition site into one peptide chain, utilizing the configuration changes of peptide as signal probe, the design of the system not only avoides labeling of the peptide, simplifies the process of the detecton, but also improves the sensitivity of OGT detection.

3.3. Selectivity of method for OGT detection

To evaluate the selectivity of this sensor, the response of the sensor towards other proteins have also been studied, in these experiments, OGT was replaced by BSA, BSM, Hb and HRP, resptectively. As shown in Fig. 2 (the blue striped bar), OGT caused a significant SPR signal, because the O-GlcNAcylated peptide affect the cleavage of proteinase K and many His-tag fragments left on the Au film to bind to Ni^{2+}. However, other proteins did not produce a significant increase in SPR signal because these proteins did not have the function like OGT to form O-GlcNAc modified proteins, which means that the sensor responds specifically to OGT. In addition, the SPR signal was significantly decreased when a well-known inhibitors of OGT, alloxan [38], was added into the OGT-catalyzed glycosylation reaction solution (Fig. 2 the blue striped bar), which means that alloxan hampered the glycosylation reaction of OGT-catalyzed and more unglycosylated peptides could be cleavaged by proteinase K, leading to the decrease of the SPR signal. This result further illustrates that OGT can transfer the O-GlcNAc to peptide and then produces the SPR signal. The specificity of OGT also makes the method highly selective, thus other biomoleculars would not interfere the OGT detection when applying in real samples, which means that this method is also suitably used for the OGT detection in complex samples.

There are two reasons why the proposed method shows a good selectivity for OGT detection. On one hand, the good specificity of OGT to transfer GlcNAc to form O-GlcNAc. On the other hand, the process of the experiment involved two enzymatic reactions and these two enzymatic reactions were separated, named pre-immobilized method (shown as Scheme 2a). This method can largely avoid the interference that come from some biomolecular added in the step of glycosylation reaction of OGT-catalyzed to the later enzymatic reaction of proteinase K. The advantage of this method would be more obviously when detect the OGT in complex samples, because more biomoleculars would be added onto the peptide modified Au film except OGT. To confirm this hypothesis, a control experiment was designed (shown as Scheme 2b). In order to better distinguish it from pre-immobilized method, this control experiment was called post-immobilized method, the details of these two methods shown as section of 3.3. In post-immobilized method, the glycosylation reaction of OGT-catalyzed was carried out in the peptide solution, and then proteinase K was added in the same peptide solution for cleavage reaction. Finally, the reaction solution was added onto the Au film for the immobilization of peptide. Compared the result of two immobilized methods, it shows clearly that the SPR signal of the post-immobilized method (Fig. S2 the red bar) was slightly higher than that of the pre-immobilized method (Fig. S2 the black bar). The higher SPR signal of the post-immobilized method may come from the following reasons: On the one hand, the glycosylation reaction of OGT-catalyzed was carried out in solution, which benifts the glycosylation reaction due to the small steric hindrance. As a result, more O-GlcNAcylated peptide could be formed and then more His-tag residues should be immobilized on the Au film to combine with Ni^{2+}, inducing a larger SPR signal. On the other hand, the peptide can be cut in two pieces (STPVSR- and -ANMKHHHHHH). Considering the amino group of lysine, the two cleaved peptide pieces both have the chance to be immobilized on the Au film. Thus, some of amino group of lysine may be immobilized on the Au film, making the result of post-immobilized method has some interference. As a result, peptide fragments containing His-tag on the Au film can bind to Ni^{2+}, inducing a larger SPR signal. However, the SPR signal of the two cleaved peptide modified Au film (curve b of Fig. S3) is much smaller than that of 1×10^{-7} M OGT treated peptide (curve a of Fig. S3), which shows that -ANMKHHHHHH peptide pieces is not easy to be immobilized on the Au film. The third reason, the cleaved peptide without His-tag and O-GlcNAcylated peptide were both immobilized on the Au film after the enzyme reaction so that the His-tag fragments dispersed well, which is also beneficial to the combination with Ni^{2+} due to the smaller steric hindrance. Thus, the peptide immobilized on the Au film can bind to more Ni^{2+}, which can calso inducing a larger SPR signal. Of course, the signal of the post-immobilization method is only about 15% larger than the pre-immobilization method, which shows that each factor has little effect. These results further prove that the method of pre-immobilization method is more suitable for the detection of OGT, which can effectively avoid the influence of the amino group on the side chain and makes the result more accurate.

It is clearly that the signals of BSA and BSM did not show big



Scheme 2. The schematic of different immobilized ways of peptide on the Au film: (a) the pre-immobilized method; (b) the post-immobilized method.

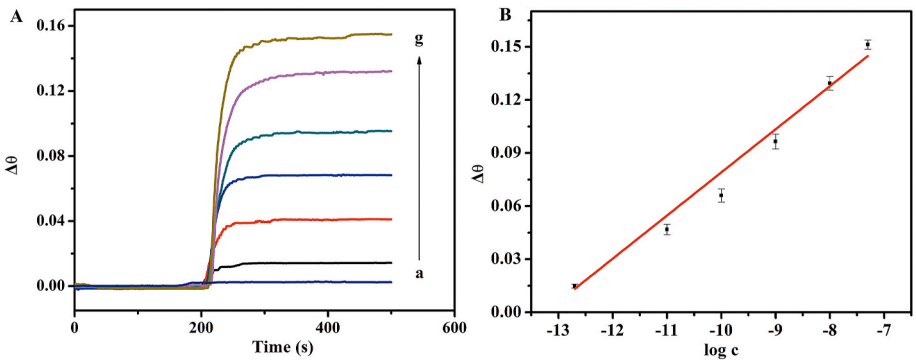


Fig. 1. (A)SPR responses of peptide-based biosensor for different concentrations (a:0, b:2.00 × 10^{-13}, c:1.00 × 10^{-11}, d:1.00 × 10^{-10}, e:1.00 × 10^{-9}, f:1.00 × 10^{-8}, g:5.00 × 10^{-8} M) OGT treated on the Au film; (B) the linear responses of the logarithm value of OGT concentration (2.00 × 10^{-13} to 5.00 × 10^{-8} M). The standard deviations were computed from at least three replicate measurements and shown as the error bars.

differences in these two methods, which means that the two proteins of BSA and BSM have little effect on the result. However, as shown in Fig. 2, the SPR signal of Hb and HRP obtained from the post-immobilized method is much higher than that from the pre-immobilized method. As we know that OGT is the only enzyme that have the function to form O-GlcNAc modified proteins and Hb or HRP do not have the function like OGT [39,40], this result was also conformed in the selectivity experiment with pre-immobilized method (blue stripe bar of Fig. 2). Then it is possible that Hb or HRP could affect the cleavage reaction of proteinase K. Because in post-immobilized method, proteinase K were added in the mixture solution after glycosylation reaction, and the two enzymatic reactions have not be separated. In fact, Hb or HRP really is a substrate of proteinase K as reported [40–42], then Hb or HRP could compete with peptide to react wtih proteinase K and then more peptide could not cut into two pieces. As a result, more His-tag fragments remained on the Au film to combine with Ni^{2+}, resulting in a larger SPR signal. Nevertheless, in pre-immobilized method, peptide immobilized on the Au film firstly, and then the reaction of OGT-catalyzed glycosylation and proteinase K cleavage be carried out step by step, the separation of two enzymatic reactions avoids the interaction of Hb or HRP to proteinase K. As is known, there are various biomolecules in real samples, including Hb and HRP, then the pre-immobilized method can effectively avoid interference from these biomoleculars to the cleavage reaction of proteinase K. Also, the method of pre-immobilization method can effectively avoid the influence of the amino group on the side chain, which makes the result more accurate. That is to say the results that obtained from the pre-immobilized method would be more accurate when some unknown biomoleculars present, especially used for the OGT detection in complex samples.

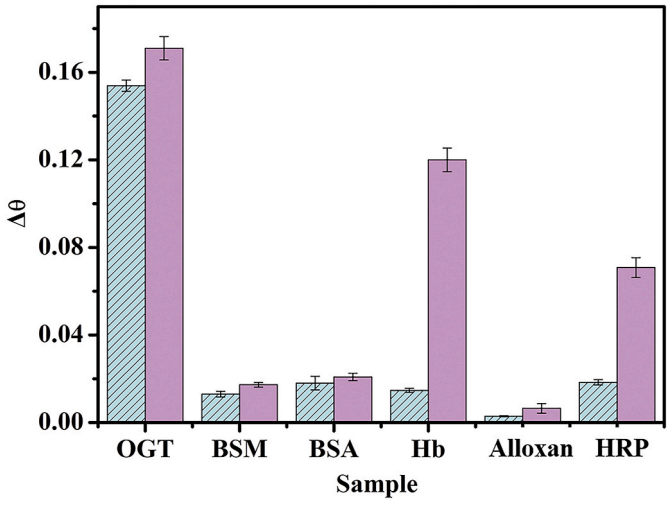


Fig. 2. SPR responses to the introduction of Ni^{2+} on different proteins interacted peptide modified Au film (OGT, alloxan, BSM, BSA, Hb and HRP) with two peptide immobilized ways: the pre-immobilized method (the blue striped bar) and the post-immobilized method (the pink bar). The standard deviations were computed from at least three replicate measurements and shown as the error bars. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Detection of OGT in blood samples

The developed method was used for OGT detection in clinical samples including six blood samples (four cancer blood samples and two healthy blood samples). It could be found that the SPR signals are different for the six samples, even these four cancer blood samples show quite different in SPR signal (Fig. 3), suggesting these blood samples contain different amount of OGT. The difference may be attributed to the different content of OGT in different types of cancer [11,36]. It is clearly seen that the SPR signal of the four cancer blood samples was larger than that of the two healthy blood samples (Fig. 3), which demonstrated that the amount of OGT in cancer blood was higher than that of in healthy blood samples. What's more, the previous study have reported that the amount of O-GlcNAc is also higher in the cancer blood samples than that of in the healthy blood samples [17], which further demonstrate that high-expression of OGT is associated with high O-GlcNAcylation. These results also indicate that the detection of OGT

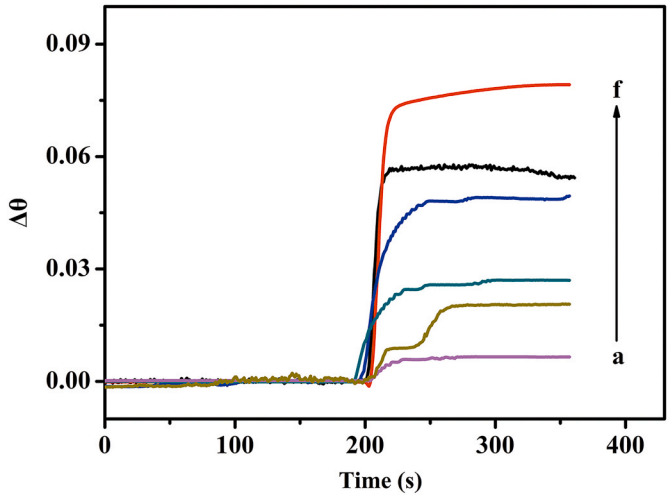


Fig. 3. SPR responses of different human blood samples (a:S1, b:S2, c:C1, d:C2, e:C3, f:C4) interacted peptide modified Au film on injecting 80 mM Ni^{2+} with the pre-immobilized method.

Table 1
The content of OGT in different clinical blood samples.

Samples	Cancer blood samples				Healthy blood samples	
	C1	C2	C3	C4	S1	S2
Δθ	0.0259 ± 0.0024	0.0490 ± 0.0032	0.0649 ± 0.0026	0.0711 ± 0.0046	0.0087 ± 0.0008	0.0203 ± 0.0011
Concentration (10^{-11})	0.07	0.60	2.69	4.84	–	0.04

Table 2
The results of two blood samples with standard addition method.

Samples	Concentration (10^{-11})	Added (10^{-11})	Found (10^{-11})	Recovery (%)
C3	2.69	5.00	7.16	90.6
S2	0.04	0.20	0.25	105.0

can promote early prevention and diagnosis of O-GlcNAc related diseases. The amount of OGT calculated from the fitted line of the four cancer blood samples were at the range from 0.07×10^{-11} to 4.84×10^{-11} M (Table. 1), which was higher than that of in the healthy blood samples. It is also can find that the amount of OGT in these blood samples varied greatly (0.04×10^{-11} to 4.84×10^{-11} M), and as point out above that our method has a wider linear range from 2.00×10^{-13} to 5.00×10^{-8} M, the concentration range of the samples is just located in the middle of the linear range, which indicate that the determination range and sensitivity of this method are both satisfy the demand of clinic sample detection. Conversely, the LOD of electrochemical method is only 10 nM, which is not sensitivity enough to detect OGT in real samples. For the FRET method can also be applied in real samples, with the LOD (3.47×10^{-13}) is just meet the requirement. But the peptide used in the FRET methods need to be labeled, which increase the complexity of the experiment. In addition, all the reactions of enzyme reaction were carried in the same solution, the effect of some biomoculers on proteinase K can not be avoided, which will result in the false signals in the experiment.

Standard addition method was used evaluate the accuracy of the method, in which two blood samples (C3 and S2) were chosen. The two samples spiked 5.00×10^{-11}M and 0.20×10^{-11}M OGT, respectively. The recovery of the two samples are 90.6% and 105.0% (Table. 2), both of them can meet the demand no matter high or low concentration of OGT added in corresponding samples, which suggesting that this method can detect accurately in a wide range. As reported above, O-GlcNAc glycosylation proteins arecomplicated since O-GlcNAc can modify to various and even unknown proteins, so it is difficult to verify the result of O-GlcNAc determination through the standard addition method. However, OGT is an enzyme that only responsible for the addition of GlcNAc and can be commercial obtained directly, so the result of OGT determination can be verified by standard addition method. That is to say OGT is suitable as a biomarker for the diagnosis of O-GlcNAc-related diseases.

4. Conclusion

In summary, a label-free SPR biosensor for sensitive detection of OGT has been developed based on one multi-functional peptide. The His-tag used as signal report probe not only avoids labeling of peptide, but also makes the method more sensitive due to the big configuration change of peptide when bind with Ni^{2+}. Moreover, the separation of two enzyme reactions can avoids the interference from other biomolecules in OGT-containing samples, thereby ensure the excellence selectivity of this method. The proposed method was successfully applied in detection of OGT in clinical samples and the recovery of the standard addition method is satisfied, which show that the new development method can meet the requirment of clinical sample detection, and has the potential to be used for early clinical diagnosis of O-GlcNAc related diseases.

Credit author statement

Nan Yin: Conceptualization, Methodology, Investigation, Writing - Original Draft, Data curation, Validation, Visualization. **Ruihuan Zhao**: Data analysis, Methodology, Writing - review & editing. **Jie Zhang**: Methodology,Writing - review & editing. **Dingding Yang**: Writing - review & editing. **Zhimin Guo**: Writing - review & editing. **Rui Liu**: Writing - review & editing. **Xin Yao**: Writing - review & editing, Funding acquisition, Resources, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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