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Surface plasmon resonance biosensor for the accurate and sensitive quantification of O-GlcNAc based on cleavage by β-D-N-acetylglucosaminidase

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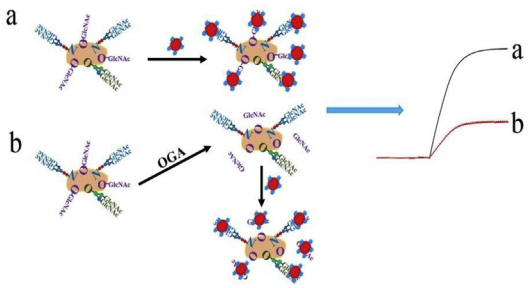
HIGHLIGHTS

- A novel SPRbiosensor for the sensitive and accuracy detection of O-GlcNAc was developed based on OGA and AuNPs.
- The method avoids the effect of other GlcNAc , sialic acid and nonspecific adsorption of AuNPs.
- O-GlcNAc concentration in cancer cells lysate and blood samples were successfully detected by this method.

GRAPHICAL ABSTRACT



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ABSTRACT

Abnormal O-linked-N-acetylglucosamine (O-GlcNAc) concentrations have been associated with a variety of diseases (e.g., cancer, Alzheimer's disease, cardiovascular disease, etc.). However, O-GlcNAc detection is complicated, time-consuming and has poor specificity, therefore, the accurate detection of O-GlcNAc is difficult. In this study, an accurate and sensitive surface plasmon resonance (SPR) biosensor for O-GlcNAc detection that is based on β-D-N-acetylglucosaminidase (OGA) and Au nanoparticles (AuNPs) was developed. In this strategy, AuNPs were used to amplify the SPR signal and improve the biosensor's sensitivity; OGA was used to cleave O-GlcNAc from O-GlcNAcylated biomolecules. The interaction between AuNPs labeled wheat germ agglutinin (AuNPs/WGA) and O-GlcNAcylated biomolecules on a modified Au film treated with and without OGA was recorded by SPR. The change of the SPR signal moves linearly with the amount of O-GlcNAc on the Au film and thus could be used for the detection of O-GlcNAc. By recording the difference of the SPR signals, this method can avoid disturbances from other sugars and nonspecific adsorption of AuNPs and thus enable the accurate detection of O-GlcNAc. The accurate detection range of O-GlcNAc was 4.65×10^{-12} to 4.65×10^{-7} M which was obtained by quantifying the amount of a standard O-GlcNAcylated peptide (O-GlcNAc-CREB), and the detection limit is 4.65×10^{-13} M. More importantly, the strategy was successfully used to detect O-GlcNAc in a real α-crystallin protein, cancer cell lysates and blood samples with satisfactory results. The study's results

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imply that this accurate and sensitive method has the potential to be applied in the early clinical diagnosis of O-GlcNAc-related diseases.

1. Introduction

O-GlcNAc is active in a type of protein post-translational modification that involves the reversible transfer of a single N-acetylglucosamine (GlcNAc) moiety to a serine or threonine residue of cytoplasmic and nuclear proteins [1,2]. Abnormal O-GlcNAc levels have been associated with various diseases, including Alzheimer's disease [3,4], diabes [5,6], breast cancer [7–9], and cardiovascular diseases [10], and so on [11–13]. The quantification of O-GlcNAc has a significant impact on biological fields [14] and facilitates the clinical diagnosis of these diseases [7,15]. However, the detection and study of O-GlcNAc has been hampered by the following issues: low cellular abundance of O-GlcNAc modification, low stoichiometry and lability of the O-glycosidic bond [14,16].

Several methods have been researched for O-GlcNAc detection. With the development of click chemistry, protein-specific labelling methods have attracted increasing attention for O-GlcNAc detection and site identification. Chu developed a selective probe metabolic chemical reporter (6-AzGlcNAc) that specifically labels O-GlcNAc in O-GlcNAcylated proteins in NIH3T3 cells [13]. This method enables the specific analysis of O-GlcNAc, the labelling efficiency is limited, and labelling methods are time-consuming, complicated and limited to highly glycosylated proteins [17–19]. Mass spectrometry (MS) has also emerged as a powerful analytical tool for O-GlcNAc detection and site mapping based on lectin or antibody enrichment [20–23]. Robert reported the detection of O-GlcNAc in the murine postsynaptic density using electron transfer dissociation mass spectrometry (ETD-MS) [21], which can better resolve the breakage of the O-glycosidic bond compared to collision-induced dissociation mass spectrometry (CID-MS) [20] with WGA enrichment. Maury investigated a novel targeted method, known as the multiple reaction monitoring mass spectrometry (MRM-MS), for the detection and quantification of native O-GlcNAc-modified peptides in a complex mixture without enrichment and labelling [14]. This method also avoids breaking of the O-glycosidic bond. MRM-MS exploits the unique capabilities of triple quadrupole (QQQ) MS that simplify the experimental steps and the process of data analysis by recognizing the target peptide, which facilitates the accurate detection of O-GlcNAc. However, this method requires knowledge of the peptide sequences, and the absence of peptide sequence information or the presence of unknown samples make it difficult to quantify the O-GlcNAc using MRM-MS impossible [24]. Recently, Liu developed a sensitive colorimetric strategy for O-GlcNAc quantification via copper deposition-enabled nonenzymatic signal amplification [25] that is based on the recognition between WGA and O-GlcNAc. In this method, PNGaseF was used to cleave N-linked GlcNAc in advance, thereby avoiding interference from the N-linked GlcNAc. However, WGA also recognizes sialic acid and other terminal GlcNAc compounds in addition to O-GlcNAc and N-linked GlcNAc, other terminal GlcNAc units and sialic acid still affect the accurate detection of O-GlcNAc [26–28]. There is also synthesized lectin which could recognize O-GlcNAc, but the recognition selectivity has not been thoroughly elucidated to date [29]. Therefore, the accurate detection of O-GlcNAc is still lacking, especially for unknown and complicated samples. Thus, the development of accurate, higher-sensitivity and simpler methods for O-GlcNAc analysis is still necessary.

SPR has been widely used in biomolecule analysis, especially for the detection of DNA, proteins, cells, enzymes, hormones and drugs [30–34]. To increase its detection sensitivity, various amplification strategies such as enzyme, polymerride and gold nanoparticles amplification methods have been developed [35–37]. Among these strategies, AuNPs are useful labels for amplifying weak signals and thus have been widely used [38,39].

In this work, a sensitive SPR biosensor for the accurate quantification of O-GlcNAc was designed based on AuNPs and OGA. The interaction between AuNPs/WGA and O-GlcNAcylated biomolecules on both OGA-treated and untreated modified Au films were recorded using SPR, and the difference in the SPR signal is linearly related to the amount of O-GlcNAc on the Au film, which can be used to detect O-GlcNAc. The linear response and detection limit of the method were investigated. The method was also successfully used to detect O-GlcNAc in α-crystallin protein, cancer cell lysates and blood samples of cancer patients. Accordingly, this simple and sensitive biosensor showed potential for applications in the clinical diagnosis of O-GlcNAc-related diseases.

2. Experimental section

2.1. Reagents and instruments

Standard O-GlcNAc modified peptide TAPT(S-O-GlcNAc)TIAPG (O-GlcNAc-CREB),TAPTN(N-N-GlcNAc)TIAPG(N-GlcNAc-CREB),TAPT(N-N-sialic acid)TIAPG (sialic acid-CREB) and TAPTSTIAPG peptide (CREB) were purchased from Bankpeptide Biological Technology Co.,Ltd. (Hefei, China). WGA was purchased from Gene Tex (USA). OGA was purchased from R&D Systems (USA). Propylamine was purchased from J&K Scientific Ltd. (Beijing, China). HEPES, α-crystallin protein, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 11-mercaptoundecanoic acid (MUA), Tween P20, and sodium hydroxide were obtained from Sigma Aldrich Co (USA). $AuCl_3 \cdot HCl \cdot 4H_2O$, $MnCl_2$, $CaCl_2$, $MgCl_2$, NaCl and H_2SO_4 were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All reagents were of analytical reagent grade and were used without further purification. Millipore Milli-Q (18.2 MΩ cm) water was used in all experiments. Unless specifically indicated, all reactions were performed at room temperature. SPR experiments were recorded on a BioNavi-200 biosensor system. UV-Vis absorption spectra were characterized by a UV-2550 spectrometer.

2.2. Preparation of samples

WGA (1 mg/mL) and α-crystallin protein stock solutions were prepared in 10 mM phosphate buffer (pH 7.6) containing 150 mM NaCl and 0.1 mM $CaCl_2$ and stored at 4 °C; 1 mg/mL freeze-dried O-GlcNAc-CREB was dissolved in a HEPES buffer (10 mM, pH 7.4) containing 1 mM $MnCl_2$, $CaCl_2$, $MgCl_2$, and 0.005% Tween P20, and the solution was stored at −20 °C. An OGA stock solution with a concentration of 0.366 mg/mL was prepared in a Tris buffer (pH = 8.0) containing 100 mM NaCl.

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2.3. Cell and blood sample preparation

Four cell lines, including a normal cell line (HEK293), breast cancer cell lines (MDA-MB-231, MDA-MB-468) and a colon cancer cell line (SW480), were used for the determination of O-GlcNAc. Cell lysates were prepared with a concentration of 2.74×10^7 cells/mL. HEK293 cells were cultured in DMEM containing 10% FBS at 37°C in 5% CO_2; MDA-MB-231, MDA-MB-468 and SW480 cells were cultured in L15 supplement with 10% FBS at 37 °C in pure air for three days. The cell density was measured with a Countess II FL Automated Cell Counter (Thermo Fisher Scientific). Next, approximately 25 min are required to prepare the cells lysates as follows: the cells were lysed with lysis buffer (0.5% Triton X-100, 1 mM EDTA, 1 mM PMSF in PBS and protease inhibitor cocktail (Roche)) for 15 min on ice. The supernatant was collected after centrifugation at 12,000 g for 10 min at 4 °C. The cell lysates were stored at −20 °C until use.

The treatment of blood samples, including three tumour patient samples and two healthy human samples requires approximately 12 h and was undertaken as follows: 1 mL samples were centrifuged for 10 min at 3000 rpm, after which the serum was discarded and approximately 0.5 mL of haematocytes was obtained and placed at −20°Covernight to break the cells. Next, 0.5 mL of PBS (pH 7.4) was added to the haematocytes, which were further broken by sonication for 30 min at 4 °C. Then the haematocytes lysates were obtained and stored at 4 °C until use.

2.4. Preparation of AuNPs and AuNPs/WGA

AuNPs (13-nm diameter) were synthesized according to the procedures described by Storhoff [40]. Before conjugation, 1 mL of a colloidal AuNP solution was adjusted to pH 7.4 with 0.1 M K_2CO_3. Next, 100 μL of WGA (1 mg/mL) were added and the mixture placed on a vortex mixer at room temperature for approximately 1 h to ensure completion of the reaction. The solution was then centrifuged for 15 min at 13,000 rpm, after which the supernatant was discarded, the red deposit was washed twice with the HEPES buffer (10 mM, pH 7.4), and the AuNPs/WGA conjugate was re-suspended in the HEPES buffer and stored at 4 °C.

2.5. Preparation of the SPR sensor surface

Modification of the SPR sensor was carried out as follows: Au film was rinsed with sulphuric acid to reduce surface contamination and thoroughly rinsed with deionized water. After that step, 20 μL of a 1 μM MUA solution was dropped onto the Au film and incubated overnight at 4 °C. Next, the modified film was thoroughly rinsed with ethyl alcohol and deionized water to remove the weakly adsorbed MUA. After that step, a mixture of aqueous solutions consisting of 40 μL O-GlcNAcylated biomolecule solution of appropriate concentration, 20 μL 0.4 M EDC solution and 20 μL 0.1 M NHS solution was dropped onto the MUA-modified Au film and allowed to react for 1 h. The modified Au film was thoroughly rinsed with deionized water. Finally, the unoccupied activated carboxylic acid sites were blocked by dropping 60 μL of 1 M propylamine onto the film and allowing it to react for 30 min, after which the modified film was thoroughly rinsed with deionized water and dried with N_2. Next the SPR sensor was used for the WGA and O-GlcNAcylated biomolecules reaction.

The O-GlcNAcylated biomolecule-incubated Au films were treated with 1.83 μg/mL of OGA at 37 °C for 16 h, prepared for the WGA and O-GlcNAcylated biomolecules reaction after being treated with OGA. Next, the modified Au films were thoroughly rinsed with a pH 8.0 Tris buffer and deionized water. The Au films were then dried with N_2 in preparation for the SPR measurements. Finally, 5 μg/mL of AuNPs/WGA solution in a HEPES buffer (10 mM, pH 7.4) containing 1 mM $MnCl_2$, $CaCl_2$and $MgCl_2$ was introduced on the O-GlcNAcylated samples modified Au film.

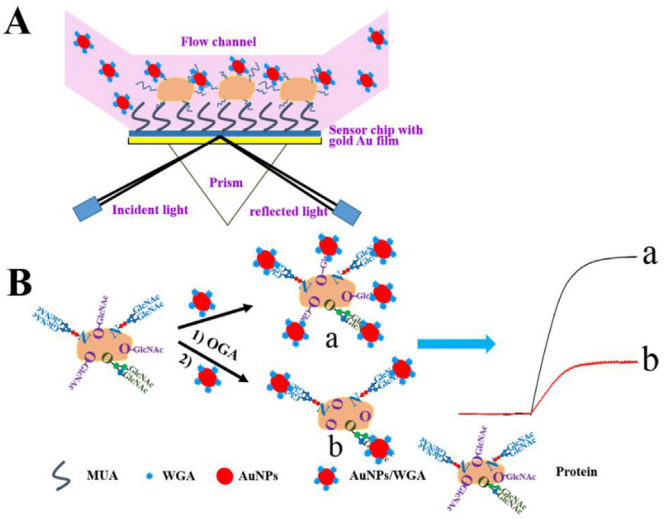
As described above, approximately 20 h are required to prepare the SPR sensor surface, and 2 h are necessary to finish a SPR signal record, therefore, this method requires approximately 22 h to obtain a measurement.

3. Results and discussion

3.1. Experimental principle

Scheme 1A presents the principle of this SPR biosensor for quantification of O-GlcNAcylated biomolecules that are based on the specific cleavage of O-GlcNAc by OGA and signal amplification by AuNPs. O-GlcNAcylated biomolecules can be immobilized on MUA modified Au films via a cross linking reaction between the amino and carboxylic acid groups. AuNPs/WGA can then be immobilized on the O-GlcNAcylated biomolecule modified Au film via recognition between O-GlcNAc and WGA, which result in a higher index change, then the SPR signal can be recorded in situ (curve a of Scheme 1B). However, as discussed above, WGA can also recognize sialic acid and the terminal GlcNAc unit of polysaccharides, which means that the recorded signal would not accurately represent the amount of O-GlcNAc in the sample.

To resolve this problem, the O-GlcNAc specific cleavage enzyme OGA was used to interact with the immobilized O-GlcNAcylated biomolecules and cleave O-GlcNAc from the Au film. Next, the AuNPs/WGA was introduced on the OGA treated film, and the SPR signal was recorded again. Less of the AuNPs/WGA would be immobilized on the OGA treated Au film due to the removal of O-GlcNAc, thus, the SPR signal would decrease (curve b of Scheme 1B). The decrease in the SPR signal should just be proportional to the amount of the O-GlcNAc on the Au film, and then the change in the signal can be used to accurately detect O-GlcNAc without interference from terminal GlcNAc and sialic acid. To show the feasibility of the designed sensor can be used to evaluate the interaction between O-GlcNAc and WGA. The SPR responses of the consecutive injections of O-GlcNAc, AuNPs/WGA and OGA were recorded (Fig. S-2), the SPR signal of the continuous flow injection of O-GlcNAc and AuNPs/WGA demonstrated that the fabricated sensor was



Scheme 1. (A) Schematic illustration of the SPR biosensor for quantification of O-GlcNAcylated biomolecules. (B) Principle used to accurately detect O-GlcNAc by OGA specific cleavage of O-GlcNAc.

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successful and can be used to evaluate the interaction between O-GlcNAc and WGA. While no signal was observed by the OGA injection, this phenomenon proves that the enzyme cleavage reaction could not occur at this condition (Details can be seen from Fig. S-6).

In addition, AuNPs are often used to increase the signals of SPR biosensors, but nonspecific adsorption of AuNPs is always a difficult problem to resolve and one that affects the results of direct detection. The use of the SPR signal difference in the quantification of O-GlcNAc avoids the effect of AuNPs nonspecific adsorption on the results. In short, the use of OGA avoids interference from other monosaccharides and nonspecific adsorption of AuNPs, which guarantees the accuracy of the detection results.

3.2. AuNPs amplification effect on the O-GlcNAc detection

O-GlcNAc-CREB was used as a model for O-GlcNAcylated biomolecules to explore the detection methods for O-GlcNAc (Fig. 1A). To confirm that the designed experiment system is used under the best conditions, the modified time of O-GlcNAc-CREB (Fig. S-3) and a concentration of AuNPs/WGA were studied (Fig. S-4). A time of 1 h for O-GlcNAcylated biomolecules immobilization and 5 μg/mL of AuNPs/WGA was used in all of the experiment. The SPR response of WGA to the 4.65×10^{-12} M O-GlcNAc-CREB modified Au film showed an obvious SPR signal upon injection of WGA into a flow cell (curve c of Fig. 1A) and the SPR signal was decreasing, which results from the different refractive indexes of the target and carrier solutions [41]. The established new baseline after the solution had been replaced by the carrier solution is higher than the original. This result demonstrates that the WGA was adsorbed onto the surface of the Au film and induced a change in the refractive index. However, a large SPR signal was again observed after injecting WGA onto the surface of MUA modified Au film (curve a of Fig. 1A), but the signal returned to the original baseline after the WGA solution passed through the flow cell. This finding means that WGA cannot be immobilized on the surface of the Au film without O-GlcNAc, which further demonstrates that the SPR signals of curve c of Fig. 1A were induced by the recognition between O-GlcNAc and WGA. The SPR dip shift increased with the increasing concentration of O-GlcNAc-CREB, which indicates that this method can be used to detect O-GlcNAc. However, these SPR signals are much smaller than that obtained upon introducing the AuNPs/WGA (Fig. 1B) to the O-GlcNAc modified Au film. After the AuNPs/WGA passed through the flow cell, the SPR signal remained stable, indicating that the interaction between O-GlcNAc and WGA is sufficiently strong to ensure that the AuNPs/WGA remain on the Au film. These results also demonstrate that the AuNPs label did not affect the interaction between WGA and O-GlcNAc.

AuNPs/WGA was also introduced to the same peptide sequence without O-GlcNAc on the modified Au film (curve a of Fig. 1B), and an obvious SPR signal (0.0062) was obtained, which should be caused by the nonspecific adsorption of AuNPs onto the Au film [30]. This finding also shows that though AuNPs can greatly increase the SPR signal, its nonspecific adsorption is still a problem to be resolved. This result further demonstrates that directly recording the signal from AuNPs/WGA would produce a false result and could not be used to detect the amount of O-GlcNAc. Curve b is the result of the introduction of AuNPs/WGA to 4.65×10^{-12}O-GlcNAc-CREB modified Au film, when comparing the signals of curves a and b in Fig. 1B, curve b is larger, which supports the specific interaction between O-GlcNAc and WGA, and it also proves that the increasing SPR signals for different concentrations of O-GlcNAc-CREB on the modified Au films comes from the greater amount of O-GlcNAc on the Au film surface.

To display the advantages of the AuNPs amplification more clearly, the SPR intensities that were produced upon injecting AuNPs/WGA (a) or WGA (b) versus the different O-GlcNAc-CREB concentrations are shown in Fig. 1C. The slope of curve a is much steeper than those without the AuNPs amplification (curve b of

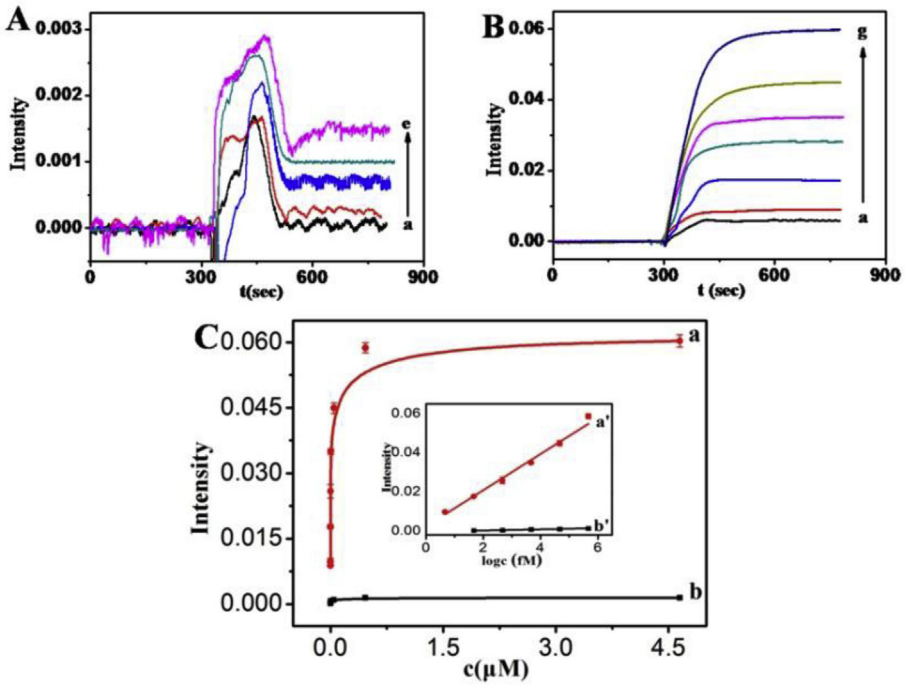

Fig. 1. SPR responses of different O-GlcNAc-CREB (a:0, b:4.65×10^{-11}, c:4.65×10^{-9}, d:4.65×10^{-8}, e:4.65×10^{-7} M) concentrations modified Au film on injecting WGA (A) and different O-GlcNAc-CREB (a:0, b:4.65×10^{-12}, c:4.65×10^{-11}, d:4.65×10^{-10}, e:4.65×10^{-9}, f:4.65×10^{-8}, g:4.65×10^{-7} M) concentrations modified Au film on injecting AuNPs/WGA (B); (C) SPR intensity versus different O-GlcNAc-CREB concentrations (4.65×10^{-13} to 4.65×10^{-6} M) upon injection of AuNPs/WGA (a) and WGA (b). Inset of C shows the fitting curve between the SPR intensity and logarithm value of O-GlcNAc-CREB concentration upon injection of AuNPs/WGA (a) and WGA (b).

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Fig. 1C) and at high concentration of O-GlcNAc, the change in the SPR signal from the AuNPs enhancement (0.060, curve a of Fig. 1C) was considerably larger than that of without AuNPs amplification (0.0015, curve b of Fig. 1C), indicating a large amplification effect from AuNPs.

A linear relationship between SPR intensity and O-GlcNAc-CREB concentration was obtained (Inset of Fig. 1C). A steeper slope in the curve a with AuNPs amplification indicates good sensitivity of the method, and the detection limit (4.65×10^{-13} M) is 10 times higher than that of the method without the AuNPs enhancement (4.65×10^{-12} M). The linear range of the method without AuNPs amplification (curve b') is 4.65×10^{-11} to 4.65×10^{-7} M, while the O-GlcNAc detection linear range of AuNPs amplification is from 4.65×10^{-12} to 4.65×10^{-7} M (curve a'). The O-GlcNAc detection range of the methods with AuNPs amplification is approximately ten times wider than that of the methods without AuNPs amplification. The wider linear range for O-GlcNAc detection with this method was also reflected in consecutive injections of the AuNPs/WGA onto the Au film modified with 4.65×10^{-9} M O-GlcNAc-CREB (Fig. S-5). With each injection, an obvious shift in the SPR dip was observed, even after the sixth consecutive injection of AuNPs/WGA. However, the total change in the SPR dip decreased with each AuNPs/WGA injection because less free O-GlcNAc was available on the Au film after introduction of the AuNPs/WGA.

3.3. Quantification of standard O-GlcNAcylated CREB peptide

The enzyme cleavage reaction methods and the cleavage conditions were optimized as shown in Figs. S-6, 7 and 8. Finally, an off-line method was selected: After the O-GlcNAc-CREB-modified Au film were treated with 1.83 μg/mL OGA for 16 h at 37 °C, AuNPs/WGA was introduced into the flow cell and the SPR signals recorded (Fig. 2B). The concentration of O-GlcNAc-CREB was the same as that before the OGA treated film (Fig. 2A), but the SPR dip shift was significantly smaller. The decrease of the SPR signal demonstrated that the O-GlcNAc on the Au film were cleaved by OGA, which limited the amount of AuNPs/WGA immobilized on the surface of the Au film and resulted in small change in the SPR dip.

To show the difference more clearly, the SPR signal changes for the OGA treated and untreated O-GlcNAc-CREB modified Au films are displayed in Fig. 2C. On the left of the figure is the SPR dip shift for the O-GlcNAc-CREB modified Au film and on the right is the SPR signal for the Au film that was treated with OGA. It can be clearly seen that the SPR signal for each concentration point on the left is significantly higher than that on right, which further demonstrated that the amount of O-GlcNAc decreased after treatment with OGA. The SPR signals should return to base line after being treated with OGA if all the O-GlcNAc on the O-GlcNAcylated biomolecules were cleavage, while obvious SPR signals were also observed for all of the SPR signals. The remaining SPR signal should be ascribed to the nonspecific adsorption of AuNPs and the remaining O-GlcNAc. It could be seen that the SPR signals were approximately the same for the samples in the O-GlcNAc-CREB concentration range from $4.65 \times 10^{-13}$to 4.65×10^{-10} M after being treated with OGA, which indicated that at a lower concentration, the O-GlcNAc units on the Au film are almost completely removed by OGA, and the remaining signal is caused by nonspecific adsorption of AuNPs. The change of the SPR signal increased with the increasing of the O-GlcNAc-CREB concentration on the Au film from 4.65×10^{-9} to 4.65×10^{-7} M. The reason for these results is that at a higher concentration, O-GlcNAc could not be cleaved completely by OGA from the Au film. Therefore, the higher SPR signals result from the nonspecific adsorption of AuNPs and the interaction between the remaining O-GlcNAc and AuNPs/WGA.

As noted in the experimental principle, the decrease of the SPR signal should be proportional to the amount of O-GlcNAc on the Au film. Thus, the change of the SPR signal (ΔR) with or without the OGA treatment at the same O-GlcNAc-CREB concentration was used to build a linear relationship with O-GlcNAc-CREB, as shown in the inset of Fig. 2C. A linear relationship between the relative SPR

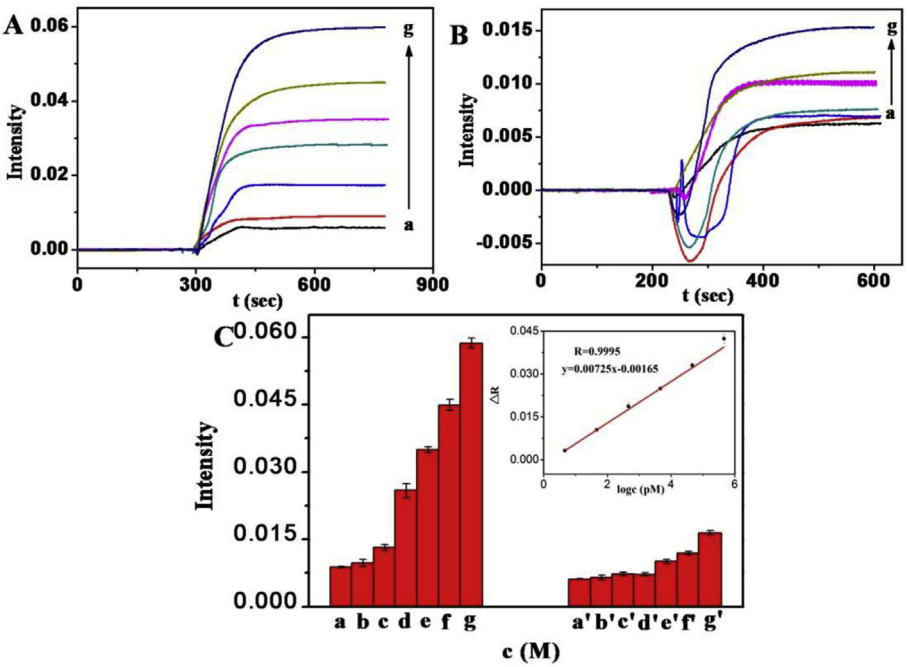

Fig. 2. SPR responses of different concentrations O-GlcNAc-CREB (4.65×10^{-13} to 4.65×10^{-7} M) modified Au film surface that was treated without (A) and with (B) OGA on injecting AuNPs; (C) Histogram of SPR intensity for different concentrations of O-GlcNAc-CR (a-g, a'-g':4.65×10^{-13} to 4.65×10^{-7} M) EB modified Au film treated without (left part) or with (right part) OGA, inset of C shows the decreased SPR intensity (ΔR) versus logarithm value of O-GlcNAc-CREB concentration in the range 4.65×10^{-12} to 4.65×10^{-7} M.

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intensity and the logarithmic value of the O-GlcNAc-CREB concentration in the range 4.65×10^{-12} to 4.65×10^{-7} M was established. The linear regression equation is $\Delta R = 0.00725\ \log C - 0.00165$ with a correlation coefficient of $R = 0.9995$, where ΔR is the decreased SPR intensity at the same O-GlcNAc-CREB concentration before and after the OGA treatment. The detection limit for O-GlcNAc was 4.65×10^{-13} M.

The proposed biosensor displayed better performance than some of the earlier methods, especially in terms of the detection range (6 orders of magnitude), which is approximately four orders of magnitude higher than that of the MRM-MS method (50−1000 pg) [14]. The proposed method has high sensitivity and a relatively wide linear range. An MRM-MS instrument was first applied to directly quantify the amount of native O-GlcNAc-modified peptides without enrichment and labelling for comparison to other MS methods [20,21]. Because the MRM-MS method requires the sequence of O-GlcNAc-CREB to be known in advance, it will be difficult to determine the amount of O-GlcNAc in unknown samples especially in complex samples. However, the method proposed here is sensitive and can accurately quantify the amount of O-GlcNAc in complex samples without knowing the peptide sequences in advance. Specifically, the change of the SPR signal is only related to the amount of O-GlcNAc on the Au film, thus, the existence of sialic acid and other terminal GlcNAc units (N-glycan and O-glycan) can not affect the signals. At the same time, the nonspecific adsorption of AuNPs is serious in the detection system, but using the decrease in the SPR signal to detect O-GlcNAc not only takes advantage of the AuNPs amplification but also avoids interference from the nonspecific adsorption of AuNPs. In summary, this method should be applicable to accurately detect O-GlcNAc in complex samples with higher sensitivity and a wider detection range.

3.4. Detection of O-GlcNAc in α-crystallin protein

The method was further used to analyse the O-GlcNAc content in α-crystallin protein. It has been reported that the detection of O-GlcNAc in α-crystallin is difficult because the protein contains only one O-GlcNAc modification site [42]. With this method, the experimental result of O-GlcNAc in α-crystallin detection is shown in Fig. 3. The SPR signal for the sample treated with OGA (violet) was smaller than that for the sample that was not treated with OGA (olive). This result indicates that O-GlcNAc in the α-crystallin protein can also be cleaved by OGA, resulting in a signal decrease. The SPR signal of the ovalbumin modified Au film that was treated with OGA was approximately the same as the film that was not treated with OGA (Fig. 3). The reason for this is that the ovalbumin protein does not contain O-GlcNAc, the observed SPR signal change can be attributed to the presence of other sugars (other terminal GlcNAc and sialic acid) and specific adsorption of AuNPs/WGA. This is because of the ability of OGA to specifically recognize O-GlcNAc, even though the ovalbumin protein may contain other terminal GlcNAc or sialic acid, but without O-GlcNAc, the SPR signal was the same before and after treated with OGA, which proves the cleavage reaction of OGA to O-GlcNAc cannot be interfered by other terminal GlcNAc and sialic acid. To further demonstrate that the method has excellent selectivity, N-GlcNAc modified peptide (N-GlcNAc-CREB), sialic acid modified peptide (sialic acid-CREB) and the peptide without modification (CREB) were synthesized. Then the samples were used to explore the selectivity and accuracy of this method (Fig. S-9). These results mean that the method has excellent selectivity for the detection of O-GlcNAcylated molecules. For the proposed method, O-GlcNAc content in 1 μg α-crystallin protein can be determined, while the other method failed to detect O-GlcNAc in α-crystallin protein even when 10 μg α-crystallin protein was used [16]. This result suggested that this high sensitivity system can be used to detect O-GlcNAc in biomolecules of low stoichiometry of O-GlcNAc modification.

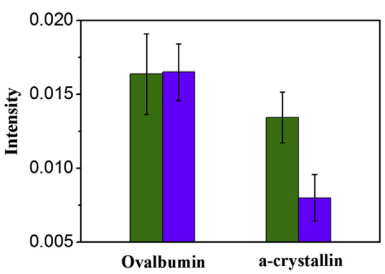

Fig. 3. Comparison of the SPR intensities of 5 μg of ovalbumin and 1 μg of α-crystallin protein modified Au film treated without (olive)or with (violet)OGA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. Determine the amount of O-GlcNAc in cells and blood samples

To investigate the potential practical application of the proposed method in complicated samples, the amount of O-GlcNAc in the lysates of HEK293, SW480, MDA-MB-231 and MDA-MB-468 cells were detected. The SPR responses of the different cell lysate modified Au films upon injection of AuNPs/WGA shown in Fig. 4A. The SPR signals exhibited different changes for the four types of cells, which indicate that they contain different amounts of O-GlcNAc or other sugars. To accurately determine the amount of O-GlcNAc, the biomolecules in the cell lysate-modified Au films were treated with OGA at 37 °C for approximately 16 h, after which the SPR signals were recorded (Fig. 4B). The SPR intensity was also smaller than that in Fig. 4A, which means that O-GlcNAc was cleaved from the biomolecules in the complex cell lysates. A histogram of SPR intensity versus the different cell lysates is shown in Fig. 4C. For all of the cell lysates, the SPR signal of the film before OGA treatment (violet) is greater than that after the OGA treatment (olive). This showed the cleavage of O-GlcNAc from the surface of the Au film after treatment with OGA, although it is not clear which biomolecules are on the surface of the Au film. These results also demonstrate that the four cells all contained the O-GlcNAcylated biomolecules, even the normal cells.

The decrease in SPR intensity (ΔR) versus the different cells is shown in Fig. 4D. The SPR intensity of the three types of cancer cells decreased more than that of the normal cells, which demonstrated that the amount of O-GlcNAc in the cancer cells was higher than that in the normal cells. This result was consistent with a previous report that found that the level of O-GlcNAcylation was significantly enhanced in cancer cells [8]. Table 1 lists the SPR intensity changes and the amount of O-GlcNAc in the different cells that were calculated from the fitted line in the inset of Fig. 2C. The level of O-GlcNAc in the normal cells was approximately 2.62×10^{-11} M, which is smaller than that of the three types of cancer cells. The amount of O-GlcNAc in these cells varied greatly (2.62×10^{-11}-1.07×10^{-9} M), but was within the detection range of this method, which suggests that the detection range is wide enough and that the sensitivity can satisfy the demand. The successful detection of O-GlcNAc in the cell lysates also indicates that the method can be used to accurately detect O-GlcNAc in unknown and even complex samples.

To demonstrate the feasibility of the clinical application of this system, the proposed method was also employed in the detection of O-GlcNAc in the blood sample of three cancer patients and two healthy individuals. The results are presented in Fig. 5 and Table 1.

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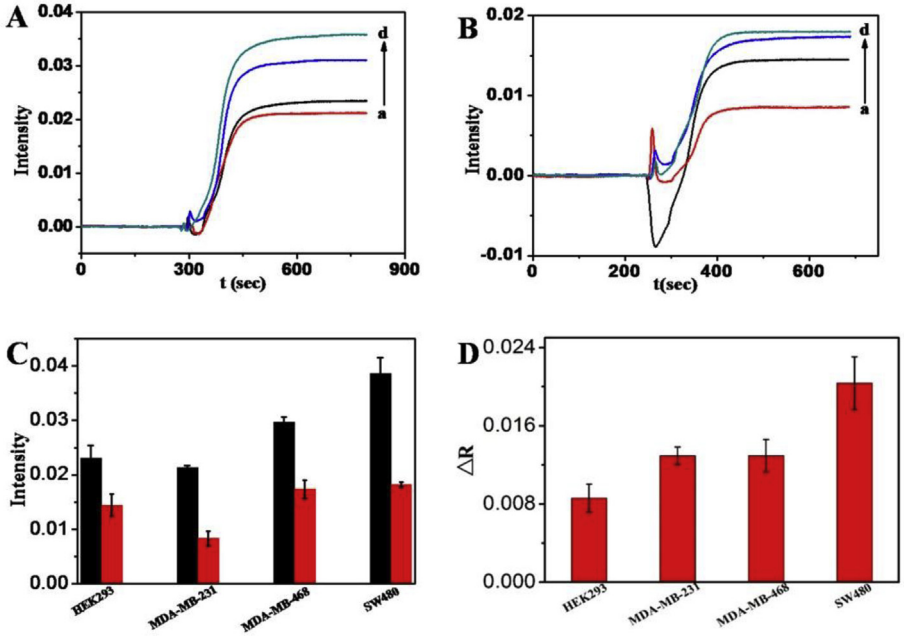

Fig. 4. SPR responses of different cells (a: MDA-MB-231, b: HEK 293, c: MDA-MB-468, d: SW480) modified Au film surface treated without (A) and with (B) OGA on injecting AuNPs/WGA; (C) Comparison of SPR intensity of different cells incubated Au film surface treated without (black) and with (red) OGA on injecting AuNPs/WGA. (D) The SPR intensity difference versus different cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

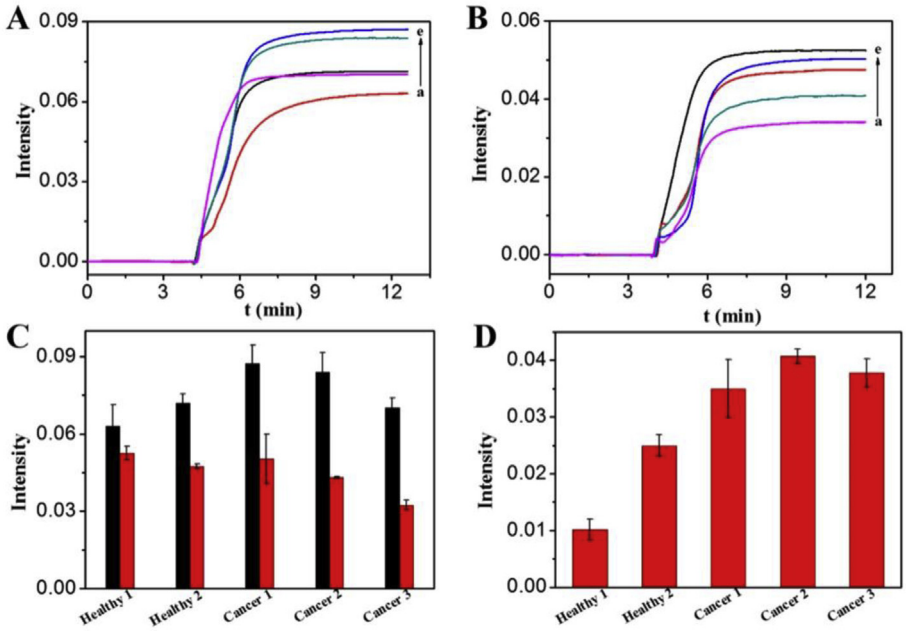

Fig. 5. SPR responses of different clinical human blood samples modified Au film surface treated without (A) and with (B) OGA on injecting 5 μg/mL AuNPs/WGA; (C) Comparison of SPR intensity of different clinical human blood samples incubated Au film surface treated without (black) and with (red) OGA on injecting 5 μg/mL AuNPs/WGA. (D) The SPR intensity difference versus different clinical human blood samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The amount of O-GlcNAc calculated from the fitted line in the inset of Fig. 2C for the healthy blood samples were 4.31×10^{-11} and 4.05×10^{-9} M, which is much lower than the amounts from the three cancer blood samples. These results further proved that the amount of O-GlcNAc was related to the presence of cancer and taken as a meaningful cancer biomarker. The amount of O-GlcNAc in the blood of the three cancer patients varied (2.21×10^{-7}, 3.03×10^{-7} and 2.75×10^{-7}), which may have contributed to the different grades of tumours [43,44]. Considering all of the aforementioned above results, the SPR biosensor provides a sensitive and accurate method for the early clinical diagnosis of O-GlcNAc related diseases.

4. Conclusion

A novel SPR biosensor was developed for the sensitive and

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Table 1
Amount of O-GlcNAc in the different cells and clinic blood samples.

	HEK293	MDA-MB-231	MDA-MB-468	SW480	Healthy blood samples			Clinic blood samples		
					1	2		1	2	3
ΔR (Intensity)	0.00860 ± 0.0014	0.0125 ± 0.0009	0.0123 ± 0.0017	0.0203 ± 0.0027	0.0102 ± 0.0018	0.0245 ± 0.0019		0.0371 ± 0.0051	0.0407 ± 0.0013	0.0378 ± 0.0025
O-GlcNAc Amount(10^{-9}M)	0.0262	0.0895	0.0840	1.07	0.0431	4.05		221	303	275

accurate detection of O-GlcNAc that was based on amplification of AuNPs and the O-GlcNAc-specific cleavage by the OGA enzyme was developed. This method not only prevents interference from other sugars but also avoids the disturbance of nonspecific adsorption of AuNPs by recording the signal difference between OGA treated and untreated O-GlcNAcylated biomolecule modified films. This method can quantify the standard O-GlcNAcylated CREB with a linear response range of 4.65×10^{-12}-4.65×10^{-7} M and a detection limit of 4.65×10^{-13} M. The sensing system was also applied to the detection of O-GlcNAc in real protein samples, cancer cell lysates and blood samples. This SPR biosensor provides a highly sensitive method for accurately quantifying O-GlcNAc in clinical blood samples and could potentially be applied in the diagnosis of O-GlcNAc related diseases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.07.058.

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