Surface Plasmon Resonance Biosensor for Sensitive Detection of HER2-positive Exosomes Based on Reformative Tyramine Signal Amplification Activated by Molecular Aptamer Beacon Conversion

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Abstract

HER2-positive exosomes play an extremely important role in the diagnosis and treatment options of breast cancers. Herein, based on the reformative tyramine signal amplification (TSA) enabled by molecular aptamer beacon (MAB) conversion, a label-free surface plasmon resonance (SPR) biosensor was proposed for highly sensitive and specific detection of HER2-positive exosomes. The exosomes were captured by the HER2 aptamer region of MAB immobilized on the chip surface, which enabled the exposure of the G4 DNA that could form peroxidase-like G4-hemin. In turn, the formed G4-hemin catalyzed the deposition of plentiful tyramine-coated AuNPs (AuNPs-Ty) on the exosome membrane with the help of H_2O_2 , generating significantly enhanced SPR signal. In the reformative TSA system, the horseradish peroxidase (HRP) as a major component was replaced with nonenzymic G4-hemin, bypassing the defects of nature enzymes. Moreover, the dual-recognition of the surface proteins and lipid membrane of the desired exosomes endowed the sensing strategy with high specificity without the interruption of free proteins. As a result, this developed SPR biosensor exhibited a wide linear range from 1.0×10^4 to 1.0×10^7 particles/mL. Importantly, this strategy was able to accurately distinguish HER2-positive breast cancer patients from healthy individuals, exhibiting great potential clinical application.

1. Introduction

Breast cancer is the most common type of cancer that causes female deaths worldwide. The number of new breast cancer cases reached 2.26 million in 2020, surpassing lung cancer (2.21 million cases) for the first time to become the world's largest cancer [1, 2]. In the diagnosis and treatment process of breast cancer, human epidermal growth factor receptor 2 (HER2) is an important prognostic indicator and predictor of HER2 targeted drugs [3, 4]. Currently, the clinical routine methods for HER2 analysis mainly include immunohistochemistry and fluorescence in situ hybridization, but these methods have several shortcomings, such as the uncertainty of the results, the high false-positive rates, and the excessively harsh requirements for specimens, etc [5]. Therefore, developing a noninvasive and reliable strategy to accurately monitor HER2 status is challenging but urgently needed.

Exosomes are 30-150 nm sized extracellular vesicles with a typical lipid bilayer membrane structure, which are secreted into the surrounding biofluids by different types of cells [6]. Through transporting various biological constituents of parent cells (lipids, nucleic acids and metabolites) into recipient cells, exosomes take part in cell-cell communication and are closely related to the occurrence and progress of disease [7]. In particular, tumor-derived exosomes not only carry cancer-specific proteins, but have high abundance and stability in body fluids, thus acting as promising biomarkers for liquid biopsy [8]. Recent reports have proved that the HER2-positive exosomes are greatly increased in patients with breast cancer, and there is the well consistency of the HER2 expression level between exosomes and tumor tissues [9, 10]. Therefore, quantitating the amounts of HER2-positive exosomes in clinical samples has the potential to improve the diagnosis and customize treatment of breast cancer.

So far, apart from traditional methods including nanoparticle tracking analysis, western blot, flow cytometry, and enzyme-linked immunosorbent assay [11], various biosensing platforms have been developed for exosomes analysis by targeting their surface proteins using the corresponding antibodies or aptamers [12–17]. Among them, surface plasma resonance (SPR) biosensor is receiving extensive attention because it is a rapid, real-time, and label-free diagnostic device [18, 19]. The sensing principle of this technique is to measure the change in refractive index around the surface of the metallic sensor chip due to alterations in mass caused by analyte-receptor noncovalent interactions [20, 21]. However, SPR biosensor for exosomes detection faces adverse conditions. For instance, the small size and low mass of exosomes cannot trigger obvious signal discrepancy, leading to the inevitable requirement of signal amplifier. Moreover, the collected exosomes are always mingled with free target proteins from serum, resulting in the generation of false-positive signal that reduces the accuracy of results. Considering these facts, we are spurred to develop a novel SPR biosensing strategy for sensitive and specific detection of HER2-positive exosomes.

Tyramine signal amplification (TSA) is a typical enzyme-assisted amplification strategy in which tyramine or reporter molecules-functionalized tyramine can be transformed by horseradish peroxidase (HRP) into a reactive oxidized intermediate with the help of H_2O_2 [22]. The intermediate then covalently conjugates and rapidly deposits on near protein residues to trigger signal enhancement [23]. In previous studies, Jung et al. developed a TSA-based surface-enhanced Raman spectroscopy sensor by modifying gold nanoparticles (AuNPs) with tyramine and Raman-active probe, exhibiting significantly enhanced sensitivity [24]. Our group proposed a cytosensor that used HRP to catalyze the deposition of tyramine-modified electroactive reporters on tumor cells [25]. Nevertheless, HRP has been suffered from complicated purification processes and poor chemical stability due to the intrinsic characters of nature enzymes [26], which compromise the clinical applicability of TSA-based strategies. G-quadruplex-hemin (G4-hemin), an HRP-mimicking DNAzyme with excellent catalytic performance, has been widely applied in the biosensing owing to its easy synthesis, high stability, and low requirement for reaction conditions [27, 28]. Therefore, G4-hemin could be a credible and promising alternative to HRP.

Herein, a label-free SPR biosensor was proposed for highly sensitive and specific detection of HER2-positive exosomes based on reformative TSA activated by target-induced molecular aptamer beacon (MAB) conversion. By combining the functions of G4 and HER2 aptamer into one molecular beacon, the engineered MABs immobilized on the sensing chip bound to HER2-positive exosomes, resulting in the structural change that accompanied the exposure of G4. Then, the formed G4-hemin catalyzed the deposition of numerous tyramine-coated AuNPs (AuNPs-Ty) on the lipid membrane of the exosomes in the presence of H_2O_2 , achieving geometrically enhanced SPR signal. Profiting from the superior catalytic ability of G4-hemin, the improved TSA had no need for HRP. What's more, the dual-identification of surface proteins and the membrane structure of exosomes was able to avoid the interference of the free proteins. Taken together, the developed SPR biosensing strategy enabled accurate detection of HER2-positive exosomes with high sensitivity and specificity.

2. Experimental

2.1. Reagents and materials

Sodium citrate powder, $\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$ and 11-mercaptoundecanoic acid (MUA) were purchased from Sinopharm Chem Co., Ltd (Shanghai, China). Hemin, tyramine, and 6-Mercapto-1-hexanol (MCH) were purchased from Sigma-Aldrich (St. Louis, USA). Hemin was dissolved in dimethyl sulfoxide as the stock solution and then diluted to the required concentration with 25 mM HEPES buffer (pH 7.4, 25 mM HEPES, 200 mM NaCl, 100 mM KCl, 1% DMSO, 0.05% Triton). Fetal Bovine Serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) were offered by Gibco (Gaithersburg, USA). N-hydroxysuccinimide (NHS) and N-(3-(dimethylamino) propyl)-N'-ethylcarbodiimide hydrochloride (EDC) were purchased from Alfa Aesar (Massachusetts, USA). Phosphate buffer (PBS) was supplied by Thermo Fisher Scientific (Wilmington, USA). All the chemicals used were of analytical reagent grade, and all the HPLC-purified oligonucleotides listed in Table S1 were prepared by Sangon Biotech. Co., Ltd (Shanghai, China). Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was utilized for the dissolution of oligonucleotides. Aqueous solutions prepared by Millipore Milli-Q gradient ultrapure water system were used (Millipore Co., MA, USA).

2.2. Instruments

All measurements were carried out on the SPR biochemical analyzer independently developed and built by our own group. The SPR analyzer is based on the principles of physical optics and mainly composed of light source, a sensing chip, flow cell, and a CCD detector. The sensorgrams are analyzed by the lab-developed program written in LabVIEW, exhibiting a time course of resonance units (RU). Transmission electron microscopic (TEM) image was performed with H-7500 transmission electron microscope (Hitachi High-Technologies Co., Japan). Nanoparticles tracking analysis (NTA) was supported by ZetaView (Particle Metrix, Germany). UV-Visible absorption spectra were carried out at a UV-2550 spectrophotometer (Shimadzu, Japan).

2.3. Extraction of cancer cell-derived exosomes

All cell lines, including HER2-positive SK-BR3 and HER2-negative HeLa, LNCaP, HepG2, and MCF-7 [29–32], were provided by the American Type Culture Collection (ATCC) (Rockville, USA). DMEM (added with 10% FBS, 1% streptomycin and penicillin) was used for all kinds of cell culture in sterile incubator containing 5% CO_2 , at 37 °C. Cell passaging was processed when growth density reached 80%. Ultimately, the cells were starved with serum-free medium for 48 h before the exosomes were extracted.

The exosomes were extracted from the cell culture medium according to the published literatures [33, 34]. Generally, after 48 h's starvation, the cell culture medium was centrifuged ($2,000 \times g$, 20 min; $10,000 \times g$, 30 min; $100,000 \times g$, 2 h. All steps were performed at 4 °C). The precipitate containing exosomes was suspended by 1 × PBS and characterized by TEM and NTA. Clinical samples were collected from the First Affiliated Hospital of Chongqing Medical University. And the extraction procedures of exosomes from clinical samples were as described above.

2.4. Preparation of AuNPs-Ty complex

Firstly, AuNPs with a particle size of about 14 nm were synthesized according to the published literatures with slight modification [35, 36]. Briefly, 88.2 mL of HAuCl_4 (1 mM) was boiled and then added rapidly to 10.1 mL of sodium citrate (34 mM). After the color changed to burgundy, the mixture was stopped heating and slowly cooled to room temperature with continued stirring. Then the prepared AuNPs and tyramine were combined by MUA that has sulfhydryl and carboxyl groups at each end. In detail, the sulfhydryl groups at one end of MUA was connected to AuNPs through Au-S bonds, and the other end was connected to tyramine through carboxyl groups, which indirectly connected AuNPs and tyramine into AuNPs-Ty complex. To begin with, add MUA (25 μL , 100 μM) to the AuNPs (5 mL) solution, gently shaking for 12 h's reaction, then centrifuge the solution twice ($2,000 \times g$, 10 min) to get AuNPs-MUA solution. Thereafter, add NHS (10 μL , 1 mg/mL), EDC (10 μL , 2 mg/mL) and tyramine (10 μL , 100 μM) to the mixed solution, gently shaking for 12 h's reaction to get AuNPs-Ty complex, stored at 4 °C for later use.

2.5. Exosomes analysis by the fabricated SPR biosensor

Firstly, immerse the gold chip and the flow cell in 75% ethanol, sonicate for 20 min at room temperature. Rinse with deionized water and blow dry with nitrogen. Then, freshly prepare the piranha solution 1 mL (H_2SO_4 : 700 μL , H_2O_2 300 μL), after cooling to room temperature, cover the chip surface for 10 min to remove the remaining impurities. Rinse the chip thoroughly with deionized water and blow dry with nitrogen. Next, dilute the capture probes of MAB to a concentration of 1.5 μM by the fixative solution (KH_2PO_4 , 1 M), modified on the surface of the chip overnight at 4 °C, and followed by the block of 1 mM MCH for 30 min. After wash and dry the chip, install the treated chip, the prism cleaned with 75% ethanol, and the flow cell on the SPR instrument for subsequent testing. Rinse the flow pipeline with cell grade 1 $\times$ PBS for 15 min to output the baseline signal (flow rate: 50 $\mu\text{L}/\text{min}$). Then, different concentrations of exosomes derived from tumor cells or clinical samples were loaded (loading volume: 150 μL , flow rate: 7 $\mu\text{L}/\text{min}$, time: 30 min). Subsequently, loading the AuNPs-Ty composite (loading volume: 150 μL , flow rate: 7 $\mu\text{L}/\text{min}$, time: 30 min). After detection, the surface of the sensing chip was regenerated with 50 mM NaOH (loading volume: 150 μL , flow rate: 20 $\mu\text{L}/\text{min}$, time: 2 min).

3. Results And Discussion

3.1. Illustration of the SPR biosensing strategy

Scheme 1. Schematic illustration for the detection of HER2-positive exosomes based on the improved TSA enabled by target-induced MAB conversion.

3.2. Characterization of the extracted exosomes, G4-hemin, and AuNPs-Ty

First, the morphology and concentration of exosomes derived from SK-BR3 cells were characterized, and the results were shown in Fig. S1. The TEM image disclosed that the exosomes were round vesicles with

a clear double-layer membrane structure (Fig. S1A). The NTA indicated that the original concentration of the exosomes was 2.75×10^9 particles/mL with a size distribution ranging from 30 to 200 nm (Fig. S1B). The above results confirmed the successful extraction of exosomes. Second, the combination of G4 and hemin was verified by UV-vis absorption spectroscopy. As we can learn from Fig. S2, 260 nm and 394 nm were the characteristic absorption peaks of G4 and hemin, respectively. As expected, in the absorption spectrum of G4-hemin, the absorption peaks of the two substances with a slight right shift were observed at the same time, indicating the successful formation of G4-hemin. Third, the characterization of AuNPs-Ty composite was performed. The TEM image showed the prepared AuNPs were uniform in volume and evenly distributed (Fig. 1A), and the UV-vis absorption spectra displayed that the typical absorption peaks of AuNPs and AuNPs-Ty were seen at 530 nm and 537 nm, respectively (Fig. 1B). These results were entirely consistent with the previous report [24], indicating the successful synthesis of AuNPs-Ty.

3.3. Feasibility of the developed biosensor

To demonstrate the feasibility of this proposed biosensor, the whole detection process was firstly analyzed. The detection steps and the corresponding sensorgrams were depicted in Fig. 2A and B, respectively. In the first step, when the HER2-positive exosomes were loaded, the SPR signal rose by about 40 RU, indicating that the exosomes were connected to the surface of the chip owing to the effective binding of the aptamer of the MAB and HER2 proteins. In the second step, once the loading of AuNPs-Ty composite, the SPR signal increased by about 200 RU, which was attributed to the deposition of plentiful AuNPs-Ty on the surface of the exosomal membrane caused by the reformative TSA. After detection, the chip surface was regenerated with 50 mM NaOH for next measurement. Subsequently, to verify that the deposition of AuNPs-Ty on the exosome surface was caused by the catalytic activity of G4-hemin rather than the nonspecific absorption, the relevant experiments were conducted. As shown in Fig. 2C, in the absence of hemin, the signal of the AuNPs-Ty was not been observed due to the fact that the exposed G4 had not peroxidase-like activity. In contrast, the AuNPs-Ty generated distinct SPR signal, demonstrating that the signal indeed originated from the reformative TSA. In addition, compared with Ty and AuNPs that produced negligible SPR signal, the signal of AuNPs-Ty increased nearly 10-fold (Fig. 2D). These results adequately demonstrated the feasibility of the proposed method for the detection of HER2-positive exosomes.

3.4. Optimization of experimental parameters

Several important reaction parameters of this biosensor were optimized to obtain good analytical performance. Owing to the effect of the density of capture probe immobilized on sensing chip on the sensitivity of the biosensor, the concentration of the MAB (C_{MAB}) was firstly optimized. As shown in Fig. S3A, the SPR signal gradually improved with the increase of the C_{MAB} ranging from 0.5 to 1.5 μM . Once the concentration was above 1.5 μM , the signal decreased rapidly, because the crowded MAB could not bind to the exosomes effectively. Therefore, 1.5 μM was chosen as the optimal C_{MAB} . When the

concentration of hemin (C_{hemin}) was 1.5 μM , being same as the C_{MAB} , the highest signal was obtained. Hence, the optimal C_{hemin} is set to 1.5 μM (Fig. S3B).

Furthermore, to avoid the nonspecific deposition of redundant signal amplifier on the chip surface which increased the background signal, the befitting concentration of the AuNPs-Ty ($C_{\text{AuNPs-Ty}}$) was evaluated. As shown in Fig. S3C, when the $C_{\text{AuNPs-Ty}}$ rose from 50 to 200 mM, the SPR signal rose synchronously and ended by a downward trend. Therefore, we chose 200 mM as the optimal $C_{\text{AuNPs-Ty}}$. Subsequently, we also optimized the reaction time between exosomes and MAB (Time_1) and the incubation time of the improved TSA (Time_2), respectively (Fig. S3D and E). When Time_1 was set as 30 min, the signal reached the peak and remained steady as time prolonged, indicating the completion of the reaction. Similarly, in the optimization experiment of Time_2 , the reaction was completed after 30 min. Therefore, we set both reaction times as 30min.

3.5. Sensitivity of the SPR biosensor

After gaining the optimal reaction conditions, we further evaluated the sensitivity of this sensing method by detecting a series of different concentrations of HER2-positive exosomes. As shown in Fig. 3A, when the concentration of exosomes increased from 0.1 to 100×10^5 particles/mL, the SPR signal also increased simultaneously, showing a good correlation. As presented in, the regression equation between the concentration of exosomes (X) and the corresponding SPR signals (Y) was $Y = 2.36 X + 18.93$ with correlation coefficient of 0.9914 (Fig. 3B). The lowest detectable concentration of the sensing strategy was 1.0×10^4 particles/mL, which was comparable to other biosensing strategies for exosomes detection, and the detailed results were shown in Table S2. The excellent analytical performance of this method is mainly due to these aspects. First, the high affinity of the aptamer to HER2 proteins allowed the G4 sequence to be fully exposed to form the G4-hemin. Second, the formed G4-hemin exerted superior peroxidase-like activity itself to catalytic the in situ deposition of numerous AuNPs-Ty on the exosomes, resulting the high SPR signal.

3.6. Specificity of the SPR biosensing strategy

The good specificity is one of the indispensable characteristics of an excellent biosensor, which directly affects the clinical application prospects of this method. Accordingly, we conducted follow-up researches to explore the specificity of this sensing strategy by choosing another four kinds of exosomes derived from different HER2-negative cancer cells (HeLa, LNCap, HepG2, and MCF-7) as interferents. As shown in Fig. 4, compared with these interferents, exosomes from HER2-positive SK-BR3 cells produced a significantly improved SPR signal. In general, the SPR signal of HER2-positive exosomes was 4.9 to 6.4 times to those of interfering substances at the same concentration (1.0×10^7 particles/mL). Besides, by detecting exosomes using the developed strategy, the cancer cells with high HER2 expression could be discriminated accurately. These results showed the excellent specificity of this method, which was mainly attributed to the dual-identification of HER2 proteins and the lipid membrane of exosomes.

3.7. Detection of exosomes in clinical samples

In order to evaluate the applicability of this method for the diagnosis of breast cancer, exosomes derived from real clinical samples were analyzed. We collected 16 serum samples from the First Affiliated Hospital of Chongqing Medical University. Among them, half of the samples were from HER2-positive breast cancer patients (P1~P8), and the rest were from healthy groups (H1~H8), which served as testing control. As shown in Fig. 5A, almost all SPR signals obtained by detecting exosomes from the serum of HER2-positive breast cancer patients were greater than 150 RU (except for P2), however, under the same circumstances, the signals obtained by detecting exosomes from healthy samples were all lower than 50 RU. In addition, the calculation of the significance probability P value also illustrated the significant difference between the two groups of data (Fig. 5B), implying the outstanding distinction ability of this method between HER2-positive breast cancer patients and healthy groups. These results shed light on the potential clinical application prospect of this sensing method.

4. Conclusions

In conclusion, a label-free SPR biosensor has been developed for highly sensitive and specific detection of HER2-positive exosomes based on the reformative TSA activated by target-induced MAB conversion. Different from the traditional TSA, the reformative TSA replacing HRP with G4-hemin fully avoids the intrinsic disadvantages of nature enzymes, thus possessing high potential for clinical transformation. And the biosensing strategy is able to simultaneously recognize HER2 proteins and the lipid membrane of the exosomes, which effectively eliminates the interference of free proteins in purified exosomes. Benefiting from the integrating of the MAB and the reformative TSA, large quantities of AuNPs-Ty are in-situ deposited on the HER2-positive exosome membrane, endowing the developed SPR biosensor with high sensitivity and specificity. Moreover, by simply changing the aptamer types, this sensing strategy can be easily extended for accurate detection of other exosome subtypes. More importantly, this method has capable of discerning patients with HER2-positive breast cancer from healthy individuals. Overall, this work offers a new SPR platform for exosome-based liquid biopsy in the diagnosis of breast cancer. Despite these results, a limitation of this method is that the experimental steps need to be further simplified to improve the flexibility in clinical applications.

Abbreviations

TSA
Tyramine signal amplification
MAB
Molecular aptamer beacon
SPR
Surface plasmon resonance
AuNPs-Ty
Tyramine-coated AuNPs

HRP
Horseradish peroxidase
HER2
Human epidermal growth factor receptor 2
AuNPs
Gold nanoparticles
G4-hemin
G-quadruplex-hemin, MCH:6-Mercapto-1-hexanol
FBS
Fetal Bovine Serum
DMEM
Dulbecco's Modified Eagle Medium
NHS
N-hydroxysuccinimide
EDC
N-(3-(dimethylamino) propyl)-N'-ethylcarbodiimide hydrochloride
PBS
Phosphate buffer
RU
Resonance units
TEM
Transmission electron microscopic
NTA
Nanoparticles tracking analysis.

Declarations

Authors' contributions

W. Chen, Z. Li, and W. Cheng performed all experimental work. J. Li, X. Li, and L. Liu conducted data analysis. T. Wu and H. Bai collected clinical samples. S. Ding and X. Li revised the manuscript and provided project guidance. X. Yu procured funding and data curation. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated and analyzed during this study are included in this article and additional file. The additional file is available. Characterization of the exosomes, characterization of G4-hemin, and Optimizations of reaction conditions (Additional file: Figures S1–S3). Sequences of oligonucleotides employed in this work (Table S1). Comparison of biosensing strategies for the detection of exosomes (Table S2).

Ethics approval and consent to participate

This study has been approved by the ethics committee of Chongqing Medical University and conducted in accordance with ethical guidelines.

Consent for publication

All authors have provided consent for the manuscript to be published.

Competing interests

The authors declare that they have no competing interests.

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Scheme 1

Scheme 1 is available in the Supplementary Files section.

Figures

Figure 1

Characterization of the signal amplifier of AuNPs-Ty. (A) TEM image of AuNPs. Scale bars = 100 nm, (B) UV-vis absorption spectra of AuNPs (black line) and AuNPs-Ty (red line).

Figure 2

Evaluation of the feasibility of the biosensing strategy. (A) Schematic diagram of the detection process. (B) Typical SPR sensorgrams of the biosensor (red line) and blank control without loading (black line). (C) SPR sensorgrams of the different conditions: (a) the intact sensing system, (b) the sensing system without hemin, and (c) blank control. (D) SPR sensorgrams of the different materials: (a) AuNPs-Ty, (b) Ty, (c) AuNPs, and (d) blank control. The concentration of HER2-positive exosomes derived from SK-BR3 cells is 1×10^7 particles/mL. All data expressed as mean $\pm$ standard variation ($n = 3$).

Figure 3

Evaluation of the sensitivity of the SPR biosensor. (A) SPR sensorgrams and (B) the calibration line for exosomes at different concentrations of 0.1, 2.5, 5, 10, 25, 50, 100 particles/mL e+5 (from a to g). All data expressed as mean $\pm$ standard variation ($n = 3$).

Figure 4

Evaluation of the specificity of the sensing strategy. (A) SPR detection sensorgrams and (B) the response signals of (a) blank control and exosomes (1.0×10^7 particles/mL) derived from (b) HeLa, (c) LNCap, (d) HepG2, (e) MCF-7, and (f) SK-BR3 cell lines, respectively. All data expressed as mean $\pm$ standard variation ($n = 3$).

Figure 5

Investigation of the clinical applicability of this biosensor. (A) Analysis of exosomes from clinical samples (breast cancer patients: P1~P8 and healthy control subjects: H1~H8) by the developed strategy and (B) the corresponding scatter plot. Significance was determined by Student unpaired t-test, ** $p < 0.05$.

Supplementary Files

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