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Highly sensitive detection of human cardiac myoglobin using a reverse sandwich immunoassay with a gold nanoparticle-enhanced surface plasmon resonance biosensor

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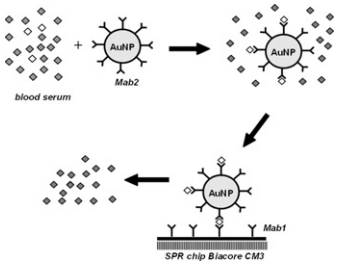
HIGHLIGHTS

- A highly sensitive immunoassay for detection of cardiac myoglobin was designed.
- A gold nanoparticles were used for SPR signal amplification.
- The limit of detection of cMb in a serum sample was found to be as low as 10 pM.

GRAPHICAL ABSTRACT



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ABSTRACT

A highly sensitive reverse sandwich immunoassay for the detection of human cardiac myoglobin (cMb) in serum was designed utilizing a gold nanoparticle (AuNP)-enhanced surface plasmon resonance (SPR) biosensor. First, a monoclonal anti-cMb antibody (Mab1) was covalently immobilized on the sensor surface. AuNPs were covalently conjugated to the second monoclonal anti-cMb antibody (Mab2) to form an immuno-gold reagent (Mab2-AuNP). The reverse sandwich immunoassay consists of two steps: (1) mixing the serum sample with Mab2-AuNP and incubation for the formation of cMb/Mab2-AuNP complexes and (2) sample injection over the sensor surface and evaluation of the Mab1/cMb/Mab2-AuNP complex formation, with the subsequent calculation of the cMb concentration in the serum. The biosensor signal was amplified approximately 30-fold compared with the direct reaction of cMb with Mab1 on the sensor surface. The limit of detection of cMb in a human blood serum sample was found to be as low as 10 pM (approx. 0.18 ng mL^{-1}), and the inter-assay coefficient of variation was less than 3%. Thus, the developed SPR-based reverse sandwich immunoassay has a sensitivity that is sufficient to measure cMb across a wide range of normal and pathological concentrations, allowing an adequate estimation of the disease severity and the monitoring of treatment.

1. Introduction

Myoglobin is a heme-containing oxygen-binding protein that is present in the cytoplasm of both cardiac and skeletal muscle cells. Cardiac myoglobin (cMb) is an early biomarker for diagnostics of acute myocardial infarction (AMI): due to its low molecular weight (17 kDa), myoglobin is rapidly released into the circulation when muscle cells are damaged. Indeed, the serum concentration of cMb is elevated above the normal range as early as 1 h after AMI [1] and correlates with the degree of the myocardial injury [2].

Currently used in medical practice immunoassays for AMI, biomarkers in blood serum have relatively low accuracy and

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sensitivity [3–5], complicating diagnostics and making the monitoring of disease treatment impossible. Therefore, new, more accurate assays are intensively being developed. Surface plasmon resonance (SPR) biosensor technology is a very promising approach [6] because it has a higher precision and reproducibility in comparison with standard enzyme-linked immunosorbent assay (ELISA) [7–10]. For example, a special comparative investigation has shown that the intra-assay coefficient of variation (CV, standard deviation/mean) and inter-assay CV for ELISA reached 22% and 38%, respectively, whereas the corresponding CV values for SPR do not exceed 0.4% and 4% (see the "Comparison of Biacore & ELISA" at http://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314774443672/litdoc28916813_20110831120514.pdf).

The SPR analysis of protein concentration based on its direct reaction with an immobilized affinity ligand (for example, a monoclonal antibody) has a detection limit of approximately 1 nM. However, the concentration level of AMI biomarkers in blood serum is low due to the extremely small damaged area of the heart muscle, and only a trace amount of protein biomarker is mixed with the large volume of circulating blood. It is for this reason that increasing the SPR biosensor sensitivity is highly important for the creation of the new SPR-based diagnostic methods. In recent years, several approaches to the amplification of an SPR signal have been reported, including a sandwich method with two antibodies [11–13], the usage of different nanoparticles [14–16] or latex particles [17], rolling-circle amplification [18] and enzymatic precipitation [19–21]. The application of gold nanoparticles (AuNPs) for SPR signal amplification seems the most promising approach, as the protein–colloidal gold complexes cause a higher refractive index than the protein or other biomolecules alone, and AuNP-enhanced SPR sandwich immunoassays were described [22–24]. AuNPs are usually used as strengthening agents that interact with an analyte previously captured on the sensor surface (direct sandwich immunoassay). At the first step, the direct reaction of the analyte with the first monoclonal antibody (Mab1), immobilized on the sensor surface, occurs when the sample is injected through the flow cell of the biosensor. The SPR signal is then amplified by sandwich formation during the binding of AuNPs conjugated with the second antibody (Mab2) [22] or biotinylated antibody and streptavidin modified AuNPs [23].

The main disadvantage of the direct sandwich assay is the long duration of the first phase (1 h or more) of analyte binding on the chip with Mab1, which is due to the extremely low concentration of the analyte in the sample and the small surface area of the chip. Therefore, this stage is actually a bottleneck in the serial analyses of samples.

The solution for this problem is to implement the analyte fishing at the first phase during the preparation of the serum samples. The molecular fishing occurs on the surface of the Mab2-AuNPs added to the serum. This approach has two significant advantages: (i) the analyte binding occurs on a considerably larger surface of the AuNPs distributed throughout the sample volume and (ii) the process of molecular fishing can be executed in parallel using many samples of serum. As a result, the average assay time can be significantly reduced whereby the biosensor analysis becomes a one-step process of binding the target protein complex to Mab2-AuNPs on the sensor surface with immobilized Mab1. In fact, this approach corresponds to a reverse sandwich immunoassay. Analogous assay was developed previously for the quantification of human tissue inhibitor of metalloproteinases-2 [24].

The purpose of this study was to develop a highly sensitive reverse sandwich immunoassay for human cardiac myoglobin (cMb) using an AuNP-enhanced SPR biosensor. This assay consists of two steps: (1) mixing the serum sample with immunogold reagent (Mab2-AuNPs conjugate) and incubation for the formation of cMb/Mab2-AuNPs complexes and (2) sample injection over the biosensor surface and evaluation of the Mab1/cMb/Mab2-AuNPs complex formation, with the subsequent calculation of the cMb concentration. The biosensor signal was amplified approximately 30-fold in comparison with the direct reaction of cMb with Mab1 on the sensor surface. The limit of detection of cMb in a human blood serum sample was found to be as low as 10 pM (approx. 0.18 ng mL^{-1}), and the inter-assay coefficient of variation (CV) was less than 3%. The measurable range covered concentrations from 1 pM to 20 nM cMb in human blood sera.

2. Materials and methods

2.1. Chemicals

The following reagents for the Biacore optical biosensor were obtained from GE Healthcare (Russia): (1) HBS buffer (150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20 and 10 mM HEPES, pH 7.4); (2) 10 mM acetate buffer (pH 5.0); (3) 10 mM glycine–HCl (pH 2.5) and (4) an amine coupling kit containing EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride), NHS (N-hydroxysuccinimide) and ethanolamine–HCl (1 M, pH 8.5).

The following materials were obtained from USBio (USA): (1) human cardiac myoglobin (cMb) (catalog no. M9800-18); (2) a matched pair of mouse anti-human cMb monoclonal antibodies Mab1 (catalog no. M9800-16) and Mab2 (catalog no. M9800-16A) and (3) human myoglobin-free serum (catalog no. S1005-14).

Human myoglobin from skeletal muscle (sMb) was obtained from Serva (Germany) (catalog no. 29895.01).

Hydrogen tetrachloroaurate ($HAuCl_4$), sodium borohydride and sodium citrate were purchased from Sigma–Aldrich.

Other reagents were of analytical grade and obtained from local suppliers.

2.2. SPR measurements

All of the SPR experiments were performed using the Biacore 3000 biosensor (GE Healthcare, USA). The instrument was operated using the "Biacore Control v.4.1.1" software, and the data were evaluated using "Biacore Evaluation v.4.1". HBS was used as the running buffer for the SPR assays. All of the binding assays were performed with Biacore CM3 sensor chips containing a short carboxymethylated dextran matrix.

The covalent immobilization of proteins (cMb, sMb, Mab1 and Mab2) on the surface of the CM3 chip was performed using the standard EDC/NHS amino-coupling Biacore protocol. In all of the experiments, the flow cell 1 (Fc1) without the immobilized protein was considered as the reference cell for the correction of the signal responses.

After each cycle of SPR measurement, the sensing surface was regenerated by the injection of 10 mM glycine–HCl (pH 2.5) for 0.5 min at a flow rate of 50 μL min^{-1}. The sensing surface was stable for more than 30 binding and regeneration cycles, thus enabling the re-use of the sensor chip.

2.3. Preparation of antibody-coated gold nanoparticles (immunogold reagent)

Gold nanoparticles were synthesized by reducing $HAuCl_4$ with sodium borohydride in the presence of sodium citrate [25]. The prepared AuNPs were stored at 4 °C in the dark.

The antibody-coated AuNPs (Mab2-AuNPs) were prepared by mixing 0.1 mL Mab2 solution (50 μg mL^{-1} in 5 mM PBS, pH 7.5) with 0.9 mL AuNPs suspension and incubating at room temperature for 60 min. AuNP-antibody complexes are formed due to the reaction of protein SH groups with gold and the formation

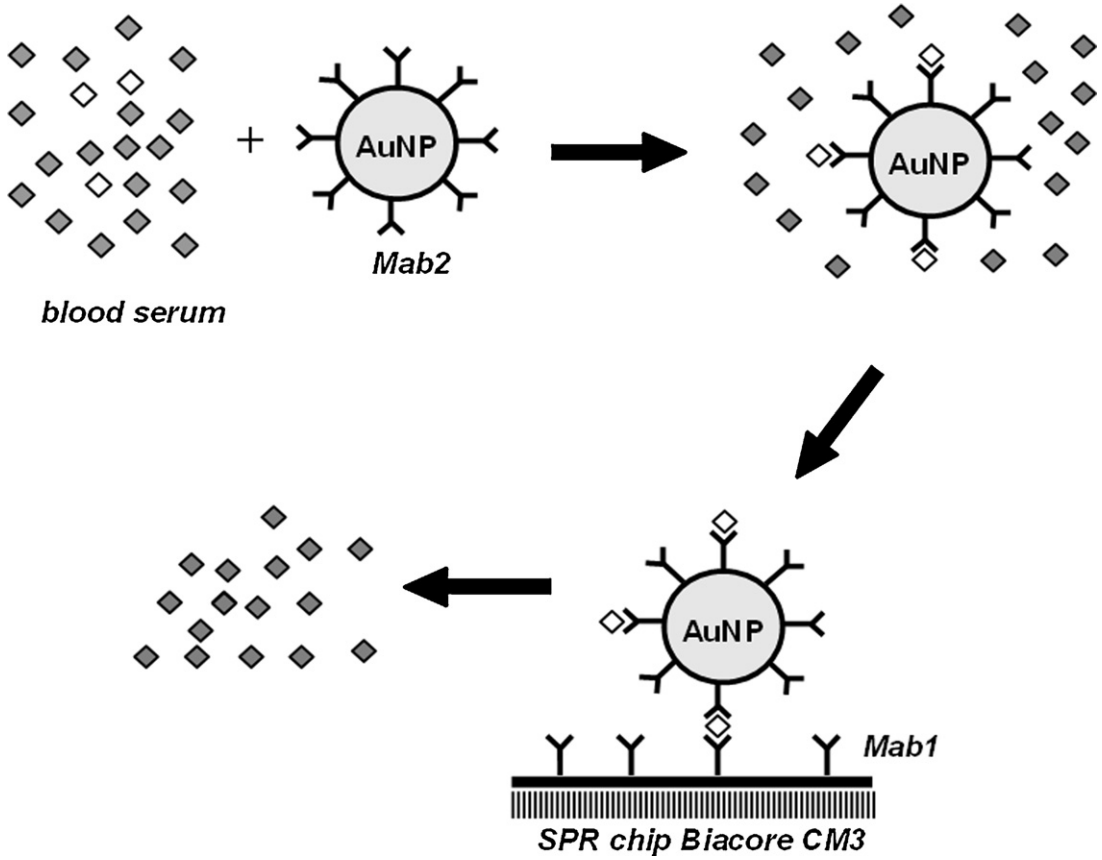

Fig. 1. A schematic diagram of the AuNP-enhanced SPR reverse sandwich immunoassay.

of Au–sulfur bonds. A blocking step was then performed by the addition of 0.2 mL BSA solution (10% [w/v] in 50 mM PBS, pH 9.0) and incubation at room temperature for 20 min. The Mab2-AuNPs were centrifuged at 16,000 × *g* for 60 min, and the pellet was re-suspended in HBS-buffer and stored at 4 °C for up to 2 weeks.

2.4. Preparation of cMb standard solutions and calibration samples of serum with given cMb concentrations

Standard solutions of cMb from 1 pM to 20 nM were obtained by diluting the stock solution by HBS.

The calibration samples of blood serum with given cMb concentrations from 1 pM to 20 nM were prepared by mixing the cMb stock solution with human myoglobin-free serum.

2.5. Evaluation of SPR signal amplification by AuNPs

For the evaluation of the SPR signal amplification, we compared the interaction of three samples containing the same cMb concentration (4 nM) with immobilized Mab1: (i) standard cMb solution in HBS; (ii) the complex cMb/Mab2 and (iii) the complex cMb/Mab2-AuNPs. Both of the complexes were prepared by mixing equal volumes of the Mab2 solution or Mab2-AuNPs suspension and standard cMb solution in HBS and incubating for 1 h at room temperature. All three of the samples were injected through the flow cell 2 (Fc2) with the immobilized Mab1 and the control flow cell (Fc1) for 5 min at a flow rate of 5 $\mu L\,min^{-1}$.

2.6. Design of the AuNP-enhanced SPR reverse sandwich immunoassay

Fig. 1 shows a schematic diagram illustrating the steps of the AuNP-enhanced SPR reverse sandwich immunoassay. First, the immunogold reagent (conjugate Mab2-AuNPs) is added to the sample of blood serum and incubated for 1 h at room temperature. The reaction between the serum cMb and immunogold reagent occurs with the formation of the primary complex cMb/Mab2-AuNPs. For serial analyses, this step can be performed simultaneously in a standard 96-well plate. Because the Biacore 3000 biosensor is compatible with such plates, the second step of the assay can be performed automatically by serial injections of the samples from the plate.

The serum mixture is injected through the biosensor flow cell (Fc2) with the immobilized Mab1 and the control flow cell (Fc1). The second reaction occurs with the formation of the sandwich Mab1/cMb/Mab2-AuNPs.

3. Results and discussion

3.1. Validation of antibody affinity and specificity

To assess the Mabs affinity and specificity, the interactions of Mab1 and Mab2 with two antigens (cMb and sMb) immobilized in different channels were analyzed in the range of the Mabs concentrations from 0.5 nM to 135 nM.

The obtained kinetics and equilibrium parameters for the antibody–antigen interactions are presented in Table 1. Mab2 interacted only with cMb, whereas Mab1 interacted with both types

Table 1
Association (k_{on}) and dissociation (k_{off}) rate constants and equilibrium dissociation constants (K_D) of the antigen–antibody complexes.

Pairs	k_{on}, M^{-1} s^{-1}	k_{off}, s^{-1}	K_D, M
Mb_{im}/Mab1	$(3.46 \pm 0.03) \times 10^5$	$(3.36 \pm 0.07) \times 10^{-3}$	$(9.7 \pm 0.2) \times 10^{-9}$
Mb_{im}/Mab2	$(1.20 \pm 0.01) \times 10^5$	$(5.7 \pm 0.5) \times 10^{-4}$	$(4.7 \pm 0.4) \times 10^{-9}$
sMb_{im}/Mab1	$(1.02 \pm 0.01) \times 10^5$	$(2.97 \pm 0.03) \times 10^{-3}$	$(2.91 \pm 0.04) \times 10^{-8}$
sMb_{im}/Mab2	No interaction		
$Mab1_{im}$/Mb	$(4.36 \pm 0.07) \times 10^4$	$(3.8 \pm 0.02) \times 10^{-3}$	$(8.7 \pm 0.1) \times 10^{-8}$
$Mab2_{im}$/Mb	$(2.02 \pm 0.07) \times 10^5$	$(9.6 \pm 0.7) \times 10^{-4}$	$(4.8 \pm 0.4) \times 10^{-9}$

of myoglobins (cMb and sMb). Consequently, Mab2 antibodies are specific and have higher affinities to cMb when compared to Mab1, which results from the difference in the dissociation rate constants of the cMb_{im}/Mab1 and cMb_{im}/Mab2 complexes. In addition, the K_D value for the cMb/Mab2 complex does not depend on which protein was immobilized. Mab2 was used for the preparation of the conjugate with AuNP.

3.2. Evaluation of the SPR signal amplification by AuNP

Mab1 was covalently immobilized on the surface of the CM3 optical chip in flow cell Fc2 at 5400 RU (equal to 5.4 ng of protein). The interactions of free cMb, complex cMb/Mab2 or complex cMb/Mab2-AuNPs with immobilized Mab1 were analyzed (see the typical sensograms in Fig. 2). Compared with the direct binding reaction of cMb with immobilized Mab1 (curve 1), complex cMb/Mab2 (curve 2) and complex cMb/Mab2-AuNPs (curve 3) amplified the biosensor signal by approximately 5- and 30-fold, respectively. It appears that the enhancement of the biosensor response using the Mab2 antibodies is not as substantial as with the conjugates of these antibodies with nanoparticles.

3.3. Implementation of AuNP-enhanced reverse sandwich SPR immunoassays

A number of sensograms for the calibration samples of blood sera with the given cMb concentrations (from 1 pM to 20 nM) was obtained for the direct and AuNP-enhanced reverse sandwich SPR immunoassays. The dependence of the biosensor signal on the cMb

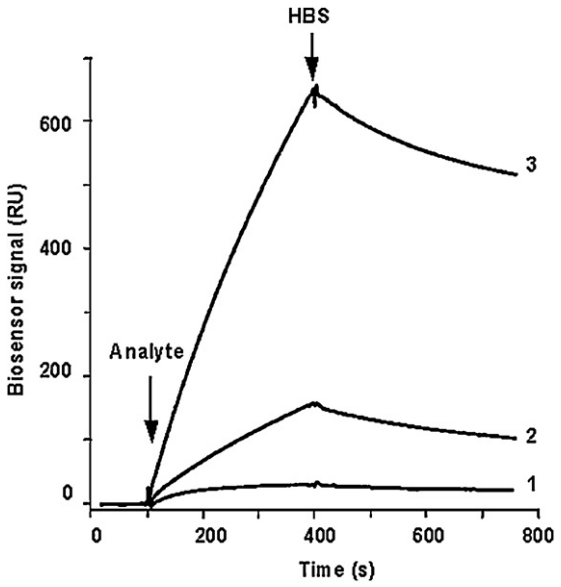

Fig. 2. Typical sensograms of the cMb (4 nM) interaction with immobilized Mab1 on the CM3 chip: free cMb (1), complex cMb/Mab2 (2) and complex cMb/Mab2-AuNP (3).

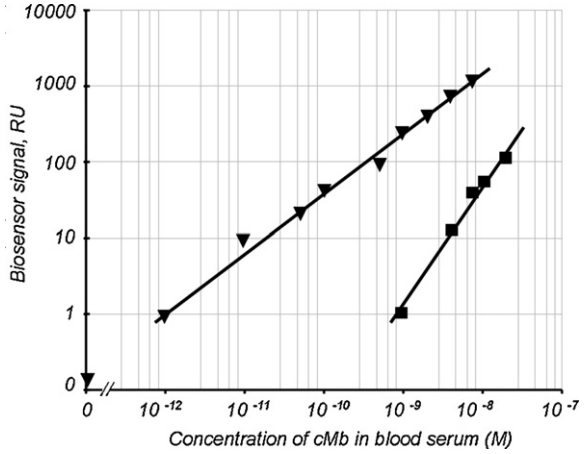

Fig. 3. Dependence of the biosensor signal on the cMb concentration in blood serum (calibration samples) in the direct SPR immunoassay (-■-) and in the AuNP-enhanced reverse sandwich SPR immunoassay (-▼-).

concentration in the calibration blood serum are shown in Fig. 3: the minimal detectable concentration of cMb decreased significantly in the case of the reverse sandwich immunoassay. In comparison with the direct assay based on the reaction of free cMb with immobilized Mab1, the reverse sandwich immunoassay exhibits a higher sensitivity due to the amplification of the biosensor signal.

The minimal detectable concentrations of cMb were determined with commonly used statistical analysis. Detection limit was defined as the smallest concentration of analyte that can be differentiated from zero with confidence. It was defined as the concentration of analyte that corresponds to a signal that is two standard deviations above the mean signal derived from multiple determinations of a sample free of analyte. The minimal concentration of biomarker that has been detected in the direct biosensor analysis was 4 nM (approx. 70 ng mL^{-1}), whereas this value was 10 pM (approx. 0.18 ng mL^{-1}) for the gold nanoparticle–antibody conjugate. Thus, the utilization of the conjugate resulted in a decrease of the limit of the minimal detected biomarker concentration by more than 2 orders. This detection result is comparable with other NP-enhanced SPR assays [23,26,27] and is better than the results for an electrochemical nanobiosensor [28]. At the same time, assay described in [24] allows to detect 0.5 pM of human tissue inhibitor of metalloproteinases-2. However, inactive form of matrix metalloproteinase-2 and monoclonal antibody to inhibitor were used as affine reagents. This assay is difficult adaptable for the analysis of other proteins. In our case, two antibodies interacting with two epitopes of the protein were used. Therefore, our approach is more universal for the analysis of any protein which has a suitable pair of antibodies.

3.4. Determination of intra- and inter-assay coefficients of variation (CVs)

The precision of the designed assay was estimated by calculating the intra-assay coefficient of variation (CV; standard deviation/mean) of multiple determinations of the same samples, all measured in a single batch. We have used multiple samples across the full range of measurable values and calculated the average intra-assay CV. The reliability of the designed assay was estimated by calculating the inter-assay CV (day-to-day variation) of multiple determinations of a single sample measured on different days. The average values of the intra- and inter-assay CVs were 0.6% and 2.8%, respectively.

4. Conclusion

A highly sensitive quantitative analysis of protein biomarkers in human blood serum is very important for the diagnoses of various diseases and the monitoring of their treatment. The low accuracy of conventional immunoassay methods results in an inadequate assessment of the severity of the disease and, in particular, the effectiveness of the treatment. These limitations apply to all of the assays for human cardiac biomarkers (cardiac myoglobin, troponin I, troponin T and creatine kinase).

In this study, we had developed a highly sensitive technology for the detection of human cardiac myoglobin in blood serum using a reverse sandwich immunoassay with AuNP-enhanced SPR biosensing for the purposes of diagnostics and the monitoring of heart attack treatment.

The developed technique can be applied for the analysis of other protein biomarkers. Moreover, this technology can be used to analyze multiple biomarkers simultaneously in one sample of blood serum using the mixture of several immunogold reagents, followed a separate analysis using different channels of the SPR biosensor with the corresponding immobilized Mab1.

Acknowledgements

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