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A highly-sensitive and rapid Surface Plasmon Resonance immunoassay procedure based on the covalent-orientated immobilization of antibodies

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Method Article

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Abstract

This method describes a highly-sensitive and rapid procedure for surface plasmon resonance \(SPR) immunoassays, which is based on the covalent-orientated immobilization of capture antibodies on 3-aminopropyltriethoxysilane \(APTES)-functionalized gold \(Au)-coated SPR chip. It involves sequentially the cleaning of Au surface; APTES-functionalization; covalent binding of protein A \(PrA) by heterobifunctional crosslinking using 1-ethyl-3-\(3-dimethylaminopropyl)-carbodiimide hydrochloride) \(EDC) and sulfo-N-hydroxysuccinimide \(sulfoNHS); blocking with 1% \(w/v) BSA; orientated immobilization of anti-human fetuin A \(HFA) by PrA; and, the detection of HFA. The developed procedure is highly sensitive, rapid and more cost-effective than the conventional procedure on commercial carboxymethyldextran-coated SPR chips. The anti-HFA antibody-bound SPR chips are prepared in 1.5 h and can be stored for one month at 4° C without any loss of functional activity. They detect HFA in the range of 0.6-20 ng mL^{-1} in only 10 min. This method is generic and can be employed for the detection of other analytes.

Introduction

Surface plasmon resonance \(SPR) is an optical technique that enables real-time and label-free detection of analytes based on their specific biomolecular interactions with antibodies bound to Au-coated SPR chip [1,2]. During the last decade, SPR has been widely employed in academia, healthcare and industries for highly diversified bioanalytical applications. The development of SPR-based immunoassays has immense utility in bioanalytical sciences and diagnostics, as they are rapid, real-time, label-free, highly reproducible, and have been demonstrated to be as sensitive as enzyme-linked immunosorbent immunoassay \(ELISA) [3-5]. We have recently demonstrated that the analytical performance of an SPR immunoassay is mainly dependent on the antibody immobilization strategy [6] as it determines the orientation, stability and affinity of immobilized antibodies [7,8]. Therefore, the screening of an appropriate antibody immobilization strategy for a particular SPR immunoassay is very important. The appropriate strategy should immobilize the antibody in an oriented manner such that its antigen binding sites are free for binding antigens [9] and its functionally active conformation is maintained [10]. The most widely used antibody immobilization chemistry for SPR immunoassays is the EDC-sulfoNHS-based heterobifunctional crosslinking of antibody by its amine groups to the carboxyl groups present on carboxymethyldextran-coated SPR chip. A wide range of carboxymethyldextran-coated SPR chips, commercially designated as CM7, CM5, CM4 and CM3, are available from GE Healthcare for various applications. This immobilization chemistry leads to a higher antibody immobilization density. However, it adversely affects the antigen detection capacity of antibodies as they may be captured via crosslinking of their amine groups present at/next to the antigen binding site [11]. Other antibody immobilization chemistries are also used, which are based on streptavidin \(SA)-biotin, nitriloacetic acid \(NTA)-poly-histidine, and thiol-Au interactions. However, these chemistries involve the modification of antibodies by poly-histidine tagging, biotinylation and thiolation, which may affect their functional activity. It will also decrease the reproducibility of SPR immunoassays as the modified antibody may vary from lot-to-lot. The

poly-histidine tag binds non-specifically to other metal-binding proteins, and suffers from continuous leeching of poly-histidine tagged antibodies bound to NTA-modified surfaces. Additionally, the SA-biotin and NTA-poly-histidine chemistries will be costly as they require the modification of carboxylmethyldextran-coated SPR chips with NTA and SA, respectively \(as done by GE Healthcare for NTA and SA chips). Several orientated antibody immobilization strategies have also been tried by researchers that involve the use of fragment crystallisable \(Fc) binding proteins such as protein A, protein G or protein A/G. These proteins bind specifically to the Fc region of antibodies keeping their fragment antigen-binding \(Fab) region free for binding antigens. The functionally active conformation of antibody is maintained in this strategy as it is bound in its native form without any chemical modification. Although multiple antigen-binding sites are present on each of these Fc binding proteins, they can only bind to a maximum of two antibodies due to steric considerations [10]. The Fc binding proteins are usually bound to the Au surface by random physical adsorption as they have high affinity towards Au. The interactions between Fc-binding protein and antibody can be broken using a regeneration solution, which regenerates the Fc binding protein-coated SPR chip, thereby enabling its multiple reuse for cost-effective immunoassays. Recently, we developed a covalent-orientated strategy for immobilizing antibodies on SPR chip, which was demonstrated to be much better in terms of its analytical performance in HFA immunoassay than the commercially-available CM5-dextran SPR chip and various other antibody immobilization strategies i.e. random, orientated and covalent [6]. The developed SPR immunoassay procedure based on this strategy is reported here. Initially the Au surface of SPR chip is cleaned by treating with a solution containing H_2SO_4 \(97.5%, v/v) and H_2O_2 \(30%, v/v) in a ratio of 2:1 \(v/v). The cleaned SPR chip is then functionalized with APTES, which forms a monolayer on the Au surface and generates free amine groups. Thereafter, the APTES-functionalized SPR chip is provided with EDC-sulfoNHS-activated PrA, which crosslinks PrA \(by its carboxyl groups) to APTES \(by its amine groups). The non-specific protein binding sites on PrA-bound SPR chip are blocked by treating with 1% \(w/v) BSA, which is followed by the orientated binding of anti-HFA antibody by PrA. The anti-HFA antibody-bound SPR chips are prepared in 1.5 h and can be stored at 4° C for one month without any loss of activity. They detect HFA in the range of 0.6-20 ng mL^{-1} in only 10 min. The developed procedure can be employed for the highly sensitive and rapid detection of analytes by immobilizing their specific antibodies on the Au surface.

Reagents

• Human Fetuin A ELISA kit \(R & D Systems, UK, cat. no. DY1184e) **\!CAUTION** Store reconstituted antibody and antigen at 4° C only if they are to be used within 2 months. Otherwise aliquot out and stored at -80° C for up to 6 months. Kit contains • Mouse anti-human fetuin A capture antibody \(720 µg mL^{-1}) • Human fetuin A/AHSG \(20 ng mL^{-1}) • Biotin-labeled goat anti-human fetuin A detection antibody • HRP-conjugated Streptavidin **\!CAUTION** Streptavidin is light-sensitive. Store in dark. • Blocker BSA in PBS \(10X), pH 7.4, 10% \(w/v) \(Thermo Scientific, Ireland, cat. no. 37525) **\!CRITICAL** Filter prior to use to remove microbial contamination. This is critical for surface blocking. • Protein A \(soluble, Cowan strain, recombinant, expressed in Escherichia coli) \(Sigma Aldrich, Ireland, cat. no. P7837).

\!CAUTION Store reconstituted protein A at 4° C only if it is to be used within 1 month. Otherwise aliquot out and stored at -20° C for up to 1 year. • Absolute ethanol \(Sigma Aldrich, Ireland, cat. no. 02856) **\!CAUTION** Highly flammable. Keep the container tightly closed. Keep away from sources of ignition. The alcohol vapors may be harmful for soft tissues like eyes or respiratory tract. Use in a safety cabinet or fume cupboard. • Sulphuric acid \(Aldrich, cat. no. 339741) **\!CAUTION** Strongly corrosive and an irritant. Avoid contact with any part of the body. Wear suitable protective clothing and safety glasses during handling. Handle in a safety cabinet or fume cupboard. In case of skin contamination, wash immediately with acid neutralizers and immediately seek medical advice. • KOH pellets \(99.99%), semiconductor grade \(Sigma Aldrich, Ireland, cat. no. 306568) **\!CAUTION** Can cause severe burns. Avoid contact with skin and eyes. Wear appropriate protection and handle in a safety cabinet. **CRITICAL** The concentration of KOH must be 1% \(w/v) in autoclaved deionised water \(DIW, 18Ω). It may affect the surface properties and antibody immobilization. • 3-aminopropyltriethoxysilane \(3-APTES) \(Sigma Aldrich, Ireland, cat. no. A3684) **\!CAUTION** Skin and eye irritant. Potential toxicity towards kidney. **CRITICAL** Prepare in autoclaved DIW \(18Ω), see REAGENT SETUP. • 1-Ethyl-3-\(3-dimethylaminopropyl)-carbodiimide hydrochloride \(EDC) \(Thermo Scientific, Ireland, cat. no. 22981) **\!CAUTION** Irritant. Handle inside a fume cupboard. EDC is hygroscopic, absorbs moisture and may lose activity. Equilibrate to room temperature \(RT) before opening the container. **CRITICAL** Store at recommended temperature \(-20° C). Reconstitute in 0.1M MES, pH 4.7, see REAGENT SETUP. • Sulfo-N-Hydroxysuccinmide \(SulfoNHS) \(Thermo Scientific, Ireland, cat. no. 24525) **CRITICAL** Store at recommended temperature \(4° C). Reconstitute in 0.1M MES, pH 4.7, see REAGENT SETUP. • BupH Phosphate Buffered Saline Packs \(0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.2) \(Thermo Scientific, Ireland, cat. no. 18372) **\!CAUTION** Avoid inhaling the powder dust. **CRITICAL** Prepare in autoclaved DIW \(18Ω), see REAGENT SETUP • BupH MES Buffered Saline Packs \(0.1 M MES \[2-\(N-morpholino)ethane sulfonic acid], 0.9 % \(w/v) sodium chloride, pH 4.7) \(Thermo Scientific, Ireland, cat. no. 28390) **\!CAUTION** Avoid inhalation. **CRITICAL** Prepare in autoclaved DIW \(18Ω), see REAGENT SETUP. • HBS-EP \(0.01 M 4-\(2-hydroxyethyl)-1-piperazineethanesulfonic acid \(HEPES) pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v surfactant P20) \(GE Healthcare, UK, cat no. BR-1001-88). • Glycine-HCl \(10 mM, pH 2.0) \(GE Healthcare, UK, cat no. BR-1003-55). • Deionized water \(18Ω DIW). **REAGENT SETUP** **PBS** Add a sachet containing 0.1M phosphate and 0.15M NaCl to 100 mL of autoclaved DIW. Dissolve well and make the volume up to 500 mL using autoclaved DIW. Each sachet makes 500 mL of PBS at pH 7.2. It can be stored at room temperature for one week and at 4° C for four weeks. **MES** Add a sachet containing 0.1M MES and 0.9% \(w/w) NaCl to 100 mL of autoclaved DIW. Dissolve well and makes the volume up to 500 mL using autoclaved DIW. Each sachet makes 500 mL of MES at pH 4.7. It can be stored at room temperature for two weeks. **APTES** The solution was supplied at 99% purity. Reconstitute in autoclaved DIW to make an effective 2% \(v/v) solution. Prepare a fresh solution for each functionalization. **EDC** Each pack contains 25 g EDC. Reconstitute in 0.1M MES buffer, pH 4.7, at a concentration of 8 mg mL^{-1}. Mix it with equal volumes of sulfoNHS solution \(22 mg mL^{-1}). Aliquots can be stored effectively for six months at -20° C. **SulfoNHS** Each sample vial contains 5 g sulfoNHS. Reconstitute in 0.1 M MES, pH 4.7, at a concentration of 22 mg mL^{-1}. Mix with equal volumes of EDC \(8 mg mL^{-1}). Aliquots can be stored effectively for six months at -20° C.

H_2SO_4 The concentrated solution is 97.5%. **H_2O_2** The concentrated solution is 30%. **Au surface cleaning solution** It is a mixture of H_2SO_4 \(97.5%, v/v) and H_2O_2 \(30%, v/v) in a ratio of 2:1 \(v/v). 60 µL of H_2SO_4 \(97.5%, v/v) is initially dispensed on the Au-coated surface followed by immediate dispensing of 30 µL of H_2O_2 \(30%, v/v). It is very similar to the piranha solution. The solution is always prepared fresh.

Equipment

• -70° C freezer \(operating range -60 to -80° C) \(New Brunswick) • 2-8° C Refrigerator \(Future, UK) • BiacoreTM 3000 surface plasmon resonance instrument \(GE Healthcare, Uppsala , Sweden) • PVC fume cupboard \(Chemflow range) \(CSC Ltd., Ireland) • Eppendorf microtubes \(1.5mL; Sigma Aldrich, cat. no. Z606340) • SIA Kit Au \(GE Healthcare, cat. no. BR-1004-05) • SigmaPlot software bundle version 11.2 from Systat for curve plotting and detailed assay analysis

Procedure

Surface cleaning and APTES functionalization TIMING ~ 1 h 2 min 1. The Au-coated SPR chip was cleaned by incubating with Au surface cleaning solution for two minutes followed by extensive washing with DIW. It oxidizes the gold surface and generates hydroxyl groups. 2. The cleaned Au-coated SPR chip was then incubated with 100 µL of 2% \(v/v) APTES for 1 h at room temperature \(RT) in a fume hood followed by five washes with DIW. The procedure functionalizes the Au surface with APTES and generates amine groups on its surface. **EDC-sulfoNHS activation of PrA TIMING ~ 15 min** 3. PrA \(990µL of 100 µg/ml in HBS) was incubated with 10 µL of cross-linking solution containing EDC \(4 mg/mL) and sulfoNHS \(11 mg/mL) in 0.1 M MES buffer, pH 4.7, at room temperature for 15 min. The procedure activates the carboxyl groups on PrA with EDC-sulfoNHS. **CRITICAL STEP** The ratio of EDC and sulfoNHS is important for optimal cross-linking. Use the recommended ratios of EDC and sulfoNHS or optimize this ratio. **? TROUBLESHOOTING** **Immobilization of anti-HFA antibody TIMING ~ 12 min** 4. Fifty microliters of EDC-sulfoNHS activated PrA \(100 µg/mL) was injected over all four flow cells of an APTES-functionalized Au chip at a flow rate of 10 µL/min. It cross-linked EDC-sulfoNHS activated protein A to the APTES-functionalized Au chip. **? TROUBLESHOOTING** 5. Twenty microliters of 1% \(w/v) BSA was then injected for blocking. CRITICAL STEP Use filtered BSA or filter the BSA solution prior to use to remove any microbial or other contaminants. **? TROUBLESHOOTING** 6. After baseline stabilization, 50 µL of anti-HFA antibody \(100 µg/mL) diluted in 10 mM HBS, pH 7.4, was injected over all the flow cells at a flow rate of 10 µL/min. **HFA-detection TIMING ~ 10 min per concentration** 7. Fifty microliters of dilution buffer \(10mM HBS, pH 7.4) was passed through all flow cells before HFA capture and the resultant changes in SPR response units \(RU), corresponding to blanks, were recorded. 8. Fifty microliters of HFA at six different concentrations \(0.6, 1.2, 2.5, 5.0, 10.0 and 20.0 ng/mL) were passed through the flow cells. The RU values obtained for blanks were subtracted from the RU values for HFA detected in the respective flow cells. 9. The SPR-based HFA detection curves were plotted with SigmaPlot software, version 11.2 using standard curve analysis based on a four parameter

logistic function. The analytical parameters such as EC_{50} and Hillslope were generated by the software analysis report.

Timing

Steps 1-2, Surface activation: 1 h 2 min **Steps 3,** PrA activation: 15 min **Steps 4-6,** Antibody immobilization: 12 min **Step 7-9,** HFA detection: 10 min for each HFA concentration

Troubleshooting

Troubleshooting advice is provided in **Table 1**.

Step	Problem	Probable reason	Solution
Step 4	No binding of protein A	Au chip may not have been cleaned properly. If the problem is not resolved, proceed to the next troubleshooting step.	Clean the Au chip again.
		APTES may have been hydrolyzed/ degraded. Determine APTES functionalization by Rutherford backscattering, FTIR spectroscopy orninhydrin assay. If the problem is not resolved, proceed to the next troubleshooting step.	Make fresh APTES solution from a different batch.
		EDC may have been hydrolyzed/ degraded. If the problem is not resolved, proceed to the next troubleshooting step.	Make fresh EDC solution from a different stock.
		SulfoNHS may have been hydrolyzed/ degraded. If the problem is not resolved, proceed to the next troubleshooting step.	Make fresh sulfoNHS solution from a different stock.
Step 6	No binding of anti-HFA antibody	Protein A may have become non-functional.	Use a different stock of protein A. As proteins are highly susceptible to storage conditions, store them properly and avoid repeatedfreeze-thaw cycles.
Step 7	High Background	Check BSA quality. BSA may have been contaminated.	Make fresh BSA solution using a different stock.
Step 8	No HFA detection	Anti-HFA antibody may have become non-functional. If the problem is not resolved, proceed to the next troubleshooting step.	Use another stock of anti-HFA antibody.
		HFA may have become non-functional.	Use another stock of HFA.

Anticipated Results

The developed SPR immunoassay procedure based on the covalent-oriented immobilization of antibodies, as shown in **Figure 1**, was employed for the detection of HFA . HFA was taken as the model system as all immunoassay components for HFA detection were commercially available. The developed SPR immunoassay had anti-HFA immobilization density of 145.1±1 ng cm^{-2} and detected HFA in the range of 0.6-20 ng mL^{-1} with an EC_{50} of 3.7 ng mL^{-1} and LOD of 0.6 ng mL^{-1}, as shown in **Figure 2**. The percentage coefficient of variance \(%CV) values for various HFA concentrations were in the range of 3.6-8.7. The anti-HFA bound SPR chips were effectively stored at 4° C for one month without any loss of functional activity, as shown in **Figure 3**. Once the SPR chip is used for HFA immunoassay, it can be reused by effectively regenerating it to PrA-bound SPR chip by treatment with 20 µL of 10 mM glycine-HCl pH 2.0. The treatment of Au-coated SPR chip with 2% APTES for 1 h was found to be the most appropriate after optimizing the concentration and duration of APTES-functionalization [6]. The greater concentrations of APTES form multilayers that decrease the SPR response as additional APTES molecules are randomly oriented in a manner that their amine groups are inaccessible for covalent binding. This was further confirmed by Rutherford back scattering [6], where the APTES density \(number of molecules per cm^2) was maximum for Au-coated SPR chips treated with 2% APTES. The developed SPR immunoassay procedure had better analytical performance than the most-widely used CM5-dextran based immunoassay procedure and various other antibody immobilization strategies [6]. The anti-HFA immobilization density of CM5-dextran based immunoassay procedure i.e. 172.8±1.2 ng cm^{-2} was greater than that of the developed procedure. But the amount of HFA detected by the developed SPR immunoassay procedure was greater than that of CM5-dextran procedure. Therefore, the developed procedure based on orientated immobilization of anti-HFA by PrA leads to more effective binding of antibodies such that their functional activity is not compromised. The EC_{50} of the developed SPR immunoassay for HFA detection was 3.7 ng mL^{-1}, which was lower than that of CM5-dextran based immunoassay i.e. 4.1 ng mL^{-1}. The lower EC_{50} signifies higher analytical sensitivity for the developed procedure. It had also better analytical performance in comparison to other immunoassay procedures based on random, covalent and orientated immobilization of anti-HFA antibody. Moreover, the long-term functional stability of the developed anti-HFA bound SPR chips will be highly useful in industrial and clinical settings, where a large number of samples need to be analyzed. The developed SPR immunoassay procedure can also be employed for the detection of other analytes by immobilizing their specific antibodies. Moreover, the devised covalent-orientated antibody immobilization strategy is multisubstrate-compatible and can be employed for developing immunoassays on a wide range of substrates \(including the inert ones) that are being used in various biosensor formats. The multisubstrate-compatibility of APTES-functionalization on different substrates has already been demonstrated by us for ELISA [7,12].

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Figures

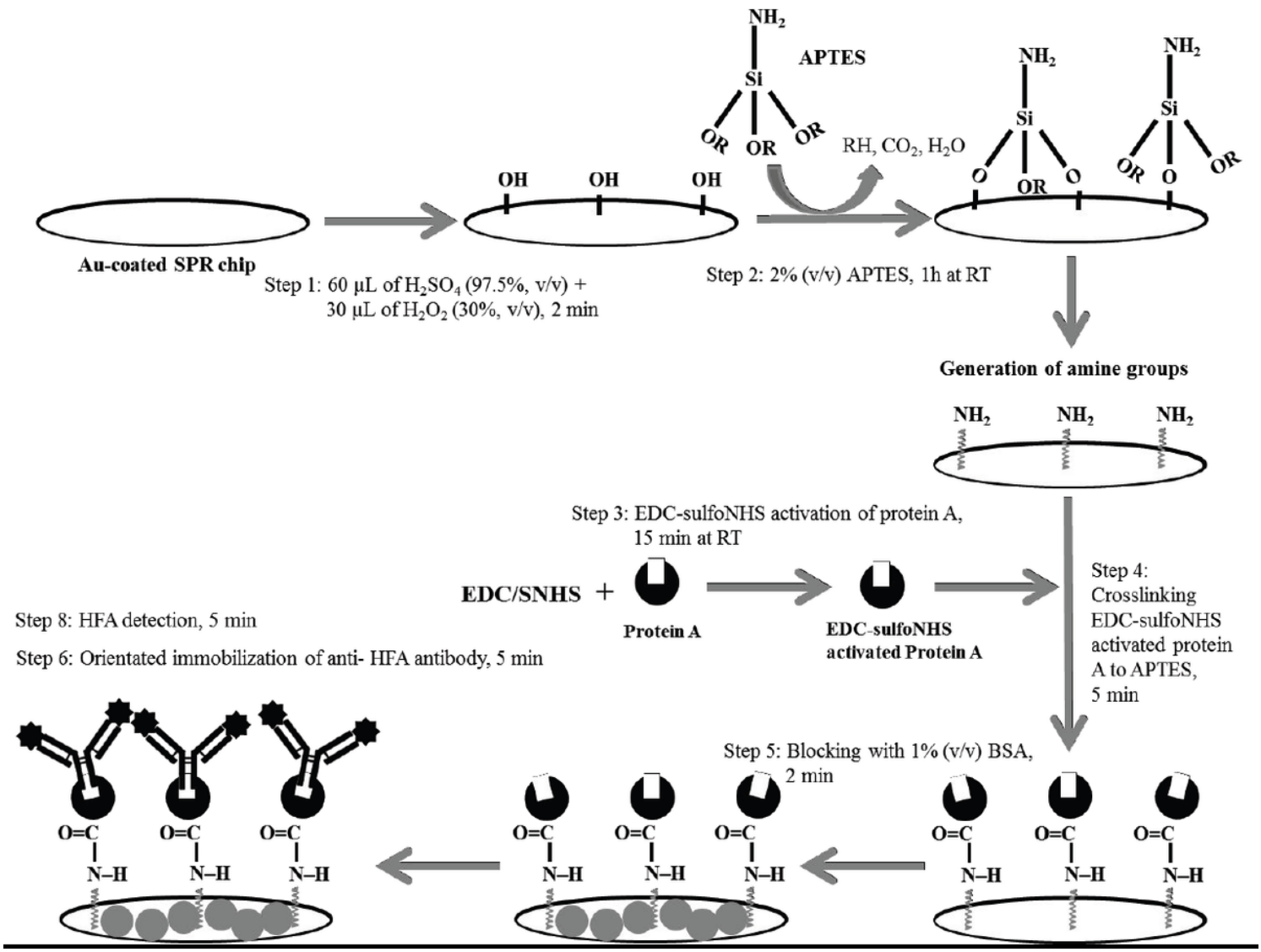

Figure 1

Schematic representation Schematic representation of the optimized covalent-orientated antibody immobilization strategy employed for SPR-based human fetuin A (HFA) immunoassay. The Au-coated SPR chip was cleaned by incubating in a cleaning solution [H~2~SO~4~ (97.5%, v/v): H~2~O~2~ (30%, v/v) = 2:1 (v/v)] for 2 min, which generates hydroxyl groups on the Au surface. It was then functionalized with APTES thereby generating free amine groups that were subsequently crosslinked to EDC-sulfoNHS activated protein A. The non-specific binding sites were blocked by 1% (w/v) BSA. The covalently-bound protein A then binds anti-HFA antibody in an orientated fashion, which is followed by the detection of HFA.

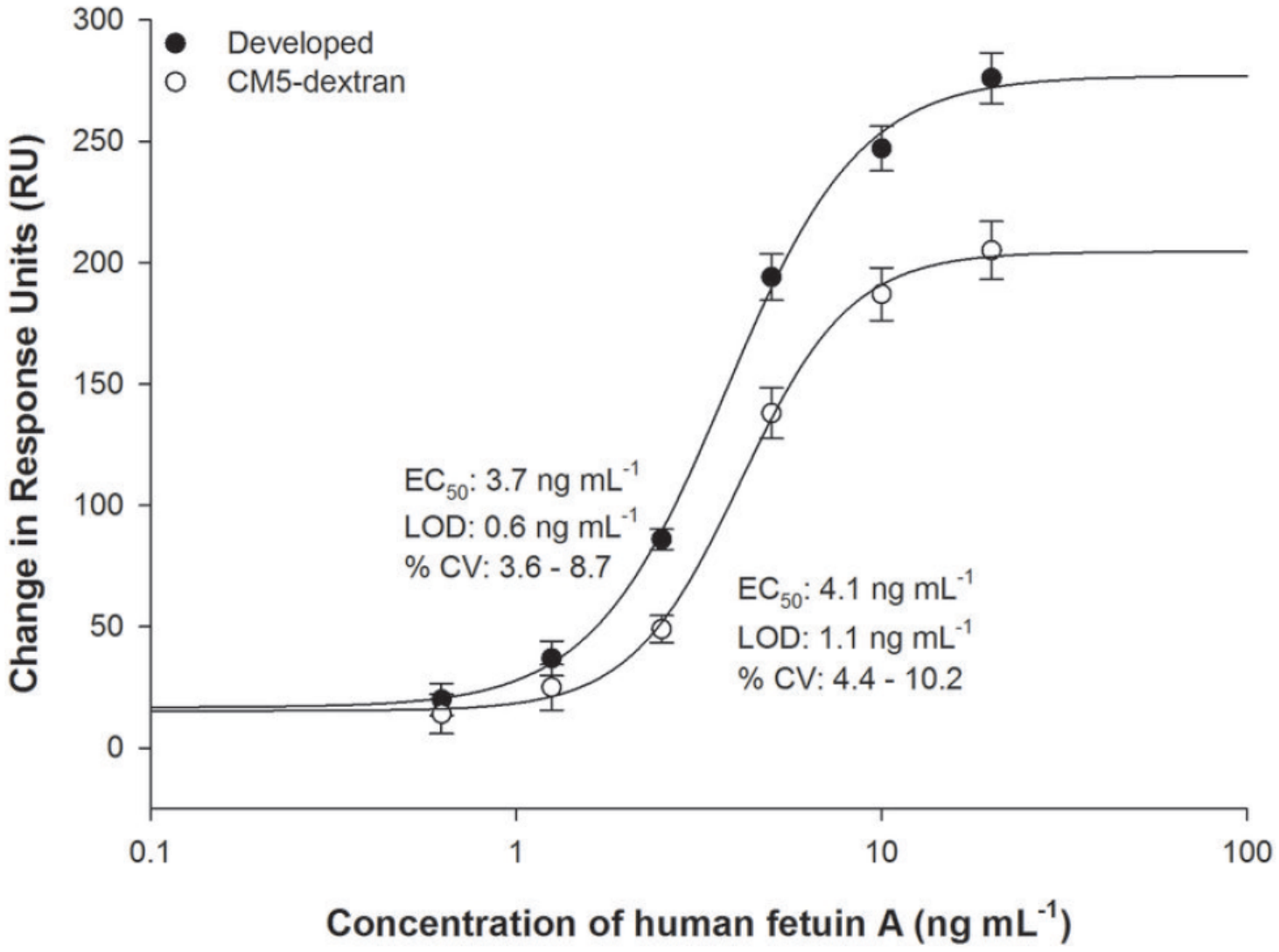

Figure 2

Comparison of developed SPR immunoassay procedure with that on CM5-dextran chip Comparison of the developed covalent-orientated antibody immobilization strategy based SPR immunoassay procedure with that on commercial carboxymethyldextran (CM5-dextran) chip. The results are shown as mean ± s.d. The half-maximum effective concentration (EC~50~) represents the concentration of HFA that generates 50% of the maximum signal on the standard curve of HFA immunoassay. LOD is the limit of detection of HFA immunoassay, while %CV is the percentage coefficient of variance in measuring the HFA concentrations in multiple repeats.

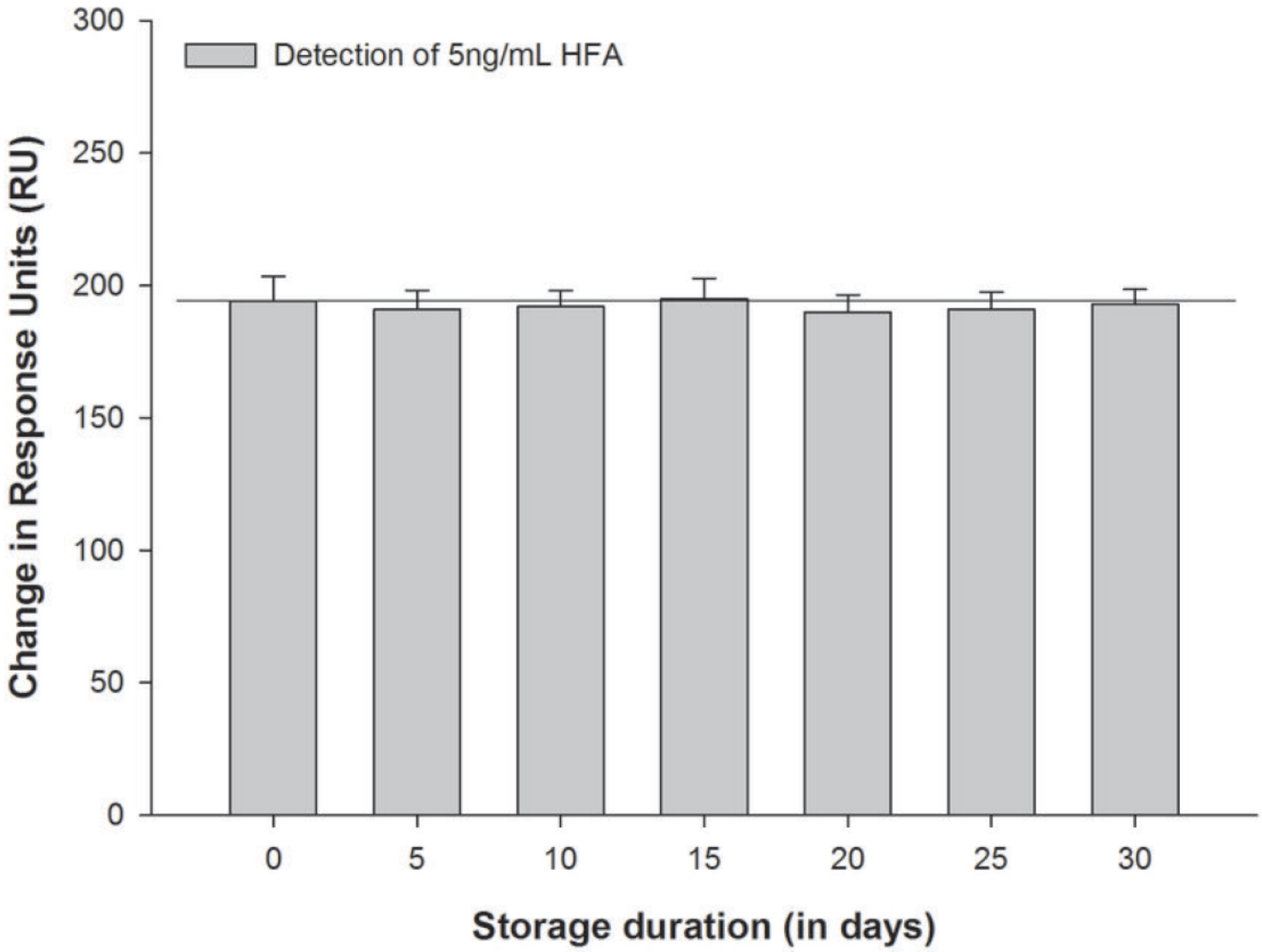

Figure 3

Stability of anti-HFA antibody bound SPR chips stored at 4 deg C for one month HFA immunoassay on the developed anti-HFA antibody-bound SPR chips stored at 4 ° C for one month. Initially seven anti-HFA antibody-bound SPR chips were prepared and 5 ng mL^-1^ HFA was detected every fifth day using a separate anti-HFA antibody-bound SPR chip.

Step	Problem	Probable reason	Solution
Step 4	No binding of PrA	Au chip may not have been cleaned properly. If the problem is not resolved, proceed to the next troubleshooting step.	Clean the Au chip again.
Step 4	No binding of PrA	APTES may have been hydrolyzed/ degraded. Determine APTES functionalization by Rutherford backscattering, FTIR spectroscopy or ninhydrin assay. If the problem is not resolved, proceed to the next troubleshooting step.	Make fresh APTES solution from a different batch.
Step 4	No binding of PrA	EDC may have been hydrolyzed/ degraded. If the problem is not resolved, proceed to the next troubleshooting step.	Make fresh EDC solution from a different stock.
Step 4	No binding of PrA	SulfoNHS may have been hydrolyzed/ degraded. If the problem is not resolved, proceed to the next troubleshooting step.	Make fresh sulfoNHS solution from a different stock.
Step 6	No binding of anti-HFA antibody	PrA may have become non-functional.	Use a different stock of PrA. As proteins are highly susceptible to storage conditions, store them properly and avoid repeated freeze-thaw cycles.
Step 7	High Background	Check BSA quality. BSA may have been contaminated.	Make fresh BSA solution using a different stock.
Step 8	No HFA detection	Anti-HFA antibody may have become non-functional. If the problem is not resolved, proceed to the next troubleshooting step.	Use another stock of anti-HFA antibody.
Step 8	No HFA detection	HFA may have become non-functional.	Use another stock of HFA.

Figure 4

Table 1 Troubleshooting Troubleshooting Table