Development of a label-free competitive ligand binding assay with human serum albumin on a molecularly engineered surface plasmon resonance sensor chip†

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The binding interactions between ligands and receptors play a vital role in their biological functions. In this work, a label-free indirect competitive binding assay based on surface plasmon resonance (SPR) detection was developed, using fatty acid–human serum albumin (HSA) as a model system. In the assay, a fatty acid analog was immobilized on the surface of an SPR gold chip by self-assembly, and was allowed to bind with HSA in solution. Addition of a ligand under investigation into the solution set up a competition with the analog for the protein, causing a reduction in the SPR signal. Due to the asymmetric structure of the ligand binding channel, HSA exhibited remarkable selectivity for the orientation of the fatty acid analog on a gold surface. When the carboxylic acid group of the analog was exposed on the sensor surface, the amount of bound HSA was very low. However, it increased by more than 7 fold when the alkyl chain was exposed. Dilution of the surface coverage of the fatty acid analog also enhanced HSA binding by reducing steric hindrance on the surface. Using a 1 : 4 mercaptoundecanoic acid–mercaptohexanol modified surface derivatized with octylamine, competitive binding assays for octanoic acid and perfluorooctane sulfonate with HSA were conducted. Using a derived kinetic equilibrium model, the equilibrium association constant was determined to be $4.57 \times 10^5 \text{ M}^{-1}$ and $2.11 \times 10^5 \text{ M}^{-1}$, respectively, which are consistent with the values measured by other established methods. The engineered sensor chip surface can be used to study the binding interactions of other chemicals with this important serum transport protein.

Introduction

The non-covalent binding interaction of small molecules, either endogenous or exogenous, with biological macromolecules such as receptors, transport proteins and DNAs is a vital process for their *in vivo* functions. Endogenous hormones modulate gene expression through the mediation of intracellular hormone receptors which act as transcription factors. These receptors are involved in the modulation of a wide variety of physiological functions including cell growth and proliferation, cell differentiation, and maintaining homeostasis. The hormone first binds with the receptor and induces its conformational change. The latter subsequently interacts with the corresponding element on DNA and initiates gene expression.^{1,2} After entering the blood, small ligands are usually associated with serum albumin as well as other more specific transport proteins such as fatty acid

binding protein or thyroid binding globulin. The distribution, bio-availability and excretion of the ligand are dictated by the extent and strength of protein binding.³ Binding interaction with DNA is also the first step in the DNA-damaging action and genotoxic process of an important class of environmental contaminants, polycyclic aromatic hydrocarbons (PAHs). In the commonly accepted chemical carcinogenesis mechanism, a PAH is converted to a reactive diol–epoxide by metabolizing enzymes, which then intercalates rapidly between the base pairs of a DNA helix, followed by covalent adduct formation to the deoxy-guanosines or deoxyadenosines in DNA.^{4,5}

Many analytical methods have been developed for the study of ligand binding interactions. One of the most frequently used methods is radio-labeled competitive ligand binding assay, in which a radio-isotope labeled ligand is employed as a tracer, and the ligand under investigation competes with the tracer for receptor binding. This method has proved to be very successful because isotope labeling imposes minimum structural change on the ligand, and the signal detection is highly sensitive. However, radio-labeled chemical reagents are often costly, and also present a safety hazard. In addition, the measurement requires physical separation between receptor-bound and free tracer, which is very inconvenient and is also a potential source of deviation from

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equilibrium.⁶ Fluorescence competitive binding assay is another extensively used method for studying ligand–receptor interactions.^{7–9} The most distinctive feature of the method is its homogeneous format, which makes the measurement very simple and convenient to perform. However, conjugation of a fluorescent group to a small ligand can potentially disrupt the original structure of the ligand and drastically change its binding properties.

The last three decades have witnessed rapid advancement and wide spread applications of surface plasmon resonance (SPR) sensors in various fields, including measurement of physical quantities, chemical sensing, and real-time biospecific interaction analysis.^{10–12} Perhaps the most outstanding advantage of the SPR biosensor is its label-free signal detection technology (without the need for radioactive or fluorescent labels), which allows for real time monitoring of the binding events at the sensor interface and straightforward extraction of kinetic information.^{13,14} As such, SPR has become a standard tool for the investigation of biomolecular interactions in life science research and drug discovery. Due to the relatively large change in refractive index at the sensor interface upon binding, in most of the studies the analyte is a high-mass molecule.

SPR ligand binding assays can be conducted in either a direct or indirect (competitive) format. In a direct assay, a biological recognition molecule such as a protein or receptor is immobilized on an SPR gold chip, and a ligand in solution is allowed to bind with the protein and subsequently be quantified. Since small molecules produce very low SPR signals, the direct assay requires high precision and high sensitivity instruments.¹⁵ In a competitive assay, a ligand analog is immobilized on the chip surface, and the protein in solution binds to the surface. Addition of a ligand under investigation to the protein solution sets up a competition with the surface analog for the protein binding, leading to a reduction in SPR signal (Scheme 1). The advantage of the competitive format is that the SPR signal originates from protein binding, and is therefore relatively easy to be measured. However, design of a ligand analog that can be immobilized on the surface and yet recognized by its protein partner is very critical and also highly challenging.^{16,17}

In the present work, an SPR-based competitive ligand binding assay was developed to study the interactions between small molecules and human serum albumin (HSA), a high abundance transport protein in human plasma. The assay relies on delicate engineering of the SPR sensor surface and controlled orientation of the HSA-binding element on the surface, which facilitates

specific protein binding and minimizes non-specific adsorption. Two representative small ligands, octanoic acid (OA, an endogenous ligand) and perfluorooctane sulfonate (PFOS, an emerging environmental contaminant), were evaluated by the SPR method. The equilibrium association constants obtained from the assays are in good agreement with previously reported values.

Experimental

Reagents

6-Mercapto-1-hexanol (MH), 11-mercaptopundecanoic acid (MUDA), 15-mercaptopentadecanoic acid (MPDA), octylamine, human serum albumin (HSA), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and PFOS (perfluorooctane sulfonate, potassium salt) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanolamine hydrochloride (EA) was obtained from Alfa Aesar (Ward Hill, MA, USA). Mouse immunoglobulin (IgG) was from Xijingke Corporation (Beijing, China), and OA was purchased from Tianjin Chemicals Co. (Tianjin, China). All other reagents were acquired from commercial vendors with analytical purity. Solutions were prepared in deionized water from a Millipore Milli-Q (Biocel) water purification system (Billerica, MA, USA).

SPR gold chip modification

Bare gold sensor chips (from Biosensing Instrument Inc., Tempe, AZ, USA) were cleaned in Piranha solution (1 : 3 mixture of 30% hydrogen peroxide and sulfuric acid) for 5 min. They were then rinsed thoroughly with deionised water, and dried in a nitrogen stream. To prepare surfaces with exposed carboxylic acid, the cleaned Au chips were immersed in a mixture of 1 mM COOH-terminated alkanethiols and 4 mM MH in ethanol for at least 12 hours. After adsorption, the gold chips were rinsed sequentially with ethanol and water, and dried with nitrogen before use. Alkyl chain exposed surfaces were prepared by *in situ* reaction on a BI-SPR 1000 system (also from Biosensing Instrument Inc.) equipped with a dual-channel flow cell. Au chips with exposed carboxylic acid were prepared first, as described above. They were then loaded into the flow cell of the SPR instrument. The carboxylic acid surface was activated for 5 min by injecting 50 μ L mixture of 100 mM NHS and 400 mM EDC, using PBS (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) as the running buffer. After surface activation, 50 μ L of 1 mM octylamine solution (in PBS with 3% DMF) was injected at a rate of 10 μ L min^{−1} to obtain a surface with exposed alkyl chain. The residual activated carboxyl groups were blocked by injection of 50 μ L EA solution (1 M in deionized water and adjusted to pH 8.5 by NaOH).

SPR measurements

To investigate the surface binding properties of HSA, 50 μ L protein in PBS was injected into the measurement channel at a flow rate of 10 μ L min^{−1} while the SPR response was recorded. After each measurement, the modified Au chip surface was regenerated by injecting 50 μ L of a mixture of acetonitrile and 0.1 M NaOH (1 : 4, v/v). For competitive binding assays of a

Scheme 1 Illustration of the SPR biosensor competitive ligand binding assay.

ligand, a 2 mM stock solution of the ligand was prepared by first dissolving it in DMF (*N,N*-dimethylformamide) and then diluting it in PBS with the final DMF content less than 3%. Ligands with different concentrations were pre-incubated with 1 μ M HSA for 60 min. A 50 μ L mixture solution containing 1 μ M HSA and different concentrations of the ligand was injected over the Au chip surface modified with octylamine. A sensorgram for each injection was recorded. Triplicate measurements were performed for each ligand concentration. The sensor chip surface was regenerated by injecting 50 μ L of a mixture of acetonitrile and 0.1 M NaOH (1 : 4, v/v) to remove surface-bound protein. After each regeneration, the SPR signal returned to the original level (base line). The SPR angle shift at different concentrations of the ligand was reproducible on repeated measurements ($n = 3$) with a standard deviation of less than 5%. The modified sensor surface was stable for at least one day with 50 injection–regeneration cycles. The SPR responses on the used sensor surface would decrease on the second day, due to the degradation and contamination of the sensor surface. Thus we always used a new sensor chip for daily experiments.

Curve fitting

Nonlinear curve fitting was performed with SigmaPlot for Windows, version 10.0, from Systat Software Inc. (Chicago, IL, USA).

Results and discussion

To develop a competitive ligand binding assay with HSA, a fatty acid analogue surface was engineered on an SPR chip. The design employed the well established approach in which a monolayer of a two alkanethiol mixture was fabricated on the gold substrate by molecular self-assembly (Fig. 1A). The active component of the monolayer is an alkanethiol with a structure similar to fatty acids, which is designed to bind selectively with HSA. The other alkanethiol is MH, which serves as a surface blocker to minimize non-specific binding of HSA to the surface. The chain length of the active alkanethiol is longer than that of the blocker so as to leave some space in the vertical direction for protein binding.

The physiological role of HSA is to transport long-chain fatty acids in blood. Both high-resolution X-ray crystallography^{18–20} and NMR studies²¹ have identified at least seven binding sites for medium and long-chain fatty acids with variable affinities. A common feature of most of the binding sites is that the carboxylate moieties of the fatty acids interact with several basic and polar side-chains at the entrance of the binding channel by electrostatic and hydrogen bonding, whereas their methylene tails extend into the inside of the mostly hydrophobic channel. In view of this binding pattern, we initially designed two SPR surfaces with MUDA and MPDA, respectively, as the active component (Fig. 1A(b) and (c)). However, as can be seen in Fig. 1B, HSA binding to these two surfaces was very low in comparison with the control surface (surface with the blocker only). In our previous work, binding of octanoic acid with HSA was evaluated by fluorescence measurement, and a relatively high affinity constant ($1.1 \times 10^7 \text{ M}^{-1}$) was obtained.²² Since the difference in chain length between MPDA and MH is 9 methylenes, the low binding

Fig. 1 (A) Schematic diagram of the four differently modified SPR sensor surfaces: (a) MH alone, (b) a 1 : 4 mixed monolayer of MUDA and MH, (c) a 1 : 4 mixed monolayer of MPDA and MH, (d) a 1 : 4 mixed monolayer of MUDA and MH derivatized with octylamine. (B) Overlaid SPR sensorgrams generated by addition of 1 μ M HSA to the four sensor surfaces.

of HSA on the SPR surface is unlikely to be due to insufficient chain length.

It then occurred to us that the two modified surfaces with fatty acid analogs might not have the right orientation for protein binding. In order for the hydrocarbon chain of the alkanethiol to get into the binding channel of the protein, the carboxylate head group would have to get inside it first, which is energetically not favored. To mimic more closely the binding geometry on the chip, a third surface was fabricated, on which the carboxylic acid of MUDA was activated and then covalently linked with octylamine by forming an amide bond. In this configuration, an 8-carbon long methylene tail oriented outward, and a polar amide group located closer to the surface (Fig. 1A(d)). As expected, the amount of HSA bound to this surface was substantially higher than the other two. Presumably, the methylene tail extends into the hydrophobic binding channel of HSA, while the amide group interacts with the polar amino acid residues. The selectivity of HSA toward the orientation of the ligand on the sensor surface is truly remarkable.

Previous studies by many research groups have found that the surface coverage of the active component in a mixed monolayer is also very important, and that a high coverage is not necessarily optimal for protein binding. A similar trend was also observed in our current work. As the ratio of MUDA to MH in the alkanethiol mixture was increased, the SPR signal initially increased, but then decreased when the ratio was higher than 1 : 4 (Fig. 2). One can imagine that, at 1 : 4 ratio, there is enough space between adjacent active alkanethiols on the surface so that there is no steric hindrance when the protein approaches the surface.

After optimization of the surface structure and coverage, the HSA concentration dependence was investigated to select an appropriate concentration for competitive assays. Fig. 3A depicts

Fig. 2 SPR angle shifts after injection of 1 μM HSA to the alkyl chain exposed sensor surfaces. The bare gold chips were immersed into solution mixtures of MUDA and MH with different molar ratios, and then covalently linked to octylamine.

the SPR angle shift of a series of injections of HSA to the flow cell. The response increased progressively with HSA concentration in the range of 0.25 μM –1 μM , but saturated at higher concentrations, with an estimated EC_{50} of approximately 1 μM . The SPR signal is found to be specific to HSA, as 1 μM mouse IgG did not produce any significant response (curve a in Fig. 3A). The sensorgrams were first fitted with different kinetic models (1 : 1 binding, 1 : 1 binding with mass transfer, bivalent analyte and

Fig. 3 (A) Overlaid sensorgrams after injection of 1 μM IgG (a) or different concentrations of HSA (from b to g, 0.25 μM , 0.5 μM , 1 μM , 2.5 μM , 5 μM , 10 μM) on a sensor surface modified with 1 : 4 MUDA–MH derivatized with octylamine, (B) plot of the equilibrium SPR angle as a function of HSA concentration. The line is the fit with the Langmuir binding isotherm.

conformation change) using BIA evaluation Software 4.1.1. The results are shown in Fig. S1 and Table S1 in the ESI.† According to Fig. S1,† the sensorgrams are not fitted well with any of these models. It is probably due to the existence of multiple binding sites on HSA with different binding affinities. We then performed equilibrium analysis using the Langmuir binding isotherm (eqn (1)), where B_{max} is the maximum binding capacity of the sensor surface, $[A]$ and K_D are the concentration of analyte in solution and the equilibrium dissociation constant, respectively. The equilibrium SPR angles (R_{eq} , in millidegrees) at different HSA concentrations are shown in Fig. 3B. The calculated value of K_D is $1130 \pm 350 \text{ nM}$. It should be noted that, according to the previous studies, HSA has one primary octanoic acid binding site with high affinity and several secondary sites with low affinity (about three orders of magnitude lower).²³ Consequently, the calculated K_D value is most likely to be associated with the high affinity site, and the competition between ligand on the surface and analyte in solution mainly occurred at this site.

$$R_{\text{eq}} = \left(\frac{[A]_{\text{free}}}{[A]_{\text{free}} + K_D} \right) B_{\text{max}} \quad (1)$$

After all the experiments described above, a competitive ligand binding assay was performed, using octanoic acid as a model ligand. A solution mixture containing 1 μM HSA and varying concentrations (1 μM –1 mM) of octanoic acid was injected into the SPR flow cell, and the response was measured. As the ligand concentration in the mixture increased, more protein was displaced from the sensor surface, resulting in a reduction of SPR angle shift. The SPR signal at a given octanoic acid concentration was normalized to the one without the ligand (R/R_0), and plotted against the ligand concentration. Such a curve is typical of a competitive binding assay, with an IC_{50} of 56 μM (Fig. 4A).

Unlike labeled direct competitive binding assays, the equilibrium association constant of octanoic acid with HSA cannot be obtained from the IC_{50} value of the SPR measurement. It is therefore necessary to design a kinetic equilibrium model and data-analysis method. For a heterogeneous interface competitive reaction, if the inclusion equilibrium between a ligand in aqueous solution (aq) and a protein (HSA) forms a complex with a stoichiometry of $m : 1$, the overall process at the solid–aqueous interface can be expressed as follows,

$$K'_A = \frac{[\text{B}]}{[\text{HSA}]_{\text{free}}[\text{Ligand}]^m} \quad (4)$$

here A and B represent the FA–HSA complex on the sensor surface and ligand–HSA complex in the solution with equilibrium association constants of K_A and K'_A respectively. From eqn (4),

$$\frac{[\text{B}]}{[\text{HSA}]_{\text{free}}} = K'_A [\text{Ligand}]^m \quad (5)$$

According to the Langmuir binding isotherm, $R_{\text{eq}0}$ (without ligand) and R_{eq} (with ligand) are given by eqn (6) and (7), in which R_{eq} is the SPR angle (in milli-degree) at equilibrium,

Fig. 4 (A) Change of the relative SPR response (R/R_0) as a function of octanoic acid or PFOS concentration in the injected solution containing $1 \mu\text{M}$ HSA. The sensor surface was modified with a solution mixture of 1 : 4 MUDA–MH and then derivatized with octylamine. (B) Plot of $[B]/[HSA]_{\text{free}}$ as a function of the ligand concentration. Solid lines are the best fitted curves.

Table 1 Data from nonlinear fit based on eqn (5)

	R^2	K_A/M^{-1}	m
OA	0.8915	$(4.57 \pm 0.27) \times 10^5$	0.7459 ± 0.0038
PFOS	0.9059	$(2.11 \pm 0.21) \times 10^5$	0.8452 ± 0.0068

$[HSA]$ is the concentration of free HAS (in nM) in solution, B_{max} is the maximum binding capacity (in milli-degree), and K_D is the reciprocal of K_A (which has been determined above).

$$R_{\text{eq}0} = \left(\frac{[HSA]_{\text{free}0}}{[HSA]_{\text{free}0} + K_D} \right) B_{\text{max}} \quad (6)$$

$$R_{\text{eq}} = \left(\frac{[HSA]_{\text{free}}}{[HSA]_{\text{free}} + K_D} \right) B_{\text{max}} \quad (7)$$

We define α as a response parameter to indicate the degree of competition, then,

$$\alpha = \frac{R_{\text{eq}}}{R_{\text{eq}0}} = \left(\frac{[HSA]_{\text{free}}}{[HSA]_{\text{free}} + K_D} \right) \left(\frac{[HSA]_{\text{free}0} + K_D}{[HSA]_{\text{free}0}} \right) \quad (8)$$

Since in our experiment the sample is continuously replenished by flow injection, $[HSA]_{\text{free}0}$ should be equal to the total

concentration of injected HSA (1000 nM); K_D is 1130 nM which was determined by the Langmuir binding isotherm. Using eqn (8), a series of $[HSA]_{\text{free}}$ values are obtained when a series of concentrations of the ligand are added into the solution. In this system, the initial concentration of HSA is fixed at 1000 nM , therefore,

$$[HSA]_{\text{free}} + [B] = 1000 \text{ nM} \quad (9)$$

Thus, a series of $[B]$ values are also obtained for a series of concentrations of the ligand.

Based on eqn (5), a plot of $[B]/[HSA]_{\text{free}}$ vs. $[B]$ yields a curve with a prefactor of K_A' and a power of m . As shown in Fig. 4(B), the best fit of the experimental data has an R^2 of 0.8915 , with $K_A = (4.57 \pm 0.27) \times 10^5 \text{ M}^{-1}$, and $m = 0.7459 \pm 0.0038$ (Table 1). The association constant between OA and HSA has been measured previously by different methods,^{22–25} and is found to be in the range of $3.4 \times 10^4 \text{ M}^{-1}$ to $1.1 \times 10^7 \text{ M}^{-1}$. The K_A value measured by SPR is in good agreement with $2.5 \times 10^5 \text{ M}^{-1}$ obtained by the centrifugal ultrafiltration method.²³ As mentioned above, HSA has up to 7 binding sites for fatty acids. Most likely, the association constant obtained in the SPR measurement is a weighted average constant at multiple sites. In addition, there might be cooperative interactions between these sites, which may lead to nominally low binding stoichiometry.

The SPR method is further validated by evaluating another small molecule, PFOS. This chemical belongs to a class of emerging environmental contaminants called perfluorinated alkyl acids (PFAAs), which are used in a wide variety of industrial and household applications. Broad distribution of PFAAs in the environment, wildlife animals and humans has been observed.²⁶ Due to their structural similarity with fatty acids, PFAAs were suspected and later confirmed experimentally to bind with serum albumins.^{27–30} The competitive binding assay for PFOS was performed in the same way as that for OA. As shown in Fig. 4(B), the best fit of the experimental data has an R^2 of 0.9059 , with $K_A = (2.11 \pm 0.21) \times 10^5 \text{ M}^{-1}$, and $m = 0.8452 \pm 0.0068$ (Table 1). For comparison, the association constant is between $1.0 \times 10^4 \text{ M}^{-1}$ and $7.6 \times 10^6 \text{ M}^{-1}$ obtained by fluorescence measurements.^{29,30} Based on these results, PFOS may bind to HSA at the same site as fatty acids with similar affinity. Therefore, the chemical in principle has the ability to compete with and displace fatty acids from the protein, and consequently disrupt the transport and distribution of fatty acids *in vivo*.

Conclusions

By designing and fabricating a fatty acid modified SPR sensor surface with a head-in, tail-out orientation, favorable binding of HSA with the surface was achieved. Surface engineering on the molecular level enabled us to develop a label-free ligand binding assay with SPR detection. The equilibrium association constants of OA and PFOS with HSA derived from the SPR measurement are consistent with the established methods. The assay does not require any labeling, and does not use any radio-active materials. Since HSA has multiple ligand binding sites, the developed method in its current design is not able to identify which site or sites OA and PFOS interact with. We are currently searching for solutions to overcome this difficulty. HSA is a very important

and most abundant transport protein in plasma. With further development, the SPR method can be used to investigate the binding interactions of a large variety of endogenous ligands, drugs and chemical contaminants with HSA for the assessment of their pharmacokinetics, toxicokinetics and bio-availability.

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