Use of a surface plasmon resonance method to investigate antibiotic and plasma protein interactions

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The pharmacologic effect of an antibiotic is directly related to its unbound concentration at the site of infection. Most of the commercial antibiotics have been selected in part for their low propensity to interact with serum proteins. These “non-specific” interactions are classically evaluated by measuring the minimal inhibitory concentration in the presence of serum. As higher throughput technologies tend to lose information, surface plasmon resonance (SPR) is emerging as an informative medium-throughput technology for hit validation. Here we show that SPR is a useful automatic tool to quantify the interaction of model antibiotics with serum proteins, and delivers precise real-time kinetic data on this critical parameter.

Keywords: Surface plasmon resonance, serum proteins, antibiotics
Serum proteins play an important role in binding to many drugs, including antibiotics. In general, serum proteins decrease the free fraction of antibiotic available to eliminate bacteria, since only the non-protein bound molecules are pharmacologically active. The proteins involved in this sequestration are mainly human serum albumin (HSA), the most abundant serum protein (4% w/v), α-1-acid glycoprotein (AGP), and γ-globulin (4, 6, 11).

Currently, most of the reports of the inhibitory effects of serum proteins on antibiotics are derived from *in vitro* studies employing the minimal inhibitory concentration (MIC) method (8, 10) or time killing curves (16). These reports correlate well with *in vivo* data (13) and are useful to evaluate the potential of a new drug candidate. However, it is also necessary to rapidly and precisely characterize how a molecule binds to serum proteins in terms of affinity constants to drive the synthesis of new and more efficient analogs. A variety of physical techniques have been proposed to measure protein binding. The most classical are ultracentrifugation (10) or dialysis (7, 10) but other alternative techniques have been used like circular dichroism (1), and extrinsic fluorescence (15). More recently, surface plasmon resonance was proposed as a medium- to high-throughput alternative to evaluate the kinetics of relatively lipophylic drugs binding to human serum proteins in real time (14).

Antibiotics are characterized by a relatively high hydrophilicity as compared to other drug classes. Consequently, antibiotics have lower affinities for serum proteins. Because the development of fast analytical methods that allow the measurement of antibiotic-serum protein interaction kinetics with a small amount of sample is desirable, we have evaluated if SPR can measure low affinities, and how SPR can be used to pre-screen rapidly libraries of antibiotic candidates for their propensity to bind to serum proteins.

**MATERIAL AND METHODS**

**Bacterial strains, antimicrobial agents and media.** *S. aureus* reference strains CIP 76.25 (ATCC 25923) was used. *S. aureus* was grown, subcultured, and quantified in Mueller-Hinton broth (MHB) and on Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA). The following antimicrobial agents belonging to different antibiotic classes were selected.
Rifampicin, vancomycin, minocycline, fusidic acid, novobiocin, lincomycin, ofloxacin, cefotaxime, erythromycin, and the nonantibiotic molecule warfarin, used as control, were purchased from Sigma-Aldrich (St Louis, MO, USA). Depending on the experiment, the MHB medium was supplemented with human serum albumin, α-1-acid glycoprotein from human plasma, or γ-globulin from human blood (Sigma-Aldrich, St Louis, MO, USA).

**Surface plasmon resonance experiments.** SPR experiments were performed at 25 °C using a BIACORE 3000 apparatus (GE Healthcare, Biacore AB, Uppsala, Sweden).

Procedure for protein immobilization: HSA diluted to 40 µg/mL in 10 mM acetate buffer, pH 5.2, was immobilized on CM5 sensor chips using amine-coupling chemistry. The surface was blocked with 1 M ethanolamine, pH 8.0, and washed with three 30 s-pulses of 50 mM NaOH to remove free HSA. Human Immunoglobulin G (γ-globulins) (Sigma-Aldrich, St Louis, MO, USA) was immobilized on flow cell 3 using the same method. PDEA-modified AGP was immobilized on flow cell 4 using a standard surface thiol coupling procedure (9). The immobilization level ranges from 9000 to 12000 RU (Resonance Unit) for γ-globulins and HSA, and 7000-10000 RU for AGP.

**Ranking experiments:** Drugs (rifampicin, vancomycin, minocycline, fusidic acid, novobiocin, lincomycin, ofloxacin, cefotaxime, erythromycin, and warfarin) were prepared as 10 mM stock solutions in 100% DMSO. They were then diluted in PBS or PBS containing DMSO to reach the final concentrations of 250 µM in 5% DMSO. Binding studies were conducted in phosphate buffered saline containing 5% DMSO with a flow rate of 50 µL/min. For ranking of drug compound binding to HSA, IgG, and AGP, randomized duplicate samples of drugs at 250 µM were injected for 30 s over the immobilized proteins or a reference surface without protein. The surfaces were then washed with the running buffer until complete regeneration was achieved. For cleaning the flow system, a bypass-wash was performed with 50% DMSO and 5% DMSO between each injection. Buffer blanks were injected before each drug injection, and binding responses were corrected for DMSO bulk differences via calibration curves (eight DMSO solutions between 4.5-5.8 % DMSO) and normalized to the same 10000 RU immobilization level for HSA, AGP, and γ-globulins.

**Kinetic experiments:** the compounds were injected at various concentrations (from 400 µM to 0.1 µM in twofold dilutions) over the reference and HSA flow cells for 30 s at a flow rate of 50
µl/min. Each cycle consisted of a blank buffer injection before the antibiotic injection (30 s association, 30 s dissociation), and the system was cleaned using a bypass-wash procedure with 50% DMSO and 5% DMSO. Data (collected at a rate of 2.5Hz) obtained in the reference flow cell was subtracted from that obtained in the HSA flow cell. Responses from injections of drug compounds were extracted 10 s after the beginning of the injection. These responses were further corrected for DMSO effects by use of the calibration curves, and the final response values were used for dose-response plotting and for K_d determinations according to Frostell-Karlsson (5). The binding level of warfarin at 50 µM was measured at different moments, before, during, and after each drug sample to check the response stability of the protein-coated surface.

*Fraction of sites occupied by the antibiotics:* the dose-response curves were obtained by plotting RU responses (corrected from DMSO bulk response) against drug concentrations, or by plotting the fraction of sites occupied against drug concentrations. The fraction of sites occupied by the antibiotics on HSA was calculated as follows: 

\[
\text{Fraction} = \frac{R_{eq} \times MW_{HSA}}{R_{im} \times MW_{drug}},
\]

where \(R_{eq}\) is the response value in RU (corrected from DMSO bulk response), and \(R_{im}\) is the HSA immobilization level in RU.

**In vitro susceptibility studies.** The MICs of rifampicin, vancomycin, minocycline, fusidic acid, novobiocin, lincomycin, ofloxacin, cefotaxime, and erythromycin for *S. aureus CIP 76.25* were determined in duplicate by standard Clinical and Laboratory Standards Institute (formerly NCCLS) microdilution methods (12).

**Protein binding.** The impact of serum protein binding was assessed by examining the impact of human serum, HSA, AGP, and \(\gamma\) globulin on the activity of 9 antibiotics in vitro. To determine the effects of protein serum on in vitro antimicrobial properties, MIC tests were performed in the presence of 50% human serum, 4% HSA, 0.1% AGP, or 1.6% \(\gamma\) globulins. Concentrations of serum protein used for in vitro studies were selected on the basis of normal physiological concentrations. A reduced potency (higher MIC) in the presence of serum or protein serum was presumed to be caused by drug binding to serum proteins. The ratio of MICs measured in the presence of plasma protein on the MIC standard was used to estimate the effect of plasma proteins.

**RESULTS**
**Ranking of the antibiotic-serum protein interactions.** Nine antibiotics with different physicochemical properties were used to set up an experimental protocol to determine HSA, AGP and γ-globulins binding levels. Some of these have been previously characterized by microbiological methods but, in most the cases, the binding kinetics and affinities are unknown. HSA, AGP and γ-globulins differ in their molecular weight and their capacity to coat the surface of the sensor chip, which impairs the direct comparison of the binding responses. An average coating of 9000-12000 RU was observed, and we have normalized the response to an average level of 10000 RU. Equilibrium responses were collected in randomized duplicates for the 9 antibiotics at 250 µM. The association and dissociation were achieved rapidly, and no regeneration of the surface was necessary. At this concentration, all of the compounds gave measurable responses. We show that novobiocin and fusidic acid gave a higher response on HSA than on AGP (Fig. 1). Vancomycin, erythromycin and lincomycin, to a lesser extent, gave a higher binding level for AGP than for HSA. Cefotaxime, minocycline, and rifampicin bind to HSA and AGP at quite similar levels. The non-antibiotic molecule warfarin was used as a control throughout the experiments. Ranking experiments on γ-globulins were less conclusive, since all the drugs exhibit low binding responses (<5) at a maximum coating level.

Antimicrobial experiments were performed to compare the SPR ranking with the inhibitory effects of serum, HSA, AGP, and γ-globulin. HSA, AGP, and γ-globulins were used at a concentration equivalent to their respective serum concentrations, though these purified proteins are unlikely to give a response strictly comparable to the one expected in a more complex environment. The strongest HSA binders, novobiocin and fusidic acid, were the most affected by the presence of HSA in the culture broth; a 256-fold increase in the MICs was observed in the presence of 4 % HSA and a 1000-fold increase in presence of serum (Table 1). Cefotaxime and minocycline demonstrate almost identical data for biosensor response in the presence of the three proteins and the effect of the proteins on antimicrobial activity against *Staphylococcus aureus* is comparable. However their values for percentage binding to serum taken from the literature rank them as high binders. This discrepancy can potentially be explained by differences in the technique used for this measurements (ie: ultracentrifugation, dialysis...) or the origin of the serum.

The strongest AGP binders (novobiocin, fusidic acid, vancomycin, lincomycin, and erythromycin) were also the most affected by the presence of AGP, with a two fold increase in
the MICs. The effect of γ-globulins on the MICs (Table 1) is almost uniform according to the SPR data and is limited to a 2-fold increase in the MIC ratio.

**Kinetics and fraction of sites occupied**

SPR is well suited to determine how many binding sites are involved in the interactions between drugs and serum proteins. HSA is known to have two main sites of drug binding, and warfarin is a well-characterized HSA ligand that binds preferentially to site I (3). Frostell-Karlsson and colleagues (5) and Rich et al. (14) used warfarin as a model molecule to validate a SPR method for drug/HSA binding constant determination from dose-response measurements. We expanded the same procedure for antibiotic molecules to determine the fraction of sites occupied on HSA and AGP.

The range of drug concentrations that allows occupancy of 0.5 to 2 sites/protein molecule is listed in the Table 2 for the nine antibiotics. Novobiocin is a high HSA binder, occupying up to 2 sites at 180 µg/ml. Fusidic acid and rifampicin occupied 1.5 sites at 215 and 320 µg/ml, respectively. Since most of the drugs occupied more than one binding site on HSA, they generate rather complex binding kinetics for a concentration range from 0.1 µg/ml to 250 µg/ml. This prevented us from evaluating the affinities for all the sites. We have only estimated the Kd for the first site from the binding curve. They range from 12-150 µM. On AGP, the fraction of sites occupied is less than one for all the drugs (data not shown).

The binding profiles of the different drugs are informative on their properties and 3 of them are shown as an example to illustrate our purpose in Fig. 2. Rifampicin gave a linear dose-response binding curve over a wide range of concentration (1-300 µg/ml) and does not saturate HSA (not more then 20 RU binding at 300 µg/ml). Novobiocin and fusidic acid the highest HSA binder in the SPR ranking experiment also showed non saturating binding profiles between 0.1 and 300 µg/ml but we have observed respectively 2 to 4 fold more binding at 300 µg/ml.

**DISCUSSION**

The technique presented here was successfully used to evaluate the propensity of reference antibiotics for HSA, AGP, and γ-globulin. Serum proteins control the availability and the distribution of most of the drugs. HSA interacts principally with acidic molecules (6) like fusidic
acid or novobiocin, while AGP is a major target for the binding of a wide variety of basic and neutral drugs (17). By binding to HSA, many hydrophobic drugs with low solubility can be transported to reach their target tissues effectively. A decrease in the concentration of molecules readily available to interact with the true target protein results in the decrease of the antimicrobial activity of the molecule. This is why the evaluation of the binding of antibiotics to serum proteins is a critical aspect in the development of this category of drugs.

In our assay, the relatively hydrophilic antibiotics tested have lower affinities for serum proteins than most of the other classes of drugs tested by SPR (5, 14). However, we show that the technique is robust enough to test this drug family. The antibiotics with the strongest ability to physically interact with serum proteins are the most affected by the presence of serum in MIC assays (our data and (2, 10)). As expected, HSA is the most abundant plasma protein, which accounts for approximately 60% of the total proteins, and is the major contributor to the binding of these reference antibiotics. The binding of drugs to multiple sites on HSA is well documented in the literature (6). This correlated with the complex binding kinetics observed in our experiments, and prevented us from evaluating the affinities for all the sites. However, the occupancy of the drug binding sites by an antibiotic at a given concentration can be readily compared to the Cmax of the antibiotic. In the case of erythromycin, a concentration of about 150 µg/ml is necessary to occupy 50% of the HSA sites. However, the Cmax of this antibiotic does not exceed a few µg/ml (2). We conclude that the binding occurs in a concentration range far higher than the concentration reached in the serum. Alternatively, one molecule of novobiocin binds one molecule of HSA at a concentration of 60 µg/ml, and its Cmax is in same range of concentration (2), which makes the observation directly relevant for the pharmacologist. In conclusion, SPR is not a substitute to classical techniques since the binding of a drug to a single purified protein like HSA does not always mimic the behavior of the same drug on serum. However HSA by far is the major contributor to these interactions and SPR discriminates efficiently between low and high HSA-antibiotic binders. These could help to rapidly and automatically prescreen larger libraries of new antibiotic candidates.

ACKNOWLEDGEMENTS
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REFERENCES


FIG. 1. Ranking of the interactions of reference antibiotics on HSA, AGP, and γ-globulins. The binding of rifampicin, vancomycin, minocycline, fusidic acid, novobiocin, lincomycin, ofloxacin, cefotaxime, and erythromycin on HSA, AGP and γ-globulins was tested by SPR. The non-antibiotic molecule warfarin was included as a control in all the experiments. Data were normalized to the same 10000 RU immobilization level for HSA (white), AGP (grey), and γ-globulins (black). The experiments were done in triplicate and are presented as a mean value ± standard error (the error is low and is not always visible).
FIG. 2. Dose response binding of rifampicin fusidic acid and novobiocin on HSA.
The concentration dependent binding of rifampicin (▲), fusidic acid (□), novobiocin (■) on HSA was followed by SPR. The binding response is presented on the left axe, and the fraction sites occupied on the protein on the right.
TABLE 1. Effects of serum proteins on different reference antibiotics.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC Standard (µg/ml)</th>
<th>+ 50% Human Serum</th>
<th>+ 4% HSA</th>
<th>+ 0.1% AGP</th>
<th>+ 1.6% γ-globuline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ofloxacin</td>
<td>0.195</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.781</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>0.391</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.195</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Minocycline</td>
<td>0.195</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.049</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.781</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>0.049</td>
<td>1024</td>
<td>256</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>0.049</td>
<td>1024</td>
<td>256</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
TABLE 2. Fraction of sites occupied on HSA.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Fraction of sites occupied on HSA for a range of drug concentrations (µg/ml)</th>
<th>Cmax (µg/ml) ***</th>
<th>% binding to serum ***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ofloxacin</td>
<td>0.5*(18-36)**, 1 (&gt;145)</td>
<td>2.6</td>
<td>25</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.5 (22-44), 1 (~180)</td>
<td>4.9-17</td>
<td>3</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>0.5 (130-180)</td>
<td>1.8-2.5</td>
<td>75</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.5 (146-220)</td>
<td>3</td>
<td>19-25</td>
</tr>
<tr>
<td>Minocycline</td>
<td>0.5 (~50), 1 (135-180)</td>
<td>2.6</td>
<td>54-80</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.5 (80-120), 1 (~245), 1.5 (&gt;325)</td>
<td>11</td>
<td>64</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.5 (~285), 1 (430-570)</td>
<td>30</td>
<td>10-19</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>0.5 (7-15), 1 (~60), 1.5 (~120), 2 (180-250)</td>
<td>62.5</td>
<td>90</td>
</tr>
<tr>
<td>Fusidic Acid</td>
<td>0.5 (~20), 1 (~160), 1.5 (215-430)</td>
<td>52</td>
<td>97-98</td>
</tr>
</tbody>
</table>

* Fraction of sites occupied on HSA. (Req*MW_HSA/Rim*MW_drug)=f

** Concentration range of the drug in brackets

*** Cmax and % binding to serum are a compilation of data obtained on different animal species (2, 10)