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Rapid screening of small ligand affinity to human serum albumin by an optical biosensor

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Abstract

Here we report the use of IAsys biosensor technology for determining the binding parameters of low molecular weight compounds, such as warfarin and bilirubin, to surface immobilized human serum albumin. The protein was covalently immobilized on the surface of the biosensor cuvette, bearing a carboxymethyl dextran layer, through a condensing reaction between the carboxyl groups of the biosensor surface and ε-amine groups of protein lysine residues. This system detects and quantifies the changes in refractive index in the vicinity of the surface of the sensor chip to which the protein is immobilized. The changes in the refractive index are proportional to the change in the absorbed mass, thus the analysis allows the monitoring of the interaction process and the determination of the binding parameters. Optical biosensor analysis, most suited for studying protein/protein or protein/nucleic acid interactions, was sensitive enough to monitor the binding of low molecular weight compounds to human serum albumin and then suitable for a rapid screening of libraries of potential drugs when bioavailability is the research target.

Keywords: Protein binding; Human serum albumin; Drugs; Optical biosensor; IAsys; Warfarin; Bilirubin

1. Introduction

Human serum albumin (HSA) plays a fundamental role in the transport of drugs, metabolites and endogenous factors [1]. As far as drugs are concerned, their binding to HSA results in controlling the free, active concentrations and in providing a reservoir for a longer action. The free concentration of a drug can change because of its interaction with co-administered drugs or because of specific variation among patients. The characterization of drug binding parameters to serum carriers results essential and this is particularly true in the design and development of new therapeutic agents [1,2].

A wide variety of biochemical techniques (ultrafiltration, ultracentrifugation and equilibrium dialysis) have been extensively applied to this purpose. These techniques are based on the

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analysis of the free drug, which can actually constitute a limit when the drug is tightly bound to the protein, as quite often occurs. Recently, biochromatography became reasonably popular for obtaining such information [3–7]. This methodology, based on the analysis of small ligand binding to immobilized albumin supports, provides several advantages over classical biochemical techniques, as it does not involve separation steps and can offer the precision, sensitivity and reproducibility of a chromatographic system. A different approach involves the use of spectroscopic techniques, such as fluorescence, UV and circular dichroism (CD) spectroscopy [8–11]. In this case, the signal allied to the drug/protein complex can be monitored, giving direct information on the binding interaction. CD, in particular, can give a deeper insight into the stereochemistry of the protein/drug adduct and then to the mechanism of binding. Very recently, optical biosensors showed their usefulness in monitoring protein binding phenomena [12–16]. This methodology results particularly promising in the frame of its application to drug protein binding screening and to kinetics investigations. A BIAcore biosensor (Uppsala, Sweden) was employed for this investigation and problems arising from the low molecular weight of the binding compounds were stressed [17,18].

In the present paper, the study of the drug binding to HSA has been approached by using the IAsys biosensor, which is based on the resonant mirror principle. This technique, as with other optical biosensors, allows interaction analysis of a wide range of biomolecules, providing information on the kinetics of a biomolecular interaction and it is also used to determine ligate concentration. In particular, we examined some low molecular weight ligates (Fig. 1), whose binding properties to HSA are well known. This was in order to establish the reliability of the determined dissociation constant by comparison with literature data. Furthermore, the stability of the anchored protein was also checked to evaluate the potential applicability of this method to the screening of compound libraries for their affinities.

2. Materials and methods

2.1. Chemicals

Carboxymethyl dextran (CMD) dual-well hydrogel cuvettes were from Labsystems Affinity Sensors (Cambridge, UK). Ethanolamine, *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-*N*,*N*-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were purchased as NHS coupling kit from Affinity Sensors. The solutions were prepared and stored as explained in the protocol. Phosphate buffered saline (PBS/T), final pH 7.4, was prepared using Sigma dry powder in foil pouches. Each pouch dissolved in 1 l of deionized water yields 0.01 M phosphate buffered saline, NaCl 0.138 M, KCl 0.0027 M, Tween-20 0.05%, pH 7.4 at 25 °C. Acetate buffer (Sigma Aldrich, Milan, Italy), 10 mM, was adjusted to pH 5 with glacial acetic acid (Sigma). All solutions were made using ultra high purity water. Iopanoic acid, iophenoxic acid, bromophenol blue, bilirubin and *rac*-warfarin were obtained from Sigma Aldrich. Human serum albumin (HSA), essentially fatty acids free, was supplied by Sigma Aldrich and used without further purification. All reagents were of analytical grade and were used without further purification.

2.2. Apparatus

All analyses were performed using IAsys Plus optical biosensor (Labsystems Affinity Sensors). This instrument employs a dual-well stirred cuvette (Fig. 2), where the sample is added in a single step, to deliver the material under investigation to the immobilized ligand. The optical biosensor was operated at 25 °C. The data were collected at the fastest data collection rate (0.3 s^{-1}) and 100% stirring in order to minimize mass transport effects.

2.3. Immobilization of HSA

The protein was immobilized through its surface amine groups via amide bonds with the CMD (Scheme 1) [17,18]. The running buffer was PBS/T: 10 mM phosphate buffered saline pH 7.4, 0.05%

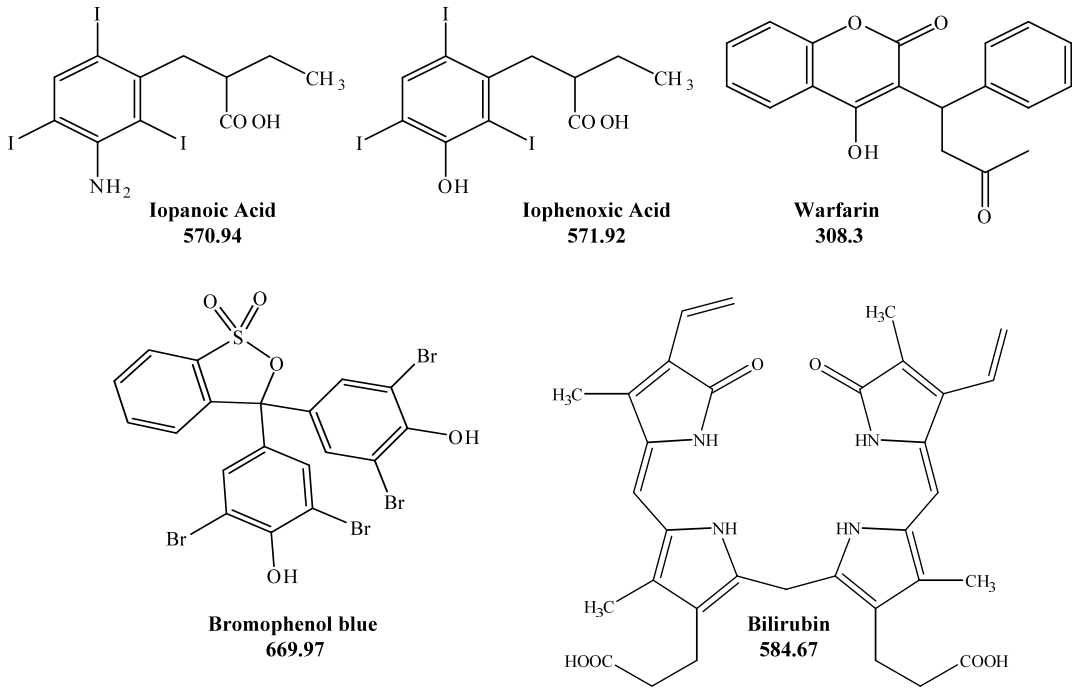


Fig. 1. Structures and molecular weights of the analysed compounds.

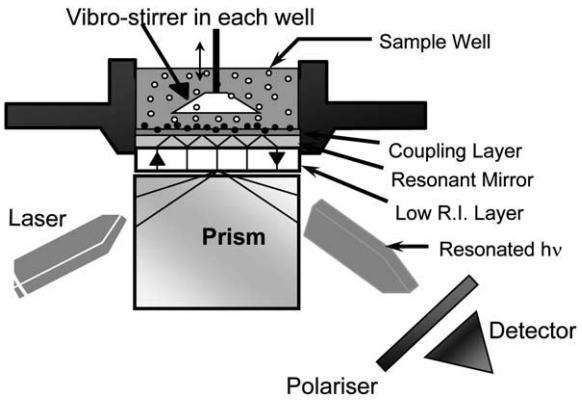
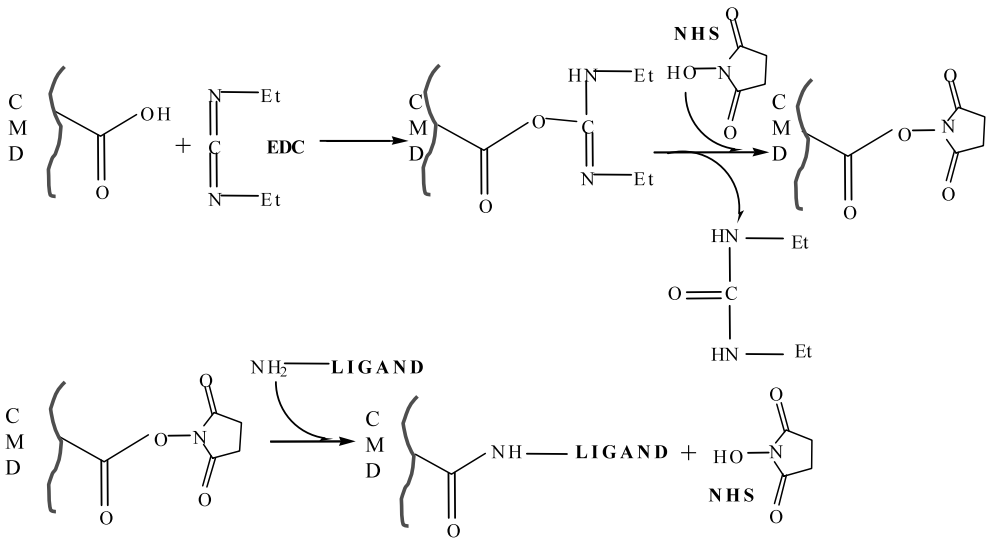


Fig. 2. Scheme of a IAsys biosensor cuvette.

Tween-20, (NaCl 136 mM, KCl 2.7 mM. CMD hydrogel in the cells was activated with a 1:1 EDC/NHS mixture (100 mM NHS and 400 mM EDC) for 7 min. HSA (concentration 0.1mg/ml in the cuvette, sodium acetate 10mM, pH 5) was allowed to react for 7 min. Unreacted NHS-esters were blocked by washing with 1 M ethanolamine, pH 8.5 for 3 min. PBS/T was added to stabilize the baseline. To determine the reproducibility of the assays, the entire experiments were replicated over different HSA surfaces: the protein was immobilized several times on different cuvettes leading to different immobilized density values averaging from 1700 to 4700 arc seconds, corresponding to 8–23 ng/mm^2 of anchored protein, respectively. The protein was immobilized only in one channel of the cuvette. The second channel was used as reference channel; the CMD surface was only activated with 1:1 EDC/NHS mixture and treated with 1 M ethanolamine in order to monitor any non-specific bindings.

2.4. Preparation of sample solutions

The entire set of samples analyzed were dissolved in PBS/T or phosphate buffer (PB) and then sonicated. The same buffer in which the solutions were prepared was used as the running buffer for the experiments. All compounds were freshly prepared as a 1 mg/ml stock solution and immediately prior to analysis were diluted with running buffer. A suitable range of concentrations



Scheme 1.

was diluted into the cuvette. The addition of samples by dilution into the cuvette (usually 1–10) avoids disturbance of the baseline. Stock solutions were prepared as follows: iopanoic acid was dissolved in 10 mM PBS/T and 10% of 0.01 M NaOH; iophenoxic acid was dissolved in 10 mM PBS/T; warfarin was dissolved in 10 mM phosphate buffer pH 7.4 and 10% of 0.1 M NaOH; bilirubin was dissolved in 10 mM PBS/T and 12.5% M NaOH; bromophenol was dissolved in 50 mM phosphate buffer, pH 7.4. In order to determine the reproducibility of the assay, the experiments were replicated over different HSA surfaces.

3. Results

Biosensor data were analyzed using Graph Fit v. 5.0.0.36 software (Erithacus Software Ltd.; author: Robin J. Leatherbarrow). GraphPad Prism 3.0 Software from GraphPad Prism was also used to fit the binding data within a non-linear regression (two site binding equation).

3.1. Immobilization of HSA and its stability

The optical biosensor is a real bio-recognition system. Enzymes or binding proteins are immobilized onto the surface of the transducer and specific interactions between the target analyte and the complementary biorecognition layer produce a physico-chemical change, which is detected and may be measured by the transducer.

Biosensor technology permits the monitoring of biomolecular interactions at the time that they occur. Most interaction analyses start up with immobilization of one of the molecules under investigation, the ligand. This allows having the molecule covalently bound onto the surface of the biosensor surface cuvette, thereby studying its interaction with analytes. After the ligand immobilization, the addition of ligates follows in order to monitor the association process; during this phase, ligand/ligate complex is formed. After the ligate binding solution is replaced by buffer, the dissociation may be monitored.

In the case of IAsys technology, the binding of molecules to the chip produces a change in the refractive index on the biosensor surface, generat-

ing an increase of response, measured in arc seconds (1/3600th of a degree). This optical technique not only detects, but also quantifies, the changes in refractive index in the vicinity of the surface of the sensor chips to which the ligand is immobilized. Since the refractive changes are proportional to the changes in the absorbed mass, this technique allows the quantitation of the analyte interacting with the ligand immobilized on the sensor chip.

The amine coupling method was used to immobilize HSA to the carboxydextran matrix. A typical sensorgram of the immobilization process is shown in Fig. 3. The amount of the immobilized protein is determined by measuring the biosensor response upon ligand binding , considering that 1 ng/mm^2 HSA gives a 200 arc seconds signal. The carboxydextran matrix of the reference channel was reacted as the experiment channel, but without anchoring the protein. This procedure is important in order to follow non specific binding phenomena on the biosensor surface. The protein was immobilized several times and the binding experiments were repeated with all the cuvettes in order to verify the repeatability of the obtained results. Moreover, the surface can deteriorate due to a decrease of bound ligand or to inactivation of ligand; in these cases, running and regeneration buffers should be very carefully chosen. To check the stability of the immobilized HSA, the biosensor response of a fixed concentration of the same ligate sample was monitored over the time. A decrease of the response was observed, the binding properties of the protein remaining higher than 70% after 2 months of use. It is worth mentioning that the binding constant values are independent of the amount of anchored protein or the amount of active protein. Indeed, the analysis of the equilibrium responses for the same set of ligate concentrations gave consistent dissociation constant values for experiments carried out at different times from the anchoring of the protein.

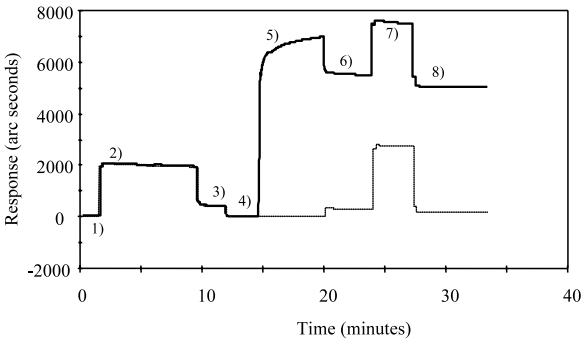


Fig. 3. Sensorgram of the HSA immobilization to the carboxymethyl dextran matrix: (1) buffer baseline stabilization; (2) EDC/NHS mixture add (7 min of reaction); (3) buffer washes to remove unreacted molecules; (4) acetate buffer re-equilibration; (5) HSA in acetate buffer add (7 min of reaction); (6) buffer washes to allow dissociation; (7) block of non-coupled activated CMD sites with ethanolamine (3 min of reaction); (8) baseline stabilization. The light line indicates the reference channel signal.

3.2. Determination of equilibrium dissociation constant (K_D) of ligates

The compounds we investigated bound reversibly to the serum carrier. Thus, it was possible to follow either the association or dissociation phases of the binding process. A typical experimental behaviour of the response plotted as a function of time is shown in Fig. 4, where *rac*-warfarin is the ligate. A steady state is actually reached in a few seconds, after the drug is added to the cuvette with the HSA modified CMD surface. Then *rac*-warfarin dissociates very rapidly when the drug solution is replaced by running buffer. The overlaid sensorgrams in Fig. 4 were obtained using different *rac*-warfarin concentrations (2×10^{-6} to 3×10^{-4} M) in the cuvette. Since dissociation

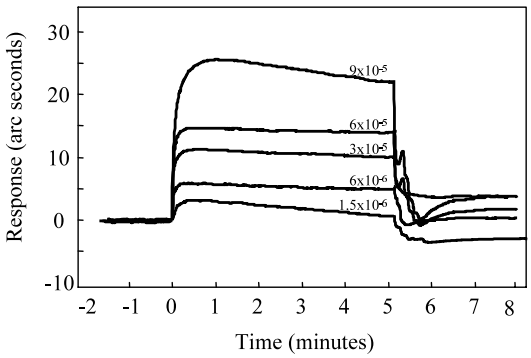


Fig. 4. Corrected sensorgram overlays for *rac*-warfarin binding to HSA. Binding and dissociation processes at the indicated *rac*-warfarin concentrations.

process is practically immediate after running buffer washes, only the association phase of the experiment was worth studying, the steady state value at every different concentration being considered the R_{eq} (response at equilibrium). These values were then corrected by subtracting the reference channel response and plotted against concentrations. Using the binding isotherm equation on GraFit v. 5.0.0.36 software, it was possible to fit equilibrium responses at different concentrations to obtain the equilibrium dissociation constant (R_{max} is the maximum capacity of the ligand for the ligate):

$$R_{eq} = \frac{R_{max}[L]}{K_D + [L]}$$

In this way, the interaction is considered to be Langmurian and so we assume that a single molecule of ligand binds one molecule of ligate, affording the only higher affinity site constant (K_{D1} 7.7×10^{-6}). *Rac*-warfarin is known to bind to different classes of binding sites and in the literature two K_D values have been reported for the high and low affinity binding sites. To better study this behaviour, data were also analyzed using a two site binding equation on GraphPad software:

$$Y = R_{max1} * [L]/(K_{D1} + [L]) + R_{max2}/(K_{D2} + [L]).$$

Two different equilibrium dissociation constants were found (K_{D1} 6.8×10^{-6}; K_{D2} 1.0×10^{-4}), the high affinity site value being consistent with the one obtained assuming a single binding site. Values are in agreement to those obtained by independent techniques [1] and also by using a BIAcore optical biosensor [18]. The related data are reported in Fig. 5 as response data at equilibrium versus drug concentration.

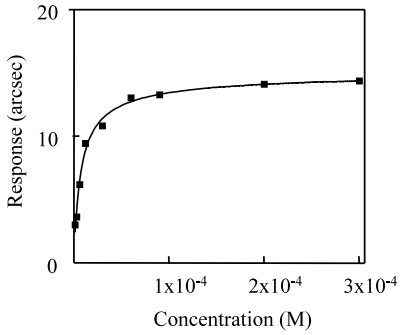


Fig. 5. Response data at equilibrium versus *rac*-warfarin concentration.

One of the main applications of this technique is to utilize biosensor as a tool for screening libraries of drugs with the ambitious aim to develop new targets considering their binding affinity to HSA. In this view, it is very important to determine the HSA binding affinity as well as the percentage of bound values, considering a fixed concentration of protein and ligate. As Myszka et al. [18] previously reported, we considered HSA concentration to be 0.68 mM to approximate the physiological concentration of HSA in serum and drug concentration equal to 10 μM, reproducing the drug concentration used in standard equilibrium dialysis assays and we then evaluated the percentage of bound ligate (values are reported in Table 1). Other low molecular weight ligates were investigated for determining their HSA binding parameters and the results are summarized in Table 1. As explained for warfarin, also with these compounds we first determined the highest affinity site by mean of binding isotherm equation. The K_D values are reasonably close to those reported in the literature [1]. Dose–responses plots are shown in Fig. 6. In the case of iopanoic acid and bromophenol blue, it was also possible to characterize a second lower affinity site: literature does not report any K_{D2} values and so a comparison to check the reliability of the biosensor method is not possible. Interesting is the case of bromophenol blue binding; the peculiarity of this colorant is to be related to its strong selectivity for binding at site I on HSA with quite high affinity. By analyzing data with a two site binding equation it was possible to determine two K_D values; however this compound appears to bind also to other lower affinity sites as suggested by a high loading on the protein at concentrations higher than 1.0×10^{-4}.

4. Conclusions

The binding responses for all the compounds we tested over HSA were reproducible even though these molecules are small compared to the size of the immobilized protein.

Table 1

Compound	K_{D1}	K_{D2}	K_{D1} (from literature)	Bound percentage
Warfarin	6.8e-006	1.0e-004	4.0 e-006[a]	99.0
Iopanoic Acid	5.6e-007	1.4e-005	1.5 e-007[b]	>99.9
Iophenoxic Acid	2.2e-008	—	1.3 e-008[b]	>99.9
Bilirubin	5.5e-008	—	1.1 e-008[c]	>99.9
Bromophenol blue	6.2e-007	5.2e-005	6.6 e-007[c]	>99.9

[a] Ref. [2]
[b] Ref. [19]
[c] Ref. [1]

This technique offers many advantages with respect to most of the other available methodologies to study biomolecular interactions. Above all is the fact that the amount of both ligand and analyte needed to obtain get informative results is low and no labeling is necessary to perform the assay. Usually 20–25 min are needed for ligand immobilization and 4–5 min for a complete characterization of a ligand–analyte interaction. The binding is a real-time analysis leading to informative results at the same time the binding itself occurs. Finally, the sensor chip could be re-used many times, being the anchored protein stable for 1 month. This fact lowers the analysis

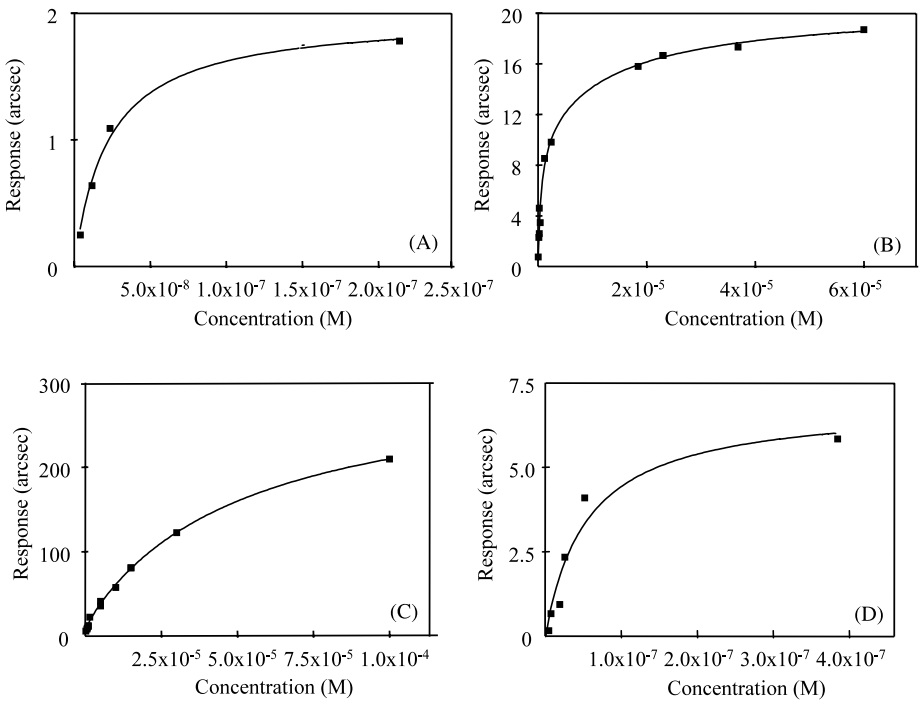


Fig. 6. Response data at equilibrium versus ligate concentration. (A) Iophenoxic acid; (B) iopanoic acid; (C) Bromophenol blue; (D) bilirubin.

costs and makes evident the potential of this method for screening libraries of compounds for their affinity to HSA.

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