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Monitoring drug–serum protein interactions for early ADME prediction through Surface Plasmon Resonance technology

Edoardo Fabini[a,*], U. Helena Danielson[b,c]

[a] Department of Pharmacy and Biotechnology, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy
[b] Department of Chemistry – BMC, Uppsala University, SE-751 23 Uppsala, Sweden
[c] Science for Life Laboratory, Uppsala University, SE-751 23 Uppsala, Sweden

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ABSTRACT

Many molecules fail to reach the market due to poor pharmacokinetic (PK) properties, rendering the potential drug virtually unavailable for the primary target despite efficient administration to the body. PK properties of endogenous and exogenous compounds in mammals are dependent, among other factors, on their ability to interact with serum proteins. The extent of binding can greatly influence their ADME (adsorption, distribution, metabolism and execration) profile. Reliable and cost-effective bioavailability studies, early in the drug discovery process, can lead to an improvement of the success rate for compounds entering clinical trials. Optical biosensors based on surface plasmon resonance (SPR) detection emerged as an efficient approach to obtain large amounts of information about the binding of small molecules to serum proteins. Simple, automated and fast assays provide a good throughput, versatility and highly informative data output, rendering the methodology particularly suited for early screening. The ability to provide basic information on PK can be easily coupled to structure–activity relationship analysis. In this review, features of the technology and its employment for the study of serum protein–small molecule interactions are presented and discussed.

1. Importance of ADME profiling early in the drug discovery process

The journey that leads to the commercialization of a new therapeutic agent usually goes through three different sequential steps: the discovery, the development and the launch on the market. The discovery phase involves the identification and the validation of a new target, the screening for compounds able to modulate the target functionality and the hit-to-lead optimization, with the final aim to produce a new chemical entity (NCE). The development phase, on the other hand, is represented by sequential clinical trials, where the NCE is tested on a representative number of human patients for effectiveness and safety. The time estimated to go through the complete process is 12–15 years with an associated cost of approximately 1 billion $ [1,2]. Nonetheless, out of all compounds entering clinical phase I, roughly 11% reach commercialization [3,4].

Oncology drugs account for more than 30% of the total fraction under development, however they have the lowest success rate of all NCE, with a likelihood of approval of only 6.4%. Vaccines are the most likely to advance from phase I to the commercialization, with an associated success rate of 14.9%, while small molecules represent the drug class with the lowest success rate of all with 7.6%. Although chances of reaching the market varies depending on the drug class and the disease considered, the overall picture emerged from the studies showed a general inefficiency during drugs development, which reflects in a big waste in term of money and resources of the pharmaceutical industry.

Inspection of the reasons underlying the high-attrition rate reveals two major causes: low efficacy of the NCE in humans and a poor pharmacokinetic (PK)/bioavailability profile [5]. Surprisingly, the latter is still today the third most common cause of attrition in Phase I, even though its contribution lowered from 39% to 16% over the last two decades [6,7]. While low therapeutic effects in human can depend on genotype differences, something that is difficult to assess before testing the drug on a representative number of patients, drug-likeness should be carefully evaluated during the discovery phase in order to ease the path toward commercialization. The era of combinatorial chemistry led to the construction of huge libraries containing larger and more lipophilic

* Corresponding author.
E-mail addresses: edoardo.fabini2@unibo.it, edoardo.fabini@gmail.com (E. Fabini).

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molecules, two characteristics affecting their pharmaceutical properties while reducing their drug-likeness [8]. Therefore, rapid and reliable bioavailability studies early in the drug discovery process can significantly help to discover better leads that enter clinical trials, with a balanced potency and improved drug-likeness profiles [9,10].

In vitro bioavailability studies involve the assessment of different properties of a NCE, including: cell permeability, chemical stability, potential drug–drug interaction, inhibition of Cyp 450 enzymes and interactions with active transporters [11,12]. The PK properties of a compound entering the human body are also related to its ability to interact with serum proteins, which markedly impacts its ADME profile. The two main actors in this framework are the human serum albumin (HSA) and the α_1-acid glycoprotein (AGP), which together constitute more than 55% of the total protein content in human blood plasma [13,14]. The extent of binding to serum proteins modifies the free concentration of drugs, and as a consequence its physiological activity. Although the therapeutic effect may be compromised by undesired removal of a compound from the circulation, the drug–protein complexes formed can constitute a reservoir that extends the time the compound resides within the circulatory system, potentially increasing its effect. Hence, binding to HSA and AGP can constitute a valid tool to forecast drug-likeness in the initial screening steps of drug development [15]. Poorly drug-like compounds can be rejected, and chemical moieties can be modified to improve both pharmacodynamics and pharmacokinetics at an early stage.

Traditionally, the binding of molecules to serum proteins has been analyzed using methods such as analytical ultracentrifugation [16,17], equilibrium dialysis [18,19], ultrafiltration [20], electrophoresis [21], spectroscopic techniques [22,23] and affinity chromatography [24,25]. These techniques are based either on the separation of the compound from the serum protein, or on a change in intrinsic parameters of the protein upon formation of a complex with the compound, such as its mobility or spectroscopic properties. These assays are well established; nonetheless, the sometimes-ambiguous interpretation of data, the relatively high material consumption and the low throughput does not facilitate their employment for routine binding analysis.

The last two decades have seen a constant growth in the application of surface plasmon resonance (SPR)-based optical biosensors for molecular recognition analysis in many different fields [26–31]. SPR biosensors are analytical platforms where one of the two interacting partners is anchored to the surface of a sensor chip (technically the ligand), while the other is flowing in solution within a small flow cell (the analyte). They bear some peculiar features, which allows the accurate measurement of the amount of analyte–ligand complex, its equilibrium dissociation constant and the association and dissociation rate of the reaction. The detection principle, based solely on refractivity at the surface, does not require any pre-labeling of molecules, avoiding time- and money-consuming steps, which could also potentially affect the interaction.

At present, the main application is arguably in structure–activity relationship (SAR) studies, where the highly informative data output is used to gather precious information on new drug lead candidate [32–35]. For instance, kinetic parameters of the drug–target reactions provide a direct tool to evaluate the rate of dissociation of the complex and a simple mean to extrapolate the residence time (τ). This parameter has gained increasing interest among medicinal chemists, and it has been demonstrated that in a wide range of biological systems τ, measured *in vitro*, remarkably correlates with the *in vivo* potency of a compound, better than the overall drug–complex steady state affinity [36,37]. SPR-based platforms have also become an elective technique for fragment-based drug discovery, and different approaches and protocols were developed to help improve experimental design and data analysis [38–40].

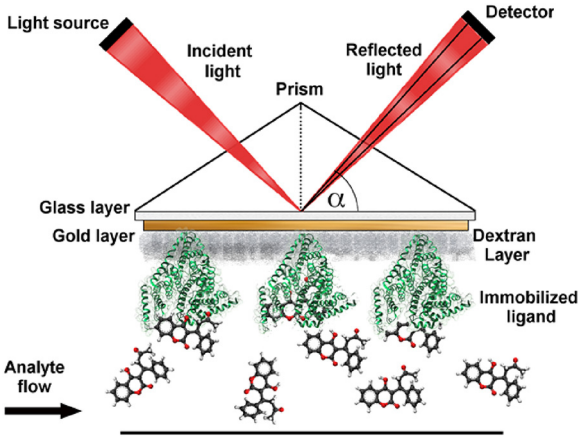

Fig. 1. Schematic representation of the SPR detection principle. The SPR angle α, when all other parameters are kept constant, is dependent only on the refractive index of the solution in proximity of the functionalized surface. Upon interaction between the analyte and the ligand, the refractivity at the surface changes, resulting in changes in α. The magnitude can be directly correlated to the amount of complex formed.

Nonetheless, optical biosensing is an intriguing approach to estimate also ADME profiles. Automated instruments minimize operator applications and increase the throughput, favoring a fast screening process, particularly useful when dealing with compound libraries. The assessment of the potency of a lead candidate can be supported with basic information on the drug-like profile. SPR technology offers the possibility to probe the binding to HSA and AGP simultaneously upon immobilization over two different spots on the same sensor chip. This provides an excellent *in vitro* mimic of the PK of a drug, whenever whole human serum is difficult to access. In this review a brief description of the technology is presented and its application for bioavailability studies is discussed.

2. SPR, description of the technique

2.1. Detection principle and instrumentation

The optical phenomenon of SPR occurs when a beam of plane-polarized light hits the surface of a thin, electron-rich, metal layer placed at the interface between two media of different refractive indices ($n_1 \neq n_2$) (Fig. 1) [41]. When the refractive index of the media n_2 is perturbed, for instance as consequence of mass accumulating at the immediate proximity of the metal layer, a signal can be detected. In a typical SPR experiment, a biological target (ligand) is tethered to the surface of a sensor chip, through chemical modification of the gold film facing the fluidic side, and the analyte is injected over the surface, free in solution. As a consequence of the ligand–analyte interaction, a response arises. This response is quantified and typically expressed as the change in refractivity (refractivity/response units, RU) over time [42].

Different optical configurations have been employed to investigate molecular binding events through SPR phenomena. The so-called Kretschmann configuration was used to develop a vast array of SPR platforms, both in the traditional and in the imaging (SPRi) format [43–46]. More recently, an alternative SPR configuration, termed localized SPR (LSPR) has emerged. With this mode, SPR is generated on the surface of a nanoparticle rater than a metal film, endowing completely new spectroscopic properties to the metal depending on nanoparticle composition, size, shape, orientation and local dielectric environment. Thanks to the miniaturization potential, LSPR-based devices are viewed as affordable, portable

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and easy-to-use tool for point-of-care testing [47,48]. Nonetheless, survey of the published literature describing serum proteins–small molecules interactions *via* SPR-based sensors, showed that the vast majority of experiments were performed through BIAcore-related instrumentations. To the best of our knowledge, they are the most relevant for the present application.

In BIAcore platforms (that employ the classical Kretschmann configuration) [49,50], the sensor chips are typically functionalized with a dextran layer, providing a flexible hydrogel for various methods of immobilization of proteins or other molecules used to define the sensor surface. A microfluidic system creates a series of flow channels wherein different ligands can be immobilized. This forms a sensor chip surface, with multiple detection spots. Analyte solutions are delivered by automated injection, and a liquid handling system that precisely controls the flow of buffer and samples. Depending on the platform employed, fluidics, sensitivity and throughput might vary, with top quality products possessing adequate characteristics for fragment library screening. Material consumption is typically very low, and for a complete analysis, only few micrograms of both the ligand and the analyte are required [26]. Taken all together, these elements make the technique well tailored for the screening of libraries of compounds that are not available in great amounts, as is the case in a typical drug development phase.

2.2. Assay set-up

SPR experiments require the immobilization of one of the two interactants on the sensing surface in order to investigate a given interaction. The functionalization of the gold layer with a hydrogel results in a three-dimensional immobilization support, and a coat that prevents non-specific adsorption of molecules to the surface [51]. The most-employed immobilization approach is to anchor the protein on the polymer and to flow the low-molecular-weight analyte over it. This represents a typical direct binding-assay, where different compounds are probed against the same target. Nevertheless, other assay formats have been developed and established [52]. The immobilization procedure can be considered the most challenging part of the experimental set-up, since the ligand needs to retain its biological activity upon anchorage to the surface. Usually, carboxylated dextran surfaces are employed and the ligand is dissolved into a weakly acidic, low ionic strength solution −1 pH unit below the pI of the ligand – to achieve enough electrostatic concentration at the dextran layer, before the linking reaction takes place. Amine coupling chemistry via exposed lysine residues is the most employed immobilization strategy for proteins, although different chemistries are readily available to meet different analysis requirements. The experimental conditions for analyzing interactions are optimized by selecting a suitable buffer and regeneration conditions, the latter needed for slowly dissociating complexes. Also the concentration range of the analyte needs to be carefully selected, as it is dependent on both the solubility of the compound and its affinity for the ligand. Whenever possible, it is important to include a positive and a negative control in a binding assay. This is to avoid artifacts due to unrelated phenomena that produce a change in refractivity, such as unspecific adsorption to the chip surface or to the protein scaffold, which results in an SPR signal. Careful experimental design and data analysis is crucial for success.

2.3. Data output

Continuous detection of the interaction results in an information-rich plot, termed "sensorgram" (Fig. 2), accurately describing the binding event in real-time. Different aspects of the molecular interaction can be studied depending on the final goal of the analysis, the quality of the data and the intrinsic behavior of

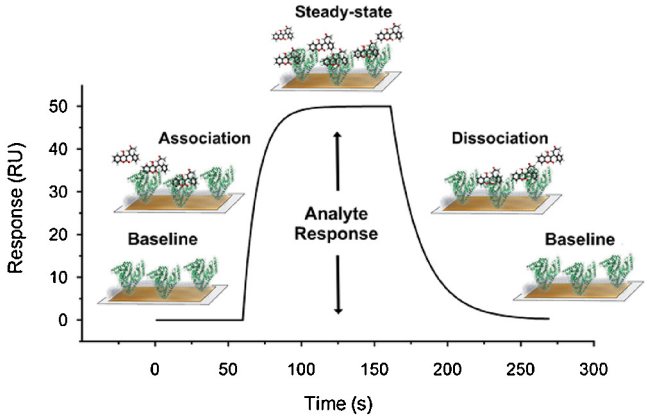

Fig. 2. Typical data output from an SPR experiment. The real-time interaction curve, named sensorgrams, represents an accurate description of the interaction between the tethered ligand and free-flowing analyte. Non-linear regression analysis of sensorgram shapes can be used to extrapolate the rate of association (k_{on}) and dissociation (k_{off}) of the binding event. Responses (expressed in response units – RU), reached at steady-state can be used to construct the isotherm of binding and determine the equilibrium dissociation constant K_D of the ligand–analyte complex.

the two binding partners [53]. Plotting responses at steady state against the injected concentrations results in a classical dose-response curve. The affinity (K_D) for a complex can be estimated by non-linear regression analysis of the data. Comparison of the maximal theoretical and experimental responses can provide information on the apparent binding capacity (often termed the "activity") of the immobilized ligand and on the stoichiometry of the interaction.

The real-time measurement shows the rate of formation and the stability of the analyte–ligand complex. Global non-linear regression analysis of a set of sensorgrams at different analyte concentrations can be used to estimate the kinetic constants for association (k_{on}) and dissociation (k_{off}). Structure-kinetic analysis, based on these parameters can provide information useful for the design of compounds with desired features. These characteristics provide valuable information which otherwise will be lost by the measurement of a simple condition of equilibrium. In addition, rational interpretation of complex sensorgrams can shed light onto reaction mechanisms that are more complicated than a simple 1:1 binding event [54,55]. In the following section, the application of the technique to the assessment of the binding to serum protein is thoroughly described.

3. Analysis of small molecule–serum protein interactions

Initial biosensor-based studies explored if the technology was amenable for studies of the binding between small molecules and immobilized serum proteins. This was expected as studies using affinity chromatography had earlier shown that interactions with stationary phases using HSA and AGP could reflect affinity data obtained using equilibrium methods with the proteins in solution [56,57]. Therefore, analysis of small molecule–serum protein interactions have been developed and standardized for SPR platforms.

3.1. Immobilization protocol and analysis conditions

Immobilization protocols are straightforward for both HSA and AGP, due to their excellent chemical stability. For HSA, high-efficient linking is achieved through classical amine coupling chemistry, using a 10 mM sodium acetate buffer with a pH of 5.2 as coupling solution [58]. Under this condition HSA is tethered to the dextran coating through its primary amine groups exposed to the surface, i.e. lysine residues. The immobilization procedure results

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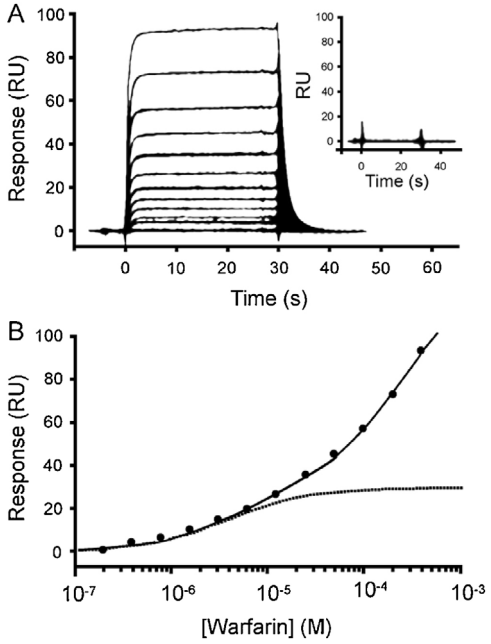

Fig. 3. Representative sensorgrams showing typical Warfarin/HSA interaction over a wide range of concentrations. (A) Overlaid sensorgrams obtained from replicate injections of warfarin (0.2–400 μM) over HSA-functionalized surface. (Inset) Responses collected from the reference surface. (B) Response at equilibrium plotted versus warfarin concentration. The solid line depicts the fit of the data to a theoretic model describing two independent-sites reaction, while the dashed line represents the saturation point of the high-affinity site. The fit yields equilibrium dissociation constants of 3.8 and 273 μM for the high-and low-affinity sites, respectively. Reprinted with permission from reference [63].

in a fully functional HSA surface, with all major binding sites readily available and accessible [58]. A different chemistry is required to immobilize AGP, and the most effective strategy involves a thiol coupling procedure. Functionalization of exposed carboxyl groups on the protein with 2-(2-pyridinyldithio) ethanolamine hydrochloride (PDEA) allows a thiol exchange with sulfuric groups on the pre-activated surface. The procedure increases the pI of AGP, improving the pre-concentration step leading to higher immobilization levels. Application of this protocol results in stable and reliable AGP sensor surfaces [58,59].

Analysis conditions for monitoring drug/serum protein interactions should be assessed on a case-by-case basis; however, a general scheme can be applied across many systems. For instance, i) phosphate buffer saline (PBS) pH 7.4, which is a good approximation of human serum composition, is the most suited running buffer in a vast array of experiments ii) injection times are short, because equilibrium is usually reached within 60 s and the dissociation is fully and rapidly reversible and iii) a calibration plot needs to be included to correct for refractive index mismatch between flow cells, whenever organic additives, such as DMSO, are supplemented in aqueous running buffer. On the other hand, parameters like concentration range are more related to the solubility of the small molecules or to the intrinsic ligand–analyte behavior and strategies to optimize these choices are presented throughout the manuscript.

3.2. Benchmark studies (primary affinity determination)

Benchmark studies with immobilized albumin have been conducted using warfarin as a reference compound (Fig. 3). The warfarin/HSA interaction was monitored under a variety of conditions: using different commercial batches of HSA, different temperatures, at different protein immobilization levels, different times from the immobilization day and different DMSO concentrations in running buffer. Within this broad range of conditions SPR data showed great robustness and good correlation with data obtained from other independent techniques [60].

In view of its consistent behavior, warfarin can be used as a standard control to examine the binding of other compounds to HSA. Data provided from warfarin–HSA interaction studies can be combined with binding data collected for other small molecules onto the same surface, in order to determine steady-state affinity (K_D) values [60]. The proposed method is based on a global fitting procedure, where a single compound concentration, typically the lowest able to give a detectable signal, is analyzed together with three warfarin calibration points. Whenever data on surface saturation for weak HSA binders are difficult to obtain, warfarin can provide information on the surface capacity, permitting K_D extrapolation with good precision and reproducibility [58,60]. For AGP, ambiguous affinity data are reported for the binding of different compounds [14]. Therefore, the employment of a standard protocol as the one developed for warfarin cannot be easily applied. Data representing the percentage of binding of reference compounds, such as dipyridamole, propranolol or alprenolol, obtained by other analytical techniques, can be used as controls to test AGP surface performances. The extent of binding to HSA and AGP of known standards can be used to classify the potential leads by dividing them into high-, medium- and low affinity binding classes.

3.3. Steady-state affinity determination

Although the previously described approach demonstrated to be solid and relevant for a broad spectrum of compounds, it is limited by the assumption that only one binding site is populated at the concentration employed. This simplifies data analysis assuming a 1:1 interaction, even if this is not always the case. The existence of more than one class of binding sites on serum proteins, have already been reported and discussed in many studies [13,14]. In particular, HSA and AGP posses not only specific pockets to bind determined chemical moieties, but also 'unspecific' binding clefts able to interact with different chemical structures with moderate affinities. They can be occupied simultaneously even at low concentrations, resulting in complex interactions hard to describe with a simple dose–response curve. Nonetheless, when interactions are not completely straightforward, careful analyses of the high-informative data output can still provide valuable information on the binding affinity.

Researchers have explored the possibility to employ complex theoretical approaches to interpret SPR data output and to precisely estimate equilibrium dissociation constants for small molecules–serum proteins complexes [61,62]. Sandblad P. and coworkers developed a rigorous process to ascertain the degree of heterogeneity of an interaction before choosing the best binding model to describe it. Scatchard plot analysis and adsorption energy distribution (AED) calculation are combined to reduce the number of possible binding models to apply and to deduce how many adsorption sites are responsible for the partitioning. Then, non-linear regression analysis is used for the data fitting procedure, considering the number of adsorption sites identified by the Scatchard plot analysis/AED calculation. This approach resulted suitable to quantify the affinities of warfarin and propranolol enantiomers to HSA and AGP respectively. Nonetheless, this methodology cannot be considered as a fast and simple tool to quantify affinities. It requires the use of a sufficiently broad range of concentrations – up to 1000-fold –, a condition that is not always achievable due to solubility problems. Moreover, the complex mathematics behind the analysis can also constitute a time-consuming step that slows down the analysis and hampers the intrinsic throughput of the technique.

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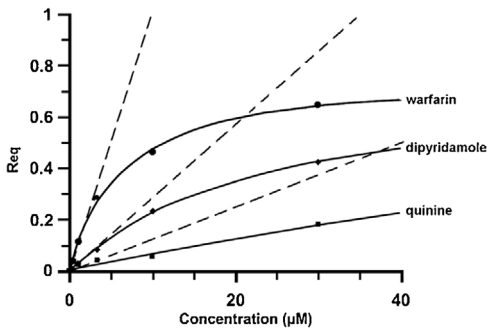

Fig. 4. Illustration of the binding efficiency concept. Saturation curves for the interaction between HSA and warfarin (circles), dipyridamole (stars), and quinine (squares). The solid curves represent the theoretical curves obtained with a 1:1 interaction model, whereas the dashed lines correspond to the binding efficiency. These represent the slope of the saturation curve at low compound concentrations. Reprinted with permission from reference [63].

In the study conducted by Gustafsson, S. S. and co-workers, a more straightforward approach has been developed with the aim to simplify as much as possible the data analysis procedure without loosing any relevant information on the binding process. The short time required to gather information on the interaction between a panel of drugs and serum proteins, was combined with a refined analysis method, with the introduction of the 'binding efficiency' concept [63]. This parameter was defined as the slope of the saturation curve at low compound concentrations (Fig. 4). It provides a simple description of the affinity of lead candidates towards serum proteins regardless of the steady-state affinity (K_D), the number of binding sites to which the compound can bind and the concentration range that can be used for poorly soluble compounds. This approach is indeed useful for the quantification of the binding of compounds with weak affinities, even when the primary K_D cannot be defined. It eliminates the controversy arising from complex interactions, giving in return valid and reproducible data, useful to compare affinities spanning over 100-fold. With this analysis method, 30 compounds, both known HSA/AGP binders and drug lead candidates, were ranked with respect to their propensity to interact with the two serum proteins.

3.4. Primary binding site and drug–drug interaction

SPR platforms have also demonstrated their utility to establish the primary binding site on serum proteins. Co-injection of a drug over a HSA sensor surface, immediately followed by the injection of the same drug together with a potential competitor, can readily show if competition for the same binding site occurs [64]. The application of SPR to test drug–drug interaction was expanded in the study by Kuroda, Y. et al. [65]. Theoretical equations were developed and applied to experimental data to discriminate between three different modes of interactions; direct competition, allosteric interaction, or independent binding. The consistency of the methods was showed by the good agreement between equilibrium dissociation constants extrapolated from the equations and previously published literature.

3.5. Cross-species studies

In addition to evaluating human serum proteins interactions, SPR biosensors can be easily employed to examine how target compounds interact with albumins from different species. This approach can roughly predict the *in vivo* bioavailability and the toxicology of a molecule before taking it into animal trials [64]. Studies reported equilibrium dissociation constants for complexes of small molecules and albumin from human, rat and mouse serum with a simple direct binding assay [66–68]. Notably, different affinities towards serum proteins from different species were identified. This highlights how administration regimens might need to be adjusted when compounds are tested on animal models, because of the different PK profiles in human and rat/mouse. In the studies, SPR analysis was coupled with cell based distribution assays, affinity chromatography and circular dichroism analyses respectively, to support the SPR binding data and to provide additional details on the molecular recognition event. The work of Giannetti et al. [69] represents an excellent example of how the implementation of SPR analysis for cross-species serum protein binding can bring valuable information during the drug development process. The atypical PK behavior of the anticancer drug, Vismodegib, was elucidated through direct SPR binding assay, in combination with molecular docking and isothermal titration calorimetry. During clinical studies, the drug exhibited an extremely long half-life (10–14 days) in humans, in contrast with half-life measured in rats, which was approximately 1.5 h. Therefore, a systematic biophysical analysis was performed to clarify this unexpected finding. Interaction of Vismodegib with both HSA and AGP from human and rat was assessed at 37 °C; findings revealed a 13 μM affinity for human AGP while no binding was detected toward AGP from rat, and while roughly same affinity was measured toward HSA from both species. Differences emerged from this study pointed out a different PK profiles for the drug binding to serum protein from different species that was not properly assed in preclinical stages. This clearly highlights how a systematic approach, as the one describe in the article from Giannetti et al., could improve translation of preclinical data and reduce attrition of drug candidates during the clinical phase of a program.

3.6. Kinetic studies

The half-life of drug–serum protein complexes is a valuable source of information on a potential lead candidate, and biosensor platforms, monitoring interaction in real-time, provide easy access to this information. Hence, different kinetic studies involving serum proteins were performed [70–72]. Among them, the interaction of ciclynopeptides (CLs) with human serum albumin was studied in relation to the use of CLs as therapeutic agents in view of their immunosuppressive and antitumor activities or as a drug carrier. Knowing the effect of small chemical modification on the rate at which CLs are loaded from the plasma and delivered to the target tissue can help to rationally design more efficient molecules. The evaluation of the interaction revealed that oxidation state of the sulfur on the methionine moiety plays a big role in determining the kinetic rates of the reaction and the overall affinity. Moreover, a structure–activity relationship could be hypothesized where the major forces stabilizing CLs–HSA complexes were found to be of hydrophobic nature.

However, it should be considered that drug–serum proteins association/dissociation events are usually of fast and often complex nature, reflecting the physiological role exploited by the transport proteins within circulatory system. HSA and AGP need to accommodate within their binding pockets different chemical scaffolds of circulating endogenous and exogenous compounds with moderate affinity, rapidly sequestrate and readily release them once the primary target is encountered. Therefore, resulting sensorgrams are characterized by a typical square-wave profile that shows fast on- and off-rate and, quite frequently, some degree of heterogeneity. In view of this behavior, association/dissociation rate constants k_{on} and k_{off} are sometimes difficult to quantify, and a traditional data fitting procedure results insufficient to provide useful and reproducible information.

Nevertheless, depending on the final scope of the analysis, the data quality and the experimental set-up, complex interaction pro-

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files can be deconvoluted into their individual components to shed light onto reaction mechanisms. This can provide a helpful aid to hypothesize the recognition process. For example, in the study by Cimitan, S. et al. the binding of different taxol analogues and anti HIV drugs to serum proteins was assessed [59]. Firstly, the binding level toward HSA and AGP were rapidly determined for the panel of drugs under investigation, and secondly employing a more rigorous experimental procedure, different binding sites were identified for the drugs binding to HSA. Time-resolved analysis clearly highlighted the presence of two distinct binding pockets on HSA. Interestingly, they were discerned into fast and slow, according to the kinetics rather than high- and low-affinity according to the stability. This behavior was ascribed to different accessibility rather than the capacity to host the binding partner.

3.7. Hyphenation with other techniques

SPR can be coupled with other, relatively old and well-established, techniques to support the kinetics extrapolated with the data fitting procedure [70] or to acquire additional information on the small molecule–serum protein complexes [73,74]. An elegant combination of SPR with complementary analytical techniques is highlighted in the study by Zhang Y. et al. [75]. An on-line system composed by SPR platform-HPLC–tandem mass spectrometry was built for the identification and characterization of HSA binders from the dried root extract of Radix Astragali. A functionalized sensor chip surface bearing a suitable amount of HSA was used to separate HSA-binders from the other components of the complex matrix. The crude extract of the herb roots is injected into the SPR system; then, after an equilibrium condition is achieved and maintained for a sufficiently long time, the eluent from the dissociation event is injected in the HPLC for separation. Eventually, the peaks obtained from the chromatographic separation are injected in the MS/MS for structural characterization. SPR separation was found to correlate consistently with data obtained by reverse ultrafiltration methodology conducted in parallel. However, the on-line SPR system bears some clear advantages over the conventional analytical ultracentrifugation approach: i) the same sensor surface can be re-used for multiple screening cycle up to 10 days; ii) false positive and matrix interferences, often detected with ultracentrifugation, are minimized here, since a mock surface can be used to probe in real-time the extent of unspecific binding on the fraction collected for the HPLC separation.

4. Conclusions

Binding to serum proteins constitutes one of the crucial aspects determining the pharmacokinetic properties of a compound circulating in human serum. Preliminary studies on the bioavailability of potential therapeutic agents can help to increase their success rate when entering clinical trials. SPR platforms are an efficient and reliable means to acquire information on the drug-likeness. The low material consumption, the short analysis time and the versatility of the technique make it suited to screen sets of molecule at an early stage of the drug discovery process. Depending on the intrinsic properties of the compounds screened, the quality of the data collected and the final aim of the study, different aspects of the binding event can be evaluated.

Despite the clear advantages brought by the implementation of SPR platforms for early PK studies, up to present days, there is not enough attention paid to it. Few cases of systematic investigation before drug development phase are reported in the literature and not enough effort is put to understand interactions with serum proteins. Moreover, SPR technology results particularly useful for collecting data on translational medicine, which otherwise needs to be assessed through time-consuming analysis that are often omitted to speed up the development process. Time lost to understand abnormal PK behavior in clinical phases could be overcome by the addition of systematic investigation on NCE–serum proteins interaction. Structure-activity relationship for serum protein binding can be constructed in short time and used to modify chemical scaffolds without hampering the activity on the primary target. This approach could ease the process of choosing the best NCE to advance to clinical trials, where poor PK/bioavailability remains one of the major causes of attrition.

From our perspective, whenever SPR platforms are employed for structure–activity relationship studies on the primary target, it should be considered as a common practice to perform *in vitro* bioavailability analysis along with it. The easy access to both HSA and AGP proteins form different species, the robust immobilization protocols and the minimal demand for operator handling render SPR biosensing a suitable methodology to construct a complete picture of the NCE under investigation, taking into consideration both pharmacodynamics and pharmacokinetic properties, and eventually increase its likelihood of success in clinical trials.

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