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On-line kinetic model discrimination for optimized surface plasmon resonance experiments

Massinissa Si Mehand, Gregory De Crescenzo*† and Bala Srinivasan†

In order to improve the throughput of surface plasmon resonance-based biosensors, an on-line iterative optimization algorithm has been presented aiming at reducing experimental time and material consumption without any loss of confidence on kinetic parameters [De Crescenzo (2008) J. Mol Recognit., 21, 256-66.]. This algorithm was based on a simple Langmuirian model to compute the confidence and predict optimal injections. However, this kinetic model is not suitable for all interactions, as it does not include mass transfer limitation that may occur for fast interaction kinetics. If a simple model was to be used when this phenomenon influenced the interactions, kinetic parameters would be biased. On the other hand, we show in this paper that data analysis with a kinetic model including a mass transfer limitation step would lead to longer experiments and poorer confidence if the interactions were simple. So, in this manuscript, we present an on-line model discrimination and optimization approach to increase the throughput of surface plasmon resonance biosensors. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: model discrimination; surface plasmon resonance; biosensor; kinetics; mass transfer limitations

INTRODUCTION

Over the last two decades, surface plasmon resonance (SPR)-based biosensors have gained in popularity and become one of the most useful tools for real-time and label-free bimolecular interaction analysis (De Crescenzo *et al*., 2008a; Holdgate *et al*., 2010; Rich and Myszka, 2010; Geschwindner *et al*., 2012). The rapid emergence of the SPR technology has lead to the commercialization of several automated instrument platforms (Rich and Myszka, 2007; Scarano *et al*., 2010) with improved detection sensitivity (Papalia *et al*., 2006; Abbas *et al*., 2011) and to the development of optimized experimental protocols and data analysis software packages (Myszka and Morton, 1998; O'Connor-McCourt *et al*., 1998; De Crescenzo *et al*., 2008b; Gorshkova *et al*., 2008; Mehand *et al*., 2012). These developments have strengthened the robustness and reliability of the SPR instruments (Geschwindner *et al*., 2012). Most of the SPR biosensors now allow for real-time measurements, thus permitting kinetic parameter identification, an asset in the drug discovery process (Swinney, 2009; Ladbury *et al*., 2010; Holdgate and Gill, 2011). SPR-based biosensing is now acknowledged as a method of choice for primary screening in drug discovery (Navratilova and Hopkins, 2011), thanks to the development of a new generation of instruments allowing for the injection of one analyte over hundreds of targets (Rich and Myszka, 2007). But the quality of the kinetic parameters determined from data analysis collected with these platforms is poor when compared with that of classical low-throughput instruments.

Experiments with low-throughput systems involve the generation of 5–10 sensorgrams by varying the concentration of the injected molecule within a range of concentrations that had been determined by the experimenter *a priori*. This experimental approach may generate more data than actually needed. Indeed, Ö'nell and Andersson (2005) showed that two adequately chosen injections are enough for the identification of kinetic parameters with the same level of confidence as from a classical set of injections. It is thus possible to drastically reduce experimentation duration and thus increase throughput; the key issue in that endeavor is to choose these two injections adequately. We have solved this problem by introducing a new on-line strategy (De Crescenzo *et al*., 2008b) to determine the optimal experiment to be performed (optimal injection duration and analyte concentration) by taking into account kinetic profile that had been already collected. This algorithm is based on one non-optimized injection; the collected data are used for a first estimation of the parameters. These parameters are used to solve an optimization problem that predicts an injection to be performed to increase the level of confidence on the kinetic parameters. The level of confidence is set equal to the confidence level of a corresponding classical SPR experiment. Our results indicated that when a simple model depicted the interaction, one or two carefully planned injections were sufficient to get comparable confidence on kinetic parameters as when deduced from the analysis of classical experiments (De Crescenzo *et al*., 2008b). We originally based our approach to simple kinetics; the use of a simple model is however not relevant when mass transfer limitation (MTL) influences the

* *Correspondence to: G. De Crescenzo, Department of Chemical Engineering École Polytechnique de Montréal, PO Box 6079, Centre-ville Station, H3C 3A7 Montréal, Québec, Canada.*
E-mail: gregory.decrescenzo@polymtl.ca

† *Both authors equally contributed to this work. To whom all correspondence should be sent.*

M. S. Mehand, G. D. Crescenzo, B. Srinivasan
Department of Chemical Engineering, École Polytechnique de Montréal, PO Box 6079, Centre-ville Station, H3C 3A7 Montréal, Québec, Canada

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kinetics of interaction (Goldstein *et al*., 1999; Mason *et al*., 1999). In this case, a simple model will not fit experimental data, and the identified kinetic parameters will be biased.

In this manuscript, we demonstrate that on the other hand, the use of a two-compartment model describing MTL (Myszka *et al*., 1998) cannot be combined to our on-line algorithm because when the interaction is simple, it will lead to longer experiments, and it may potentially result in decreased confidence levels on kinetic parameters. We investigated the domain of application of each model based on the conclusions of Goldstein and colleagues, (1999). On the basis of these conclusions and the work of De Crescenzo *et al*., 2008b, we designed an algorithm to improve the throughput of the SPR experiments while selecting the adequate model to depict the interactions. This algorithm was shown to be almost as fast as the optimization strategy we originally proposed without any model discrimination.

THEORY

Modeling of the interaction

After the immobilization of one of the interacting species (the ligand, later denoted B) at the biosensor surface, standard Biacore experiments (the most widely used SPR biosensors now sold by GE Healthcare) consist in injecting its binding partner (the analyte A), followed by the injection of a buffer solution to monitor the dissociation of the ligand/analyte complexes. If the dissociation is not complete, an additional regeneration step must be included in the protocol. All these steps (complex formation, dissociation and regeneration) constitute a sensorgram. A set of sensorgrams can be obtained by repeating these steps while varying the length of the analyte/buffer injections or the analyte concentration.

When MTL does not influence the interaction, the following simple model describes the interactions:

$$\dot{R} = k_a C_T (R_{\max} - R) - k_d R \tag{1}$$

where k_a and k_d are the related association and dissociation rate constants expressed in $M^{-1}s^{-1}$ and s^{-1}. R is the resulting recorded signal in resonance unit (RU), corresponding to the surface accumulation of A resulting from complex formation (AB). R_{max} is the maximal amount of analyte that can specifically bind to the surface in RU. C_T is the concentration of injected analyte.

For molecular systems displaying fast kinetic rates, the interactions may be influenced by MTL; the following model describes them:

$$\begin{cases} \dot{C}_A = -k_a C_A \left(\widetilde{R}_{\max} - \widetilde{R}\right) + k_d \widetilde{R} + \widetilde{k}_M (C_T - C_A) \\ \dot{R} = k_a C_A (R_{\max} - R) - k_d R \\ \widetilde{R} = \dfrac{R}{h},\ \widetilde{R}_{\max} = \dfrac{R_{\max}}{h}, \widetilde{k}_M = \dfrac{k_M}{h} \end{cases} \tag{2}$$

where C_A is the concentration of the analyte at the vicinity of the surface where the interaction takes place (in M) and $\widetilde{k}_M$ corresponds to the normalized mass transfer coefficient (in s^{-1}). In the case of the MTL model, note that Goldstein *et al*. (1999) demonstrated that the value of h did not influence the kinetic parameters, and the data were insensitive as well; h was thus set to 1 RU M^{-1} for simplicity sake. In Equation 2, k_M is thus in RU $M^{-1}\,s^{-1}$. Using the conversion factor 1 RU = 10^{-10} g cm^{-2} and taking the molecular weight (MW in g mol^{-1}) of the analyte into consideration, on can thus obtain k_M in cm s^{-1} by multiplying $\widetilde{k}_M$ by (10^{-7}/MW).

Parameter identification problem

To identify k_a and k_d, sensorgrams are generated by varying the concentration of the analyte A. Each sensorgram consists of an on-period (analyte injection, t_a) and an off-period (buffer injection, t_d). From the experimental data, the following least squared identification algorithm is used to compute the required parameters:

$$\min_{\lambda} \sum_{i=1}^{N} \frac{1}{P_i} \sum_{j=1}^{P_i} \left(R^i_{meas}(j) - R^i_{pred}(j) \right)^2 \tag{3}$$

where λ is the parameter vector, $\lambda = [k_a\ k_d\ R_{max}]$ for the simple model and $\lambda = [k_a\ k_d\ R_{max}\ k_M]$ for the MTL model. N is the number of sensorgrams, P_i is the number of data points in the i^{th} sensorgram, R_{meas} is the value of a given data point, R_{pred} its related value predicted from the model presented in Equation (1) or (2).

The confidence on kinetic parameters is computed using the standard error and the Fisher information matrix, as already described in the previous work (De Crescenzo *et al*., 2008b).

Optimization and model discrimination

The optimization algorithm proposed by De Crescenzo *et al*. (2008b) is based on the satisfaction of the confidence level on two parameters of the simple model (kinetic constants). As already discussed, this model does not describe the interactions that are mass transfer limited. If such is the case, the simple model will return biased kinetic parameters. The MTL model is more appropriate to avoid this error. However, this model comprises an additional parameter. Hence, if the MTL model is used while analyte diffusion is not limiting (i.e., if data may be adequately fit with the simple model), the confidence on kinetic parameters will be decreased when compared with that determined using the simple model because the number of parameters influences negatively the confidence. As the experimental time of the optimized protocol originally proposed by De Crescenzo *et al*. (2008b) depends on the level of confidence, the choice of the model to depict the data is thus important. The direct replacement of the simple model by the MTL model is not an optimal solution, as demonstrated later. The introduction of model discrimination within the optimization algorithm is thus essential to avoid biased kinetic parameters (by always using the simple model) and unnecessary data collection (by always using the MTL model).

In this work, the optimization approach proposed by De Crescenzo *et al*. (2008b) is extended to find a compromise between model discrimination and optimization. Several criteria have been proposed in the literature to find the optimal design for model discrimination (Atkinson, 2008; Schwaab *et al*., 2008; Abd El-Monsef and Seyam, 2011; Alberton *et al*., 2011). Here, model discrimination is not an end; rather, it is just a means to shorten the experiment. To do so, we used the dimensionless number below:

$$\theta = \frac{\widetilde{R}_{\max} k_a}{\widetilde{k}_M} \tag{4}$$

Of interest, if θ is low (i.e., $\theta < 0.1$), MTL is negligible and both simple and MTL models are equivalent; the use of a simple

model is thus sufficient to analyze the experimental data. On the contrary, for θ values higher than 0.1, MTL significantly affects the kinetics of binding even when the fit with simple model gives good residuals. The latter occurs for $0.1 < \theta < 1$. In that case, as shown by Goldstein *et al.* (1999), $k_a^e = \frac{k_a}{1+\theta}$ and $k_d^e = \frac{k_d}{1+\theta}$. Thus, for $\theta > 0.1$, the use of the MTL model is compulsory in order to get error on kinetic parameters of less than 10%.

The discrimination step consists of raising the confidence on θ in order to choose the relevant model. To compute the confidence on θ, the following equation was used:

$$\frac{\Delta\theta}{\theta} = \sqrt{\frac{\Delta\varepsilon_{k_a}{}^2}{k_a{}^2} + \frac{\Delta\varepsilon_{R_{\max}}{}^2}{R_{\max}{}^2} + \frac{\Delta\varepsilon_{k_M}{}^2}{k_M{}^2}} \tag{5}$$

We could then define $(\theta_{min}, \theta_{max}) = \theta \pm \Delta\theta$.

Our optimization algorithm is presented in Figure 1. The objective of this algorithm is to minimize the length of the experiments while choosing the model that describes the interaction in order to avoid unnecessary data collection and biased kinetic parameter. To optimize the experiment and discriminate between the models, values of the parameters of the models have to be known. However, such is not the case experimentally: to evaluate these parameters, our optimization algorithm is based on a first non-optimized experiment. Once the parameters are computed, the following problem is solved to predict the injection that will discriminate between the models:

$$\min_{t_a,t_d,C_A} (t_a + t_d)$$
$$s.t \begin{cases} \xi_a \le \delta_a, \xi_b \le \delta_b \\ \text{if } \theta < 0.1 \text{ then } \theta_{\max} < 0.1 \\ \text{f } \theta > 0.1 \text{ then } \theta_{\min} < 0.05 \\ t_a + t_d < T_{\max}, \quad t_a < t_{a\max}, \quad t_d < t_{d\max}, \quad C_A < C_{\max} \end{cases} \tag{6}$$

where t_a and t_d are the duration of the association and the dissociation phase of the experiment, respectively; C_A is the concentration of analyte; $t_{a\ \max}$ and $t_{d\ \max}$ are the maximal durations allowed for the association and dissociation phases, respectively; $C_{A\ \max}$ is the maximal concentration of analyte allowed and $T_{\max}$ the maximum total time allowed for the experiment. The standard errors ξ_a and ξ_d are computed based on the Hessian information. δ_a and δ_d are the constraints on kinetic parameters during the discrimination step. Note that this algorithm was designed as we observed that the confidence on θ was always very poor.

Once the model is chosen according to the algorithm presented in Figure 1, the optimization problem, expressed as follow, is used to optimize the experiment with the correct model to focus in the identification of the kinetic parameters with the desired level of confidence:

$$\min_{t_a,t_d,C_A} (t_a + t_d)$$
$$s.t \begin{cases} \xi_a \le \beta_a, \xi_b \le \beta_b \\ t_a + t_d < T_{\max}, \quad t_a < t_{a\max}, \quad t_d < t_{d\max}, \quad C_A < C_{\max} \end{cases} \tag{7}$$

where β_a and β_d are the desired standard errors on the estimated parameters k_a and k_d, respectively. Within this manuscript, the desired standard errors (β_a and β_d) were set equal to those we

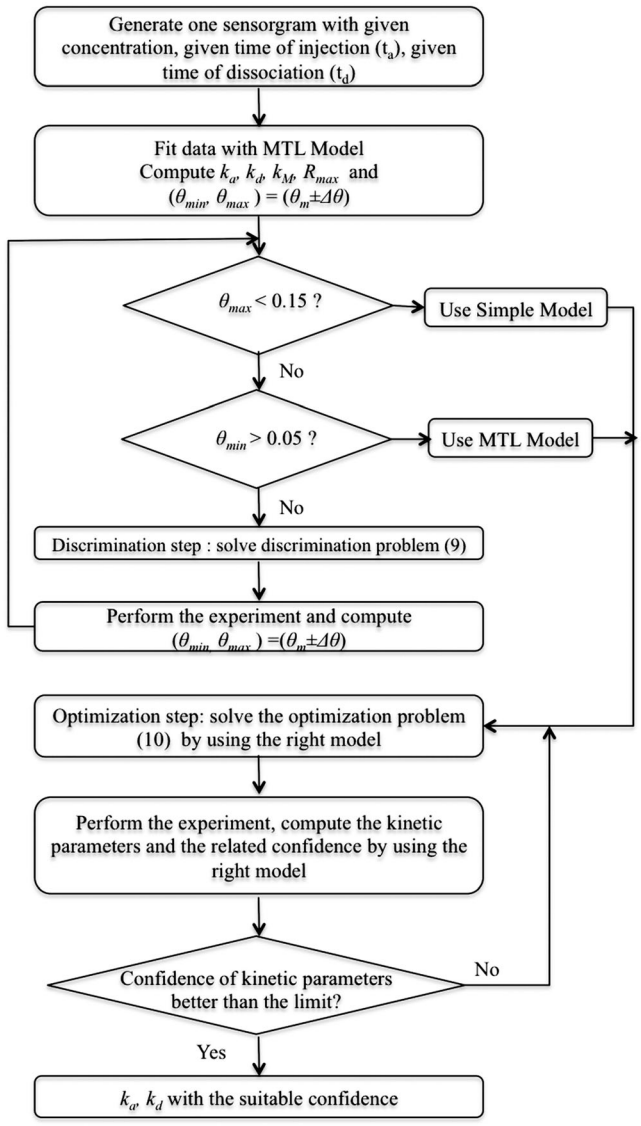

Figure 1. Algorithm corresponding to the "optimization with model discrimination" approach.

obtained from the analysis of the corresponding classical set of sensorgrams (De Crescenzo *et al.*, 2008b). For the first non-optimized experiment, the choice of the concentration as well as the duration of the injections is critical to provide the software with data allowing for a reasonable estimation of the parameters. However, the next two experiments will choose concentrations that are appropriate, considering the dynamics and the mass transfer into account. Because the experimental planning is so performed to bring out the key model characteristics, no weighting of the data is performed in the calculation of the sum of the squared residuals for data corresponding to optimized injections.

MATERIALS AND METHODS

Materials

Experimental data were generated with a BIACORE T100 optical biosensor equipped with research-grade CM5 sensor chips (GE Healthcare, Baie d'Urfe, QC). HBS-EP buffer, acetate buffer and ethanolamine were purchased from GE Healthcare. N-ethyl-N'-(3-

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dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), carbonic anhydrase isozyme II (CAII) that had been purified from bovine erythrocytes, 4-carboxybenzenesulfonamide (CBS), sulfanilamide, 1,3-benzenedisulfonamide (BDS), acetazolamide, dimethyl sulfoxide (DMSO) and phosphate buffer saline (PBS, 10 mM, pH 7.4) were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON).

Biosensor surface preparation

Biosensor surface preparation (CAII and blank surfaces) was performed according to published protocols (Navratilova *et al*., 2007). CAII surfaces were prepared at a density of approximately 5000 RU. After CAII immobilization or blank surface generation, the system was extensively primed with buffer (HBS-EP).

Biacore sample injections

Analyte and buffer preparations

Analytes and buffer were prepared according to protocol reported in Navratilova *et al*., 2007. HBS-EP with 3% of DMSO was used as running buffer. All the analytes were dissolved in pure DMSO ([CBS] = 1.76 mM, [BDS] = 0.35 mM, [sulphanilamide] = 1.67 mM and [acetazolamide] = 10.39 mM). These stocks were diluted by mixing 30 μl with 970 μl of pure HPS-EP, resulting in concentrations of 52.98, 10.49, 50.27 and 311.83 μM for each analyte, respectively, with 3% of DMSO in order to match the amount of DMSO in the running buffer. Thirty microliter of the new solution of acetazolamide were mixed with 970 μl HPS-EP containing 3% of DMSO to produce a new stock solution of 9.35 μM.

Analyte injections

Prior to analyte injections, three prime procedures and buffer injections were performed to stabilize the baseline of the instrument. All injections of CBS and sulphanilamide were performed in duplicate at a flow rate of 100 μl min^{-1} with a data collection rate set at 10 Hz, at 18°C. All injections of BDS and acetazolamide were performed in duplicate at a flow rate of 20 μl min^{-1} with a data collection rate set at 10 Hz, at 18°C. For preliminary classical kinetic experiments, CBS, sulphanilamide, BDS and acetazolamide were injected at concentrations comprised between 264.90 nM and 52.98 μM, 251.37 nM and 50.27 μM, 52.45 nM and 10.49 nM and 5.21 nM 9.35 μM, respectively, in addition to six buffer solutions (for double referencing purposes), for 60 s across both control and CAII surfaces, followed by a maximum of 350 s injection of HBS-EP running buffer at 18°C. As complete dissociation was observed in each case, no regeneration procedure was performed, in agreement with previous reports (Day *et al*., 2002; Navratilova *et al*., 2007).

Data analysis

All simulations and data were analyzed with an in-house software package developed with the MATLAB 7.7.0.471 (R3008b) software platform (The Mathworks, Natick, USA). The least-square problem presented in (2) was solved with the standard simplex program available in the optimization Toolbox 4.1 of MATLAB. The optimization problems were solved by the sequential quadratic programming program available in the Toolbox 4.1 of MATLAB.

RESULTS AND DISCUSSION

Direct optimization with mass transfer limitation model

The optimization algorithm developed by De Crescenzo *et al*., 2008b was originally based on a simple kinetic model that did not take into account any potential MTL. As the MTL model (Myszka *et al*., 1998) is more general, our first attempt consisted in replacing the simple model by the MTL model in our original algorithm.

Three scenarios were then simulated to test the validity of such an approach: (i) MTL does not influence the interaction ($\theta = 0.05$), (ii) MTL weakly influences the interaction ($\theta = 0.5$) and (iii) MTL strongly influences the interaction ($\theta = 2$). In all cases, k_a, k_d and R_{max} values were fixed at $10^5\ M^{-1}\ s^{-1}$, $10^{-3}\ s^{-1}$ and 50 RU, respectively. The first non-optimized injections (from which our algorithm predicts which injection to be performed next) were simulated with an analyte concentration of 1.2 μM, t_a of 60 s and t_d of 440 s (Figure 2, panels A–C). For all simulations, a Gaussian noise with a standard deviation of 0.2 RU (corresponding to the background noise of the Biacore T100 instrument) was added (Figure 2). For each scenario, the constraints on confidence were computed by considering a set of sensorgrams corresponding to the classical method (i.e., simulation of 10 sensorgrams corresponding to concentrations varying from 10 nM to 2 μM with a noise of 0.2 RU being added; Figure 2, panels D–F) as described in De Crescenzo *et al*., 2008b. The model truly describing the interactions was then used to compute these constraints: simple model for $\theta = 0.05$ and MTL model for $\theta = 0.5$ and $\theta = 2$. Table 1 lists the duration of the various optimized injections that have been proposed by the modified optimization algorithm.

As can be seen in Table 1, the shortest optimized experiment corresponded to the case where mass transfer strongly influenced the interaction ($\theta = 2$); whereas the longest optimized experiment was obtained when MTL did not influence the interaction ($\theta = 0.05$). Its duration was almost as long as a classical experiment (Table 1). This observation is directly related to the inappropriateness of the MTL model in this specific case, combined to the fact that the MTL model has an additional parameter when compared with the simple one (the more parameters a model has, the poorer the level of confidence is). Also, when simulated data corresponded to simple kinetics, the algorithm that used the MTL model was not able to propose optimized conditions to reach the desired confidence level (i.e., the level of confidence one would have had when analyzing the corresponding "classical" set of sensorgrams). Altogether, this preliminary case study indicated that the direct replacement of the simple model used in the original algorithm of De Crescenzo *et al*., 2008 by the MTL model would lead to unnecessarily long experiments for all the cases where MTL does not influence the interaction. The extension of the original algorithm to interactions being potentially mass transfer limited must thus include a discrimination strategy so as to use the MTL model only when necessary.

The "optimization with model discrimination" approaches

To achieve this goal, two approaches combining optimization and model discrimination were proposed and tested with simulated data sets corresponding to the three scenarios we described earlier (Figure 2). These approaches are based on the algorithm presented in Figure 1. In both cases, the first proposed injection aims to discriminate between simple and MTL models,

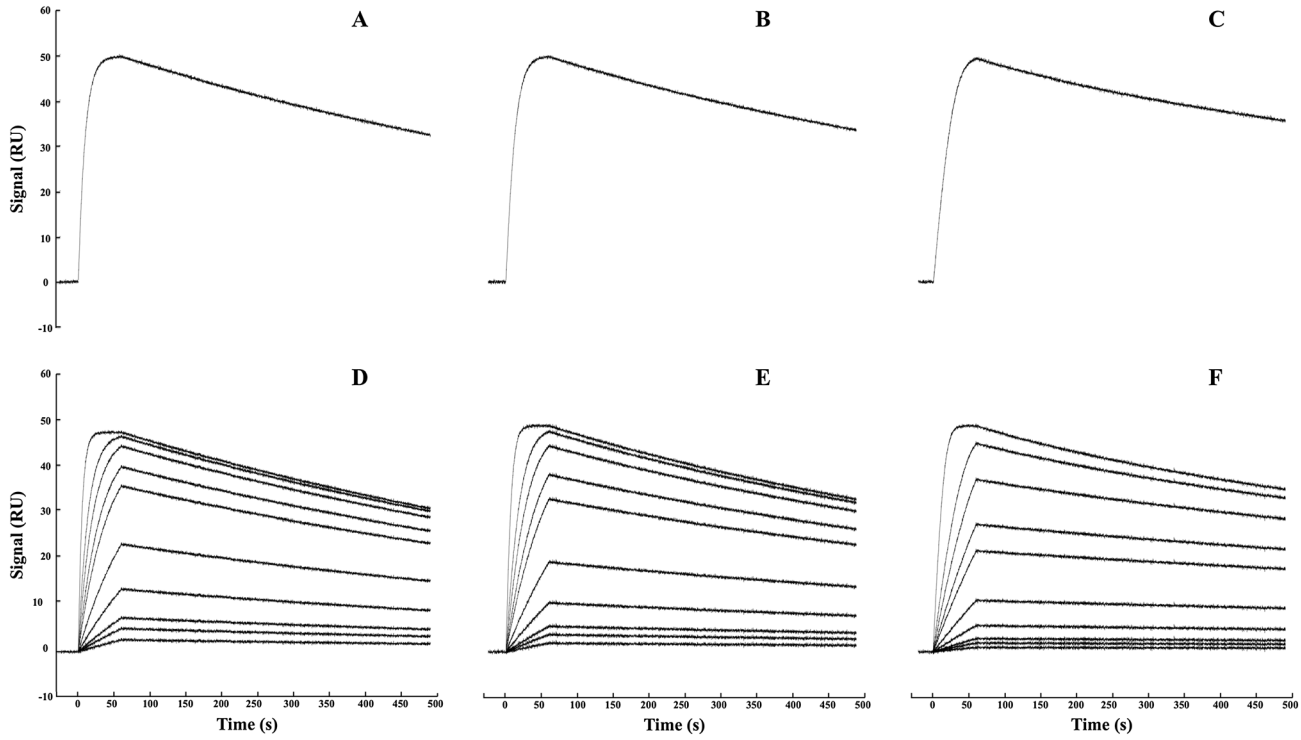

Figure 2. Simulated sensorgrams corresponding to the non-optimized data used in this manuscript. Panels (A), (B) and (C) correspond to the single sensorgrams that were simulated to test the direct replacement of the simple model by the mass transfer limitation (MTL) one in the original algorithm by De Crescenzo *et al*. (2008). Panels (D), (E) and (F) correspond to the "classical" sets of sensorgrams that were used as reference to determine the level of confidence on the kinetic parameters to be used as constraints by the different algorithms. All simulations were performed to cover the following three scenarios (i) MTL does not influence the interaction, $\theta = 0.05$ (panels A and D); (ii) MTL weakly influences the interaction, $\theta = 0.5$ (panels B and E); and MTL strongly influences the interaction, $\theta = 2$ (panels C and F). In all cases k_a, k_d and R_{max} were set equal to $10^5\,M^{-1}\,s^{-1}$, $10^{-3}\,s^{-1}$ and 50 RU, respectively. The injection and dissociation times were set equal to 60 and 440 s, respectively.

Table 1. Direct optimization with the mass transfer limitation model

θ	Number of on-line optimized injections (s)	Duration of the on-line optimized injections (s)	Duration of the on-line optimized experiment*(s)	Duration of the classical experiment*
2	2	313	5 504	11 000
0.5	3	503	8 025	11 000
0.05	3	719	10 190	11 000

*In order to calculate the duration of the simulated experiments, the duration of the sensorgrams corresponding to analyte injections was doubled (duplicate injection), and the time corresponding to four buffer injections for calibration and double referencing purpose has been added (consistent with calculations reported in De Crescenzo *et al*., 2008b).

subsequent injection(s) aim(s) to refine parameter identification to reach the desired level of confidence on kinetic parameters (taking into account the information contained in the first two injections). Both approaches only differed on constraints imposed on kinetic parameters for the first injection. For approach 1, the algorithm presented in Figure 1 was first applied with the constraints on kinetic parameters confidence being completely relaxed (i.e., $\delta_a = \delta_d = 100\%$) in an effort to predict the shortest injection that would allow to discriminate between the simple and MTL models, that is, to give θ_{min} and θ_{max} values permitting discrimination (Figure 1). Approach 1 was determined to be very efficient for model discrimination because the algorithm returned very short first injections (Table 2). However, as no significant improvement was made on the confidence on kinetic parameters with this injection, the subsequent optimized injections were longer or more than one optimized injections were required, eventually resulting in relatively long experimental time (Table 2).

As a trade-off between discrimination and confidence improvement, approach 2 was then designed. It corresponded to the case where the same algorithm is first applied with $\delta_a = \delta_d = 30\%$ in order to discriminate between models as well as gain on confidence on the kinetic parameters from the very first predicted injection. Thus, whereas in approach 1, the first

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Table 2. Simulations results related to approaches 1 and 2

	θ	Number of injections*	Duration of the on-line optimized injections(s)**	Duration of the on-line optimized experiment (s)***	Duration of classical experiment***
Approach 1	2	1 + 3	60 + 296	6 322	11 000
($\delta_a = \delta_d$ =100%)	0.5	1 + 2	60 + 241	5 288	11 000
	0.05	1 + 1	73 + 120	4 158	11 000
Approach 2	2	1 + 1	143 + 60	4 218	11 000
$\delta_a = \delta_d$ = 30%)	0.5	1 + 1	70 + 60	3 780	11 000
	0.05	1 + 1	117 + 82	4 194	11 000

*The first number corresponds to the number of the injection for model discrimination and the second one to the subsequent injection(s) for experiment optimization.

**The first number corresponds to the duration of the injection for model discrimination and the second one to the subsequent injection(s) for experiment optimization.

***In order to calculate the duration of the simulated experiments, the duration of the sensorgrams corresponding to analyte injections was doubled (duplicate injection), and the time corresponding to four buffer injections for calibration and double referencing purpose has been added (consistent with calculations reported in De Crescenzo *et al*., 2008).

proposed injection focused solely on discriminating between kinetic models, the first proposed injection within approach 2 aimed to increase confidence both on θ and kinetic parameters.

As can be seen in Table 2, approach 2 resulted in longer initial injections than those reported for approach 1. However, this slight increase allowed to reduce significantly the number and the duration of subsequent injections, resulting in global experimental durations of less than 40% of that of the corresponding classical experiment (4218 s versus 11 000 s in the worst case, see Table 2). On the basis of these encouraging simulations, approach 2 was then experimentally tested.

Experimental validation

On the basis of the literature (Cannon *et al*., 2004; Navratilova *et al*., 2007; Mehand *et al*., 2012), biological systems corresponding to carbonic anhydrase isozyme II (CAII) interactions with various drugs were selected to generate data corresponding to the three aforementioned scenarios. Kinetics corresponding to CAII interacting with CBS, sulfanilamide and BDS at 18°C with a flow rate of 100 μl min^{-1} have already been reported to follow a simple model (Navratilova *et al*., 2007), whereas those corresponding to acetazolamide have been reported to include MTL when experiments were performed at 25°C (Cannon *et al*., 2004). In order to explore various scenarios, CBS and sulfanilamide were injected at 100 μl min^{-1}, whereas BDS and acetazolamide were injected at 20 μl min^{-1} to increase MTL and thus cover the three scenarios we had simulated.

Classical data generation and kinetic analysis were performed to compute kinetic parameters related to these interactions using both simple and MTL models. Global fits and related kinetic parameters are presented in Figure 3 and Table 3. As expected, experimental data covered all possible scenarios: no MTL for CBS and sulfanilamide (i.e., $\theta = 0.06$ and $\theta = 2.61$ *10^{-5}, respectively), limited MTL for BDS injected at low flow rate ($\theta = 0.28$) and strong MTL for acetazolamide (i.e., $\theta = 4.28$). For BDS (low MTL), differences in kinetic parameter values when using a simple model rather than the MTL model were of 15% (although fits emanating from the use of the simple model were judged as good). This deviation increased to 74% for acetazolamide; in this case, fits were not as perfect as visually determined (data not shown).

For experimental validation, approach 2 was applied as follows: quadruplicate injections of buffer and duplicate injections of analyte (non-optimized injections) were performed at analyte concentration of 3.17 μM, 3.01 μM, 0.62 μM and 22.50 nM for CBS, sulphanilamide, BDS and acetazolamide, respectively. Temperature, flow rate and duration of the injection were identical to those performed for the generation of the classical data sets (Figure 3). For each drug, double-referenced sensorgrams were then used as input in our in-house software package (Figure 1). Kinetic parameters, θ and their related confidence were computed with the MTL model. As a poor confidence was observed for each drug, a first optimized injection was proposed by our software in order to discriminate between kinetic models; this injection was then performed in duplicate. As for the non-optimized injection, quadruplicate buffer injections were also performed for double referencing purpose. Kinetic parameters, θ and their related confidence were computed from all sensorgrams (non-optimized and optimized). In all cases, this unique injection was sufficient to choose the correct model. Once the model was chosen, the problem of optimization (7) was solved to improve the confidence on the kinetic parameters. In all cases, one injection was predicted by the software and was experimentally performed. Subsequent analysis confirmed that desired confidence levels on kinetic parameters had been reached. Experimental data corresponding to this on-line optimization approach are presented in Figure 4 and resulting kinetic parameters are summarized in Table 3. A comparison of these parameters with those emanating from classical experiments or from the literature (Navratilova *et al*., 2007; Mehand *et al*., 2012) indicated that our proposed approach was successful for kinetic parameter identification. Furthermore, approach 2 allowed noticeable reduction of experiment duration as indicated by the time reduction reported in the last column of Table 3 (this value corresponds to the ratio between the durations of optimized experiment and its related classical experiment). This ratio ranged between 2.15 and 3.09. Of salient interest, these values were found to be very similar to those we previously reported when performing on-line optimization

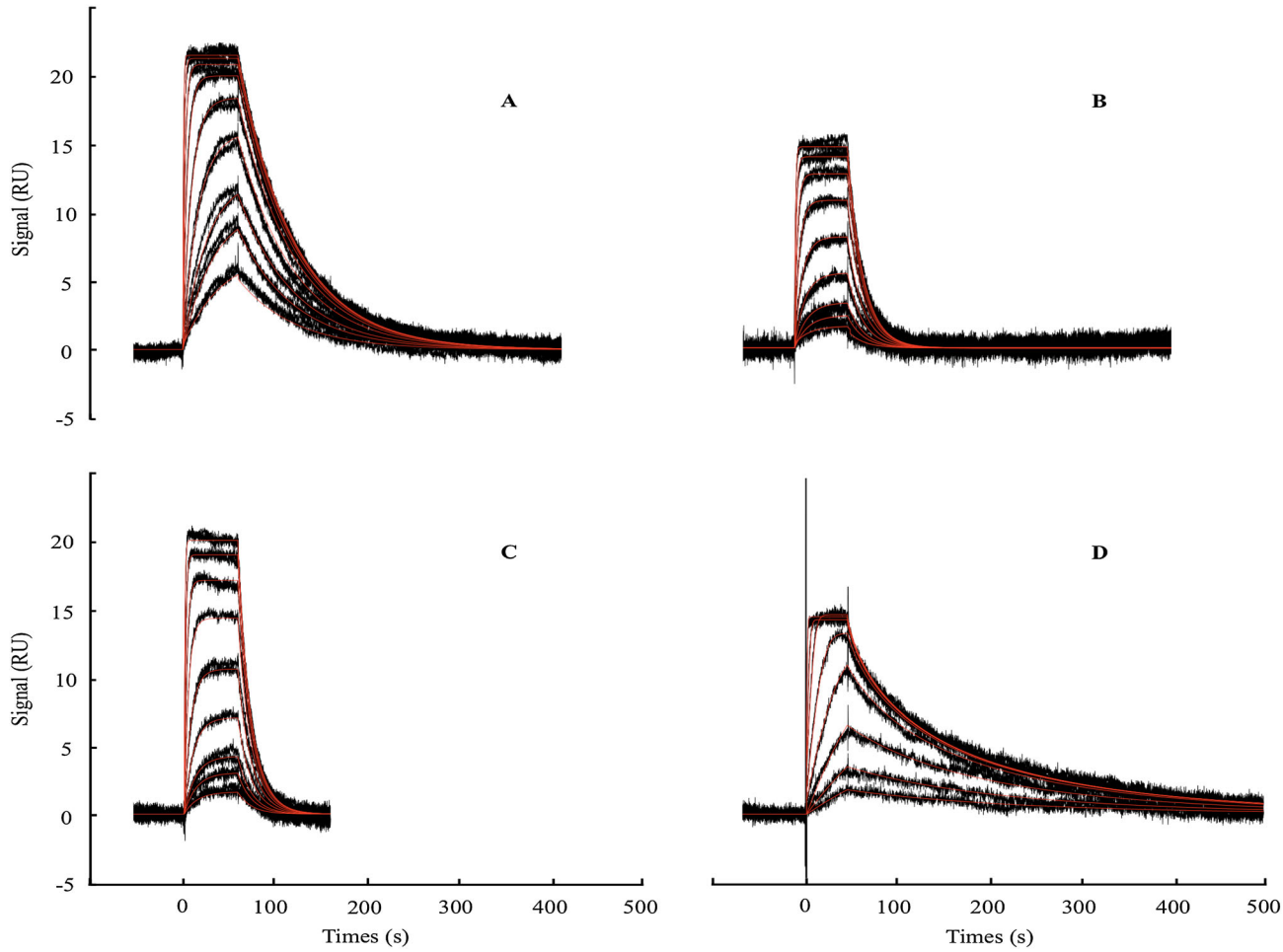

Figure 3. Classical kinetic analysis of CBS, Sulfanilamide, BDS and acetazolamide binding to CAII surfaces. Black dots correspond to control-corrected and double-referenced sensorgrams for all case studies. Panels (A)–(D) correspond to the injection of CBS, sulfanilamide, BDS and acetazolamide over CA II (5000 RU), respectively. Red lines correspond to global fits using the simple model for panels (A) and (B) and the mass transfer limitation model for panels (C) and (D).

Table 3. Kinetic constants determined by the classical method and approach 2 (discrimination/optimization)

	Classical method		Approach 2		Time reduction ratio
	$k_a \times 10^4$ ($M^{-1} s^{-1}$)	$k_d \times 10^{-3}$ (s^{-1})	$k_a \times 10^4$ ($M^{-1} s^{-1}$)	$k_d \times 10^{-3}$ (s^{-1})	
CBS 100 µl min^{-1}	2.9 ± 0.3	16 ± 1	3.2 ± 0.3	17 ± 1	2.52
Sulfanilamide 100 µl min^{-1}	2.0 ± 0.2	58 ± 4	2.2 ± 0.2	60 ± 4	2.15
BDS 20 µl min^{-1}	12 ± 2	73 ± 10	12 ± 2	76 ± 8	2.85
Acetazolamide 20 µl min^{-1}	238 ± 47	19 ± 4	282 ± 56	23 ± 3	3.09

without any model discrimination (De Crescenzo *et al*., 2008b; ratio ranging from 1.8 to 3.2).

Altogether, because our approach can be easily automated and because it is compatible with both ligand capture and covalent immobilization protocols, data presented in this manuscript highly suggest that the algorithm we have developed may be valuable to increase the throughput of current biosensors when screening large sets of analytes binding to a given ligand. However, for optimal performance, the proper implementation of our approach would require that (a) the collected data are free of experimental artefacts but mass transport limitations (e.g., those related to heterogeneous surfaces resulting from random coupling) and (b) the biological system under study to be characterized by a simple kinetic mechanism of binding. While a careful experimental approach based on the recommendations summarized by Myszka (1999) will eliminate these artefacts and

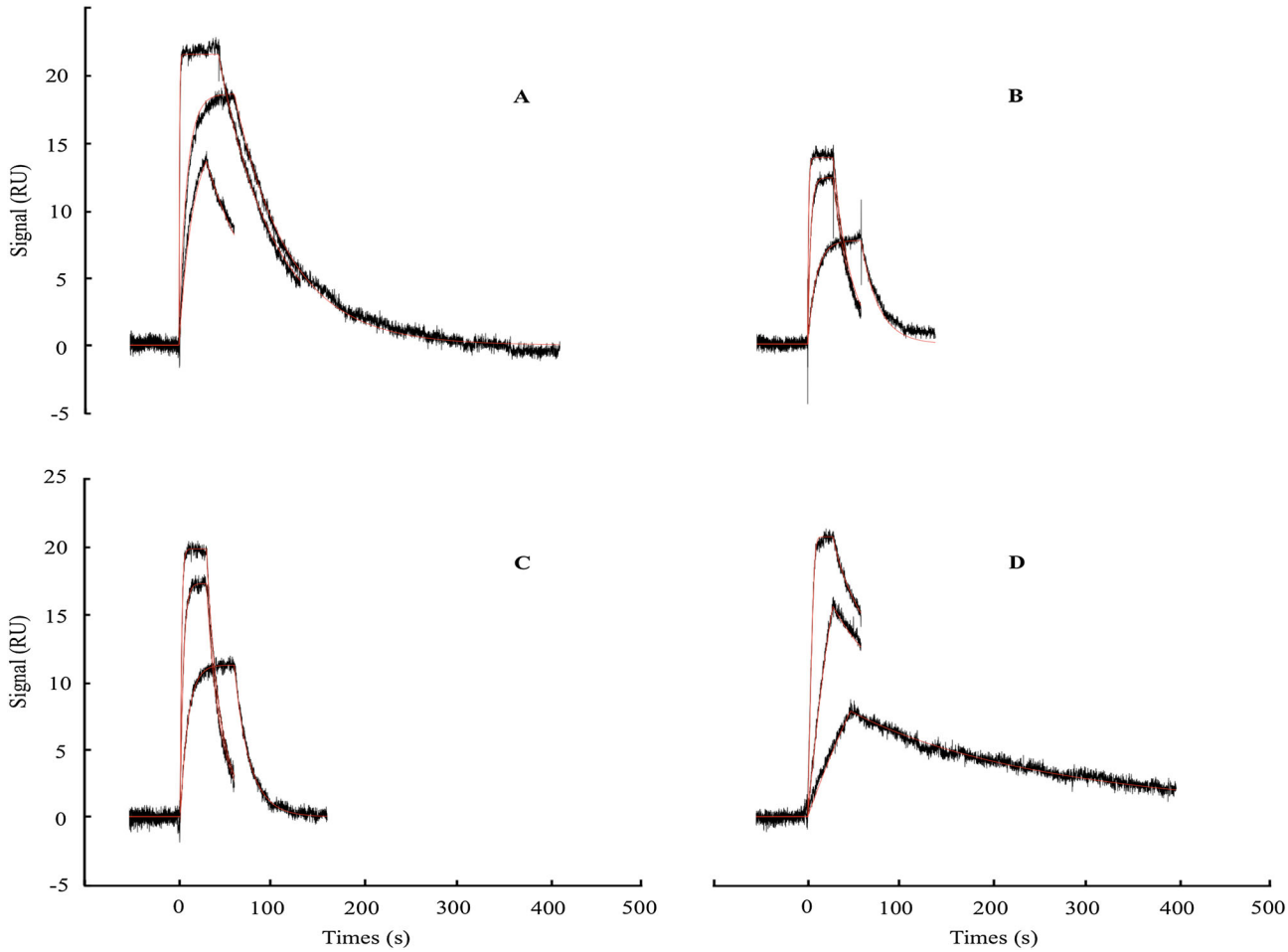

Figure 4. Experiments performed according to approach 2 for kinetic analysis of CBS, sulfanilamide, BDS and acetazolamide binding to CAII surfaces using a BIACORE T100 biosensor. Black dots correspond to control-corrected and double-referenced sensorgrams for all case studies. Red lines correspond to global fits using the simple model in (A) and (B) and model one in (C) and (D). Panels (A)–(D) correspond to the injection of the following analytes: CBS, Sulfanilamide, BDS and Acetazolamide at 18°C over CA II (5000 RU).

will diminish mass transport limitations, complex mechanisms of interactions being intrinsic to the nature of binding partners would require the use of more sophisticated kinetic models to be combined to complex experimental strategy for proper characterization. In that case, model discrimination is unlikely to be automated without prior information on the system. Our approach may thus be of salient interest for biological systems for which preliminary data suggest that deviation from a 1:1 interaction is unlikely to occur. Those may include the kinetic characterization of the interactions between a tethered protein and (a) its binding partner at different temperatures, (b) small-MW compounds that have been selected after a first round of *in vitro* screening and (c) single-point mutants of a protein/peptide known to interact with the ligand with a 1:1 stoichiometry.

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