Talanta 114 (2013) 211–216

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Short communication

Simulated single-cycle kinetics improves the design of surface plasmon resonance assays

William Palau [a,b], Carmelo Di Primo [a,b,*]

[a] Université de Bordeaux, laboratoire ARNA, F-33000 Bordeaux, France
[b] INSERM, U869, laboratoire ARNA, F-33600 Pessac, France

ARTICLE INFO

Article history:
Received 22 February 2013
Received in revised form
29 March 2013
Accepted 8 April 2013
Available online 15 April 2013

Keywords:
Optical biosensor
SPR
Simulation
Kinetics
Single-cycle kinetics
Nucleic acids
Interactions

ABSTRACT

Instruments based on the surface plasmon resonance (SPR) principle are widely used to monitor in real time molecular interactions between a partner, immobilized on a sensor chip surface and another one injected in a continuous flow of buffer. In a classical SPR experiment, several cycles of binding and regeneration of the surface are performed in order to determine the rate and the equilibrium constants of the reaction. In 2006, Karlsson and co-workers introduced a new method named single-cycle kinetics (SCK) to perform SPR assays. The method consists in injecting sequentially increasing concentrations of the partner in solution, with only one regeneration step performed at the end of the complete binding cycle. A 10 base-pair DNA duplex was characterized kinetically to show how simulated sensorgrams generated by the BiaEvaluation software provided by Biacore™ could really improve the design of SPR assays performed with the SCK method. The DNA duplex was investigated at three temperatures, 10, 20 and 30 °C, to analyze fast and slow rate constants. The results show that after a short obligatory preliminary experiment, simulations provide users with the best experimental conditions to be used, in particular, the maximum concentration used to reach saturation, the dilution factor for the serial dilutions of the sample injected and the duration of the dissociation and association phases. The use of simulated single-cycle kinetics saves time and reduces sample consumption. Simulations can also be used to design SPR experiments with ternary complexes.

1. Introduction

Surface plasmon resonance (SPR)-based instruments are powerful tools that are routinely used in pharmaceutical and academic laboratories for investigating biomolecular interactions. The major advantage of this label-free technique is that the signal depends only on changes in mass that occur upon formation of a complex between a target immobilized on a sensor chip and a partner injected over the surface, in a continuous flow of buffer [1,2]. Some commercial optical biosensors have become so sensitive that, for instance with the Biacore™ T200 instrument, according to the manufacturer, there is no limit to the mass that can be detected. Nowadays, SPR has become the 'gold standard' for label-free detection in real time of virtually all kinds of compounds [3].

In a classical SPR experiment one partner is first immobilized onto the sensor chip while the other one, namely the analyte, is injected over the surface. Several injections of the analyte at increasing concentrations are generally performed in order to determine the rate and the dissociation equilibrium constants [4]. If the complex does not dissociate rapidly, a regeneration step is required after each sample injection to remove the analyte that interacted with the immobilized target. The association and dissociation rate constants, k_a and k_d, respectively, are determined by direct curve fitting of the sensorgrams (signal as a function of time) according to models that describe the complex formation. In most cases a Langmuir 1:1 model of interaction is used. The dissociation equilibrium constant, K_D, is calculated as k_d/k_a.

In 2006, Karlsson and co-workers introduced a new method for performing SPR experiments with Biacore™ instruments, the most commonly used commercial optical biosensors. This method known today as single-cycle kinetics (SCK) consists in injecting sequentially over the functionalized sensor chip surface one partner of the interaction, the analyte, at increasing concentrations, without regeneration steps between each sample injection [5]. The SCK method is faster than the classical one because the regeneration step is performed at the end of a series of concentrations and not between each concentration injected. The method is not restricted to Biacore instruments and can be used with

Abbreviations: SPR, surface plasmon resonance; SCK, single-cycle kinetics; SCKODS, single-cycle kinetics on a decaying surface; SSCK, simulated single-cyle kinetics.

* Corresponding author at: Université de Bordeaux, laboratoire ARNA, F-33000 Bordeaux, France. Tel.: +33 540003050.
E-mail address: carmelo.diprimo@inserm.fr (C. Di Primo).

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.04.022

any optical biosensor instrument that can perform successive injections in a single cycle.

When Biacore™ commercialized their instruments, they provided a software package that included a programme named BiaSimulation, to simulate binding curves by the classical method. Surprisingly, in the last generation Biacore™ instruments that include the SCK method, a user-friendly programme is missing to simulate SCK sensorgrams. In 2006, Karlsson and co-workers performed simulations with the CLAMP software developed by Myszka and Morton [6] to validate the SCK method [5]. The authors simulated classical and SCK sensorgrams with a random noise added of 0.5 resonance units (RU). Not surprisingly global fitting of these simulated curves retrieve the rate constants that were used to simulate the sensorgrams. The authors also mentioned in one sentence that simulations could be performed with the BiaEvaluation 4.1.1 software from Biacore. In this work a 10 base-pair DNA duplex was characterized kinetically to show how simulations of SCK sensorgrams made with the BiaEvaluation software could really improve the design of surface plasmon resonance assays. The DNA duplex was investigated at three temperatures, 10, 20 and 30 °C, to analyze fast and slow rate constants. After a short obligatory preliminary experiment, simulations provide users with the best experimental conditions to be used, saving time and reducing sample consumption. Simulations can also be used to prepare SPR experiments with ternary complexes.

2. Materials and methods

2.1. Oligonucleotides

The DNA oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany). The biotinylated DNA, 5′CTCACAACAG3′, synthesized with a biotin-TEG tag at its 5′ end, was purified by HPLC. The complementary strand, 5′CTGTTGTGAG3′, was of high purity salt free grade (HPSF, Eurofins MWG Operon). Upon receipt, the oligonucleotides were resuspended in 100 μl of milliQ water and passed through G-25 spin columns to remove any traces of chemical compounds. The concentration of the samples was determined at 260 nm with a Nanodrop ND-1000 spectrophotometer (Labtech, France) using molar extinction coefficients calculated at http://www.eurofinsdna.com/home.html.

2.2. Surface plasmon resonance experiments

SPR experiments were performed with a Biacore™ T200 apparatus (GE Healthcare Life Sciences, Uppsala, Sweden). The experiments were performed on CM5 sensor chips (Biacore™) coated with 1000–2000 RU of streptavidin (Roche Applied Sciences, Roche Diagnostics, Meylan, France). 10 RU of the biotinylated DNA were immobilized on one flow cell of the sensor chip. A flow cell left blank was used for double-referencing of the sensorgrams [7]. Spikes still present after that were not removed because they did not affect the results, as previously shown [8,9]. The DNA samples were prepared in the running buffer, 20 mM sodium phosphate buffer, pH 7.2 at 20 °C, containing 70 mM sodium chloride and 0.050% Tween-20, and were injected, in duplicate at 25 μl/min. The regeneration of the surface was achieved with a 2-min pulse of 20 mM NaOH. The association and dissociation rate constants, k_a and k_d, respectively, were determined by direct curve fitting of the sensorgrams to a Langmuir 1:1 model of interaction, as described in the Supplementary Data. The dissociation equilibrium constant, K_D, was calculated as k_d/k_a.

2.3. Simulation of SCK sensorgrams

The formulas used to generate simulated sensorgrams were written in the formula entry window available in the BiaEvaluation 4.1.1 (Biacore™) software, for a Langmuir 1:1: model of interaction, as previously described [5].

The formulas for SCK sensorgrams with three sequential injections are:

AB+R_1*$1+$R_2$*$2+R_3*$3; $1=(sign(t−(ton1))−sign(t−(ton1+c_time)))/2; $2=(sign(t−(ton2))−sign(t−(ton2+c_time)))/2; $3=(sign(t−(ton3))−sign(t−(ton3+c_time)))/2; $4=kt($1*Conc/F^2+$2*Conc/F+$3*Conc−A); $5=$k_a$*A*B−$k_d$*AB; A=$4−$5|0; B=−$5|R_{max}; AB=$5|0

The formulas for SCK sensorgrams with five sequential injections are:

AB+R_1*$1+$R_2$*$2+R_3*$3+$R_4$*$4+R_5*$5; $1=(sign(t−(ton1))−sign(t−(ton1+c_time)))/2; $2=(sign(t−(ton2))−sign(t−(ton2+c_time)))/2; $3=(sign(t−(ton3))−sign(t−(ton3+c_time)))/2; $4=(sign(t−(ton4))−sign(t−(ton4+c_time)))/2; $5=(sign(t−(ton5))−sign(t−(ton5+c_time)))/2; $6=kt($1*Conc/F^4+$2*Conc/F^3+$3*Conc/F^2+$4*Conc/F+$5*Conc−A); $7=$k_a$*A*B−$k_d$*AB; A=$6−$7|0; B=−$7|R_{max}; AB=$7|0

In these formulas (written without added formatting), t is the time variable; AB is the complex formed on the surface; R_1, R_2, R_3, R_4 and R_5 are the bulk refractive index of the first, second, third, fourth and fifth injections of the analyte, respectively; ton1, ton2, ton3, ton4 and ton5 are the start times of the first, second third, fourth and fifth injections of the analytes; k_a and k_d are the association and dissociation rate constants of the AB complex formation, respectively; kt is the mass transport rate constant; c_time is the injection time in seconds; Conc is the highest concentration of analyte injected; F is the dilution factor; R_{max}, the maximum response.

The procedure to simulate SCK sensorgrams requires first to generate and to display a baseline in the project window of the BiaEvaluation software. This can be achieved by opening a flat

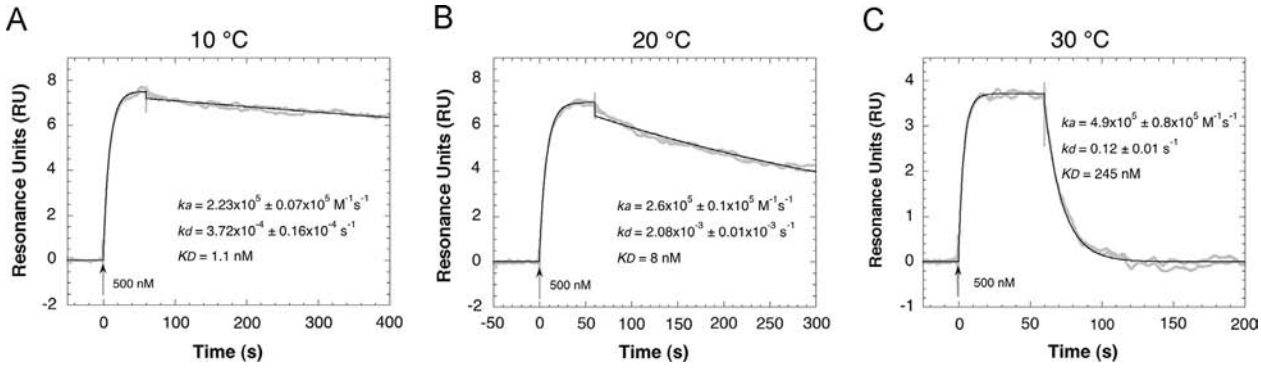

Fig. 1. Kinetic analysis of the DNA duplex formation. The SPR experiments were performed at three different temperatures, 10 °C (A), 20 °C (B) and 30 °C (C). The complementary DNA oligonucleotide was injected in duplicate over the functionalized surface at 500 nM. The grey curves represent the experimental data and the black ones the fit of the sensorgrams to a Langmuir 1:1 model of interaction. The association and dissociation rate constants, k_a and k_d, respectively, and the calculated dissociation equilibrium constant, $K_D=k_d/k_a$, were determined as described in the Supplementary data.

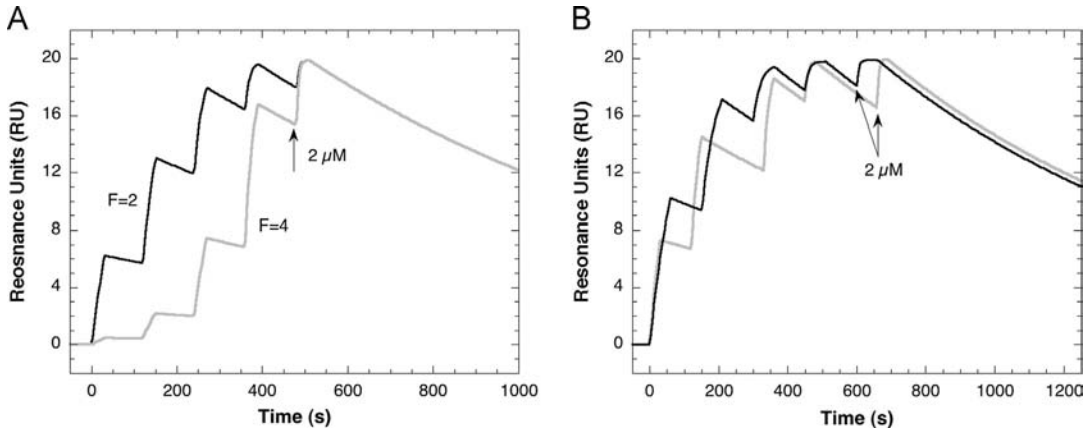

Fig. 2. Simutated SCK sensorgrams with five sequential injections. The procedure used for generating these sensorgrams are detailed in Section 2. The association and dissociation rate constants used for these simulations were fixed arbitrarily at 10^5 M^{-1} s^{-1} and 10^{-3} s^{-1}, respectively. The maximum concentration injected is equal to 2 μM (arrow). (A) Effect of the dilution factor, *F*. In these simulations the association and dissociation phases were fixed at 30 and 60 s, respectively. The dilution factor was fixed either at 2 (black line) or 4 (grey line). (B) In these simulations the dilution factors were fixed at 2. The simulated sensorgrams were generated with association and dissociation phases lasting 60 s (black line), or with an association phase lasting 30 s and a dissociation phase of variable duration (grey line).

sensorgram from any previous experiment or by creating it. A baseline (zero signal as a function of time) can be easily created with Excel. The values are then copied in a new sheet, in BiaEvaluation. A curve is then created from this table (Create Curve, in the menu). The new curve of zero signal as a function of time will appear in the project window of the BiaEvaluation programme. Simulated SCK (SSCK) sensorgrams will actually consist in 'fitting' the baseline with the SCK models describing three or five sequential injections. The only difference with a normal fit is that in this case, the parameters are constant values known by the user, as already mentioned [5]. In the simulations reported in this work, the bulk refractive indexes were fixed at zero and the mass transport rate constant, kt, was fitted globally with initial value of 10^8 s^{-1} (kt can also be left constant, values above 10^7 s^{-1} have no influence on the simulation). R_{max} can be fixed at any value. In this work, R_{max} was fixed at 20 RU (Fig. 2) and at 7 RU (Fig. 3), a value close to that expected knowing that 10 RU of biotinylated target was immobilized on the sensor chip surface.

2.4. Simulation of single-cycle kinetics on a decaying surface (SCKODS) sensorgrams

The formulas to generate simulated SCKODS sensorgrams for a ternary complex are

R0∗exp(−k_b(*t*−*t*0))+Offset+ BC+R_1∗$1+$R_2$∗$2+R_3∗$3; $1=(sign(*t*−(ton1))−sign(*t*−(ton1+c_time)))/2; $2=(sign(*t*−(ton2))−sign(*t*−(ton2+c_time)))/2; $3=(sign(*t*−(ton3))−sign(*t*−(ton3+c_time)))/2; $4=kt($1∗Conc/*F*^2+$2∗Conc/*F*+$3∗Conc−B); $5=$k_c$∗B∗C−kd∗BC; B=$4−$5|0; C=−$5|R_{max}; BC=$5|0

This model of interaction has been already described [9]. BC is the complex formed by injecting the analyte C during the dissociation phase of the target B from the anchor A (Fig. 4A). Briefly, a dissociation phase (in bold) is added to a model that describes three sequential injections (or more). R0 is the response in RU at the start of the fit; k_b is the dissociation rate constant of the captured target B from the anchor A; *t*0 is the time at the beginning of the fit; k_c and k_d are the association and dissociation rate constants, respectively, of C binding to B; Offset is the residual response at infinite time. This parameter should be fixed at zero, unless some rebinding is simulated. The procedure to generate simulated SCKODS is similar to that described for SCK sensorgrams. The user knows all the parameters. If a complete simulation not related to experiments is performed, the user fixes them all. If simulations are used to help design an experiment, the rate constants are first determined in a preliminary experiment. They are imposed in the simulations. Then the user can vary all the other parameters to find out the best conditions to be used.

3. Results and discussion

The SCK method introduced by Karlsson and co-workers in 2006 really makes easier SPR experiments [5]. The method is faster than the classical one and requires less regeneration steps. The immobilized target and the surface last longer in particular when harsh regeneration conditions have to be used to dissociate the analyte from the target immobilized on the sensor chip. However the SCK method requires preliminary experiments to find out which concentrations, dilution factors and durations of the association phases are best to reach saturation and to generate binding steps well distributed between zero and the maximum responses. This is time and sample consuming. With the help of simulations, an SPR experiment that uses the SCK method will consist of three steps. First, an experiment is carried out to generate a sensorgram at one concentration only of analyte injected. The rate constants of the binding reaction are determined from this preliminary experiment. Then, the values of these constants are used to simulate SCK sensorgrams in order to find the best experimental conditions (dilution factor, maximum concentration, and duration of the phases of the sensorgrams). Lastly, the SPR experiments are performed. A 10 base-pair DNA duplex was characterized kinetically to illustrate how simulated single-cycle kinetics can really help design SPR experiments with sequential injections. The DNA duplex was analyzed at three different temperatures, 10, 20 and 30 °C, to investigate and to simulate slow and fast rate constants.

The first step consists in performing a preliminary experiment in order to determine the rate constants of the binding reaction. At this stage there is no need to perform extensive experiments. The goal is to generate a sensorgram containing enough kinetic information, *i.e.* enough curvature for the association phase and long enough dissociation phase in particular when the rate of dissociation is slow, so that the deduced rate constants describe reasonably well the binding reaction. The results obtained when a complementary DNA oligonucleotide was injected at 500 nM over the DNA-functionalized sensor chip are reported in Fig. 1, at three temperatures. The association and dissociation rate constants, k_a and k_d, respectively, were determined by direct curve fitting of these sensorgrams, assuming a Langmuir 1:1: model of interaction. As expected, the stability of the DNA duplex increases when the temperature decreases, due to slower rates of dissociation (see also Supplementary Data, Table 1).

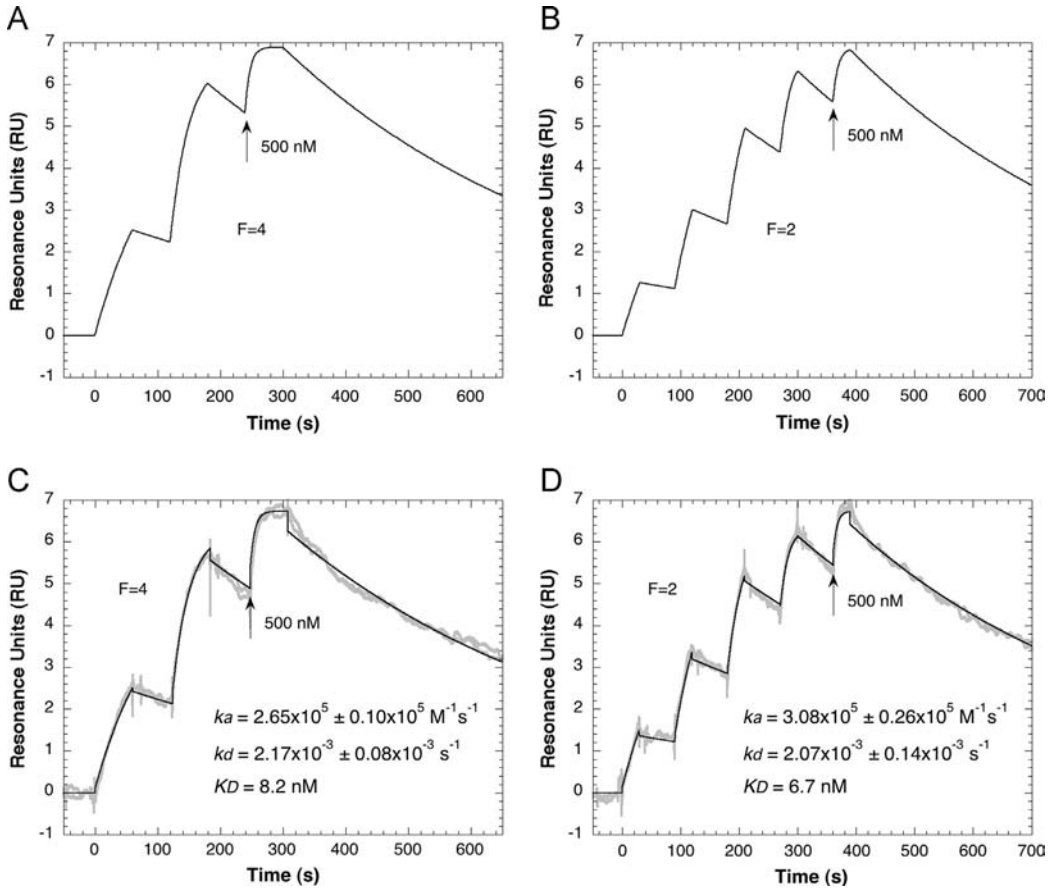

Fig. 3. Simulated and experimental sensorgrams for the DNA duplex formation at 20 °C. The simulated sensorgrams, either with three (A) or five (B) sequential injections of the complementary DNA strand, were generated using the rate constants determined in the preliminary experiments, at 20 °C (Fig. 1B). The results for the other temperatures (10 and 30 °C) are reported in the Supplementary Data (Table 1 and Figs. S1 and S2). The maximum concentration injected was fixed at 500 nM. Two dilutions factor were used, 4 (A) and 2 (B). The experimental sensorgrams (C and D) were obtained by using the same parameters (dilution factor, duration of the phases, maximum concentration) than those used for the simulations. The grey curves represent the experimental data (injection performed in duplicate) and the black ones the fit to a Langmuir 1:1 model of interaction. The binding constants are indicated in the figures. The values are the average and standard deviation of three independent experiments with duplicate injections of the analyte.

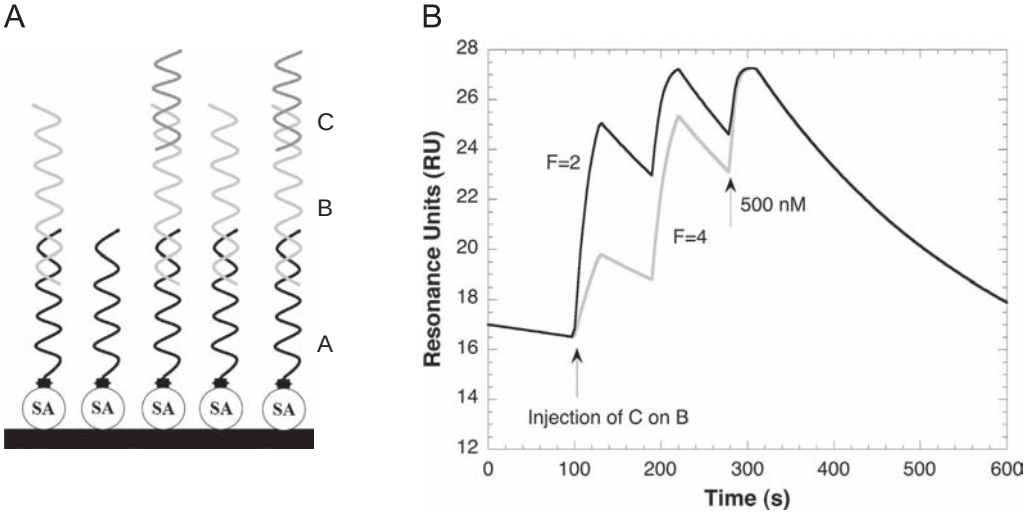

Fig. 4. Simulated SCKODS sensorgrams. (A) Ternary DNA complex. The biotinylated anchor A that was immobilized on the sensor chip surface by biotin–streptavidin (SA) coupling captures B which is able to recognize C. (B) The simulated sensorgrams were generated using the association and dissociations rate constants, $k_c = 3.9 \times 10^5\ M^{-1}\ s^{-1}$ and $k_d = 3.9 \times 10^{-3}\ s^{-1}$, respectively, determined for the ternary DNA complex A–B–C referred to as A–B_{13}–C_{10} in a previous work [9], with C forming 10 base pairs with B, and B forming 13 base pairs with the biotinylated anchor A. In these simulations C was injected (first arrow) while B dissociated from the anchor A with a dissociation rate constant k_b of $3 \times 10^{-4}\ s^{-1}$. The dissociation phase of B from A, before the injection of C, is shown between 0 and 100 s (before the first arrow). The second arrow indicates the maximum concentration injected (500 nM). The injection time was fixed at 30 s and the dissociation phase between the injections at 60 s. The effect of the dilution factor F is reported: $F=4$ (grey line) and $F=2$ (black line).

The next step consists in simulating SCK sensorgrams using the rate constants that have been determined in the preliminary experiment. We used the Biacore™ BiaEvaluation 4.1.1 software to simulate SCK sensorgrams. Assuming a Langmuir 1:1 model of interaction, we wrote the formulas corresponding to three and five sequential injections of the analyte as described previously by Karlsson and co-workers and more recently [5,9]. In an SCK experiment, direct curve fitting of the experimental sensorgrams are achieved with the rate constants, k_a and k_d, the maximum response level, R_{max}, and the bulk refractive index, R_i, as floating parameters. The dilution factor, F, the maximum concentration injected, Conc, and the injection time, c_time, are set by the user and, therefore, are known constants. The start time of the injections, ton, is also a constant value known by the user. They are either imposed by the instrument (Biacore™ T200) or can be chosen by the user (Biacore™ 2000, Biacore™ 3000 and other equivalent SPR instruments). In the first case the time-lapse between the end of an injection phase and the beginning of the next one is constant, in the second case it can vary. In both cases, these durations correspond to the dissociation phase. The SSCK sensorgrams are obtained by converting the floating parameters to constant ones as described in Section 2. To illustrate the procedure, four simulated sensorgrams with five sequential injections are reported in Fig. 2. The rate constants, k_a and k_d, were chosen arbitrarily, equal to 10^5 M^{-1} s^{-1} and 10^{-3} s^{-1}, respectively. In these simulations we analyzed the effects of changes in the dilution factor, F, in the injection time, c_time, and in the start time of injection, ton, while the other parameters, Conc (2 μM), R_{max} (20 RU) and R_i (0), were left constant. The sensorgrams reported in Fig. 2A were obtained by simulating association phases lasting 30 s with a time-lapse between the end of an injection and the beginning of the next one, *i.e.* the dissociation phase, equal to 60 s. This simulation shows the effect of changing the dilution factor from 2 to 4, used to prepare the samples by serial dilution from the concentration, 2 μM, which permits to reach saturation. In a new simulation (Fig. 2B), sensorgrams were generated with association and dissociation phases equal to 60 s, or with association and dissociation phases of 30 s and of variable duration, respectively.

SSCK binding curves obtained using the rate constants determined for the formation of the DNA duplex, at 20 °C, for three and five sequential injections of the analyte, are reported in Fig. 3 (see Supplementary Data for the results obtained at 10 and 30 °C, Figs. S1 and S2). These sensorgrams were obtained with a maximum concentration equal to 500 nM, a concentration above the expected dissociation equilibrium constant, as shown previously [9]. The association phase durations were fixed at 60 (Fig. 3A) and 30 s (Fig. 3B). The start time of the injections was left constant to simulate equal dissociation phases imposed by the Biacore™ T200 instrument, except for the last one, which always lasts longer to provide enough kinetic information. Two dilution factors for the serial dilution of the samples injected were tested: $F=4$ (Fig. 3A) and 2 (Fig. 3B).

SPR experiments were then performed with the parameters that were used for the simulations. The results obtained at 20 °C are reported in Fig. 3C and D (see Supplementary Data for 10 and 30 °C, Figs. S1 and S2, respectively). The experimental sensorgrams look very similar to those that were simulated. This indicates that the rate constants that were determined from the preliminary experiment, with just one concentration of analyte injected, described very well the binding reaction. This was further confirmed when the binding constants from these preliminary experiments were compared with those obtained from experiments with three or five sequential injections, performed after the simulations (see Supplementary Data, Table 1).

Recently we introduced an extension of the SCK method. We showed that it was possible to determine the binding constants by the SCK method of a bimolecular complex that was dissociating from an immobilized anchor [9]. This method was named single-cycle kinetics on a decaying surface (SCKODS). We showed that in a ternary complex A–B–C (Fig. 4A), C could be injected sequentially at increasing concentrations, *i.e.* using the SCK method, to form the complex B–C, while the captured target B was dissociating from an anchor A that was immobilized on the sensor chip surface. The sensorgrams obtained by the SCKODS method can also be simulated using the BiaEvaluation software. We used the rate constants and the experimental parameters from the SPR assay with a DNA ternary complex published previously [9] to simulate SCKODS sensorgrams corresponding to C binding to B, while B was dissociating from the anchor A. Simulated SCKODS sensorgram are reported in Fig. 4B. The procedure for such simulations is similar to that described for bimolecular complexes except that the rate of dissociation of B from the immobilized anchor A is an additional required parameter. In a manner comparable to that described for bimolecular complexes, a preliminary experiment can be performed to determine this rate constant together with those corresponding to the captured bimolecular complex B–C. SCKODS sensorgrams can be then simulated to find out the best experimental conditions to be used. The effect of changing the dilution factor F for the analyte C is shown in Fig. 4B.

In conclusion, simulations are very useful to design SPR experiments by the SCK method. It requires knowing the rate constants of the binding reaction, which can be determined in a preliminary experiment. In principle this step should not necessitate more than one or two attempts with one concentration injected, even when the equilibrium constant of the reaction is not known. A way to perform this preliminary experiment is to inject manually the analyte over the functionalized surface. If the rate of association is apparently fast, the injection can be stopped as soon as enough curvature is recorded. Conversely, if the rate is slow, the association phase is let progress until a significant curvature of the sensorgram is obtained. As regards the dissociation phase, the recording of the SPR signal should last enough, in particular when the rate of dissociation is slow. Anyone who wants to simulate SCK or SCKODS sensorgrams can copy the formulas reported in the Section 2 and past them in the formula entry window available in the BiaEvaluation software, after having created a Langmuir 1:1: model of interaction. This software is available from Biacore™ but it does not require owning a Biacore™ instrument. Therefore, it can be used to design SPR experiments with any optical biosensor instrument that can perform successive injections in a single cycle. Simulations provide users with the best experimental conditions to be used, saving time and reducing sample consumption.

Acknowledgements

We are grateful to the structural biology facility (UMS 3033/US001) of the Institut Européen de Chimie et Biologie (Pessac, France) for access to the Biacore™ T200 instrument that was acquired with the support of the Conseil Régional d'Aquitaine, the GIS-IBiSA and the Cellule Hôtels à Projets of the CNRS. We thank the Institut National de la Santé et de la Recherche Médicale and the Agence Nationale de Recherche sur le Sida et les hépatites, Grant #ASA12007GRA, for financial support. We thank Dr. Andrew Goldsborough and Dr. Christophe Quétard for reading the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.04.022.

References

[1] L.G. Fagerstam, A. Frostell, R. Karlsson, M. Kullman, A. Larsson, M. Malmqvist, H. Butt, J. Mol. Recognition 3 (1990) 208–214.

[2] S. Löfås, B. Johnsson, J. Chem. Soc. Chem. Commun. 21 (1990) 1526–1528.
[3] R.L. Rich, D.G. Myszka, J. Mol. Recognition: JMR 24 (2011) 892–914.
[4] T.A. Morton, D.G. Myszka, Methods Enzymol. 295 (1998) 268–294.
[5] R. Karlsson, P.S. Katsamba, H. Nordin, E. Pol, D.G. Myszka, Anal. Biochem. 349 (2006) 136–147.
[6] D.G. Myszka, T.A. Morton, Trends Biochem. Sci. 23 (1998) 149–150.
[7] D.G. Myszka, J. Mol. Recognition: JMR 12 (1999) 279–284.
[8] C. Di Primo, J. Mol. Recognition 21 (2008) 37–45.
[9] W. Palau, C. Di Primo, Biochimie 94 (2012) 1891–1899.