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1. Kinetics

1.1 Introduction

An important application of SPR biosensors is the possibility to perform kinetic analysis on interaction plots. The binding kinetics characterize a biomolecular interaction quantitatively by the rate constants and by the equilibrium constants.
An SPR interaction plot generally contains three phases: the association phase, the dissociation phase and the regeneration phase (see figure 1.1). The association phase and the dissociation phase can be used to determine the rate constants and thereby the equilibrium constants.

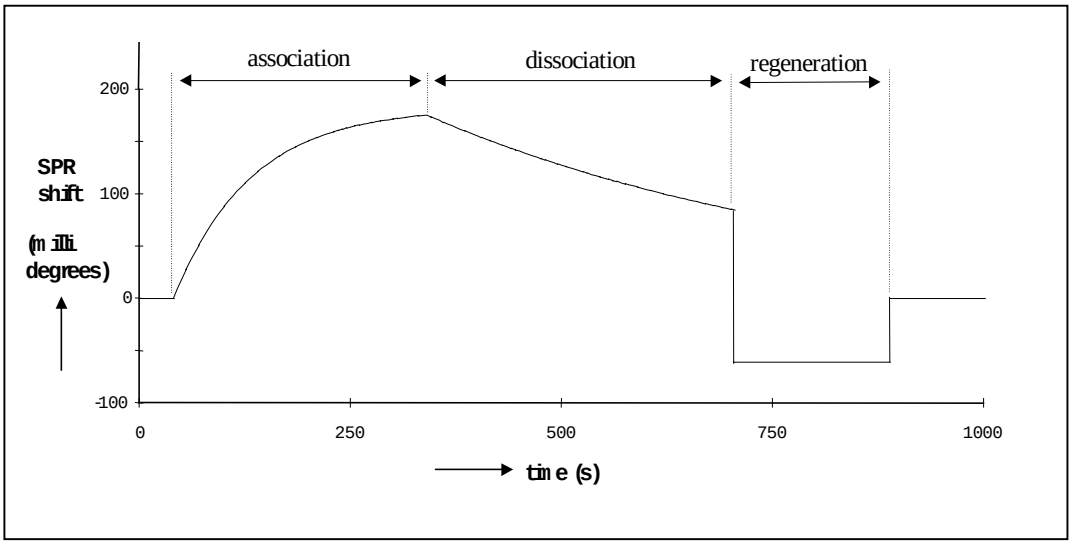


Figure 1.1 Example of an SPR interaction plot. The association phase of the interaction plot shows the complexation of interactants analyte A and immobilized ligand B. The complex AB is measured as SPR shift and expressed as signal R (in milli degrees). The dissociation phase of the interaction plot shows dissociation of complex AB into A and B, and thereby a decrease in R. The regeneration phase shows total dissociation or regeneration of the biospecific surface. After the regeneration phase, a next interaction plot can be measured.

In an interaction plot, the maximal amount of analyte response (R_{max}) is proportional to the ratio of the molecular weights of analyte and ligand according to

$$R_{max} = \frac{MW_{analyte}}{MW_{ligand}} \cdot valence \quad (1)$$

A theoretical introduction in kinetics and kinetics applied for SPR biosensors is discussed in this chapter.

The binding reaction equation of two macromolecular interactants (A and B) for a

one-to-one binding reaction is written as :

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB \quad (2)$$

with

$$K_A = \frac{k_a}{k_d} = \frac{[AB]}{[A][B]} \qquad K_D = \frac{k_d}{k_a} = \frac{[A][B]}{[AB]}$$

where k_a and k_d are the rate constants, k_a is the association rate constant in $M^{-1}s^{-1}$ and k_d is the dissociation rate constant in s^{-1}, and where K_A and K_D are the equilibrium constants, K_A is the association constant in M^{-1} and K_D is the dissociation constant in M.
The association part of the binding reaction equation is written as:

$$A + B \xrightarrow{k_a} AB \quad (3)$$

with

$$\frac{d[AB]}{dt} = k_a[A][B] \quad (4)$$

The dissociation part of the binding reaction equation is written as:

$$AB \xrightarrow{k_d} A + B \quad (5)$$

with

$$\frac{d[AB]}{dt} = -k_d[AB] \quad (6)$$

The equilibrium constants do not contain any information about the rates of complexation. Information about the association rate constant is obtained from the association phase, and kinetic information about the dissociation rate constant is obtained from the association and the dissociation phase.

1.2 Kinetic models

1.2.1 Association phase

The observed rate of formation is the sum of both reaction rate equations:

$$\frac{d[AB]}{dt} = k_a[A][B] - k_d[AB] \quad (7)$$

The concentration of ligand B ($[B_t]$), is described in time by:

$$[B]_t = [B]_{t=0} - [AB]_t \quad (8)$$

Substitution this equation into equation 7 results in

$$\frac{d[AB]}{dt} = k_a[A]\left[[B]_{t=0} - [AB]_t\right] - k_d[AB] \quad (9)$$

The complex formation of AB is measured real-time with the SPR instrument, and is called signal R. Due to negligibly small concentration differences of analyte A during complexation, concentration C is assumed to be constant in time. Therefore, complex formation is considered as pseudo first order kinetics in SPR biosensors[1-3], and equation 9 can be rewritten as:

$$\frac{dR}{dt} = k_a C(R_{max} - R_t) - k_d R_t \quad (10)$$

rearranged into

$$\frac{dR}{dt} = k_a C R_{ma} - (k_a C + k_d) R_t \quad (11)$$

Equation 11 can be regarded as a straight line $dR/dt = -k_s R_t + b$ with $k_s = (k_a C + k_d)$, and $b = k_a C R_{max}$. Parameters k_s and b can be determined by linear regression of a plot of dR/dt values versus R_t values. The determined k_s value is a concentration-dependent parameter. This method is called the linearization method[4], because the data are presented in such a way, that the relevant parameter k_s can be determined by linear regression (see figure 1.2).

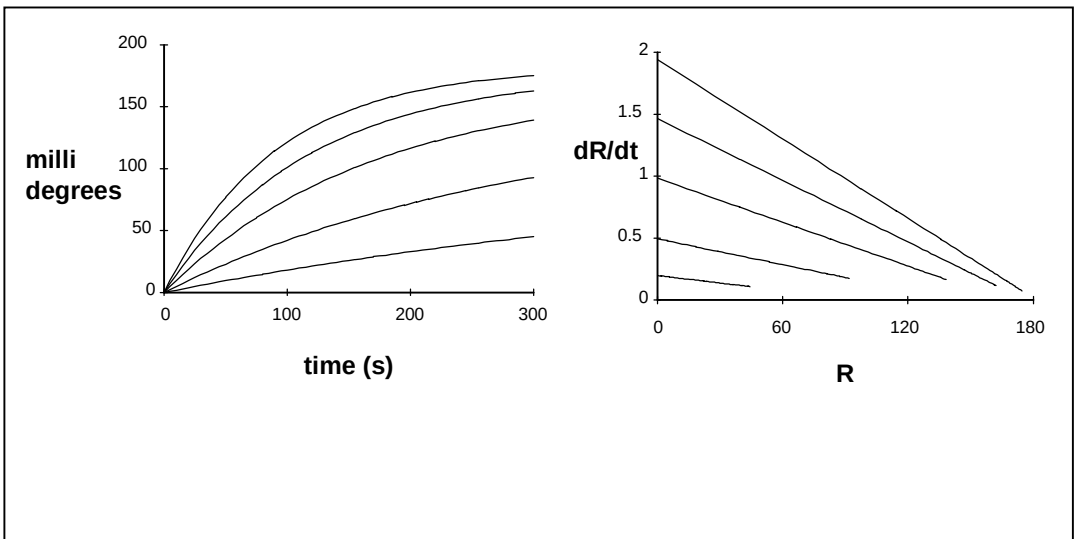


Figure 1.2 Determination of k_s values by linearization method. Simulated curves of the association phase of a bimolecular interaction are shown in an overlay plot (left figure). The parameters for the simulation were: $k_a = 1.10^5$ $M^{-1}s^{-1}$, $k_d = 1.10^{-3}$ s^{-1}, $R_{max} = 200$, and concentrations of 10, 25, 50, 75 and 100 nM.
k_s values are derived from a plot of dR/dt versus R. The slope of that straight line is equal to k_s. An overlay plot is shown for five concentrations (right figure).

Kinetic rate constants are determined by a plot of k_s values versus concentration. Linear regression reveals the association rate constant from the slope of the plotted straight line, and the dissociation rate constant from the y-intercept of the plotted line (see figure 1.3).

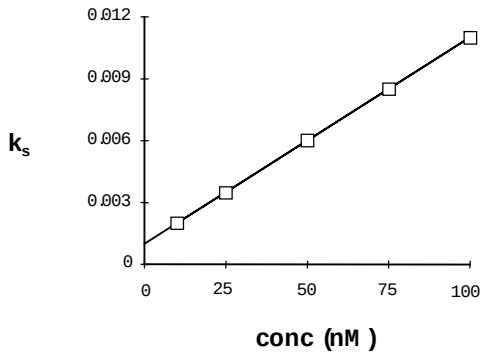


Figure 1.3 Determination of kinetic parameters from a plot of k_s (= $k_aC + k_d$) versus concentration. The slope of the plotted line is equal to the association rate constant and the y-intercept is equal to the dissociation rate constant. Data points of the plot were simulated. The parameters for the simulation were: $k_a = 1.10^5$ $M^{-1}s^{-1}$, $k_d = 1.10^{-3}$ s^{-1}, $R_{max} = 200$, and concentrations of 10, 25, 50, 75 and 100 nM. The calculated k_s values for this simulation were 0.002, 0.0035, 0.006, 0.0085, and 0.011,

The y-intercept of the plot k_s versus concentration becomes close to zero, if the dissociation rate constant is low ($k_d<10^{-3}$ s^{-1}). Consequently, the associated errors are large in the determination of low dissociation rate constants by this approach[2].

The kinetic rate parameters can also be determined by nonlinear regression of the data points (t, R_t) to the integrated form of equation 11. This method, the integrated rate analysis method[5], uses untransformed data points in contrast to the linearization method, which uses transformed data points. The integrated rate equation is written as

$$R_t = \frac{k_aCR_{max}\,[1-e^{-(k_aC + k_d)t}]}{k_aC + k_d} + R_0 \quad (12)$$

The integrated rate equation of the association phase possesses three time-dependent parameters, and can therefore be considered as

$$R_t = E\,[1-e^{-k_st}] + R_0 \quad (13)$$

In this equation, E is the maximal extent of change in response at a certain concentration and is equal to $k_aCR_{max}/(k_aC + k_d)$, k_s is equal to ($k_aC + k_d$), and R_0 is the response at t=0. These parameters are revealed by non-linear curve fitting of measured data points to equation 13. The relevant kinetic information is obtained from parameter k_s. A plot of k_s values versus concentration is used for linear regression to obtain the association rate constant from the slope and the dissociation rate constant from the y-intercept. For this calculation, it is necessary to measure interactions at several concentrations.

The integrated rate equation 13 is a model to describe the association phase of a binding curve for ideal cases. It describes the one-to-one interaction of a macromolecular complex for one phase, and therefore one k_a or k_d. However, it is possible that the interaction is not adequately described by a monophasic model based on one k_a or k_d. In some cases, the interaction is described by a

biphasic model based on two equilibrium constants, K_A or K_D:

$$R_t = E_1 \left[-k_{s(1)}t \right] + E_2 \left[-k_{s(2)}t \right] + R_0 \quad (14)$$

This integrated rate equation is an extension of the integrated rate equation 13 with parameters indicated by the subscript 1 by a second phase with parameters indicated by the subscript 2.

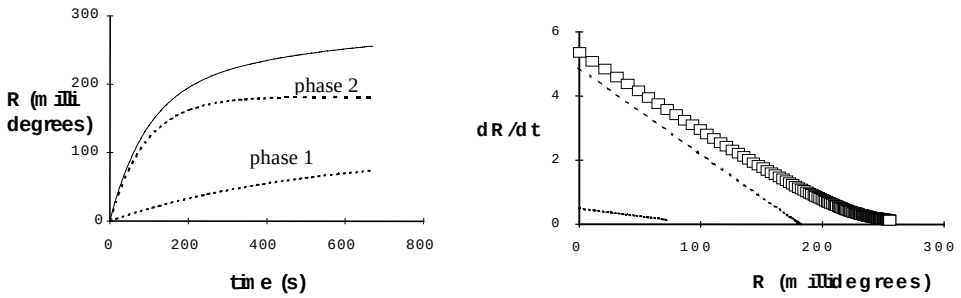


Figure 1.4 Biphasic interaction of biomolecules. The association phase of an interaction was simulated for a biphasic interaction model (right figure). The simulated biphasic interaction curve is made up of two monophasic interaction curves, phase 1 and phase 2, respectively. The parameters for the simulation were for phase 1: $k_a = 1.10^5\ M^{-1}s^{-1}$, $k_d = 1.10^{-3}\ s^{-1}$, $R_{max} = 200$ mdeg, and for phase 2: $k_a = 1.10^4\ M^{-1}s^{-1}$, $k_d = 1.10^{-3}\ s^{-1}$, $R_{max} = 200$ mdeg for the concentration 100 nM. Biphasic interaction can be visualized by a plot of dR/dt versus R for the determination of k_s by the linearization method. Deviations from a straight line indicate the occurrence of biphasic interaction (figure on the right). The two monophasic plots of dR/dt versus R are straight lines, but the overall plot is not.

Nonlinear regression of measured data points of the association phase of a binding curve to equation 14 results in 5 parameters (E_1, $k_{s(1)}$, E_2, $k_{s(2)}$, and R_0). The kinetic information is obtained from the parameters $k_{s(1)}$ and $k_{s(2)}$. Plots of $-k_s$ versus concentration will be used to determine the association rate constants and dissociation rate constants.

The occurrence of biphasic interactions is generally caused by heterogeneity of the immobilized ligand or by heterogeneity of the analyte. Ligand heterogeneity may be caused by impurities of the ligand or by the immobilization procedure. Analyte heterogeneity may be caused by analyte impurity or by the ability to bind the ligand by more than one binding site with different affinities. It should be emphasized, that the biphasic model presented here describes the interactions of two independent phases only.

1.2.2 Dissociation

The dissociation part of the binding reaction equation is written as:

$$AB \xrightarrow{k_d} A + B \quad (5)$$

with

$$\frac{d[AB]}{dt} = -k_d[AB] \quad (6)$$

Under SPR biosensor conditions[1-3], this equation is written as:

$$\frac{dR}{dt} = -k_d R \quad (15)$$

This equation can be rearranged to:

$$\frac{dR}{R} = -k_d t \quad (16)$$

This equation can be solved by the linearization method and by the integrated rate analysis method. Equation 16 can be integrated and rearranged to:

$$\ln\left[\frac{R_{t=0}}{R_t}\right] = k_d(t - t_o) \quad (17)$$

The kinetic rate constant can be determined from equation 17 by linear regression. A plot of ln ($R_{t=0}/R_t$) values versus ($t–t_0$) values reveals a slope which is equal to the dissociation rate constant. Data points are transformed by this method

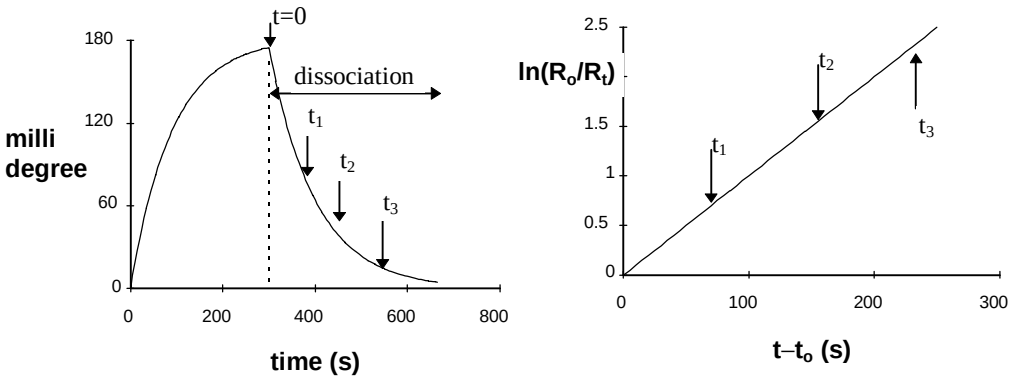


Figure 1.5 Determination of the dissociation rate constant from the dissociation phase by the linearization method. An example of a dissociation phase is simulated with a dissociation rate constant of 10^{-2} s^{-1} (figure left). The transformed data ($\ln(R_0/R_t)$) are plotted against the time to determine the dissociation rate constant (figure on the right). The plotted line is described as: $\ln(R_0/R_t) = k_d\,(t–t_o)$. The slope of the plotted line is equal to the dissociation rate constant k_d.

The dissociation rate constant can also be determined directly from the data points (t,R_t) by the integrated rate method[5] using nonlinear regression, because equation 17 can be rearranged to:

$$R_t = R_0 e^{-k_d t} + R_\infty \quad (18)$$

Curve fitting of data points to the integrated dissociation rate equation 18 results in three parameters. The parameter R_0 resembles the amount of bound complex to be dissociated in time, the parameter R_∞ resembles the signal after infinite time, and the parameter k_d is the dissociation rate constant.

Similar to the occurrence of two phases in the association phase of binding curves, the dissociation phase may possess two phases. Biphasic dissociation is described as:

$$R_t = R_{0(1)} e^{-k_{d(1)} t} + R_{0(2)} e^{-k_{d(2)} t} + R_\infty \quad (19)$$

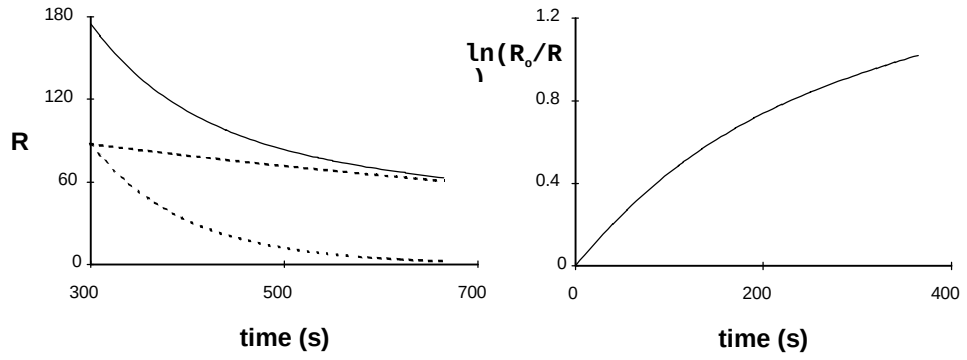


Figure 1.6: Biphasic dissociation of a bimolecular complex. Dissociation of complex (175 milli degree) was simulated for two phases. The first phase (87.5 milli degree) dissociates with a dissociation rate constant of 10^{-2} s^{-1} and the second phase (87.5 milli degree) dissociates with a dissociation rate constant of 10^{-3} s^{-1}. Biphasic dissociation is the sum of both monophasic dissociations (left figure).
Biphasic dissociation can be visualized by a $\ln(R_0/R_t)$ versus time plot (figure right). Such a plot should result in a straight line with a slope of the dissociation rate constant. Severe deviation of a straight line for a biphasic dissociation can easily be observed.

This equation is based on the assumption, that both dissociation reactions are independent events. Biphasic dissociation may occur for complexes with a stoichiometry other than one, or may be caused by impurities in ligand or analyte. It can also occur when reassociation of the released analyte takes place.
Both dissociation rate constants $k_{d(1)}$ and $k_{d(2)}$ are determined by nonlinear regression of data points to equation 19.

1.2.3 Equilibrium

The observed rate of formation (dR/dt) is zero at equilibrium[2,3].

$$\frac{dR}{dt} = k_a C(R_{max} - R_{ec}) - k_d R_{eq} = 0 \quad (20)$$

In this equation, R_{max} is the maximal amount of complex and R_{eq} is the amount of complex at equilibrium. This equation can be rearranged to

$$\frac{R_e}{C} = K_A R_{max} - K_A R_{eq} \quad (21)$$

A plot of R_{eq}/C as y-values versus R_{eq} as x-values results in a straight line with a slope of $-K_A$ and an y-intercept of $K_A R_{max}$. Therefore, the association constant K_A can be determined by linear regression from transformed data points at equilibrium.

1.2.4 Kinetic analysis methods

Two different methods were presented in this chapter to analyze interaction plots of SPR biosensors. Data can be analyzed by the linearization method and by the integrated rate method[4].
The first-used method for kinetic analysis of SPR data was the linearization method[1-3]. However, this method is only suitable for the interaction of simple bimolecular interaction in ideal cases. Another disadvantage is that it transforms the error associated with the parameter estimates[5]. The advantage of this method is that deviation of an ideal bimolecular interaction is visualized by the data transformation in a dR/dt versus R plot for the association phase or a $\ln(R_0/R_t)$ versus $t-t_0$ plot for the dissociation phase.
The integrated rate method was introduced in 1993 by O'Shannesy *et al*[5], and is applied in the kinetic analysis software of SPR biosensors today. Deviations of the ideal bimolecular interaction can be analyzed by the integrated rate analysis. SPR data are analyzed by this method in the SPR kinetic evaluation software. The linearization method is used to visualize possible deviations of the ideal bimolecular interaction case, and to determine the start values of the parameters used in the nonlinear regression analysis of the integrated rate method.

1.3 Mass transport

1.3.1 Theory

The kinetic theory presented in the previous sections of this chapter are based on the assumption, that the concentration of the analyte at the sensor surface is equal to the bulk concentration. However, a concentration gradient from the bulk solution to the sensor surface is always present[6-8]. The flux of molecules to the sensor surface or out from the surface, is described by the mass transport rate k_m.
For a bimolecular reaction where ligand B is immobilized to the sensor surface, the analyte A is transported to the surface by convection and diffusion in the surface layer. The overall rates of complex formation (k_f and k_r) is therefore a function of the mass transport rates (k_m and k_{-m}) and of the reaction rates (k_a and k_d).

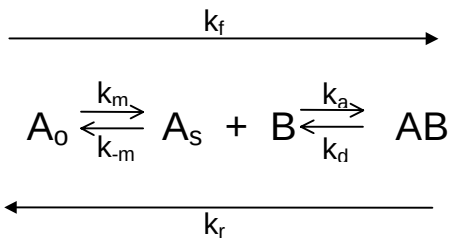


Figure 1.7: Bimolecular binding of A and B at the sensor surface. A is the analyte and B is the immobilized ligand. Difference in concentration of A is indicated by the subscript o (A_o) for the bulk concentration and by the subscript s (A_s) for the surface concentration. The overall rate constants k_f and k_r are determined by the mass transport rate constants k_m and k_{-m} and by the reaction rate constants k_a and k_d.

This means that the rate of binding and dissociation may be limited by the binding reaction itself, or by mass transport to the sensor surface. In practice, three possibilities can be distinguished[6]: (1) reaction-limited system, (2) mass transport-limited system, (3) combination of (1) and (2).
In the first system, concentration differences are balanced much faster by mass transport than created by the reaction at the sensor surface. In the second and third system, surface concentration is different from the bulk concentration, because the mass transport rate is too low to compensate for concentration differences. Under these conditions, the overall rates of complex formation differ from the true rate constants of the binding reaction. For a kinetic analysis of a biomolecular interaction, it is important that the system is limited by the reaction and not by mass transport. It is also important to notice that the dissociation phase as well as the association phase may be limited by mass transport. Mass transport effects in the dissociation phase will favor rebinding of the released analyte.

The kinetic rate equation for a bimolecular interaction is described by

$$d[AB]/dt = k_a[A_s][B] - k_d[AB] \quad (22)$$

In this equation [B] is the concentration of uncomplexed ligand at the surface as

$$[B] = [AB_{max}] - [AB] \quad (23)$$

Due to the assumption that the fluxes from and out of the sensor surface are equal, the mass transport can be described as[7]

$$k_m[A_0] - k_m[A_s] = k_a[A_s][B] - k_d[AB] \quad (24)$$

Combination this equation and the previous equation 23 results in

$$[A_s] = (k_m[A_0] + k_d[AB])/(k_m + k_a([AB_{max}] - [AB])) \quad (25)$$

Combining of this equation with the kinetic rate equation gives[7]

$$d[AB]/dt = k_f[A_0]([AB_{max}] - [AB]) - k_r[AB] \quad (26)$$

with

$$k_f = k_a k_m/(k_m + k_a[B]) = k_a/(1+k_a[B]/k_m)$$
$$k_r = k_d k_m/(k_m + k_a[B]) = k_d/(1+k_a[B]/k_m)$$

A kinetic interaction takes place if the mass transport flux is much higher than the association rate, when $k_m >> k_a[B]$. Then k_f becomes k_a and k_r becomes k_d and equation 26 becomes a true kinetic rate equation. By introducing a limit coefficient, LC, mass transport-limited and kinetic-limited binding rates can be differentiated.

$$LC = k_a[B]/ k_m \quad (27)$$

If LC is much greater than 1 k_f and k_r become k_a and k_d. The effect of LC values on interaction curves can be estimated by simulating curves based on equation 26. The observed binding kinetics approximate true kinetics for LC values < 0.5. If LC > 0.5 mass transport significantly contributes to the observed kinetics.
Mass transport limitations during the dissociation phase are governed by the same factors as the ones during the association phase. This means that if mass transport-limitation should occur in the association phase, it is always followed by mass transport-limitation in the dissociation phase.

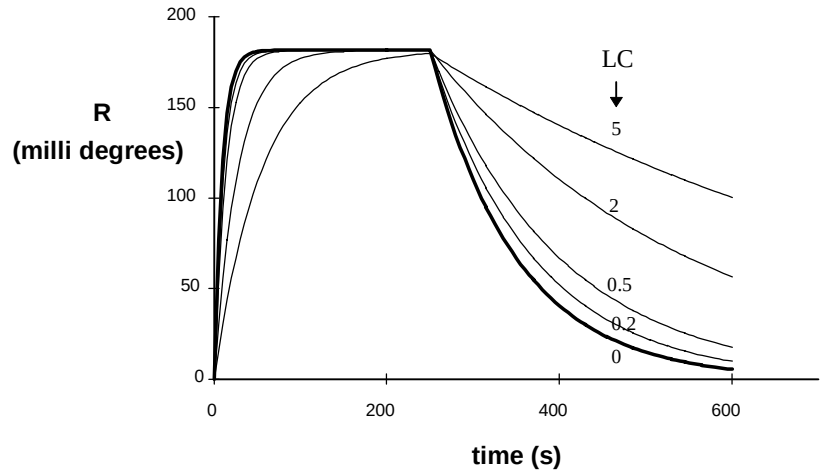


Figure 1.8 Simulated interaction plots[7] with different values of the mass transport limit coefficient (LC). The effect of the ratio of the interaction-limited association rate and the mass transport rate on the bimolecular interaction with the parameters $k_a = 1.10^6$ $M^{-1}s^{-1}$, $k_d = 1.10^{-2}$ s^{-1}, C= 100 nM, R_{max}= 200 and LC = 0 is shown for LC values 0.2, 0.5, 2 and 5. The bold curve (LC = 0) represents the fully interaction-controlled kinetics.

When the reaction rate is fully limited by mass transport, the mass transport coefficient can be described in a flow system as[7]

$$k_m = 0.98(D/h)^{2/3}(f/bx)^{1/3} \quad (28)$$

The parameters in this equation are D for diffusion coefficient of the analyte, f for flow rate, and the flow cell dimensions h for height b for width and x for the distance from the flow cell entrance. It should be noted that the hydrodynamic conditions in the cuvette are not the same as with the flow cell.
When the reaction rate is fully limited by mass transport, the rate can be described as:

$$\frac{d[AB]}{dt} = k_m[A_o] \quad (29)$$

which is for SPR biosensors equal to

$$\frac{d[R]}{dt} = k_m C \quad (30)$$

Under mass transport limitation, the reaction rate is independent of the amount of complex AB. This in contrast to reaction-controlled conditions, where the reaction rate depends on the amount of complex AB according to equation 10:

$$\frac{dR}{dt} = k_a C(R_{max} - R_t) - k_d R_t \quad (10)$$

The initial association rate can be calculated from this equation by setting R_t to zero:

$$\text{initial association rate} = k_a C R_{max} \quad (31)$$

The initial association rate will be proportional to immobilization level of the ligand, but if the immobilization level is increased above a certain level the initial association rate will be limited by mass transport. It will become constant and independent of R_{max} according to equation 30.

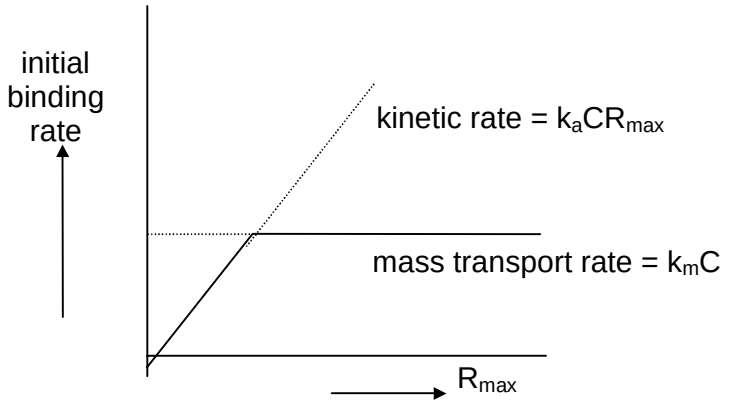


Figure 1.9 Illustration of the immobilization level of the ligand on the initial binding rate for a bimolecular interaction. The observed initial binding rate will reflect interaction kinetics on surfaces with a low immobilization level, and will be proportional to the immobilization level. Upon increase of the immobilization level and thereby increase of R_{max}, the binding rate will approach the mass transport limiting binding rate. As a result, the observed binding rate will not be affected anymore, even if the immobilization level is still increased.

Kinetic measurements should be performed under binding reaction-controlled conditions. This means that low immobilization levels of the ligand are preferred to eliminate or to reduce possible mass transport effects. Mass transport effects in the dissociation phase can also be eliminated or reduced by including a free ligand of low molecular weight in the dissociation buffer[9]. The free ligand competes with the immobilized ligand for binding the released analyte, and reduces therefore the effective amount of immobilized ligand (R_{max} is reduced).

Another possibility to reduce mass transport effects is to decrease the thickness of the diffusion layer. The thickness of the diffusion layer, d, can be determined from the diffusion coefficient and the mass transport coefficient by:

$$d = D / k_m \quad (32)$$

The thickness of the layer depends on the diffusion coefficient and thereby on the flow rate, which is the mix speed in the SPR cuvette system. The layer thickness will be reduced by increasing the mix speed. Consequently, mass transport effects will be reduced.
From a practical point of view, it is very important to distinguish mass transport-controlled binding reactions from kinetic-controlled binding reactions. Mass transport limitation can be visualized by two different plots.

The plot used for the determination of k_s values by the linearization method, the plot of dR/dt versus R, will indicate the occurrence of mass transport by that part of the plotted line that is independent of R. In other words, the horizontal part of the line. This part corresponds to the initial part of the association curve, where the initial binding rate is higher than the mass transport rate.
The other plot used for visualization of mass transport effects is a plot of ln(dR/dt) versus t. The relationship between t and ln(dR/dt) is derived from equation 12, and is described by

$$\ln(dR/dt) = \ln(k_a C R_{max}) - (k_a C + k_d)t \quad (33)$$

Similar to a dR/dt versus R plot, this plot will be a straight line with slope $-(k_a C + k_d)$ for interaction-limited kinetics. Mass transport-limited kinetics will be identified by that part of the line that is independent of time, the horizontal part of the line. Identification of the mass transport-limited region of a binding curve proceeds similar to that of a dR/dt versus R plot. In the kinetic evaluation software, a ln(dR/dt) versus t plot is used for the selection of the kinetic part of a binding curve.

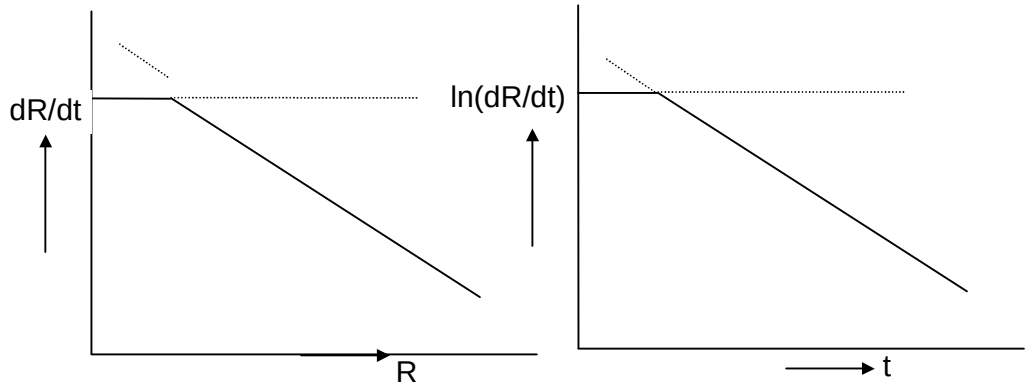


Figure 1.10 Visualization of mass transport effects in the association phase of a binding curve. Horizontal parts in a dR/dt versus R plot (left plot) or in a ln(dR/dt) versus t plot (right plot) identify the mass transport-limited region of a binding curve.

1.3.2 Concentration determination

Under fully mass transport-limited conditions, the observed reaction rate is directly proportional to the analyte concentration according to equation 30. Under these conditions, the observed reaction rate is a direct measurement of analyte concentration[8]. Mass transport-limited conditions are favored by high immobilization levels of the ligand and by low mixing rates. Conditions that are not suitable for kinetic measurements.

Concentrations of samples can be determined by a standard plot of mass transport-limited binding rate (dR/dt) versus concentration (C). The concentration of samples with different affinities than the analyte used in the standard plot and with molecular weights equal to the standard analyte can be determined by the use of the standard plot as reference.

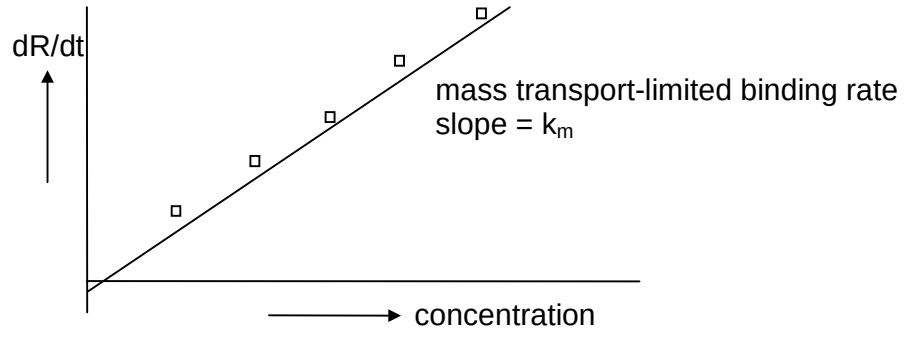


Figure 1.11: Standard plot for concentration determination. The concentration of samples with equal molecular weight as the sample used for the standard plot can be determined by one measurement using the standard plot as reference.

1.4 Guidelines for kinetic measurements

Purity of ligand and analyte: Contaminations of ligand or analyte may complicate the interaction and influence the binding curve. Therefore, the kinetic analysis may also be complicated. It is recommended to use ligand and analyte of high purity for kinetic analysis or concentration determination.

Stoichiometry: One-to-one interactions are the most simple interactions and also the most simple interactions to analyze. Other interactions may contain binding sites with different affinities. If that is the case, kinetic analysis will be much more complicated to analyze than the analysis of a one-to-one interaction. Therefore, it is recommended to study one-to-one interactions for kinetic analysis. The stoichiometry can be determined experimentally by the determination of R_{max}.

Immobilization level: A low immobilization level is recommended for kinetic measurements to reduce mass transport effects. The experimentally determined association rate constant should be independent of R_{max}. If the apparent association rate constant depends on R_{max}, the true association constant is higher due to the occurrence of mass transport limitation of the binding rate. This means that R_{max} should be reduced in a kinetic measurement till the experimentally determined association rate constant is independent of R_{max}.
The situation is reversed for concentration determinations: a high immobilization is recommended to achieve a fully mass transport-limited system.

Mix speed: A high mix speed is recommended for kinetic analysis to reduce mass transport effects, and a low mix speed should be used for concentration determinations.

Contact times: It is advised to measure the interaction till equilibrium for kinetic measurements, because the obtained curves can be analyzed better by nonlinear regression than for (small) parts of the curves. This means that the contact time depends on the kinetic constants of the interaction and of the analyte concentration. In case of the ideal monophasic bimolecular interaction it is not necessary to measure till equilibrium.
Short contact times are advised for concentration determinations, because the initial binding rate is reflected by mass transport-limited binding rate in general.

Dissociation. High analyte concentrations are recommended to analyze the dissociation phase, because rebinding of the released analyte is minimized due to the lack of available free binding sites at the beginning of the dissociation process. Rebinding of the released analyte can also be prevented by adding a low molecular weight competing ligand to the dissociation buffer. Theoretically, the determined dissociation rate constant should be independent of the analyte concentration.

1.5 References

1. **Malmborg, A.-C., A. Michaëlson, M. Ohlin, B. Jansson, and C.A.K. Borreback.** 1992. Real time analysis of antibody-antigen kinetics. Scan. J. Immunol. **35:** 643-650.
2. **Karlsson, R., A. Miachaelsson, and L. Matsson.** 1991. Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor based analytical system. J. Immunol. Methods, **145:** 229-240.
3. **Fägerstam, L.G., Å. Frostell-Karlsson, R. Karlsson, B. Persson, and I. Rönnberg.** 1992. Biospecific interaction analysis using surface plasmon resonance detection applied to kinetic, binding site and concentration analysis. J. Chromatogr. **597:** 397-410.
4. **Morton, T.A., D.G. Myszka, and I. Chaiken.** 1995. Interpreting complex binding kinetics from optical biosensors: a comparison of analysis by linearization, the integrated rate equation, and numerical integration. Anal. Biochem. **227:** 176-185.
5. **O'Shannessy, D.J., M. Brigham-Burke, K.K. Soneson, P. Hensley, and I. Brooks.** 1993. Determination of rate and equilibrium binding constants for macromolecular interactions using surface plasmon resonance: use of nonlinear least square analysis methods. Anal. Biochem. **212:** 457-468.
6. **Glaser, R.W.** 1993. Antigen-antibody binding and mass transport by convection and diffusion to a surface: A two-dimensional computer model of binding and dissociation kinetics. Anal. Biochem. **213:** 152-161.
7. **Karlsson, R., H. Roos, L. Fägerstamm, H. Nilshans, and B. Persson.** 1994. Kinetic and concentration analysis. Methods: a companion to Methods in Enzymology, **6:** 99-110.
8. **Karlsson, R., L. Fägerstamm, H. Nilshans, and B. Persson.** 1993. Analysis of active antibody concentration. Separation of affinity and concentration parameters. J. Immunol. Methods, **166:** 75-84.
9. **Panayotou, G., M.D. Waterfield, and P. End.** 1993. Riding the evanescent wave. Current Biology, **3:** 913-915.

2 SPR Kinetic Evaluation software

2.1 Introduction

Experimentally obtained SPR data can be evaluated with the SPR kinetic evaluation software. This chapter describes how to use the software to analyze data of interaction plots. The software is explained by the analyses of three data sets. These data sets include examples of monophasic interaction, biphasic interaction. Analysis of SPR data involves the following steps:

1. Making overlay plots of several interaction curves
2. Selection of analysis region.
3. Selection of interaction model and curve fitting
4. Storing of results of the fitting procedure into an analysis results file.

2.2 SPR Kinetic Evaluation screen and workspace

The Kinetic evaluation software is installed as a program map on your win95 PC. Double click the kinetic evaluation icon to start the program. The program starts with the *Kinetic evaluation* window.

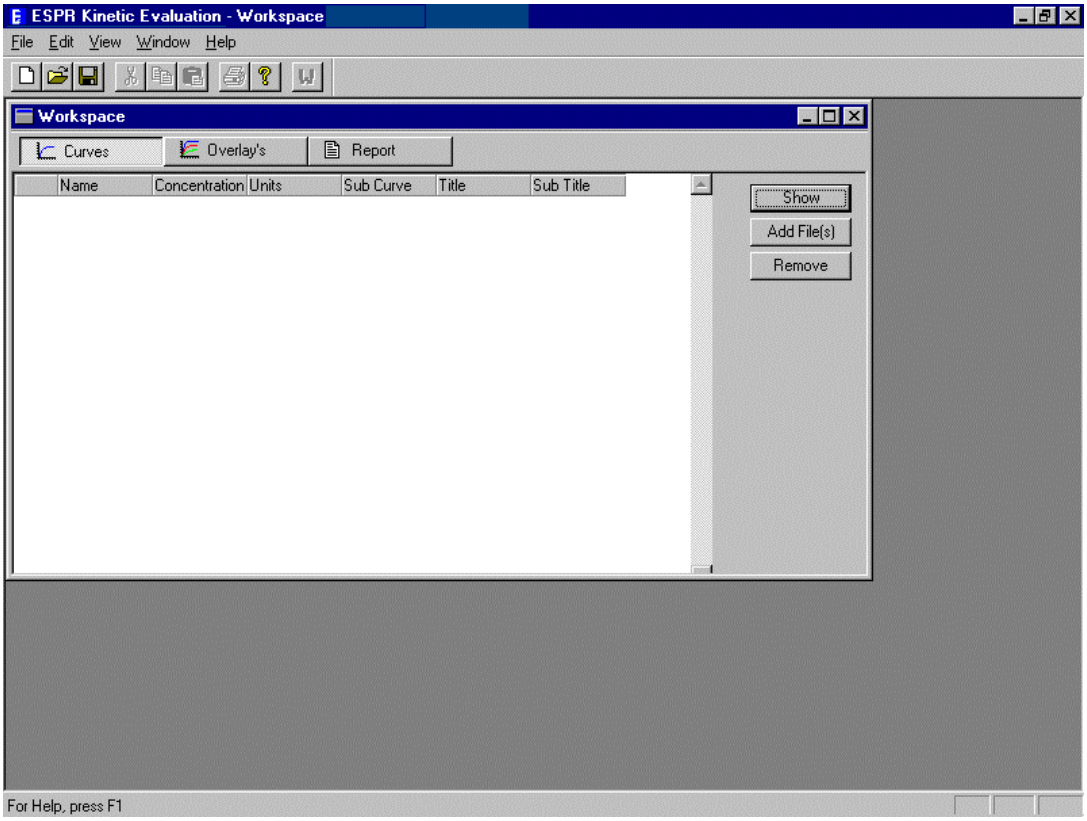
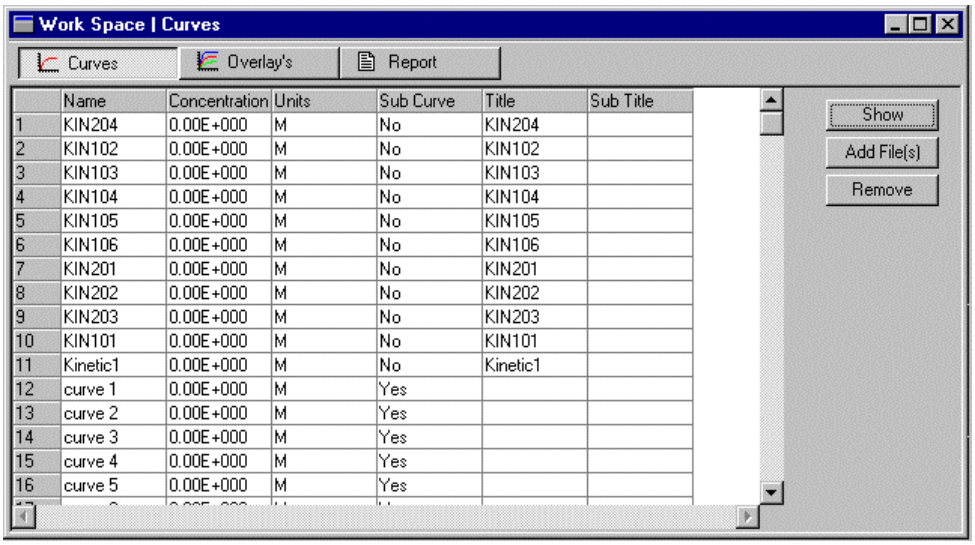


Figure 2.1: Kinetic evaluation screen

The screen contains the menu bar, the tool bar, and the workspace.
The workspace is the central part of the program. It manages your files.

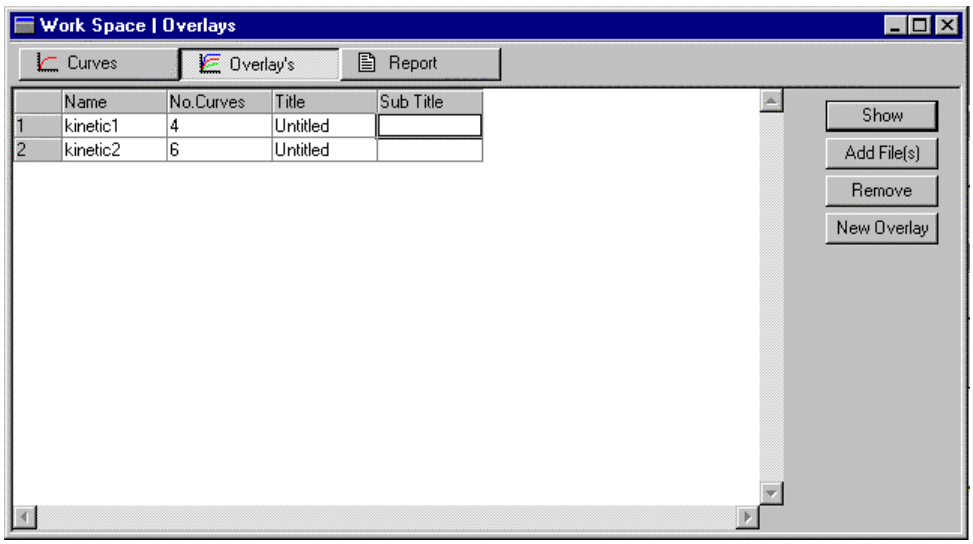
All data files are located in the View option Curves of the Workspace



	Name	Concentration	Units	Sub Curve	Title	Sub Title
1	KIN204	0.00E+000	M	No	KIN204	
2	KIN102	0.00E+000	M	No	KIN102	
3	KIN103	0.00E+000	M	No	KIN103	
4	KIN104	0.00E+000	M	No	KIN104	
5	KIN105	0.00E+000	M	No	KIN105	
6	KIN106	0.00E+000	M	No	KIN106	
7	KIN201	0.00E+000	M	No	KIN201	
8	KIN202	0.00E+000	M	No	KIN202	
9	KIN203	0.00E+000	M	No	KIN203	
10	KIN101	0.00E+000	M	No	KIN101	
11	Kinetic1	0.00E+000	M	No	Kinetic1	
12	curve 1	0.00E+000	M	Yes		
13	curve 2	0.00E+000	M	Yes		
14	curve 3	0.00E+000	M	Yes		
15	curve 4	0.00E+000	M	Yes		
16	curve 5	0.00E+000	M	Yes		

Figure 2.2: Workspace\Curves table

Data files can be viewed by the Show button, can be added to the list by the Add File(s) button and can be removed from the list by the Remove button.
Overlay plots created by the Kinetic Evaluation program are listed in table belonging to the View Overlay button of the Workspace (second button).



	Name	No.Curves	Title	Sub Title
1	kinetic1	4	Untitled	
2	kinetic2	6	Untitled	

Figure 2.3: Workspace\Overlays table

Overlay plots can be shown by the Show button, they can be added from disk by the Add file(s) button, they can be removed from the list by the Remove button, and finally they can be created by the New Overlay button.
The third button of the Workspace, the Report button contains a list of report files of the fit results.

2.3 Overlay plot

Interaction plots of SPR data are represented in two ways mainly. Either several interaction plots are stored into one data (*.ibo) file, or only one interaction plot per file is present.
When you have measured manually, you probably have more than one interaction plot per file. By performing the data acquisition with the autosampler, it is most likely that you have only one interaction plot per file.
Therefore, there are several ways to create an overlay plot

I By Subcurves (manualy obtained data files)

- Begin with an empty Workspace.
- Switch to Workspace\Curves, and click Add files button.
- In the Select File(s) window open the file kinetic1.ibo in SPR\data directory. The kinetic1.ibo file will appear on the screen. The file contains a graph of six interaction plots/curves.

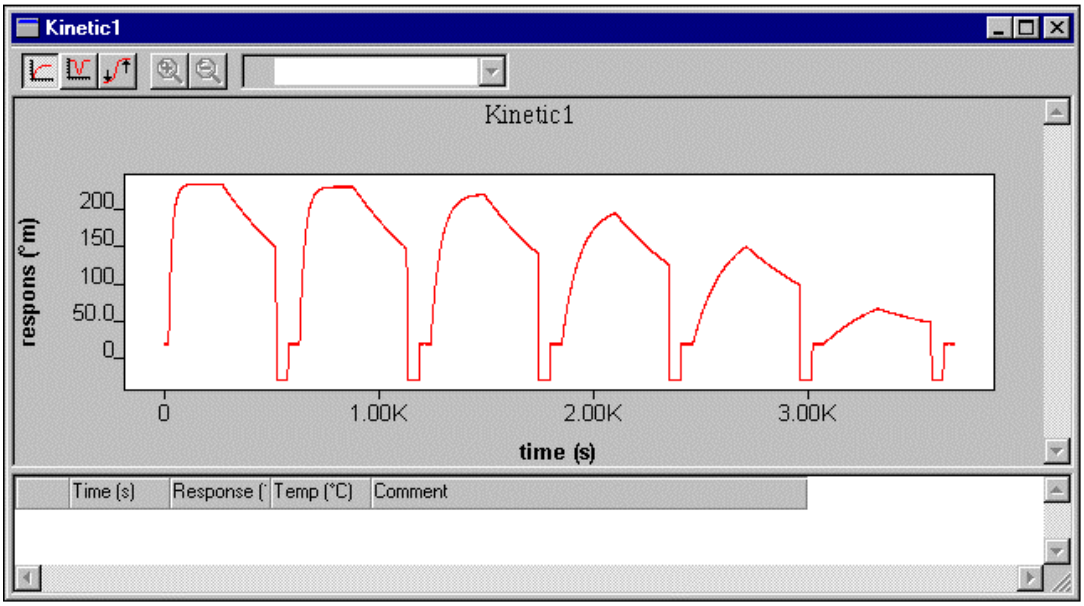


Figure 2.4: data file window of kinteic1.ibo

- Select the first curve with the left mouse button. Zoom in by clicking the magnifying button in the toolbar of the kinetic1 screen.
- Select the entire first curve with the left mouse button. The selected region will be shown in black (figure 2.5).

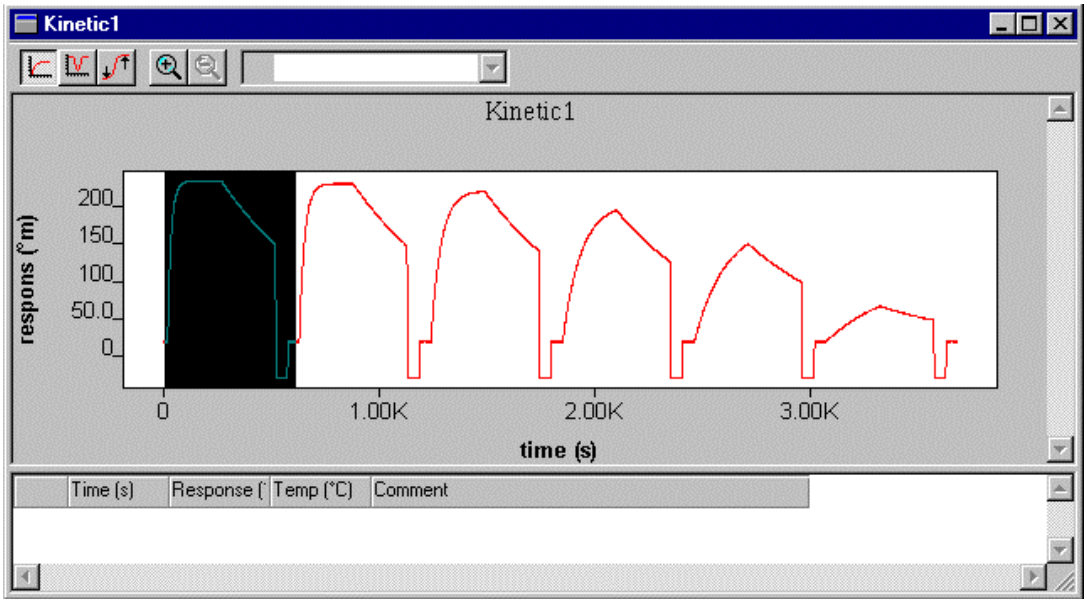


Figure 2.5: Selection of the first interaction plot

- Click with the right mouse button on the curve. The shortcut menu will be shown on the screen.

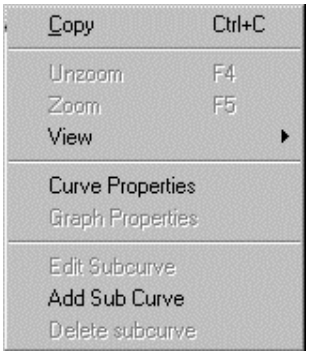


Figure 2.6: Shortcut menu shown by clicking the right mouse button

- Select the option Add Sub Curve to open the *Add subcurve* window.

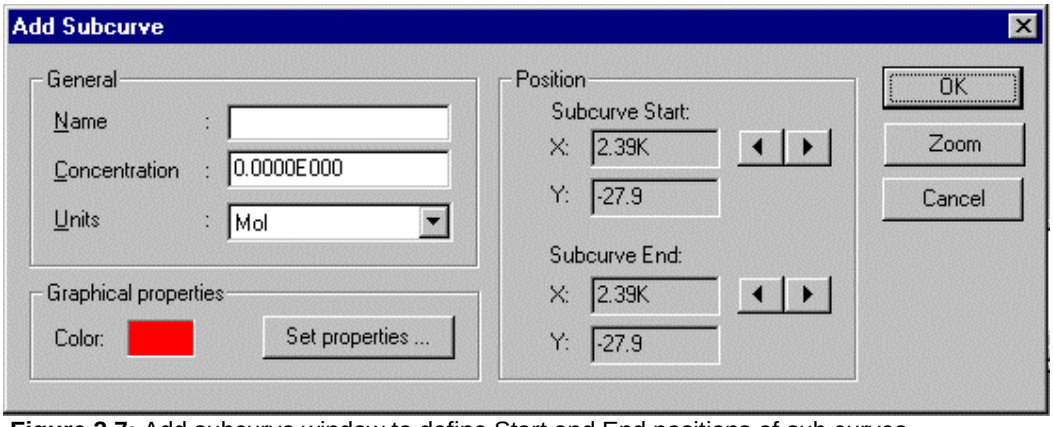
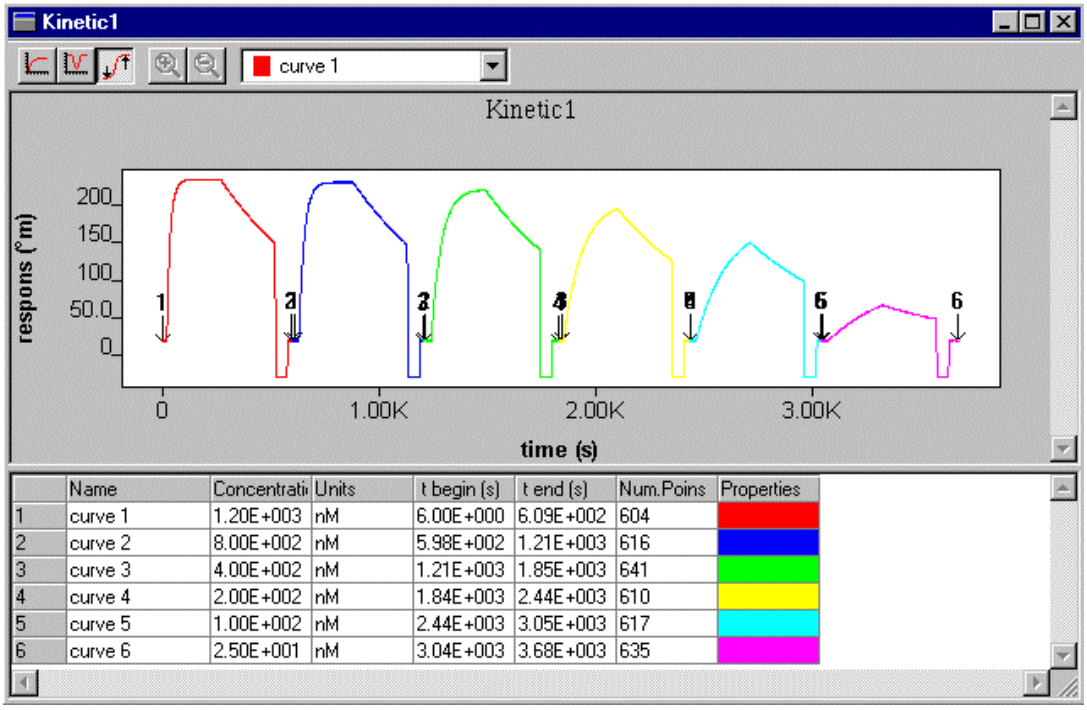


Figure 2.7: Add subcurve window to define Start and End positions of sub curves

- Adjust the subcurve start and end postions by scrolling and define the General properties of the subcurve.

Fill in Name : curve1
Concentration : 1200
Units : nM

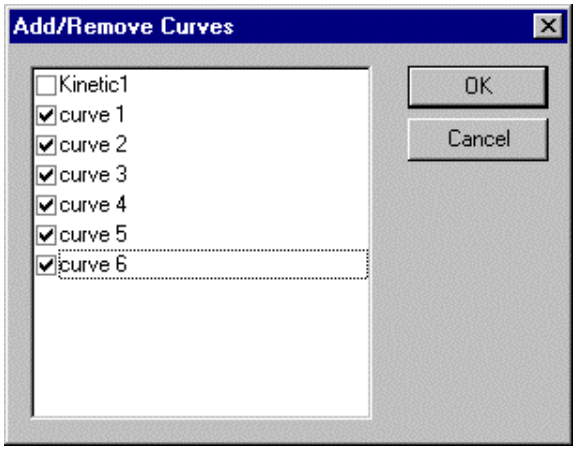
- After closing the *Add subcurve* window, the *kinetic1 curve* window shows the added subcurve in the entire curve between two markers 1, and in the table below the graph. The toolbar of the kinetic 1 window shows that the curve screen has switched from curve presentation (first tool) to subcurve presentation (third tool)
- Repeat the procedure to add subcurves for all concentrations and name them curve2 till curve 6, respectively. Enter the concentrations and the units for every curve. (1200 nM, 800 nM, 400 nM, 200 nM, 100 nM and 25 nM for curve 1 till 6 respectively).



	Name	Concentrati	Units	t begin (s)	t end (s)	Num.Poins	Properties
1	curve 1	1.20E+003	nM	6.00E+000	6.09E+002	604	
2	curve 2	8.00E+002	nM	5.98E+002	1.21E+003	616	
3	curve 3	4.00E+002	nM	1.21E+003	1.85E+003	641	
4	curve 4	2.00E+002	nM	1.84E+003	2.44E+003	610	
5	curve 5	1.00E+002	nM	2.44E+003	3.05E+003	617	
6	curve 6	2.50E+001	nM	3.04E+003	3.68E+003	635	

Figure 2.8: Sub curve presentation of kinetic1 window shows 6 defined sub curves in the plot and in the corresponding table.

- Create an overlay plot of the add sub curves by File: New overlays or by the first tool on the toolbar. As a result, the *Add/Remove Curves* window will



appear on the screen.

Figure 2.9: Add/Remove Curves window for selection of curves to create an overlay plot

- Select the subcurves curve1, curve2, curve3, curve4, curve5, curve 6 in the *Add/Remove Curve* window. The overlay plot window will be shown

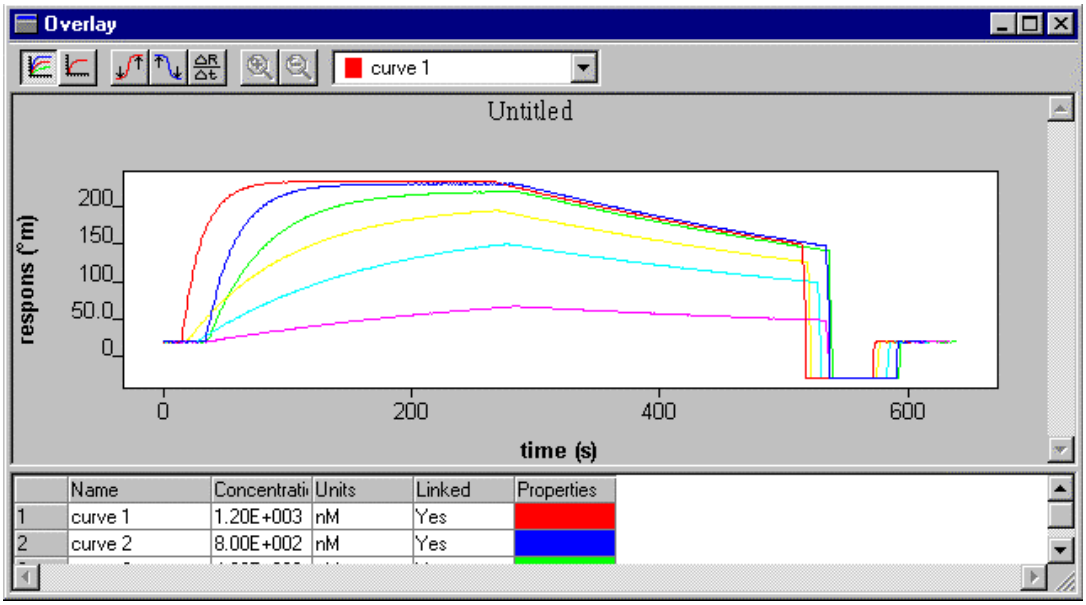


Figure 2.10: Overlay plot of 6 curves. Injections have not been synchronized yet.

The sample injection times of the curves are not equal. To synchronize the curves, determine the curve with the shortest sample injection time. The other cuves will be synchronized to this time.

- For every curve separately, select the region with left mouse button between the two sample injection times (region between sample injection time of reference curve, and sample injection time of another curve).
- Select the curve to be synchronized in toolbar of overlay window.
- Select Analyse: Change All Points (F3) in menu bar.

- Select the Synchronize option (first option) in the Change All Points window, and click Apply button. The selected curve will be synchronized. You can undo this operation by clicking the Undo button.

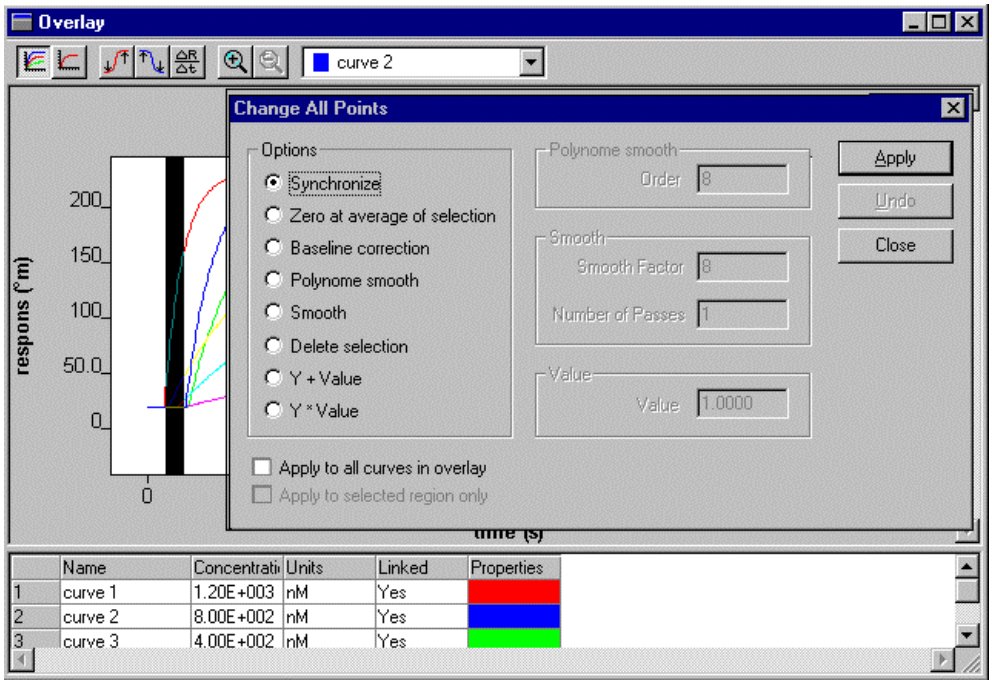


Figure 2.11: Synchronization of injections in the overlay plot.

- Repeat the synchronization procedure for all curves.
- Save the overlay plot with File: Save as kinetic1.iko.

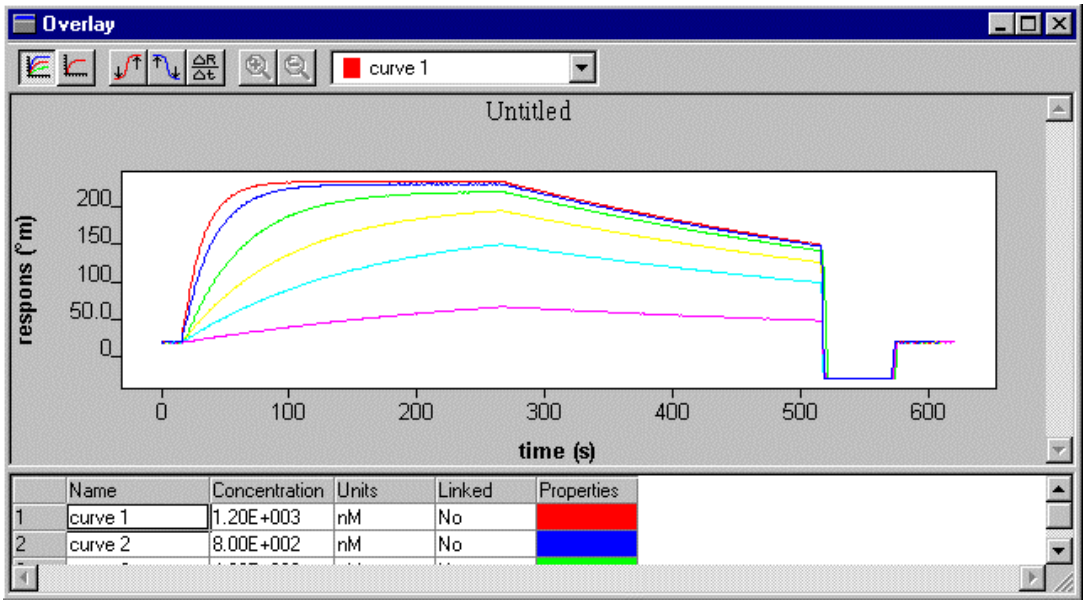


Figure 2.12: Overlay plot of 6 curves after synchronization of injections.

II. By Copy / Paste (manually obtained data files)

- Begin with an empty Workspace.
- Switch to Workspace\Curves, and click Add files button.
- In the Select File(s) window open the file kinetic1.ibo in SPR\data directory. The kinetic1.ibo file will appear on the screen. The file contains a graph of six interaction plots/curves (see figure 2.4).
- Select the first curve with the left mouse button. Zoom in by clicking the magnifying button in the toolbar of the kinetic1 screen.
- Select the entire first curve with the left mouse button. The selected region will be shown in black (figure 2.5).
- Select the Edit: Copy command of the main menubar or click the copy button of the main menu toolbar.
- Open a new overlay plot by File : New Overlay.
- Click the OK button of the Add/Remove window without selecting any curve. As a result, an empty overlay window will be shown on the screen.
- Select the Edit:Paste command of the main menubar or click the Paste button of the main toolbar. The first curve will be pasted into the overlay window.
- Repeat the copy / paste procedure for all curves of the file.
- The injection times should be synchronized according the procedure described for figure 2.10.

III Multiple curves simultaneously (autosampler obtained data files)

- Begin with an empty workspace.
- Switch to Workspace\ Curves, and click Add files button .
- In the Select File(s) window select the files kin101.ibo, kin101.ibo, kin102.ibo, kin103.ibo, kin104.ibo, kin105.ibo, and kin106.ibo in SPR\data directory. (Use the Shift or Ctrl key for selection of the files). Click the Open button to open all files.

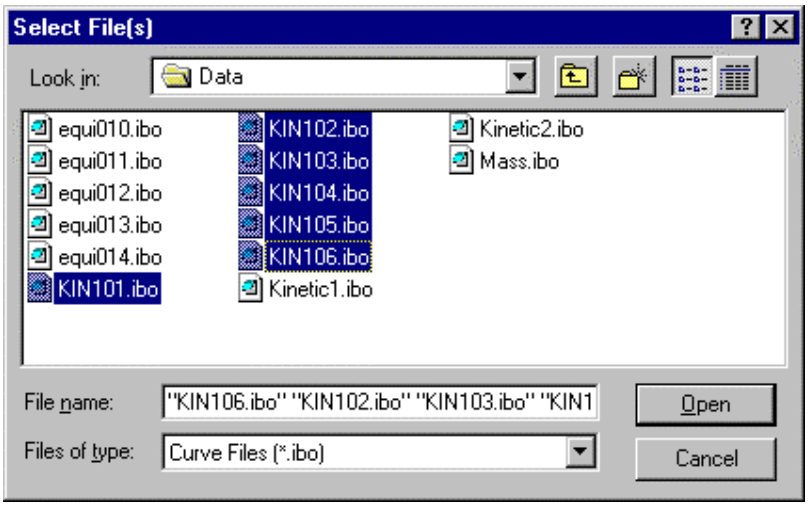


Figure 2.13: Select File(s) window to open multiple files simultaneously .

- All files will be opened and will be shown. Close all files separately or simultaneously by Window: Close All. The curves are listed in the table of Workspace\Curves.
- Create an overlay plot of the six curves by File: New Overlay or by the first tool of the toolbar. Select the six curves in the Add/Remove Curve window and click OK button.

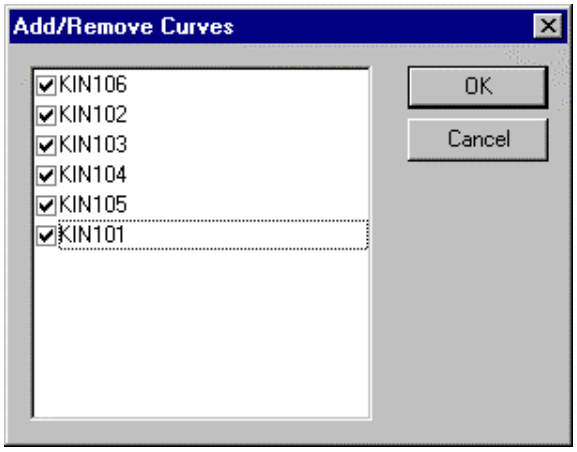


Figure 2.14: Selection of files for creation of an overlay plot in the Add/Remove Curve window.

The overlay plot will be shown in the overlay window. The curve properties can be entered in the table below the graph.

- Enter the concentrations and the units for every curve. (1200 nM, 800 nM, 400 nM, 200 nM, 100 nM and 25 nM for curve 1 till 6 respectively).

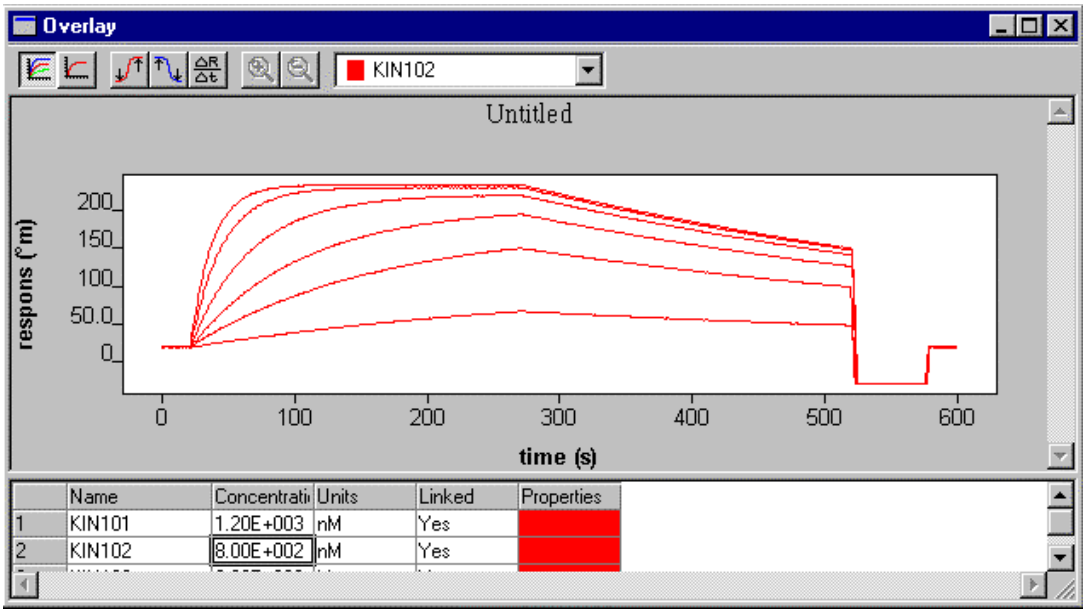


Figure 2.15: Overlay plot of 6 autosampler curves.

IV Double channel data files

Three types of data sets can be obtained with the double channel instrument. Subsequently, the data of channel 1, channel 2, or the difference signal of channel 1 - channel 2 can be analysed kinetically. Therefore, overlay plots of these data sets have to be created in different ways.
An example SPR II data set are files SII01, SPRII02, SPRII03, SPRII04, and SPRII05. The concentrations of the analytes are 1200, 800, 400, 200, and 100 nM, respectively.

CHANNEL 1 representation
The normal curve window views the data of channel 1 only. By opening the an SPRII file, non- corrected interaction plots are shown. Figure 2.16 shows this for file SII01.

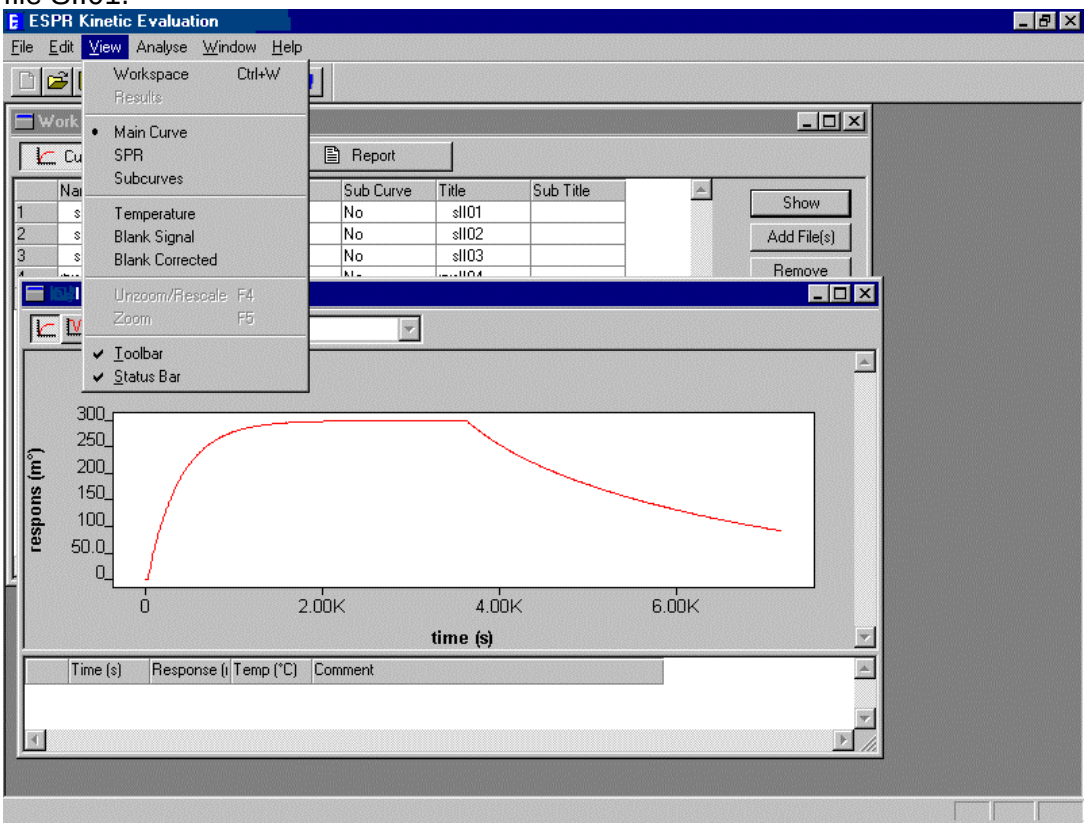


Figure 2.16: SII01.ibo data file viewed in default settings. (The view options blank signal and blank corrected are not in use)

CHANNEL 2 representation

The data of the second channel are the signals of the blank experiment. The signals of the analyte with a surface without ligand. It shows the non-specific interaction.

Channel 2 could also have been used as a second experiment with different interaction partners. In this case the data is not used as blank data.

However, in the software the second channel is viewed as blank signal. It is shown in the curve window together with the data of channel 1. It is not possible to show it without data of channel 1. Figure 2.17 shows the data of both channels of file SII01. Use the command View: Blank signal for it.

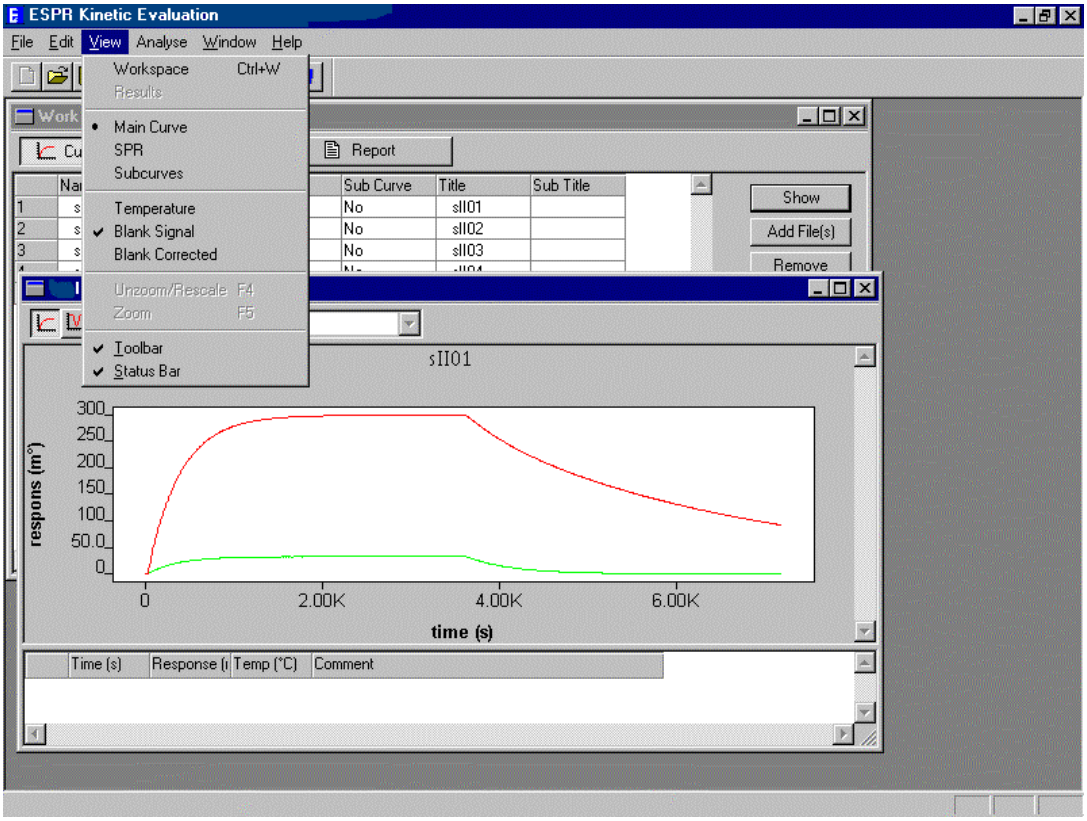


Figure 2.17: Representation of channel 1 and channel 2 of file SII01.ibo

CHANNEL 1 - CHANNEL 2 representation

The difference signal of channel 1 - channel 2 can be shown by the command View: Blank Corrected. Notice: it is not possible to view the difference signal of channel 2 - channel 1. See figure 2.18 for an example of file SII01.

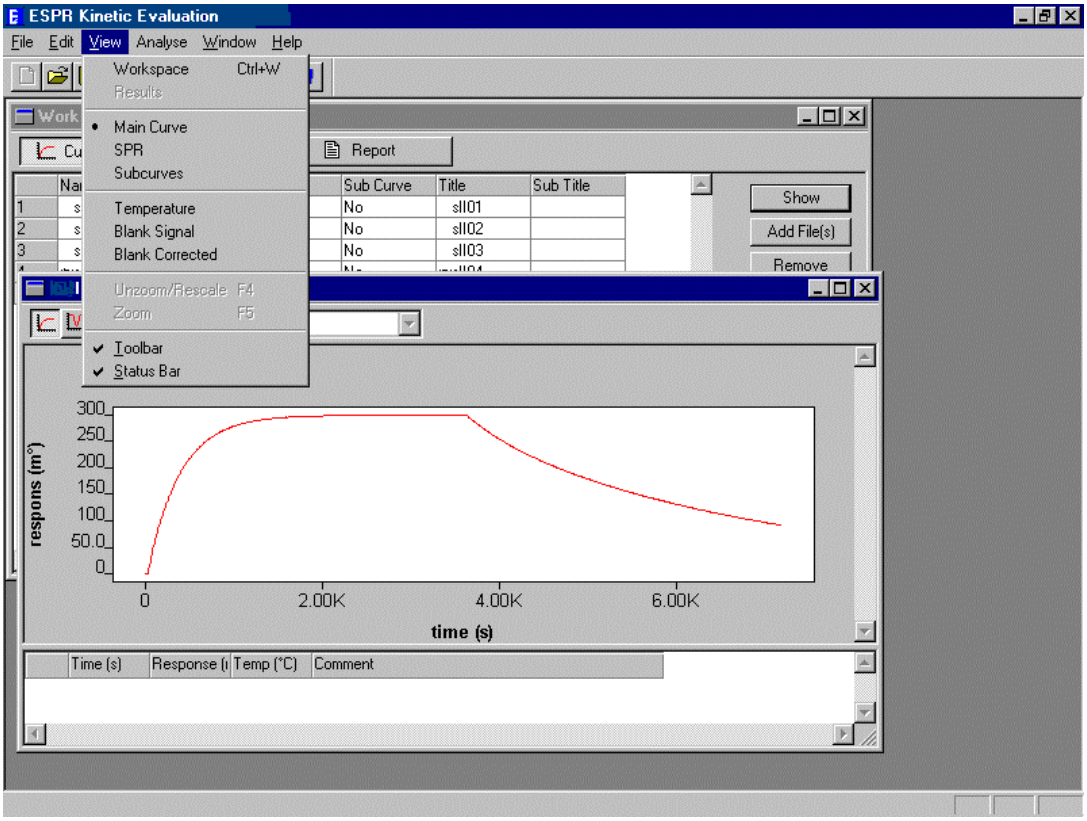


Figure 2.18: Blank corrected representation of file SII01. The screen shows an interaction plot of data channel 1 - data channel 2. The data of channel 1 have been taken from the interaction of an analyte with a ligand, and the data of channel 2 have been taken simultaneously from the analyte with a surface without the ligand.

Overlay plots of the three data set representations are created according to:

CHANNEL 1 data

Overlay plots of files SII01/05 are created similar to the procedure of SPR I data files, described in section 2.3.I, II, and III. The procedure described in section 2.3. III is recommended, because it is the fastest and easiest method.
In short, add the files to Workspace\Curve and open a new overlay (File: New Overlay). Then, select the files in the *Add/Remove Curves window*. The overlay plot of channel 1 will be shown (figure 2.19)

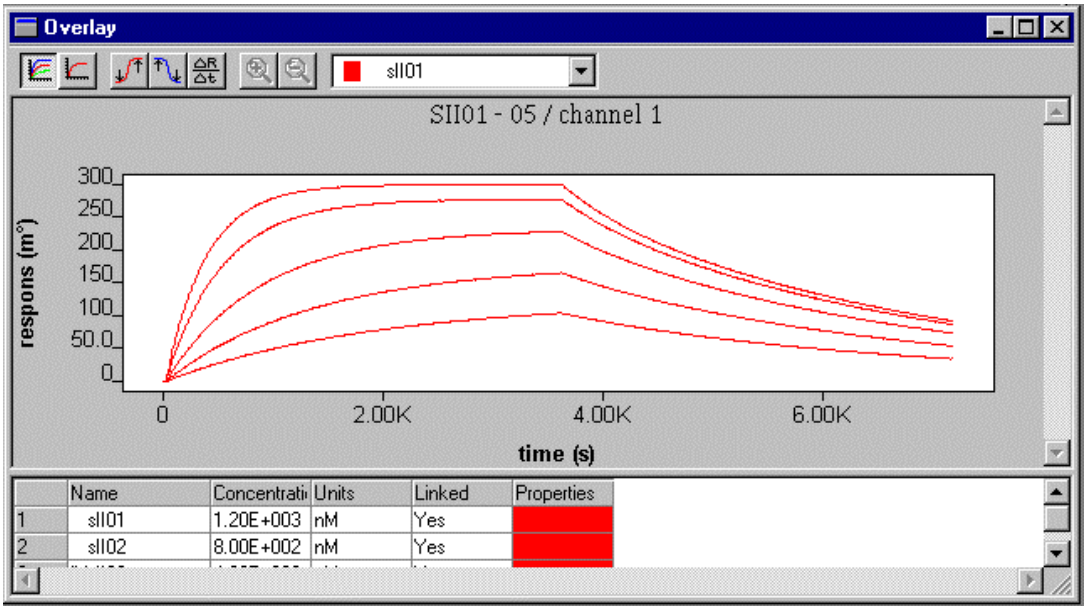


Figure 2.19: Overlay plot of channel 1 data of SPR II data set

CHANNEL 2 data

The Copy / Paste procedure used in section 2.3.II should be applied to create an overlay plot of data of channel 2. The command Paste special is used instead of the command Paste to paste the data of channel 2 from the clipboard into an overlay plot. If the command Paste is used, then data of channel 1 will be pasted.

The procedure will be explained for the SPRII data set which has been added to the Workspace previously.

- In Workspace\Curve select a curve and click the Show button to show the file.
- Select View:Blank signal option of main menu bar. The data of channel 2 will also be plotted in the curve window.
- Select the entire curve with the left mouse button (click and drag)
- Select the option Edit: Copy

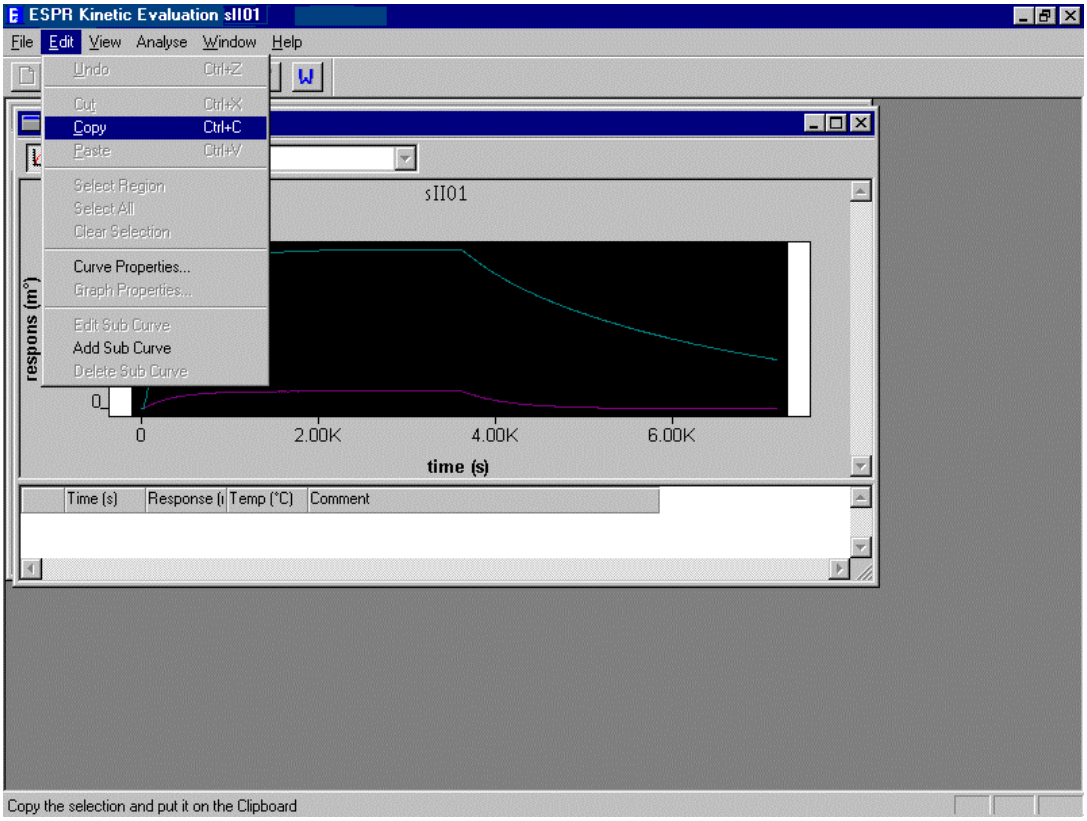


Figure2.20: Copy data of channel 1 and channel 2 into the clipboard of file SII01, when the curve window is in the View option Blank signal.

- Open an empty overlay plot by File: New Overlay and afterwards click the OK button of the Add/Remove curves window.
- Paste data of channel 2 in the active overlay window by Edit: Paste special

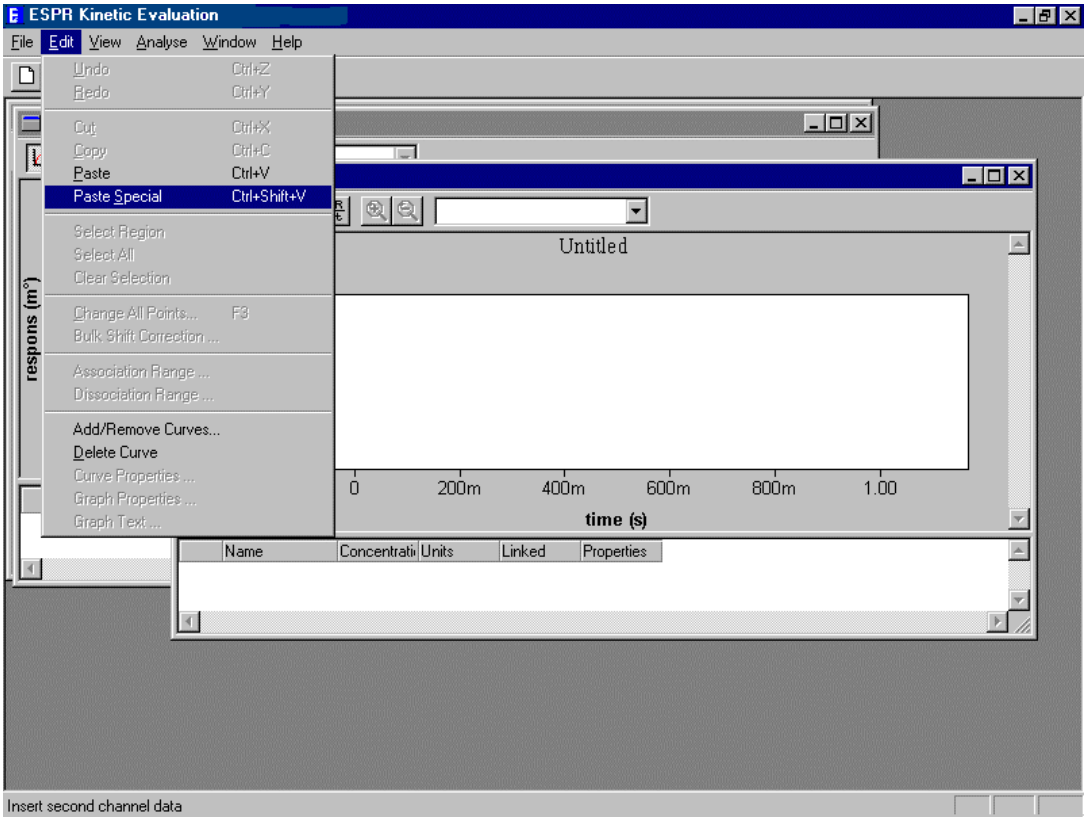


Figure 2.20: Pasting data of channel 2 into an overlay plot by the command Paste Special

- Repeat the Copy / Paste special procedure for all 5 SPRII data files.
- The overlay plot contains data of channel 2 only.(figure 2.21)

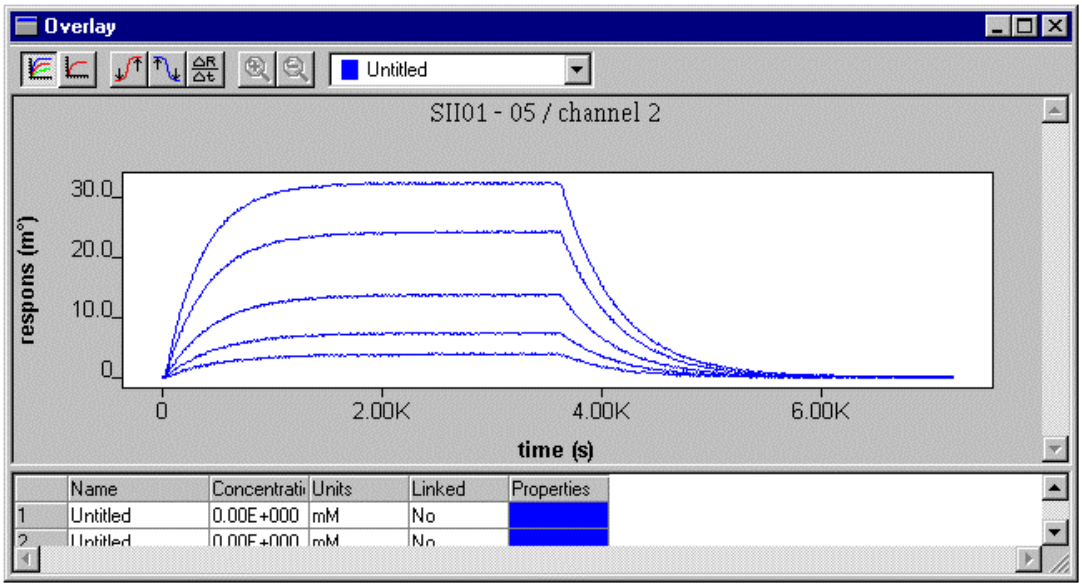


Figure 2.21: Overlay plot of SII01/05 channel 2 data set. The interaction plots resemble the non-specific interaction of the analyte with a blank surface.

CHANNEL 1 - CHANNEL 2 data

This procedure is similar to the procedure to create an overlay plot of channel 2 data. The Copy / Paste method is applied for curves in the blank corrected representation. The method is explained for the SPRII data set already present in the workspace.

- Switch to Workspace\Curve and show the SII01 curve
- Select View: Blank corrected to switch to the difference signal representation
- Select the entire curve with your left mouse button (click and drag)
- Select Edit:Copy option of main menu
- Open a new empty overlay window (File: New overlay, and click the OK button of the *Add/Remove curves window*)
- Select Edit: Paste option of main menu
- Repeat procedure for all files, which will result in an overlay plot shown in figure 2.22.

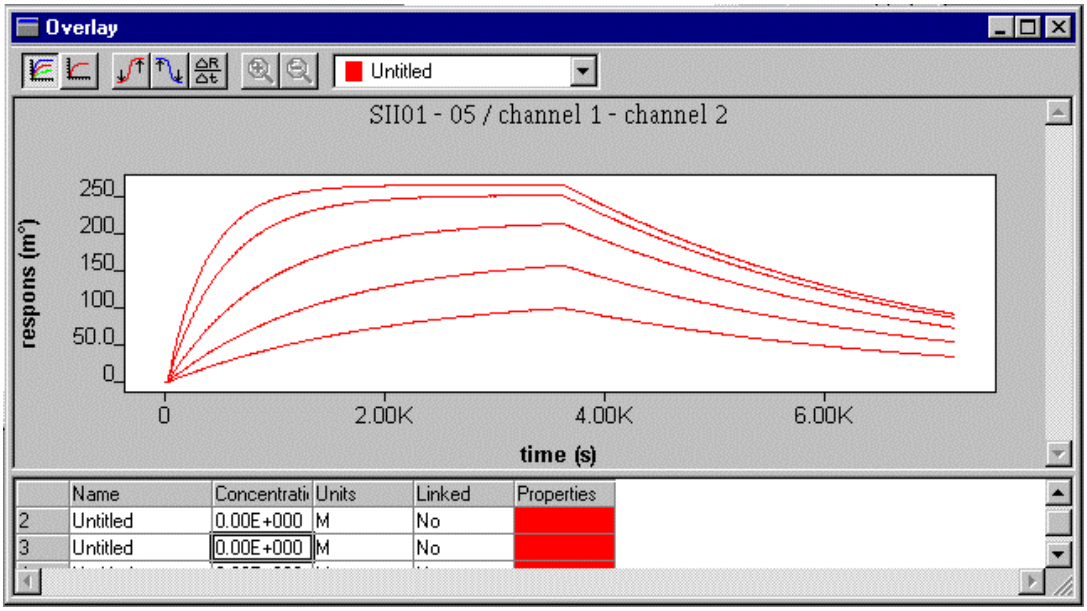


Figure 2.22: Overlay plot of difference signals (channel 1 - channel 2) of SPR II data set. The difference signals are the interaction plots corrected for non-specific binding of the analyte with a blank surface.

2.4 Monophasic association

An example of the analysis of a monophasic association is given for the analysis of data files kin101.ibo, kin102.ibo, kin102.ibo, kin104.ibo, kin105.ibo, kin106.ibo. These files contain 6 monovalent interactions of analyte A with ligand B. The concentrations of analyte A are 1200 nM, 800 nM, 400 nM, 200 nM, 100 nM, and 25 nM, respectively. The kinetic analysis of the association phase will reveal the association and dissociation rate constants, and thereby the equilibrium constants. The analysis is performed by:

- Create an overlay plot of the six binding curves (see 2.3) or open a saved overlay file (*.iko)
- Switch to Workspace\Overlay and show the overlay file.
- Click the dR/dt button (tool 5) of the Overlay window to see if there is a masstransport-limited region in the curve (see 1.2.5 for theory). Check all curves, and click on tool 5 again to switch to the standard overlay presentation.

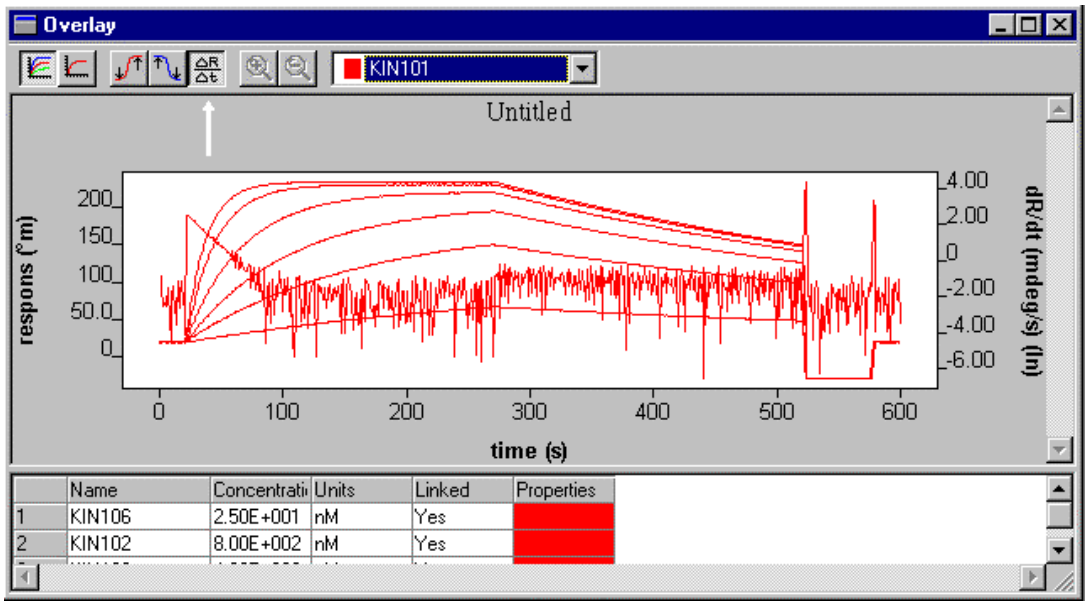


Figure 2.23: Overlay plot containing 6 curves. One curve KIN101 is shown as ln dR/dt versus time plot. The View option is activated by tool 5, which is indicated by an arrow in the figure.

The ln(dR/dt) plot is used for the selection of the kinetic phase of the association phase. Look at the beginning of the association phase, if the ln(dR/dt) is horizontal, the binding rate is limited by mass transport. For a kinetic analysis, this part of the curve should be left out.

- Select the region to be analyzed with the left mouse button. Click the left mouse button at the beginning of the association phase and drag the mouse pointer to the end of the association phase, and finally release the mouse button. The selected region is indicated in black.

- Open the shortcut menu with the right mouse button.

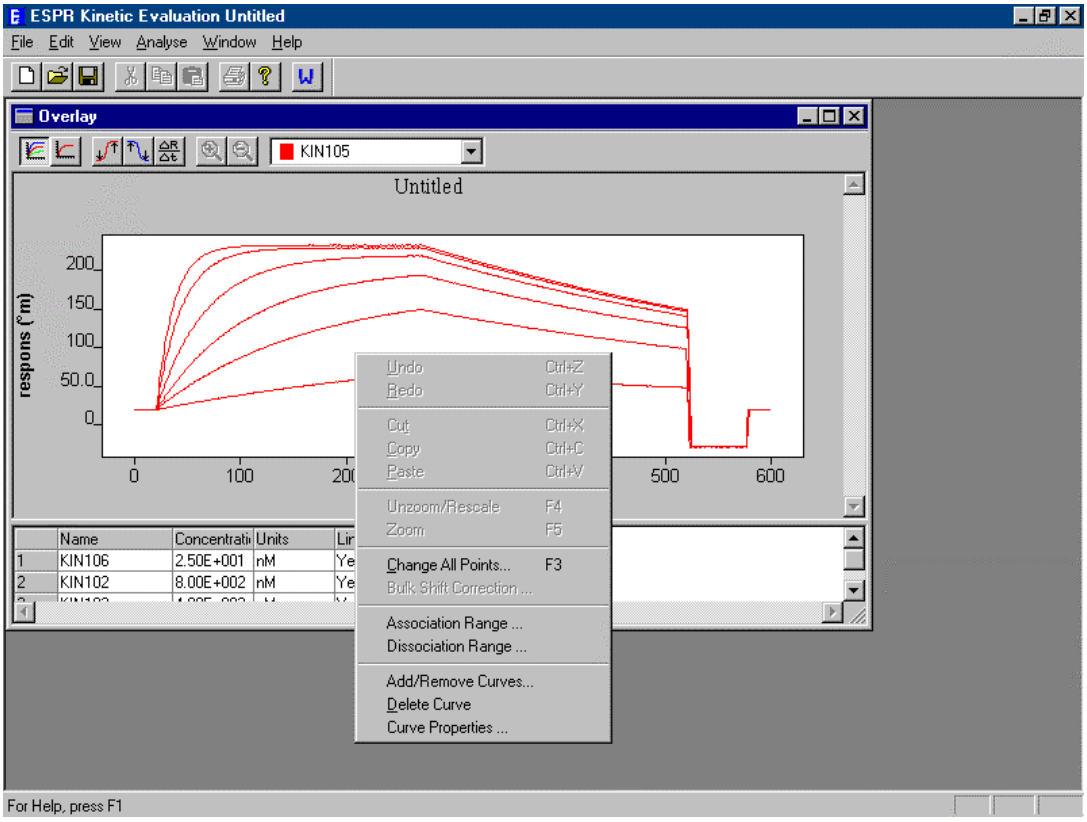


Figure 2.24: Shortcut menu is shown after clicking with the right mouse button on the overlay plot.

- Select association range to open set *association range* window.

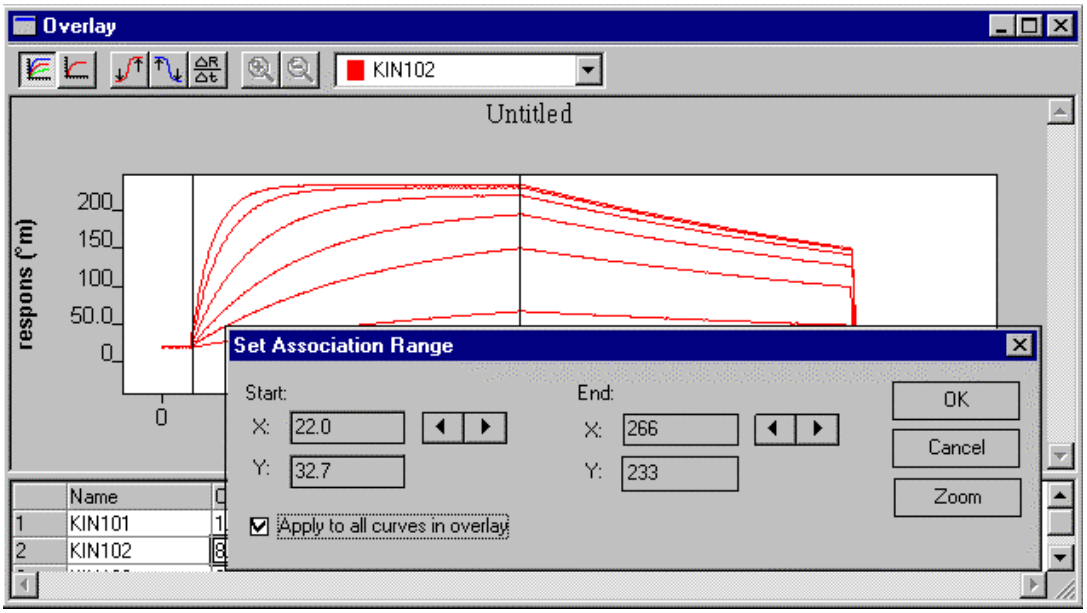


Figure 2.25: Define association ranges by the Set Association Range window.

- Adjust the association ranges by scrolling, mark apply to all curves in overlay

and click OK button. The association ranges in the overlay plot can be shown by clicking the third tool of the overlay window. (Click again to switch to the standard overlay window).

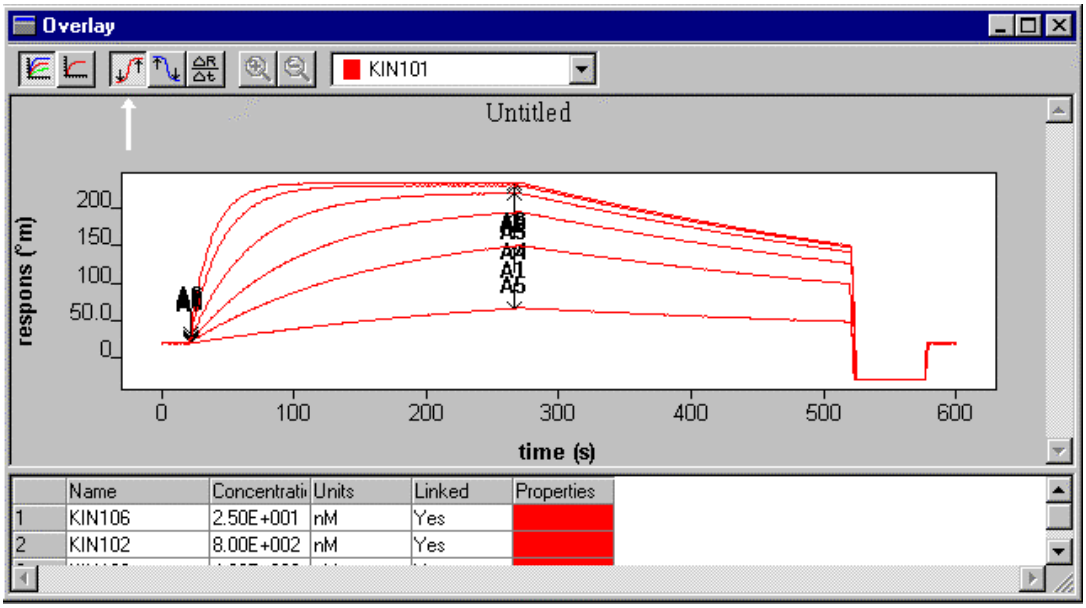


Figure 2.26: Defined association ranges are viewed by the third tool, which is indicated by an arrow in the figure.

- Start the association fitting procedure by Analyse: Association (or CtrA). The *Analyse Association Phase* window will be opened.

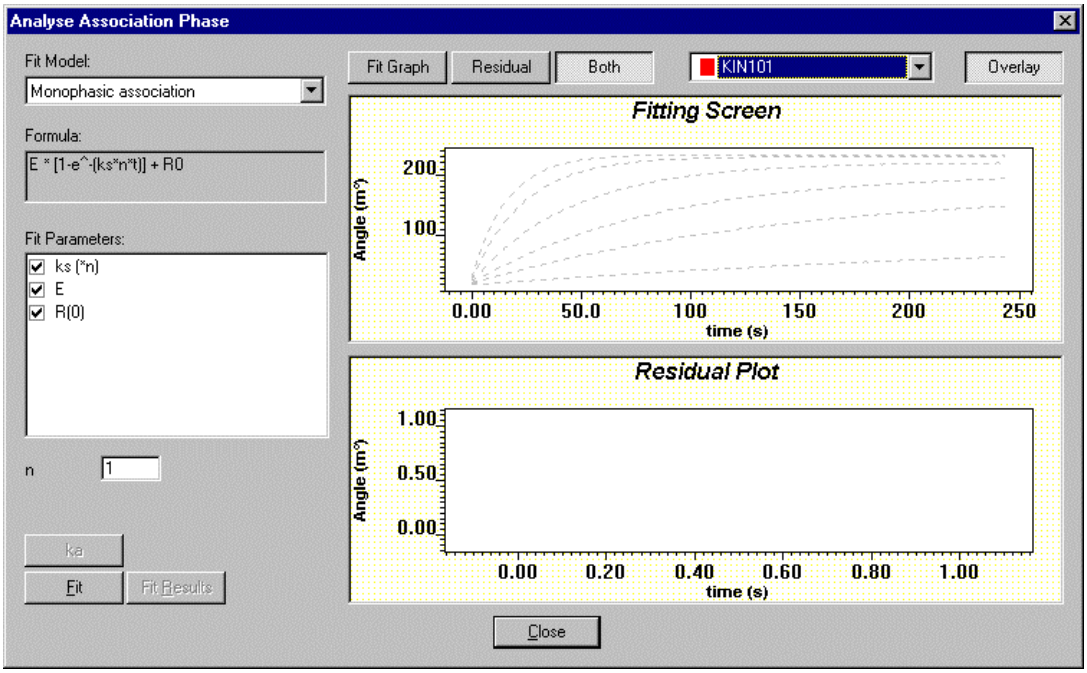


Figure 2.27: Analysis association window for fitting the curves of the overlay plot.

In the right part of the window the fitting screen is presented. Three presentations of the fitting procedure are possible:

Fit graph, which shows the fitted curves only
Residual Plot, which shows the residual* plots only
Both, which shows the fitted curves and residual plots together

*) The residual plot is a plot, which presents the fitted curve as a straight line at the x-axis (y = 0) and the measured data points as residuals (y-value data points - fitted y-value). Data points should be located randomly around the fitted curve.

- Click the Both button.
- Select Fit Model: Monophasic association. Data will be fit according the model given in Formula:
  R =E * [1-exp(-ks*n*t)] + R0
  and the Fit parameters
  E
  ks
  R0

(see 1.2.1 for a mathematical description of this model)

By default all fit parameters are marked, and are involved in the fitting procedure.
It is possible to fix a fit parameter by removing its mark.

- Press the Fit button and the *Fitting* window will appear.

- Click Fit all button

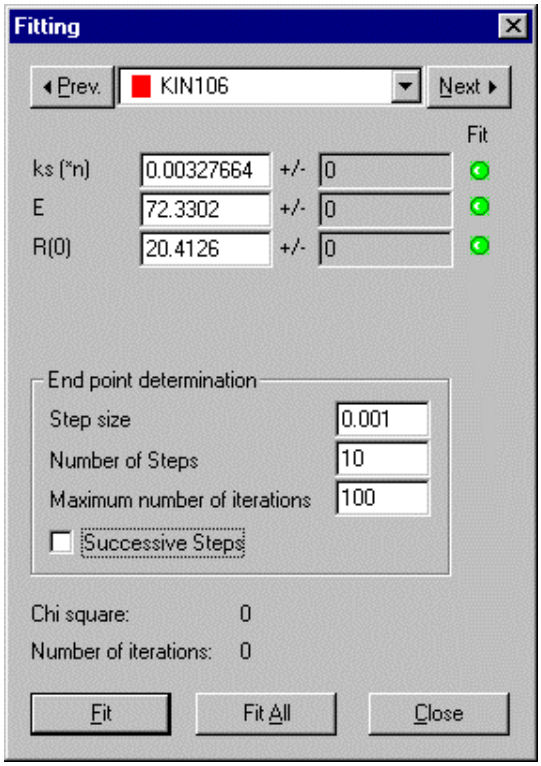


Figure 2.21: Fitting window to start fit procedure

The fitting procedure will be applied for all curves. The performance of the fitting is displayed in the residual plot. If data points are distributed randomly around the x-axis, then the applied model is correct. The residual plots of the 6 curves

can be viewed by the Next button of the *Fitting* window.

- Click Close button of *Fitting* window. The underlying *Analyse Association Phase* window is activated now.
- Click Fit results button to open *Fit results* window. The fitting results of all curves are displayed in a table. Click To report button to store results in a Report file. Click Close button to go back to the *Analyse Association Phase* window.
- Click ka button to calculate the kinetic rate constants by linear regression. The *Linear Fit* window shows the ks versus Concentration plot and the association rate constant ka and the dissociation rate constant kd. Click To report button to store linear fit results.
- Click Close button of *Analyse Association phase* window to go back to the overlay plot.
- To see all results, switch to the Report option of the Workspace. Use Ctrl W, View: Workspace option of menu bar, or the W button of toolbar to show the Workspace. Selection of the Report option results in an overview of results.

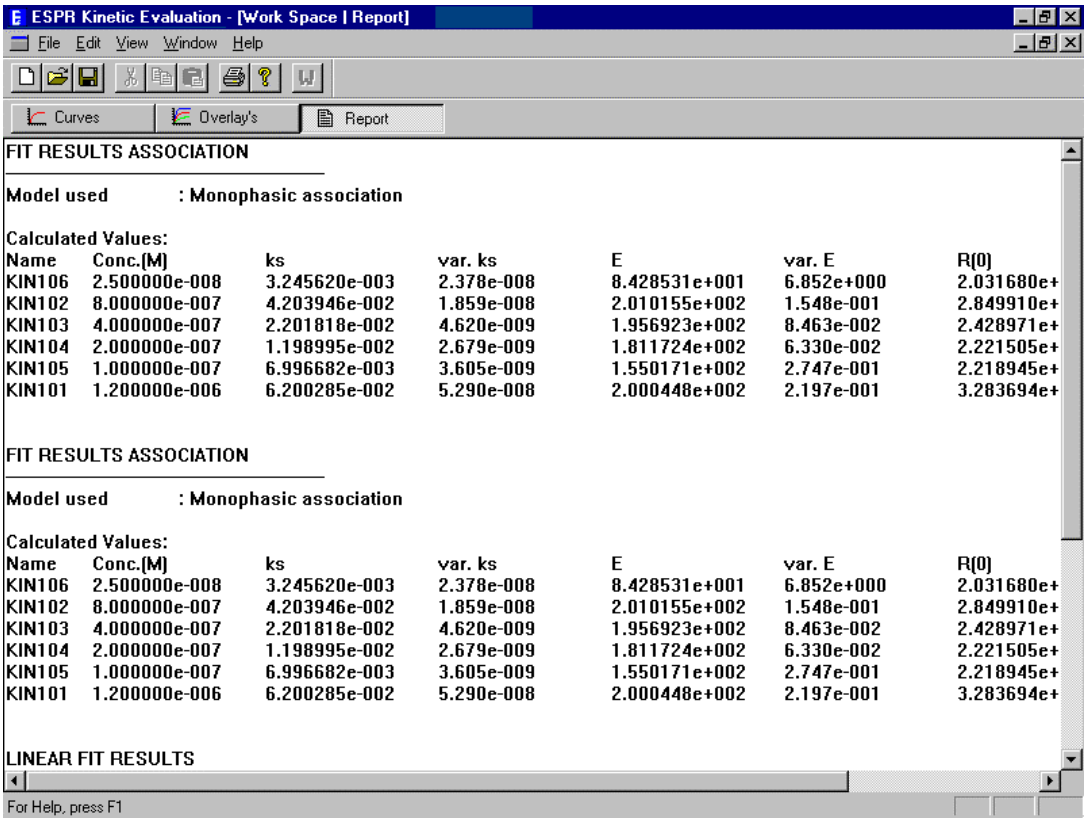


Figure 2.28: Workspace\Report presentation of stored fit results.

2.5 Monophasic dissociation

An example of the analysis of a monophasic dissociation is given for the analysis of the same data files of the monophasic association 2.4. This file contains 6 monovalent interactions of analyte A with ligand B. The concentrations of analyte A are 1200 nM, 800 nM, 400 nM, 200 nM, 100 nM, and 25 nM. The kinetic analysis of the dissociation phase will reveal the dissociation rate constant only. The analysis is performed by:

- Create an overlay plot of the six binding curves or open the created overlay file (*.iko)
- Select the dissociation range of the overlay plot. Click with left mouse button at the beginning of the dissociation phase and drag the mouse pointer to the end of the dissociation phase. Release the mouse button. The selected region is shown in black.
- Click the right mouse button to open the shortcut menu and select the option Dissociation Range to open *Set Dissociation Range* window. The borders of the dissociation range can be defined precisely. The procedure is similar to the one of the association range in 2.4.
- View the dissociation ranges with tool 4 of the toolbar of the overlay window. Click tool 4 again to undo the view option.

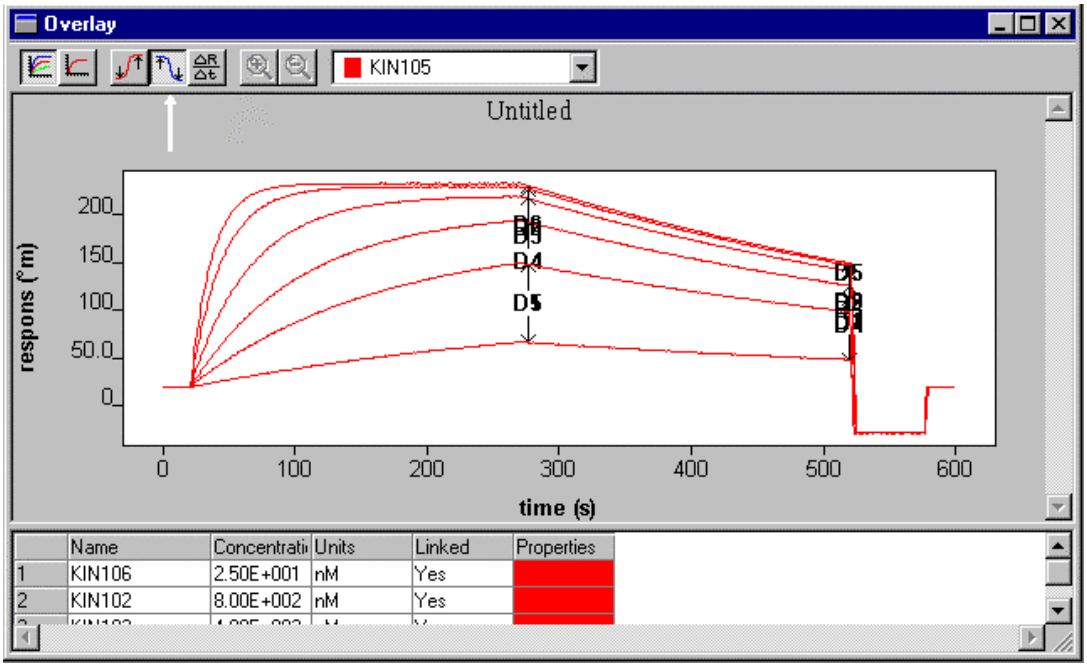


Figure 2.29: Presentation of the dissociation ranges in the overlay plot by tool 4, which is indicated by an arrow in the figure.

- Select Analysis: Dissociation constants (or Ctrl + D) to open the *Analyse Dissociation Phase* window.

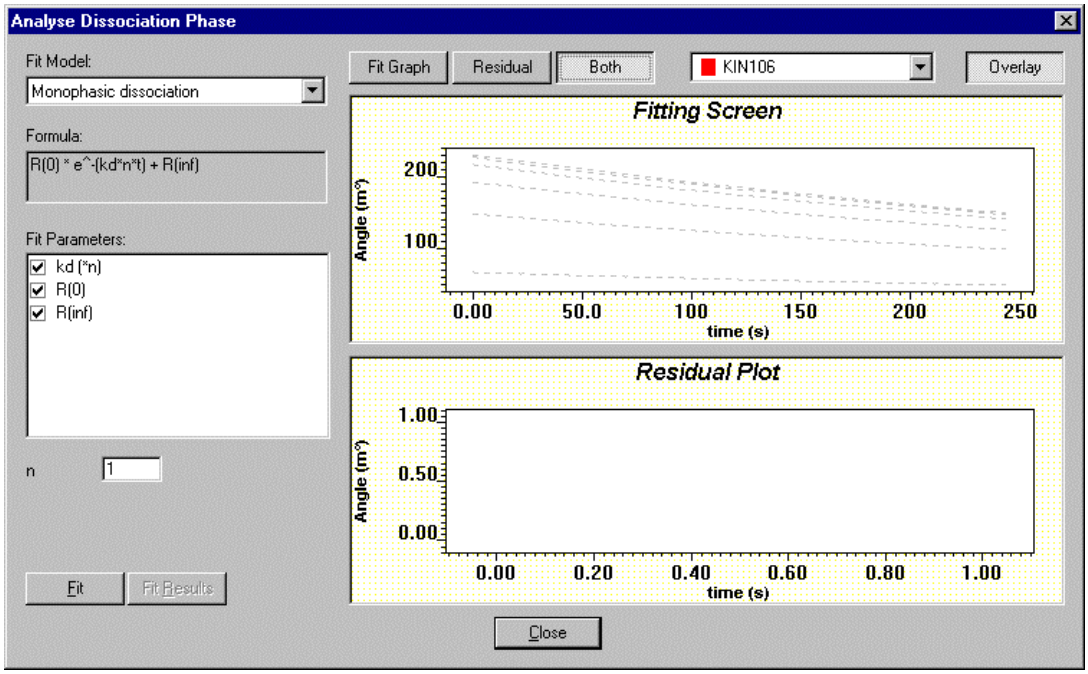


Figure 2.30: Analyse Dissociation Phase window to fit the curves according to the monophasic dissociation model.

This window is similar to the one of the association phase. Select the Both button to view the fitted curves as well as the residual plots.

- Click the Fit button to start the fitting. The *Fitting* window will appear.
- Click Fit all button to fit all curves to the monophasic dissociation model.
- Click Fit results button to open *Fit results* window, which contains a table with the fit results. Click To report button to store the results in the Report file of the Workspace.
- Click Close button to go back to the overlay plot.

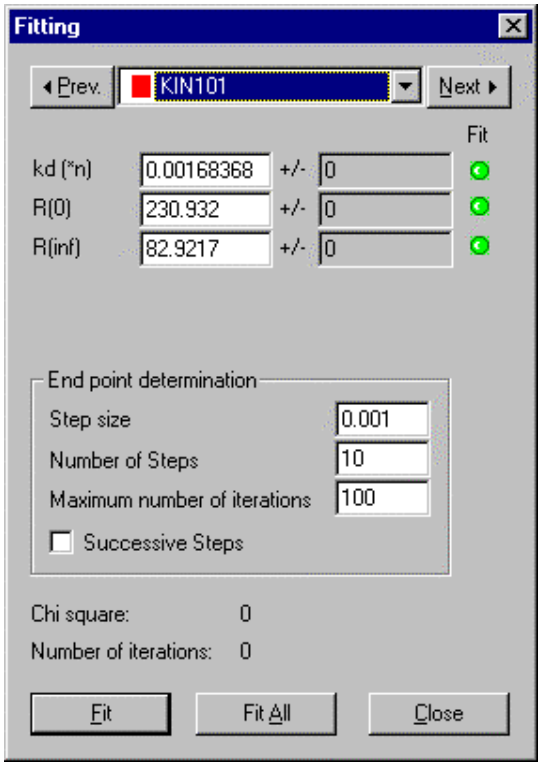


Figure 2.31: Fitting window

2.6 Biphasic association

An example of the analysis of a biphasic association is given for the analysis of data files kin201.ibo, kin202.ibo, kin203.ibo, kin204.ibo. These files contains 4 monovalent interactions of analyte A with ligand B. The concentrations of analyte A are 1200 nM, 800 nM, 400 nM, and 200 nM. The kinetic analysis of the association phase will reveal two association rate constants and two dissociation rate constants, and thereby the equilibrium constants. The analysis is performed similar to the procedure described in 2.3 for monophasic association, and is therefore not described step by step:

- Create an overlay plot of the four binding curves and save the file (see 2.3). Enter the concentrations in the table of the overlay plot.
- Select the association phase region, use the procedure mentioned in chapter 2.3.
  Fit the data to the monophasic association model and look at the residual plots of the fitted curves.

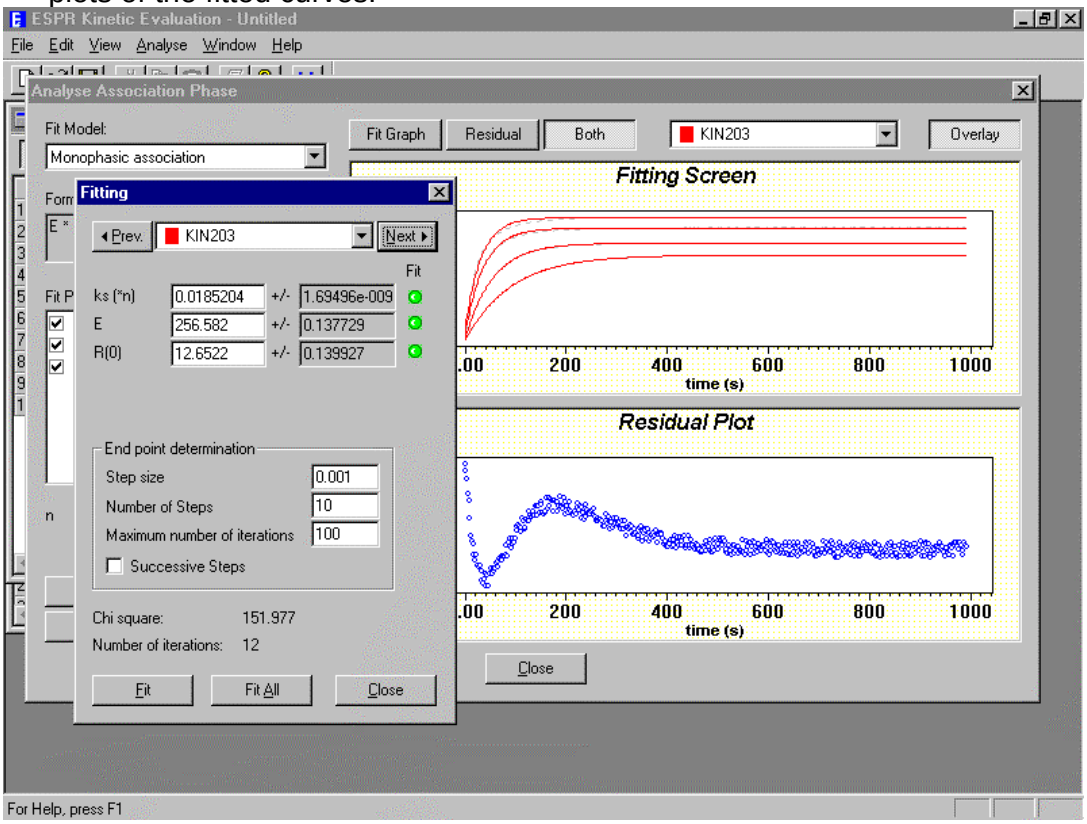


Figure 2.32: Monophasic association phase fit result of curve KIN203.

The applied monophasic model does not fit the data correctly. The data set is not distributed randomly around the x -axis in the residual plots.

Therefore, select the bipahsic association model in the Fit model section of the *Analyse Association Phase* window and fit again.

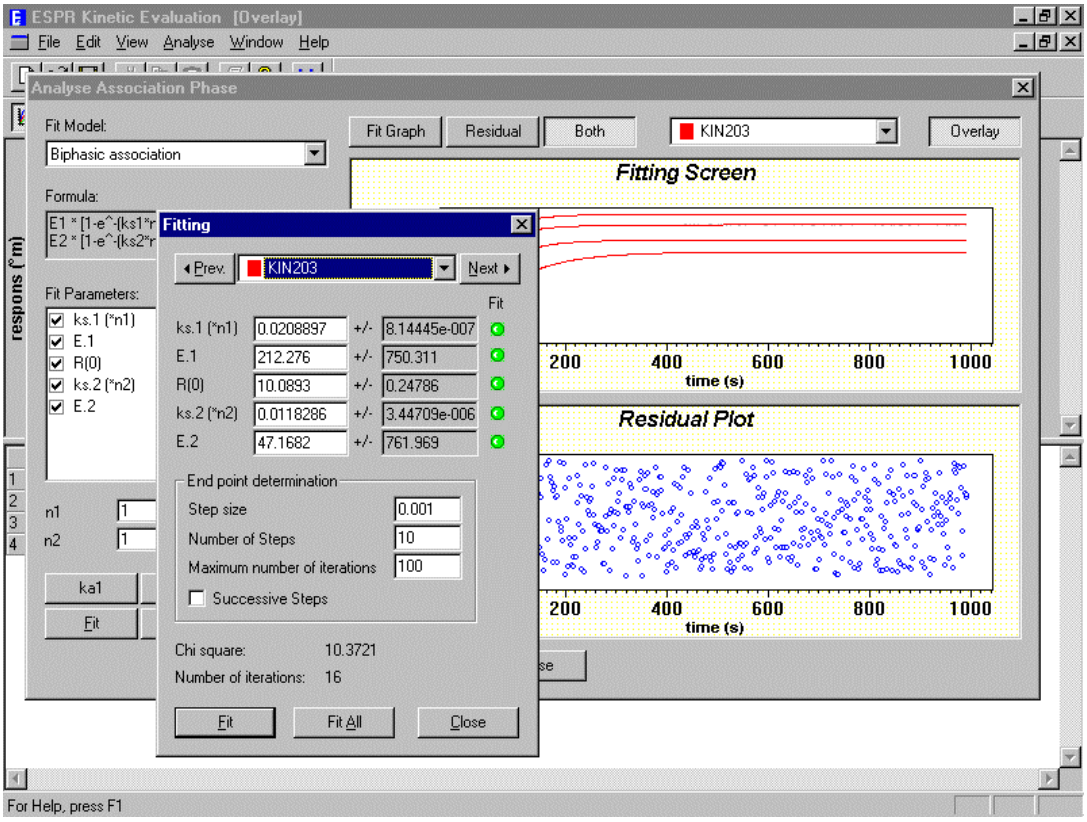


Figure 2.33: Biphasic association phase fit result of curve KIN203.

From the residual plots it appears that the biphasic model describes the data better than the monophasic model.

- To calculate the kinetic rate constants of the first phase click the ka1 button of the *Analyse Association Phase* window, and to calculate the constants of the second phase click the ka2 button.

When to use the biphasic association model?

– Data points of the residual plot of monophasic association model are not randomly distributed around the fitted monophasic interaction, and the data points of the biphasic model are distrubeted randomly.

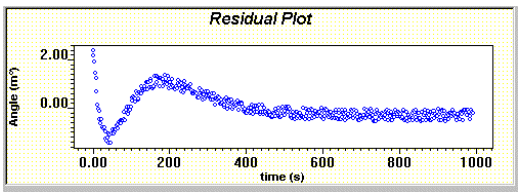


Figure 2.34a: incorrect residual plot

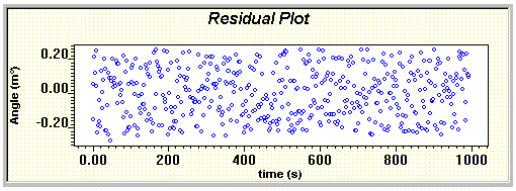


Figure 2.35b: Correct residual plot

2.7 Biphasic dissociation

An example of the analysis of a biphasic dissociation is given for the analysis of the same data set used for the analysis of biphasic association. The concentrations of analyte A are 1200 nM, 800 nM, 400 nM, and 200 nM. The kinetic analysis of the dissociation phase will reveal two dissociation rate constants. The analysis is performed similarly to the procedure described in 2.5 for monophasic dissociation. The analysis is performed by:

- Create an overlay plot of the four binding curves or open the created overlay file (*.iko)
- Select the dissociation range of the overlay plot. Click with left mouse button at the beginning of the dissociation phase and drag the mouse pointer to the end of the dissociation phase. Release the mouse button. The selected region is shown in black.
- Click the right mouse button to open the shortcut menu and select the option Dissociation Range to open *Set Dissociation Range* window. The borders of the dissociation range can be defined precisely. The procedure is similar to the one of the association range in 2.4.
- View the dissociation ranges with tool 4 of the toolbar of the overlay window. Click tool 4 again to undo the view option.
- Select Analysis: Dissociation constants (or Ctrl + D) to open the *Analyse Dissociation Phase* window. This window is similar to the one of the association phase. Select the Both button to view the fitted curves as well as the residual plots.
- Click the Fit button to start the fitting. The *Fitting* window will appear.
- Select the biphasic model in the *Analyse Dissociation Phase* window and click Fit all button to fit the data.
- Click Fit Results button to show the fit results of both phases.

2.8 Equilibrium Analysis

Steady state levels of the association phase can be analysed to an equilibrium model described in chapter 1.2.3. This model calculates the association constant K_A.

The equilibrium analysis procedure will be shown for data set equi010.ibo, equi011.ibo, equi012.ibo, equi013.ibo, and equi014.ibo. The concentrations of the analytes are 1200, 800, 400, 200, and 100 nM respectively. To perform the analysis it is essential that the absolute values of the steady state levels are known. This means that the association phase should be corrected for a bulk shift, and that the baseline values are set to zero. Therefore, the procedure is:

- Add files equi010- equi014 to the Workspace\Curve and create an overlay plot according the procedure mentioned in section 2.3.III. Enter the concentration values of the curves in the table below the overlay plot.

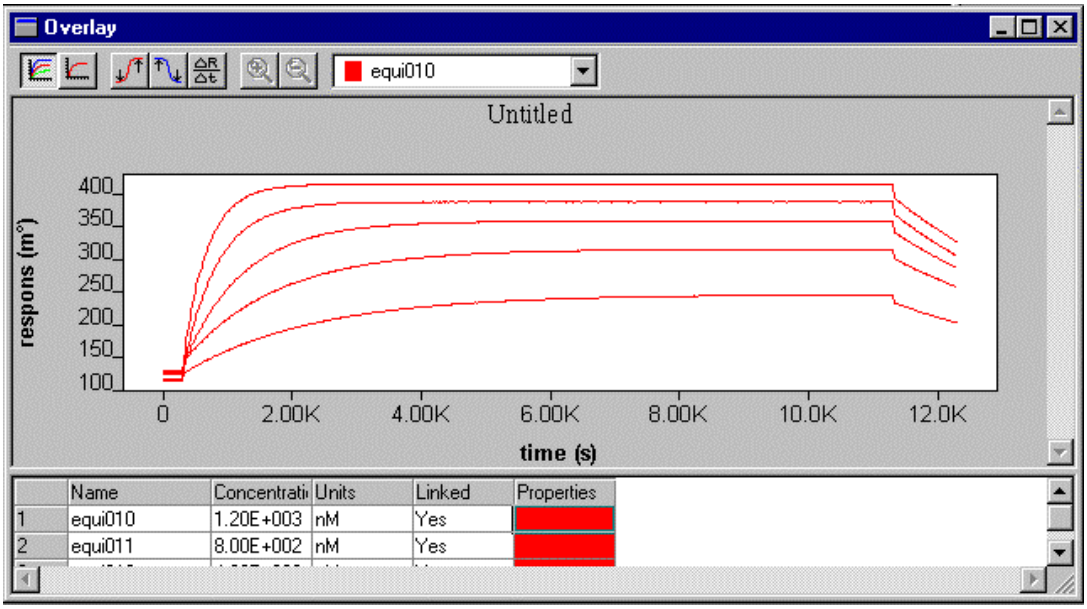


Figure 2.35 : Overlay plot of data set equi010.ibo - equi014.ibo

Select the association phase of the overlay plot with the left mouse button (click and drag) and choose the option Edit: Bulk Shift Correction

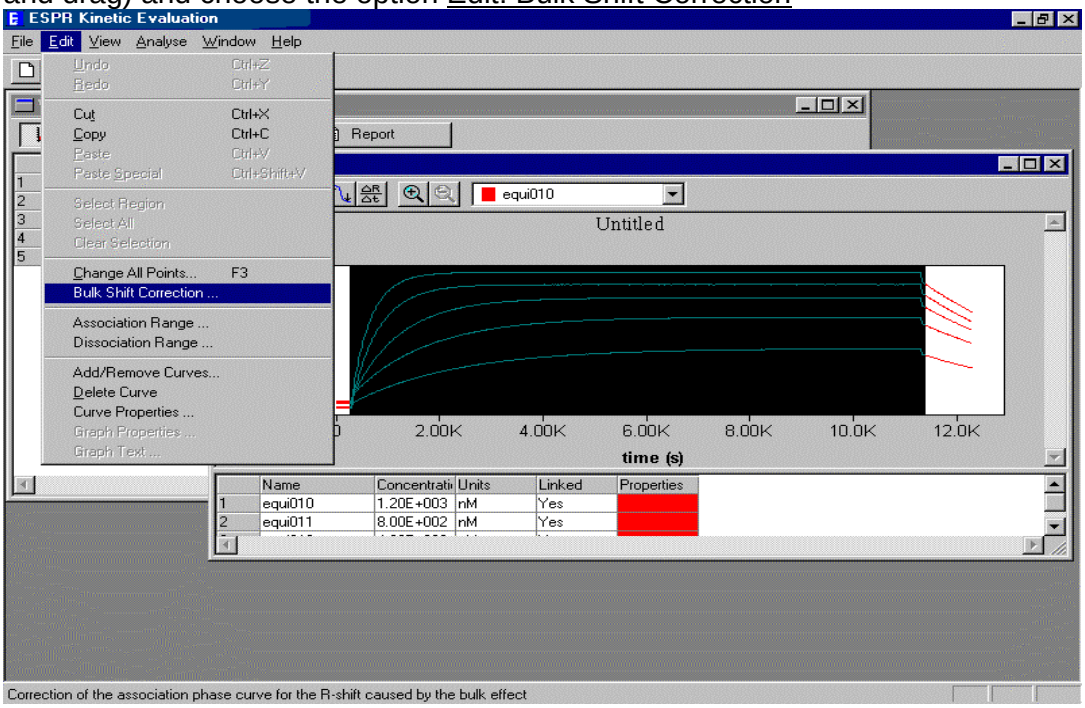


Figure 2.36: Bulk Shift Correction option applied to the association phase

- After the Bulk Shift Correction command, the *Bulk Shift Correction window* will appear on the screen to set the start and the end of the association phase and to set the end of the bulk shift correction which is the start of the dissociation phase

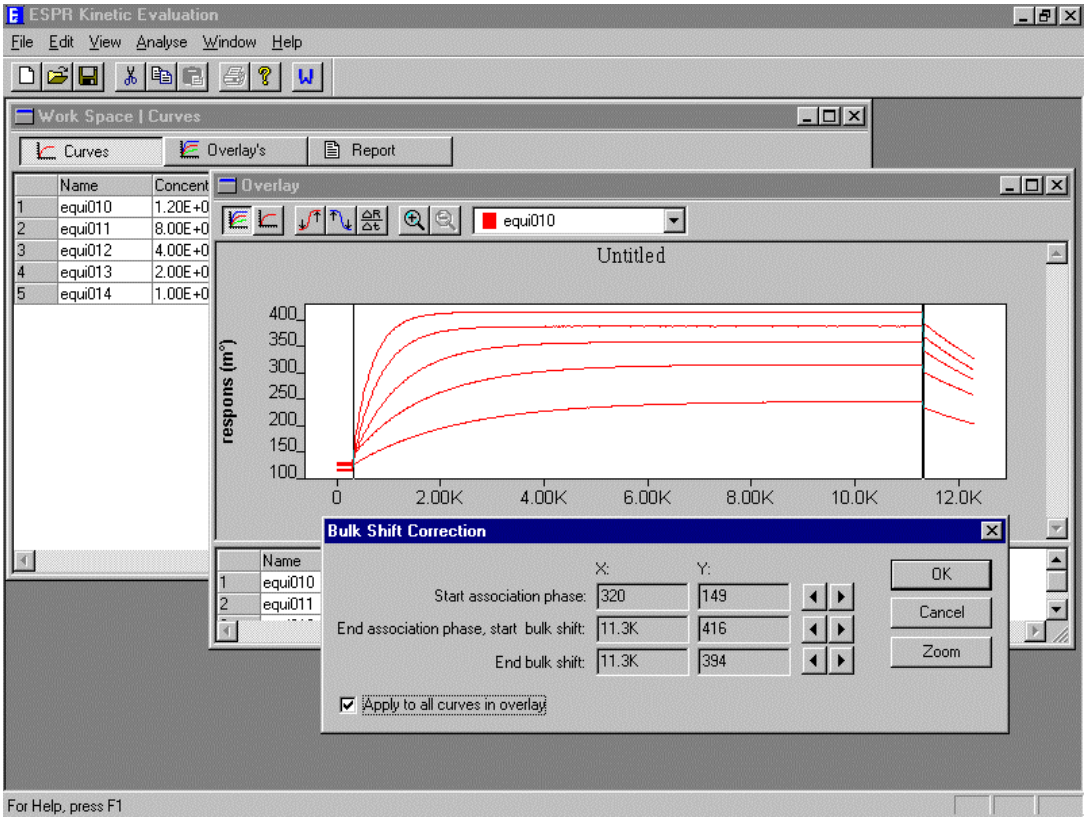


Figure 2.37: Bulk Shift Correction window to define the start of association phase, the end of the association phase, and the beginning of the dissociation phase

The bulk shift correction operation calculates the response difference between the end of the association phase (beginning of bulk shift correction) and the beginning of the dissociation phase (end of bulk shift correction). The difference response is subtracted from the association phase, which is defined by at the *Bulk Shift Correction window* from the start of the association phase till the end of the association phase. The result of this operation is shown in figure 2.38.

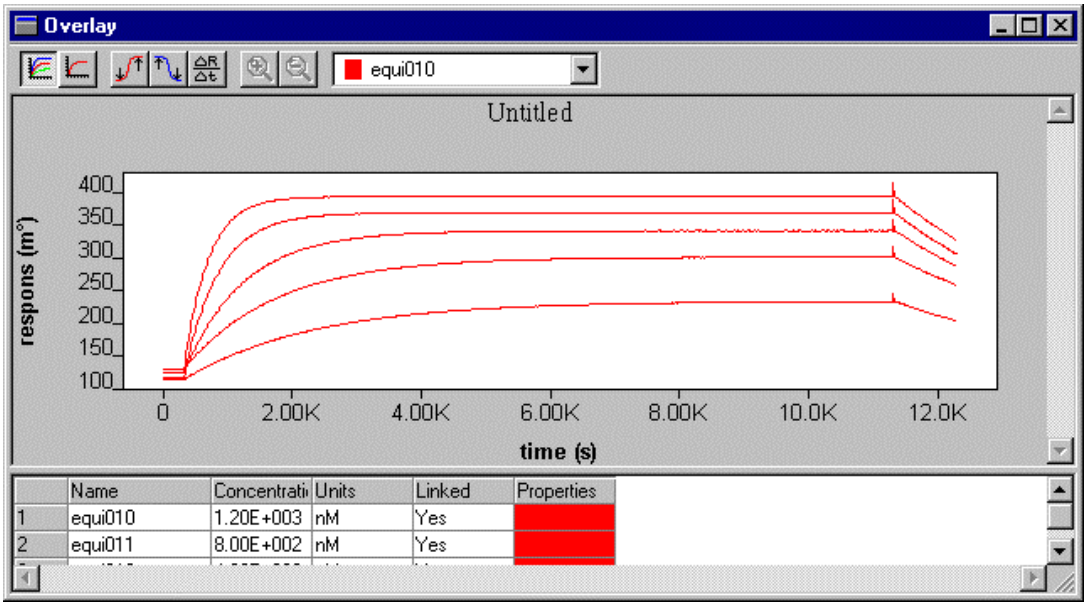


Figure 2.38: Overlay plot of data set equi010.ibo - equi014.ibo after bulk shift correction.

- Set the baseline values to zero by Edit: Change All Points after selection of the baseline area with the left mouse button.

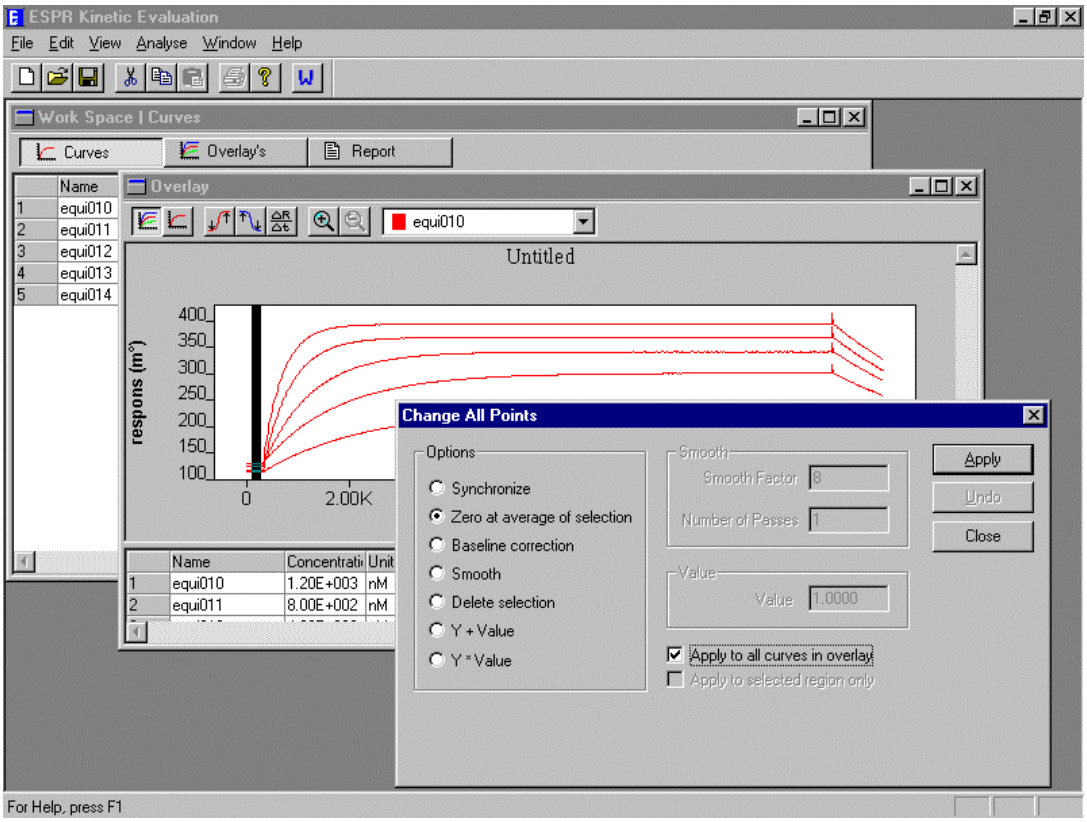


Figure 2.39: Set baseline to zero of data set equi010.ibo - equi014.ibo

- Now, the overlay plot is ready for the equilibrium analysis. Select the steady state area of the association phase with the left mouse button (click and drag). For each curve, the average y value of the selected area will be

calculated and used in linear regression of a plot of Req / C versus C. Then, select the option Analysis: Equilibrium

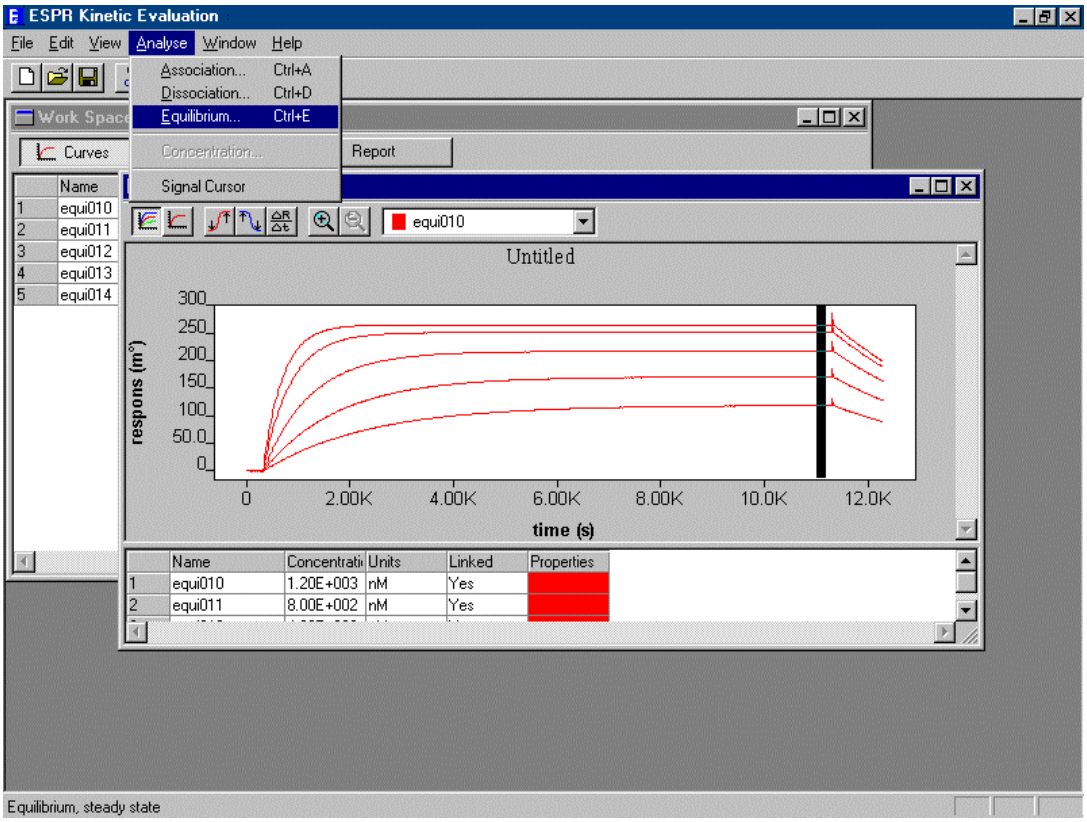


Figure 2.40: The Equilibrium Analysis operation is applied on the selected area of the steady state or equilibrium levels of the interaction plots.

The Linear Fit window will appear on the screen with the final result K_A.

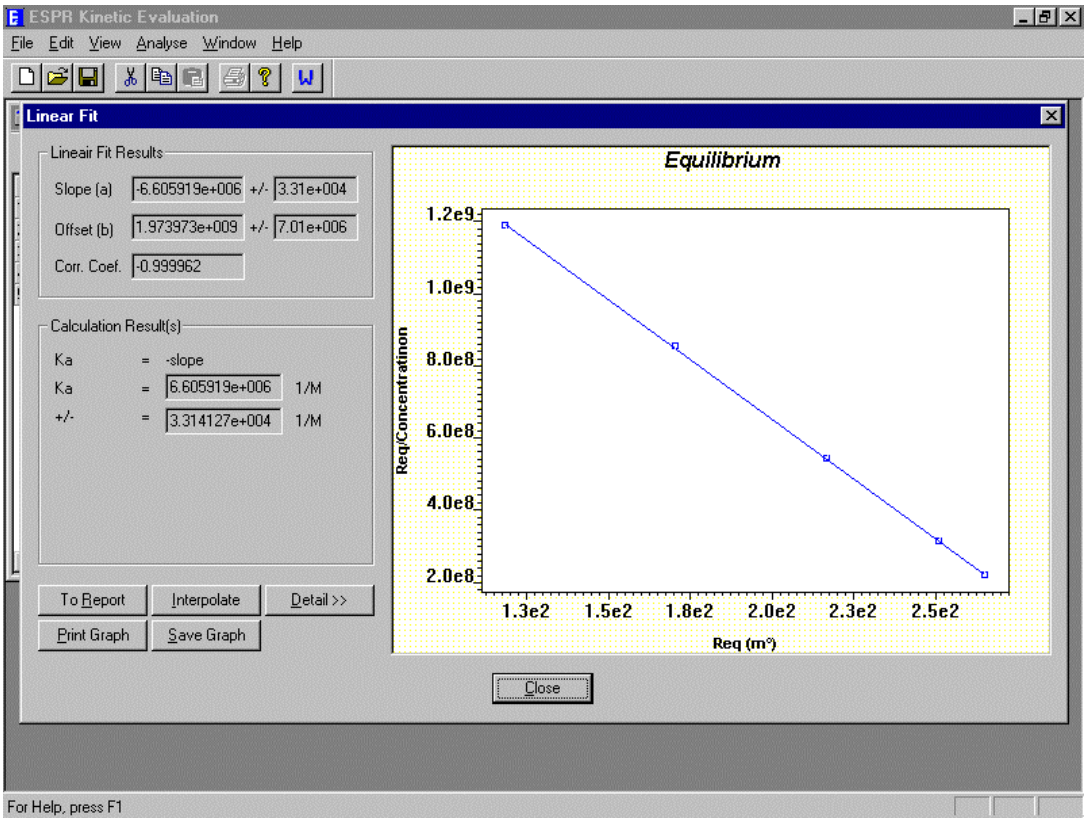


Figure 2.41: Linear Fit window to calculate K_A from the slope of the plot of Req/C vs Req

The buttons of the *Linear Fit window* are:

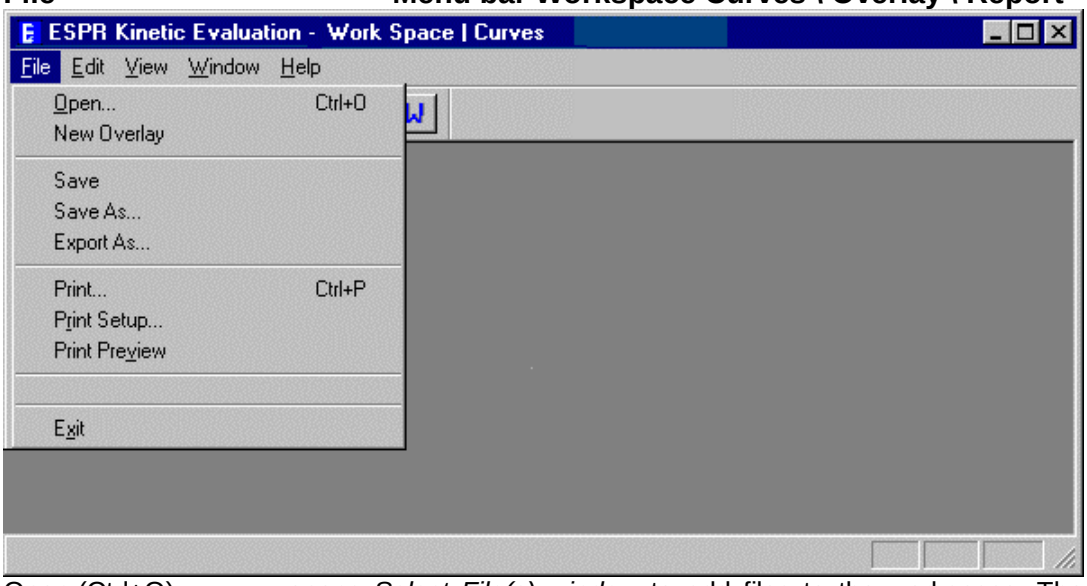
To report	sent linear fit results to Result Page of Workspace
Interpolate	shows Interpolate window to find X or Y values
Detail	shows data points in Req/C vs Req plot. You can skip data points for the linear regression procedure
Print Graph	prints the Req/C vs Req plot
Save Graph	saves graph as *.emf file
Close	closes *Linear Fit window*

2.9 Kinetic Evaluation files

Curve files (*.ibo)	Original data files obtained from SPR measurements
Sub curve files (*.ikc)	Sub curve data files created by Kinetic Evaluation software
Overlay files (*iko)	Overlay plots created by KE software
Result files (*ikr)	Fitting result file created by KE software

2.10 Menu options

File **Menu bar Workspace Curves \ Overlay \ Report**



Open (Ctrl+O) opens *Select File(s) window* to add files to the workspace. The directory can be selected by the directory tool of the window. SPR Kinetic Evaluation files are selected by the option File Type. The file types are Curve files (*.ibo), Sub Curve files (*.ikc), Overlay files (*.iko), Result files (*.ikr), and All Files (*.*).

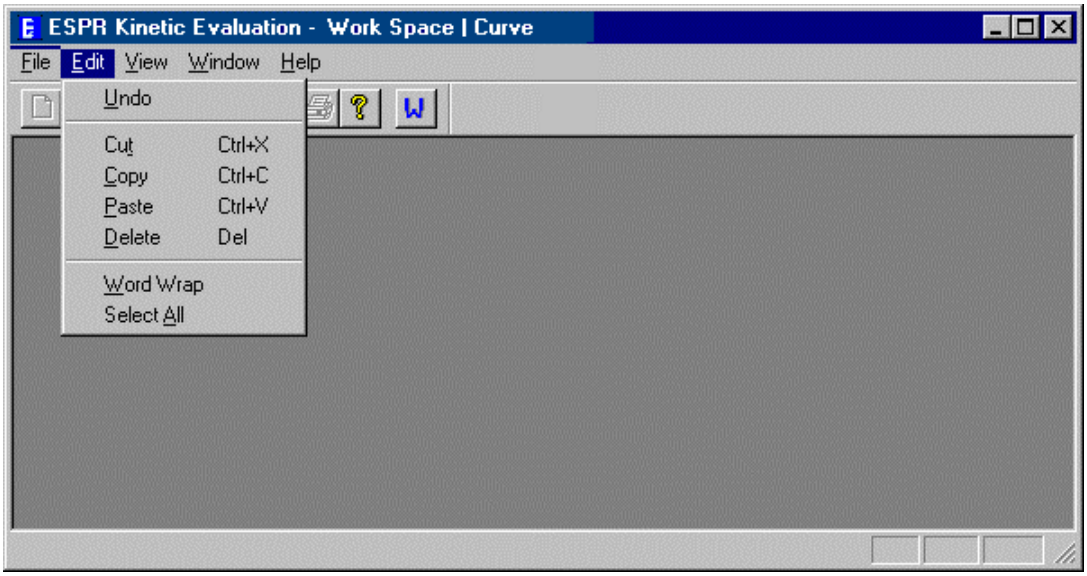
New Overlay opens *Add / Remove Curves window* to create a new overlay plot. The window shows a list of curve files (*.ibo) and sub curve files (*.ikc) present in the workspace. Curves are added to the overlay plot by selection of the curves and clicking the OK button.

Save saves file by current file name. If the current file is untitled, then the procedure of the Save as option will take place.

Save as opens *Save as window* to store files on disk The directory to save the file can be selected by the directory tool of the window. SPR Kinetic Evaluation files are saved as Sub Curve files (*.ikc), Overlay files (*.iko), Result files (*.ikr), or Report files (*.txt). Fill in a File name, and click the Save button to save the file.

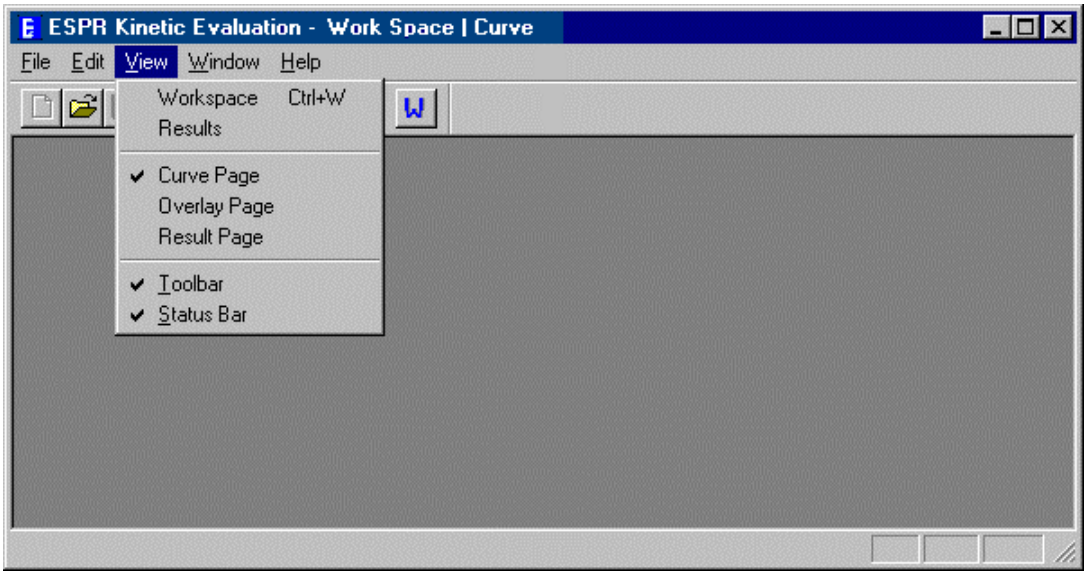
Export as	Text opens *Export as Text window* to export curve files as text files.Graphic opens *Export as graphic window* to export graphic as windows enhanced metafile (*.emf).
Print (Ctrl+P)	opens *Print window* to print file
Print Setup	opens *Print Setup* window to select a printer, paper size and page layout
Print Preview	shows preview of print on screen
Exit	closes the Kinetic Evaluation program

Edit **Menu bar Workspace Curves \ Overlay \ Report**



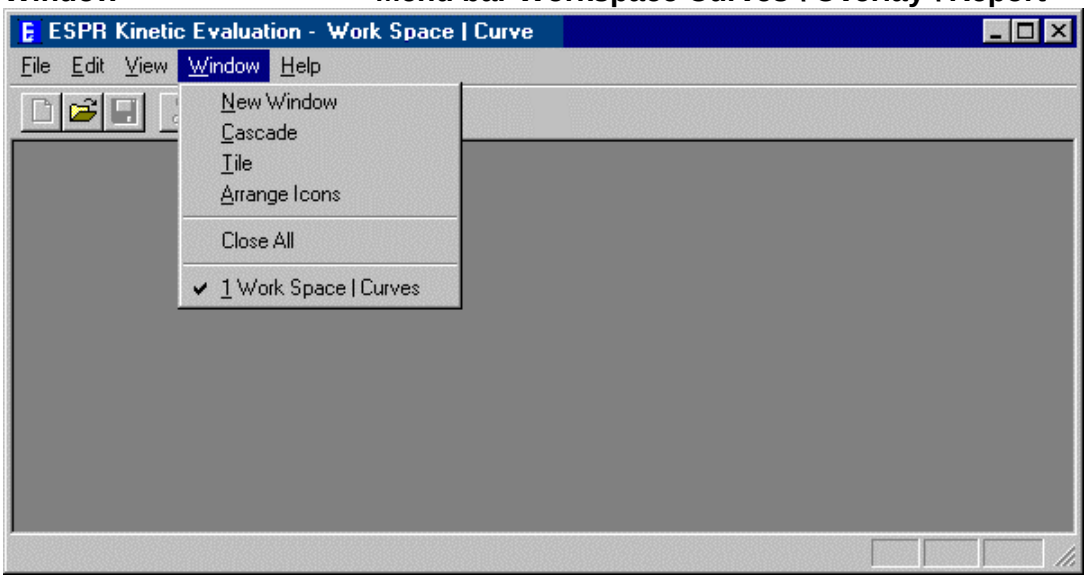
Undo	undo previous action
Cut (Ctrl+X)	cut selected object (text or (sub)curve)
Copy (Ctrl+C)	copy selected object (text or (sub)curve) to clipboard
Paste (Ctrl+V)	paste selected object (text or (sub)curve) from clipboard
Delete	(Del) cut selected object (text or (sub)curve)
Word wrap	wraps your text in active window
Select All	select all objects

View **Menu bar Workspace Curves \ Overlay \ Report**



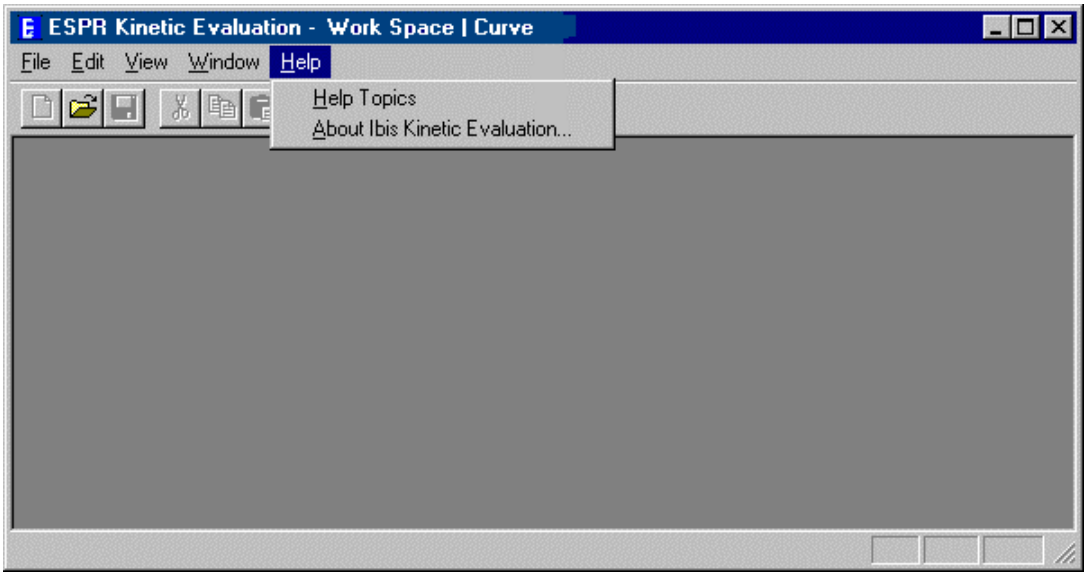
Workspace (Ctrl+W)	opens workspace window
Results	inactive
Curve Page	views Curve Page of Workspace
Overlay Page	views Overlay Page of Workspace
Result Page	views Result Page of Workspace
Toolbar	views toolbar
Status Bar	views status bar

Window **Menu bar Workspace Curves \ Overlay \ Report**



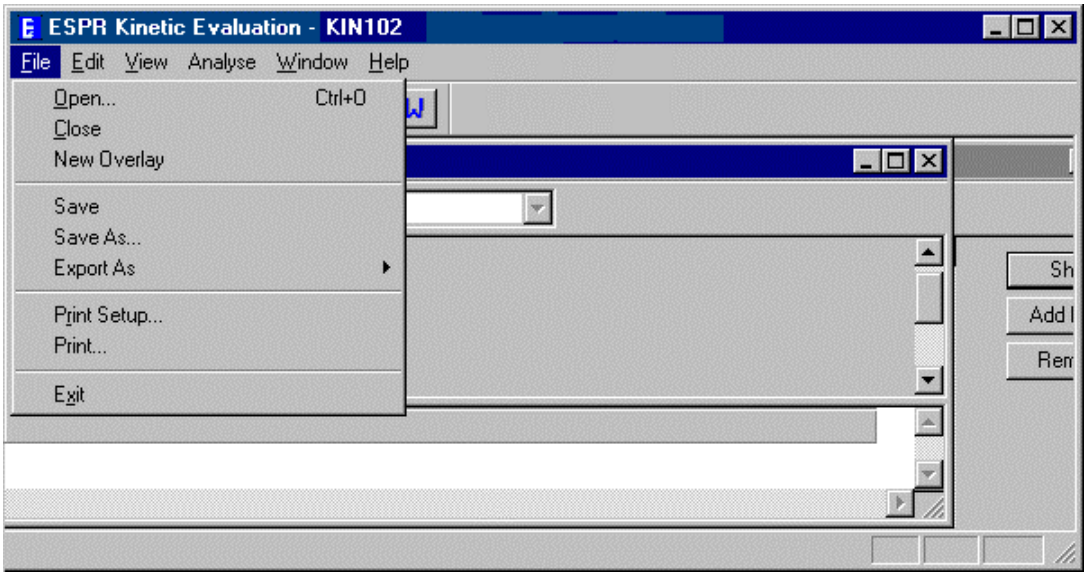
New Window	copies active window
Cascade	cascades all windows
Tile	tiles all windows
Arrange Icons	arranges icons
1 Work Space|Curves	active Page of Workspace

Help **Menu bar Workspace Curves \ Overlay \ Report**



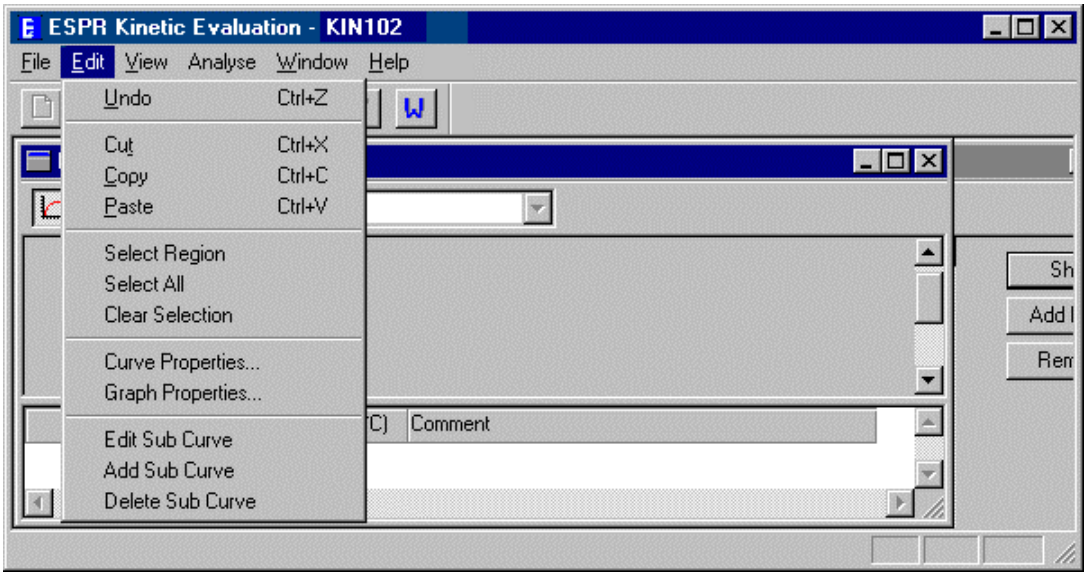
Help Topics	Shows the available topics in the helpfile
About Kinetic Evaluation	SPR KE version

File **Menubar - Main Curve**



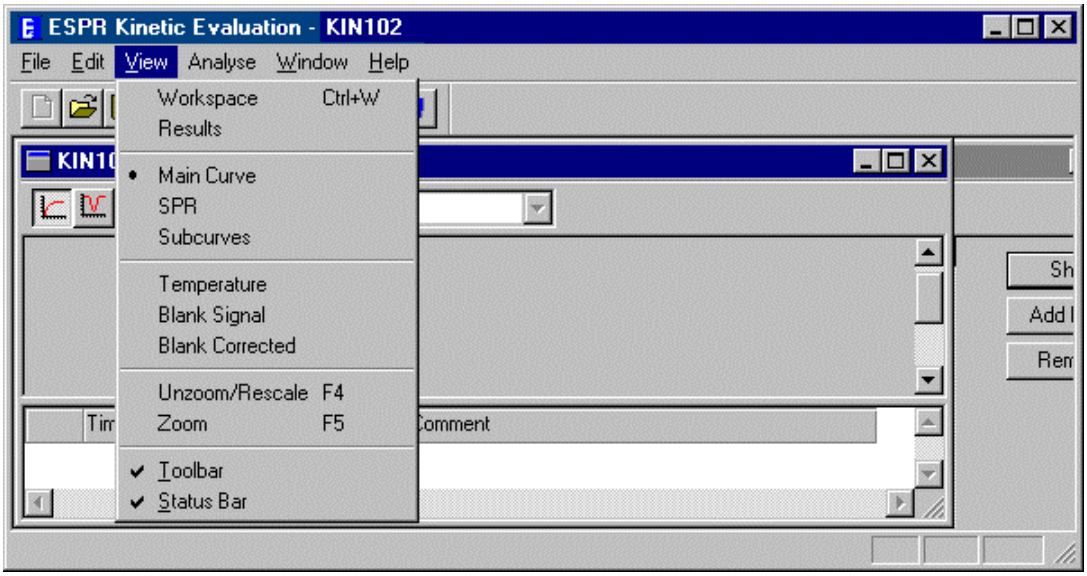
Open	opens *Select File (s) window*, select your directory and your file. With File type you can distinguish *.ibo, *.ikc, *.iko, *ikr, and *.* files.
Close	closes opened data file . The file is still listed in Curve Page of Workspace.
New Overlay	opens *Add / Remove window* with files data present in Workspace listed. Select the files to create an overlay and press the OK button.
Save	saves active data file
Save as	opens *Save as window* to save active file. Select directory and fill in file name
Export as	TEXT exports data file as text file. The text file can be imported into other programs (fitting programs, or spreadsheet programs) GRAPHIC exports data file as picture. The graphic file can be imported into a word processor
Print Setup	Used to configure your printer
Print	Print graph
Exit	Closes KE program

Edit **Menubar - Main Curve**



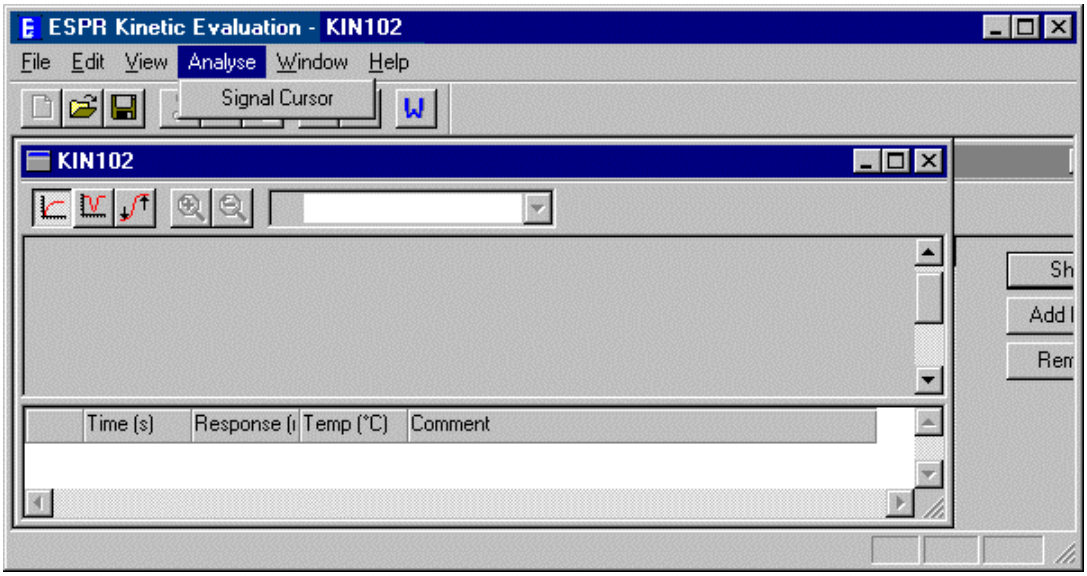
Undo	(Ctrl+Z)	undo previous action
Cut	(Ctrl+X)	cut selected object (text or (sub)curve)
Copy	(Ctrl+C)	copy selected object to clipboard
Paste	(Ctrl+V)	paste selected object from clipboard
Select Region	not active	
Select All	not active	
Clear Selection	not active	
Curve Properties	opens *Curve Properties window* to set name of curve, concentration and its units, and graphical properties	
Graph Properties	not active	
Edit Sub Curve	opens the *Edit Subcurve window* to edit the general properties (name, concentration, and units) and graphical properties of the current subcurve. You can also edit the start and end position of the subcurve by scrolling.	
Add Sub Curve	opens *Add subcurve window*. Before you open this window, select the region of the subcurve with left mouse button. Enter the general properties and adjust the start and end position of the subcurve by scrolling. The added subcurve will be listed in the Curve Page of the Workspace as a new curve.	
Delete Sub Curve	deletes the active subcurve after confirmation	

View **Menubar - Main Curve**



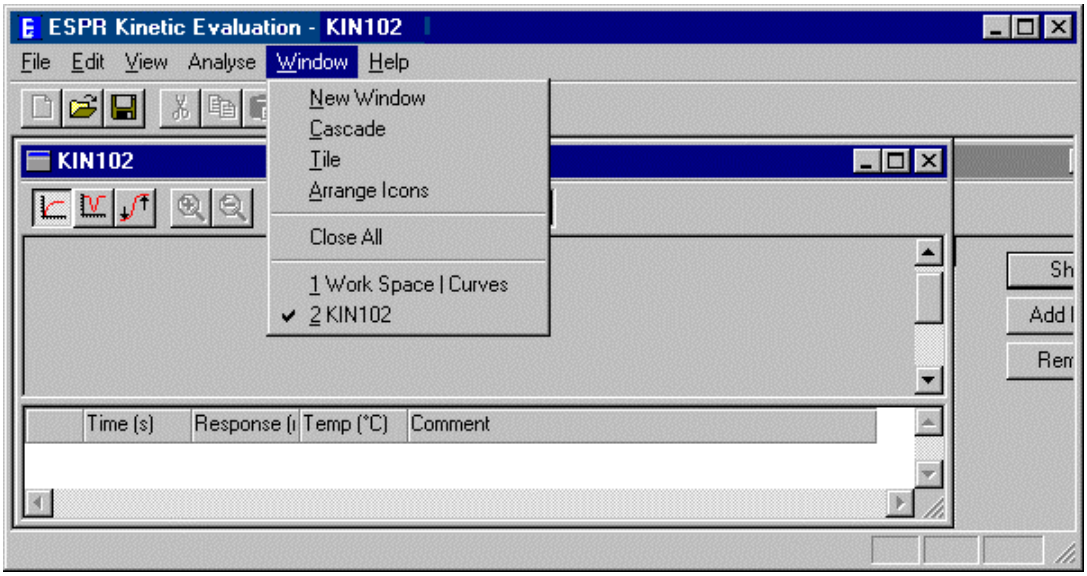
Workspace	(Ctrl+W)	switches to the Workspace
Results	not active	
Main Curve	switches to curve presentation of data acquisition window	
SPR	switches to SPR plot presentation of data acquisition window	
Subcurves	switches to curve presentation of data acquisition window	
Temperature	shows the temperature during the measurement in the Main Curve presentation. Not active in SPR and subcurve presentation	
Blank Signal	shows the data of channel 1 and 2 of SPR II data in the Main Curve presentation. Not active in SPR and subcurve presentation	
Blank Corrected	Shows the difference signal of channel 1 and channel 2 in the Main Curve presentation. Data of channel 1 - channel 2 together with data of channel 1 are shown. Not active in SPR and subcurve presentation	
Unzoom / Rescale	F4	rescales a zoomed area of the curve. Only active when an area has been zoomed in before
Zoom	F5	zooms in a selected area of the curve. Not active when an area is not selected with the left mouse button (click and drag)
Toolbar	views toolbar of data acquisition window (not the main toolbar)	
Status Bar	views status bar of main window (not the data acquisition window)	

Analyse **Menubar - Main Curve**



Signal Cursor — opens *Signal Cursor window* in Curve presentation window. By scrolling you can walk through the data points, which are shown in the *Signal Cursor window*

Window **Menubar - Main Curve**



New Window — copies active window

Cascade — cascades all windows

Tile — tiles all windows

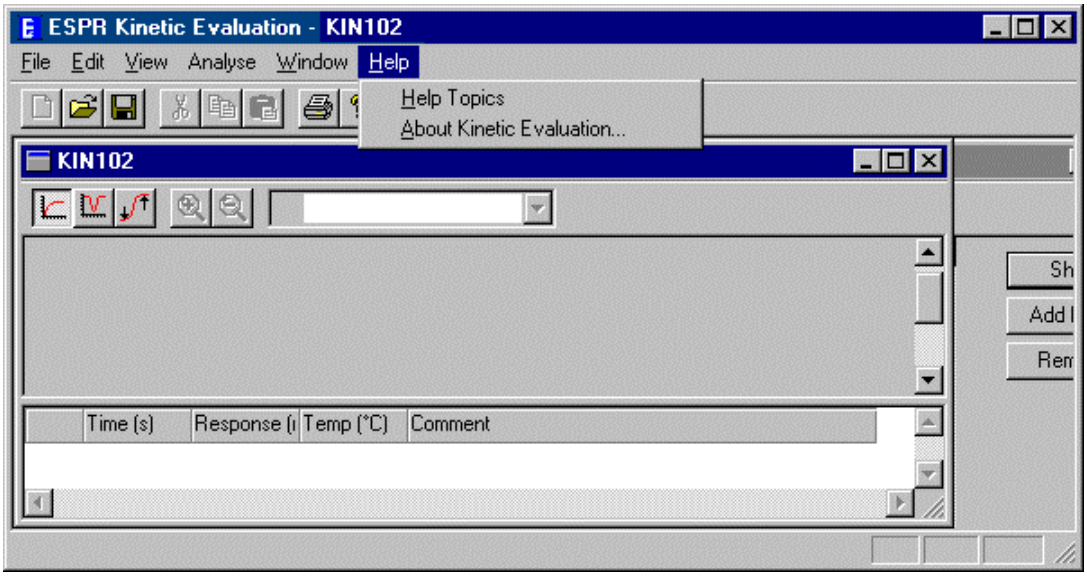
Arrange Icons — arranges icons

Close all — closes all windows

1. Work Space | Curves switches to Curve Page of Workspace

2. KIN102 switches to data acquisition window (file kin102.ibo)

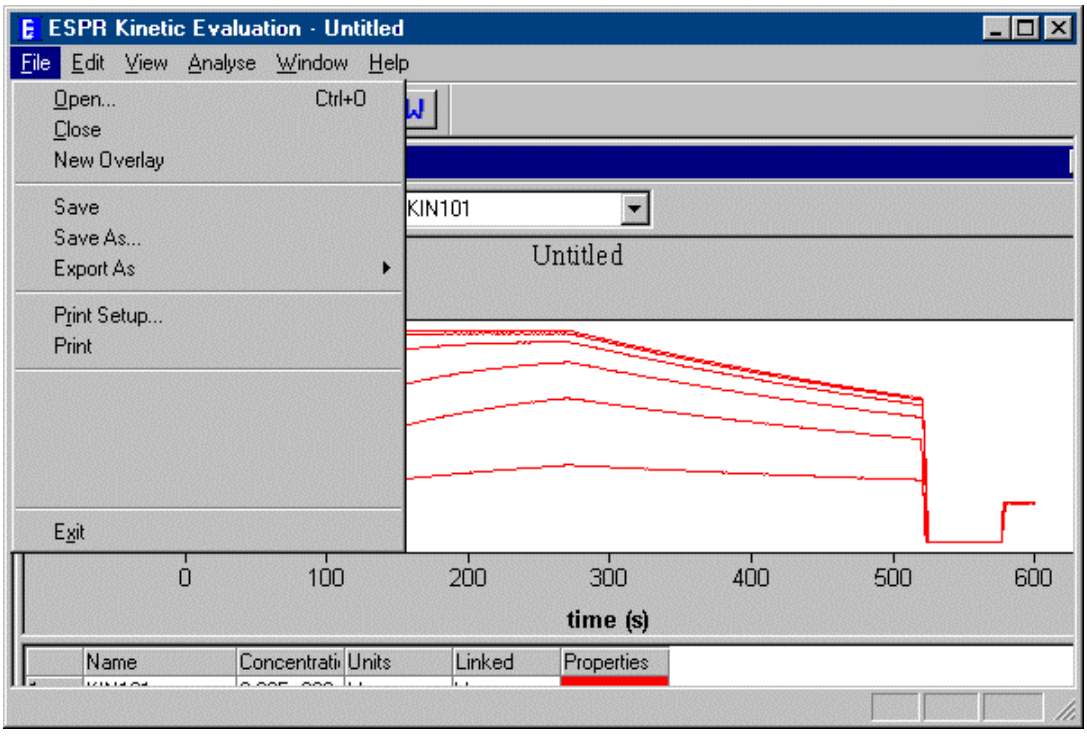
Help **Menubar - Main Curve**



Help Topics Show Topics in Help File

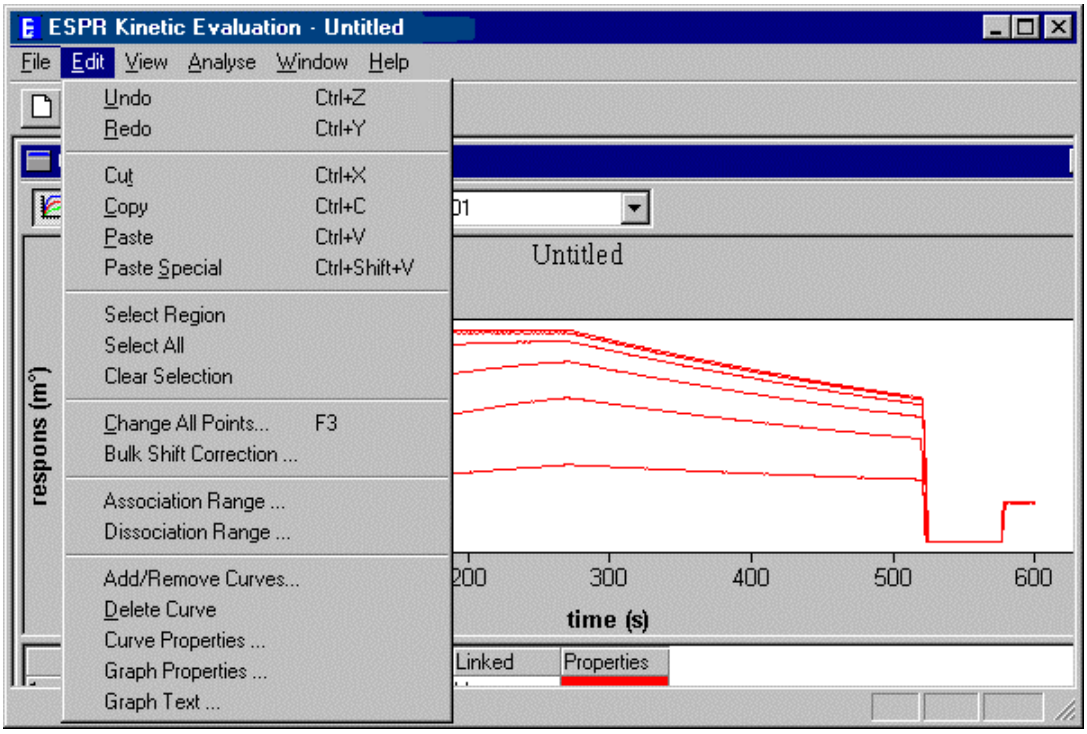
About SPR Kinetic Evaluation KE version information

File **Menubar - Overlay window**



Open	opens *Select File (s) window*, select your directory and file. Select File type between *.ibo, *.ikc, *.iko, *ikr, and *.* files.
Close	closes opened overlay file . The file is still listed in the Overlay Page of Workspace.
New Overlay	opens *Add / Remove window* with files data present in Workspace listed. Select the files to create an overlay and press the OK button.
Save	saves active overlay file
Save as	opens Save as window to save active file. Select directory and fill in file name
Export as	TEXT exports overlay file as text file. The text file can be imported into other programs (fitting/spread sheet programs) GRAPHIC exports overlay file as picture. The graphic file can be imported into a word processor
Print Setup	sets up your printer
Exit	closes KE program

Edit **Menubar - Overlay window**



Undo	(Ctrl+Z)	Undo previous action.
Redo	(Ctrl+Y)	Redo previous action

Cut (Ctrl+X) cut selected region of curve

Copy (Ctrl+Y) copy selected region of curve

Paste (Ctrl+V) paste selected region of curve

Paste Special paste curve of channel 2 (blank signal) of SPR II data (Ctrl+Shift+V)

Select Region not active

Select All not active

Clear Selection not active

Change All Points opens *Change All points* window to change data points by mathematical operations:

- Synchronize: two curve are synchronized by selection of the region between two sample injections (between the start of two assiociation phases). Activate the curve where the longest sample injection time in the toolbar of the overlay window. Close the *Change All Points* window before, and reopen it afterwards with F3. Select the synchronize option and click the Apply button.
- Zero at average of selection: select baseline with left mouse button and select this option and set baseline at zero and correct entire curve by this value. To perform this operation for all curves, select the option 'apply to all curves' before executing this operation
- Baseline correction: use this option if you have a linear drift in the baseline as well as in the binding curve. Select the baseline involved with left mouse button and execute this option. The entire binding curve will be adjusted.
- Smooth: use this option if you want to smooth binding curves. Select the area to be smoothed with left mouse button and execute this option. Select 'apply to selected region only' option if you do not want to smooth the entire curve. The amount of smoothing can be set by the smooth factor and number of passes.
- Delete selection: delete selected area of curve
- Y + value: adds the entered Y value to the entire curve. If you want to add a value to a part of the curve then select it by the left mouse button and select the option 'apply to selected region only' before executing the operation
- Y * value: multiplies the entered Y value to the entire curve. If you want to multiply a part of the curve then select it by the left mouse button and select the option 'apply to selected region only' before executing the operation

Bulk Shift Correction use this option to correct an interaction lot by the bulk shift in the association phase. Select the association phase with the left mouse button before selection of this option. After selection of this option, the *Bulk shift correction window* will be opened. It contains three scroll bars with X and Y values. These scroll bars define the start of the association phase, the end of the association phase, which defines the beginning of the bulk shift, and finally the end of the bulk shift, with is the beginning of the dissociation phase. The selected association phase will be

corrected for the shift in Y values between the second and third scroll bar after executing this option by the Apply button.
After opening the *bulk shift correction window* scroll with the third bar (End of bulk shift) to the right. As a result, two vertical lines will appear which define the beginning and end of the bulk shift. Scroll with the second bar to the left to define the end of the association phase, and scroll with the third bar to the beginning of the dissociation phase. Finally scroll with the first bar to the beginning of the association phase to define the entire region of the association phase. Then, click the Apply button for the bulk shift correction operation.

Association Range — select the association range with the left mouse button before you select this option. This option shows the *Set Association Range window.* The Start and End values of the association phase are selected by two scroll bars. Define the association range precisely with the upper scroll bar for the start of the association phase, and with the lower scroll bar for the end of the association phase. Click the OK button to set the range for the active curve. Select the option 'Apply to all curves in overlay' to set equal association ranges for all curves. The set association range can be viewed in the overlay window by clicking the third tool of the overlay window toolbar

Dissociation Range — select the dissociation range with the left mouse button before you select this option. This option shows the *Set Disociation Range window.* The Start and End values of the dissociation phase are selected by two scroll bars. Define the dissociation range precisely with the upper scroll bar for the start of the dissociation phase, and with the lower scroll bar for the end of the dissociation phase. Click the OK button to set the range for the active curve. Select the option 'Apply to all curves in overlay' to set equal dissociation ranges for all curves. The set dissociation range can be viewed in the overlay window by clicking the fourth tool of the overlay window toolbar.

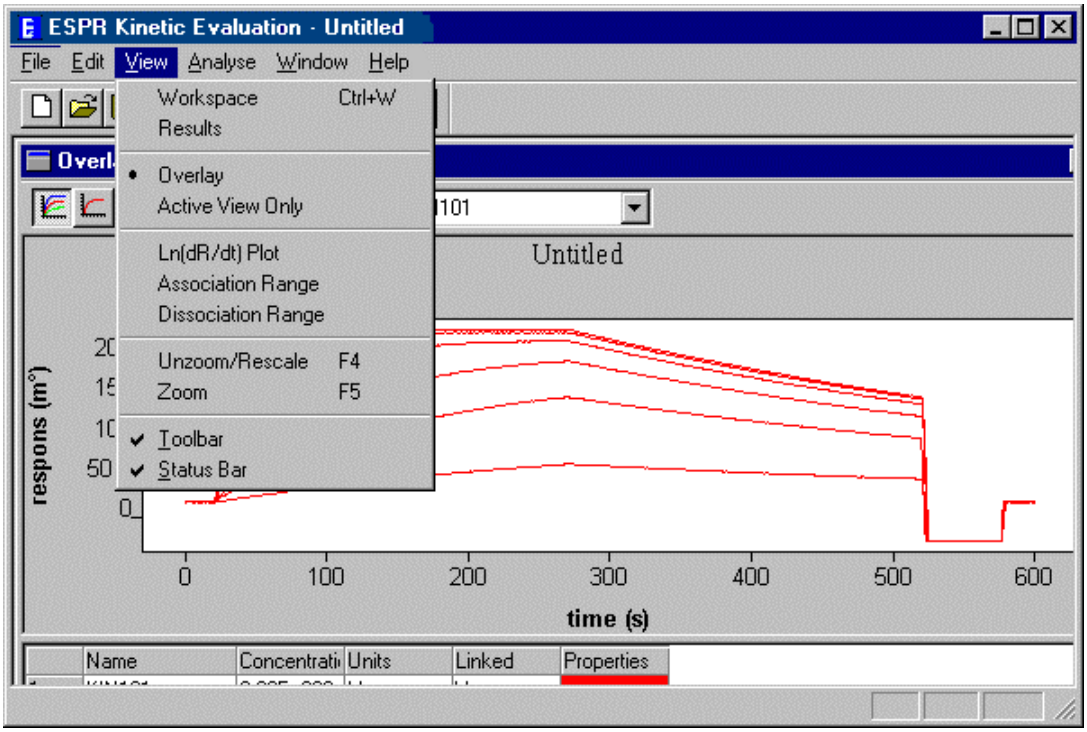
Add / Remove Curves — shows *Add/Remove Curves window* with all curves present in table of Curve Page of the Workspace. Marked curves are present in the current overlay plot. Add or remove curves to this overlay by marking or unmarking of the curves in the *Add/Remove Curves window*.

Delete Curves — deletes active curve from overlay plot after confirmation

Curve Properties — opens *Curve Properties window* to define general properties (name, concentration, and units) and graphical properties (line color, line style, line type, and scatter type) of curve

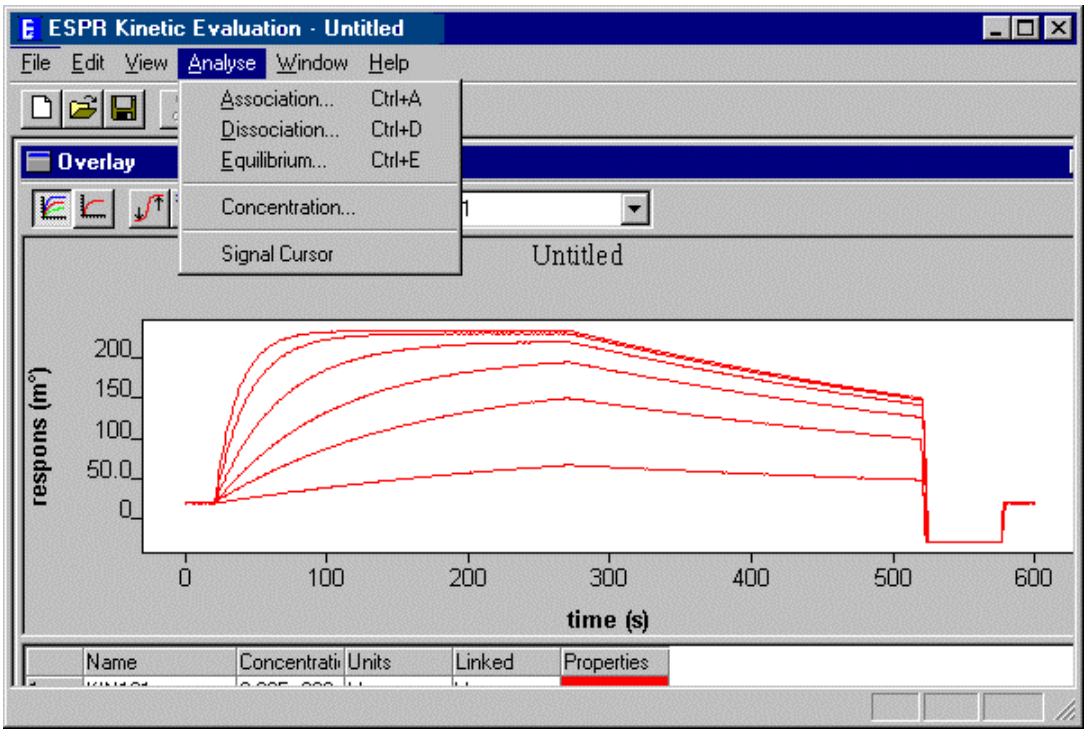
Graph Properties — not active

View **Menubar - Overlay window**



Workspace	views Workspace
Results	not active
Ln(dR/dt) Plot	views ln (dR/dt) versus t plot of active curve in overlay plot. This view option enables you to select the kinetic region of the association phase of the binding curve. The mass transport - limited region gives a horizontal ln (dR/ dt) vs t plot. If it is present, then it is seen just after sample injection
Association Range	shows the set association ranges in the overlay plot
Dissociation Range	shows the set dissociation ranges in the overlay plot
Unzoom / Rescale	rescales a zoomed area (F4)
Zoom	zooms in a selected area
Toolbar	views toolbar of overlay window
Status Bar	views statusbar of main window

Analyse **Menubar - Overlay window**



Association (Ctrl+A) Use this analyse option after setting the association phase range, otherwise no data points are available for the fitting procedure. After selection of this option, the *Analyse Association Phase window* is shown on the screen. It contains:

Fit model:	monophasic/biphasic model; choose one
Formula:	equation of association fit model
Fit parameters:	parameters used in fit model, 3 or 5, mono or biphasic model resp. The fit parameters are all selected to participate as free variables in the fit procedure. If you want to fix a parameter at a certain value, deselect the parameter of the list and enter the value in the next window, the *Fitting window*
n:	number of binding sites; default = 1
Graph:	a plot of the fitted curves is available in four presentations. These possibilities are shown on the screen as buttons called:
Fit graph	presentation of fitted curves
Residual	presentation of residual plots
Both	presentation of fitted curves and residual plots
Overlay	overlay or curve presentation
Fit	button to start the fit procedure. Click this button after selection of model and graph type.

The *Fitting window* appears on top of the *Analyse Association Phase window* after click Fit button. It contains:

-Fit parameters and start values, adjust values if the fit procedure fails, set value for fixed parameters for every curve. Click the Next button to switch to the next curve. A fixed parameter is defined in the previous *Analyse Association Phase window*. Free parameters are marked by a green cirkel, and fixed parameters not.
-End point determination values:
-step size (defines step size between iterations)
-number of steps (number of steps with defined step size)
-maximum number of iterations
-Successive steps on or off (on is more stringent)
-Chi square value (zero before fitting)
-Number of iterations value (zero before fitting)
-Three buttons:

Fit	fit active curve
Fit all	fit all curves
Close	close fitting window, back to *Analyse Association Phase window*

Click Fit or Fit all button to start the fitting procedure. The fitted curve will be plotted in the fitting screen and residual plot of the underlying *Analyse Association Phase Window.*The goodness of fit is judged by the chi-square value. A good fit is obtained with a low chi- square value.

After fitting, click the Close button to return to the *Analyse Association Phase window*.

In this window, the button ka and Fit results are available now for use. Click Fit results button to activate the *Fit results window*, which shows a table with the fitted parameters. To save the fit results click the To report button. This send the fit results to the Result Page of the Workspace. Click the Close button to leave the *Fit results window.*

Click the ka button to calculate the association rate constant ka and the dissociation rate constant kd from the association phase by a plot of ks versus C. The ks value has been determined by the fitting procedure. (For the biphasic model, two ka buttons are shown: the ka1 button for the first phase and the ka2 button for the second phase).

The *Linear Fit window* is shown after clicking the ka button. It contains a plot of ks vers C. The slope of the plot is equal to ka and the y intercept is equal to kd. The rate constants are calculated by linear regression. By default, linear regression is performed with use of standard deviation of data points. Therefore, the contribution of the data points to the linear regression results is not equal. You can switch this option off.

The buttons of the *Linear Fit window* are:

To report	sent linear fit results to Result Page of Workspace
Interpolate	shows Interpolate window to find X or Y values
Detail	shows data points in ks vs C plot. You can skip data points for the linear regression procedure
Print Graph	prints the ks vs C plot
Save Graph	saves graph as *.emf file
Close	closes *Linear Fit window*

Click the Close button to return to the *Analyse Association Phase window.*
Click the Close button of the *Analyse Association Phase window* to return to the overlay plot window

Dissociation (Ctrl+D) Use this analyse option after setting the dissociationphase range, otherwise no data points are available for the fitting procedure. After selection of this option, the *Analyse Dissociation Phase window* is shown on the screen. It contains:

Fit model: monophasic / biphasic model; choose one
Formula: equation of dissociation fit model
Fit parameters: parameters used in fit model, 3 or 5, mono or biphasic model resp.
The fit parameters are all selected to participate as free variables in the fit procedure. If you want to fix a parameter at a certain value, deselect the parameter of the list and enter the value in the next window, the *Fitting window*
n: number of binding sites; default = 1
Graph: a plot of the fitted curves is available in four presentations. These possibilities are shown on the screen as buttons called:

Fit graph presentation of fitted curves
Residual presentation of residual plot
Both presentation of fitted curves and residual plots
Overlay overlay or curve presentation
Fit button to start the fit procedure. Click this button after selection of model and graph type.

The *Fitting window* appears on top of the *Analyse Dissociation Phase window* after click Fit button. It contains:

-Fit parameters and start values; adjust values if fit procedure fails,set value for fixed parameters for every curve. Click the Next button to switch to the next curve. A fixed parameter is defined in the previous *Analyse Dissociation Phase window*. Free parameters are marked by a green cirkel, and fixed parameters not.
-End point determination values:
-step size (defines step size between iterations)
-number of steps (number of steps with defined step size)
-maximum number of iterations

-Successive steps on or off (on is more stringent)
-Chi square value (zero before fitting)
-Number of iterations value (zero before fitting)
-three buttons:

Fit fit active curve
Fit all fit all curves
Close close fitting window, back to *Analyse Dissociation Phase window*

Click Fit or Fit all button to start the fitting procedure.The fitted curve will be plotted in the fitting screen and residual plot of the underlying *Analyse Dissociation Phase window.* The goodness of fit is judged by the chi- square value. A good fit is obtained with a low chi- square value.

After fitting, click the Close button to return to the *Analyse Dissociation Phase window*. In this window, Fit results button is available now for use. Click Fit results button to activate the *Fit results window*, which shows a table with the fitted parameters. To save the fit results click the To report button. This send the fit results to the Result Page of the Workspace. Click the Close button to leave the *Fit results window.*

Click the Close button of the *Analyse Dissociation Phase window* to return to the overlay plot window

Equilibrium (Ctrl+E) Use this option to analyse equilibrium levels of interaction plots only. Before you use this option, it is necessary the set the data points of the entire interaction plot at its absolute values. The baseline should be averaged at zero by the option Edit: Change All Points (F3), and the bulk shift in the association phase should be corrected by Edit: Bulk Shift Correction.

Select the equilibrium levels of the overlay plot with the left mouse button (click and drag). For every curve, the area that is selected will be used to calculate the average Response value, which is equal to the equilibrium value.

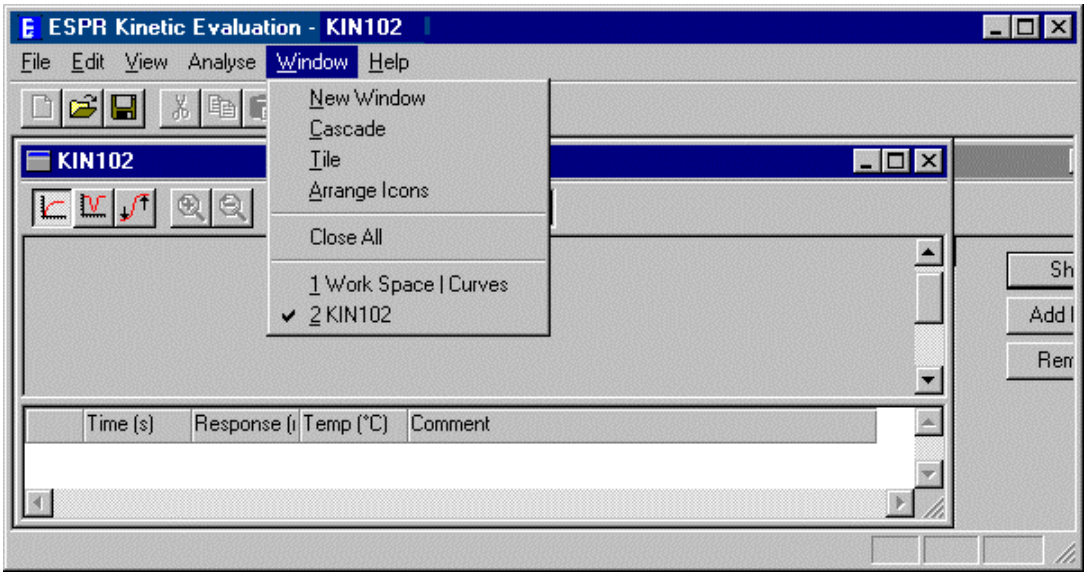
Now you can select this option, which will show the *Linear Fit window* on the screen. It contains a plot of Req/C versus Req with a slope of - K_A in M^{-1}.

The buttons of the *Linear Fit window* are:

To report sent linear fit results to Result Page of Workspace
Interpolate shows Interpolate window to find X or Y values
Detail shows data points in Req/C vs Req plot. You can skip data points for the linear regression procedure
Print Graph prints the Req/C vs Req plot
Save Graph saves graph as *.emf file
Close closes *Linear Fit window*

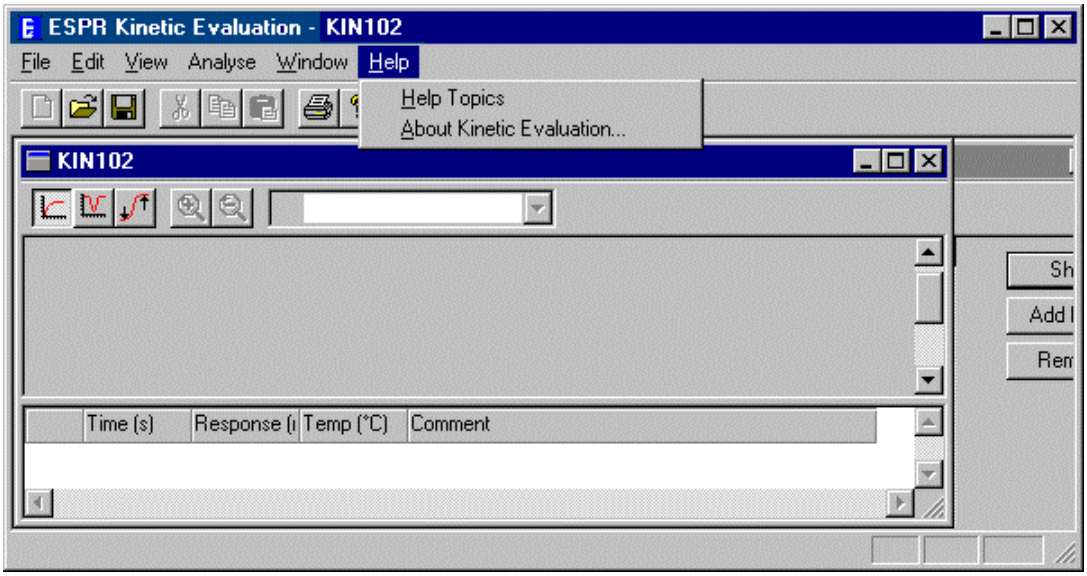
Signal Cursor	opens *Signal Cursor window* in overlay window.The overlay plot automatically switches from overlay presentation to curve presentation. Thereby, only the active curve is shown. By scrolling you can walk through the data points of that particular curve, which are shown in the *Signal Cursor window.*

Window **Menubar - Overlay window**



New Window	copies active window
Cascade	cascades all windows
Tile	tiles all windows
Arrange Icons	arranges icons
Close all	closes all windows
1. Work Space | Overlays	switches to Overlay Page of Workspace
2. Overlay	switches to overlay window

Help — Menubar - Overlay window



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2.11 Toolbar explanation

Main	Commands Main Menu
	File: New
	File: Open
	File: Save
	Edit: Cut
	Edit: Copy
	Edit: Paste
	File: Print
	Help: Help Topics
	View: Workspace
Curve window	
	View curve
	View SPR plot
	View subcurves
	Zoom in selected area
	Zoom out selected area

Overlay window

Overlay plot representation

Curve representation

View association ranges

View dissociation ranges

View ln (dR/dt) versus t plot

Zoom in selected area

Zoom out selected area