

REVIEW

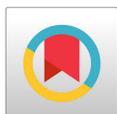
AFFINImeter Software: from its Beginnings to Future Trends A Literature review

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INTRODUCTION

Molecular recognition is one of the most important events in biological systems. The characterization of molecular interactions is a fascinating research area, fundamental to understand the function of biomolecules and to develop new bioactive compounds (drugs). The comprehensive thermodynamic and kinetic characterization of a binding event requires monitoring the formation of the complex(es) as a function of the concentration of reactants or as a function of time, and the subsequent data analysis using a mathematical model that describes the binding process monitored. During the past years, the growing interest in the field of molecular recognition has been reflected in a considerable improvement in the instruments sensitivity of well established biophysical techniques and in the development of new ones; yet less has been investigated in the development and optimization of new analysis tools for a reliable understanding of binding data, and here is where the software AFFINImeter has stepped in.

AFFINImeter [1] is a shareware software for the general analysis of binding experiments. It was born upon the need of a tool for the analysis of isothermal titration calorimetry (ITC) measurements that could handle complex interactions in an easy-to-use way. Since it was released as a cloud-based software in 2015 many scientist from academic labs, research institutes and pharmaceutical companies are being benefited from the advanced tools that AFFINImeter offers for the reliable characterization of interactions by ITC; at present, AFFINImeter has been adapted for the analysis of titration data generated from different biophysical techniques popular in drug design and discovery programs such as nuclear magnetic resonance (NMR), general spectrometric methods or Microscale thermophoresis (MST).

This review is based on the research published up until now in which AFFINImeter has been used for the characterization of molecular interactions by ITC, and it has two main purposes: on the one hand we show the evolution that AFFINImeter has experienced since its beginnings to the present time and near future, motivated by the need of versatile analysis tools to exploit the full potential of ITC and other biophysical techniques. On the other hand, we highlight with selected examples the helpfulness of advanced analytical tools and a proper use of them, for the reliable characterization of molecular interactions. In the review we have included published

works and a few examples worth to mention that, despite they are not officially published yet, they have been already accepted for publication or presented in scientific conferences.

The beginnings of AFFINmeter and first published papers

AFFINmeter was officially released as a cloud software for ITC data analysis in 2015 by our company, Software 4 Science Developments (S4Sd). However, the very beginning starts with sulTCas[e] [2], a simple web application developed in 2012 in the Soft Matter Group of the Applied Physics Department of the University of Santiago de Compostela for the design and optimization of ITC experiments that offered a couple of basic stoichiometric binding models. This code was internally expanded to fit experimental ITC data as well as to consider an unlimited amount of models [3]. In view of the increasing demand of custom models by researchers sulTCas[e] gave rise to a preliminary private version of AFFINmeter, which implemented a user-friendly graphic interface to design models (the “model builder” tool), to set up the fitting parameters and minimization algorithm, as well as the possibility to perform global fitting of multiple ITC isotherms. The utility of this version of the software was evidenced in two research papers. The first work was focused on the microcalorimetric study of the inclusion of monovalent and divalent metal cations by p-sulfonatocalix[4]arene [4]. Here, a refined competitive 1:1 model was used to consider the presence of Na⁺ as counterion in the calixarene solution, and that the ions of the titrating solution compete with Na⁺ for binding to the calixarene. This model allowed the collection of true affinity constants instead of just apparent constants where the presence of the counterion is not considered. In the second work, higher order dissociation models were generated and used to investigate protein self-association by ITC [5]. The first commercial publicly released version of AFFINmeter was focused on the advanced analysis of ITC isotherms making use of models based on equilibria between stoichiometric species or models based on an independent sites approach, the “model builder” tool, and the possibility to perform global analysis. Besides, it included a potent simulation tool in which advanced binding models can be applied. The second public version of AFFINmeter (2016) incorporated the automated processing of raw data from Microcal and TA platforms (the two largest manufacturers of ITC instruments) and the KinITC tool, an exclusive method to retrieve kinetic information from standard ITC titrations. Since then, the usefulness of AFFINmeter has been reflected in a number of papers describing research where the software has been used. In the following sections we review these research works, organized according to the main analysis tools available in the software.

Analyses based on a stoichiometric equilibria approach: the model builder

Stoichiometric models are of common use for evaluating binding data. This approach uses reaction schemes based on equilibria between stoichiometric species and it is applicable to a wide range of binding processes. The model builder is a user-friendly tool with which researchers can generate and apply tailored binding models in an easy way. It uses a three letter code to define the titrate (M), the titrant (A) and a third species (B) that may participate in the interaction and that can be located in the syringe mixed with A, in the cell mixed with M or in both places. In the models, the term “FS” refers to “free species” (Figures 1A, 2A and 4A). In this way, researchers can have access to, basically, any stoichiometric model to suitably describe complex interactions. Besides, three parameters r_M , r_A and r_B are included in the models. These are parameters exclusive of AFFINmeter and can be fitted in the analysis, if necessary, to correct for potential differences between nominal and true functional/active concentration of M, A and B, respectively. This way, a value of 1, i.e. for r_M , indicates that the nominal concentration of M matches with its true functional concentration while values lower or higher than 1 point towards a concentration of M that is lower or higher, respectively, than the nominal concentration. So far, several labs have published research work where AFFINmeter stoichiometric

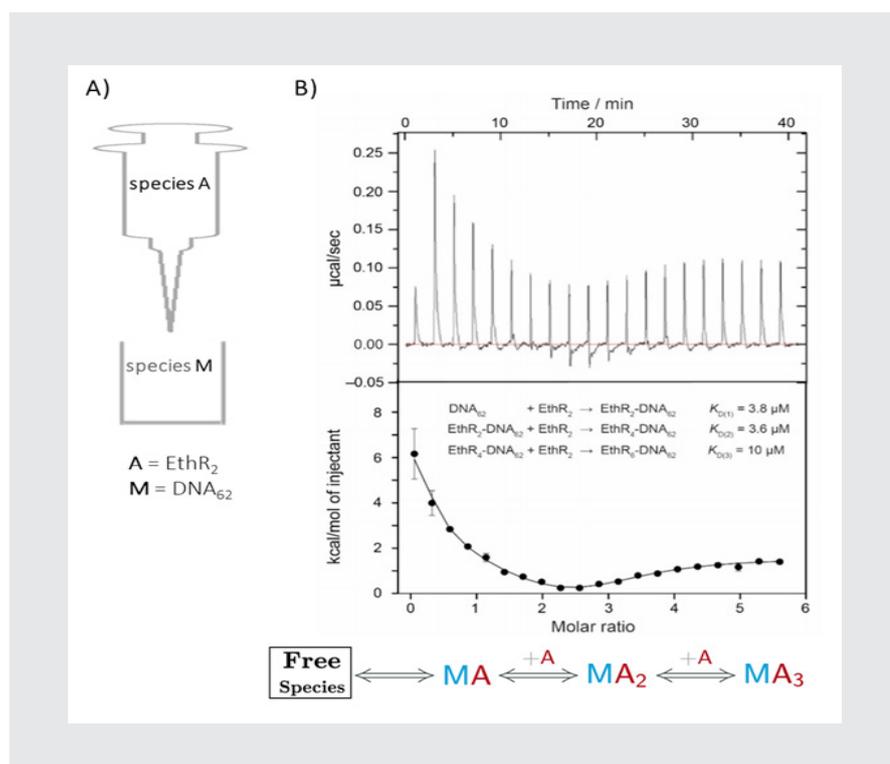


Figure 1. A) Schematic representation of the ITC titration of EthR into DNA62 based on the MAB code of AFFINImeter where “A” is the EthR dimer (in the syringe) and “M” is DNA62 (in the calorimetric cell). B) ITC data for the titration showing the thermogram (upper panel) and the fitted isotherm (lower panel). The AFFINImeter tailored model used is shown at the bottom of the figure. Adapted with permission from figure S12 of [18], published by The Royal Society of Chemistry.

models are used [4, 5, 6-22]. While some only required standard models to characterize simple 1:1 interactions [6-15], a number of them made use of tailored models to unravel complex mechanisms of binding and/or to fit ITC data from assays performed under a non-standard experimental setup [4, 5, 16-22]. An interesting example of tailored binding model was published by Shiu-Hin Chan et al., who reported ITC data of the titration of the transcriptional repressor EthR into DNA [18]. Prior to ITC, native electrospray-ionization mass spectrometry experiments confirmed the dimeric nature of EthR in solution and that six subunits of EthR were able to bind to its DNA operator sequence. Based on this information, a tailored model $FS \leftrightarrow MA \leftrightarrow MA_2 \leftrightarrow MA_3$ was used to fit the ITC data where A is the EthR dimer and M is the DNA monomer (**Figure 1B**). Analysis of the ITC data using this model confirmed the stoichiometry and provided a thermodynamic characterization of the binding of the first, second, and third EthR dimer to the DNA.

An illustrative example of the use of AFFINImeter model involving three interacting species, M, A, B, is in the work performed by Marques-Carvalho et al. [19], where ITC was utilized in a thoughtful study to characterize the interaction Ca^{2+} /calmodulin (CaM) with the different sites of the EAG1 potassium channel. Part of this research consisted of ITC experiments to characterize the binding between CaM with a large channel fragment of EAG1 (CNbHD-BDC1-BDC2). Titration of CaM into CNbHD-BDC1-BDC2 yielded a two-stepped isotherm that was successfully fitted using a sequential model $FS \leftrightarrow MA \leftrightarrow M_2A$, demonstrating that CaM binds as a bivalent species through its C- and N- terminal lobes.

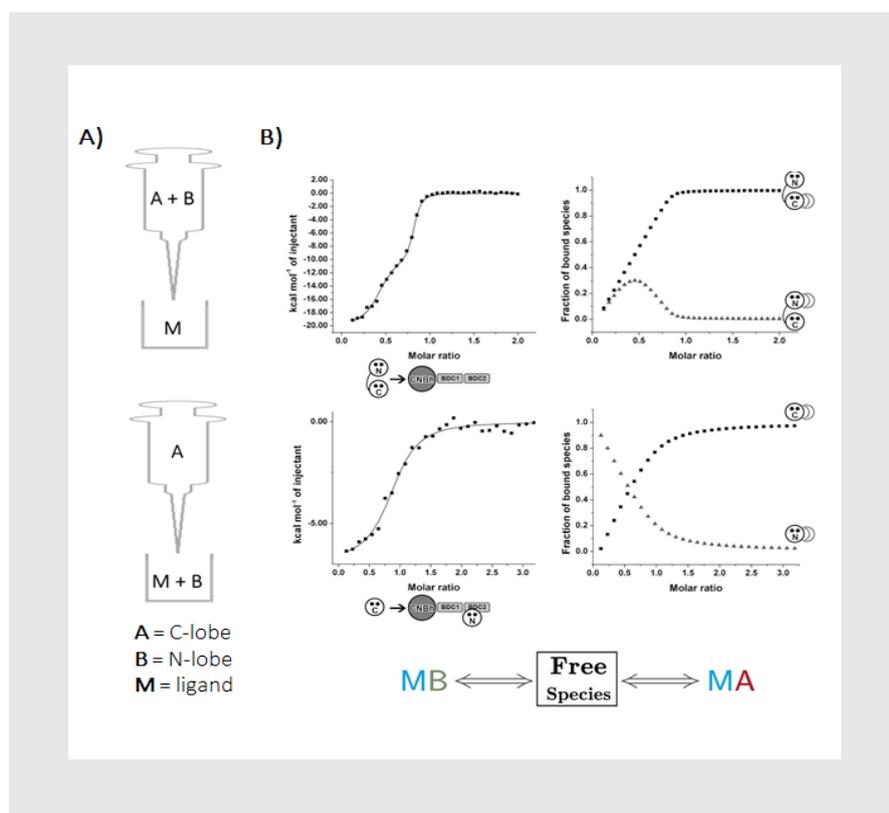


Figure 2. A) Schematic representations based on the AFFINImeter MAB code, of the ITC titration of CaM into CNBhD-BDC1-BDC2 (up) and the titration of C-lobe into a mixture of CNBhD-BDC1-BDC2 and N-lobe (down). B) Fitted isotherms of the two titrations (left) and species distribution plots (right). The AFFINImeter competitive model used is shown at the bottom of the figure. Adapted with permission from figure 7 of [19], published by Cell press.

Alternatively, a competitive model $MB \leftrightarrow FS \leftrightarrow MA$ was used to impose that the N- and C- lobes interact independently with the same or overlapping channel regions allowing to extract site-specific thermodynamic parameters. Here, species A and B of the model represent C- and N- lobes of CaM, as if they were independent competing species mixed in the syringe sample (**Figure 2, upper part**). The same model was used in a competitive experiment with a different experimental setup consisting of a displacement assay in which the C-lobe was titrated into CNBhD-BDC1-BDC2 premixed with a saturating concentration of N-lobe. This time species B (N-lobe) was mixed with M (CNBhD-BDC1-BDC2) in the calorimetric cell (**Figure 2, lower part**). The analogous results obtained using both approaches supported the model of a single site in CNBhD-BDC1-BDC2 with which the N- and C- lobes of CaM interact independently. Interestingly, the authors provide a thoughtful interpretation of the results obtained based on species distribution plots, which show the formation/disassociation of the stoichiometric complexes formed, MA and MB, throughout the titration experiments (**Figure 2, right**).

It is also worth mentioning a work performed by the AFFINImeter scientific team in collaboration with the Pharmaceutical Company ROVI, to develop an original protocol based on ITC experiments and data analysis with AFFINImeter, towards the evaluation of anti-coagulant activity of low molecular weight Heparins (LMWH). ITC titrations of LMWH into the protein antithrombin yielded two stepped isotherms that were fit using the competitive

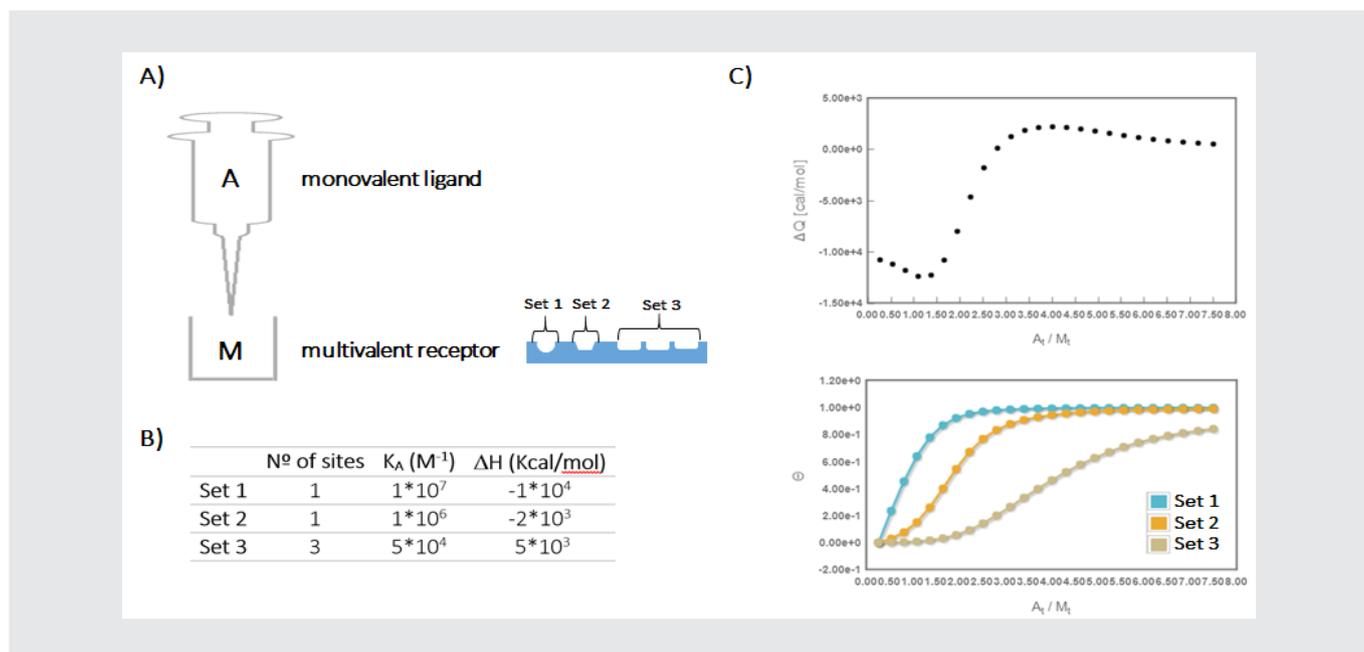


Figure 3. A) Schematic representation of an ITC titration involving a monovalent ligand in the syringe and a multivalent receptor in the cell. The receptor is characterized by three sets of independent sites where set 1 and set 2 include one site each, and set 3 include 3 sites that are equivalent. B) Table of K_A and ΔH values describing each set of sites. C) Simulation of ITC data performed in AFFINImeter following the independent sites approach for the interacting system described. The corresponding ITC isotherm (up) and fractional occupancy of the sites (down) are shown.

model $MB \leftrightarrow FS \leftrightarrow MA$ to account for the presence of a high affinity pentasaccharide sequence with anticoagulant activity in the LMWH molecule (A) and other non-anticoagulant low affinity sequences (B). Here, the main goal consisted in determining the amount of high affinity sequence. Using the r_A and r_B parameters in the fitting process it was possible to get this valuable information of pharmaceutical interest. This protocol has been accepted for publication as a book chapter in *Methods in Molecular Biology* [23].

Analyses based on an independent sites approach

The independent sites (IS) approach is based on site-specific equilibrium constants and it is focused on the analysis of a ligand binding to a multivalent receptor with a number "n" of independent binding sites. The sites can be equivalent (and therefore grouped in a single set of sites) or different (grouped in different sets of sites) and the number of sites of each set can be considered as a fitting parameter. Therefore, this approach is a good choice for the analysis of interactions involving multivalent receptors and the formation of complexes of unknown stoichiometry. Into this approach, the advantage that AFFINImeter offers over other software packages is that the number of sets is not limited to just one or two sets of sites. Besides, it includes the possibility to analyze competition experiments where a second ligand (B) competes with a primary ligand (A) for binding to all or certain sets of sites. An illustrative example is shown in **Figure 3**, of a simulation performed with AFFINImeter of a monovalent ligand binding to a multivalent receptor with 3 sets of different sites. So far, the number of publications performed with AFFINImeter IS approach has been modest [21, 24-27]. It has been recently used by Silva et al. in the study of a cecropin A2 melittin hybrid antimicrobial peptide binding with a 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho69 (1'-rac-glycerol) model membrane system, in a research focused

on the understanding of the mechanism of action of antimicrobial peptides [24]. In this study the ITC titrations of Lipid membrane into peptide ITC yielded a sigmoidal curve that was fit to a 1 set of independent sites where the number of sites “n” calculated provided information about the extension of the interaction between the lipids and the peptide. The IS approach has also been used in the study of ligand-receptor interactions where the resulting isotherms present a non-sigmoidal shape, manifesting a higher degree of complexity. Two of them are centered in the study of the interaction between flavonoids with salivary proteins [25, 26] and a third one focused on structural and thermodynamic basis of polyphenol interactions with peptides relevant in celiac disease [27]. In all cases the experimental data was fitted to a theoretical titration curve of two sets of independent sites, each with different binding strengths, in order to obtain the site-specific equilibrium constants characterizing each set of sites, and the number of sites per set and molecule.

Analyses using Global fitting

Global analysis consists of fitting two or more data sets simultaneously, given that these data share information of the binding event(s) under study. Then, increasing the amount of experimental information and putting it together to provide a single set of binding parameters is an efficient way of reducing the number of degrees of freedom in the fitting model, thus increasing the reliability of the results. It is a matter of fact that global analysis offers a much more robust approach than individual analysis, but it is not routinely used yet by the scientific community as a required method for the characterization of binding interactions. In this sense, a key limitation has been that global analysis is not implemented in many software packages and, if so, it is not always provided as an easy to use tool. In AFFINImeter, the global analysis is easily available in such a way that two or more data sets can be uploaded into the same fitting project and restrictions can be imposed in a straightforward way to connect fitting parameters between the data.

Thus far, researchers have taken advantage of the global analysis tool of AFFINImeter at different levels of complexity. It has been applied in the thermodynamic characterization of simple 1:1 interactions where the ITC titration was repeated three times [8, 13, 17]. Here the global analysis was a preferred option over the individual fitting in order to yield a single set of K_A and ΔH values. Besides, it has been used in the analysis of complex interactions where advanced binding models involving more than just one equilibrium are required, to reduce the number of degrees of freedom. For non-trivial interaction mechanisms, the number of fitting parameters increases (at least, a set of K_A and ΔH for each equilibrium has to be determined) and the global analysis of various data sets providing complementary information becomes a necessity in order to avoid over-parameterization. An example is provided by Shiu-Hin Chan et al. [17], already described in the previous section of this review (on the subject of Analyses based on a stoichiometric equilibria approach). In order to retrieve the K_A and ΔH values of the equilibria that describes the sequential incorporation of three ligand molecules (EthR dimer) into the multi-site receptor (DNA) a global analysis of two isotherms was performed. Importantly, the two isotherms were obtained from ITC titrations where different concentrations of EthR and DNA were used. Therefore, a distinct distribution of the complex species along the titrations is monitored, which increases the amount of information. Unfortunately, in this article only one of the isotherms is shown whereas a figure of the two globally fitted isotherms should be presented to graphically illustrate the global analysis performed. Another example is in the protocol previously mentioned, for the evaluation of anticoagulant activity of low molecular weight heparins [22] where the use of global analysis was a requirement for the successful characterization of the LMWs. In one hand, three ITC titrations were performed using different concentrations of LMWH, for a better definition of the regions of the isotherm corresponding to high and low affinity binding modes; on the other hand, analogous titrations were performed using unfractionated heparin, instead of LMWH, as a model substance that carries the sequence of high affinity binding with anticoagulant properties. A global

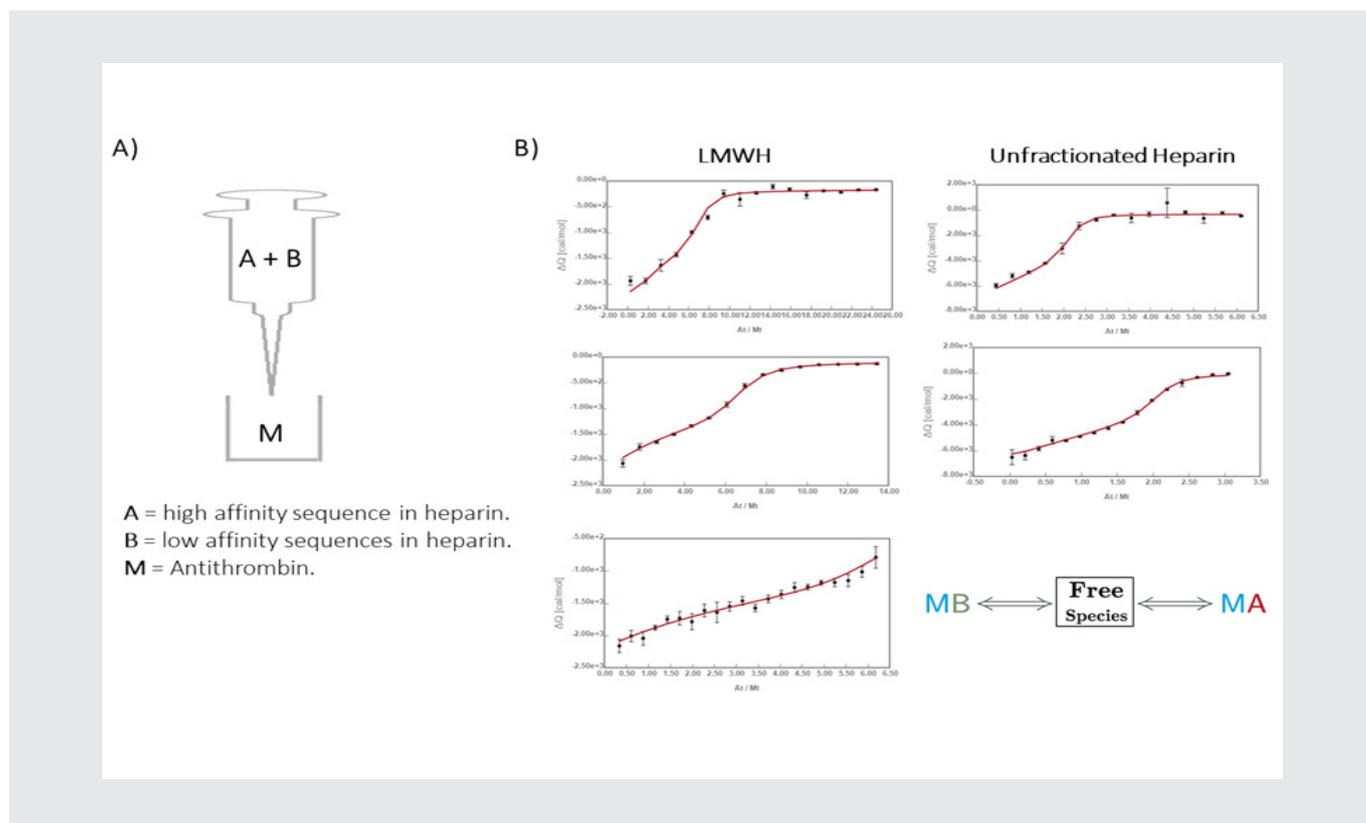


Figure 4. A) Schematic representation of the ITC titrations of heparin (LMWH or unfractionated heparin) into antithrombin based on the MAB code of AFFINImeter where “A” is the high affinity sequence with anticoagulant activity, B represents low affinity sequences and M is the antithrombin. B) Global analysis of the ITC isotherms from three titrations performed with a different concentration LMWH in the syringe (left) and two titrations with different concentration of unfractionated heparin (right). The AFFINImeter tailored model used is shown at the bottom of the figure.

analysis was performed including all these data series where K_A and H of the high affinity binding are common parameters between isotherms, imposing the condition that the same anticoagulant sequence of the unfractionated heparin is also present in the LMWH (**Figure 4**).

Finally, a higher level of complexity comprises the use of mathematical equations to establish relationships between fitting parameters. Although this possibility has not been extensively exploited it brings the possibility to analyze systems of high complexity, not only to get a thermodynamic characterization but also to elucidate complex binding mechanisms. A study taking advantage of this feature of AFFINImeter was carried out by our scientific team in collaboration with Dr. Daniella Hishimaru and Dr. Mirko Enning, and has been recently presented at the 3rd European MicroCal meeting [28]. In this study a series of ITC titrations were conducted to investigate the origin of the efficiency of the interaction between the heterogeneous nuclear ribonucleoprotein (hnRNP) E1 and single-stranded (ss), poly(C)-rich nucleic acid sequences. Here, a global analysis was performed in which site constants of individual domains and stoichiometric constants of the model describing the multivalent interaction of the full protein are related through mathematical relationships that were the key to reveal and characterize a synergistic effect between all three KH modules within hnRNP E1. The corresponding manuscript is now under preparation for publication.

Combining AFFINImeter analysis tools to obtain thermodynamic and mechanistic information of non-trivial interactions

The previous sections of this review show the relevance of combining the model builder tool and global analysis in the same fitting when the time comes to characterize non-trivial interactions. But the full potential of AFFINImeter is reached when a comprehensive study is achieved, making use of the various analysis and validations tools offered. Ultimately, this approach can provide structural and mechanistic information, besides thermodynamic information. A well conducted example of such complete study has been recently published by Mazzotta et al [21] in which ITC was used to characterize the interaction of Ca^{2+} /Calmodulin with dCRY, a light responsive flavoprotein, and with INAD, a photoreceptor-specific protein. The titration of dCRY into CaM denoted the presence of two binding sites in CaM and the corresponding isotherm was fit in two ways: using a stoichiometric model $\text{FS} \leftrightarrow \text{MA} \leftrightarrow \text{MA}_2$, or an IS approach where two different independent sites are considered. The comparison of the results obtained using the two approaches confirmed that the two sites act independently; one of the sites, of high affinity (HA), binds dCRY with K_D in the nanomolar range, while the second site, of low affinity (LA) is three orders of magnitude weaker. The titration of INAD into CaM showed a weak interaction between these two species and the corresponding isotherm didn't show an optimal sigmoidicity to provide a reliable determination of the stoichiometry. For this, a 1:1 model was assumed in the in-

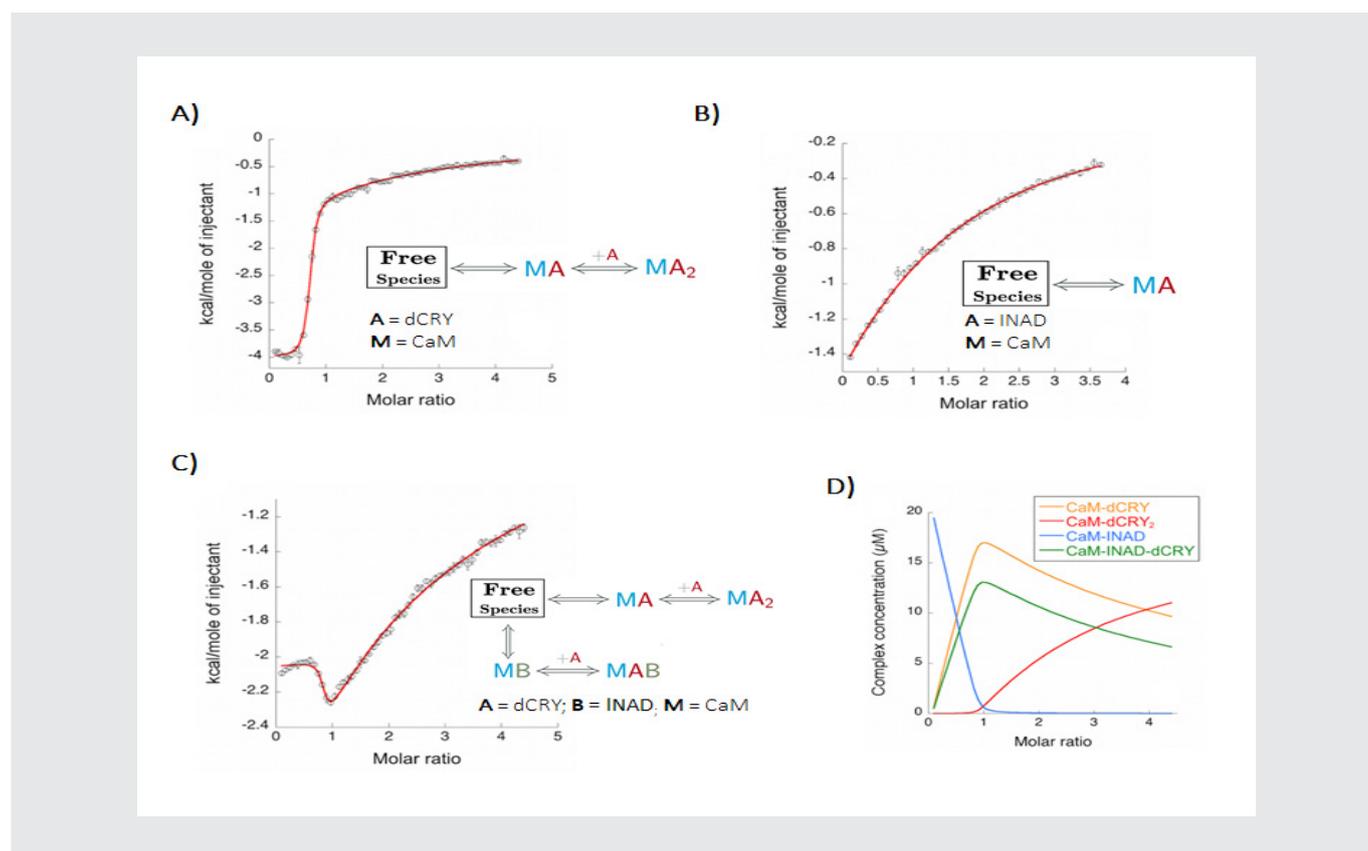


Figure 5. Global analysis of ITC isotherms from A) titration of dCRY into CaM, B) titration of INAD into CaM and C) competitive experiment of dCRY into CaM pre-incubated with INAD. The AFFINImeter models applied to each isotherm in the global fitting are shown next to the isotherms. D) species distribution plot corresponding to the competition experiment. Adapted with permission from figure 3 of [21], published by Frontiers.

dividual fit of this data. In order to understand whether the site of CaM occupied by INAD corresponds to the HA or LA site observed for dCRY, competitive ITC experiments were performed. One of these experiments consisted of a titration of dCRY into CaM pre-incubated with an excess of INAD. Here, the visual inspection of the corresponding isotherm suggested that INAD competes with dCRY for binding to the HA site. In order to achieve a detailed quantitative thermodynamic characterization the global analysis of the three data series (standard titrations of dCRY and INAD into CaM and competitive experiment) was performed, imposing restrictions where common K_A and ΔH parameters are shared between data series (Figure 5).

The authors demonstrated that the global analysis is satisfactory only if a model $MBA \leftrightarrow MB \leftrightarrow FS \leftrightarrow MA \leftrightarrow MA_2$ is used to fit the competitive experiment data, which implies that a ternary complex CaM-INAD-dCRY can be formed. The results confirmed the 1:1 stoichiometry of the CaM-INAD complex and showed that the affinity of dCRY for the LA site in CaM is enhanced by the presence of INAD bound to the HA. Importantly, the authors make use of the AFFINImeter validation and interpretation tools to evaluate the results reliability:

1. the goodness of fit (a normalized chi-square parameter with % units) to assess how close the theoretical curve is to the sigma-width gaussian distribution of experimental data;
2. the standard/statistical errors of each fitted parameter to determine the uncertainty associated to the value;
3. the table of local minima to check for potential over-parameterization;
4. the species distribution plot, as a tool for results interpretation.

KinITC: delivering kinetic information of binding events from Isothermal Titration Calorimetry data

ITC has been used traditionally to determine the complete thermodynamic profile and stoichiometry of binding interactions through the analysis of the binding isotherm produced from the ITC titration. Besides, it has been established as a technique to measure kinetics of enzyme-catalyzed reactions because the thermal power generated is a real-time direct measure of the reaction progress. Likewise, the raw thermogram of a standard ITC titration can be used to obtain the kinetic profile of binding events. This property has been extensively studied by Prof. Philippe Dumas and Dr. Eric Ennifar, who developed the method KinITC for obtaining joint thermodynamic and kinetic data of binding interactions using ITC. The method was first published in 2012 [29] and implemented in the software AFFINImeter three years later, as a result of a fruitful collaboration of P. Dumas and E. Ennifar with S4Sd. Up to date, a number of articles and book chapters [29-32] have been published where the KinITC method is explained in detail, including an application note of Malvern-Microcal where the implementation of KinITC in AFFINImeter is described [30]. In AFFINImeter, a simplified version of the method (lately named as KinITC-ETC) has been implemented for the analysis of 1:1 and 1:n interactions, characterized by a simple one-step kinetic profile. It consists of an analysis of the peak broadening observed throughout the titration, based upon the variation of the equilibration time from injection to injection, represented as a function of titrant to titrate molar ratio (Figure 6). The resulting equilibration time curve (ETC) is globally analyzed together with the binding isotherm to directly determine the thermodynamic association constant (K_A) and the dissociation rate constant (k_{off}). The association rate constant (k_{on}) is calculated as the product of K_A and k_{off} . At present, AFFINImeter has incorporated the KinITC-ETC method into an automated protocol that, upon data uploading, processes the raw thermogram and analyzes the resulting isotherms and ETC to retrieve thermodynamic and kinetic information. This protocol has been described in detail in a book chapter that has been accepted for publication in *Methods in Molecular Biology* [33].

Since KinITC-ETC was released a number of research works have been published that manifest the potential and robustness of the method. Early studies were conducted for

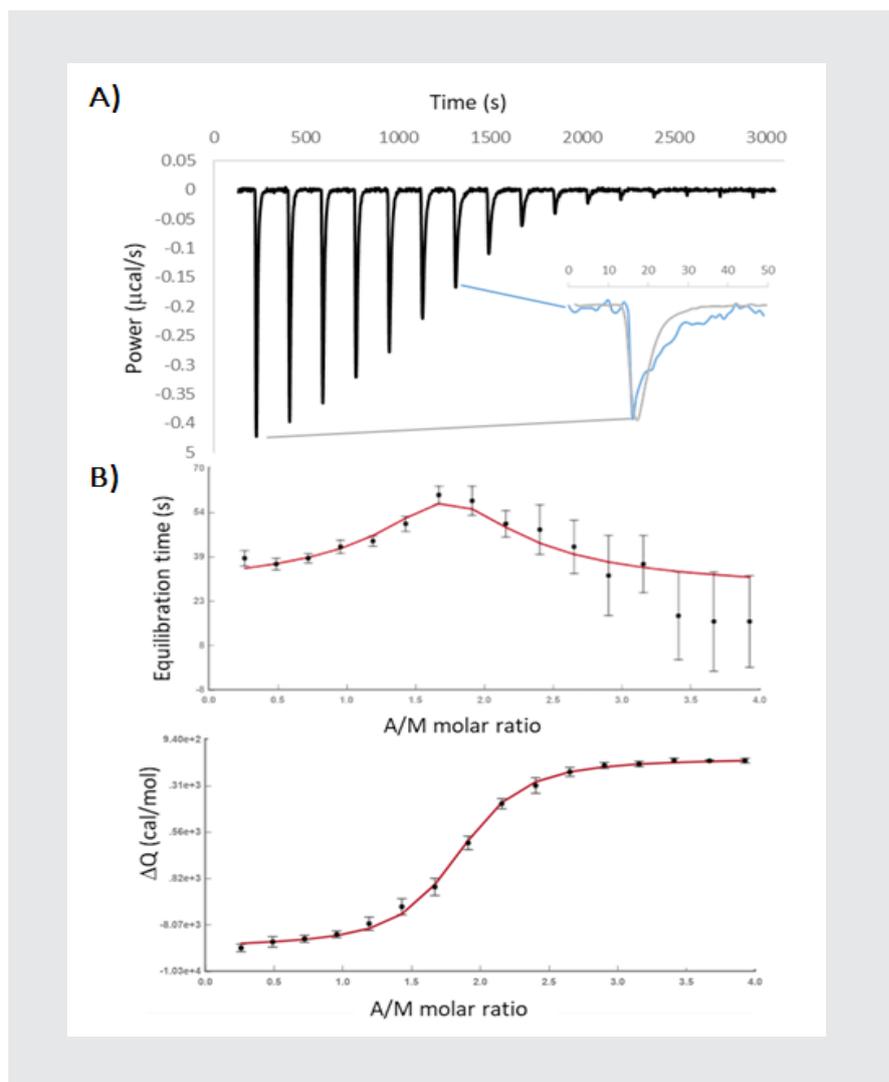


Figure 6. ITC data of a 1:1 binding interaction used for thermodynamic and kinetic analysis. A) Thermogram and overlaid of two peaks from the beginning and mid-titration to highlight the peak broadening observed at mid-titration. B) Equilibration time curve (up) and binding isotherm (down) obtained from the thermogram. These two curves are globally fitted in KinITC-ETC to retrieve thermodynamic and kinetic information.

the kinetic analysis using ITC of the interaction of carbonic anhydrase and the inhibitor 4-carboxybenzenesulfonamide [29], a well characterized model system widely utilized to validate the performance of kinetic methods of analysis like surface plasmon resonance (SPR). Later on, it has been successfully used for the thermodynamic and kinetic analysis of different kinds of interacting systems including nucleic acid–ligand [34–36], protein–ligand [37, 38], antibody–antigen [39], and small molecule–cation interactions [40]. In some of the works the kinetic information obtained with KinITC-ETC was successfully compared with other techniques like SPR [29, 35, 36, 38], fluorescence [34] or rotating droplet electrochemistry [34]. The results published indicates that KinITC yields limits of detection for k_{on} and k_{off} close to those stated for SPR, and permits the kinetic characterization of interactions with affinity ranging from micromolar to nanomolar. In general, there

is a good match between KinITC and other techniques for kinetic characterization. Into this subject, it is worth highlighting the outstanding work recently published by Pascal Zihmann et al. focused on testing the reliability of KinITC-ETC to retrieve kinetic information [38]. Using AFFINImeter, the authors re-analyzed ITC data published in previous years of 29 mannosides binding to the bacterial adhesion FimH, covering a wide range of binding affinities with K_D values from low micromolar (100 μM) to high nanomolar (1 nM). A subset of 4 ligands were chosen for a thoughtful comparison between KinITC-ETC and SPR observing a good agreement between techniques. For the on-rate constants an excellent agreement was observed with values differing by less than a factor of two between ITC and SPR. Differences in off-rate values were at most of a factor of six, which can be easily justified by the potential presence of experimental artifacts inherent to both techniques. This work constitutes the first systematic kinetic analysis of protein–ligand interactions using KinITC-ETC and stresses the potential of ITC together with this analytical tool in studies to elucidate structure-kinetic relationships.

Current view and future perspectives

Since AFFINImeter was developed in 2013 (first public release in 2015) a total number of 30 research articles have been published where the software has been used to analyze ITC data. Although it is not a number representing all the research that has been carried out with AFFINImeter (i.e. research performed in pharmaceutical companies is not included and a large number of measurements are currently being analyzed and are expected to be published at the short term) it provides an illustrative view on to what extent and how the different analysis tools are being exploited. In **Figure 7** we have included a bar diagram where the software performance is shown in terms of publication rate (average of the articles published per month, and grouped per year of publication), and a circular chart showing the percentage of articles in which AFFINImeter was used for simple data analysis (applied to 1:1 interactions and individual analysis), for advanced thermodynamic analysis (use of complex models and/or global fitting) and for kinetic analysis. From these graphs it

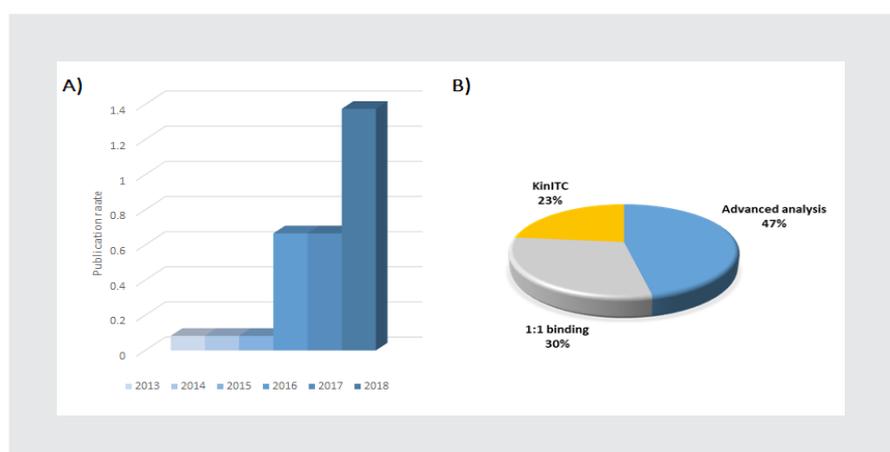


Figure 7. Overview of the articles published where AFFINImeter has been used since its development (2013) to present (August 2018). A) Bar diagram of publication rate (average of the articles published per month, and grouped per year of publication) B) Circular chart of the percentage of articles where AFFINImeter was used for simple data analysis (applied to 1:1 interactions and individual analysis), for advanced thermodynamic analysis (use of complex models and/or global fitting) and for kinetic analysis.

can be seen that there is a clear tendency, with an increase in the publication rate since the software was released to the present time (August 2018) where about two thirds of the research presented (70 %) have used AFFINImeter for analysis other than individual fitting to a 1:1 model. 47 % of the articles made use of advanced models and/or global analysis and 23 % made use of KinITC to retrieve kinetic information.

Besides research articles, there is a number of publications dedicated to the description of KinITC that have been essential to offer a detailed explanation of this new method. At present, an article is being prepared focused on a detailed description of the main features and technical details of the software, with special emphasis on explaining the implementation of thermodynamic binding models.

Based on the information received from these articles and feedback from AFFINImeter users it seems clear that the advanced analysis tools available in AFFINImeter are facilitating the performance of new experimental designs, the reliable interpretation of complex data, and expanding the range of applications of the technique. Still, we consider that the full potential of the software has not been exploited yet; for instance, there are cases where analysis of data from 1:1 binding interactions is limited to a thermodynamic characterization while using the same titration experiment a kinetic analysis could be performed without extra-effort. Also, the IS approach has only been used, so far, for the analysis of interactions involving two sets of sites while with this approach it is possible to extend to a larger, user-defined, number of sets and even the possibility to incorporate a competitor molecule. These characteristics make the IS approach an attractive tool for the characterization of polymer/nanoparticle–ligand interactions, relevant in biomedical applications. The use of tailored models and global analysis has to be accompanied by a careful evaluation of the reliability and robustness of the results. In this sense, AFFINImeter includes a series of validation tools whose use should be included and described in the articles as a part of the analysis performed in order to provide the best proofs of reliability. Definitely, when the amount of sample is not a limitation, several measurements of the same system at different conditions (concentrations or temperatures) should be performed and they should be analyzed altogether with restraints between the parameters of each experiment in order to produce highly robust results. The availability of species distribution plots is another feature that provides interesting information in order to understand interaction mechanisms. Last, but not least, when using KinITC for kinetic analysis detailed information of the analysis result and reliability should be presented, including graphs of the fitted isotherm and ETC. This should always be a requirement when the time comes to publish fitted data but, it takes specially relevance when the method applied is new, as it is the case of KinITC.

A couple of limitations of the current version of the software that some users have pointed out are, on the one hand, that the model builder does not offer the possibility to directly generate equilibria based on reversible first-order reaction to describe i.e. conformational changes; on the other hand, the KinITC-ETC method is available only for 1:1 and 1:n interactions.

The current and future trends of the software AFFINImeter are oriented, on the one hand, to overcome existing limitations of the current version and to continue extending the range of applications for ITC data analysis and, on the other hand, to expand the use of the advanced analysis tools to other biophysical techniques. In this way, new tools are being developed for the analysis of ITC experiments and they are expected to be implemented in AFFINImeter at the short term. Some examples are the analysis of micellization/aggregation experiments [41], the implementation of global multi-temperature analysis making use of the Van't Hoff equation, hybrid stoichiometric-equilibria/independent-sites approaches and optimization of models to include more general analyses. Besides, we have recently released two new applications, AFFINImeter-NMR and AFFINImeter-spectroscopy. The first one is addressed to the processing and analysis of 2D-NMR titrations and works jointly with the module MBinding of the MNova NMR software [42]. The second

one is a broader application where binding curves generated from different spectroscopic techniques can be uploaded, allowing the advanced analysis using complex models and global fitting of curves obtained from the same, or different experiments/techniques. With AFFINImeter-spectroscopy an integrative data approach is offered, useful in orthogonal biophysical methods that provides overlapping information from different perspectives.

Conclusions

In this review, we have summarized the research work published where AFFINImeter has been used in the analysis of ITC data since it was made available in 2013 until the present time. To achieve this, the literature presented has been organized according to the main advanced tools offered in the software, with the aim to highlight the relevance that these tools have in the thoughtful characterization of binding events. We have remarked, with selected examples, the necessity of using advanced tools like the model builder and global analysis for the reliable characterization of non-trivial interactions, where two or more equilibria are present. Besides, with the implementation of the method KinITC-ETC in AFFINImeter we have put this potent tool for the kinetic analysis using standard ITC titrations easily available to the scientific community. Herein, the works published until now have demonstrated the reliability of KinITC, which is gaining ground as a well-established method for kinetic characterization. Despite the satisfactory results observed of the use of AFFINImeter by researchers, we believe that the exploitation of its full potential is yet to come.

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