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Working Group 2, Task Group 5

Recommended procedure for proper data collection and analysis in isothermal titration calorimetry experiments: the case of multiple host-guest equilibria

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Recommended procedure for proper data collection and analysis in isothermal titration calorimetry experiments: the case of multiple host-guest equilibria

Giuseppina D. G. Santonoceta,[a] Jeremy Brandel,[b] Carmelo Sgarlata[a]

[a]*Dipartimento di Scienze Chimiche, Università degli Studi di Catania, V.le A. Doria 6, 95125, Catania, Italy; email: sgarlata@unict.it*

[b]*Université de Strasbourg, CNRS, IPHC UMR 7178, F-67000 Strasbourg, France*

in collaboration with research groups from the Universities of Catania, Italy (Davide Russo, Lidia Zuccarello); Ferrara, Italy (Denise Bellotti, Maurizio Remelli); Firenze, Italy (Matteo Savastano, Antonio Bianchi); Ljubljana, Slovenia (Žiga Medoš, Marija Bester-Rogac); Messina, Italy (Clemente Bretti, Gabriele Lando); Strasbourg, France; Udine, Italy (Martina Sanadar, Andrea Melchior) and Wroclaw, Poland (Paulina Potok, Slawomir Potocki)

NECTAR NETWORK FOR EQUILIBRIA AND CHEMICAL THERMODYNAMICS ADVANCED RESEARCH
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Abstract

Isothermal titration calorimetry (ITC) is a widely employed technique for the study of binding processes occurring in solution as it enables the simultaneous determination of equilibrium constant and standard enthalpy change values through a single experiment. Despite its widespread use, there is a lack of standard/best practices to be followed for accurate experimental design, execution and data analysis thus leading to great variability and often potential inaccuracy of the results obtained. Some efforts have been made in the literature to address these issues and evaluate the instrumental uncertainty and proper data comparison. However, many challenges and questions still remain open, mostly dealing with complex speciation and data treatment when simultaneous equilibria occur in solution.

In this context, the Working Group 2, Task Group 5 of the NECTAR Cost Action (CA18202), involving several research groups from different European universities, focused on a multi-laboratory exercise for the calorimetric study of multiple host-guest complex formation intending to define key aspects and offer recommendations for speciation and data treatment in solution thermodynamics and ITC. Procedures, data and results preliminary obtained by the participating laboratories of Catania, Italy and Strasbourg, France are presented and discussed in this document.

Introduction

Solution equilibria are involved in different research fields including chemical speciation, coordination and supramolecular chemistry. The properties of molecules depend on their interactions in a given system; consequently, to explain which factors and forces control the binding processes in solution and determine whether or not they take place, a complete thermodynamic profile assessing the strength and the nature of these interactions is required.

Nowadays, isothermal titration calorimetry (ITC) is considered a gold-standard technique[1] and an invaluable tool[2] for the study of binding/chemical processes in solution with high sensitivity (e.g. acid-base reactions, metal-ligand complexation, host-guest recognition process and biomolecular interactions) through the measure of the heat, released or absorbed, upon reaction. This makes calorimetry an extremely versatile technique which does not require modification of the compounds to be studied or the addition of any reagent as opposed to, for example, spectroscopy. Moreover, titration calorimetry is the only technique that allows the simultaneous determination of both the equilibrium constant and the standard enthalpy change values which means that both the enthalpic and entropic components of the standard free energy term can be determined from a single experiment. The complete thermodynamic profile thus obtained often reveals striking aspects of the forces driving the interaction processes and their dependence on the properties of the interacting species.

Although widely employed by the scientific community, there is still a lack of good laboratory practices for the experimental design, setup and execution of calorimetric titrations as well as for data analysis that should be customarily applied to obtain accurate, reproducible and trustworthy results.

Over the years, some papers dealt with standard operating procedures for the study of the binding processes through calorimetric measurements. For example, Velázquez-Campoy *et al.* proposed a series of protocols that should be followed to characterize moderate, very low or very high binding processes involving macromolecules and ligands.[3]

More recently, recognizing the importance of careful experimental design and execution as well as data treatment as key elements for the appropriate implementation of the scientific method, a multi-laboratory benchmark study was conducted.[4] The work aimed to evaluate *inter- and intra-laboratory basal levels of uncertainty from instruments* using two test reactions for a simple 1:1 binding process (the complexation of Ca^{2+} or Mg^{2+} with EDTA in different buffer conditions) to assess the important factors that can influence both the data collected by ITC experiments (mostly focusing on the «response» obtained by different instruments) and the thermodynamic parameter values derived therefrom. This study was further used to prepare a standard operating protocol discussing aspects and conditions that are fundamental for an optimal experimental design and to produce reliable and scientifically correct results.[5]

However, in the field of solution thermodynamics, there is still a need for guidelines that may help to properly design a calorimetric titration dealing with the formation of multiple complex species (i.e.,

with stoichiometry other than the simple 1:1) and to accurately analyze ITC data when simultaneous equilibria occur in solution.

Speciation can be a complicated matter and often requires careful planning and paying attention to a variety of factors. For example, when multiple species having different stoichiometries and thermodynamic parameters are formed, the best speciation models cannot be envisaged from a single calorimetric measurement and thus a series of titrations have to be set up by optimizing simultaneously the titrant/titrand ratio needed to promote the formation of a given species and the related standard reaction enthalpy or heat to be detected.[6] Moreover, appropriate chemical speciation requires that the exact stoichiometry of each formed species is known rather than relying on the "*n* value" often included as a parameter to be refined in the binding models used by most commercially available software. These are just some of the issues that make the collection and interpretation of calorimetric data/results challenging.

Within this framework, the activity of Working Group 2, Task Group 5 (involving researchers from the Universities of Catania, Ferrara, Firenze, Ljubljana, Messina, Strasbourg, Udine and Wroclaw) of the NECTAR Cost Action (CA18202) focused on a multi-laboratory exercise for the study of host-guest complex formation via solution calorimetry. This deliverable contains and discusses procedures and data obtained from the laboratories of Catania, Italy and Strasbourg, France as a preliminary example of the workflow of Task Group 5.

Calorimetric titrations, parameters refinement and critical comparison/discussion of the results obtained are currently ongoing in the various laboratories participating to the Action with the final goal of filling the gap and solving issues still open on the examination of complex equilibria in solution and on the analysis of calorimetric data dealing with the formation of multiple species to finally provide a simple but authoritative operating protocol to the scientific community.

General procedures

Although a list of the preliminary operations and checks needed to perform accurate ITC measurements is beyond the scope of this work, we think that offering some recommendations of general applicability can be useful.

1. Chemical and electric calibration of an ITC instrument

Before proceeding with calorimetric measurements, the instrument has to be correctly calibrated as improper calibration can be the main source of systematic errors. Calibration methods are usually reported in the getting-started guide provided by the manufacturer of the instrument. Various chemical systems have been proposed for the chemical calibration of calorimeters.[7,8,9] Among these, the protonation of 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) and the complexation of Ba^{2+} with 1,4,7,10,13,26-hexaoxacyclooctadecane (18-crown-6) are among the most widely suggested reactions for calibration. If chemical standards are not available, electrical pulse calibrations are generally suitable as a second choice. However, it is good to keep in mind that the latter method produces less accurate and precise results than those obtainable from a chemical calibration as the heat released during electrical calibration may not be exactly comparable to the heat generated or absorbed by a chemical process.

2. Baseline and water into water titration

The instrument has to be equilibrated until the baseline (defined as "*the value recorded for the calorimetric signal when no thermal power is evolved in the reaction vessel*") reaches typically recommended slope and peak-to-peak standard deviation values.[8] These parameters give an idea of the "drift" and the "noise", respectively, and thus allow to determine the acceptability limits set by a researcher. Also, a baseline drift may indicate that a chemical is being absorbed or released by the cell walls or may be a warning for a bad power compensation.
The detection limit of the calorimeter (defined as "*twice the standard deviation of the average heat per injection*") can be determined by titrating water into water and fitting the data points obtained to a straight line.[7,8] Moreover, this experiment can be useful to verify if the burette is operating properly (i.e., no systematic errors occur during the titration) and if the reaction vessel is clean.

3. Experimental setup

An accurate experimental setup is essential for properly running a calorimetric titration and obtaining reliable results. Some key points to pay attention to when planning a calorimetric titration are listed below.

- *Estimation of the concentrations of titrant and titrate*

To design a calorimetric titration, any available information on the species, stoichiometry, binding affinity and enthalpy changes from similar chemical systems has to be seek out in the literature. With these preliminary indications in hands, titrations can be simulated through proper software[10,11] to decide the concentrations of titrant/titrate to be used as well as the final molar ratio to be achieved in the cell for the formation of fair amounts of the species of interest.

The determinability and the accuracy of K and ΔH will depend on the "Wiseman c value" defined as "*the product of the binding constant (K_f) times the total macromolecule concentration (C_R)*" in the reaction vessel.[12] The value of "c" is related to the degree of completion of the reaction at the equivalence point; the experimental data that affect mostly the enthalpy and the association constant values are those collected at the beginning and around the equivalence point. The *c* interval (experimental window) to obtain accurate results has been debated extensively in the literature.[13,14]

In general, it is suggested that:

a) 100 is the ideal value for the $K_f * C_R$ product;

b) $K_f * C_R$ should never be <50 or >500 (and must be kept below 1000) since values <50 would determine large errors in ΔH^0 and values over 500 would result in large errors in K_f.[15]

Concentrations generally used in modern ITC instruments are in the order of millimolar both for the reactant in the burette and in the cell although issues related to solubility, aggregation or precipitation phenomena have not to be overlooked.

- *Preparation of the titrant and titrate solutions*

When defining the experimental conditions, the stability and solubility of the reactants as well as factors such as temperature, pH and ionic strength have to be considered. Stock solutions of both titrant and titrate have to be prepared with great care, under identical conditions and with the same composition (for example in the case of mixed solvents or when using a buffering agent) to avoid/minimize unspecific heat effects due to solution mismatches; reactants have to be pure and free from contaminants. Before running a titration, all solutions have to be degassed (usually under vacuum and stirring for about 10-20 min) to rule out the formation of bubbles in the burette or the sample cell during the experiment.

- *Calorimetric titration setup*

The total volume of titrant to be added and the volume of each injection have to be defined so as to collect a sufficient number of points that enables the formation of each expected species in a range between 20% and 80%. Ideally, the end of the titration is signalled by a plateau region in the binding isotherm with very small heat effects indicating the completeness of the reaction.

The injection volumes have to produce well-defined and significantly detectable calorimetric signals. It is recommended to add the same injection volume for all the titration points unlike for the first

addition, which should deliver a very small volume of titrant (to be thrown-away in the refinement process) to overcome the long-debated problems related to diffusion phenomena and, mostly, backlash effect caused by the mechanical properties of the syringe system.
The time interval between the injections depends on the kinetics of the binding reaction and has to be chosen to guarantee equilibrium conditions before each subsequent addition thus avoiding any overlap between neighbour signals or an incomplete collection of the heat released/absorbed per injection. Calorimetric titrations must be performed under stirring for ensuring homogeneous conditions; the stirring speed (rpm) can be properly selected according to the features of the investigated systems.

- *"Blank" (dilution) titration*

The heats recorded in a calorimetric titration contain both the amount released or absorbed by the chemical reaction occurring in the cell and some contributions due to the dilution of titrant and titrate as well as other "non-chemical" thermal effects (stirring, friction, etc.). Correctly measuring and subtracting these "non-chemical" heat terms from the raw titration data is essential for accurately determining the heat associated solely to the chemical reaction of interest. The heat of dilution of both titrant and titrate needs to be considered. The dilution of the titrate (cell) may have a negligible thermal effect, though this needs to be checked. Conversely, the heat of dilution released/absorbed when a reagent is added from the burette to the cell, which contains all the other components (buffer, background salt, etc.) but the titrate, may not be negligible and has to be subtracted from the gross titration heat to yield the net heat of reaction.

4. Data analysis

Calorimetric data to be submitted to the software analysis are the net heats of reaction obtained as described above. The concentration of both titrant and titrate, the injection volumes as well as the volume of the cell are usually fixed parameters that need to be known with great accuracy. Using these (known) parameters and an initial guess value of the binding constant, the software will start minimizing the discrepancy between calculated and experimental heat values iteratively until the convergence criteria are satisfied (non-linear least-square analysis); this will result in the determination of both the association constant and the enthalpy change values for the examined reaction.
However, most of the software makes use of a variety of "binding models" that do not satisfactorily handle the formation of multiple species in solution. This is a fundamental aspect when choosing the tool for treating solution equilibria: a good software must enable all the species and their stoichiometries to be unequivocally determined and the chemical model to be precisely defined. The program should avoid the use of "built-in binding models" which provide, as a refined parameter, the

questionable *n* value which is often improperly attributed to the possible binding sites of the ligand or the stoichiometry of the complex.

It is also recommended that a reasonable number of titrations (better if performed at different experimental conditions) are analysed simultaneously for obtaining more robust binding constant and enthalpy change values. Uncertainties associated to the refined parameters, as obtained by the data fitting process, have always to be displayed and are expected to be as small as possible; in case of large errors, they should be critically examined to find out their source and improve either the experimental design/execution or the binding model applied to the dataset.

Results and discussion

Inspired by the intriguing results reported for similar host-guest systems,[6] a water-soluble, anionic macrocyclic receptor having a three-dimensional cavity was used as the host (*p*-sulfonatocalix[4]arene, C4TS) while two molecules bearing a C10 alkyl chain linked to one (decyltrimethylammonium bromide, Dec-Me_3N^+ Br^-) or two (decamethonium bromide, Dec-$(Me_3N)_2^{2+}$ $2Br^-$) charged moieties were selected as the guests (*Figure 1*). It is expected that these two cationic molecules are able to form a 1:1 or a 1:1 along with a 2:1 host-guest inclusion complexes, respectively, in aqueous solution.

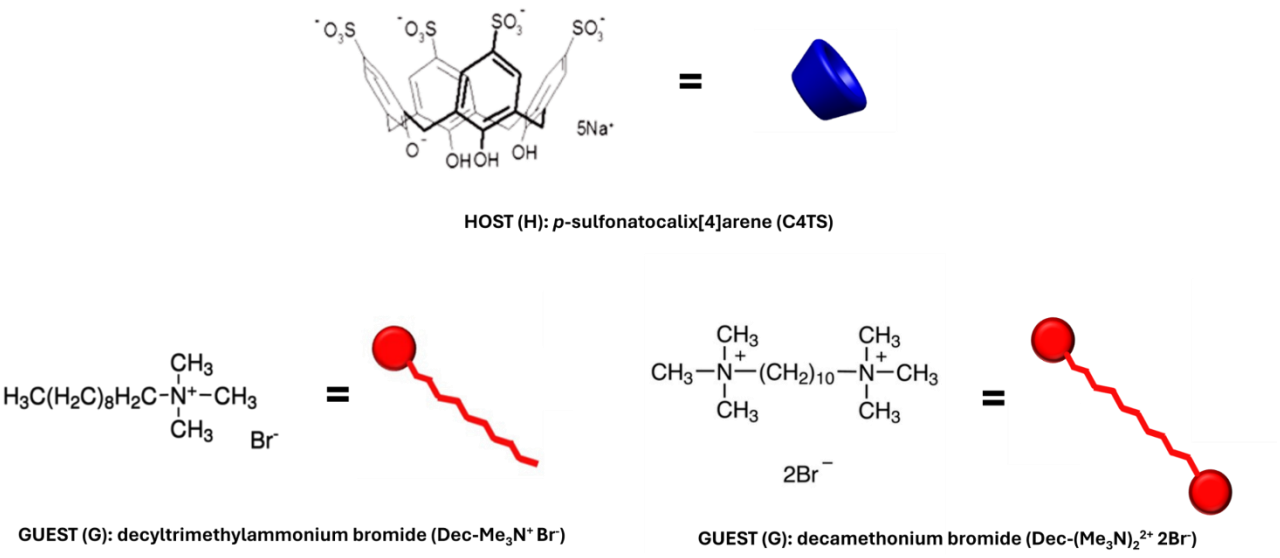

Figure 1. Chemical structures of the selected host and guest molecules

The *C4TS – Dec-(Me$_3$N)$^+$* system

Following the recommended procedure described above, the experimental set-up was defined to find the best conditions for the study of the interaction occurring between the receptor and the monocationic guest. Based on the stoichiometry, binding constant and ΔH^0 values already reported in the literature for analogous systems, the concentrations of host and guest, the number and volume of injections and the final titrant/titrate ratio were chosen to optimize the formation of the complex during the titration, as illustrated in the species distribution diagram in *Figure 2*.

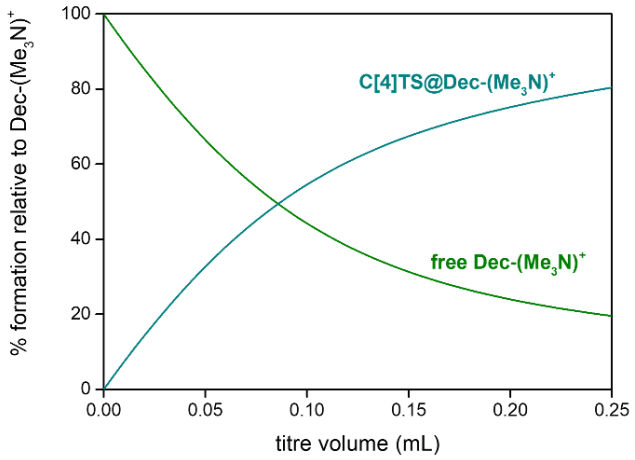

Figure 2. Species distribution diagram computed with HySS by using a guess/expected stability constant based on values reported for similar systems (C_H = 3.00 mM, C_G = 0.30 mM)

An example of calorimetric titration of a C4TS solution into the single charged guest solution and the corresponding dilution/blank experiment are shown in *Figure 3a* and *3b*, respectively. Both measurements started with a small 'throwaway' injection (*ca.* 2 µL) to account for the backlash effect occurring in the first injection due to the mechanical properties of the syringe system.[7] Subsequent additions were performed at a constant volume of 8 µL each. *Figure 3a* indicates that both the signals in the thermogram and the shape of the calorimetric curve are well-defined. Conversely, although the peaks are clearly distinct in the blank experiment too (*Figure 3b*), the baseline is here quite noisy due to the small heat associated with the dilution events. The upward-pointing peaks indicate an exothermic process taking place for both experiments in *Figure 3*.

Figure 4 shows the corresponding binding isotherm (integrated net heat values as a function of the molar ratio of the two reagents) whose sigmoidal shape and inflection point may provide a very preliminary indication of the stoichiometry and the binding affinity of the host-guest complex.

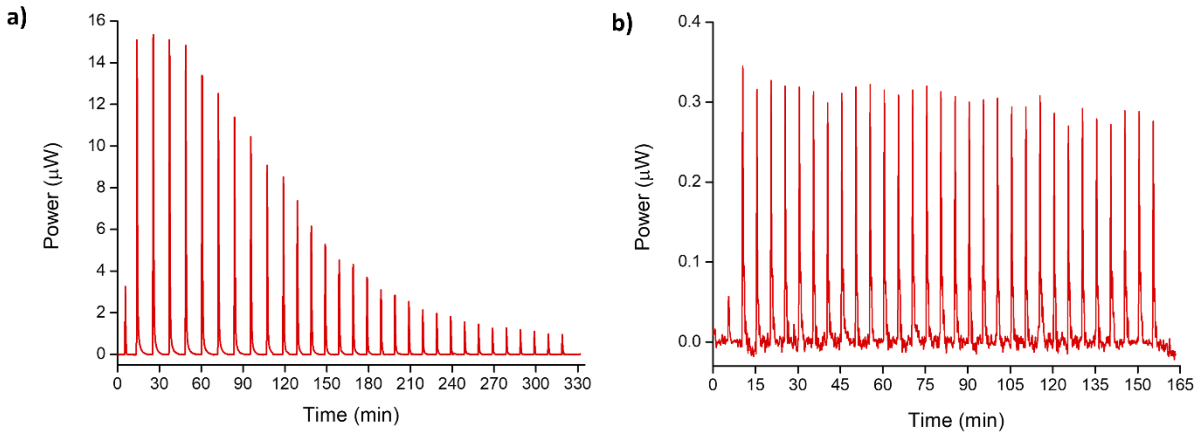

Figure 3. Typical ITC titration of a C4TS solution (3.03 mM) into a Dec-$(Me_3N)^+$ solution (0.24 mM) (*a*) or a phosphate buffer solution (50 mM, pH 6.70, blank/dilution experiment) (*b*) at 25 °C

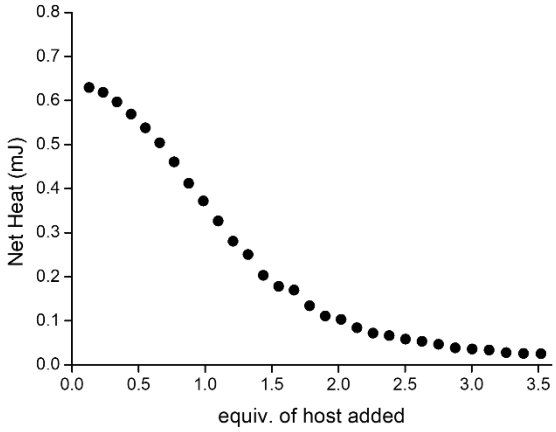

Figure 4. Molar ratio plot (net heat curve) for the C4TS – Dec-$(Me_3N)^+$ system

The non-linear least-squares analysis of the net heat data through the HypCal software[16] allowed for the accurate determination of the exact stoichiometry of the host-guest complex, the conditional binding constant and the thermodynamic parameters which refers to the equilibrium in *Equation 1*:

$$H + G \rightleftarrows HG \qquad \textit{Equation 1}$$

The macrocycle forms a 1:1 complex species with the monocationic guest as can be expected by the structural features of both the host and the guest (i.e., the host cavity is unable to include more than one guest molecule, *Figure 5*). Other stoichiometries and their combinations have been included in the chemical model but they were always rejected by the software.

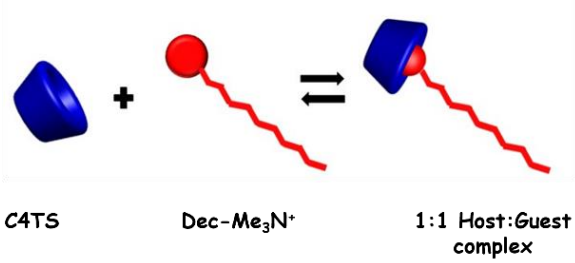

Figure 5. Graphical representation of the 1:1 host-guest equilibrium

The data refinement output (*Figure 6*) reveals a good repeatability of the performed titrations and a satisfactory overlap between the experimental and calculated heat values. Noteworthy, the appropriate experimental design allowed us to optimize the formation of the complex during the titration (almost 90% of the complex species is formed at the end of the experiment).

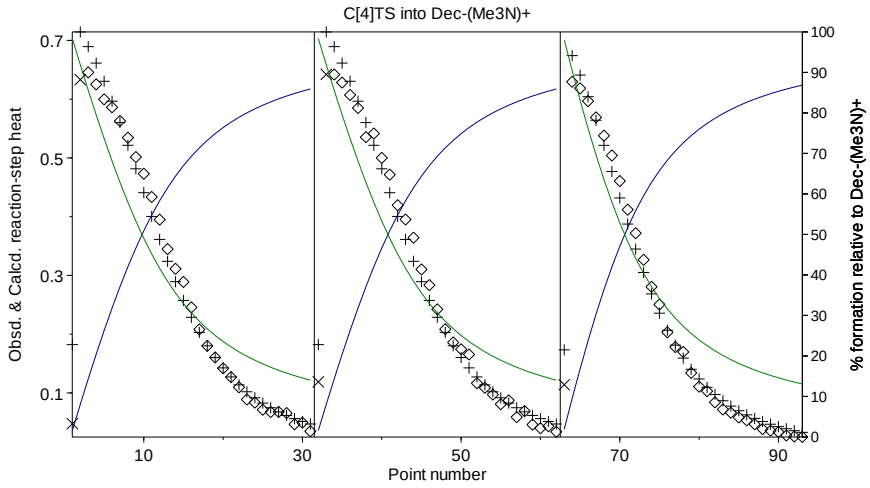

Figure 6. HypCal output (◊: Q_{obs}; +: Q_{calc}) obtained from the analysis of the net heat of reaction (raw reaction heat – dilution/blank heat) for the formation of the C4TS@Dec-$(Me_3N)^+$ complex. Data from three different titrations are analyzed together. The species distribution diagram [C4TS@Dec-$(Me_3N)^+$ complex (blue), free guest (green)] is also calculated by the software.

The refined parameters, namely the conditional stability constant and the standard enthalpy change values obtained from data collected and treated either in Catania or Strasbourg for the formation of the 1:1 host-guest complex at 25 °C and pH 6.70, are shown in *Table 1*. Both the refined parameters are accurate, with small uncertainties and nicely fit the expected values for similar supramolecular systems in water.

Table 1. Conditional stability constant and standard enthalpy change values for the C4TS@Dec-$(Me_3N)^+$ complex formation at 25 °C and pH 6.70 (phosphate buffer, 50 mM)

Lab	Species	Log K[a]	ΔH^0 [a,b]
Catania	HG	4.12 (4)	-37.73 (1)
Strasbourg	HG	4.16 (5)	-31.4 (8)

[a] standard deviation in parenthesis; [b] in kJ mol $^{-1}$

The *C4TS – Dec-$(Me_3N)_2^{2+}$* system

The experimental set-up was first defined to optimize the conditions to be used to run the calorimetric measurements; an example of titration simulation is shown in *Figure 7*. The structural features of the two reacting molecules and the literature reports on similar systems let us hypothesize the formation of two host-guest complex species described by the equilibria in *Equations 2 and 3*.

$$H + G \rightleftarrows HG \qquad \textit{Equation 2}$$

$$HG + H \rightleftarrows H_2G \qquad \textit{Equation 3}$$

Once the 1:1 complex is formed, the second cationic moiety at the end of the alkyl spacer of the guest is still able to interact with a further host molecule to form a capsular structure in which two calixarene cavities complex both the guest charged ends (*Figure 8*).

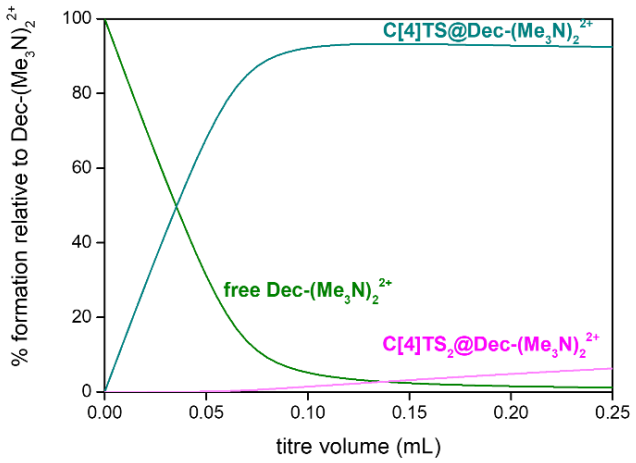

Figure 7. Species distribution diagram computed with HySS by using guess/expected stability constants based on values reported for similar systems (C_H = 3.00 mM, C_G = 0.15 mM)

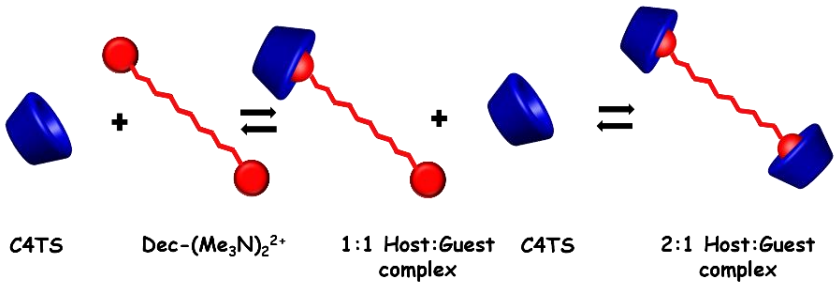

Figure 8. Graphical representation of the 1:1 and 2:1 host-guest equilibria

Typical calorimetric titration and dilution experiments for the C4TS- Dec-$(Me_3N)_2^{2+}$ system are shown in *Figure 9* and the corresponding molar ratio plot is displayed in *Figure 10*. Again, all titrations started with a small throw-away injection of titrant (2 µL) and continued with subsequent injections of 8 µL each.

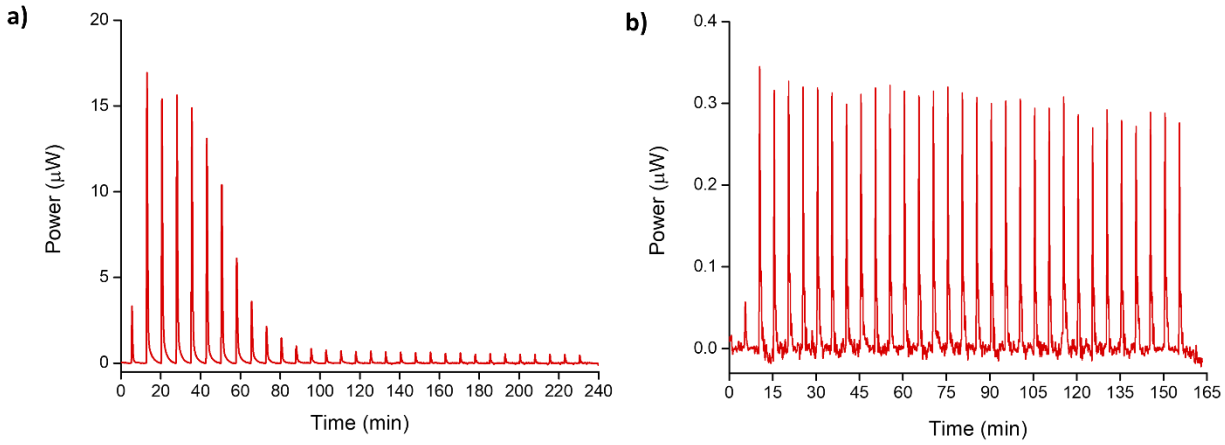

Figure 9. Typical ITC titration of C4TS solution (3.00 mM) into a Dec-$(Me_3N)_2^{2+}$ solution (0.15 mM) (*a*) or a phosphate buffer solution (50 mM, pH 6.70, blank/dilution experiment) (*b*) at 25 °C

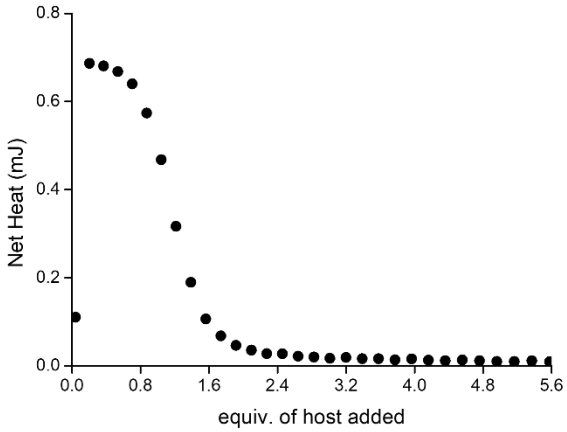

Figure 10. Molar ratio plot (net heat curve) for the C4TS – Dec-$(Me_3N)_2^{2+}$ system

The binding process between the C4TS and Dec-$(Me_3N)_2^{2+}$ is exothermic as indicated by the positive peaks in the calorimetric curve. However, the magnitude of the heat released drops dramatically to very small values after the addition of one equivalent of C4TS to the guest solution. An example of curve fitting performed by HypCal is shown in *Figure 11*. Despite the apparently satisfactory overlap between the experimental and calculated heat values, the refined parameters (especially the ΔH^0 values) have large errors and are highly correlated with each other. This disappointing but not surprising outcome can be attributed to the significant difference in the magnitude of the heat released/recorded when the first or the second calixarene unit is complexed to the double-headed guest. As can be inferred by the different regions of the calorimetric curve (*Figures 9a* and *10*), the heat developed in the second complexation step is too small for an accurate data refinement.

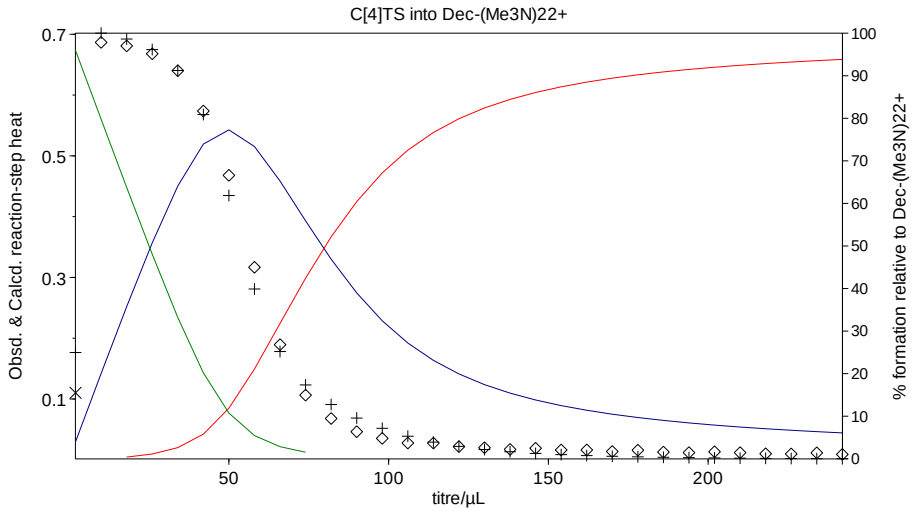

Figure 11. HypCal output (◊: Q_{obs}; +: Q_{calc}) obtained from the analysis of the net heat of reaction (raw reaction heat – dilution heat) for the formation of the C4TS@Dec-$(Me_3N)_2^{2+}$ and $C4TS_2$@Dec-$(Me_3N)_2^{2+}$ complex species. The species distribution diagram [C4TS@Dec-$(Me_3N)_2^{2+}$ complex (blue), $C4TS_2$@Dec-$(Me_3N)_2^{2+}$ complex (red), free guest (green)] is also calculated by the software.

A possible strategy to handle disparities in experimental values obtained from calorimetric titrations would require assigning lower weights to the data points in the second part of the titration curve to reduce the "role/contribution" of potentially less reliable data points in the curve fitting process. However, there are arguments both for and against this approach, likely due to concerns about possible bias introduced by assigning different weights to data points.[16,17]

An alternative and most effective procedure is to split the calorimetric curve into two portions and choose the concentrations of titrant and titrate (and thus their final ratio) in such a way that the heats generated in the second titration would be somehow comparable to those from the first titration curve. The simultaneous refinement of two titration curves using the suitable software will then produce more accurate and reliable results. Treating the two calorimetric curves together offers the advantage of easily facing intricate chemical systems in which more species are in equilibrium with each other. Overall, the so-called global analysis of multiple titration curves not only helps in handling disparities in the calorimetric experimental data collected but also ensures that the complexity of multi-species equilibria is properly addressed in the data refinement process.

Accordingly, a new experimental design was proposed to optimize the formation of each complex species which also takes into proper account the heat differences found for the two complexation events (*Figure 12*). Examples of calorimetric titration of C4TS into the dicationic guest at conditions that highlight the formation of the desired species (HG or H_2G) are shown in *Figures 13* and *14*, respectively.

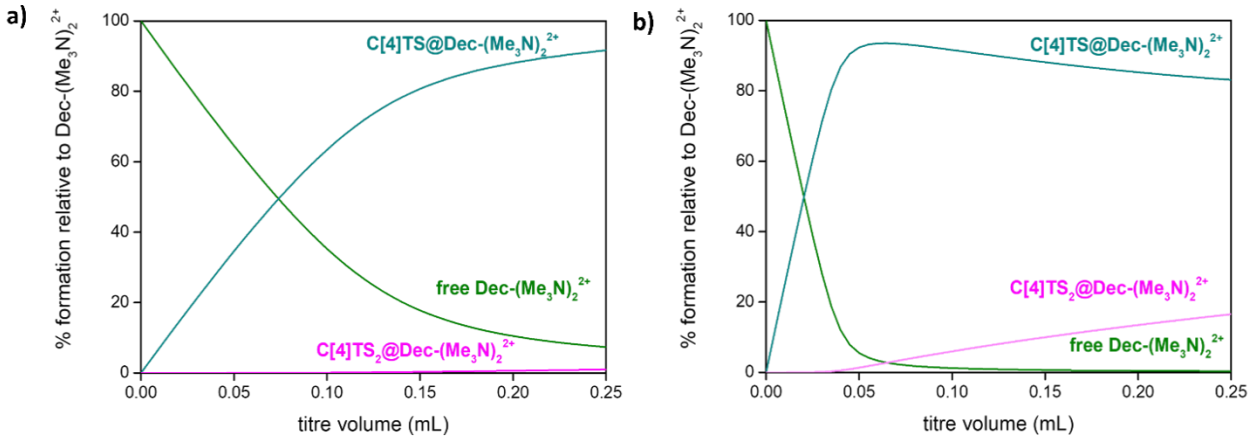

Figure 12. Species distribution diagram computed with HySS by using guess/expected stability constants based on values reported for similar systems. *a)* C_H = 1.00 mM, C_G = 0.15 mM; *b)* C_H = 11.5 mM, C_G = 0.45 mM

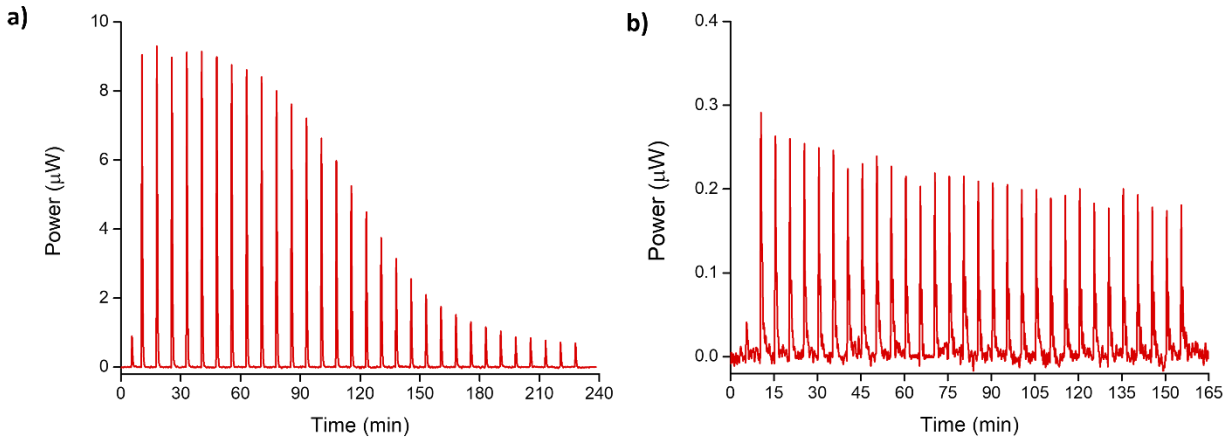

Figure 13. Typical ITC titration of C4TS solution (1.10 mM) into a Dec-$(Me_3N)_2^{2+}$ solution (0.15 mM) (*a*) or a phosphate buffer solution (50 mM, pH 6.70, blank/dilution experiment) (*b*) at 25 °C

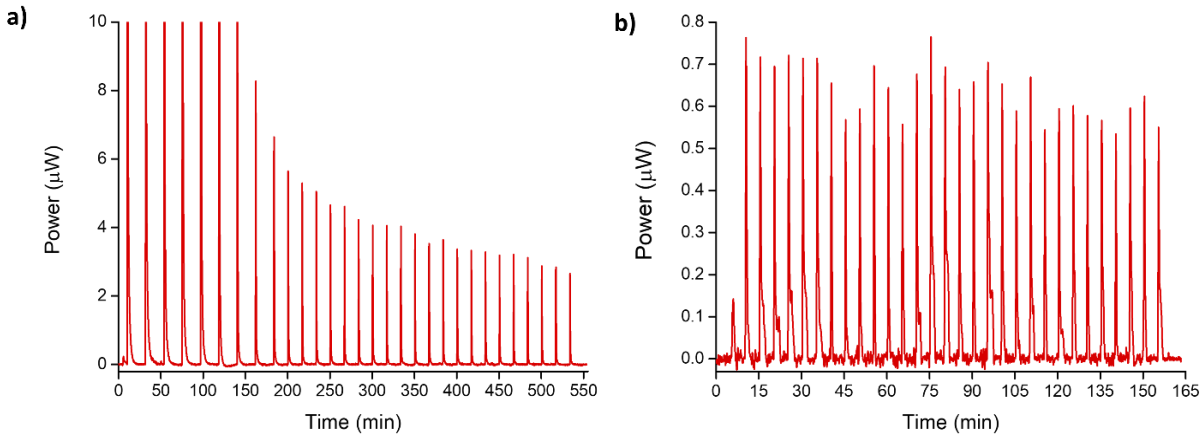

Figure 14. Typical ITC titration of C4TS solution (11.58 mM) into a Dec-$(Me_3N)_2^{2+}$ solution (0.45 mM) (*a*) or a phosphate buffer solution (50 mM, pH 6.70, blank/dilution experiment) (*b*) at 25 °C

The chosen conditions ensured the full formation of the HG complex in the first 5-6 injections for the "second" titration (*Figure 14a*). The corresponding signals in the thermogram are out of the power scale as the released heat is too high and, consequently, these points will be excluded from the data refinement. *Figure 15* shows the binding isotherms (net heat curves) for the two titration sets. Remarkably, the careful design of ITC experiments, by appropriately adjusting concentrations and volume of injections, allowed us to record an appropriate number of data points describing the formation of each of the two complex species and, at the same time, to achieve comparable observed heat values for both the complexation events.

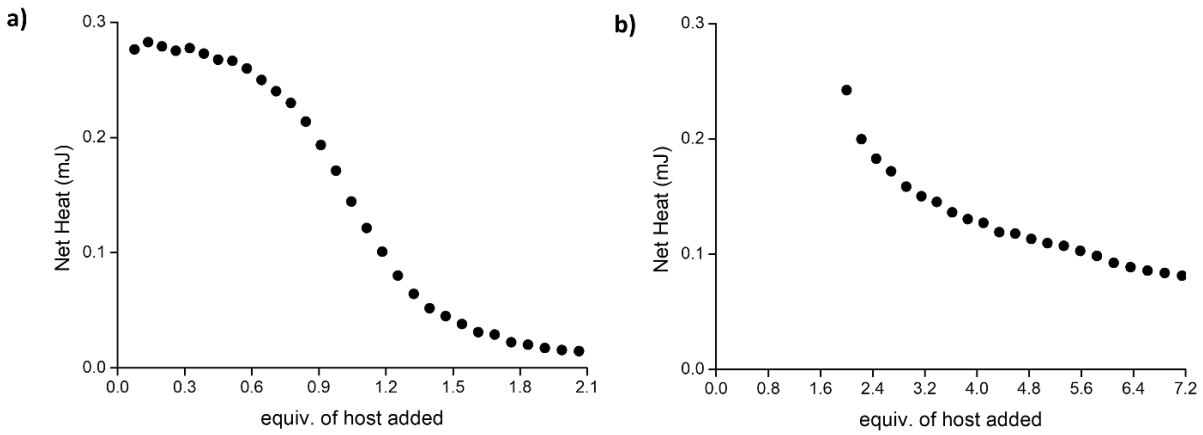

Figure 15. Molar ratio plots (net heat values) obtained for the first and the second titration curve for the C4TS – Dec-$(Me_3N)_2^{2+}$ system

The simultaneous refinement of the net heat data (*Figure 16*) from a set of 8 different titrations performed at different experimental conditions (see *Table 3* in the Experimental section) allowed the determination of the species, conditional binding affinities and thermodynamic parameters listed in *Table 2*. Results obtained through the analysis of datasets from the Catania and Strasbourg labs are shown for the sake of comparison.

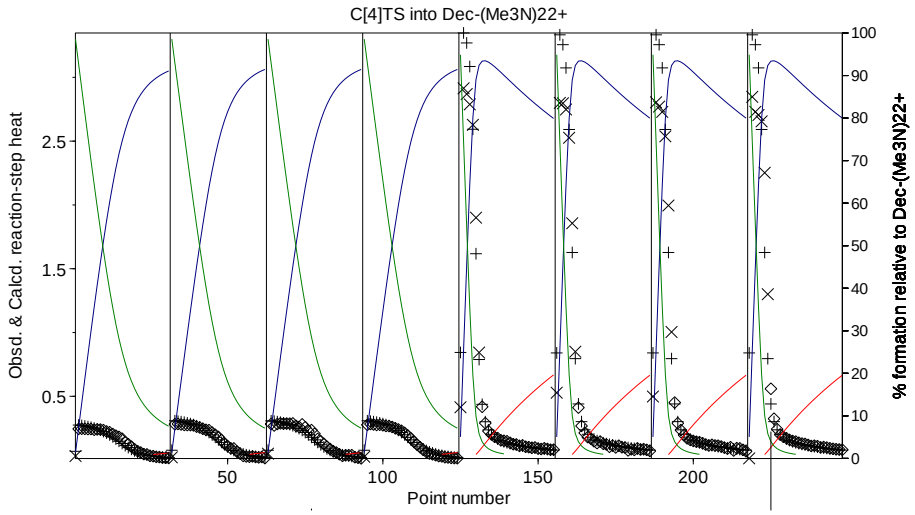

Figure 16. HypCal output (◊: Q_{obs}; +: Q_{calc}) obtained from the analysis of the net heat of reaction (raw reaction heat – dilution/blank heat) for the formation of the C4TS@Dec-$(Me_3N)_2^{2+}$ and $C4TS_2$@Dec-$(Me_3N)_2^{2+}$ complex species. Data from eight different titrations, carried out at different conditions (see Table 3) are analyzed together. The species distribution diagram [C4TS@Dec-$(Me_3N)_2^{2+}$ complex (blue), $C4TS_2$@Dec-$(Me_3N)_2^{2+}$ complex (red), free guest (green)] is also calculated by the software.

Table 2. Conditional stability constant and standard enthalpy change values for the formation of the C4TS – Dec-$(Me_3N)_2^{2+}$ complexes at 25 °C and pH 6.70 (phosphate buffer, 50 mM)

Lab	Species	Log K[a]	ΔH^0 [a,b]
Catania	HG	4.99 (4)	-37.31 (1)
	H_2G	2.1 (1)	-38.24 (4)
Strasbourg	HG	5.10 (4)	-30.2 (4)
	H_2G	1.9 (1)	-51.99 (5)

[a] standard deviation in parenthesis; [b] in kJ mol $^{-1}$

Unlike what was observed in the case of a single titration encompassing the formation of both HG and H_2G species (*Figure 10*), splitting the calorimetric curve into two different titration steps to be refined together with the proper software allowed us to obtain both a satisfactory overlap between the experimental and calculated heat values and more accurate and reliable results in terms of binding constants and enthalpy changes exhibiting small errors. The uncertainty on the log K value determined for H_2G might look a bit larger and can be attributable to the small value of the binding constant determined for this complex which makes difficult the formation of large % of this species during the titration.

The complexation of both the Dec-$(Me_3N)^+$ and the Dec-$(Me_3N)_2^{2+}$ molecules by the calixarene receptor in buffered aqueous solution are in all cases enthalpy favoured and driven processes as a result of favourable interactions (mostly electrostatic, cation-π and CH–π) between the positively charged guests and the host; however, a detailed discussion on the driving forces of the binding reactions is beyond the scope of the present document.

General comment on the Catania *vs.* Strasbourg refined parameters

The log K values obtained by the two different labs for both host-guest systems show great reproducibility (even in the significant digits) considering the difference in the operator, instrument model (Nano-ITC, TA Instruments *vs.* ITC200, GE Healthcare), calorimeter setup (sample cell volume: 1 mL *vs.* 0.2 mL; typical injection volume: 8 µL vs. 1 or 2 µL) and laboratory settings (room having double walls with rock wool, no windows, double door and fine control of the temperature by two independent air conditioning systems[7] *vs.* non air-conditioned room). These results highlight the great significance of the design and proper execution of the experiment, which ultimately affects the shape of the binding isotherm and thus the determination of species and stability constants.
The differences observed in the refined ΔH^0 values could be ascribed to differences in the laboratory settings and, more likely, to the diverse response/accuracy of the instruments as a consequence of a different calibration operation. The Strasbourg team is currently performing some chemical calibration experiments following the recommended procedures.[7] Once the calibration factor is determined, data from the Strasbourg lab will be corrected accordingly (and possibly further titrations will be carried out with the newly calibrated instrument) and the results compared again with the Catania ones as well as with those provided meanwhile by the other labs participating to the exercise.

Conclusions

Within the NECTAR COST Action (CA18202), the WG2 - Task Group 5 aimed to describe and critically discuss experimental and data fitting aspects concerning the speciation and thermodynamic characterization of solution equilibria dealing with multiple host-guest complexes by making use of titration calorimetry. Proper experimental conditions/design and data treatment procedures are key factors in determining reliable equilibrium constant and enthalpy change values for a complete thermodynamic picture of the solution equilibria.

Using a simple host-guest system able to form either 1:1 or 1:1 and 2:1 complex species, the Task Group 5 focused on the rational experiment design and appropriate analysis of calorimetric curves; this work highlighted the importance of using models which include all the equilibria occurring in solution and the exact stoichiometry of the species formed rather than resorting to ambiguous “binding models” often refining unnecessary parameters such as the *n* value.

The preliminary results described in this document will be complemented and critically compared to those obtained by the other participating research groups (thus benefiting from a variety of instruments, software and expertise) to prepare detailed guidelines for

- improving the general knowledge of isothermal titration calorimetry as a valuable technique for the determination of the overall binding parameters of sometimes complicated solution equilibria
- providing recommendations on how to properly design an experiment and analyse its data so as to increase the reproducibility, accuracy and reliability of the results that are intended to be published.

Experimental

Materials

The host (H), *p*-sulfonato-calix[4]arene (C4TS), the guests (G), decyltrimethylammonium bromide (Dec-$(Me_3N)^+$ Br^-) and decamethonium bromide (Dec-$(Me_3N)_2^{2+}$ $2Br^-$), sodium phosphate monobasic and sodium phosphate dibasic were purchased from Sigma-Aldrich. The buffer solution (phosphate buffer, 50 mM, pH 6.70) was prepared by weighing equimolar amounts of the two phosphate salts; the pH of the obtained buffer solution was checked potentiometrically. Both host and guests were dried at 100 °C for 2 hours to remove possible hydration water and the stock solutions were prepared by dissolving the properly weighed amounts in 50 mM phosphate buffer (pH 6.70). The buffer was used to keep the calixarene in the penta-anionic form and to minimize any contribution resulting from the interaction of either the host or the guests with the proton. High-purity water (Millipore, Milli-Q Element A 10 ultrapure water) and A-grade glassware were employed throughout.

ITC titrations – CaSAC Lab, University of Catania

ITC titrations were carried out at 25 °C with a nano-isothermal titration calorimeter Nano-ITC (TA Instruments, USA) having an active cell volume of 0.988 mL and equipped with a 250 µL injection syringe. The calorimeter was calibrated chemically by a test HCl/TRIS reaction according to the reported procedure[7] and further checked through an electrical calibration. Measurements were run in the overfilled mode which does not require any correction for liquid evaporation and for the presence of the vapor phase.[15] The reference cell was always filled with ultrapure water. Before each titration experiment, all solutions were degassed with gentle stirring under vacuum for about 15 min. The reaction mixture in the sample cell was stirred at 250 rpm during the titration and the injection time intervals were chosen to guarantee equilibrium conditions before each subsequent addition.

C4TS – Dec-$(Me_3N)^+$ system: ITC measurements for the HG complex formation were carried out by titrating an aqueous buffered solution of C4TS into an aqueous buffered guest solution (*Table 3*).

C4TS – Dec-$(Me_3N)_2^{2+}$ system: ITC measurements for an overall screening including the formation of both the expected complex species were initially carried out by titrating an aqueous buffered solution of C4TS (3.00 mM) into an aqueous buffered guest solution (0.15 mM). Subsequently, proper experimental conditions were selected to suitably investigate the two different complexation equilibria and highlight the formation of the HG or the H_2G species (*Table 3*). For each titration, an appropriate number of data points was collected to obtain a satisfactory fit of both the first and last portion of the curve; the two portions were then analyzed together to obtain the final parameter values.

Table 3. Experimental conditions for the calorimetric titrations carried out in Catania for the C4TS – guest systems at 25 °C and pH 6.70 (phosphate buffer, 50 mM)

Guest	Complexation step	Number of titrations	Cell (guest, mM)	Burette (host, mM)	Injection volume (uL)	Number of injections	Final host/guest ratio
Dec-(Me$_3$N)$^+$		3	0.24	3.03	2 8	1 30	3.5
Dec-(Me$_3$N)$_2^{2+}$	Overall screening	1	0.15	3.00	2 8	1 30	5.6
	1st step	4	0.15	1.10	2 8	1 30	2.0
	2nd step	4	0.45	11.58	2 8	1 30	7.2

The heats of dilution for all the examined systems were determined in separate blank experiments by titrating solutions of C4TS into a solution containing phosphate buffer only. The power curve was integrated by using the NanoAnalyze software (TA Instruments, USA) to obtain the gross heat evolved in the reaction. The net heats of reaction were obtained by subtracting the heat evolved in the blank experiments.

ITC titrations – RePSeM Lab, University of Strasbourg

ITC titrations were carried out at 25 °C with a nano-isothermal titration calorimeter ITC200 (GE Healthcare, USA) having an active cell volume of 0.2022 mL and a 50 µL injection syringe. Measurements were run in the overfilled mode. The reference cell was always filled with ultrapure water. Prior to the experiments, all solutions were degassed by stirring under vacuum for about 15 min. The reaction mixture in the cell was stirred at 500 rpm; the injection time intervals always guaranteed equilibrium conditions before each subsequent addition.

ITC measurements for examining the complex formation equilibria for both *C4TS – Dec-(Me$_3$N)$^+$* and *C4TS – Dec-(Me$_3$N)$_2^{2+}$* systems were carried out as described above for the Catania Lab using the experimental conditions in *Table 4*.

The heats of dilution, determined in separate blank experiments by titrating solutions of C4TS into a solution containing phosphate buffer only, resulted to be negligible or not detectable and consequently the raw heat values were used “untreated” for data fitting purpose. The power curve was integrated by using the Origin built-in software.

Table 4. Experimental conditions for the calorimetric titrations carried out in Strasbourg for the C4TS – guest systems at 25 °C and pH 6.70 (phosphate buffer, 50 mM)

Guest	Complexation step	Number of titrations	Cell (guest, mM)	Burette (host, mM)	Injection volume (uL)	Number of injections	Final host/guest ratio
Dec-(Me$_3$N)$^+$		2	0.21	2.94	0.5 2	1 19	3.0
Dec-(Me$_3$N)$_2^{2+}$	Overall screening	1	0.11	2.94	0.5 1	1 38	6.0
	1st step	3	0.11	1.12	0.5 1	1 38	2.5
	2nd step	3	0.34	11.90	0.5 1	1 38	7.0

Data analysis

The heats of reaction (corrected for the dilution/blank effect, if any) were analyzed by HypCal,[16] a software specifically designed for the determination of equilibrium constant and formation enthalpy values of complex species formed in solution through a non-linear least-squares minimization of the function

$$U = \Sigma(Q_{obs} - Q_{calc})^2$$

where Q_{obs} is the observed heat for a given reaction step, while Q_{calc} is calculated as

$$Q_{calc} = -\Sigma(\delta n \Delta H^0)$$

where δn is the change in the number of moles of a reaction product and ΔH^0 is the molar formation enthalpy of the reaction product. The summation is carried out over all the reaction steps of the specific chemical system. The squared residuals $(Q_{obs} - Q_{calc})^2$ are summed over all the titration points. For each host-guest system, log K values and thermodynamic parameters are determined by simultaneously analyzing calorimetric data obtained from multiple titrations.

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