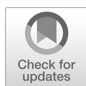

# Chapter 15

# The Use of ITC and the Software AFFINImeter for the Quantification of the Anticoagulant Pentasaccharide in Low Molecular Weight Heparin

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## Abstract

In this chapter, we describe an original protocol based on ITC experiments and data analysis with the software AFFINImeter to get information of heparin-AT interactions relevant for the elucidation of the anticoagulant activity of heparins. This protocol is used to confirm the presence of the bioactive pentasaccharide with anticoagulant activity in heparins and to determine the amount of this pentasaccharide in the sample. Here we have applied this protocol to the characterization of low molecular weight heparins.

**Key words** ITC, AFFINImeter, Low molecular weight heparin, Pentasaccharide, Antithrombin, Data analysis, Heterogeneous mixtures, Anticoagulant activity, Biosimilars

## 1 Introduction

Isothermal titration calorimetry (ITC) has long been considered the gold standard for the thermodynamic characterization of molecular recognition events [1]. Moreover, the ITC technique offers much more information than just the thermodynamic profile of interactions; protocols based on a careful experimental design together with the use of appropriate software tools for ITC data analysis are now making possible the elucidation of detailed structural and kinetic information from standard ITC titrations and the explanation of mechanisms involved in complex binding events of biological relevance [2–4].

As an illustrative example, in this chapter we describe an original protocol based on ITC experiments and data analysis with the software AFFINImeter [5] toward the evaluation of anticoagulant activity of low molecular weight heparins (LMWHs). Ultimately, this protocol is of value in the identification and characterization of biosimilars.

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Heparin is a naturally occurring linear heterogeneous polysaccharide, well known for being the most widely used anticoagulant drug in the world [6]. The anticoagulant activity of heparin is mediated primarily through the interaction of a specific pentasaccharide sequence present in the polysaccharide chain with the serine protease antithrombin (AT). Therefore, the anticoagulant potency of heparin is directly related with the amount of this bioactive pentasaccharide (BP) existing in the sample [6, 7].

LMWHs are derived from unfractionated heparin by controlled depolymerization processes, fractionation methods, or both. Thus, once prepared, the characterization of the LMWHs in terms of structure and anticoagulant activity is of particular practical interest for their successful use as antithrombotic drugs [6, 8]. The difficulty of determining the amount of BP present in LMWHs (and heparins in general) arises from the inherent binding heterogeneity of the saccharide and its interaction with AT; AT binds BP with high, nanomolar affinity, but it also binds other heparin sequences with lower affinity [9].

Herein we describe a protocol based on ITC experiments of heparin-AT interactions that provide information of the high-affinity and low-affinity binding events. Analyses of these experiments with the software AFFINImeter are used to determine the amount of BP present in heparins. The main steps of the protocol consist of (1) the realization of various ITC titrations of LMWH into AT at different experimental conditions of concentration, optimized to monitor high- and low-affinity interactions; (2) similarly, the realization of ITC experiments with unfractionated heparin (UFH) as control experiment; and (3) global analysis of these isotherms with the advanced tools of the AFFINImeter software. Among these tools are the use of a tailored binding model that considers the presence of two competing ligands (the BP and low-affinity polysaccharide sequences) in the syringe binding to AT in the calorimetric cell (Fig. 1), the incorporation of two additional fitting parameters in the analysis that account for the molar fractions of BP and low-affinity sequences present in UFH and LMWH samples, the global fitting of all the isotherms using the tailored binding model mentioned, and sharing common binding parameters (enthalpies and equilibrium constants for the same thermodynamic processes) among isotherms. In this way, the protocol provides confirmation of the presence of the BP in the LMWH and the thermodynamic characterization of its binding to AT. Besides, it provides the fraction of BP present in UFH and LMWH.

This protocol is for the particular case of ITC analysis of heparin-AT interactions; it is worth mentioning the publication of a more general protocol for the ITC analysis of glycosaminoglycan-protein interaction that offers additional practical information to this chapter [10].

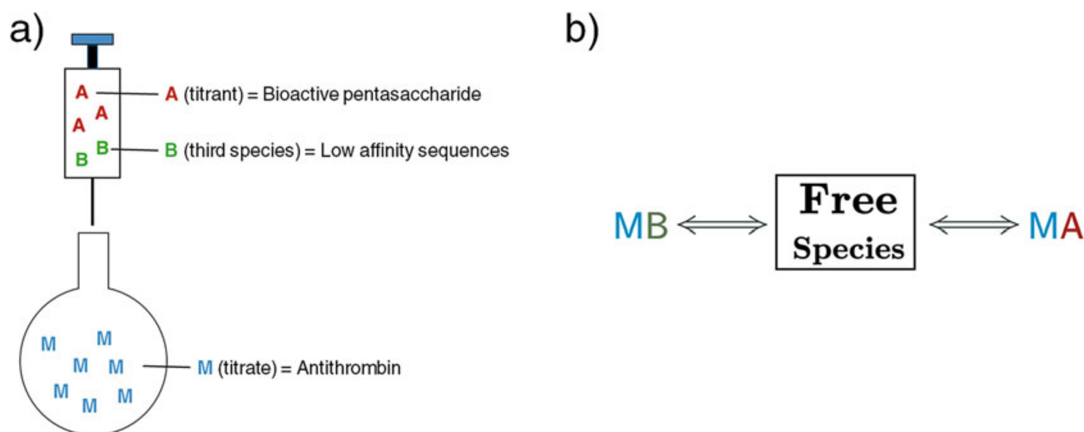



**Fig. 1** (**a**) Schematic representation of the ITC titration experiment of heparin (LMWH or UFH) into AT. AFFINImeter uses a letter code to describe the species participating in the interaction where M is the titrate (AT), A is the titrant (pentasaccharide), and B is the third species that represent the sequences in heparin that bind AT with low affinity. (**b**) Competitive binding model used in the analyses consisting in the formation of the complexes MA (pentasaccharide-AT) and MB (low-affinity sequence-AT) from FS (free species). As an illustrative example, in this chapter, we describe an original protocol based on ITC experiments and data analysis with the software AFFINImeter toward the structural characterization and evaluation of anticoagulant activity of low molecular weight heparins (LMWHs). Ultimately, this protocol is of value in the identification and characterization of biosimilars

## 2 Materials

Prepare all buffered solutions of LMWH, UFH, and AT using ultrapure water. The solutions are prepared just prior to use, and all the experiments are performed the same day and with the same AT solution (*see* **Note 1**). All the solutions must be degassed before being placed in the ITC instrument to avoid the presence of air bubbles. All the ITC experiments are performed at 25 °C.

### *2.1 Titrants*

LMWH (Av. MW = 4300 g mol$^{-1}$) is provided as solution sample ~100 mg mL$^{-1}$ in water; UFH, unfractionated heparin sodium (Av. MW = 12,000 g mol$^{-1}$), is provided as a solid white powder.

### *2.2 Titrate*

AT (MW = 58,000 g mol$^{-1}$) is a commercial sample from Chromogenix consisting on a lyophilized powder in glass vials (1 mg AT, containing 12 mg of albumin and 9 mg of NaCl per vial).

### *2.3 Buffer Used to Prepare the Solutions*

Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 10 mM, pH 7.4 containing 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant tween 20 (*see* **Note 2**).

### *2.4 ITC Equipment*

MicroCal ITC200, highly sensitive low volume isothermal titration calorimeter. Cell volume, 202.9 μL; syringe volume, 40 μL.

## 3 Methods

### 3.1 Preparation of LMWH, UFH, and AT Solutions

1. Prepare a 16.2 μM AT solution adding 1 mL of buffer into the AT vial containing 1 mg of the protein. Shake gently the vial to obtain a homogeneous solution (*see* **Note 3**).
2. Prepare a 0.5 mM UFH solution dissolving 6 mg of UFH per 1 mL of buffer in a clean, empty vial. Prepare one more UFH solution at 0.25 mM, by dilution of the 0.5 mM solution with the necessary amount of buffer (*see* **Note 4**).
3. Prepare a 2 mM LMWH solution by dilution of the original LMWH sample (100 mg $mL^{-1}$ in water) with the necessary amount of buffer in a clean, empty vial. Prepare two more LMWH solutions at 1.1 mM and 0.5 mM by dilution of the 2 mM solution with the necessary amount of buffer.

### 3.2 Experimental Setup for ITC Experiments

1. Load the ITC sample cell with 200 μL of the titrate (16.2 μM AT solution) and the syringe with 40 μL of the titrant (UFH or LMWH solutions). Perform the ITC experiment with a first injection of 0.5 μL followed by 15 injections of 2.49 μL (*see* **Note 5**).
2. Clean cell and syringe thoroughly before the next titration using the standard protocol for the ITC instrument.
3. Perform two titrations of UFH into 16.2 μM AT solution using the UFH solutions 0.5 mM and 0.25 mM and three titrations of LMWH into 16.2 μM AT solution using the LMWH solutions 2 mM, 1.1 mM, and 0.5 mM.
4. A dilution blank titration of UFH/LMWH into buffer can be performed for each titration (*see* **Note 6**).

### 3.3 Data Analysis with the Software AFFINImeter

1. Upload the raw ITC files into the AFFINImeter software to perform the automatic processing of the thermograms and to obtain the corresponding binding isotherms (*see* **Note 7**). This processing includes thermogram baseline correction, noise removal, peak integration, and determination of the corresponding uncertainties. Save the isotherms into the "dataseries" folder.
2. Select one dataseries (isotherm), and edit the experimental settings to consider the presence of two ligands mixed in the syringe, BP, and low-affinity sequences (Fig. 2). By default, the titrant is represented by the species "A" in AFFINImeter, and the titrate is represented by the species "M." Include the second ligand as species "B" at the same concentration as for the species "A" (*see* **Note 8**). Follow the same procedure for all the isotherms.

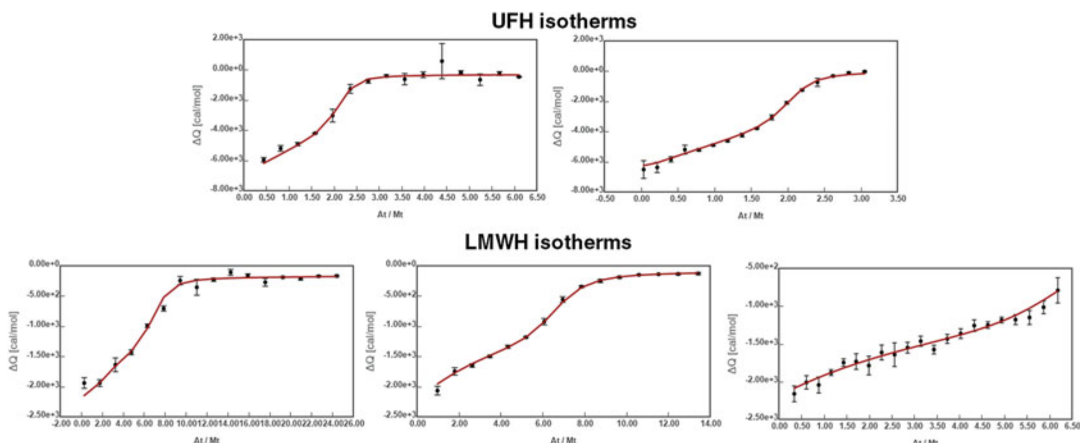
**Fig. 2** Example of the dataseries corresponding to the ITC titration of LMWH (1.1 mM) into AT (16.2 mM) and how it must be edited for data analysis; the fields corresponding to the co-solute should be completed with the name of the species B (i.e., low-affinity sequences) and the concentration of LMWH used in the experiment (1.1 mM) placed in the syringe

3. Generate a global fitting project including the five isotherms, and use the same binding model, MB↔FS↔MA, to fit all of them; the model consists of two equilibria to reflect the formation of the BP-AT complex (MA) from free species (FS) and the formation of the low-affinity sequence-AT complex (MB) from FS (*see* **Note 9**).
4. In the analysis, the following fitting parameters are considered: binding constant ($K_A$) and binding enthalpy ($\Delta H$) of each equilibrium as well as the parameters Qdil, rA, and rB of each isotherm (*see* **Note 10**).
5. Include the following restrictions: (a) $K_A$ and $\Delta H$ of the equilibrium FS↔MA are shared parameters between all the isotherms; (b) $K_A$ and $\Delta H$ of the equilibrium FS↔MB, rA, and rB are shared parameters between the isotherms of the three experiments with LMWH and the condition rB = 1 − rA must be included; (c) similarly, $K_A$ and $\Delta H$ of the equilibrium FS↔MB, rA, and rB are common parameters between the isotherms of the two experiments with UFH and the condition rB = 1 − rA must be included (*see* **Note 11**).
6. Run the analysis.

### 3.4 Validation of the Fitting

Checking for the goodness of the fit (GoF) is a necessary step to determine if the result obtained is reliable (Fig. 3). For this, use the following steps:

1. By visual inspection, check if the theoretical curve of each dataseries approaches the measured data.




**Results**

**Table:** reaction parameters

| Reaction | | $K_A$ ($M^{-1}$) | ΔH (Kcal·mol$^{-1}$) |
|---|---|---|---|
| UFH | FS ↔MA | (8.39 ± 0.29)·10$^6$ | -9.42 ± 0.04 |
| | FS ↔MB | (1.52 ± 0.19)·10$^4$ | -14.17 ± 1.73 |
| LMWH | FS ↔MA | 8.39·10$^6$ | -9.42 |
| | FS ↔MB | 1.52·10$^4$ | -3.08 ± 0.41 |

**Table:** correction parameters

| | rA | rB |
|---|---|---|
| UFH | 0.51048 ± 0.00011 | 0.48952 |
| LMWH | 0.15679 ± 0.00006 | 0.84321 |

**Fig. 3** Analysis result of the global fitting of isotherms from titrations of UFH into AT and titrations of LMWHp into AT. The results of fitting parameters are shown in blue; the results of parameters that are linked to other are shown in black

2. Use the GoF value calculated in the analysis. The value of GoF provided by AFFINImeter is normalized; this means that the GoF value for a perfect fit would be 100%.
3. Check the value of each fitted parameter and also the corresponding uncertainty. The uncertainty should be significantly lower than the parameter (at least one order of magnitude lower is commonly accepted). If the parameter converges to the maximum or the minimum value allowed for it, then it is recommended to fix its value or to increase the range allowed for it.
4. Revise the list of local minima provided by AFFINImeter to confirm that the analysis is not overparameterized (*see* **Note 12**).

### 3.5 Result Interpretation

Based on our experience using this protocol to analyze many heparin samples, the following information can be obtained from the fitting:

1. A good fitting confirms that $K_A$ and $\Delta H$ of the equilibrium FS↔MA are the same in UFH and LMWH and confirms the presence of the BP in the LMWH.
2. The values obtained of $K_A$ and $\Delta H$ of the equilibrium FS↔MA provide a good thermodynamic description of the interaction between the BP and AT.

3. The values obtained of rA provide consistent information of the molar fraction of BP present in the UFH and LMWH samples.
4. The values of $K_A$ and $\Delta H$ of the equilibrium FS↔MB and the values obtained of rB are only estimates of the interaction of the low-affinity sequences with AT because of the inherent structural and binding heterogeneity of these sequences.
5. Overall, with this protocol it is possible to determine the percentage of bioactive species in heterogeneous mixtures, being this information key in the analysis of biosimilars.

## 4 Notes

1. The use of the same AT solution freshly prepared (five samples, 1 mL) is to ensure that exactly the same concentration of AT is used in all the experiments.
2. We used this buffer composition to make it coincide with surface plasmon resonance (SPR) experiments we run in parallel with the ITC experiments. But, for the ITC assays, the buffer doesn't necessarily have to include the additives EDTA and surfactant.
3. Since the commercial AT sample contains excipients (albumin and NaCl), it is particularly important to dissolve the full content of the vial to get a homogeneous solution to be used in all the ITC experiments.
4. The UFH sample can be prepared in higher amount, and the remaining volume can be stored at 4 °C to be used in future ITC assays with other LMWHs.
5. The experimental setup has been optimized using the simulation tool of the software AFFINImeter. It has helped to define the conditions under which the two binding events are differentiated in the isotherms.
6. It is always advisable to perform the blank dilution experiment. However, based on our experience and others [10], titration of the commercial UFH and the LMWH into buffer results in negligible heat release.
7. AFFINImeter allows uploading multiple raw data files simultaneously, for a fast raw data processing and generation of all the isotherms. Although these steps are performed automatically, manual processing is also possible.
8. The nominal concentration of A and B indicated in the settings coincides with the concentration of the LMWH/UFH sample; afterwards, the true concentration of A and B is calculated during the analysis.

9. This binding model is available in the default list of binding models of AFFINImeter. Or, it can be created directly using the AFFINImeter tool "Model Builder."
10. The parameters rA and rB account for the molar fractions of BP and low-affinity sequences, respectively, present in UFH and LMWH. The parameter Qdil accounts for potential heat changes due to dilution of the titrant into the titrate solution or buffer mismatch. Herein the heat of dilution of the UFH or LMWH samples is negligible [10], but a heat change effect has been observed most probably due to buffer mismatch (due to the excipients of the AT sample). To correct this effect, the parameter Qdil is set as a fitting parameter in all isotherms.
11. Global analysis yields a more robust result as compared to individual analysis, as it provides the possibility to share common fitting parameters between the binding isotherms. Here, $K_A$ and $\Delta H$ of the equilibrium FS↔MA are common parameters between all the isotherms because it represents the interaction between the pentasaccharide and AT. This restriction is to confirm that the BP is present in the LMWH prepared and therefore it binds with the same thermodynamic profile as the BP of the UFH. $K_A$ and $\Delta H$ of the equilibrium FS↔MB are not necessarily shared between UFH and LMWH isotherms because the structure (and therefore binding thermodynamic properties) of the low-affinity sequences can vary between the two compounds. rA and rB are not shared between UFH and LMWH isotherms because the fraction of BP and low-affinity sequences can (and in fact should) vary between the two compounds. The restriction rB = 1−rA is to impose the condition that the overall amount of LMWH or UFH is the sum of the molar fractions of BP and low-affinity sequences. This condition is an approximation because we don't know the exact molecular weight of the low-affinity sequences.
12. AFFINImeter allows performing multiple independent minimizations for a given analysis, each starting from a different set of random seeds for the fitting parameters. It is recommendable to perform at least 10–20 independent minimizations (repeats in the advance settings menu) to ensure that the final result is not a local minimum and that the employed model is not overparameterized. Ideal analyses are those with high GoF values (close to 100) and several minimizations converging to the same set of parameters. Multiple solutions with similar GoF and different parameter values are suspicious of overparameterization in the employed model.

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