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14. ABSTRACT

Isothermal titration calorimetry (ITC) is a microcalorimetry technique used to study interactions between biomolecules and ligands in aqueous solution. This ARO equipment grant was provided to enable the purchase of an ITC instrument, foster the development of ITC expertise at the recipient institution, and examine the interactions of divalent metal cations with specific proteins using microcalorimetry techniques. An ITC instrument was purchased, specifically the model PEAQ-ITC from the MicroCal division of Malvern Instruments. This is a new

15. SUBJECT TERMS
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Report Title

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Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received	Paper

TOTAL:

Number of Manuscripts:

Books

Received	Book

TOTAL:

Received Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:...... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:...... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):...... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:...... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:...... 0.00

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

SCIENTIFIC PROGRESS

(1) Foreword (optional): Not applicable.

(2) Table of Contents (if report is more than 10 pages): Not applicable.

(3) List of Appendixes, Illustrations and Tables:

The PDF Attachment contains 2 Figures and 1 Table.
Figure 1: ITC data for interaction of Mg2+, Be2+, Ca2+, and Mn2+ with EDTA at pH 6.0.
Figure 2. ITC data for interaction of Be2+ and Mg2+ with NTA at pH 6.0.
Table 1: Summary of ITC data for Figure 1 experiments.

(4) Statement of the problem studied

This award was provided through the DoD HBCU/MI equipment grant program. The entire amount of the award is dedicated to the purchase of one equipment item, the ITC (isothermal titration calorimetry) instrument. ITC is a microcalorimetry technique used to study interactions between biomolecules and ligands in aqueous solution. Prior to this award, there was no ITC instrument within the state of Nevada. The goals for this project are to purchase an ITC, develop expertise in using the ITC, and expose faculty, graduate, and undergraduate students to this technology. The ITC will be used for a variety of research and educational activities. The initial research objectives associated with this award are to use the ITC as a tool to investigate metal-protein interactions, and especially to investigate the interaction of Be2+ ion with GSK3b protein, which is hypothesized to be an unusually stable metal-protein association.

(5) Summary of the most important results

This grant was originally written to purchase a MicroCal iTC200 instrument. Fortunately, we were able to purchase instead the newer MicroCal model PEAQ-ITC, which is the upgraded successor to the iTC200. MicroCal, a division of Malvern Instruments, is one of only two companies that manufacture ITC equipment suitable for biochemical analyses. The MicroCal company released the iTC200 in Oct. 2008, then released the PEAQ-ITC in March 2015. Thus, the new PEAQ-ITC is the first improvement in the ITC product line in almost 7 years. The thermal core of the two units is essentially unchanged. However, the PEAQ-ITC has an integrated washing module, an improved mixing mechanism, improved signal-to-noise performance, and vastly improved software for instrument control and data analysis. The details of the new PEAQ-ITC model had been kept proprietary until the product launch date of March 3, 2015. Although the PEAQ-ITC is a little more expensive than the iTC200, the PI was able to purchase the newer instrument at a reduced price to accommodate the funding available from the ARO grant.

The instrument was installed on June 23-24, 2015 during a two day site visit by the vendor's field engineer. Initial calibration and performance verification runs were conducted. The performance of the thermal core and the quality of the new instrument software were very impressive. However, an intermittent "low vacuum" error was observed during the instrument's wash cycle operation. The appearance of this error forces abrupt termination of the run, so the instrument could not be used reliably for experimental work. Over a period of several months, diagnostic procedures were conducted in collaboration with the vendor's field engineer to troubleshoot the low vacuum problem. A second site visit by MicroCal's field engineer took place on July 8, 2015. A third site visit occurred on August 24, 2015 in which the entire wash module including the vacuum pump was replaced. However, none of these steps solved the problem. The vacuum system and wash module were new features introduced with the PEAQ-ITC model; these had not been part of the older iTC200 systems. Our lab was among the very first to obtain the model PEAQ-ITC, so it is not surprising that our lab had to be at the forefront of field testing and troubleshooting the new system. The field engineer told me that our system was the first he had ever installed, and I noticed that the serial number on our original wash module was 0007, a very low number (I expect that some of the units 0001 to 0006 would have been kept at the vendor's facility for in-house use during product development). So, we were in essence beta-testing a brand new wash module format. As PI, I was highly motivated to figure out the cause of the problem and solve it, and eventually I was able to do so. I made two changes to the system, one involving the physical hardware, and the other with the software.

There is a place in the wash module program where the ITC's microprocessor-controlled internal vacuum pump runs for a 90 second period, then it evaluates the vacuum pressure attained, and if that value meets spec it proceeds. If the spec is not attained, the cycle aborts and an error message is displayed. After careful observation, I had the impression that the pump is just barely able to reach the expected pressure within the specified time, and that this explained the intermittent nature of the problem: sometimes the pressure is adequate and sometimes it is close but not quite enough. So, anything that would improve the vacuum pump performance even slightly might help to solve the problem. I knew that the pump itself was not the problem, because the vendor had replaced the original pump with a new one and that gave no improvement. The PEAQ-ITC wash module contains a trap that collects water and methanol solutions that are used to wash the ITC cell and injection syringe. I reasoned that the vacuum pump would work more effectively with a secondary trap installed downstream of the main trap and upstream of the pump. I discussed this with the vendor's engineers, and they assured me that the geometry of the tubing prevented liquid from getting to the pump, and moreover that this type of pump was designed to be able to tolerate moisture. The cell wash cycle uses only water, whereas the syringe wash cycle uses methanol and water at different steps. Therefore, the liquids in the main trap are water and methanol in varying proportion, depending on which types of washes had been conducted recently. I observed that the methanol, which has a lower vapor pressure, could volatilize under vacuum and create a slightly visible "fog" in the tubing that goes to the pump. The fog represents liquid condensation, and made me think that more liquid goes to the pump under real-life usage conditions than the vendor engineers had anticipated. So, I obtained fittings that were compatible with the specialized small diameter instrument tubing and installed my own customized secondary trap between the main trap and the vacuum pump. I made dust-free desiccant packets from Drierite and placed them in the secondary trap. By pumping dry air rather than wet air, the vacuum pump could attain a high vacuum threshold more rapidly (i. e. reliably within the 90 second time window).

The other innovation I came up with was software-related. I reasoned that the "low vacuum" error message occurs when an internal vacuum reading fails to meet a programmed threshold value. So, I wanted to get into the software code for the instrument control software and re-set the threshold value that triggers the error to be less stringent. I proposed this solution to the vendor's lead engineer, who gave me instructions on how to access this section of code. I changed the "low vacuum" error trigger from -70 kPa to -64 kPa. Note that the actual vacuum pressure attained by the system is unaffected, this software modification simply changes the threshold needed to avoid triggering the cycle abort. These two modifications (installation of the secondary trap and adjustment of the parameter within the instrument control code) have completely solved the problem. At its worst, the wash cycle abort was occurring up to 50% of the time. Since instituting these changes, we have conducted more than 250 runs without any problem. For a while, this was a frustrating situation that involved months of interactions with the vendor, trying different possible solutions, etc. However, now the instrument is working very reliably. Thankfully, the timing for the purchase worked out so that we ended up with the significantly improved PEAQ-ITC instead of the older iTC200 model.

Our research using the ITC to study metal interactions with biomolecules is still at an early stage, but we are proceeding on two parallel tracks. One track is to use model compounds as metal-binding partners, and the second is to use proteins such as GSK3b. So far, more effort has gone into using model compounds such as EDTA (ethylenediaminetetraacetic acid), the well-known metal chelating agent. We have had some difficulty producing purified GSK3b protein in large quantity, so for our first ITC experiments it has seemed prudent to gain experience with the ITC technology using materials that are less precious. We have been using model compounds to validate our techniques, to learn about artifacts that can arise during ITC and strategies to avoid them, to gain expertise with various aspects of data analysis, and to see how varying buffer composition, pH, and run temperature affects experimental outcomes. A sample of the work we have done on the PEAQ-ITC with EDTA in MES (2-(N-morpholino)ethanesulfonic acid) buffer is shown (Figure 1 and Table 1). The ITC results for metal ion binding with EDTA in 100mM MES pH 6.0 produced the expected rank-order binding affinity relationship Mn2+ > Ca2+ > Be2+ > Mg2+. The association of EDTA with manganese or calcium ions is exothermic (producing downward peaks for each injection), whereas the magnesium and beryllium associations are endothermic (upward peaks). In aqueous solution, the Mg2+ and Be2+ ions have strong, well-ordered interactions with water molecules from the solvent; the release of these water molecules upon binding with EDTA is entropically favorable and this drives the reaction. The unfavorable enthalpy (positive deltaH) observed for the binding of these two ions with EDTA arises from the need to break the strong metal-solvent bonds. For Mg2+, deltaH = +22.5 kJ/mol; for Be2+, deltaH = +36.8 kJ/mol (Table 1). This indicates that the metal-oxygen bonds in solvated beryllium are much stronger than those for magnesium. The endothermic nature of the Mg2+-EDTA interaction is known [Kandeel et al., 2013; O'Brien et al., 2015]. The endothermic nature of the Be2+-EDTA interaction, shown here, has not been previously reported. Of the 13 different metal ions examined for EDTA interaction via ITC by Kandeel et al., only Mg2+ was endothermic, showing that this is an unusual characteristic.

Our research focused on Be2+ in particular, as this divalent cation has received the least study and is hypothesized to exhibit interesting behavior in its interactions with biologically relevant macromolecules. The dissociation constant (Kd) that we observed for the interaction of Be2+ with EDTA at pH 6.0 is in good agreement with that expected from literature values. An expected value for this interaction under these conditions is not available directly. However, in a table of metal-EDTA formation constants at 20ºC and 0.1M ionic strength, Be2+ is listed as log Kf = 9.2 [Harris, 1982]. This indicates that the equilibrium constant for the reaction with the fully deprotonated EDTA species (Y4-) = 1.58 x 109. The value of alphaY4- at pH 6 = 2.3 x 10-5, so adjusting the equilibrium constant for pH 6 gives (1.58 x 109)(2.3 x 10-5) = 3.63 x 104, which corresponds to Kd = 2.75 x 10-5 M. This is very close to the Kd = 2.60 x 10-5 M (± 0.08 x 10-5 M) that we observed for Be2+-EDTA at pH 6 (Table 1).

EDTA displayed quantitative, but not qualitative, differences in its interactions with the metal ions. We also wished to employ an ITC model system that could provide a qualitative distinction between Be2+ and its nearest neighbor Mg2+. NTA (nitrilotriacetic acid) is known to interact with Mg2+ only weakly [Souaya et al., 2000]. Using 0.2 mM NTA in 100 mM MES, pH 6.0, we observed a binding interaction for 2 mM BeSO4, but not for 2 mM MgSO4 (Figure 2). For the Be2+-NTA interaction, the observed Kd = 4.77 x 10-5 M (± 0.38 x 10-5 M), and the observed N (binding sites per macromolecule) was 0.509. The N value near one-half indicates that, under the conditions employed, a single Be2+ ion coordinates two NTA molecules to form a 2:1 NTA:metal complex. Among the alkaline earth metals (group IIA), Be2+ and Mg2+ are the "hardest" (HSAB theory) and have the smallest ionic radii, but Mg2+ has a principal coordination number of 6, whereas Be2+ coordinates 4 ligands with tetrahedral geometry. Our ITC results suggest that a pair of carboxylate groups from each NTA molecule can be positioned around the central metal atom in a sterically-comfortable tetrahedral arrangement, providing a structural basis for the selectivity of Be2+ over Mg2+ in this coordination system.

Compared to the other metal salts, Be2+ is challenging to work with, as it tends to precipitate as beryllium hydroxide if either the concentration or the pH is too high. Speciation of aqueous beryllium salt is more complex than for other solvated metal ions, which can affect apparent stoichiometry in binding studies. And, unlike MgSO4, CaSO4, or MnSO4, a stock solution of BeSO4 dissolved in pure water is very acidic, due to solvent hydrolysis. Inadequate pH control can lead to spurious ITC results, and we have determined that the concentration of MES buffer needs to be no lower than 100 mM (i.e. a 50:1 molar ratio relative to BeSO4) in order to avoid pH-related artifacts when working with beryllium. Investigators studying other metal salts via ITC typically use buffer concentrations in the 10-25 mM range. Our results with simple model compounds demonstrate that we are capable of generating meaningful data with Be2+ in ITC experiments.

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(7) Appendixes: Not applicable.

Technology Transfer

Final Report

Final Report: High Stability Metal-Protein Interactions Evaluated by Microcalorimetry

Contract Number: W911NF-15-1-0043

Contract Period: Feb. 1, 2015 - Jan. 31, 2016

Report Date: April 29, 2016

Author: Ronald K. Gary (PI)

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SCIENTIFIC PROGRESS

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Our research focused on Be^{2+} in particular, as this divalent cation has received the least study and is hypothesized to exhibit interesting behavior in its interactions with biologically relevant macromolecules. The dissociation constant (K_d) that we observed for the interaction of Be^{2+} with EDTA at pH 6.0 is in good agreement with that expected from literature values. An expected value for this interaction under these conditions is not available directly. However, in a table of metal-EDTA formation constants at 20ºC and 0.1M ionic strength, Be^{2+} is listed as log $K_f = 9.2$ [Harris, 1982]. This indicates that the equilibrium constant for the reaction with the fully deprotonated EDTA species (Y^{4-}) = 1.58 x 10^9. The value of α_{Y4-} at pH 6 = 2.3 x 10^{-5}, so adjusting the equilibrium constant for pH 6 gives (1.58 x 10^9)(2.3 x 10^{-5}) = 3.63 x 10^4, which corresponds to K_d = 2.75 x 10^{-5} M. This is very close to the K_d = 2.60 x 10^{-5} M (± 0.08 x 10^{-5} M) that we observed for Be^{2+}-EDTA at pH 6 (Table 1).

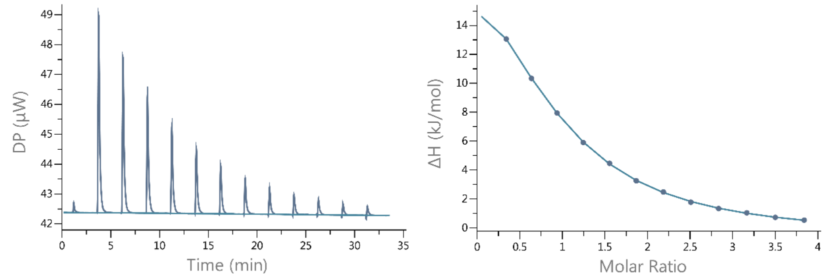
EDTA displayed quantitative, but not qualitative, differences in its interactions with the metal ions. We also wished to employ an ITC model system that could provide a qualitative distinction between Be^{2+} and its nearest neighbor Mg^{2+}. NTA (nitrilotriacetic acid) is known to interact with Mg^{2+} only weakly [Souaya et al., 2000]. Using 0.2 mM NTA in 100 mM MES, pH 6.0, we observed a binding interaction for 2 mM $BeSO_4$, but not for 2 mM $MgSO_4$ (Figure 2). For the Be^{2+}-NTA interaction, the observed K_d = 4.77 x 10^{-5} M (± 0.38 x 10^{-5} M), and the observed N (binding sites per macromolecule) was 0.509. The N value near one-half indicates that, under the conditions employed, a single Be^{2+} ion coordinates two NTA molecules to form a 2:1 NTA:metal complex. Among the alkaline earth metals (group IIA), Be^{2+} and Mg^{2+} are the "hardest" (HSAB theory) and have the smallest ionic radii, but Mg^{2+} has a principal coordination number of 6, whereas Be^{2+} coordinates 4 ligands with tetrahedral geometry. Our ITC results suggest that a pair of carboxylate groups from each NTA molecule can be positioned around the central metal atom in a sterically-comfortable tetrahedral arrangement, providing a structural basis for the selectivity of Be^{2+} over Mg^{2+} in this coordination system.

Compared to the other metal salts, Be^{2+} is challenging to work with, as it tends to precipitate as beryllium hydroxide if either the concentration or the pH is too high. Speciation of aqueous beryllium salt is more complex than for other solvated metal ions, which can affect apparent stoichiometry in binding studies. And, unlike $MgSO_4$, $CaSO_4$, or $MnSO_4$, a stock solution of $BeSO_4$ dissolved in pure water is very acidic, due to solvent hydrolysis. Inadequate pH control can lead to spurious ITC results, and we have determined that the concentration of MES buffer needs to be no lower than 100 mM (i.e. a 50:1 molar ratio relative to $BeSO_4$) in order to avoid pH-related artifacts when working with beryllium. Investigators studying other metal salts via ITC typically use buffer concentrations in the 10-25 mM range. Our results with simple model compounds demonstrate that we are capable of generating meaningful data with Be^{2+} in ITC experiments.

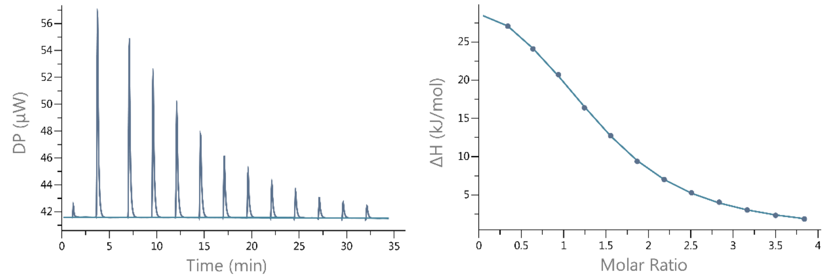
Table 1: Summary of ITC Data for Figure 1 Experiments.

	[Syr] (M)	[Cell] (M)	Control Type	N (sites)	KD (M)	ΔH (kJ/mol)	ΔG (kJ/mol)	-TΔS (kJ/mol)
$MgSO_4$	2.00E-03	1.00E-04	Single	0.915	4.83E-05	22.5	-24.7	-47.1
$BeSO_4$	2.00E-03	1.00E-04	Single	1.3	2.60E-05	36.8	-26.2	-62.9
$CaSO_4$	1.00E-03	1.00E-04	Single	1.16	1.02E-06	-14.7	-34.2	-19.5
$MnSO_4$	1.00E-03	1.00E-04	Single	1.09	1.37E-08	-9.9	-44.9	-35.0

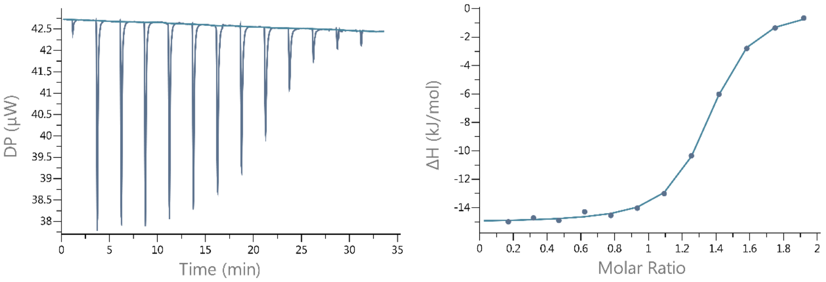
2mM $MgSO_4$ in 100mM MES pH 6.0 vs 0.1mM EDTA in 100mM MES pH 6.0



2mM $BeSO_4$ in 100mM MES pH 6.0 vs 0.1mM EDTA in 100mM MES pH 6.0



1mM $CaSO_4$ in 100mM MES pH 6.0 vs 0.1mM EDTA in 100mM MES pH 6.0



1mM $MnSO_4$ in 100mM MES pH 6.0 vs 0.1mM EDTA in 100mM MES pH 6.0

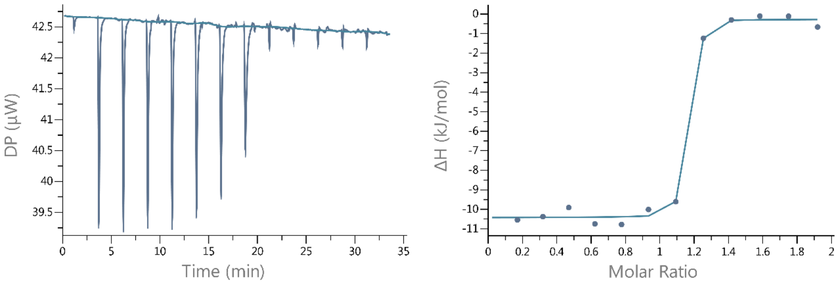
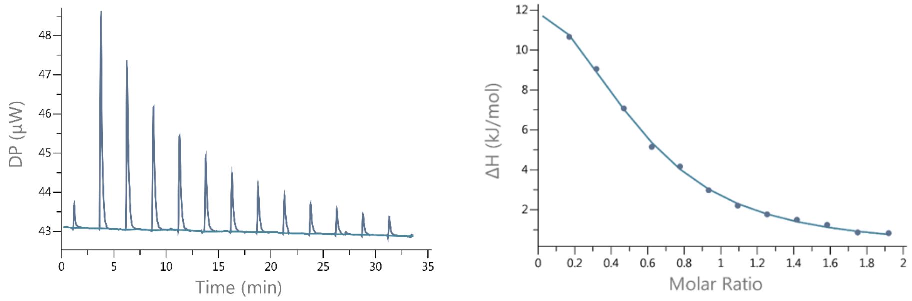


Figure 1. ITC data for interaction of Mg^{2+}, Be^{2+}, Ca^{2+}, and Mn^{2+} with EDTA at pH 6.0. The association of EDTA with Mn^{2+} or Ca^{2+} ions was exothermic (producing downward peaks for each injection), whereas the Mg^{2+} and Be^{2+} associations were endothermic (upward peaks).

2mM $BeSO_4$ in 100mM MES pH 6.0 vs 0.2mM NTA in 100mM MES pH 6.0



2mM $MgSO_4$ in 100mM MES pH 6.0 vs 0.2mM NTA in 100mM MES pH 6.0

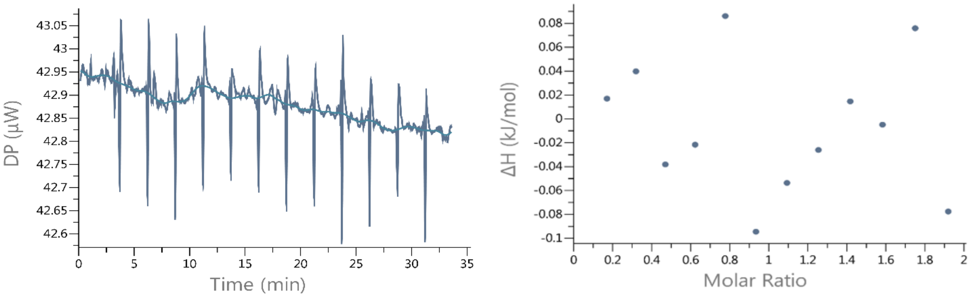


Figure 2. ITC data for interaction of Be^{2+} and Mg^{2+} with NTA at pH 6.0.
The association of NTA with Be^{2+} was endothermic (upward peaks) but overall favorable (ΔG = -24.7 kJ/mol). Under comparable conditions, Mg^{2+} exhibited no meaningful affinity for NTA (note the large difference in y-axis scale in the plots for each metal).

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(7) Appendixes: Not applicable.