RESEARCH NOTE

Preliminary investigation of deoxyoligonucleotide binding to ribonuclease A using mass spectrometry: An attempt to develop a lab experience for undergraduates [version 1; peer review: 2 approved]

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Abstract

Deoxyoligonucleotide binding to bovine pancreatic ribonuclease A (RNase A) was investigated using electrospray ionization ion-trap mass spectrometry (ESI-IT-MS). Deoxyoligonucleotides included CCCCC (dC₅) and CCACC (dC₂AC₂). This work was an attempt to develop a biochemistry lab experience that would introduce undergraduates to the use of mass spectrometry for the analysis of protein-ligand interactions. Titration experiments were performed using a fixed RNase A concentration and variable deoxyoligonucleotide concentrations. Samples at equilibrium were infused directly into the mass spectrometer under native conditions. For each deoxyoligonucleotide, mass spectra showed one-to-one binding stoichiometry, with marked increases in the total ion abundance of ligand-bound RNase A complexes as a function of concentration, but the accurate determination of dC₅ and dC₂AC₂ dissociation constants was problematic.

Keywords

education, biochemistry lab, protein-ligand interactions, mass spectrometry, ribonuclease A

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Abbreviations

dC ₅	deoxyoligonucleotide with the sequence: CCCCC
dC ₂ AC ₂	deoxyoligonucleotide with the sequence: CCACC
RNase A	bovine pancreatic ribonuclease A
ESI-IT-MS	electrospray ionization ion-trap mass spectrometry
nESI-Q-TOF-MS	nanoelectrospray ionization quadrupole time-of-flight mass spectrometry
RNase A+dC ₅	ligand-bound form of RNase A (with one dC ₅ ligand)
RNase A+dC ₂ AC ₂	ligand-bound form of RNase A (with one dC ₂ AC ₂ ligand)
RSD	relative standard deviation

Introduction

Bovine pancreatic ribonuclease A (RNase A) is an endoribonuclease (EC 3.1.27.5) that hydrolyzes RNA. It is a small single chain polypeptide (124 amino acids) containing four disulfide bridges and is known for its significant stability¹. RNase A has been called “the most studied enzyme of the 20th century” and it has seen wide use as a model protein in biochemical and biophysical experiments¹. Undergraduate life-science majors often learn of RNase A as part of a biochemistry course in the context of the Nobel Prize winning protein folding experiments performed by Christian Anfinsen². Students may also be familiar with the need to inhibit ribonucleases when working with RNA in the lab, often accomplished with diethyl pyrocarbonate, or will have learned about the role of ribonucleases in microRNA biology³. Still others may recognize RNase A as an example of an enzyme that employs general acid-base catalysis as part of its chemical mechanism⁴. Thus, RNase A is an excellent model for undergraduate lab experiments, not only because it has been extensively studied, but also because its use presents an opportunity to reemphasize important concepts in biochemistry and biology.

The application of mass spectrometry to the analysis of biomolecules has made an enormous impact in the life sciences. Protein identification, the characterization of protein modifications, and the quantification of biomolecules using mass spectrometry are commonplace. Of these, protein identification is the most established in an undergraduate teaching lab^{5–10}. Numerous other biological applications of mass spectrometry have existed for many years, but some of these are arguably, less broadly appreciated, and this is especially true for undergraduates. Native mass spectrometry is an approach based on electrospray ionization, where biomolecules are sprayed from a non-denaturing solvent¹¹. Under such conditions, protein-ligand complexes can be maintained and a dissociation constant (K_d) can be determined via a titration experiment^{12–14}.

Previously, nanoelectrospray ionization quadrupole time-of-flight mass spectrometry (nESI-Q-TOF-MS) was used to investigate ligand binding to RNase A^{12,15,16}. These studies used nESI ionization for its superior sensitivity and relied on the TOF mass analyzer for its high mass range^{12,15,16}. In Zhang *et al.*, free RNase A and the ligand-bound forms of RNase A populated three charge states (+8, +7, and +6) at pH 6.6, with most of the signal (~90%) coming from the +7 charge state, which exceeded m/z 2000 in the ligand-bound forms¹². Similarly, in Sundqvist *et al.*, focus was placed on the +7 charge state of free RNase A and its ligand-bound forms¹⁵. In contrast, Yin *et al.* reported the most abundant charge state of free and ligand-bound forms of RNase A to be +8 at pH 6.6¹⁶. Unfortunately, California State University-Chico does not own a nESI-Q-TOF-MS as employed by each of these research groups. Instead, we have an electrospray ionization ion-trap mass spectrometer (ESI-IT-MS), which by comparison to nESI-Q-TOF-MS, offers a lower sensitivity and mass range (50–2000 m/z). Consequently, at the outset of this preliminary investigation, it was recognized that observation of the +7 and +6 charge states of ligand-bound RNase A would not be possible with our instrument.

This work was an attempt to develop a biochemistry lab experience that would introduce undergraduate life-science majors to the use of mass spectrometry for the analysis of protein-ligand interactions. Two deoxyoligonucleotides, CCCCC (dC₅) and CCACC (dC₂AC₂), were investigated for their ability to bind RNase A. Titration experiments were performed using a fixed RNase A concentration and variable deoxyoligonucleotide concentrations. Samples at equilibrium were infused directly into our ESI-IT-MS under native conditions. The relative simplicity of the sample preparation and instrument operation (by direct infusion) were viewed as desirable features for an undergraduate teaching lab. Data analysis was also straightforward. Herein is described the results of this preliminary investigation. This work differentiates itself from the abovementioned RNase A ligand binding studies (using mass spectrometry) by the experimental conditions employed, which includes the identity of the investigated ligands and the type of mass spectrometer used^{12,15,16}.

Methods

Sample preparation

A stock solution of bovine pancreatic ribonuclease A (#R6513, Sigma-Aldrich, St Louis, MO, USA) was prepared at 5.60 mg/mL in LC-MS grade water (Thermo-Fisher Scientific, Waltham, MA, USA). Ammonium acetate (NH₄OAc) was LC-MS grade (#73594, Sigma-Aldrich). HPLC-purified deoxyoligonucleotides with the sequence “CCCCC” (dC₅) and “CCACC” (dC₂AC₂) were obtained from ThermoFisher and the stock solutions (200 μ M) were prepared in LC-MS grade water. Samples were prepared in 1.5 mL microcentrifuge tubes as indicated in Table 1. Six replicates were prepared and analyzed for “Sample 1” whereas “Samples 2–5” were prepared and analyzed in

triplicate. Each sample was mixed by micropipetting, and incubated at room temperature for ten minutes, prior to analysis.

Mass spectrometry

Samples were analyzed with a Thermo LCQ Advantage ion-trap mass spectrometer equipped with an electrospray ionization source. The instrument was operated in positive ion mode using a 4.5 kV spray voltage, 60°C capillary temperature, 200 ms inject time, 10 microscans, and nitrogen sheath and aux gas settings of 30 and 15, respectively. The instrument was tuned on the +8 charge state of free RNase A at m/z 1723.7 (Table 2). Each sample was subjected to direct-infusion at 2.5 $\mu\text{L}/\text{min}$ using the LCQ syringe pump and full-scan mass spectra

(m/z 1500-1950) were collected for two minutes. The upper m/z range was capped at 1950 to exclude the +7 charge state of free RNase A, which in its various adduct forms, began at m/z 1955.5 (Table 2). The rationale was that the +7 charge state of the ligand-bound forms of RNase A were above m/z 2000, which made +7 data incomplete and unusable (Table 3).

Determination of total ion abundance

To facilitate determination of total ion abundance, tables of predicted m/z values for free RNase A (Table 2) and the ligand-bound forms of RNase A (RNase A+dC₅ and RNase A+dC₂AC₂) (Table 3) were constructed. A series of 98 Da adducts were included in Table 2 and Table 3 due to their presence in the

Table 1. Sample preparation.

Component	Sample #				
	1	2	3	4	5
RNase A (5.60 mg/mL) ¹ (μL)	10	10	10	10	10
LC-MS grade H ₂ O (μL)	40	37.5	35	30	20
20 mM NH ₄ OAc, pH 6.00 (μL)	50	50	50	50	50
200 μM deoxyoligonucleotide ² (μL)	0	2.5	5	10	20
Total Volume (μL)	100	100	100	100	100
Overall [deoxyoligonucleotide²] (μM)	0	5	10	20	40
Overall [RNase A] (μM)	40.9	40.9	40.9	40.9	40.9

¹409 μM RNase A; calculated with the MW_{av} (13,690.3) for PDB ID: 1RTA (Ref. 17).

²Either dC₅ or dC₂AC₂.

Table 2. Predicted m/z values for free RNase A with P_i adducts (X)¹. The +8 charge state used in this work is highlighted.

Ion	Free RNase A					
	X=0	X=1 P _i	X=2 P _i	X=3 P _i	X=4 P _i	X=5 P _i
[M+X+H] ⁺	13682.3	13780.3	13878.3	13976.3	14074.3	14172.3
[M+X+2H] ²⁺	6841.7	6890.7	6939.7	6988.7	7037.7	7086.7
[M+X+3H] ³⁺	4561.4	4594.1	4626.8	4659.4	4692.1	4724.8
[M+X+4H] ⁴⁺	3421.3	3445.8	3470.3	3494.8	3519.3	3543.8
[M+X+5H] ⁵⁺	2737.3	2756.9	2776.5	2796.1	2815.7	2835.3
[M+X+6H] ⁶⁺	2281.2	2297.6	2313.9	2330.2	2346.6	2362.9
[M+X+7H] ⁷⁺	1955.5	1969.5	1983.5	1997.5	2011.5	2025.5
[M+X+8H]⁸⁺	1711.2	1723.4	1735.7	1747.9	1760.2	1772.4
[M+X+9H] ⁹⁺	1521.2	1532.0	1542.9	1553.8	1564.7	1575.6
[M+X+10H] ¹⁰⁺	1369.1	1378.9	1388.7	1398.5	1408.3	1418.1

¹Where X=0 (no phosphate adduct), X=1 P_i (+98), X=2 P_i (+196), X=3 P_i (+294), X=4 P_i (+392), X=5 P_i (+490).

Table 3. Predicted m/z values for the ligand-bound¹ forms of RNase A with P_i adducts (X)². The +8 charge state used in this work is highlighted.

Ion	RNase A+dC ₅						RNase A+dC ₂ AC ₂					
	X=0	X=1 P _i	X=2 P _i	X=3 P _i	X=4 P _i	X=5 P _i	X=0	X=1 P _i	X=2 P _i	X=3 P _i	X=4 P _i	X=5 P _i
[M+L+X+H] ⁺	15066.2	15164.2	15262.2	15360.2	15458.2	15556.2	15090.3	15188.3	15286.3	15384.3	15482.3	15580.3
[M+L+X+2H] ²⁺	7533.6	7582.6	7631.6	7680.6	7729.6	7778.6	7545.7	7594.7	7643.7	7692.7	7741.7	7790.7
[M+L+X+3H] ³⁺	5022.7	5055.4	5088.1	5120.7	5153.4	5186.1	5030.8	5063.4	5096.1	5128.8	5161.4	5194.1
[M+L+X+4H] ⁴⁺	3767.3	3791.8	3816.3	3840.8	3865.3	3889.8	3773.3	3797.8	3822.3	3846.8	3871.3	3895.8
[M+L+X+5H] ⁵⁺	3014.1	3033.7	3053.3	3072.9	3092.5	3112.1	3018.9	3038.5	3058.1	3077.7	3097.3	3116.9
[M+L+X+6H] ⁶⁺	2511.9	2528.2	2544.5	2560.9	2577.2	2593.5	2515.9	2532.2	2548.6	2564.9	2581.2	2597.6
[M+L+X+7H] ⁷⁺	2153.2	2167.2	2181.2	2195.2	2209.2	2223.2	2156.6	2170.6	2184.6	2198.6	2212.6	2226.6
[M+L+X+8H]⁸⁺	1884.2	1896.4	1908.7	1920.9	1933.2	1945.4	1887.2	1899.4	1911.7	1923.9	1936.2	1948.4
[M+L+X+9H] ⁹⁺	1674.9	1685.8	1696.7	1707.6	1718.5	1729.4	1677.6	1688.5	1699.4	1710.3	1721.2	1732.0
[M+L+X+10H] ¹⁰⁺	1507.5	1517.3	1527.1	1536.9	1546.7	1556.5	1509.9	1519.7	1529.5	1539.3	1549.1	1558.9

¹Where RNase A+dC₅, L = +1383.9 (MW_{av}) for one dC₅, and RNase A+dC₂AC₂, L = +1408.0 (MW_{av}) for one dC₂AC₂.

²Where X=0 (no P_i adduct), X=1 P_i (+98), X=2 P_i (+196), X=3 P_i (+294), X=4 P_i (+392), X=5 P_i (+490).

mass spectra of this work, and that of earlier studies^{12,15}. These adducts have been suggested to be either H₂SO₄ or H₃PO₄¹⁸. Other RNase A studies have assigned these adducts as phosphate, and so each 98 Da adduct (X) in this work was designated as “P_i” (Table 2 and Table 3)^{12,15}. Although mass spectra showed that free RNase A had up to 8 P_i adducts (Figure 1A and 1F), only the 0-5 P_i adduct forms of free RNase A and its ligand bound forms were used. This restraint was necessitated by the predicted m/z overlap of the ligand-bound forms of RNase A (with P_i adducts >5) with the m/z of free RNase at the +7 charge state. The “Qual Browser” feature of Xcalibur 1.4 SR1 software (Thermo) was used for analysis of each *.raw file. For each sample, mass spectra comprising the two-minute data collection were averaged. The “spectrum list view” was used to obtain intensity data for all of the ions in the ranges comprising the +8 charge state (with 0-5 P_i adducts) for free RNase A (m/z 1710.7-1772.9), RNase A+dC₅ (m/z 1883.7-1945.9), and RNase A+dC₂AC₂ (m/z 1886.7-1948.9). The intensity data for all ions in each m/z range were added to give the “total ion abundance” of the free (*Ab*_(P)) and ligand-bound forms (*Ab*_(PL)) of RNase A. The total ion abundance for the ligand-bound forms (RNase A+dC₅ and RNase A+dC₂AC₂) were plotted as a function of [deoxyoligonucleotide] using GraphPad Prism 7.

Calculation of total ion abundance ratio and K_d

The total ion abundance ratio was determined at each [deoxyoligonucleotide] using the method described by Kitova *et al.*¹³, where for a 1:1 protein-ligand complex, the total ion abundance ratio (*R*) is calculated using the total abundance of all ligand-bound ions (*Ab*_(PL)) and the total abundance of all free protein ions (*Ab*_(P)) as shown in Equation 1:

$$R = Ab_{(PL)} / Ab_{(P)} = [PL]_{eq} / [P]_{eq} \quad [1]$$

The total ion abundance ratio (*R*) is used with the initial ligand concentration ([L]₀) and initial protein concentration ([P]₀) to calculate the association constant (*K_a*) using Equation 2¹³:

$$K_a = R / ([L]_0 - ((R / (1 + R)) [P]_0)) \quad [2]$$

The *K_d* can then be calculated as the reciprocal of the *K_a* value.

Results

Table 1 indicates that samples contained an overall [RNase A] of 40.9 μM. Relatively low signal intensities observed for the +8 charge state of free and ligand-bound forms of RNase A necessitated this concentration, which was higher than the 5–20 μM RNase A used by others in nESI-Q-TOF-MS experiments^{12,15,16}. Table 2 and Table 3 present predicted m/z values for free RNase A and the ligand-bound forms of RNase A (RNase A+dC₅ and RNase A+dC₂AC₂) with multiple P_i adducts, which correlated well with observed m/z values (Figure 1). Upon increasing the concentration of dC₅, the total ion abundance of free RNase A was found to decrease in intensity while the total ion abundance of RNase+dC₅ was found to increase in intensity, which suggested 1:1 stoichiometry for the dC₅:RNase A interaction (Figure 1A–E). Similar results were seen for the titration using dC₂AC₂ (Figure 1F–J). Table 4 presents total ion abundance data for free RNase A in samples that contained no added deoxyoligonucleotide. Total ion abundance data for free RNase A across six replicates gave a RSD of 16.4% (Table 4). Table 5 contains total ion abundance data for free RNase A and the ligand-bound forms of RNase A in samples that contained various concentrations of dC₅ or dC₂AC₂. Total ion abundance data across three replicates at each [deoxyoligonucleotide] exhibited RSD values of approximately 20% or less (Table 5). A plot of the total ion

Figure 1. Mass spectra showing free RNAse A and ligand-bound forms as a function of added [deoxyoligonucleotide]. The +8 charge state is shown. (A & F) no added deoxyoligonucleotide, (B) 5 μ M dC₅, (C) 10 μ M dC₅, (D) 20 μ M dC₅, (E) 40 μ M dC₅, (G) 5 μ M dC₂AC₂, (H) 10 μ M dC₂AC₂, (I) 20 μ M dC₂AC₂, and (J) 40 μ M dC₂AC₂. The number of phosphate adducts ($P_i = 0-5$) are indicated in four representative mass spectra (A, D, F, and I).

Table 4. Total ion abundance for free RNAse A in samples that contained no added deoxyoligonucleotide. Data is for the +8 charge state.

Replicate	Free RNAse A
1	71,438,882
2	80,188,529
3	70,622,004
4	94,929,471
5	61,169,836
6	65,198,871
Average	73,924,599
SD	12,135,483
%RSD	16.4

abundance for free RNAse A, RNAse A+dC₅, and RNAse A+dC₂AC₂ as a function of [deoxyoligonucleotide] is shown in Figure 2. The total ion abundance for RNAse A+dC₅ and RNAse A+dC₂AC₂ increased until 20 μ M deoxyoligonucleotide, but decreased at 40 μ M (Figure 2). Table 6 presents the calculated total ion abundance ratio (R) and dissociation constant (K_d) at each [deoxyoligonucleotide]. Samples containing <40 μ M deoxyoligonucleotide unexpectedly produced negative K_d values (Table 6). By contrast, Table 6 shows that samples containing 40 μ M deoxyoligonucleotide produced consistent positive values where the average K_d for dC₅ was 2.2 ± 0.1 μ M and the average K_d for dC₂AC₂ was 1.0 ± 0.1 μ M.

Dataset 1. LCQ *.raw data files for all samples

<http://dx.doi.org/10.5256/f1000research.14268.d198373>

Data files 1–6 are for samples that contained free RNAse A (6 replicates), Data files 7–18 are for samples that contained RNAse A and dC₅ (3 replicates per [dC₅]). Data files 19–30 are for samples that contained RNAse A and dC₂AC₂ (3 replicates per [dC₂AC₂]).

Table 5. Total ion abundance for free RNase A and the ligand-bound forms vs. [deoxyoligonucleotide]. Data is for the +8 charge state.

[dC ₅] (μ M)	Replicate 1		Replicate 2		Replicate 3		Statistics			
	Free RNase A	RNase A +dC ₅	Free RNase A	RNase A +dC ₅	Free RNase A	RNase A +dC ₅	Free RNase A		RNase A+dC ₅	
							Average	SD	Average	SD
									%RSD	%RSD
5	65,099,625	18,794,425	68,544,428	23,145,457	78,972,474	25,147,375	70,872,176	7,223,420	10.2	14.5
10	47,825,661	27,545,273	46,350,320	31,619,901	43,525,177	30,298,740	45,900,386	2,185,262	4.8	7.0
20	30,107,821	45,426,668	23,925,313	39,437,219	21,135,712	32,082,196	25,056,282	4,591,732	18.3	17.1
40	7,997,701	28,282,560	5,843,389	21,520,555	6,539,939	23,879,435	6,793,676	1,099,341	16.2	14.0
[dC ₂ AC ₂] (μ M)	Replicate 1		Replicate 2		Replicate 3		Statistics			
	Free RNase A	RNase A +dC ₂ AC ₂	Free RNase A	RNase A +dC ₂ AC ₂	Free RNase A	RNase A +dC ₂ AC ₂	Free RNase A		RNase A + dC ₂ AC ₂	
							Average	SD	Average	SD
									%RSD	%RSD
5	51,636,536	14,389,383	42,294,232	12,579,820	42,446,013	13,959,865	45,458,927	5,350,505	11.8	6.9
10	36,684,700	21,676,041	24,124,562	15,498,871	32,045,216	21,127,649	30,951,493	6,351,098	20.5	17.6
20	15,246,271	25,179,158	18,296,917	33,389,720	20,579,066	38,415,859	18,040,751	2,675,610	14.8	20.7
40	4,941,720	26,053,318	5,332,933	30,339,897	4,343,254	23,458,825	4,872,636	498,443	10.2	13.1

Figure 2. Total ion abundance for free RNase A and the ligand-bound forms vs. [deoxyoligonucleotide]. (A) [dC₅] and (B) [dC₂AC₂]. The data is from Table 5, where points represent the average (n=3) ± standard deviation.

Table 6. The total ion abundance ratio (*R*) and dissociation constant (*K_d*) calculated at each [deoxyoligonucleotide]. Data used for calculations was from Table 5.

[dC ₅] (μM)	Replicate 1		Replicate 2		Replicate 3	
	<i>R</i>	<i>K_d</i> for dC ₅ (μM)	<i>R</i>	<i>K_d</i> for dC ₅ (μM)	<i>R</i>	<i>K_d</i> for dC ₅ (μM)
5	0.29	-14.4	0.34	-15.8	0.32	-15.3
10	0.58	-8.6	0.68	-9.7	0.70	-9.7
20	1.51	-3.0	1.65	-3.3	1.52	-3.1
40	3.54	2.3	3.68	2.1	3.65	2.2
[dC ₂ AC ₂] (μM)	Replicate 1		Replicate 2		Replicate 3	
	<i>R</i>	<i>K_d</i> for dC ₂ AC ₂ (μM)	<i>R</i>	<i>K_d</i> for dC ₂ AC ₂ (μM)	<i>R</i>	<i>K_d</i> for dC ₂ AC ₂ (μM)
5	0.28	-14.0	0.30	-14.7	0.33	-15.6
10	0.59	-8.8	0.64	-9.3	0.66	-9.5
20	1.65	-3.3	1.82	-3.5	1.87	-3.6
40	5.27	1.1	5.69	0.9	5.40	1.0

Conclusions

This preliminary work demonstrates the potential and pitfalls of a LCQ ESI-IT-MS instrument to investigate protein-ligand interactions in an undergraduate teaching lab. Even though dC₅ and dC₂AC₂ binding to RNase A are clearly illustrated in Figure 1, the presence of the P_i adducts complicated the mass spectra and broadened the signals for free RNase A and the ligand-bound forms of RNase A. In-source collision-induced dissociation was explored to reduce P_i adduct formation, but it appeared to disrupt the RNase A+dC₅ and RNase A+dC₂AC₂ complexes, and so this approach was abandoned (data not shown). It is unclear why the decrease in the total ion abundance for the ligand-bound forms of RNase A was observed at higher deoxyoligonucleotide concentrations (Figure 2). Previously, the ion intensity ratio of free RNase A to the RNase A+cytidine 2'-monophosphate (2'-CMP) complex was observed to vary with charge state as follows: +8 (0.65), +7 (0.73), +6 (1.1)¹².

This led Zhang *et al.* to suggest that either the binding of ligand, or the presence of ligand in the analyzed RNase A samples, created a change of the charge state distribution for the protein-ligand complex¹². In the present work, the binding of deoxyoligonucleotide, or the presence of deoxyoligonucleotide in samples, could have shifted some of the total ion abundance of free and/or ligand-bound RNase A from the +8 charge state to lower charge states, which were beyond the mass range of our ion-trap mass analyzer. This highlights an inherent limitation of this work, which was the inability to gather data for all free and ligand-bound RNase A charge states. Kitova *et al.* stated the importance of including all ligand-bound and free protein ions in the calculation of *R*, and emphasized that the “sometimes-used practice” of employing a particular charge state to determine *K_a* should be avoided¹³. Thus, the lack of data for the +7 and +6 charge states of RNase A hindered accurate collection of total ion abundance data, which may have

affected calculations of R and led to the negative K_d values at low ligand concentrations (Table 6). The positive K_d values in Table 6 are of similar magnitude to those determined by Zhang *et al.* for 2'-CMP and CTP, via a nESI-Q-TOF-MS titration experiment, which were $1.7 \pm 0.3 \mu\text{M}$ and $0.8 \pm 0.2 \mu\text{M}$, respectively¹². They are also in the neighborhood of in solution K_d measurements (3–24 μM) observed for the binding of short fluorescein-labeled deoxyoligonucleotides to RNase A¹⁹. In conclusion, while RNase A is an excellent model for many experiments, instructors wishing to use a LCQ ESI-IT-MS instrument to investigate protein-ligand interactions are encouraged to consider other protein-ligand systems that would enable all charges states (of the free and ligand-bound protein) to be observed.

Data availability

Dataset 1. LCQ *.raw data files for all samples. [10.5256/f1000research.14268.d198373](https://doi.org/10.5256/f1000research.14268.d198373)²⁰

Data files 1–6 are for samples that contained free RNase A (6 replicates), Data files 7–18 are for samples that contained

RNase A and dC₅ (3 replicates per [dC₅]), Data files 19–30 are for samples that contained RNase A and dC₂AC₂ (3 replicates per [dC₂AC₂]).

Competing interests

No competing interests were disclosed.

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Ryan N. Jackson

Department of Chemistry and Biochemistry, Utah State University, Logan, UT, USA

In this manuscript, author Dan Clark describes an experimental protocol aimed at introducing undergraduate biochemistry students to mass spectrometry methods. Native electrospray ion-trap mass spectrometry (ESI-IT-MS) was used to produce mass spectra of unbound ribonuclease A (RNase A) and ligand – bound RNase A at differing concentrations of added oligonucleotides. Dissociation constants were determined using observed abundance ratios of bound and unbound RNase A. However, dissociation constants with small concentrations of oligonucleotides were inaccurate, while the largest concentration produced a dissociation constant similar to that previously published. The author explains that these discrepancies likely result from limitations in the mass spectrometry equipment available, and concludes that a different protein – ligand combination may be better suited for the desired experiment.

Overall this was a well-conducted investigation and meets an acceptable standard for publication. Contradictory to the conclusion of the author that another protein – ligand combination may be better suited to teach undergrads about mass spec, I found strong educational merit in the failures of this protocol to determine all dissociation constants. As an instructor of undergraduates I have found that students can often learn more when things do not work exactly as expected. The experiment presented here offers an opportunity for students to understand equipment limitations and may help students obtain a stronger understanding of how the diversity of charged states impacts the ability to collect an accurate mass spectra.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biochemistry, Structural Biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 09 April 2018

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Samuel J. Allen

BioElectron Technology Corporation, Mountain View, CA, USA

General Comments: This work describes an experimental approach to introduce native mass spectrometry to undergraduate students. The author provides sufficient context for the choice of Ribonuclease A as the target protein, and describes the use of mass spectrometry under “native” conditions. This work also introduces undergraduates to practical use of mass spectrometry by providing “expected” m/z tables and shows the effect of adducted species on mass spectrometry signal. Plainly stated, the proposed experiment did not meet the initial hypothesis of the author. One major limitation to this study was the use of an ion trap mass spectrometer, which the author states as being the only available instrument at the academic institution. Additionally, the data from this study results in negative dissociation constant values for the lowest ligand concentrations, but reasonable dissociation constants at the highest ligand concentration. The author attempts to describe these results relative to other similar studies. As a result, this work describes the “potential and pitfalls” of attempting this experiment, which is useful for undergraduate students that are early in their scientific career.

Suggestion (no change requested): For future investigations and undergraduate studies, it would be beneficial to use centrifugal desalting columns in an attempt to remove the phosphate adducts. This would result in improved ion response, less convoluted spectra, and would introduce undergraduate students to common sample preparation used in native-like protein MS experiments.

Suggestion (no change requested): For future investigations and undergraduate studies, a native MS technique that has been used to address the upper m/z limitation of ion trap is to “supercharge” proteins¹. This can be done by adding as low as 1% v/v sulfolane or m-nitrobenzyl alcohol to the sample.

Additional comments to conclusion (minor revisions requested): There are two issues that the authors addresses regarding the data from this study. (1) The observation of negative dissociation constants and (2) decreasing PL abundance at the highest L concentration. To point (1), although these experiments are being performed under native conditions (i.e. non denaturing solvents), there are still other factors during electrospray ionization that need to be considered during native experiments. For example, Benkestock, et al² show data that suggests that the “capillary-to-cone” distance and the electrospray probe internal diameter can affect the PL to L ratios. To point (2), the decrease in PL abundance at the highest L concentration may be due to non-specific binding as described in the already cited Kitova et al. (2012)³(Section 2.4 and Figure 3). The author should add a couple sentences to the conclusion addressing how “non-ideal” ionization conditions and non-specific binding could have affected the measurements.

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Native Mass Spectrometry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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