RESEARCH NOTE

REVISED Preliminary investigation of deoxyoligonucleotide binding to ribonuclease A using mass spectrometry: An attempt to develop a lab experience for undergraduates [version 2; referees: 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 (dC_5) and CCACC (dC_2AC_2). 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 dC_5 and dC_2AC_2 dissociation constants was problematic.

Keywords

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

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REVISED **Amendments from Version 1**

I am very grateful to the reviewers for their comments and suggestions. In this new version of the manuscript, I have expanded the Conclusions section as requested by Dr. Allen. Two sentences were added that refer to centrifugal desalting as a method that could have reduced phosphate adduct formation and would be useful in training students. Five more sentences were added to address how non-ideal ionization conditions and non-specific binding could have affected the measurements. The expansion necessitated inclusion of a new reference (Benkestock *et al.*, 2004) suggested by Dr. Allen.

See referee reports

Abbreviations

dC_5	deoxyoligonucleotide with the sequence: CCCCC
dC_2AC_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_2AC_2	ligand-bound form of RNase A (with one dC_2AC_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 stability[1]. 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 experiments[1]. 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 Anfinsen[2]. 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 biology[3]. Still others may recognize RNase A as an example of an enzyme that employs general acid-base catalysis as part of its chemical mechanism[4]. 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 solvent[11]. 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 forms[12]. Similarity, in Sundqvist *et al.*, focus was placed on the +7 charge state of free RNase A and its ligand-bound forms[15]. 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 6.6[16]. 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 (dC_5) and CCACC (dC_2AC_2), 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 (NH_4OAc) was LC-MS grade (#73594, Sigma-Aldrich). HPLC-purified deoxyoligonucleotides with the sequence "CCCCC" (dC_5) and "CCACC" (dC_2AC_2) were obtained from ThermoFisher and the stock solutions (200 µM) 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 µL/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+dC_5 and RNase A+dC_2AC_2) (Table 3) were constructed. A series of 98 Da adducts were included in Table 2 and Table 3 due to their presence in the mass spectra of this work, and that of earlier studies[12,15]. These

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_2O (µL)	40	37.5	35	30	20
20 mM NH_4OAc, 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 [deoxyoligonucleotide[2]] (µM)	**0**	**5**	**10**	**20**	**40**
Overall [RNase A] (µM)	40.9	40.9	40.9	40.9	40.9

[1]409 µM RNase A; calculated with the MW_{av} (13,690.3) for PDB ID:1RTA (Ref. 17).
[2]Either dC_5 or dC_2AC_2.

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

[1]Where 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-bound[1] 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						RNaseA+dC_2AC_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

[1]Where RNase A+dC_5, L= +1383.9 (MW_{av}) for one dC_5, and RNase A+dC_2AC_2, L= +1408.0 (MW_{av}) for one dC_2AC_2.

[2]Where 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).

adducts have been suggested to be either H_2SO_4 or H_3PO_4[18]. 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+dC_5 (m/z 1883.7-1945.9), and RNase A+dC_2AC_2 (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+dC_5 and RNase A+dC_2AC_2) 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:

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

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 2[13]:

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

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+dC_5 and RNase A+dC_2AC_2) with multiple P_i adducts, which correlated well with observed m/z values (Figure 1). Upon increasing the concentration of dC_5, the total ion abundance of free RNase A was found to decrease in intensity while the total ion abundance of RNase+dC_5 was found to increase in intensity, which suggested 1:1 stoichiometry for the dC_5:RNase A interaction (Figure 1A–E). Similar results were seen for the titration using dC_2AC_2 (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 dC_5 or dC_2AC_2. Total ion abundance data across three replicates at each [deoxyoligonucleotide] exhibited RSD values of

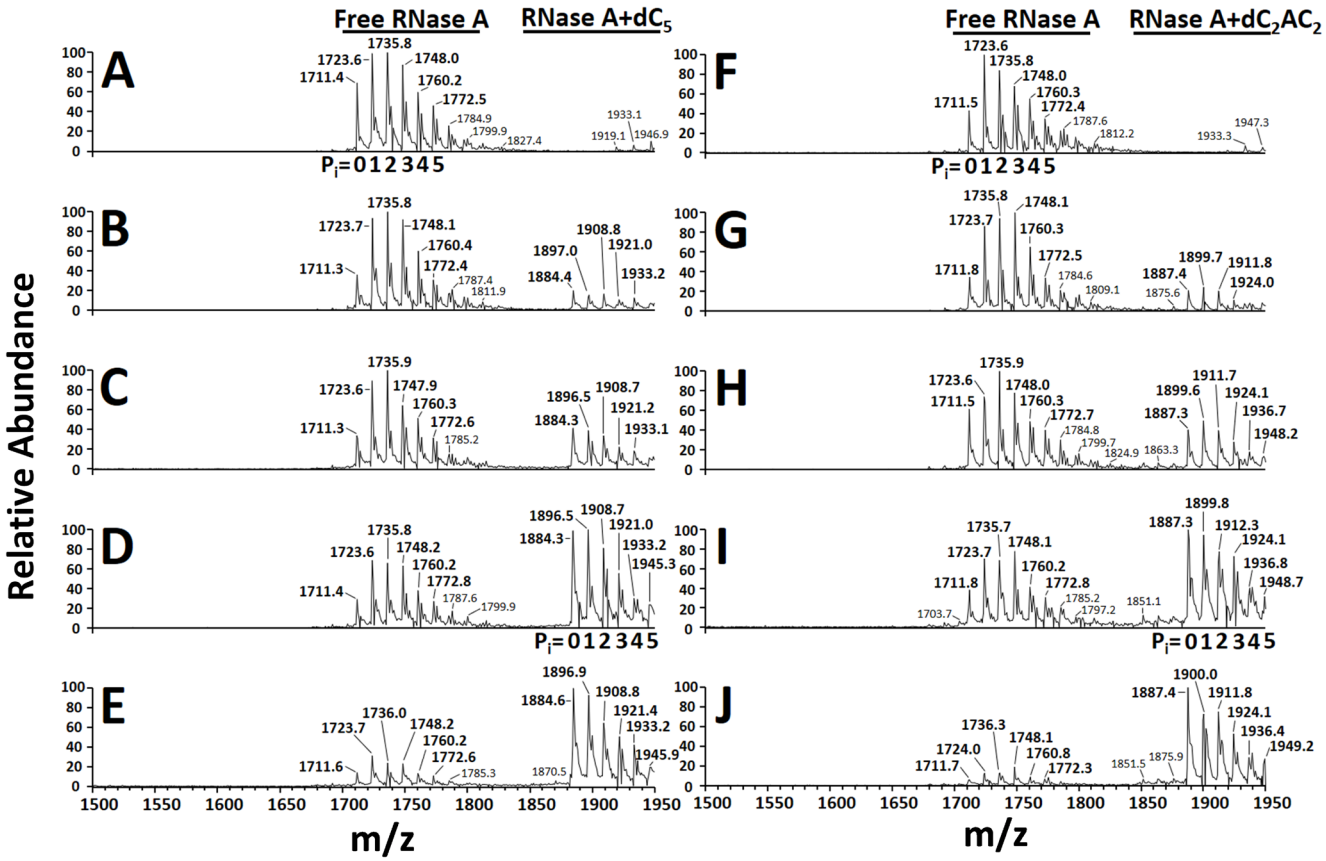

Figure 1. Mass spectra showing free RNAase A and ligand-bound forms as a function of added [deoxyoligonucleotide]. The +8 charge state is shown. (**A** & **F**) no added deoxyoligonucleotide, (**B**) 5 µM dC_5, (**C**) 10 µM dC_5, (**D**) 20 µM dC_5, (**E**) 40 µM dC_5, (**G**) 5 µM dC_2AC_2, (**H**) 10 µM dC_2AC_2, (**I**) 20 µM dC_2AC_2, and (**J**) 40 µM dC_2AC_2. 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

approximately 20% or less (Table 5). A plot of the total ion abundance for free RNase A, RNase A+dC_5, and RNase A+dC_2AC_2 as a function of [deoxyoligonucleotide] is shown in Figure 2. The total ion abundance for RNase A+dC_5 and RNase A+dC_2AC_2 increased until 20 µM deoxyoligonucleotide, but decreased at 40 µM (Figure 2). Table 6 presents the calculated total ion abundance ratio (R) and dissociation constant (K_d) at each [deoxyoligonucleotide]. Samples containing <40 µM deoxyoligonucleotide unexpectedly produced negative K_d values (Table 6). By contrast, Table 6 shows that samples containing 40 µM deoxyoligonucleotide produced consistent positive values where the average K_d for dC_5 was 2.2 ± 0.1 µM and the average K_d for dC_2AC_2 was 1.0 ± 0.1 µM.

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 dC_5 (3 replicates per [dC_5]), Data files 19–30 are for samples that contained RNase A and dC_2AC_2 (3 replicates per [dC_2AC_2]).

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] (μM)	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	%RSD	Average	SD	%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	**22,362,419**	3,248,054	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	**29,821,305**	2,078,847	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	**38,982,028**	6,683,871	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	**24,560,850**	3,432,116	14.0
[dC_2AC_2] (μM)	Replicate 1		Replicate 2		Replicate 3		Statistics					
	Free RNase A	RNase A +dC_2AC_2	Free RNase A	RNase A +dC_2AC_2	Free RNase A	RNase A +dC_2AC_2	Free RNase A			RNase A+ dC_2AC_2		
							Average	SD	%RSD	Average	SD	%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	**13,643,023**	945,474	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	**19,434,187**	3,419,096	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	**32,328,246**	6,681,887	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	**26,617,347**	3,475,037	13.1

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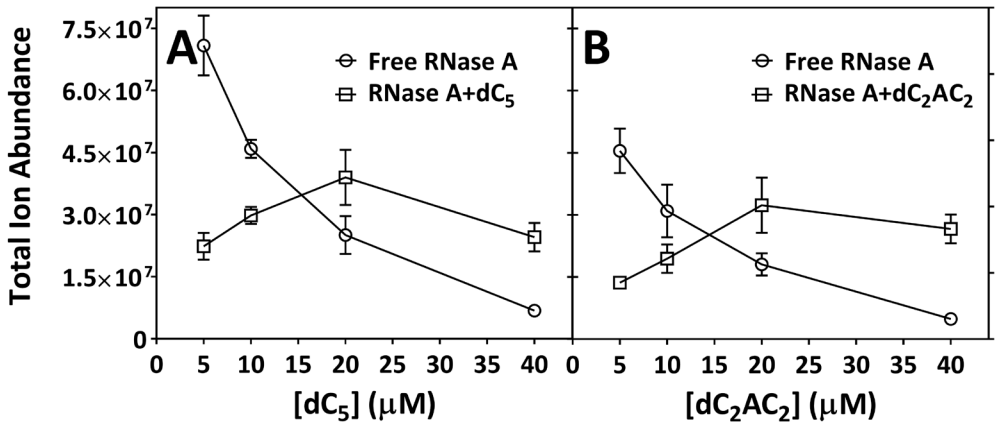

Figure 2. Total ion abundance for free RNase A and the ligand-bound forms vs. [deoxyoligonucleotide]. (**A**) [dC_5] and (**B**) [dC_2AC_2]. The data is from Table 5, where points represent the average (n=3) ± standard deviation.

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	
	R	K_d for dC_5 (μM)	***R***	K_d for dC_5 (μM)	***R***	K_d 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_2AC_2] (μM)	Replicate 1		Replicate 2		Replicate 3	
	R	K_d for dC_2AC_2 (μM)	***R***	K_d for dC_2AC_2 (μM)	***R***	K_d for dC_2AC_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 dC_5 and dC_2AC_2 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+dC_5 and RNase A+dC_2AC_2 complexes, and so this approach was abandoned (data not shown). Although it was not attempted, centrifugal desalting of the RNase A stock solution might have eliminated P_i adducts and improved the quality of the mass spectra in Figure 1. As an added benefit, in the context of an undergraduate lab, desalting would also introduce students to a common sample preparation technique. 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 complex[12]. 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 avoided[13]. 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). Other factors to consider, that could have affected measurements, include non-ideal ionization conditions and non-specific ligand binding. Benkestock *et al.* showed that instrument-derived parameters (e.g. capillary-to-cone distances) could affect the protein-ligand complex to free protein ratio[19]. They also demonstrated that compared to pneumatically assisted ESI, which was used in this work, nESI better reflects the equilibrium between free protein and protein–ligand complexes in solution. Furthermore, Kitova *et al.* noted that changes in the magnitude of K_a, with changes in ligand concentration, might indicate nonspecific ligand binding[13]. As seen in Table 6, K_d values varied with the deoxyoligonucleotide concentration. Therefore, it is reasonable to suspect that non-specific binding may have contributed to the decreased total ion abundance of the ligand-bound forms of RNase A at higher ligand concentrations (Figure 2). Notwithstanding these possibilities, 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 ± 0.3 μM and 0.8 ± 0.2 μM, respectively[12]. 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 A[20]. 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[21]

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 dC_5 (3 replicates per [dC_5]), Data files 19–30 are for samples that contained RNase A and dC_2AC_2 (3 replicates per [dC_2AC_2]).

Competing interests

No competing interests were disclosed.

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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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References

1. Marshall GR, Feng JA, Kuster DJ: **Back to the future: ribonuclease A.** *Biopolymers.* 2008; **90**(3): 259–277.
PubMed Abstract | Publisher Full Text
2. **Press Release: The 1972 Nobel Prize in Chemistry.** 1972, (accessed March 1, 2018).
Reference Source
3. Wahida F, Shehzada A, Khan T, *et al.*: **MicroRNAs: synthesis, mechanism, function, and recent clinical trials.** *Biochim Biophys Acta.* 2010; **1803**(11): 1231–1243.
PubMed Abstract | Publisher Full Text
4. Cuchillo CM, Nogués MV, Raines RT: **Bovine pancreatic ribonuclease: fifty years of the first enzymatic reaction mechanism.** *Biochemistry.* 2011; **50**(37): 7835–7841.
PubMed Abstract | Publisher Full Text | Free Full Text
5. Counterman AE, Thompson MS, Clemmer DE: **Identifying a Protein by MALDI-TOF Mass Spectrometry: An Experiment for the Undergraduate Laboratory.** *J Chem Educ.* 2003; **80**(2): 177–180.
Publisher Full Text
6. Reimann CT, Mie A, Nilsson C: **Introduction to biological mass spectrometry: Determining identity and species of origin of two proteins.** *J Chem Educ.* 2005; **82**(8): 1215–1218.
Publisher Full Text
7. Albright JC, Dassenko DJ, Mohamed EA, *et al.*: **Identifying gel-separated proteins using in-gel digestion, mass spectrometry, and database searching: Consider the chemistry.** *Biochem Mol Biol Educ.* 2009; **37**(1): 49–55.
PubMed Abstract | Publisher Full Text
8. Short M, Short A, Vankempen R, *et al.*: **Using HPLC-mass spectrometry to teach proteomics concepts with problem-based techniques.** *Biochem Mol Biol Educ.* 2010; **38**(4): 242–246.
PubMed Abstract | Publisher Full Text
9. Wilson KA, Tan-Wilson A: **Seed storage proteins as a system for teaching protein identification by mass spectrometry in biochemistry laboratory.** *Biochem Mol Biol Educ.* 2013; **41**(2): 79–86.
PubMed Abstract | Publisher Full Text
10. Alty LT, LaRiviere FJ: **Peptide mass fingerprinting of egg white proteins.** *J Chem Educ.* 2016; **93**(4): 772–777.
Publisher Full Text
11. Boeri Erba E, Petosa C: **The emerging role of native mass spectrometry in characterizing the structure and dynamics of macromolecular complexes.** *Protein Sci.* 2015; **24**(8): 1176–1192.
PubMed Abstract | Publisher Full Text | Free Full Text
12. Zhang S, Van Pelt CK, Wilson DB: **Quantitative determination of noncovalent binding interactions using automated nanoelectrospray mass spectrometry.** *Anal Chem.* 2003; **75**(13): 3010–3018.
PubMed Abstract | Publisher Full Text
13. Kitova EN, El-Hawiet A, Schnier PD, *et al.*: **Reliable determinations of protein-ligand interactions by direct ESI-MS measurements. Are we there yet?** *J Am Soc Mass Spectrom.* 2012; **23**(3): 431–441.
PubMed Abstract | Publisher Full Text
14. Ishii K, Noda M, Uchiyama S: **Mass spectrometric analysis of protein-ligand interactions.** *Biophys Physicobiol.* 2016; **13**(2016): 87–95.
PubMed Abstract | Publisher Full Text | Free Full Text
15. Sundqvist G, Benkestock K, Roeraade J: **Investigation of multiple binding sites**

on ribonuclease A using nano-electrospray ionization mass spectrometry. *Rapid Commun Mass Spectrom.* 2005; **19**(8): 1011–1016.
PubMed Abstract | Publisher Full Text

16. Yin S, Xie Y, Loo JA: **Mass spectrometry of protein-ligand complexes: enhanced gas-phase stability of ribonuclease-nucleotide complexes.** *J Am Soc Mass Spectrom.* 2008; **19**(8): 1199–1208.
PubMed Abstract | Publisher Full Text | Free Full Text

17. Birdsall DL, McPherson A: **Crystal structure disposition of thymidylic acid tetramer in complex with ribonuclease A.** *J Biol Chem.* 1992; **267**(31): 22230–22236.
PubMed Abstract

18. Chowdhury SK, Katta V, Beavis RC, *et al.*: **Origin and removal of adducts (molecular mass = 98 u) attached to peptide and protein ions in electrospray ionization mass spectra.** *J Am Soc Mass Spectrom.* 1990; **1**(5): 382–388.
PubMed Abstract | Publisher Full Text

19. Benkestock K, Sundqvist G, Edlund PO, *et al.*: **Influence of droplet size, capillary-cone distance and selected instrumental parameters for the analysis of noncovalent protein-ligand complexes by nano-electrospray ionization mass spectrometry.** *J Mass Spectrom.* 2004; **39**(9): 1059–67.
PubMed Abstract | Publisher Full Text

20. Fisher BM, Grilley JE, Raines RT: **A new remote subsite in ribonuclease A.** *J Biol Chem.* 1998; **273**(51): 34134–34138.
PubMed Abstract | Publisher Full Text

21. Clark DD: **Dataset 1 in: Preliminary investigation of deoxyoligonucleotide binding to ribonuclease A using mass spectrometry: An attempt to develop a lab experience for undergraduates.** *F1000Research.* 2018.
Data Source

Open Peer Review

Current Referee Status: ✔ ✔

Version 2

Referee Report 27 April 2018

doi:10.5256/f1000research.16067.r33468

✔ **Samuel J. Allen**
BioElectron Technology Corporation, Mountain View, CA, USA

The revised report meets the suggested changes.

Competing Interests: No competing interests were disclosed.

Referee Expertise: Native Mass Spectrometry

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

Version 1

Referee Report 12 April 2018

doi:10.5256/f1000research.15529.r32249

✔ **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.

Referee Expertise: Biochemistry, Structural Biology

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

Referee Report 09 April 2018

doi:10.5256/f1000research.15529.r32248

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" proteins[1]. 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 al[2] 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.

References

1. Going CC, Xia Z, Williams ER: New supercharging reagents produce highly charged protein ions in native mass spectrometry.*Analyst*. 2015; **140** (21): 7184-94 PubMed Abstract | Publisher Full Text
2. Benkestock K, Sundqvist G, Edlund PO, Roeraade J: Influence of droplet size, capillary-cone distance and selected instrumental parameters for the analysis of noncovalent protein-ligand complexes by nano-electrospray ionization mass spectrometry.*J Mass Spectrom*. 2004; **39** (9): 1059-67 PubMed Abstract | Publisher Full Text
3. Kitova EN, El-Hawiet A, Schnier PD, Klassen JS: Reliable determinations of protein-ligand interactions by direct ESI-MS measurements. Are we there yet?. *J Am Soc Mass Spectrom*. 2012; **23** (3): 431-41 PubMed Abstract | Publisher Full Text

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.

Referee Expertise: Native Mass Spectrometry

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