

# Quantitative Determination of Noncovalent Protein-Ligand Interactions Using Automated Nanoelectrospray Mass Spectrometry

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## Overview

### Purpose:

To demonstrate the capability of an automated chip-based nanoelectrospray ionization mass spectrometry (nanoESI/MS) method for quantitative determination of noncovalent interactions between proteins and ligands.

### Methods:

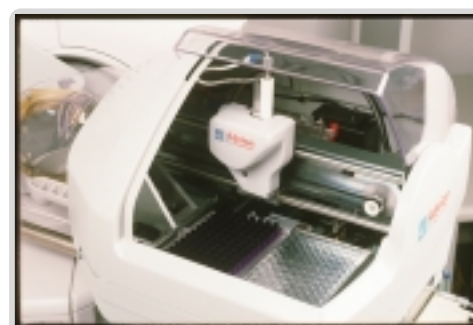
- Titration and competitive binding experiments were performed using two protein-ligand systems.
- A well-characterized model system, ribonuclease A complexed with both cytidine 2'-monophosphate (2'-CMP) and cytidine triphosphate (CTP), was used for demonstrating the method.
- An inactive mutant of the endoglucanase catalytic domain (Cel6A D117Ac) of the *T. fusca* bacterium and two oligosaccharide ligands were used for further validation of the method and automated nanoESI/MS system.

### Results:

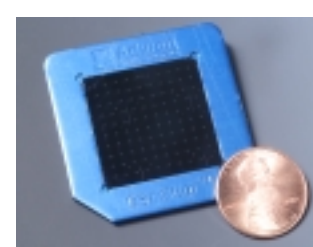
- A nanoESI/MS approach and a method for quantitative noncovalent protein-ligand interactions were developed.
- The determination of micromolar and submicromolar dissociation constants by automated nanoESI/MS analysis agreed with data provided by conventional techniques.<sup>(1-4)</sup>

## Introduction

Investigations of noncovalent protein-ligand interactions by electrospray ionization mass spectrometry (ESI/MS) are of great interest because of their relevance to molecular recognition and to combinatorial ligand library searching. The advantages of the ESI/MS technique include high sensitivity and the capability of obtaining stoichiometric information. For analysis of quantity-limited protein samples, the conventional method uses pulled-capillary nanoelectrospray, a time-consuming technique that is tedious, has poor reproducibility, and requires a skilled user. However, an automated nanoelectrospray system has been developed recently - the NanoMate™ 100 and ESI Chip. This system offers a simple, one-time spray optimization for 100 samples and is more user-friendly than conventional techniques. Other advantages of the system include low sample consumption, the ability to conserve sample not consumed in the analysis, enhanced spray stability, and no carryover. To demonstrate the capability of the nanoESI/MS platform, the results for quantitative determination of noncovalent interactions for two different protein-ligand systems are presented and discussed.



NanoMate 100



ESI Chip  
Nozzle-side View

## Methods

### Titration Experiments:

The RNase protein was kept at 10 and 4 μM in 10 mM NH<sub>4</sub>HAc for titration of 2'-CMP (1 to 20 μM) and (1 to 8 μM), respectively. The Cel6A D117Ac protein was kept at 25 and 12 μM in 0.05% acetic acid for titration of cellotriose (G3, 7.5 to 50 μM) and cellotetraose (G4, 3 to 48 μM), respectively. Both proteins were incubated at room temperature for 15 minutes prior to MS analysis.

### Competitive Binding Experiments:

Equal molar amounts of 2'-CMP and CTP (4 μM) were mixed with 4 μM of RNase in 10 mM NH<sub>4</sub>HAc pH 6.8. Equal molar amounts of G3 and G4 (18 μM) were mixed with 18 μM of Cel6A D117Ac protein in 0.05% acetic acid. All were incubated at room temperature for 15 minutes prior to MS analysis.

### Automated Nanoelectrospray:

Using the NanoMate system, 3 μL of each liquid sample were aspirated from a 96-well plate with a separate conductive pipette tip. Each sample was infused through an ESI Chip nozzle at a flow rate of 100 nL/min at an inlet pressure of 0.3 psi and a spray voltage of 1.5-1.7 kV.

### Mass Spectrometry:

The Micromass Q-TOF micro was operated in TOF/MS positive ion mode with sample cone voltage of 30 V and a source temperature of 45 °C. Data for each sample were acquired for 2 minutes in the mass range between 1000-3500 Da with a scan rate of 1 scan per 2 seconds. All data were processed using the MassLynx software.

### Data Analysis:

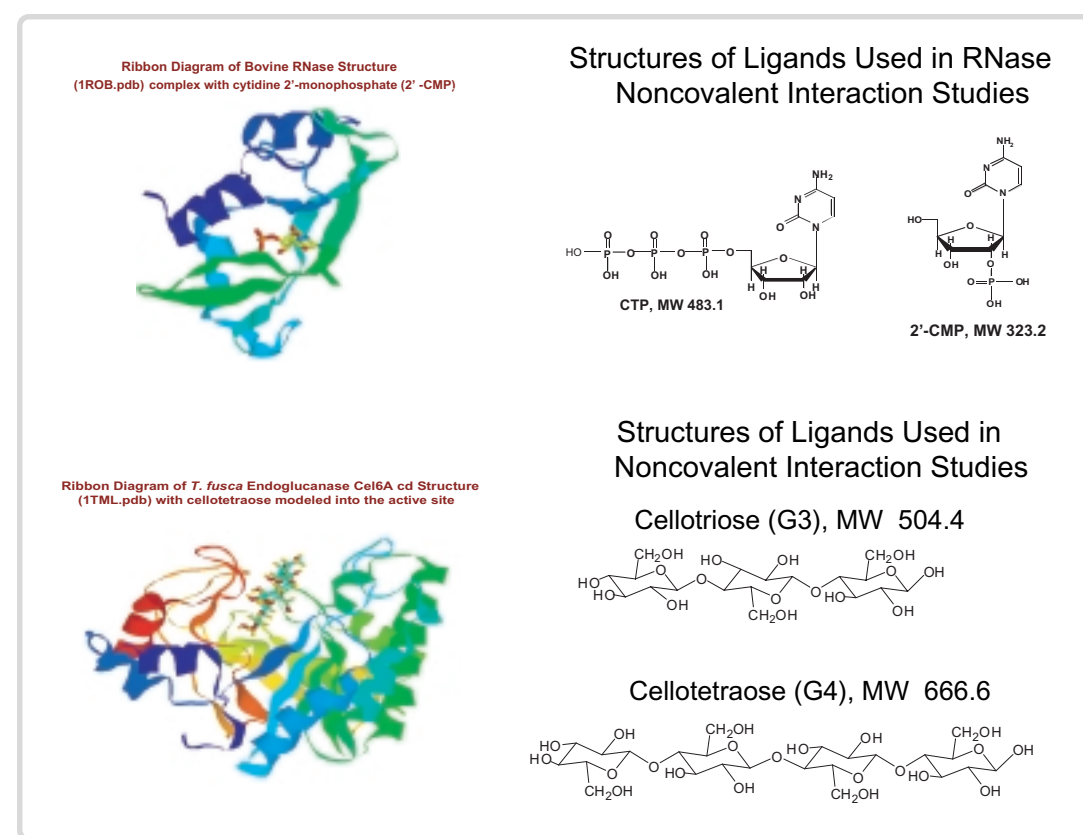
#### Determination of Dissociation Constants (K<sub>d</sub>)

$$\text{Titration Experiments} \quad K_d = \frac{[R] \times [L]}{[RL]} = \frac{[R] \times ([L] - [RL])}{[RL]}$$

$$[RL]/[R] = 1/K_d \times ([L] - [RL])$$

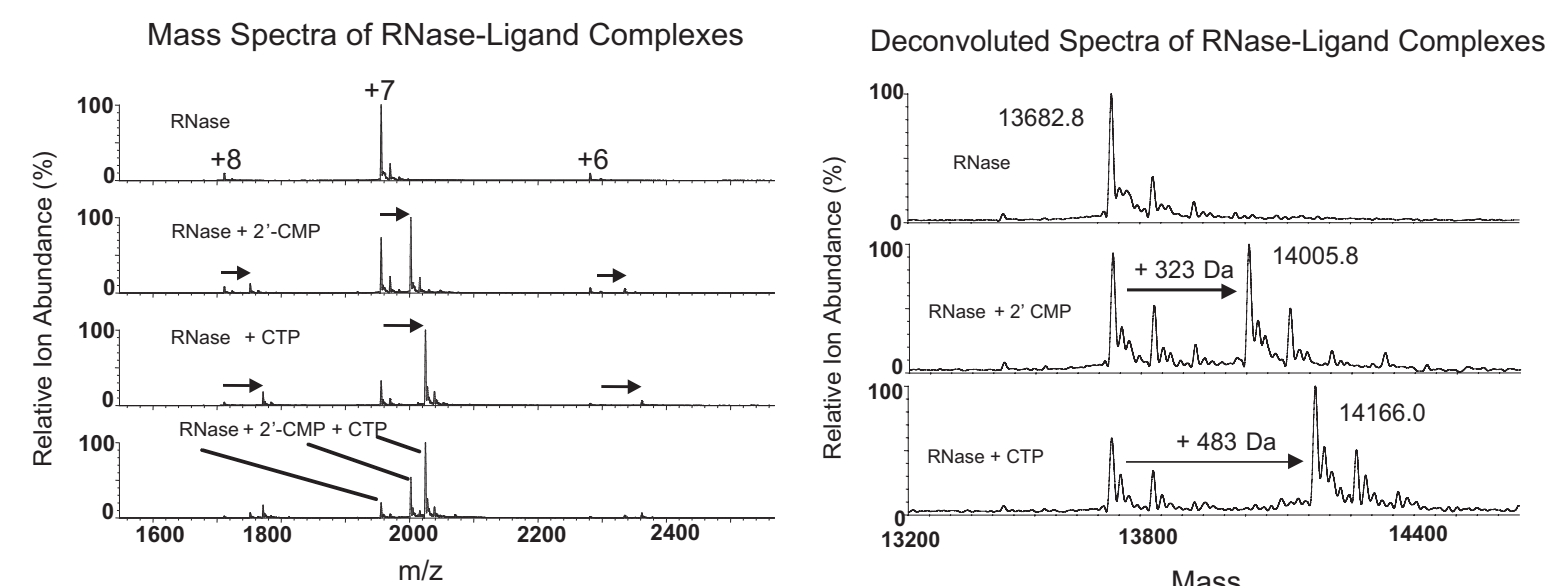
$$\text{Competitive Binding Experiments} \quad K_{dRL1} = [R] \times ([L_1] + [RL_2]) / [RL_1]$$

$$K_{dRL2} = [R] \times ([L_2] + [RL_1]) / [RL_2]$$

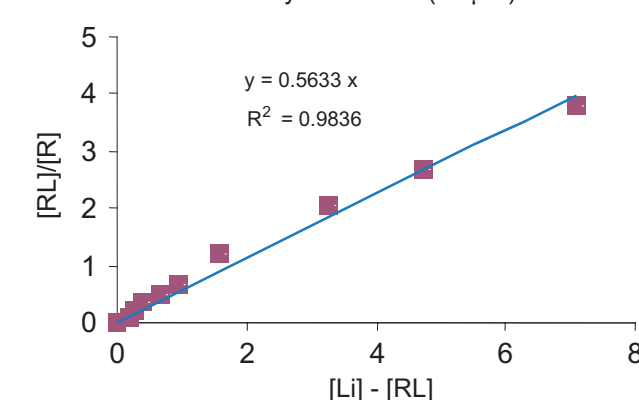


## Results

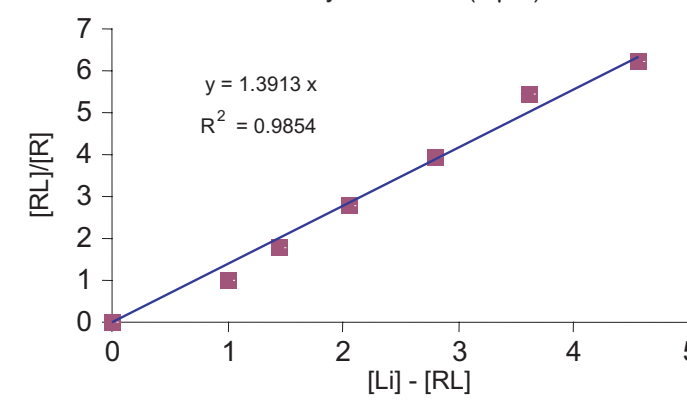
### RNase



Titration Assay for RNase (10 μM) with 2'-CMP



Titration Assay for RNase (4 μM) with CTP

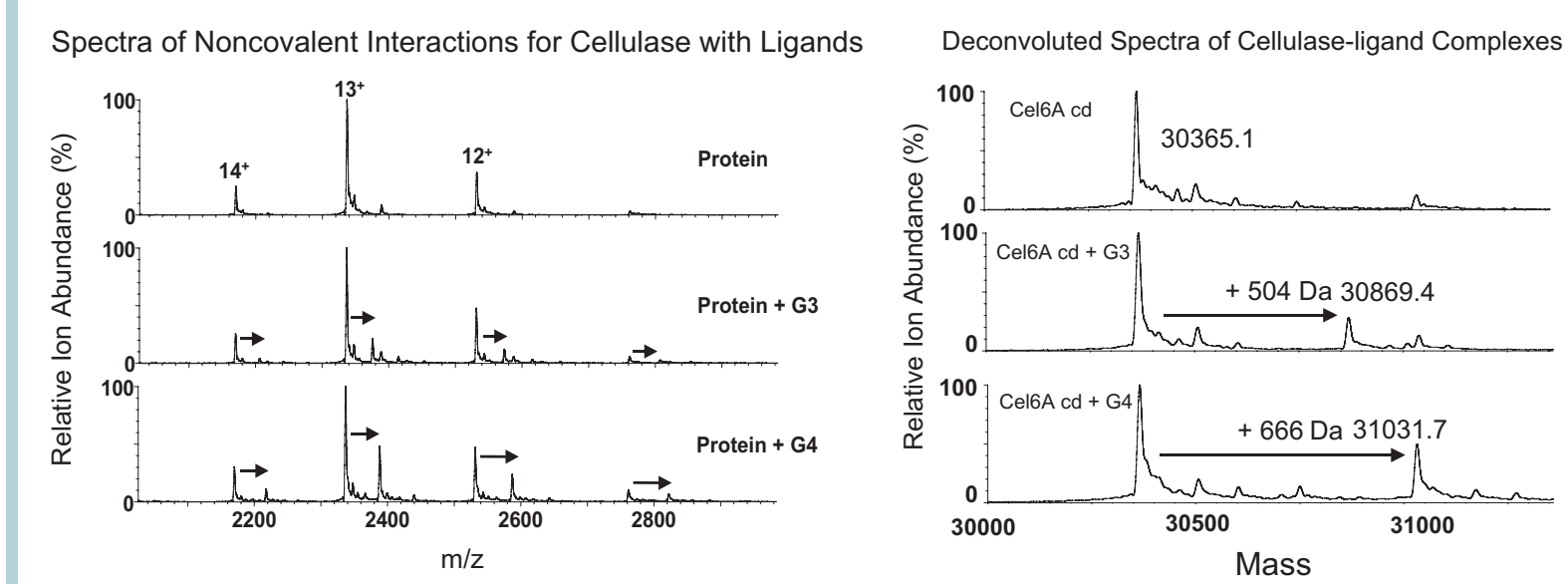


Summary of Binding Assay for RNase and Cytidine Nucleotide Ligands Using Automated NanoESI/MS

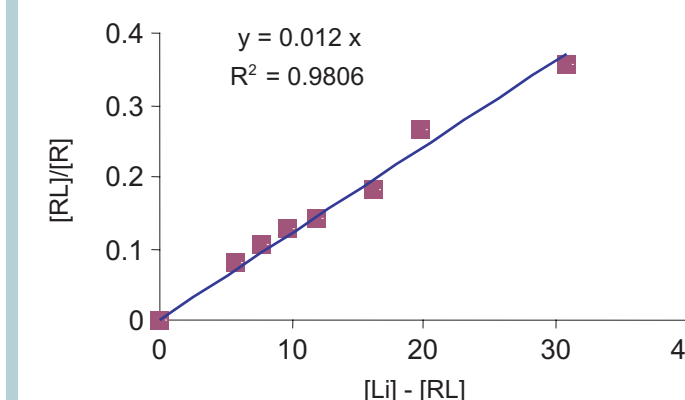
Ligand	K <sub>d</sub> (μM)		
	Titration Experiment		Competitive Binding Experiment
	Avg. of ind. points	Plot	
2'-CMP*	1.71 ± 0.33	2.00 ± 0.43	2.30 ± 0.40
CTP	0.80 ± 0.20	0.74 ± 0.30	0.75 ± 0.40

\*The K<sub>d</sub> of RNase 2'-CMP is 1.6 ± 0.4 as determined by Circular Dichromism<sup>(1)</sup> and is 1.0 ± 0.8 as determined by Isothermal Titration Calorimetry<sup>(2)</sup>.

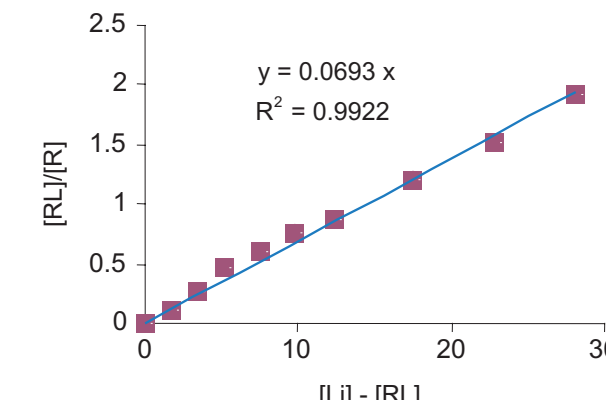
### Cel6A D117Ac



Titration Assay for Cel6A D117Ac (25 μM) with Cellotriose



Titration Assay for Cel6A D117Ac (12 μM) with Cellotetraose



Summary of Binding Assay for Cel6A cd and Its Oligosaccharide Ligands

Ligand	K <sub>d</sub> (μM)		
	Titration Experiment		Competitive Binding Experiment
	Avg. of ind. points	Plot	
Cellotriose (G3)*	77.50 ± 9.20	78.40 ± 3.50	76.90 ± 6.30
Cellotetraose (G4)	13.55 ± 1.19	14.43 ± 1.50	11.46 ± 1.20

\*The K<sub>d</sub> of Cel6A cd is 68.0 ± 6.6 as determined by displacement titration with 4-methylumbelliferyl β-cellobioside as an indicator ligand using a spectrofluorimeter<sup>(3)</sup>, while the K<sub>d</sub> of Cel6A D117A to G3 is the same as that of Cel6A wild type<sup>(4)</sup>.

## Conclusions

- The determination of micromolar and submicromolar dissociation constants by automated nanoESI/MS analysis was in agreement with data obtained using conventional techniques.<sup>(1-4)</sup>
- The nanoESI/MS method can be used to measure solution-binding constants for the complexes of two different protein-ligand systems.
- The fully-automated nanoESI/MS platform used in this study is a proven valuable system for noncovalent binding studies with many advantages over conventional ESI/MS and pulled capillary nanoESI/MS approaches.
- The automated nanoESI/MS platform offers a great potential for rapid screening of compound libraries in drug discovery programs.

### References:

- 1.) Jones, C.L.; Fish, F.; and Muccio, D.D. *Anal Biochem* **2002**, *302*, 184-190.
- 2.) Straume, M. and Freire, E. *Anal Biochem* **1992**, *203*, 259-268.
- 3.) Barr, B.K.; Wolfgang, D.E.; Piens, K.; Claeysens, M.; and Wilson, D.B. *Biochemistry* **1998**, *37*, 9220-9229.
- 4.) Wolfgang, D.E. and Wilson, D.B. *Biochemistry* **1999**, *38*, 9746-9751.

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