

# 1) Overview

## Goal

To investigate specific non-covalent complexes of A-site RNA and neomycin as a model system for the development of screening methods using relatively simple mass spectrometric methods.

## Method

To use mass spectrometry and compare the complex formation for three different techniques (Electrospray LCQ and Q-TOF, as well as UV-MALDI-MS).

## Result

All three mass spectrometric techniques enable the analysis of non-covalent complexes. The dissociation constant  $K_d$  is determined to be in the range of 156 nM.

## 2) Introduction

A general goal of our research is the development of simple mass spectrometric methods to screen small compounds (e.g. as potential therapeutic agents) with respect to their interaction with DNA and RNA.

To complement earlier work on DNA/drug interactions, we chose the non-covalent interaction of the aminoglycoside neomycin with the prokaryotic ribosomal A-site RNA as a model [1].

Instrumentation included an Electrospray ion trap, an Electrospray quadrupole/orthogonal time of flight instrument and UV-MALDI-MS.

### 3) Methods

The 27 nucleotide model of prokaryotic ribosomal A-site RNA was synthesized by Dharmacon, Inc. The A-site RNA carried a biotin label in some cases. This did not affect the binding. Neomycin sulfate was obtained from Sigma. Binding was investigated by titrating the A-site RNA (final concentration of 0.5, 1, and 10  $\mu\text{M}$ ) with increasing amounts of neomycin. Electrospray (Q-TOFII, Micromass and LCQ, Finnigan) was performed in the negative-ion mode using 20% acetonitrile to assist ionization. The presence of 20 - 80 mM ammonium acetate helped maintain the secondary structure of the RNA and reduced the effects of unwanted cation attachment. MALDI (Voyager RP-DE, PerSeptive) was performed in both ion modes, using 3-hydroxypicolinic acid (HPA), 6-aza-2-thiothymine (ATT) and a mixture of 2,3,4- and 2,4,6-trihydroxyacetophenone (THAP) as matrices.

## 4) Results

We found 10  $\mu\text{M}$  RNA to be an appropriate concentration for screening various small compounds for their ability to form non-covalent complexes. Signals consistent with specific non-covalent binding of neomycin to A-site RNA (see Figure 1 for structures) were observed using both Electrospray and MALDI. An approximately linear relationship between the amount of neomycin used and the amount of complex was observed over a wide range of neomycin concentrations.

### Electrospray

Results obtained with the Q-TOF and LCQ are consistent and reproducible (see Figure 2). Using the mass range  $m/z$  250-2400 for the analyses of the complexes, the Q-TOF offers a greater dynamic range. This enables the analysis of the complex with a neomycin concentration down to 250 nM

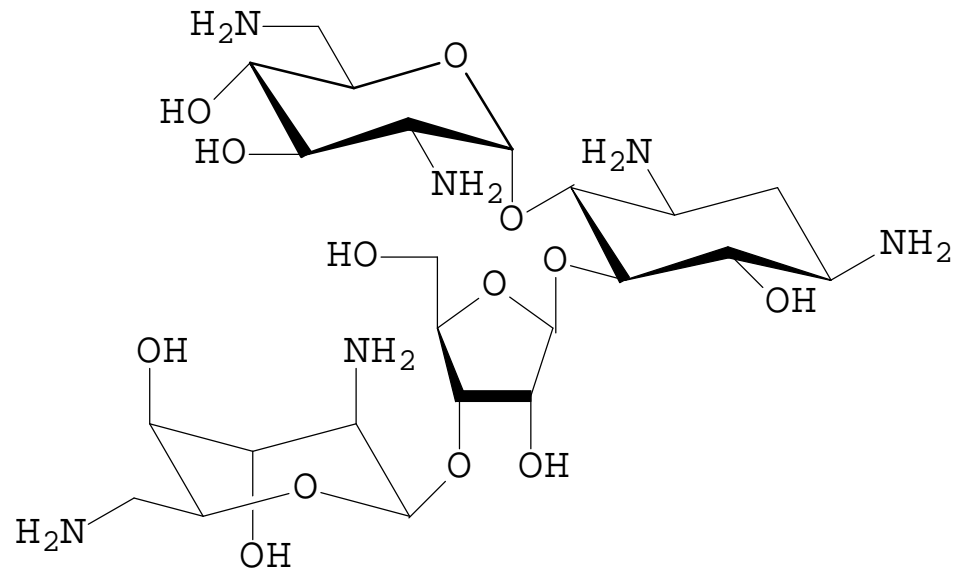
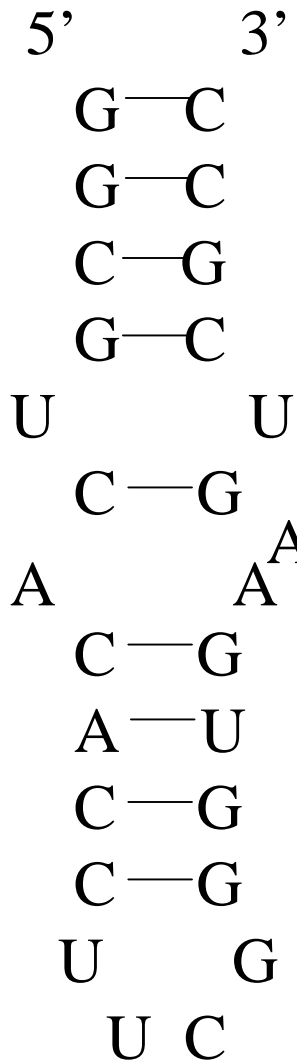
# Figure 1

A-site RNA (27 nt)

$M_{\text{average}} = 8639.23$

$M_{\text{monoisotopic}} = 8635.17$

A-site RNA with 5' biotin label:  $M_{\text{average}} = 9042.23$



Neomycin B M=614.31

(Q-TOF, Figure 3) and down to 750 nM using the LCQ and holding the RNA concentration fixed at 10  $\mu$ M.

It appears that multiple binding does not occur until all available RNA molecules have been complexed with a single neomycin. If a second, stoichiometric quantity of neomycin is added at this point, a second binding site on the RNA molecules appears to be occupied. A similar observation is made with respect to a third binding site (Figure 4). This lack of random distribution of substrate/host binding combinations suggests that binding is specific and that the three binding sites (particularly the first one) may have very different affinities for neomycin. This behavior is consistent with literature reports of similar aminoglycosides [2].

No binding of more than 3 neomycin molecules to A-site RNA was observed, even when neomycin was added at a concentration of 100  $\mu$ M compared to 10  $\mu$ M A-site RNA (Figure 4d).

Figure 2

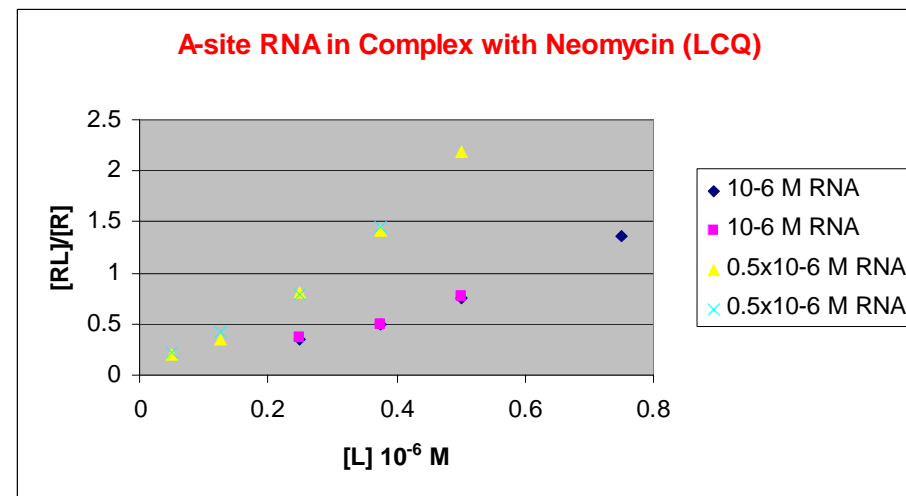
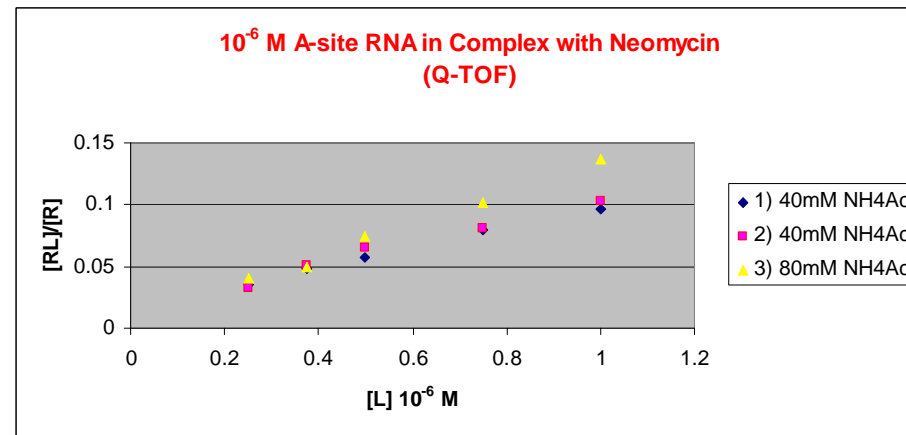
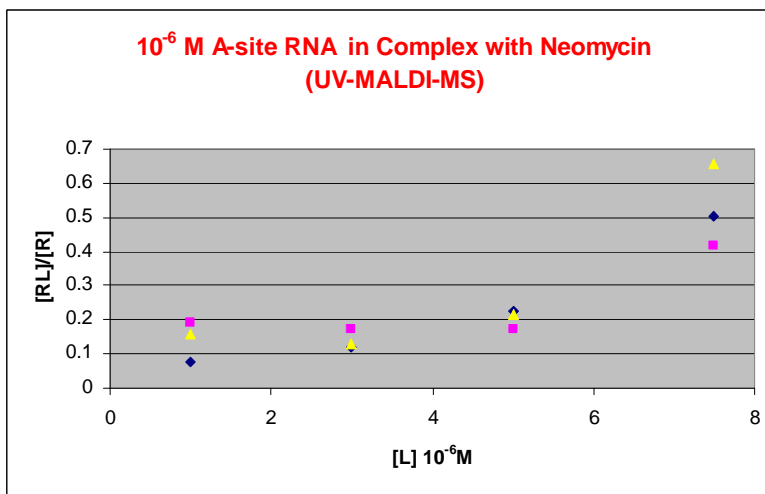
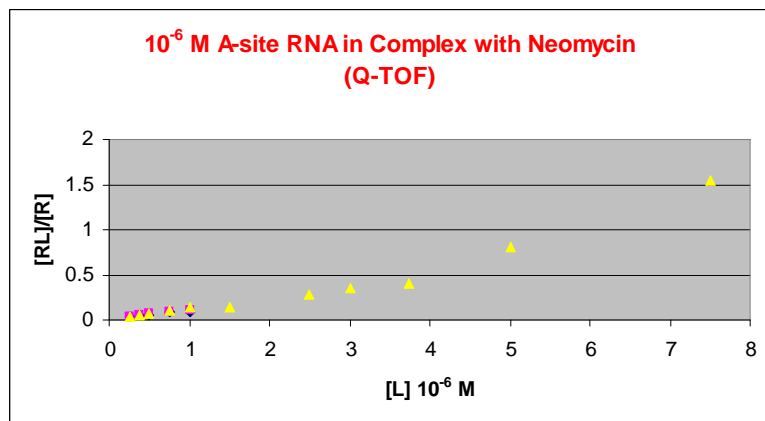
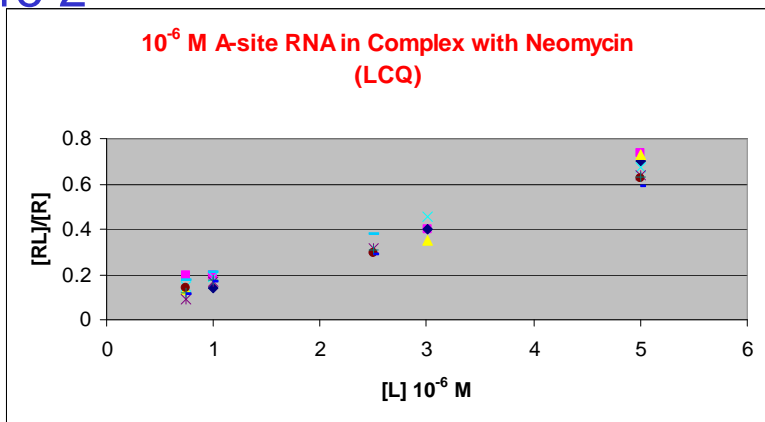


Figure 3 10  $\mu$ M RNA and 250 nM neomycin

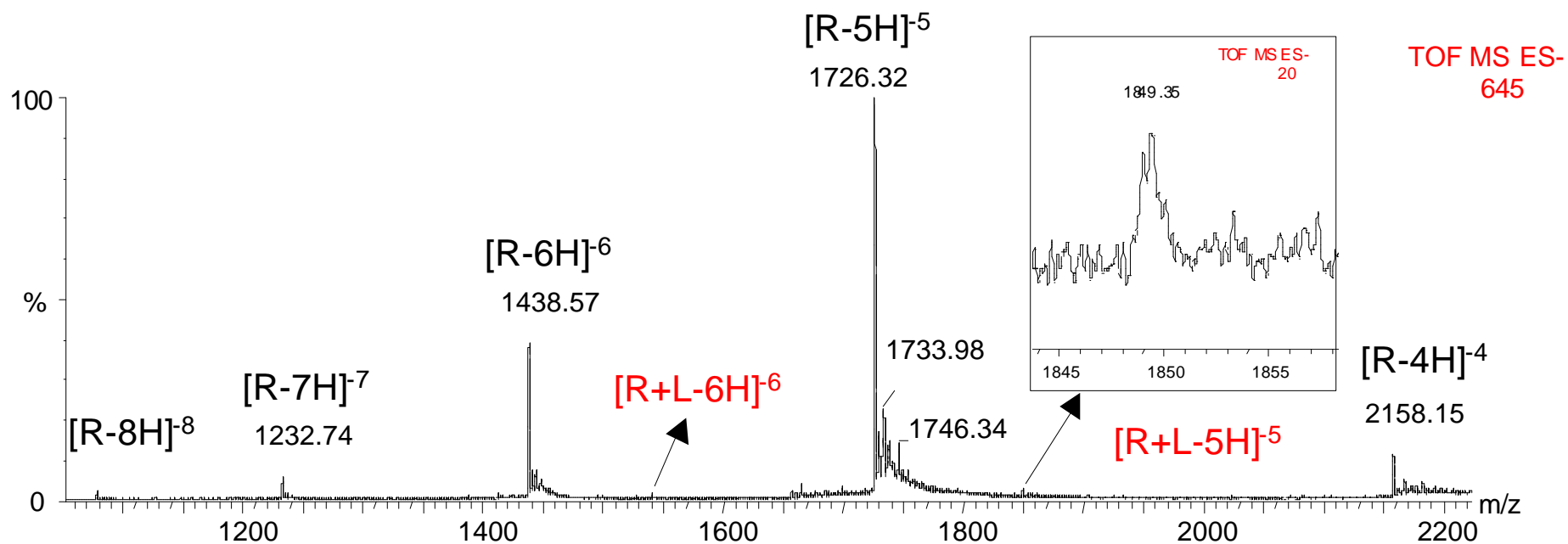
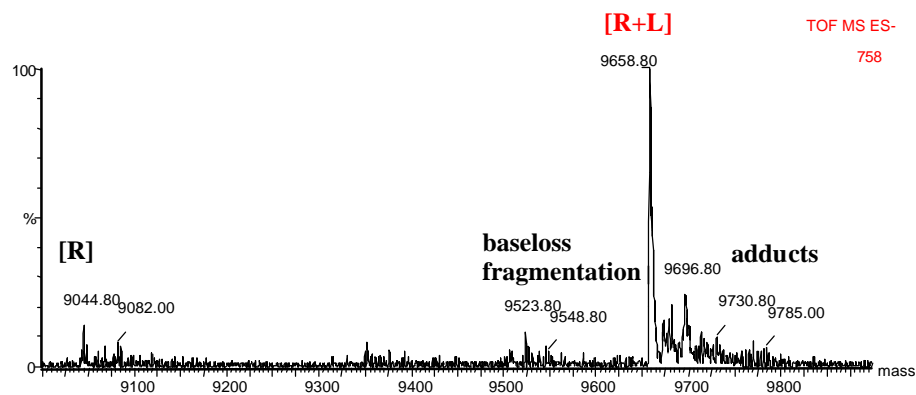
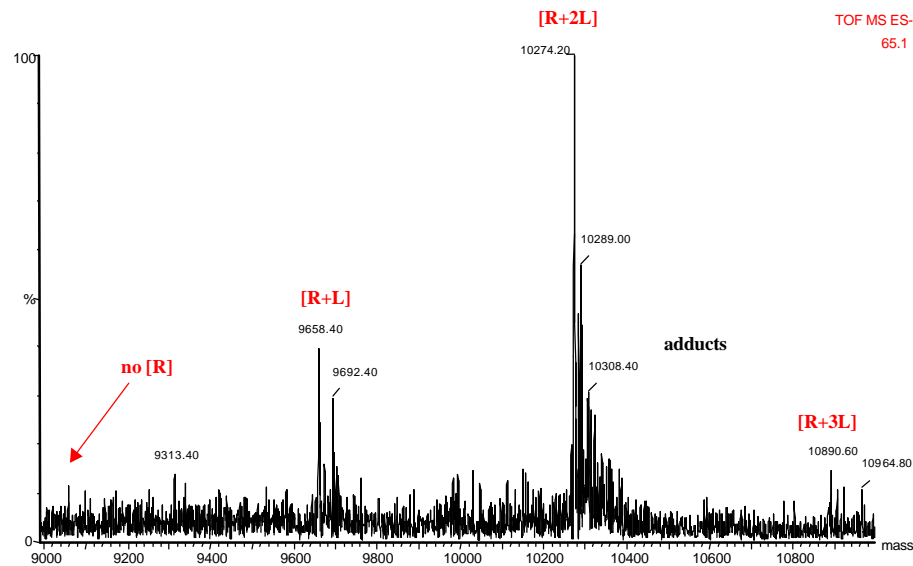


Figure 4 Deconvoluted spectra (Q-TOF)

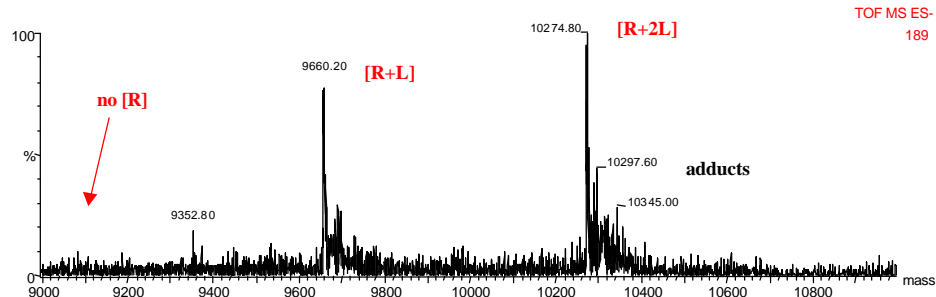
a) 10  $\mu$ M RNA (5' biotin) and 10  $\mu$ M neomycin



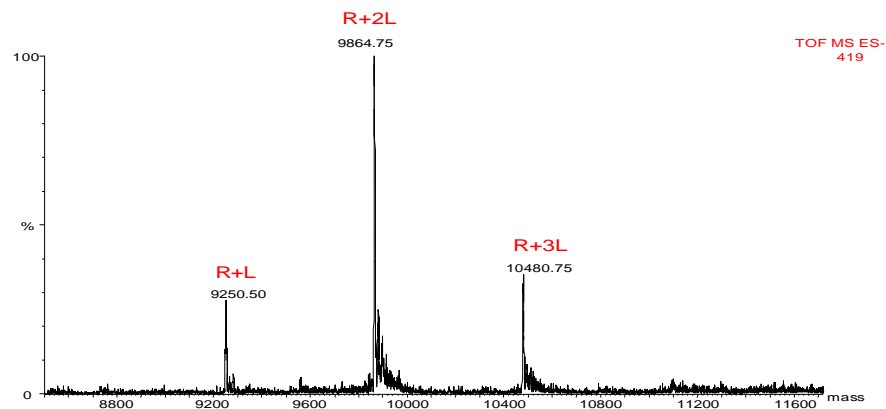
c) 10  $\mu$ M RNA (5' biotin) and 30  $\mu$ M neomycin



b) 10  $\mu$ M RNA (5' biotin) and 20  $\mu$ M neomycin



d) 10  $\mu$ M RNA and 100  $\mu$ M neomycin



## UV-MALDI-MS

There are a few, if any examples reported dealing with MALDI analysis of non-covalent complexes between RNA and small organic ligands.

Here we demonstrate that the A-site RNA/neomycin complex can be investigated by UV-MALDI-MS using the matrices ATT and THAP in the positive as well as negative-ion mode. The absence of signals for the complex using the 3-HPA matrix as well as the absence of multiple binding of neomycin to A-site RNA when applying less than the stoichiometric quantity of ligand suggests that the non-covalent complex is specific. No “first-shot-phenomenon” was observed for the complexes.

This is in agreement with the literature [3].

The MALDI data show a larger dispersion over the same range of neomycin concentration, due to heterogeneous sample morphology and the more disruptive nature of the ionization process.

Consistent with the Electrospray data, no more than 3 binding sites were observed when applying excess neomycin concentrations (figure 5a and b).

We obtained the best results using the ATT matrix and collecting data in the negative ion-mode.

Although UV-MALDI is normally viewed as unsuitable for  $K_d$  determination, it, unlike Electrospray, does enable a fast and carry-over-free screening for tight binders to target RNA.

### Determination of the dissociation constant $K_d$

To determine the  $K_d$  for the first binding site of neomycin to A-site RNA, we began with the protocol of Hofstadler [2] and developed a new approach using the Mathcad (version 8.0) software. The process of determining the  $K_d$  from the experimental data involves the least square fit between the measured degree of binding ( $[RL]/([R]+[RL])$ ) [4] and the modeled degree of binding as a function of the initial ligand concentration for a postulated  $K_d$ .

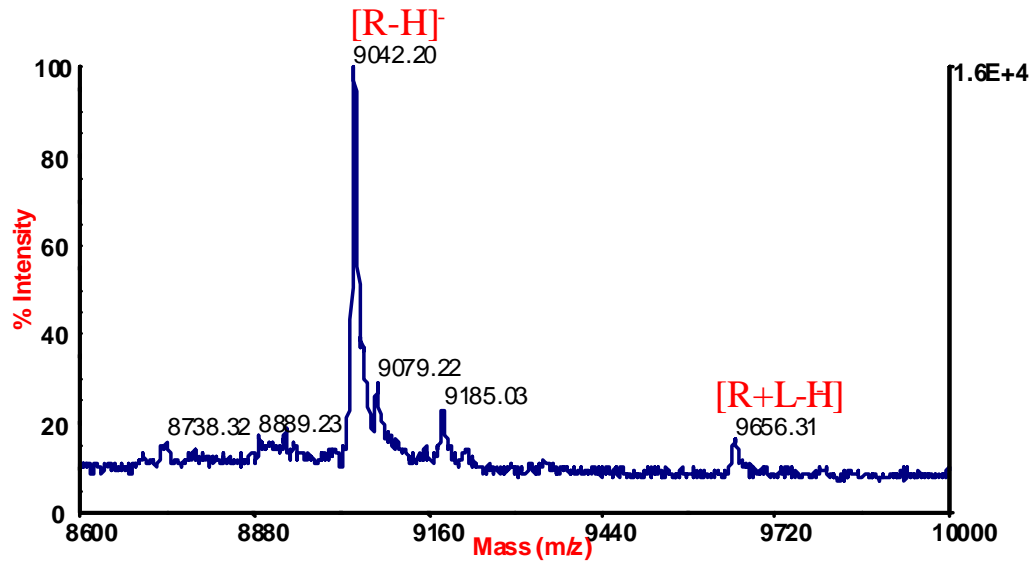
The method involves:

- a guess of the  $K_d$
- this guessed  $K_d$  implies the experimental conditions (initial RNA and ligand concentrations)
- the performed experiment gives a new  $K_d$  value (Figure 6)
- the experimental  $K_d$  may be subject to a large error when the initial RNA concentration is not optimal and small changes in binding produce large differences in  $K_d$
- if necessary, the experiment is redesigned by changing the initial RNA concentration and corresponding initial ligand concentration to maximize the changes in modeled degree of binding as a function of  $K_d$  in the neighborhood of the last  $K_d$ .

Using this approach we determined the  $K_d$  for the first binding site of neomycin to A-site RNA to be in the range of 156 nM, which is in good agreement with the literature [5].

Figure 5

3 a) 10 mM A-site RNA (5' biotin label) and 1 mM neomycin



3 b) 10 mM A-site RNA (5' biotin label) and 30 mM neomycin

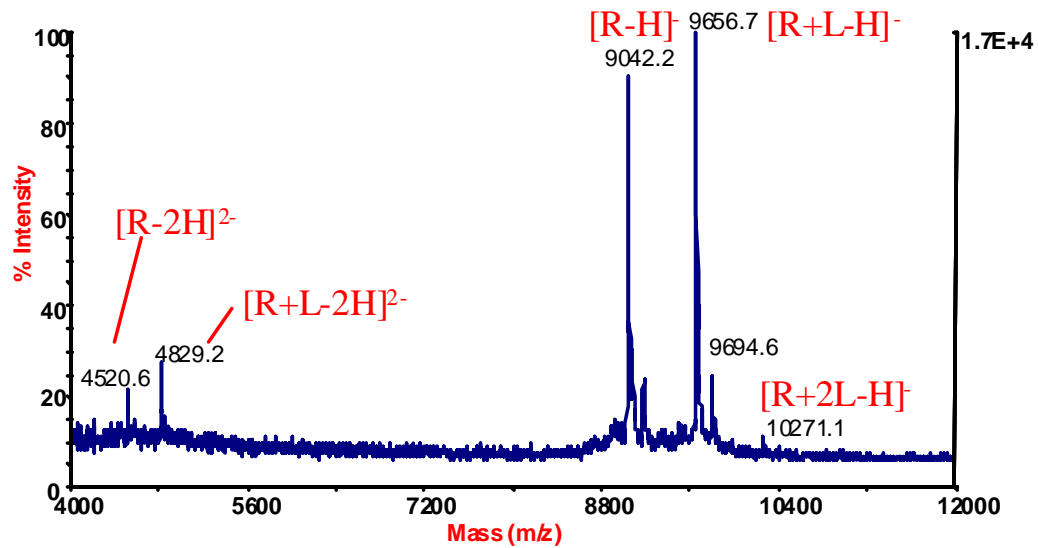
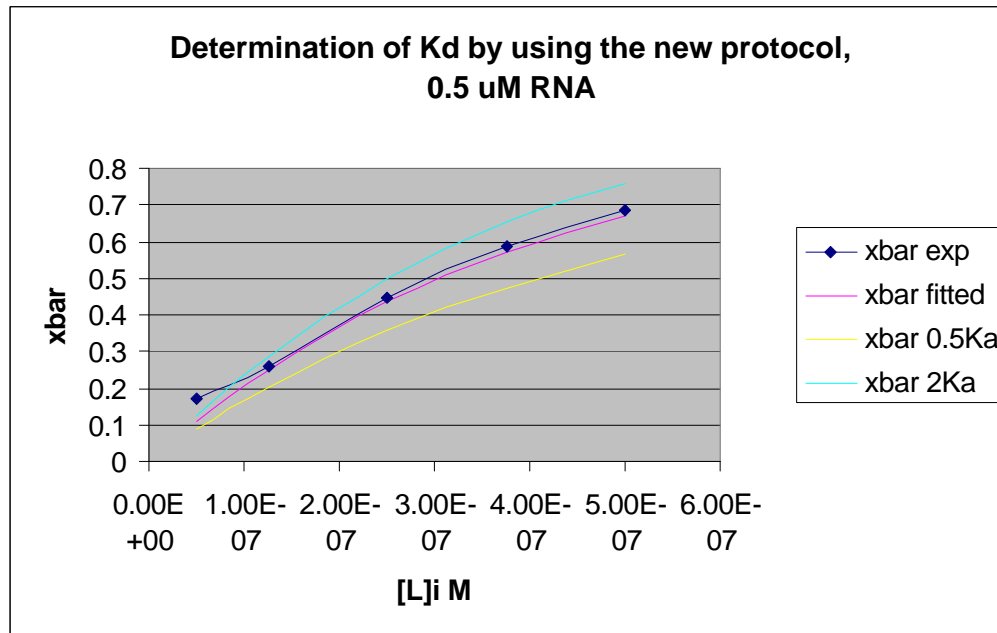
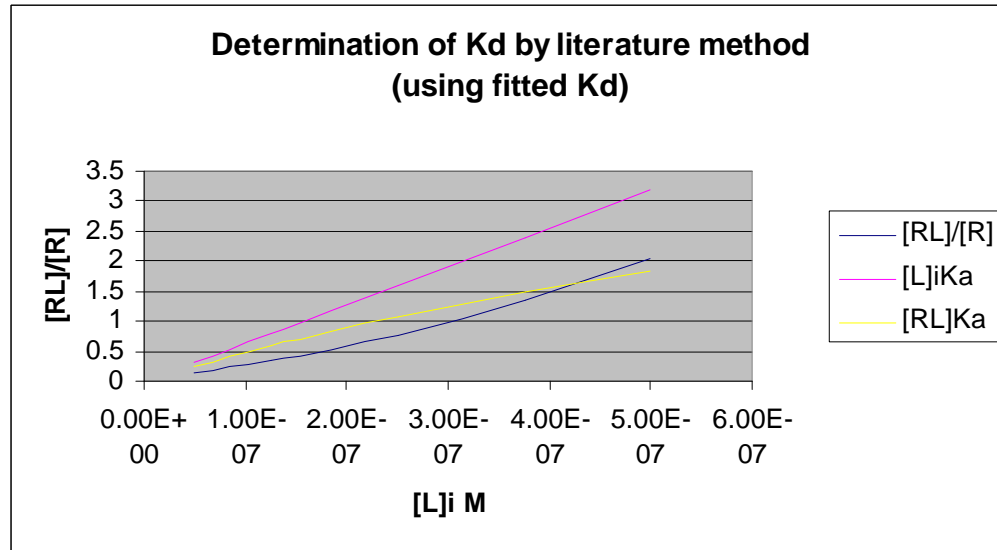


Figure 6



## 5) Conclusions

All three mass spectrometric techniques are useful to screen compounds for their ability to form complexes with the target RNA. However, the more disruptive nature of the ionization process and the heterogeneous preparation morphology introduce a larger dispersion in complex formation.

We developed a Mathcad protocol to determine the  $K_d$  value for binding of neomycin to A-site RNA. This protocol makes use of the fact that the greatest certainty in determining the  $K_d$  is dependent on the initial RNA concentration. Initial RNA concentrations in the range of 0.5-10  $\mu\text{M}$  can be used to determine the  $K_d$ . However, small changes in the degree of binding for an initial RNA concentration of 10  $\mu\text{M}$  lead to large changes in the fitted  $K_d$ . As was reported earlier [2] initial RNA concentrations should be used in the range of the  $K_d$ .

## 6) Acknowledgements

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## 7) References

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