

## RESEARCH ARTICLE

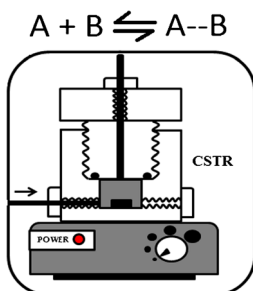
# Determination of Noncovalent Binding Using a Continuous Stirred Tank Reactor as a Flow Injection Device Coupled to Electrospray Ionization Mass Spectrometry

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**Abstract.** Described is a new method based on the concept of controlled band dispersion, achieved by hyphenating flow injection analysis with ESI-MS for noncovalent binding determinations. A continuous stirred tank reactor (CSTR) was used as a FIA device for exponential dilution of an equimolar host-guest solution over time. The data obtained was treated for the noncovalent binding determination using an equimolar binding model. Dissociation constants between vancomycin and Ac-Lys(Ac)-Ala-Ala-OH peptide stereoisomers were determined using both the positive and negative ionization modes. The results obtained for Ac-L-Lys(Ac)-D-Ala-D-Ala (a model for a Gram-positive bacterial cell wall) binding were in reasonable agreement with literature values made by other mass spectrometry binding determination techniques.

Also, the developed method allowed the determination of dissociation constants for vancomycin with Ac-L-Lys(Ac)-D-Ala-L-Ala, Ac-L-Lys(Ac)-L-Ala-D-Ala, and Ac-L-Lys(Ac)-L-Ala-L-Ala. Although some differences in measured binding affinities were noted using different ionization modes, the results of each determination were generally consistent. Differences are likely attributable to the influence of a pseudo-physiological ammonium acetate buffer solution on the formation of positively- and negatively-charged ionic complexes.

**Keywords:** Binding constants, CSTR, Electrospray ionization mass spectrometry, Controlled band dispersion, Vancomycin, Alanine stereoisomers

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## Introduction

Binding constant determination is of great importance in the drug discovery process. It can be used to gain information about the binding affinity of, for example, new antibiotics to microbial targets, and to aid in the development of new therapeutics [1]. Along these lines, vancomycin is one of the most studied glycopeptide antibiotics. It is known for its ability to

block cell wall synthesis in Gram-positive bacteria. This antibiotic binds stereospecifically to the carboxy-terminal D-Alanine-D-Alanine sequence of peptidoglycan precursors and inhibits new enzymatic crosslinking at this terminal sequence [1–4]. Numerous studies about noncovalent binding determination between vancomycin and different cell wall model targets have been performed [1, 3, 5–7]. In an effort to build from the knowledge gained, this model has also been used to facilitate the discovery of new antibiotic leads that might exhibit their activity through the same mechanism [8, 9].

Noncovalent binding can be evaluated by numerous methods, including those featuring UV spectroscopy [10], nuclear magnetic resonance (NMR) [10, 11], surface plasmon resonance (SPR) [12, 13], calorimetry [14, 15], and electrospray ionization mass spectrometry (ESI-MS) [5, 16–

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19]. The output of such measurements is usually an association constant ( $K_a$ ). UV spectroscopy, NMR, and calorimetry methods require large amounts of sample and are time-consuming. This makes them inappropriate when high throughput binding measurements are desired. Compared with ESI-MS, SPR provides little information on interaction stoichiometry [20], whereas NMR only allows the study of proteins with a molecular mass lower than 30,000Da [16]. ESI-MS is the most specific and sensitive technique of the mentioned methods as it allows for the direct and independent detection of analytes and complexes down to the picomole to femtomole range [16]. The lowest described dissociation constant ( $K_d$ ) value in the literature determined using ESI-MS was approximately  $10^{-15}$ M [20].

Previous studies have proven that noncovalently bound complexes formed between species in solution can be transferred to and preserved in the gas phase, which allows the determination of binding constant values by MS [1, 16, 21–23]. Using MS, association constants can be obtained by different approaches, such as titration [24–28], competitive binding [1, 17], or hydrogen/deuterium exchange [16, 29, 30] experiments. Among these approaches, titration is the most used method for the determination of binding constants. In a titration experiment, typically the concentration of one component is held constant while the concentration of its binding partner is varied. Then, the intensities of an unbound component and formed complex are often recorded. The binding constants may be determined from this data by linearization using a Scatchard plot or by fitting the data using a nonlinear model [20]. Although titration is simple to perform, it can also be a tedious and time-consuming procedure since it requires the preparation of multiple samples with varied concentrations [3, 6, 31].

To improve throughput, Fryčák and Schug developed a different titration method that uses a single solution of host and guest and is based on controlled band-dispersion [24, 25, 32, 33]. The controlled band-dispersion concept appeared together with the development of flow injection analysis. Flow injection analysis (FIA) was first described in 1975 by Ružička and Hansen [34] as a means to automate chemical analysis, lessen sample and reagent consumption, and improve throughput relative to classical methods. This technique provides automation of the solution handling operations, which decreases human error. Basically, FIA consists of the injection of a known volume of sample into a continuous flow of solution. As the injected sample is propelled, it disperses in the flowing stream and promotes the mixing between sample and any reagents present in the flow stream. The product of the reaction is measured in the detector placed downstream. The use of FIA and controlled dispersion for MS-based binding determinations has been termed dynamic titration [24, 25, 32, 33]. This method involves the injection of the guest solution into a flowing stream. The host solution, present in the flowing stream at constant concentration, mixes with the guest during band dispersion to create a time-dependent compositional gradient of guest in the presence of host. A binding constant can be

determined either by fitting the dispersion profile data using an appropriate (e.g., Gaussian) function combined with a simple binding model [32] or by integrating the degree of association across the observed signal peak [24, 25]. The use of these strategies is facilitated by in-house developed software; however, a drawback of the curve fitting procedure is that an appropriate function must be chosen to best match the shape of the dispersed band.

The objective of this work was to develop a new MS-based noncovalent binding determination method. For that purpose, a continuous stirred tank reactor (CSTR) [35] was used as a flow injection device. An analyte pulse-injected into a CSTR, which has been optimized to provide near-ideal mixing, will undergo an exponential dilution over time, which is easily modeled. In the current embodiment, an equimolar solution of host and guest was pulse-injected into the device. By combining an exponential dilution model with a previously established equimolar binding model [36], binding constants could be determined for host–guest complexation reactions with a single injection. This work-flow was successfully modeled and then applied as a proof-of-principle for the determination of binding constants between vancomycin and Ac-Lys(Ac)-Ala-Ala tripeptide stereoisomers. The use of both positive and negative ionization mode ESI-MS was demonstrated and the obtained results compared favorably with literature values.

## Experimental

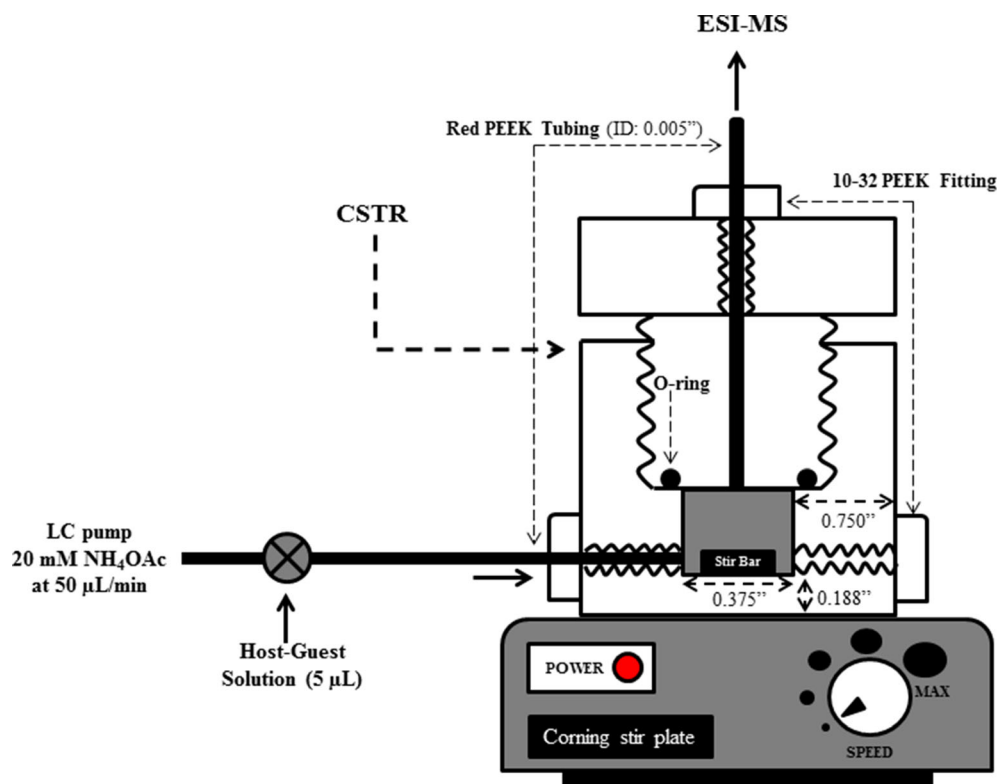
### *Continuous Stirred Tank Reactor (CSTR) Theory, Construction, and Operation*

In this work, we demonstrated the use of a CSTR to enable FIA and the measurement of binding constants for host–guest interaction partners by dynamic titration and ESI-MS. The experimental set-up is shown in Figure 1. In general, when an analyte is pulse-injected into a CSTR, placed in-line between a pump and a detector, the analyte plug is initially diluted into the chamber volume. If mixing is ideal, dilution happens instantaneously. From that point, the analyte will be continuously diluted over time. As fractions of the solution in the CSTR chamber leave the device, they are transported to the detector and replaced by fresh solvent. The initial maximum diluted concentration of the analyte ( $C_0$ ) registered at the detector can be calculated from:

$$C_o = \frac{C_{sample} \times V_{injected}}{V_{CSTR}} \quad (1)$$

where  $C_{sample}$  is the concentration of the analyte injected,  $V_{injected}$  is the injected sample volume, and  $V_{CSTR}$  is the CSTR volume. The concentration of the analyte in the device at any point in time  $C(t)$  is given by:

$$C(t) = C_o \exp\left(-\frac{Q}{V_{CSTR}} t\right) \quad (2)$$



**Figure 1.** Schematic of the experimental manifold for noncovalent binding determinations using a continuous stirred tank reactor (CSTR) and ESI-MS

where  $t$  is time and  $Q$  is volumetric flow rate. Thus, as time progresses under a constant flow rate, the analyte experiences an exponential dilution; analyte concentration and resultant detector signal thus decay exponentially in a highly predictable and easily modeled fashion.

The CSTR device schematized in Figure 1 was machined in The University of Texas at Arlington Machine Shop from a 1.875 in diameter rod of polyether ether ketone (PEEK) (Plastics International, Eden Prairie, MN, USA). The completed device consists of a housing body with two inlets drilled on either side of the base of a cored chamber. A 5×2mm Teflon-coated stir bar (Bel-Art Products, Wayne, NJ, USA) was placed in the bottom of the inner chamber. A matching threaded top was machined, through which an outlet channel was cored to accommodate 1/16" o.d. tubing. An o-ring (0.489 in×0.070"; 70-Durometer nitrile rubber; Hecht Rubber Corporation, Jacksonville, FL, USA) was inserted onto the base of the threaded cap to prevent leakage. PEEK fittings (10-32) and PEEK tubing (red; 0.005" i.d. and 1/16" o.d.) (Idex Health & Science, Oak Harbor, WA, USA) were used to make connections to the device. For these experiments, only one inlet was used. The other inlet was capped. Assembled to an operational state,  $V_{CSTR}$  for this device was determined to be  $0.70\pm 0.01$  mL (average±standard deviation;  $n=8$ ) based on water density at 21.9°C.

The CSTR was connected to standard liquid chromatography instrumentation components. A LC-20AD pump (Shimadzu Scientific Instruments (SSI), Columbia, MD,

USA) was connected to a LC-20AD autosampler (SSI) and interfaced with the CSTR device. The flow rate was 50 μL/min. A 20mM ammonium acetate ( $\text{NH}_4\text{OAc}$ ) solution was used as the mobile phase. An injection volume of 5 μL was used to introduce the equimolar mixtures of host and guest. The CSTR was placed on a Corning stir plate and the stir speed was set to 75% of the maximum speed. Detection was obtained using a Shimadzu ITTOF ion trap-time-of-flight mass spectrometer (SSI) equipped with a conventional electrospray ionization source (ESI). The source was operated in both positive and negative ionization modes. Nitrogen gas from a liquid nitrogen dewar was used as nebulizing gas at a flow rate of 1.5L/min. The detector voltage was set to 1.64kV and the interface voltages were set to 4.5kV (positive mode) and -4.0kV (negative mode). The CDL and heat block temperatures were 200°C. Ion accumulation time in the ion trap was optimized for sensitivity and signal quality and set to 80ms. The ion trap was set to scan from  $m/z$  400 to 2000. Data were acquired using LCMS Lab Solutions ver. 3.60.361 (SSI). Run times were set to 100min to accomplish exponential dilution of injected samples and appropriate cleansing of the system between injections. Data analysis and simulations were performed using Microsoft Excel.

### *Equimolar Binding Model*

In order to determine the dissociation constant ( $K_d$ ) (the inverse of  $K_a$ ) between each of the tripeptides and vancomycin at an

equivalent concentration, the equimolar binding model developed by Gabelica et al. [36] was used. An association constant is calculated by:

$$K_a = \frac{1 + R \frac{i(H)}{i(HG)}}{\left(\frac{i(H)}{i(HG)}\right)^2 C(t)} \quad (3)$$

where  $R$  is the relative response factors between the guest–host complex and the free vancomycin host, and  $i$  represents the ESI-MS intensities of the free host ( $i(H)$ ) and the host–guest complex ( $i(HG)$ ).

When operating in the linear response regime of the ESI-MS instrument (e.g., concentrations kept below 100  $\mu$ M to avoid ion suppression), the response factor ( $R$ ) can be assumed to remain constant through the CSTR dilution. Importantly, it is very difficult to empirically determine the response factor of the host–guest complex; so, it is not uncommon to assume that the response factors for the host and the guest are similar (e.g.,  $R=1$ ).

Indeed, if the relative response factor ratio could be accurately determined, a highly accurate binding constant could also be determined. For example, in the work by Raji et al. [37], this assumption was demonstrated to be a poor one for assessing binding between a series of similarly sized peptides. In the case of vancomycin binding, the tripeptide bacterial cell wall model, this assumption has been shown to be reasonably valid [9]. In fact, this assumption was also made by Jørgensen et al. [1], where they considered that the response factor for the doubly protonated antibiotic–peptide complex was similar to the response factor for the doubly protonated “free” antibiotic. They obtained binding constants consistent with previous studies that used non-MS-based binding determination techniques.

Based on these assumptions, Equation 4 reduces to

$$K_a = \frac{1 + I}{I^2 C(t)} \quad (4)$$

where  $I$  is the intensity ratio between the host and host–guest complex,

$$I = \frac{i(H)}{i(HG)} \quad (5)$$

### Chemicals and Reagents

LC-MS grade water was purchased from J.T. Baker (Phillipsburg, NJ, USA). Ammonium acetate and vancomycin hydrochloride hydrate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tripeptide stereoisomers of diacetyl-lysyl-alanyl-alanine (Ac-*L*-Lys(Ac)-*L*-Ala-*L*-Ala, Ac-*L*-Lys(Ac)-*D*-Ala-*L*-Ala, Ac-*L*-Lys(Ac)-*D*-Ala-*D*-Ala, and Ac-*L*-Lys(Ac)-*L*-Ala-*D*-Ala) were synthesized and purified to >95% purity

(according to the manufacturer) by Pepmic Co., Ltd. (Suzhou, China).

A stock solution of 1.00mM vancomycin was prepared in 40mM  $\text{NH}_4\text{OAc}$  in LC-MS water. Solutions of 1.00mM tripeptide were prepared in LC-MS water. Stock solutions were kept at  $-20^\circ\text{C}$  prior to use. Working solutions were made by mixing equal aliquots of 1.00mM stock solutions of the tripeptide and vancomycin into a HPLC sample vial to 0.50mM concentration ( $C_{\text{sample}}$ ).

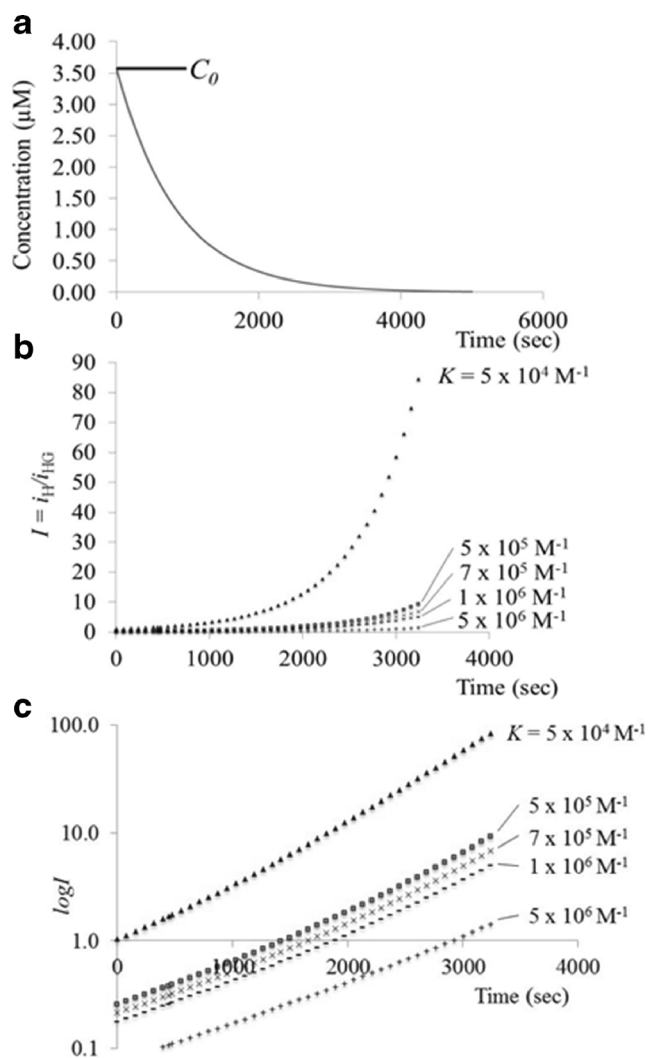
## Results and Discussion

In this work, a CSTR device was coupled to ESI-MS and used for determination of binding constants. Because this device allows an exponential dilution of the host–guest solution over time, a simple model could be developed to fit the obtained data. This approach was aided by the use of an equimolar titration model [36]. The technique was first simulated. Next, optimization studies were performed for a  $K_a$  determination of vancomycin binding Ac-Lys(Ac)-Ala-Ala stereoisomers. The technique was shown to provide results consistent with literature determinations of this well-studied noncovalent binding system, particularly those performed previously using other MS-based methods.

### Data Simulation

The developed dynamic titration consists of a single injection of an equimolar solution of host and guest at a known flow rate. A multipoint titration is obtained as ideal mixing between host–guest solution and the mobile phase is achieved in a CSTR device, which has a well-known volume. An exponential dilution of the host–guest solution is created by an influx of pure solvent into the device and efflux of its contents into the detector. Thereby, using Equation 2, a range of host concentrations is obtained over time as shown in Figure 2a.

Figure 2a represents a well-defined concentration gradient obtained by exponential dilution using Equations 1 and 2. The initial maximum concentration is denoted  $C_0$ . Figure 2b and c show simulated data for a dynamic titration of theoretical host–guest contributions at different levels of affinity. Figure 2b shows that as the dilution occurs, the relative amount of free host increases, as the amount of complex decreases. Figure 2c represents the change in ion intensities on a logarithmic scale. Thus, the predictable dilution of the host–guest system in the CSTR makes it possible to easily distinguish different degrees of binding affinity. Of course, in practice, differences in response factors for the free species and the formed complexes would alter the titration curves. A more complex model taking into account relative response factors would be a simple extension; however, as mentioned previously, empirical determination of response factors for complexes is a more difficult undertaking.



**Figure 2.** Simulated data for the dynamic titration of an equimolar solution of host and guest: (a) host concentration profile; (b) host:complex relative intensity profile; and (c) host:complex logarithmic relative intensity profile

### Optimization Studies

Different optimization studies for  $K_d$  determination using the Ac-L-Lys(Ac)-D-Ala-D-Ala tripeptide were performed. This peptide was chosen since its binding with vancomycin has been well studied and described in previous literature [1, 3, 5–7].

Flow rates of 50, 125, 250, and 500  $\mu\text{L}/\text{min}$  were studied. The choice of different flow rates would be expected to provide a compromise between ESI-MS ionization efficiency (higher at lower flow rates) and throughput (better at higher flow rates). For the CSTR, according to Equation 1,  $C_0$  is determined using the injected analyte concentration and the sample and CSTR volumes. This assumes that the solution is instantaneously diluted in the CSTR vessel because of near-ideal mixing.  $C_0$  should be established at the same moment the diluted analyte begins to exit the device. Ideal mixing is impossible to achieve in a flowing system. Yet, if higher flow rates are used, it is

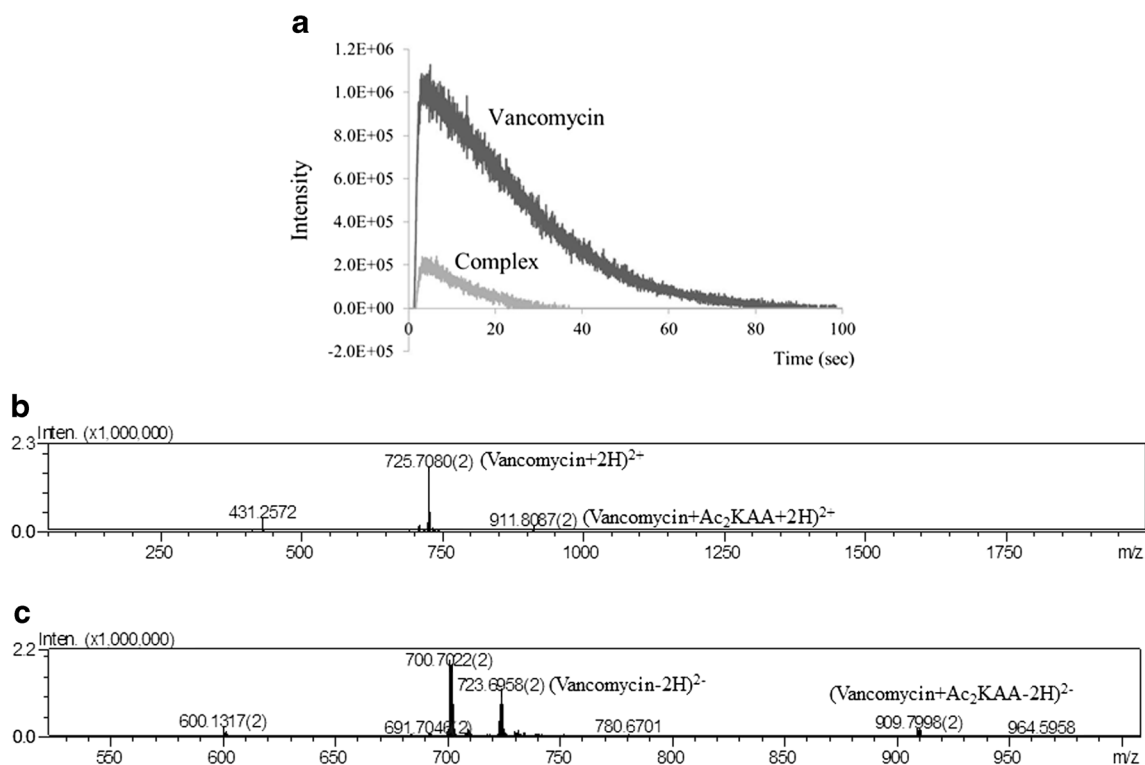
possible that the mixing is further from ideal, which would cause errors in the  $K_a$  determination. On the other hand, if a large volume injection is used at lower flow rates, the analyte will exit the vessel before all of it is loaded. Different injection volumes of 5, 25, and 40  $\mu\text{L}$  were also tested. Because we were less concerned with high throughput in these experiments, a lower flow rate value of 50  $\mu\text{L}/\text{min}$  was chosen (Supplementary Table S1). With this setting, as expected, compared with previous literature, similar  $K_d$  values were obtained with the lowest injection volume, 5  $\mu\text{L}$ .

Accumulation time in the ion trap has a significant influence on the qualitative and quantitative performance of the mass spectrometer. According to Liu et al. [38], the number and signal of the ions were increased with an increase in the accumulation time in the range of 10–100 ms. Therefore, different ion accumulation times were studied. Ion accumulation times of 30, 60, 80, and 100 ms were tested. With lower accumulation times (30 and 60 ms), it was not possible to determine the  $K_d$  value for all the stereoisomers. For accumulation times of 80 and 100 ms,  $K_d$  values were determined for all the stereoisomers and the results were generally similar (Supplementary Table S2). However, more variability was experienced with a 100 ms accumulation time and, thus, 80 ms was chosen for the final experimental measurements described below.

### Binding Determinations for Vancomycin and Ac-Lys(Ac)-Ala-Ala Stereoisomers

Solutions containing vancomycin and the respective Ac-L-Lys(Ac)-X-Ala-X-Ala stereoisomers (where X is D or L) in equimolar concentrations were injected in the CSTR-ESI-MS system. Since both guest and host undergo a dilution through time, vancomycin and tripeptide concentrations ( $C_{\text{sample}}$ ) of 0.50 mM were injected in the system in order to obtain a  $C_0$  of 3.57  $\mu\text{M}$  (5  $\mu\text{L}$  of sample was diluted into the 700  $\mu\text{L}$  vessel). The intensities of both free host and complex were recorded.

A representative profile for the dynamic titration is shown in Figure 3a. As the host–guest solution was injected, it was near-ideally mixed with the mobile phase in the CSTR and an exponential dilution of the equilibrium mixture was obtained. It was assumed that equilibrium is established rapidly as fractions leave the device and are transferred to the detector. Typical spectra for the measured reactants and products in the positive and negative ionization modes are shown in Figure 3b and c. Even though the IT-TOF accommodates polarity switching (50 Hz) during the course of an analysis, in our experiments, full data sets were collected separately for each ionization mode. The complex and free host were present predominantly as doubly protonated ion forms in the positive ionization mode ( $[\text{V}+\text{Ac}_2\text{KAA}+2\text{H}]^{2+}$  and  $[\text{V}+2\text{H}]^{2+}$ ) with corresponding peaks at  $m/z$  ratios 912 and 725, respectively. In the negative mode, they were present as doubly deprotonated ion forms ( $[\text{V}+\text{Ac}_2\text{KAA}-2\text{H}]^{2-}$  and  $[\text{V}-2\text{H}]^{2-}$ ) with corresponding peaks at  $m/z$  ratios 910 and 723, respectively. Equation 6 was fit to the measured data. Figure 4



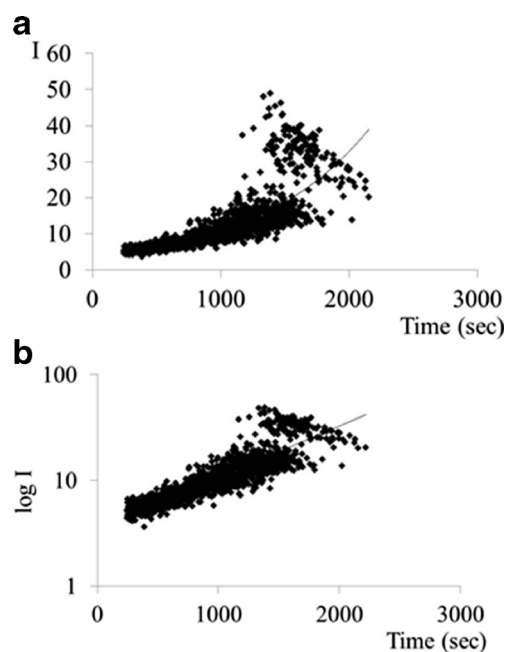
**Figure 3.** Equimolar CSTR titration profile (a) for an equimolar mixture of vancomycin host and Ac-L(Ac)-Lys-D-Ala-D-Ala guest in solution (0.50 mM). ESI-MS spectra are shown using the positive ionization mode (b) and the negative ionization mode (c)

shows the plots obtained by fitting the intensities of both the free host and the host–guest complex.

The experimental profiles shown in Figure 4 are similar to the simulated profiles shown in Figure 2b and c, with exception of contributions from experimental error in the former. As the concentration decreases, because of the exponential dilution, a wider spread of values is obtained. As expected, the precision of the measurement suffers as lower concentrations are monitored. To limit the contributions from potential non-ideality near  $C_0$  and contributions from increased error at the greatest dilutions, values of  $I$  obtained between 300 and 800s were used for  $K_d$  determination.

$K_d$  values determined using the equimolar binding model developed by Gabelica et al. [36], and combined with the CSTR dilution model, are presented in Table 1. The results obtained for Ac-L-Lys(Ac)-D-Ala-D-Ala are similar to those reported in prior literature [1, 6, 7, 13], where a variety of different analytical methods were used. However, the reported literature values do vary quite a bit, and this is most certainly due to slight differences in solution conditions and associated methodological accuracies. In many cases, precision of some binding determination techniques, especially depending on the magnitude of binding, are known to vary by as much as 50%. Two of the works referenced used mass spectrometry for binding determination and presented similar binding values to the ones obtained in this work. Actually, binding constants obtained previously by the Zenobi group using mass spectrometry and surface plasmon resonance have been compared [39]. The authors concluded that the agreement between the two methods was not ideal. The

authors also described the limitations of the SPR method, which could have a negative influence on binding determinations.



**Figure 4.** Modeled results for an equimolar solution of vancomycin and Ac-L(Ac)-Lys-D-Ala-D-Ala in the positive ionization mode: (a) the correlation between  $I$  and time; and (b) the correlation between  $\log I$  and time

**Table 1.** Values of Dissociation Constants,  $K_d$  (M), Obtained Using the Developed Method

Tripeptide	This work		[1]	[6]	[7]	[13]
	(+)	(-)				
Ac-L-Lys(Ac)-D-Ala-D-Ala	$8.6 \times 10^{-5} \pm 3.0 \times 10^{-5}$	$6.6 \times 10^{-6} \pm 1.9 \times 10^{-6}$	$1.25 \times 10^{-6}$	$2.03 \times 10^{-6}$	$6.67 \times 10^{-7}$	$6.5 \times 10^{-5}$
Ac-L-Lys(Ac)-D-Ala-L-Ala	$3.0 \times 10^{-3} \pm 1.7 \times 10^{-3}$	$1.35 \times 10^{-4} \pm 7.6 \times 10^{-5}$	-	-	No binding	-
Ac-L-Lys(Ac)-L-Ala-D-Ala	No binding	$5.9 \times 10^{-3} \pm 2.1 \times 10^{-3}$	-	-	No binding	-
Ac-L-Lys(Ac)-L-Ala-L-Ala	No binding	$3.3 \times 10^{-3} \pm 1.6 \times 10^{-3}$	-	-	No binding	-

Some variation in  $K_d$  values were obtained when the experiment was run using the positive and negative ionization modes. The dissociation constant value of Ac-L-Lys(Ac)-D-Ala-D-Ala obtained using the negative ionization mode was closest to the values presented in previous literature. No binding was observed for Ac-L-Lys(Ac)-L-Ala-L-Ala and Ac-L-Lys(Ac)-L-Ala-D-Ala using the positive ionization mode; binding for these was observed and quantified (albeit with high error) only while using the negative ionization mode. This difference observed between the negative and positive ionization modes can be due to several factors. Indeed, with respect to response factors, these can vary between the different ionization modes. Regarding the solvent, ammonium acetate was chosen to mimic physiological conditions. However,  $\text{NH}_4\text{OAc}$  is not usually chosen for analysis in the positive mode, as it is a slightly basic solution that is commonly used to promote efficient formation of negative ions in the negative ionization mode of ESI. On the other hand, according to Jørgensen et al. [40], the gas-phase stabilities of the vancomycin–tripeptide complexes observed in positive and negative ionization modes are also different. In fact, the addition and removal of protons during the ESI process might interfere with the structurally specific interactions between vancomycin and the tripeptides. The addition of protons to a gas-phase complex can be associated with a decrease in pH during the ESI process, which causes protonation of the anionic C-terminus of the ligand. This may lead to a weakening of the hydrogen bonds formed between ligand and the host. Therefore, retention of the ligand in the binding pocket is much less favorable in the positive mode than in the negative mode. In the end, the ESI solution and instrumental parameters used in this work favored the doubly deprotonated complex, which originated more accurate results using the negative ionization mode.

Clearly, the results show that Ac-L-Lys(Ac)-D-Ala-D-Ala and Ac-L-Lys(Ac)-D-Ala-L-Ala bind more tightly to vancomycin than the other two isomers. This is explained by the fact that vancomycin has a configuration that is specific for the D-Ala-D-Ala unit, which is present in the cell wall of Gram-positive bacteria. This is one of the main reasons why vancomycin is an effective antibiotic; whereas bacteria freely use D-amino acids in their biochemistry, eukaryotes predominantly rely on L-amino acids. Thus, the stereospecific preference for binding by vancomycin allows it be an effective antibiotic therapy. The results are further in agreement with a previous study by Jørgensen et al. [5], which showed that complexes of vancomycin with Ac-L-Lys(Ac)-D-Ala-D-Ala are significantly stronger than those with Ac-L-Lys(Ac)-L-Ala-L-Ala.

The developed method further allowed the determination of noncovalent binding for the Ac-Lys(Ac)-Ala-Ala-OH peptide stereoisomers that have been previously described as having no binding [7]. This could be explained by the fact that the developed method is more sensitive compared with the UV spectroscopy method used by Nieto and Perkins [7]; additionally, the IT-TOF mass spectrometer used in this work may be more sensitive than mass spectrometers used in previous studies. In this work, dissociation constants were determined in both the positive and negative ionization modes. Previous studies only describe the use of positive ionization mode for vancomycin  $K_d$  determination. Here, the negative ionization mode gave better and more comprehensive results for binding determinations in this system as explained previously. The ability to monitor complexation in both the positive and negative ionization modes, and their relative agreement, provides an inherent check on the validity of obtained values. Since the formed complexes must evaporate from the electrosprayed droplet unaltered relative to their equilibrium state, the fact that this can be done from ESI droplets that are either predominantly positively charged or negatively charged means that this is a fairly robust system for investigation by this and related mass spectrometric titration methods.

## Conclusions

The developed method allowed the determination of dissociation constants between vancomycin and Ac-Lys(Ac)-Ala-Ala tripeptide stereoisomers in both the positive and negative ionization modes. The hyphenation of FIA with ESI-MS by using a CSTR device allowed controlled exponential dilution of the equimolar host–guest solution, which was easily modeled. Determination of dissociation constants was possible by calculating host concentrations over time and by applying a previously developed equimolar titration model.

In contrast to classic titration methods, the developed method avoids the need for preparation of several guest solutions at different concentrations. With a single injection, the dynamic titration method gives a multipoint calibration in minutes, whereas the classic titration method requires many hours to perform and replicate. The simplicity of this method is its major advantage for the noncovalent binding studies field. The use of the CSTR is a simple flow injection device, which makes it a good option for determination of association constants. As has been demonstrated in other FIA techniques, the CSTR could also be combined with other detection methods for similar determinations.

This method should also be suitable for use with more complicated binding models such as non-equimolar titrations or competitive binding determinations. A different binding model would have to be used to account for the relative proportions of interactants as well as the presence of other guests and complexes. Although this method is designed for systems exhibiting 1:1 binding stoichiometry, it would be possible to modify it to accommodate higher order binding. In general, the predictable exponential dilution provided by the CSTR simplifies the determination of host or guest concentration along the temporal composition gradient generated.

In spite of its advantages, this method could present some limitations related to the determination of lower or higher dissociation constants. In order to do so, lower or higher concentrations of host and guest would be needed, which in this work would not be advisable as concentrations in the  $\mu\text{M}$  range should be used to guarantee operation in the linear response region. Another limitation is the potential influence of on-off binding kinetics on the measured binding constants (particularly, the off rate). If the system cannot equilibrate on the time scale of the CSTR dilution, an accurate measurement of the proportion of complex cannot be made. This (and mass ranges on the instrument) might limit the application to much larger systems, particularly if they have slow rates of dissociation. Also, some improvements could be made to the developed method in order to improve its performance. A higher throughput could be obtained by testing different CSTR devices with lower internal volume. Smaller CSTR devices would decrease the time necessary for diluting the injected solution, which therefore would increase the determination rate. It would also require lower working concentrations, since the dilution factor between the injected volume and the CSTR volume would be reduced. A great deal of prior work on the use of tank reactors in chemical engineering studies can be found in the literature. In general, many of these configurations could be miniaturized and adapted so that an information-rich detection system, such as mass spectrometry, can be used for interaction monitoring.

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