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Affinity Capillary Electrophoresis Can Simultaneously Measure Binding Constants of Multiple Peptides to Vancomycin[1]

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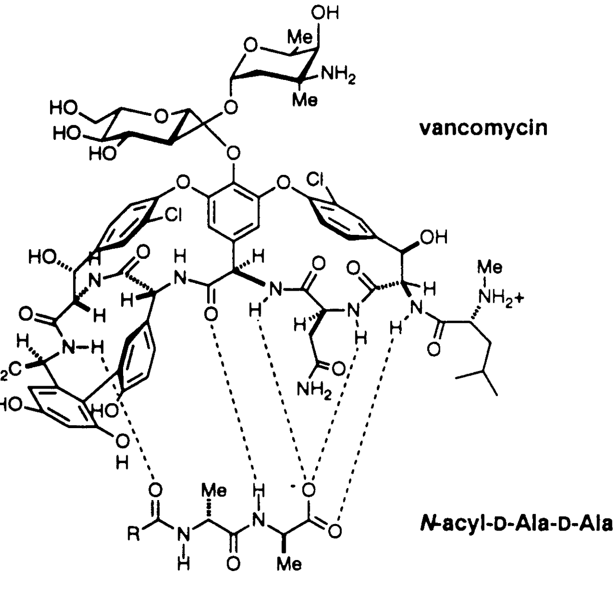
Summary: Affinity capillary electrophoresis is a sensitive and convenient technique for studying molecular recognition involving low molecular weight receptors.

This paper reports the use of affinity capillary electrophoresis (ACE) in the measurement of binding constants involving low molecular weight receptors, using vancomycin as an example.[2,3] In this system, it was straightforward to measure binding constants of four compounds (two pairs of enantiomeric peptides) to vancomycin simultaneously, and the technique can, in principle, be extended to larger numbers of ligands. We used vancomycin obtained from *Streptomyces orientalis* and *N*-acyl-D-(L)-alanyl-D(L)-alanines as a model system.[4] This system for molecular recognition has been extensively studied using other techniques, and a body of information concerning binding constants is available for comparison with constants estimated by ACE.[4]



The electrophoretic mobility μ (cm^2 V^{-1} s^{-1}) of a species in capillary electrophoresis (CE) has a relationship to its mass (M) and net charge (Z) of the approximate form $\mu \approx Z/M^{2/3}$.[5] We have carried out two types of experiments. In one, we set the electrophoresis buffer at pH 7.1, a value at which vancomycin carries a partial positive charge. We then included a range of concentrations of negatively charged peptides in the buffer and measured the corresponding change in the mobility of vancomycin, μ_v. In these experiments, the change in μ_v was due primarily to the change in charge of vancomycin (from partial positive to partial negative) on binding the peptide.[6] By setting the pH so that vancomycin and the vancomycin–ligand complex were oppositely charged, we avoided overlap with a neutral compound (mesityl oxide, MO) added as an internal standard to measure the contribution of electroosmosis to the retention times. Figure 1 shows representative electropherograms. We call this type of experiment an "R_L" experiment, meaning one in which we observe the receptor (R) and vary the concentration of the ligands (L).

Table I. Binding Constants (K_b) of *N*-Acyl-D(L)-Ala-D(L)-Ala Ligands to Vancomycin Measured by Affinity Capillary Electrophoresis (ACE)

		confign of	K_b (mM^{-1}) method		
ligand	R	peptide	R_L	L_R	other
1a	methyl	D,D	8.7[a]	4.8[a]	16,[b] 20,[c] 18[d]
b	methyl	L,L[e]			
2a	diacetyl-L-Lys	D,D	240[f]		1000,[b] 1500,[c] 48[d]
3a	succinyl	D,D	9.3[f]		
b	succinyl	L,L[e]			
4a	Fmoc-Gly	D,D	30[f]	19[f]	
b	Fmoc-Gly	L,L[e]			
5a	Fmoc-Gly-Ala	D,D,D		14[f]	
b	Fmoc-Gly-Ala	L,L,L[e]			

[a] Obtained in 10 mM sodium phosphate buffer (pH 7.1). [b] Obtained from the data of Popieniek and Pratt using a fluorescence-based assay in 100 mM phosphate buffer (pH 7.0).[8] [c] Obtained from the data of Nieto and Perkins using a UV-difference binding assay in 20 mM citrate (pH 5.1).[9] [d] Obtained from the data of Bugg et al. using a UV-difference binding assay in 20 mM citrate (pH 5.1).[10] [e] The L,L- and L,L,L-peptides showed no detectable binding, using the L_R method, at the concentration of vancomycin up to 150 μM. [f] Obtained in 20 mM sodium phosphate buffer (pH 7.5).

In a second, complementary, type of experiment, we observed the mobility μ_L of the ligands and varied the concentration of vancomycin included in the electrophoresis buffer (an "L_R" experiment). These experiments were carried out at pH 7.5, where vancomycin is essentially electrically neutral. The change in the mobility of the negatively charged peptides was due primarily to a change in mass (that is, hydrodynamic drag) on complexation with vancomycin.[6] These L_R experiments—in which the receptor is used as a component of the buffer—will often be more practical with ACE than with other analytical techniques, since CE uses only small quantities of materials. In principle, this type of procedure has the useful capability to measure binding constants of a number of ligands simultaneously. In the example shown in Figure 1, a single

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[1] This research was supported by the NIH (GM 39589) and by the NSF through the M.I.T. Biotechnology Processing Engineering Center (Cooperative Agreement CDR-88-03014).

[2] Reviews of capillary electrophoresis: Frenz, J.; Hancock, W. S. *TIBTECH* **1991**, *9*, 243–250. Kuhr, W. G. *Anal. Chem.* **1990**, *62*, 403R–414R. Novotny, M. V.; Cobb, K. A.; Liu, J. *Electrophoresis* **1990**, *11*, 735–749. Gordon, M. J.; Huang, X.; Pentoney, S. L., Jr.; Zare, R. N. *Science* **1988**, *242*, 224–228.

[3] For a related study involving protein receptors, see: Chu, Y.-H.; Whitesides, G. M. *J. Med. Chem.*, submitted for publication.

[4] Bugg, T. D. H.; Wright, G. D.; Dutka-Malen, S.; Arthur, M.; Courvalin, P.; Walsh, C. T. *Biochemistry* **1991**, *30*, 10408–10415. Popieniek, P. H. and Pratt, R. F. *J. Am. Chem. Soc.* **1991**, *113*, 2264–2270. Williams, D. H.; Waltho, J. P. *Biochem. Pharmacol.* **1988**, *37*, 133–141. Malabarba, A.; Trani, A.; Ferrari, P.; Pallanza, R.; Cavalleri, B. *J. Antibiotics* **1987**, *40*, 1572–1587.

[5] This expression is approximate, and a number of functional forms have been suggested to describe the relationship between μ, Z, and M: Rickard, E. C.; Strohl, M. M.; Nielsen, R. G. *Anal. Biochem.* **1991**, *197*, 197–207. Grossman, P. D.; Colburn, J. C.; Lauer, H. H. *Anal. Biochem.* **1989**, *179*, 28–33. Deyl, Z.; Rohlicek, V.; Adam, M. *J. Chromatogr.* **1989**, *480*, 371–378.

[6] Vancomycin (MW = 1450) is essentially electrically neutral at pH 7.5 (vancomycin and MO have the same migration time in 20 mM sodium phosphate buffer at pH 7.5). All ligands we used have smaller molecular weights (202, **1**; 372, **2**; 260, **3**; 439, **4**; 510, **5**) and carry one negative charge.

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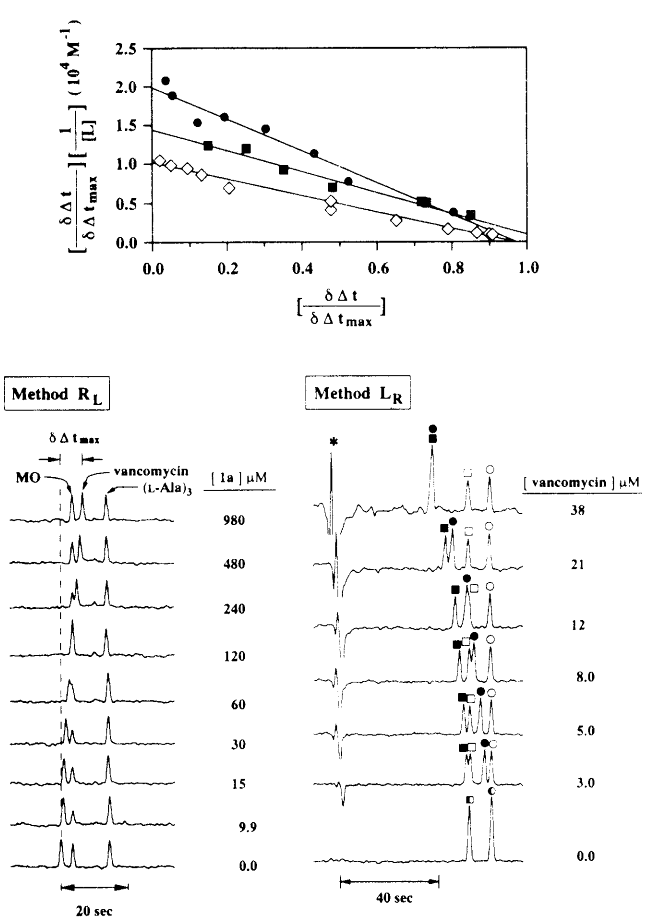

Figure 1. Method R_L: affinity capillary electrophoresis (ACE) of vancomycin in 10 mM sodium phosphate buffer (pH 7.1) containing various concentrations of *N*-acetyl-D-Ala-D-Ala (**1a**) (◇). The neutral marker mesityl oxide (MO) and the tripeptide L-Ala-L-Ala-L-Ala were used as internal standards. Method L_R: ACE of *N*-Fmoc-Gly-D-Ala-D-Ala (**4a**) (●), *N*-Fmoc-Gly-D-Ala-D-Ala-D-Ala (**5a**) (■), *N*-Fmoc-Gly-L-Ala-L-Ala (**4b**) (○), and *N*-Fmoc-Gly-L-Ala-L-Ala-L-Ala (**5b**) (□) in 20 mM sodium phosphate buffer (pH 7.5) containing various concentrations of vancomycin. The asterisk (*) indicates the position of the peak for unidentified neutral species carried through the capillary by electroosmotic flow. The total analysis time in each experiment was ~2.5 min (method R_L) and ~4.0 (method L_R) at 30 kV using a 45-cm (inlet to detector), 50-μm uncoated fused silica capillary. The graph is a Scatchard plot of the data according to eq 1.

set of measurements determined values of K_b for **4a**, **4b**, **5a**, and **5b**.[7]

By measuring the appearance time (t) of the peak due to vancomycin (method R_L) or to peptides (method L_R) as a function of the concentration of additive (L or R) present in the CE buffer, it was possible to determine binding constants (K_b). Equation 1 gives a convenient

$$(\delta\Delta t/\delta\Delta t_{\max})(1/[\mathrm{B}]) = K_b - K_b(\delta\Delta t/\delta\Delta t_{\max}) \qquad (1)$$

form for Scatchard analysis: here $\Delta t_{[\mathrm{B}]}$ is the difference between appearance time of the species of interest and the internal standard at concentration [B] of the additive in buffer, $\delta\Delta t = \Delta t_{[\mathrm{B}]} - \Delta t_{[\mathrm{B}]=0}$, and $\delta\Delta t_{\max}$ is the value of $\delta\Delta t$ at saturating concentrations of B. Table I summarizes results.

Figure 1 shows Scatchard plots (eq 1) derived from both R_L and L_R experiments. The values of K_b for **1a** and **4a** obtained using method R_L compare well with those obtained by method L_R. The values of binding constants measured by ACE also fall in the range of those values obtained from other assays.[8-10]

Affinity capillary electrophoresis has several advantages as a method of measuring binding constants. First, it requires only small quantities of receptor and ligand. Second, when using method R_L, neither high purity for the receptor nor an accurate value of its concentration is required. Third, when using method L_R, it may be possible to measure binding constants of several ligands to a single receptor in the same set of experiments. When used with relatively low molecular weight species, the adsorption of the capillary wall that complicates experiments with proteins is unlikely to occur.[11]

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Supplementary Material Available: Experimental details for the preparation of **1**–**5** (7 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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[7] Vancomycin absorbs strongly at 200 nm. High concentrations (≥50 μM) decreased the S/N in the L_R method. We used Fmoc-derivatized peptides to increase sensitivity.

[8] Popieniek, P. H.; Pratt, R. F. *Anal. Biochem.* **1987**, *165*, 108–113.

[9] Nieto, M.; Perkins, H. R. *Biochem. J.* **1971**, *123*, 789–803.

[10] Bugg, T. D. H.; Wright, G. D.; Dutka-Malen, S.; Arthur, M.; Courvalin, P.; Walsh, C. T. *Biochemistry* **1991**, *30*, 10408–10415.

[11] In an R_L experiment, partial adsorption of the receptor on the wall of the capillary has no influence on the measured binding constants, provided that its extent is independent of the concentration of L. The equilibration between solution and capillary wall simply changes the effective mobility, but not the form of the Scatchard analysis. In an L_R experiment, concentration-dependent adsorption may have more potential to cause error. It should, however, be detectable in most cases in nonlinearity in the Scatchard plot.