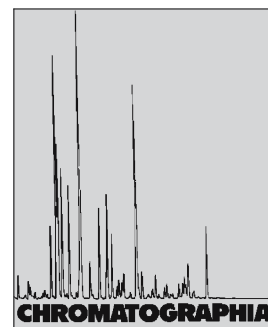


Determination of Binding Constants of Polyethylene Glycol Vancomycin Derivatives to Peptide Ligands Using Affinity Capillary Electrophoresis



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Abstract

Vancomycin (Van) from *Streptomyces orientalis* has been derivatized with polyethylene glycol [PEG; PEG-550 (1), 750 (2), 1,100 (3), 2,000 (4), 5,000 (5), and 8,000 (6) g mol⁻¹] at the N-terminus of the glycopeptide backbone and their binding to D-Ala-D-Ala terminus peptides assessed using affinity capillary electrophoresis (ACE). Utilizing ACE, a plug of Van-PEG and non-interacting standards are injected and electrophoresed. Analysis of the change in the relative migration time ratio of the Van-PEG species, relative to the non-interacting standards, as a function of the concentration of peptide, yields a value for the binding constant (K_b). Values of K_b for N-acetyl-D-Ala-D-Ala, **7** to the Van-PEG derivatives are weaker than those for N_α,N_ε-diacetyl-Lys-D-Ala-D-Ala, **8** (for example, values of K_b for **7-1** and **8-1** are 1.8 and $47.7 \times 10^3 \text{ M}^{-1}$, respectively). These results demonstrate that derivatization of Van with PEG has little effect on the affinity of D-Ala-D-Ala peptide ligands to it. The findings further prove the versatility of ACE and its ability to estimate binding parameters of ligands to antibiotics.

Keywords

Affinity capillary electrophoresis
Vancomycin
Binding constants
Polyethylene glycol

Introduction

The discovery of new proteins, made possible by the completion of the human genome, has provided scientists with the opportunity to parlay these findings into potential effective pharmaceuticals. These

proteins, of the recombinant type, are limited in their chemical and physical stability hindering their usage. One method to stabilize them in the liquid state is through chemical modification to form a conjugated protein. Recently, polyethylene glycol (PEG) has been

effectively utilized in protein conjugation studies to yield potentially stable and robust therapeutic drugs [1–7].

There is a great need to develop new analytical techniques that can quantify the extent of interaction between biological species. At present, all techniques used for estimating the affinity of one molecule to another are based on the measurement of changes in the physicochemical properties of the drug. Given the dependence of drug delivery and drug interaction quantification on each other new techniques focusing on the latter will greatly expedite new drug therapies needed in the treatment of many human diseases.

Capillary electrophoresis (CE) is a versatile analytical technique that has gained widespread use in many areas of research but specifically in the analysis of biomolecules [8]. CE has been successfully used to separate, for example, anions, cations, proteins, DNA, and viruses [9]. CE separates analytes based on differences in the apparent velocities of charged solute species under the influence of an applied electric field.

One technique that has found great success in the study of biomolecular non-covalent interactions and in determining binding parameters is affinity CE (ACE) [8–38]. For example, Ding et al. [10] examined the interaction between anti-human immunodeficiency virus type 1 (anti-HIV-1) active compounds with *trans*-activation response (TAR) RNA by

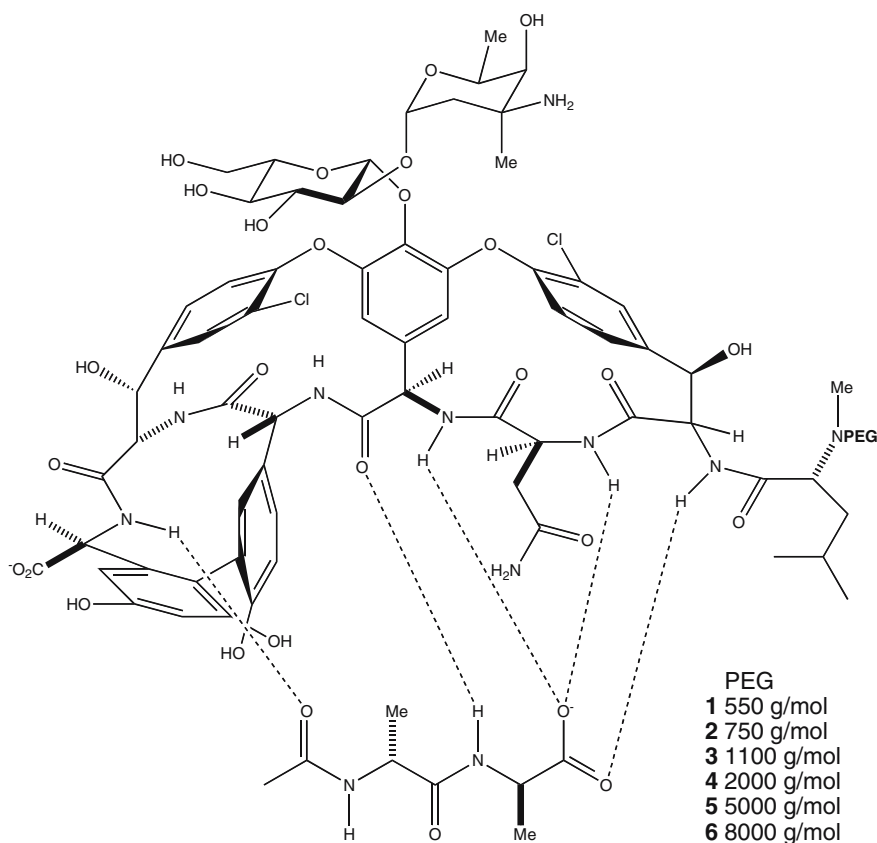


Fig. 1. Van-PEG compounds and ligands used in this study

ACE. Zavaleta et al. [11] developed multiple injection ACE to probe the binding of the antibiotics to small peptides. Finally, Varenne et al. [12] used ACE to examine the binding of fucoidan, an anticoagulant polysaccharide of marine origin, to antithrombin.

Vancomycin (Van) from *Streptomyces orientalis* is a parenteral glycopeptide antibiotic that kills bacterial cells by inhibiting peptidoglycan biosynthesis (Fig. 1) [38–46]. It functions by binding to the terminal D-Ala-D-Ala dipeptide of bacterial cell wall precursors, thereby, impeding further processing of these intermediates into peptidoglycan [39, 40]. Historically, Van has been the drug of choice in treating infections caused by bacterial resistant to other types of antibiotics.

Unfortunately, Van resistant enterococci (VRE) has emerged which is of major concern to the biomedical community. For example, the evolution of methicillin-resistant *Staphylococcus aureus* (MRSA), resistant to all established antibiotics, is now a serious worldwide problem. At present, the only treatment for MRSA is Van [39]. Van resistance is now also common in *Enterococcus fae-*

calis, hence, it is becoming increasingly important to develop new Van-group antibiotics, to study their physicochemical parameters, and to examine their activity against VRE.

In this paper, we extend the use of ACE to estimate binding constants of D-Ala-D-Ala terminus peptides to a series of pegylated-Van antibiotics (Fig. 1). The data obtained by ACE are in agreement with previous studies with underivatized Van and demonstrate the advantages of using ACE to estimate affinity parameters between peptides for antibiotics [9, 11, 22, 27, 30]. This study demonstrates the effect of modification of Van by a chemical group used in drug delivery has on its affinity for target molecules.

Experimental

Chemicals and Reagents

All chemicals were analytical grade. Vancomycin from *Streptomyces orientalis*, N-acetyl-D-Ala-D-Ala, **7**, N_α,N_ε-diacetyl-Lys-D-Ala-D-Ala, **8**, and carbonic anhydrase B (CAB, EC 4.2.1.1, containing CAA and CAB isozymes, from bo-

vine erythrocytes), were purchased from Sigma Chemical Company (St. Louis, MO, USA) and used without further purification. Polyethylene glycol (PEG) ($M_w = 550, 750, 1,100, 2,000, 5,000,$ and $8,000 \text{ g mol}^{-1}$) were obtained from Fluka (Milwaukee, WI, USA). Mesityl oxide (MO) was purchased from Calbiochem (San Diego, CA, USA). Stock solutions of Van (1.0 mg mL^{-1}) and bovine carbonic anhydrase B (1.0 mg mL^{-1}) were each prepared by dissolving the lyophilized species in buffer (192 mM glycine–25 mM Tris; pH 8.3).

Apparatus

The capillary electrophoresis (CE) system used in this study was a Beckman Model P/ACE 5510 (Fullerton, CA, USA). The capillary tubing (Polymicro Technologies, Inc., Phoenix, AZ, USA) was of uncoated fused silica with an internal diameter of 50 μm , length from inlet to detector of 47.0 cm and a length from detector to outlet of 6.5 cm. Data were collected and analyzed with Beckman System Gold software. The conditions used in CE were as follows: voltage, 20 kV; current, 4.5 μA depending on the capillary length; detection, 200 nm; temperature, $23 \pm 0.5 \text{ }^\circ\text{C}$.

Procedures

A sample of increasing concentrations of peptide in buffer was vacuum injected into the capillary for 1.0 min at high pressure followed by a sample (3.6 nL) of solution for 2 s containing 0.2 mg mL^{-1} of Van, 0.8 mg mL^{-1} of CAB, and $0.4 \mu\text{L}$ of MO in buffer. The electrophoresis was carried out using a tris–gly buffer and repeated at increasing concentrations of the peptide ligand (0–150 and 0–1,000 μM for **7** and **8**, respectively) for 2.0 min.

Synthesis of Vancomycin Derivatives

m-PEG Activation

Synthesis of PEGylated compounds followed previous procedures [47–49]. Approximately 5 g ($\sim 1.0 \text{ mmol}$) of m-PEG was dissolved in 50 mL anhydrous methylene chloride. The solution was

cooled to 0 °C, and 4 eq. of triethylamine (TEA) and 4 eq. of *p*-nitrophenyl chloroformate was slowly added. After the addition the solution was allowed to slowly warm up to room temperature while maintaining the pH at 7.5–8.0 by additions of TEA. The pH of the solution was checked by placing a drop of solution on a piece of wet pH-paper. After stirring for 3 h in vacuum, a few drops of base were added to the solution. The solution was filtered and added to diethyl ether (100 mL). The solution was filtered and the solvent evaporated. The product was then purified by recrystallization in diethyl ether (3 × 100 mL) at –10 °C. The typical yield of the reaction was 75%.

PEGylation of Vancomycin

To 1 mL of phosphate buffer pH 8.5 (0.1 M) was added 25 mg (0.17 mol) of vancomycin and 1 equivalent of the activated m-PEG. The solution was stirred for 4 h. The solution was dialyzed against water using a M.W. 1,000 membrane (Spectra/Por), until the yellow color had disappeared. The typical yield of the reaction was 61%.

Results and Discussion

Binding constants were obtained for two D-Ala-D-Ala terminus peptides, *N*-Acetyl-D-Ala-D-Ala (**7**) and *N,N*-diAc-Lys-D-Ala-D-Ala (**8**) to Van-PEG using ACE. Van contains in its glycopeptide backbone an *N*-terminus that is susceptible to nucleophilic attack. In our studies, PEG of varying molecular weights was first converted to the corresponding activated esters. The *N*-terminus amine on the glycopeptide backbone is susceptible to electrophilic attack at pH 8.5. No derivatization of the amine on the sugar moiety is possible since its pK_a is above 8.5. In our studies no derivatization was observed.

In our initial study we examined the interaction of Van-PEG750 (**2**) and two small peptides. In this study a plug of sample containing **2**, CAB, and MO were injected into the capillary column at low pressure for 2 s. The sample was then subjected to electrophoresis in buffer containing increasing concentrations of **7**. CAB and MO are non-interacting standards used in the data analysis. Neither marker has any affinity to **2** or the peptide ligands used in this study.

Figure 2 shows a representative series of electropherograms of **2** in capillaries filled with increasing concentrations (0–1,000 μM) of **7**. Upon electrophoresis a dynamic equilibrium is achieved between **2** and **7** resulting in a shift in migration time of the **2–7** complex. The complexation between **2** and **7** resulted in an increasing negative charge and the complex is detected later than the uncomplexed form. In ACE, changes in migration time of a receptor on complexation to a ligand are dependent on the change in the charge-to-mass ratio ($Z/M^{2/3}$) of the receptor. Complexation to a ligand more greatly affects the charge of the receptor than a change in its mass. On the other hand, a large ligand (along the order of the receptor), and, especially one that was neutral, would have had a reverse effect and the complex would have had a lower mobility than peptide **2** alone. As can be seen in Fig. 2, all of the electropherograms had the same elution pattern. Each concentration of peptide was run in multiplate and over two separate days. Figure 2 confirms the existence of some unreacted Van (25% of total Van content in sample). Still, CE is able to differentiate **2** from Van as both compounds have different charge-to-mass ratios. The versatility of this technique allows for analysis of **2** to be unaffected by the impurity as long as the peak is well defined.

Figure 3 is a Scatchard plot of the data for **2**. In this form of analysis termed the relative migration time ratio (RMTR) (Eq. 1), the binding constant is estimated using two non-interacting markers injected with the sample [25]. Here, t_r , t_s , and $t_{s'}$

$$RMTR = (t_r - t_{s'}) / (t_s' - t_s) \quad (1)$$

are the measured migration times of the Van-PEG compound, and the two non-interacting standard peaks, respectively. In the present experiments, t_s and $t_{s'}$ are the migration times of CAB and MO, respectively. A Scatchard plot can be obtained via Eq. 2.

$$\Delta RMTR_{R,L} / [L] = K_b \Delta RMTR_{R,L}^{\max} - K_b \Delta RMTR_{R,L} \quad (2)$$

Here, $\Delta RMTR_{R,L}$ is the magnitude of the change in the relative migration time ratio as a function of the concentration of peptide ligand. Equation 2 allows for the estimation of K_b on a relative time scale

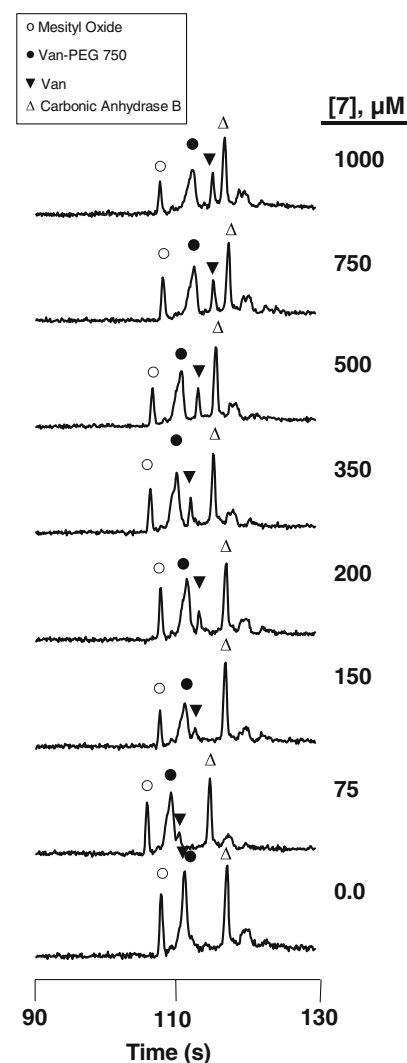


Fig. 2. A representative set of electropherograms of **2** in 0.192 M glycine–0.025 M Tris buffer (pH 8.3) containing various concentrations of **7**. The total analysis time in each experiment was 4.0 min at 20 kV (current: 4.0 μA) using a 40.5-cm (inlet to detector), 50-μm I.D. open, uncoated silica capillary. Mesityl oxide (MO) and carbonic anhydrase B (CAB and CAA isozymes) were used as internal standards

using two non-interacting standards and compensates for fluctuations in electroosmotic flow (EOF), background electrolyte (BGE) viscosity and electric field variations in the capillary column.

To establish that little change in the affinity of D-Ala-D-Ala terminus peptides to Van-PEG species occurs upon changing the size of the PEG moiety on Van we derivatized Van with PEG2000 (**4**). Figure 4 shows a representative series of electropherograms of **4** to ligand **8**. Upon increasing the concentration of **8** in the running buffer the peak for **4** shifts to a greater migration time similar to that

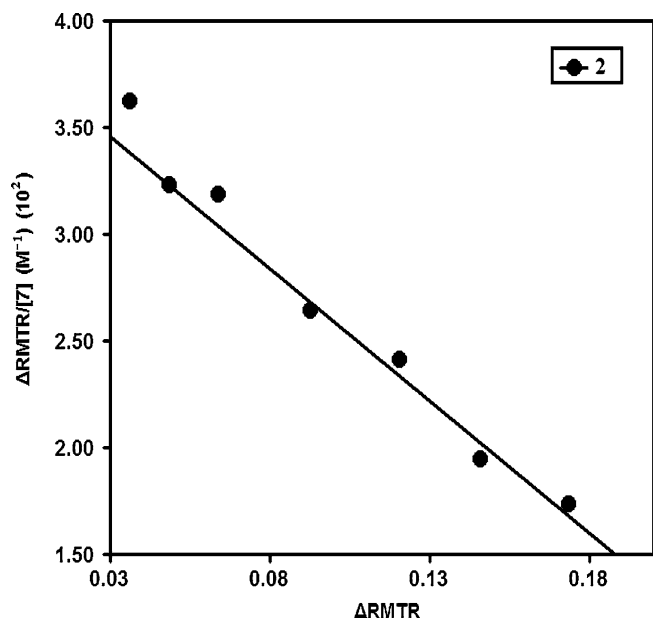


Fig. 3. Scatchard plots of **2** according to Eq. 2

found for the binding of **2** to **7**. Figure 5 shows the Scatchard plots of **4** according to Eq. 2.

Table 1 is the data for the binding of Van-PEG derivatives **1–6** to peptides **7** and **8**. For ligand **7** all of the values for the binding constants to the Van-PEG species were of the same order (10^3) although there was some variance in the extent of binding. There is no trend (either increasing or decreasing) in the values of K_b obtained herein. For ligand **8** the values for K_b ranged from 10^4 to 10^5 in magnitude. Of particular interest are the large binding affinities of ligand **8** for compounds **4** and **5**. There is no chemical reason why PEG's of molecular weight 2,000 or 4,000 g mol^{-1} would influence the affinity of D-Ala-D-Ala terminus peptides to Van. The value of the binding constant for the larger compound **6** for **8** is comparable to that found for compounds **1–3** so one cannot reason that larger PEG moieties influence the binding of peptides to Van. Similar is born out with ligand **7** to **1–6**. It should also be noted that there is no big difference in the binding constants for Van found in the literature versus that of the present Van-PEG species.

ACE has several advantages as a method for measuring interactions between receptors and ligands. First, it requires smaller quantities of material than in other assay techniques. Second, puri-

fication of the sample is not necessary as long as the component can be distinguished and separated from other species in the mixture. Third, it does not require radiolabeled or chromophoric ligands. Fourth, the commercial availability of automated CE instrumentation and the high reproducibility of the data make it experimentally convenient and appropriate for high-throughput applications.

Conclusion

Binding constants between peptide ligands and Van-PEG derivatives may be estimated both expeditiously and accurately using affinity capillary electrophoresis (ACE). In these experiments the capillary was first filled with Van-PEG followed by a plug of sample containing peptide ligand and non-interacting standards and electrophoresed. Upon application a dynamic equilibrium is achieved between Van and the ligand. The use of the relative migration time ratio yielded values for binding constants of the order of those obtained by other assay techniques with underivatized Van antibiotics. It was apparent that PEGylation of Van does not significantly affect the binding constants to the ligand used in this study. Further work to demonstrate the versatility of ACE in examining

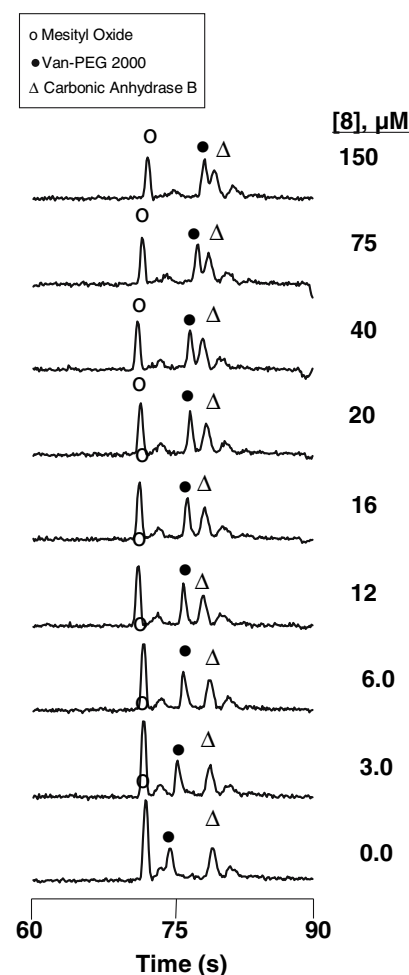


Fig. 4. A representative set of electropherograms of **4** in 0.192 M glycine–0.025 M Tris buffer (pH 8.3) containing various concentrations of **8** in the running buffer. The total analysis time in each experiment was 2.0 min at 20 kV (current: 4.5 μA) using a 30.5-cm (inlet to detector), 50- μm I.D. open, uncoated silica capillary. Mesityl oxide (MO) and carbonic anhydrase B (CAB and CAA isozymes) were used as internal standards

antibiotic–ligand interactions is in progress.

Acknowledgments

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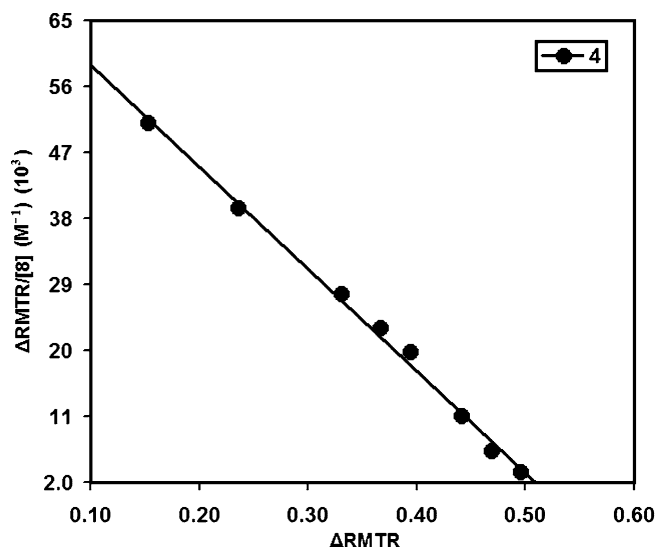


Fig. 5. Scatchard plots of **4** according to Eq. 2

Table 1. Experimental values of binding constants K_b (10^3 M^{-1}) and standard deviations of Van-PEG derivatives and ligands **7** and **8** measured by ACE

Van-PEG derivatives	Ligands	
	7	8
	K_b^a (standard deviations)	
1	1.836 (10.4%)	47.660(2.7%)
2	3.431 (5.0%)	41.691 (5.0%)
3	3.767 (6.4%)	16.324 (7.8%)
4	2.717 (9.0%)	142.779 (0.7%)
5	1.897 (2.3%)	152.028 (1.4%)
6	5.862 (6.4%)	36.610 (8.2%)

^aThe reported binding constants are the average values from at least two experiments for each ligand

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