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On-column synthesis coupled to affinity capillary electrophoresis for the determination of binding constants of peptides to glycopeptide antibiotics

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Abstract

Binding constants of the glycopeptide antibiotics teicoplanin (Teic), ristocetin (Rist), and vancomycin (Van), and their derivatives to D-Ala–D-Ala terminus peptides were determined by on-column ligand and receptor synthesis coupled to affinity capillary electrophoresis (ACE) or partial filling ACE (PFACE). In the first technique, 9-fluorenylmethoxycarbonyl (Fmoc)–amino acid–D-Ala–D-Ala species are first synthesized using on-column techniques. The initial sample plug contains a D-Ala–D-Ala terminus peptide and two non-interacting standards. Plugs two and three contain solutions of Fmoc–amino acid–*N*-hydroxysuccinimide (NHS) ester and buffer, respectively. Upon electrophoresis, the initial D-Ala–D-Ala peptide reacts with the Fmoc–amino acid NHS ester yielding the Fmoc–amino acid D-Ala–D-Ala peptide. Continued electrophoresis results in the overlap of the glycopeptide in the running buffer and the plug of Fmoc–amino acid–D-Ala–D-Ala peptide and non-interacting markers. Subsequent analysis of the change in the electrophoretic mobility (μ) or relative migration time ratio (RMTR) of the peptide relative to the non-interacting standards, as a function of the concentration of the antibiotic, yields a value for the binding constant. In the second technique, derivatives of the glycopeptides Teic and Rist are first synthesized on-column before analysis by ACE or PFACE. After the column has been partially filled with increasing concentrations of D-Ala–D-Ala terminus peptides, a plug of buffer followed by two separate plugs of reagents are injected. The order of the reagent plugs containing the antibiotic and two non-interacting standards and the anhydride varies with the charge of the glycopeptide. Upon electrophoresis, the antibiotic reacts with the anhydride yielding a derivative of Teic or Rist. Continued electrophoresis results in the overlap of the derivatized antibiotic and the plug of D-Ala–D-Ala peptide. Analysis of the change in RMTR of the new glycopeptide relative to the non-interacting standards, as a function of the concentration of the D-Ala–D-Ala ligand yields a value for the binding constant.

Keywords: Binding constants; Affinity capillary electrophoresis; Glycopeptides; Peptides; Antibiotics; Vancomycin; Ristocetin; Teicoplanin

1. Introduction

Glycopeptide antibiotics of the vancomycin group are a class of naturally occurring antibiotics produced by the fermentation of microorganisms [1,2]. They inhibit the growth of Gram-positive bacteria by hindering cell wall peptidoglycan biosynthesis [3–8]. These drugs bind to the D-Ala–D-Ala portion of peptidoglycan intermediates and inhibit the transglycosylation reaction required for cross-linking of the cell wall, thereby, resulting in bacteriostasis or bacterial cell death.

The history of the fight against infectious bacteria focuses on the recurring problem of drug resistance. For example, the evolution of methicillin-resistant *Staphylococcus aureus* (MRSA), resistant to all established antibiotics, is now a serious worldwide problem [9]. At present, the only treatment for MRSA is vancomycin (Van). Unfortunately, among the most dramatic and worrisome examples of resistance to antimicrobial agents in recent years has been the emergence and spread of Van resistance in enterococci (VRE).

Van (Fig. 1A) from *Streptomyces orientalis* has been called the antibiotic of last resort because of its effectiveness in treating infections caused by bacteria resistant to other antibiotics. Like Van, teicoplanin (Teic) (Fig. 1B) from *Actinoplanes teicomyceticus* and ristocetin (Rist) (Fig. 1C)

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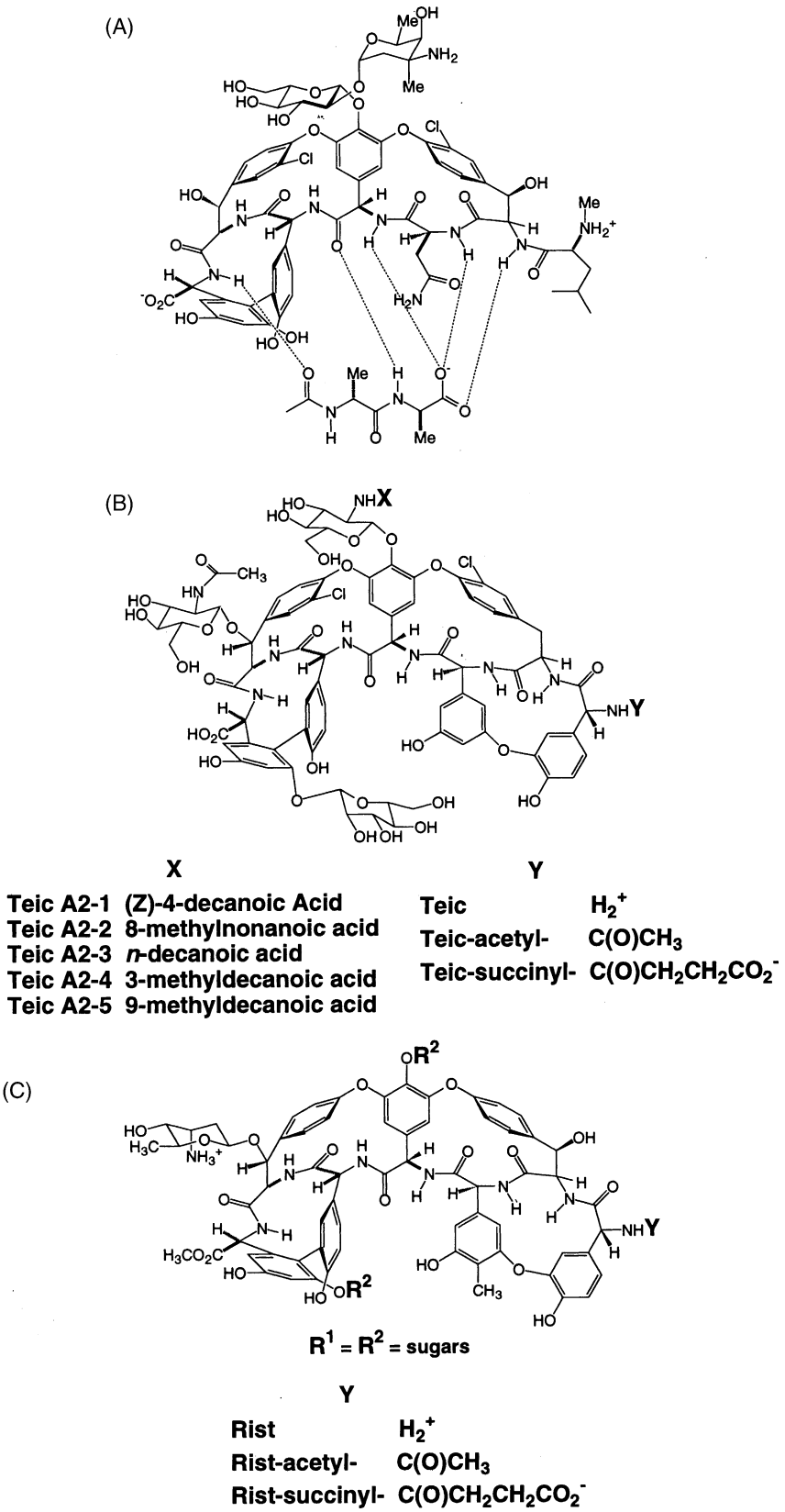

Fig. 1. Structures of (A) vancomycin complexed with *N*-Ac–D-Ala–D-Ala (**6**), (B) teicoplanin, and (C) ristocetin.

from *Nocardia lurida*, inhibit cell wall synthesis by impeding the action of transglycosylases and transpeptidases [10–12]. It is becoming increasingly important to develop new Van-group antibiotics, study their physicochemical parameters, and to examine their activity against VRE. Of equal importance is the development of new analytical methods that would allow for high-throughput synthesis and binding analysis of potentially important drug targets. Current glycopeptide research focuses on one, the design of new antibiotics to better understand the factors which promote and hinder binding to their target molecules and, two, the development of new peptides to mimic surface modifications on the bacterial cell wall. In order to analyze these compounds, it is important to develop efficient analytical techniques to examine the effect the derivatization of glycopeptides and D-Ala–D-Ala terminus peptides has on the binding event.

Capillary electrophoresis (CE) is an important analytical technique in many research laboratories primarily because of its small sample size requirement and high speed of analysis. CE separates molecules based on their charge-to-mass ratio upon application of a voltage gradient. During the past decade CE has evolved into a powerful analysis technique focusing on water-soluble ionic species.

Recent advances in molecular biology have aided in the determination of a great number of molecular interactions including protein–peptide, protein–DNA, and antibody–antigen. Affinity capillary electrophoresis (ACE) is one technique that has been used to characterize a number of receptor-ligand interactions [14–44]. For example, De Lorenzi et al. used ACE to characterize the interaction between drugs and transthyretin [25]. Progent et al. used ACE to determine binding constants between anionic polydispersed polymers and peptides [26]. Finally, Kiessig et al. probed the binding of the human immunodeficiency virus (HIV) capsid protein p24 to cyclophilins using ACE [27]. In ACE, free and bound forms of a receptor can be distinguished as a function of the concentration of free ligand in the electrophoresis buffer. Unlike other forms of binding assays, ACE does not require that free and bound receptor/ligand be known, hence, ACE simplifies the estimation of K_b. In a traditional ACE study, the receptor is subjected to increasing concentrations of ligand in the running buffer thereby causing a shift in the migration time of the receptor. Analysis of this change in migration time relative to a standard(s) yields a value for the binding constant.

Herein, we combine on-column derivatization and ACE to estimate binding constants of D-Ala–D-Ala terminus peptides to the glycopeptide antibiotics Teic, Rist, and Van (Fig. 2). The data described here demonstrate the advantages of using on-column derivatization and ACE to estimate binding parameters between ligands and antibiotics. These techniques can be further developed to evaluate a variety of on-column modified species.

2. Experimental

2.1. Chemicals and reagents

All chemicals were analytical grade. 9-Fluorenylmethoxy carbonyl (Fmoc)–Gly–*N*-hydroxysuccinimide (NHS) ester, Fmoc–Ala–NHS, Gly–Ala–Ala–D-Ala–D-Ala, and D-Ala–D-Ala–D-Ala–D-Ala were purchased from Bachem California (Torrance, CA, USA) and used without further purification. 4-Carboxybenzenesulfonamide (CBSA) was purchased from Aldrich (Milwaukee, WI, USA). D-Ala–D-Ala, D-Ala–D-Ala, nicotinamide adenine dinucleotide (NAD), vancomycin (Van), *N*-acetyl–D-Ala–D-Ala, **6**, and N_α,N_ϵ-diacetyl–Lys–D-Ala–D-Ala, **7**, were purchased from Sigma (St. Louis, MO, USA). Teicoplanin·HCl was purchased from advanced separation technologies (Whippany, NJ, USA) and was used without further purification. Ristocetin was purchased from Bio Data Corp. (Horsham, PA, USA) and was used without further purification. Mesityl oxide (MO) was purchased from Calbiochem (San Diego, CA, USA). Fmoc–Gly–D-Ala–D-Ala, **1**, was synthesized off-column based on literature procedures [13]. For on-column ligand synthesis, stock solutions of Rist, Teic (100 μmol/l), and Van (200 μmol/l), Gly–Ala–Ala–D-Ala–D-Ala (0.001 mol/l), D-Ala–D-Ala–D-Ala–D-Ala (0.001 or 0.01 mol/l), D-Ala–D-Ala (0.001 or 0.01 mol/l), D-Ala–D-Ala–D-Ala (0.001 or 0.01 mol/l), NAD (2.5 mmol/l), and CBSA (1 or 3 mmol/l) were each prepared by dissolving in buffer (20 mM phosphate buffer; pH 7.5). For on-column receptor synthesis, stock solutions of NAD (1 mg/ml), Teic (0.4 mg/ml), and Rist (1 mg/ml), were each prepared by dissolving the samples in buffer (20 mM phosphate, pH 6.9). Stock solutions of acetic anhydride and succinic anhydride were prepared by dissolving the compounds in acetonitrile.

2.2. 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 40.5 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: For on-column ligand synthesis coupled to ACE—voltage: 24 kV; current: 35.4 μA; detection: 205 nm; temperature: 23 ± 0.1 °C. For on-column receptor synthesis coupled to ACE—voltage: 20 kV; current: 22 μA; detection: UV detection at 200, 214, 254 nm; temperature: 23 ± 0.1 °C.

2.3. Procedures

On-column ligand synthesis coupled to ACE: plugs containing a sample of D-Ala–D-Ala terminus peptide and

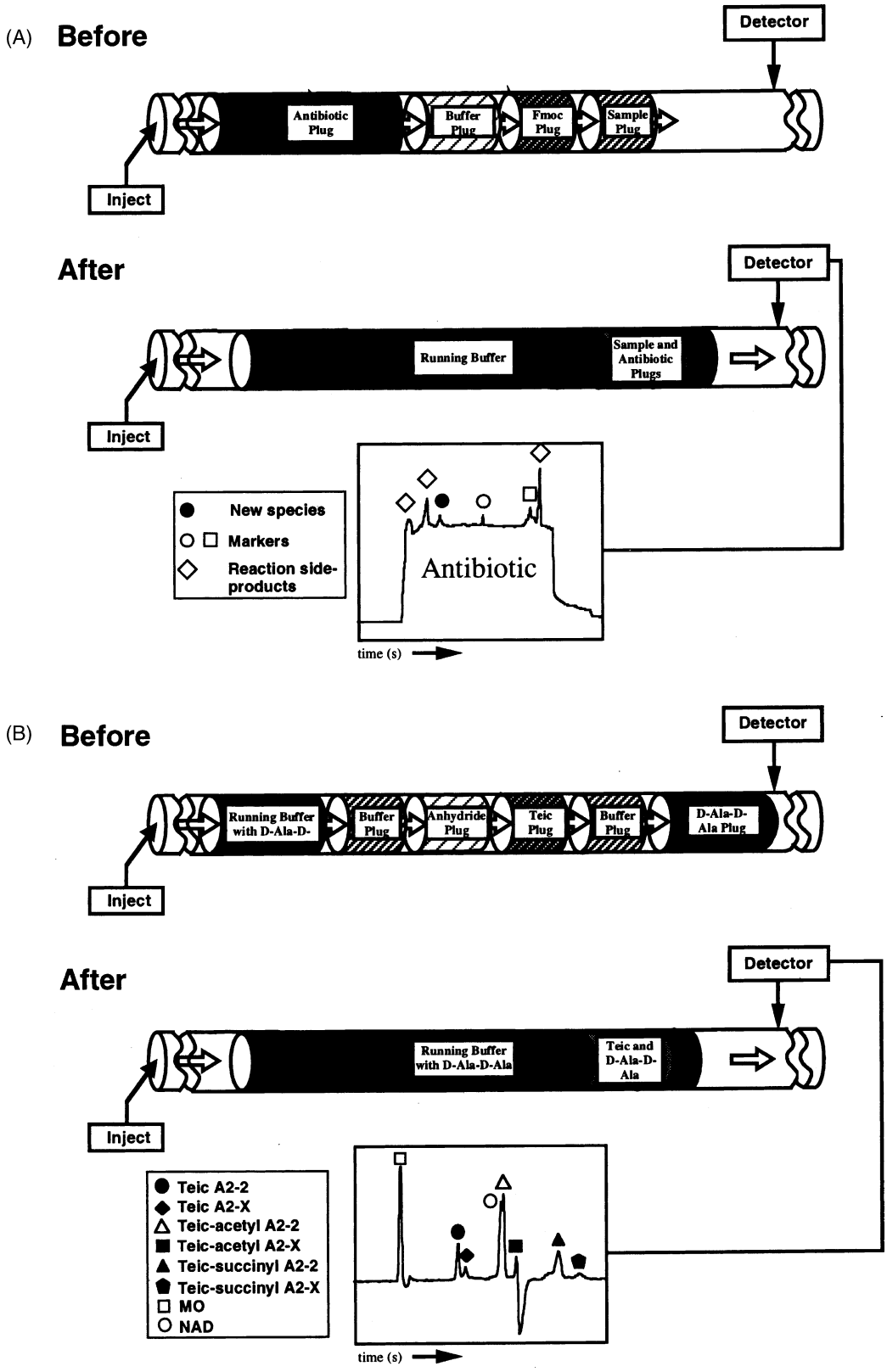

Fig. 2. Schematic of on-column (A) ligand and (B) receptor derivatization using ACE.

CBSA (also NAD in some studies) (1.2 nl) and Fmoc-amino acid-NHS in acetonitrile (1.2 nl) were vacuum injected into the capillary. For the Van studies, an extra plug of buffer was injected (1.2 nl) to separate zones of sample from Van. Electrophoresis was carried out using 20 mM phosphate buffer (pH 7.5) for 5.0 min. In the binding studies, electrophoresis was carried out with Rist dissolved in the buffer solution at increasing concentration (0–80 or 100 μmol/l) 24 kV for 5 min. A similar procedure was used for Teic and Van except the concentration of Teic and Van in the running buffer was between 0 to 25 μmol/l and 0 to 40 μmol/l, respectively.

On-column receptor synthesis coupled to PFACE: A sample of D-Ala–D-Ala ligand was vacuum injected into the capillary for 8.0–12.0 s. Plugs containing buffer (3.6 nl), Teic (1.2–2.4 nl), and acetic and succinic anhydride (1.2–2.4 nl) were then introduced by vacuum injection. Electrophoresis was carried out using 20 mM phosphate buffer (pH 6.9) for 5.0 min. A similar procedure with Rist was used except that the anhydride plug preceded that of the Rist plug. On-column receptor synthesis coupled to ACE: the capillary was first equilibrated with buffer (20 mM phosphate; pH 6.9) at increasing concentrations of ligand. Plugs containing buffer (3.6 nl), Teic (1.2–2.4 nl), and acetic and succinic anhydride (1.2–2.4 nl), and buffer (3.6 nl) were then introduced by vacuum injection. Electrophoresis was carried out using increasing concentrations of the D-Ala–D-Ala ligands for 5.0 min.

3. Results and discussion

We have examined the on-column synthesis of Fmoc-derivatized peptides coupled to standard ACE techniques and estimated their binding to Teic and Rist. In these experiments, separate plugs of D-Ala–D-Ala–D-Ala and non-interacting standards, and Fmoc-amino acid NHS ester are injected and electrophoresed in a buffer solution containing an increasing concentration of antibiotic (in this case, Teic). From run to run, a reproducible quantity of derivatized peptide is synthesized since the duration of contact between the two zones of liquid in the capillary column is constant.

Upon synthesis of the new derivatized species, **2**, it subsequently interacts with Teic forming a complex on migrating through the capillary column. Fig. 3A is a series of electropherograms of **2** in increasing concentrations of Teic in the capillary column. Increasing concentrations of Teic in the electrophoresis buffer causes a greater shift in migration time of **2** to shorter migration times. Several other peaks are observed in the electropherograms and were found to be starting material and hydrolyzed Fmoc reagent.

Fig. 3B is a Scatchard plot of the data for **2** using the change in the electrophoretic mobility μ_L of a ligand (L) on complexation with a receptor (R) present in the buffer can be correlated to the binding constant K_b [16,30–32].

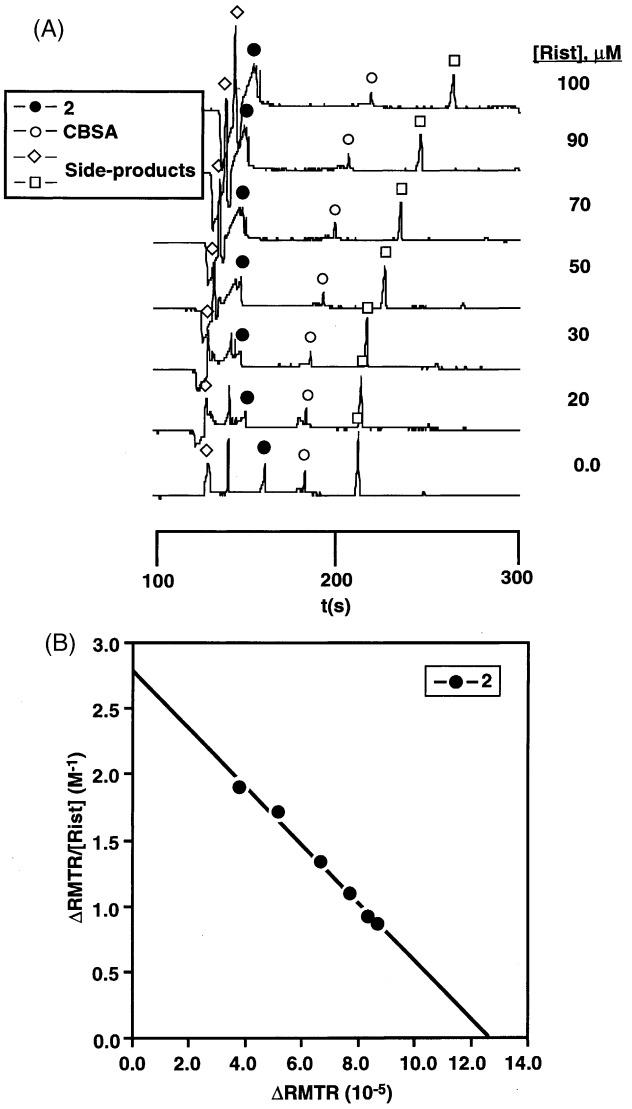

Fig. 3. (A) A representative series of electropherograms of Fmoc–Gly–D-Ala–D-Ala-D-Ala, **2**, in 20 mM phosphate buffer (pH 7.5) at 205 nm containing various concentrations of Van using the column synthesis PFACE technique. The total analysis time in each experiment was 5.0 min at 24 kV (current: 35.4 μA) using a 40.5 cm (inlet to detector), 50 μm i.d. open, uncoated quartz capillary. Carboxybenzenesulfonamide (OBSA) was used as an internal standard. (B) Scatchard plot of the data for **2** according to Eq. (1).

Analysis of the magnitude of the change in mobility $\Delta\mu_{L,R}$ as a function of the concentration (R) of receptor yields K_b (Eq. (1) is used for Scatchard analysis.

$$\frac{\Delta\mu_{L,R}}{[L]} = K_b\Delta\mu_{L,R}^{max} - K_b\Delta\mu_{L,R} \quad (1)$$

We have also examined the on-column derivatization of a series of D-Ala–D-Ala terminus peptides with Fmoc amino acid NHS esters and estimated the binding constants of the new Fmoc products to Van using PFACE. In these experiments plugs of D-Ala–D-Ala–D-Ala–D-Ala and two non-interacting markers dissolved in phosphate

buffer, Fmoc–Ala–NHS ester dissolved in acetonitrile, buffer, and Van in phosphate buffer partially-filled into the capillary column, are injected and electrophoresed. The plug of buffer is intended to prevent overlap of the Van and reaction plugs prior to synthesis of the new Fmoc species. Upon electrophoresis overlap of the separate zones of species occurs thereby yielding the new Fmoc derivatized product. The zone of Van migrates into the zone of Fmoc–Ala–D-Ala–D-Ala–D-Ala–D-Ala, **3**, where a dynamic equilibrium is established between the two species. We used NAD and CBSA as markers in the Scatchard analysis. Neither of these species interacts with any of the synthesized compounds in the running buffer during electrophoresis. The migration times of the standards remain constant during the course of the binding experiment.

Fig. 4A shows a representative series of electropherograms of **3**, in a capillary partially-filled with increasing concentrations of Van at 205 nm. The addition of increasing concentrations of Van in the running buffer shifts the peak for **3** to the left of its original place relative to the internal standards. The Van-**3** complex has a greater molecular weight than the ligand itself and, thereby, has less drag than the individual non-complexed molecules. A 40 s shift in **3** is observed between uncomplexed and complexed forms at the highest concentrations of Van. The height of the Van plateaus in Fig. 4A increase due to the increased concentration of Van partially-filled in the capillary column. The conversion of D-Ala–Ala–D-Ala–D-Ala to **3** is constant throughout the duration of the experiment as demonstrated by a reproducible Peak area for **3** at all concentrations of Van. Three other peaks (A–C) are observed in the electropherograms and are identified as (A) unreacted Fmoc–Ala–NHS ester, (B) D-Ala–D-Ala–D-Ala–D-Ala and, (C) Fmoc–Ala acid. D-Ala–Ala–D-Ala–D-Ala has some affinity for Van but it is small relative to **3**. D-Ala–Ala–D-Ala–D-Ala lacks a carbonyl moiety alpha to the *N*-terminus which greatly reduces its affinity to glycopeptides.

Fig. 4B is a Scatchard plot of the data for **3** using the RMTR, as the basis for the analysis [21]. In experiments employing two non-interacting standards, K_b can be estimated by relating the two standards to the species being studied using the RMTR (Eq. (2)). Here, t_r, t_s, and t'_s are the measured migration times of the:

$$\mathrm{RMTR} = \frac{t_r - t'_s}{t'_s - t_s} \tag{2}$$

peptide ligand and the two non-interacting standards, respectively. A Scatchard plot can be obtained using Eq. (3). Here, $\Delta\mathrm{RMTR}_{L,R}$ is the magnitude of the change in the RMTR as a:

$$\frac{\Delta\mathrm{RMTR}_{L,R}}{[L]} = K_b\Delta\mathrm{RMTR}^{max}_{L,R} - K_b\Delta\mathrm{RMTR}_{L,R} \tag{3}$$

function of the concentration of peptide. Eq. (3) allows for the estimation of K_b on a relative time scale using two non-interacting standards and compensates for fluctuations

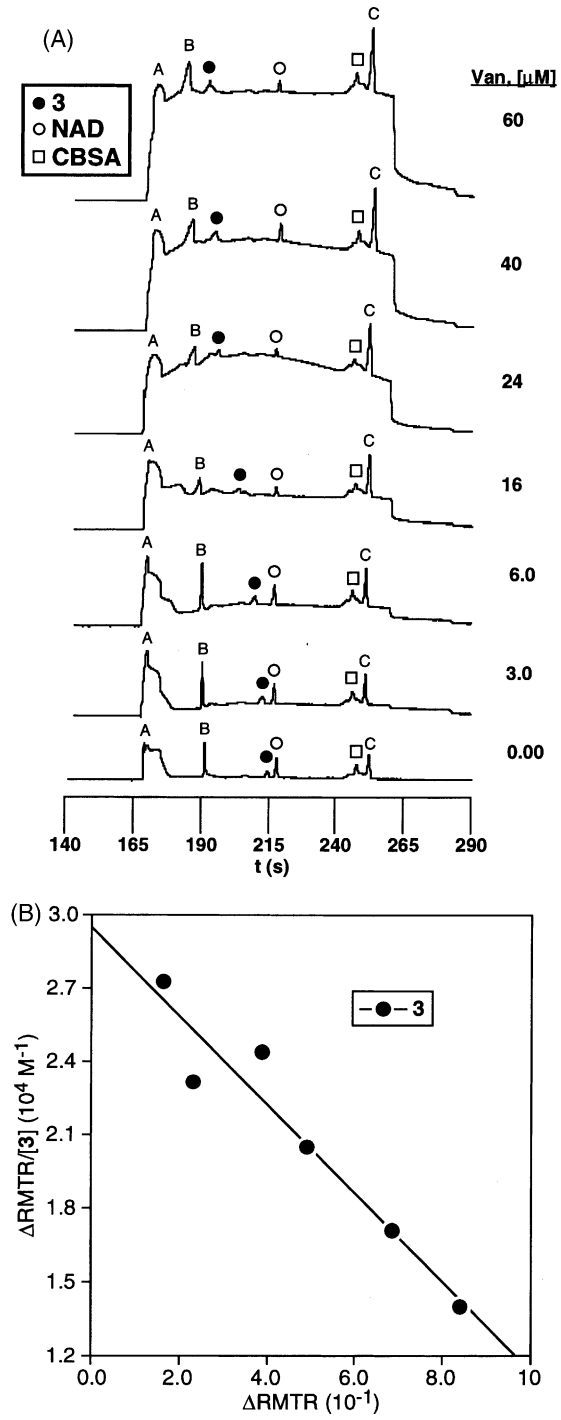

Fig. 4. (A) A representative series of electropherograms of Fmoc–Ala–D–Ala–D-Ala–D-Ala, **3**, in 20 mM phosphate buffer (pH 7.5) at 205 nm containing various concentrations of Van using the on-column synthesis PFACE technique. The total analysis time in each experiment was 5.0 min at 24 kV (current: 35.4 μA) using a 40.5 cm (inlet to detector), 50 μm i.d. open, uncoated quartz capillary. Nicotinamide adenine dinucleotide (NAD) and 4-carboxybenzenesulfonamide (CBSA) were used as internal standards. A–C are explained in the text. (B) Scatchard plot of the data for **3** according to Eq. (3).

Table 1
Experimental values of binding constants K_b ($10^3\,M^{-1}$) of ligands 1–5 to glycopeptide antibiotics measured by the on-column ligand synthesis ACE technique

Ligand	Peptide	Antibiotic	K_b[a]
1	Fmoc–Gly–D-Ala–D-Ala	Van	41.6[b]
2	Fmoc–Gly–D-Ala–D-Ala–D-Ala	Van	14.9[c]
2	Fmoc–Gly–D-Ala–D-Ala–D-Ala	Teic	17.9
2	Fmoc–Gly–D-Ala–D-Ala–D-Ala	Rist	21.9
3	Fmoc–Ala–D-Ala–D-Ala–D-Ala–D-Ala	Van	22.9
3	Fmoc–Ala–D-Ala–D-Ala–D-Ala–D-Ala	Teic	44.2
4	Fmoc–Ala–D-Ala–D-Ala	Van	174.5
4	Fmoc–Ala–D-Ala–D-Ala	Teic	420[d]
5	Fmoc–Ala–D-Ala–D-Ala	Van	34.3
5	Fmoc–Ala–D-Ala–D-Ala	Rist	51.8

[a] The reported binding constants are the average value from two or three experiments for ligands 1–5.
[b] Previous estimate [14]: $K_b = 19.0 \times 10^3\,M^{-1}$.
[c] Previous estimate [14]: $K_b = 19.0 \times 10^3\,M^{-1}$ and $30 \times 10^3\,M^{-1}$.
[d] Study conducted at pH 8.3.

in the capillary column induced by electrophoresis. In the present experiment, t_s and t_s' are the migration times of NAD and CBSA, respectively. Table 1 summarizes the binding data for D-Ala–D-Ala terminus peptides to Van obtained by Eq. (3).

In the next series of experiments, we examined the derivatization of the glycopeptides Teic and Rist by anhydrides. Teic is sold as a mixture of five compounds with the major and minor components designated as Teic A2-2 and A2-X ($X = 1$, 3–5), respectively. We examined the binding interactions between Teic to the peptide, *N*-acetyl-D-Ala–D-Ala, **6** using PFACE. A plug of sample containing increasing concentrations of **6** was initially injected to partially fill the capillary. Initial experiments had correlated the amount of sample injected to pressure and length of injection. A plug consisting of electrophoresis buffer was then vacuum injected into the capillary to separate the reagents from the ligand plug. The buffer plug serves as a barrier between Teic and the D-Ala–D-Ala terminus peptides so mixing does not occur prior to ACE analysis. The third injection contained Teic and the non-interacting standards MO and NAD. The final injection contained an equimolar mixture of succinic and acetic anhydrides. Upon electrophoresis, the anhydrides and Teic plugs overlap and react forming new Teic derivatives. The Teic derivatives then migrate into the zone of **6** and a dynamic equilibrium is achieved upon electrophoresis.

Fig. 5A shows a representative series of electropherograms of Teic and its derivatives. Upon increasing concentrations of **6** (0–500 μM) partially filled in the column a box is formed due to the increased absorbance of the ligand in the capillary column. The box is not observed below [***6***] = 200 μM as the peptide is only slightly chromophoric at 200 nm. All sample compounds used in this study are negatively charged at pH 6.9 and elute alter the neutral marker MO.

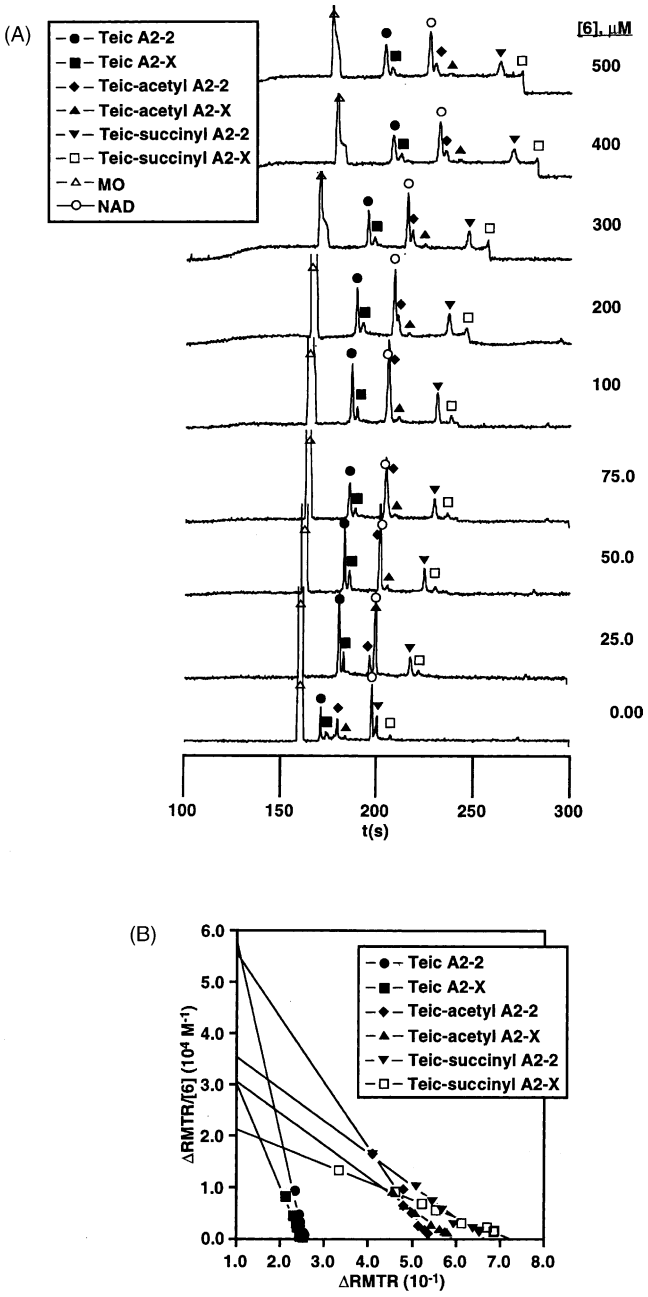

Fig. 5. (A) A representative series of electropherograms of Teic and its derivatives in 20 mM phosphate buffer (pH 6.9) containing various concentrations of **6**, using on-column receptor synthesis coupled to PFACE. The total analysis time in each experiment was 5.0 min. at 20 kV using a 46.5 cm (inlet to detector) 50 μm i.d. open, uncoated quartz capillary. (B) Scatchard plot of Teic and its derivatives with **6** using Eq. (3).

Upon addition of increasing concentrations of **6** in the running buffer, the migration times of Teic and its derivatives shift to greater migration times. The complexation between **6** and the Teic derivatives resulted in an increasing negative charge on the compounds and the complexes are detected later than the uncomplexed form. At the point of saturation, the Teic peaks no longer shift to the right despite

Table 2
Experimental values of binding constants K_b ($10^4\ M^{-1}$) of Teic and Rist with ligands **1**, **6**, and **7** measured by the on-column receptor synthesis PFACE/ACE technique

Antibiotic	K_b		
	1	6	7
Teic	140	37	65
Teic-A2-X	140	20	130
Teic-acetyl-A2-2	16	13	7.9
Teic-acetyl-A2-X	7.6	6.2	8.7
Teic-succinyl-A2-2	5.0	6.2	12
Teic-suocinyl-X	nd	3.4	11
Rist	nd	4.8	10
Rist-acetyl	nd	1.7	3.1
Rist-succinyl	nd	0.9	1.8

nd: not determined.

increasing concentrations of **6** in the running buffer. Fig. 5B is a Scatchard plot of the data for **6** using the RMTR form of analysis.

Similar studies were conducted with ligands **1** and **7** but using standard ACE techniques. Here, the column is equilibrated with ligand, separate plugs of buffer, Teic sample (also containing the non-interacting standards), anhydride in actonitrile, and buffer. Electrophoresis proceeds in increasing concentrations of peptide in the running buffer. Overlap of the separate zones of reagent plugs yield the new derivatized Teic derivatives. The zone of ligand then migrates into the zone of the Teic derivatives and a dynamic equilibrium is established prior to detection.

Table 2 summarizes the binding data for Teic and its derivatives to ligands **1**, **6**, and **7**. As can be seen, a decrease in binding is found upon derivatizing Teic-A2-2 and A2-X. The binding constant decreases between the glycopeptides and the peptide ligands upon derivatization consistent with previous reports [1]. The data demonstrate that modifying the charge on the terminus does change the ability of Teic to complex with D-Ala–D-Ala terminus peptide ligands.

It has been proposed that the charged *N*-terminus plays a critical role in stabilizing the initial weak coulombic complex between the glycopeptide and D-Ala–D-Ala ligand [33].

This complex exists for a short time before undergoing a conformational change to its fully bonded state involving five hydrogen bonds.

We then examined the on-column acetylation and succinylation of Rist and subsequent binding constant analysis for the new derivatives using PFACE. The C-terminus of Rist is esterified and, hence, it is positively charged at pH 6.9 and elutes prior to MO. Upon derivatizing the *N*-terminus, Rist-acetyl and Rist-succinyl are formed. They are both negatively charged and differ in charge from the unmodified Rist by a charge of one and two, respectively. In these experiments, we examined the interaction of Rist and its derivatives to peptides **6** and **7**.

A plug containing increasing concentrations of **7** was injected to partially fill the capillary. A buffer plug was then vacuum injected into the capillary to separate the reagents from the ligand plug. The third injection contained a mixture of succinic and acetic anhydride while the fourth plug contained Rist, MO, and NAD. Upon electrophoresis, the overlap of the separate zones of reagent plugs yields the Rist derivatives. Ligand **7** is more negative than the Rist derivatives used in this experiment and, hence, PFACE was used in this study.

Fig. 6A shows a representative series of electropherograms of Rist in buffer plugs containing increasing concentrations of **7**. The plug of **7** is not observed in these electropherograms because unlabeled D-Ala–D-Ala ligands minimally absorb at the detection wavelength of 254 nm at the low concentrations used in this study. Upon addition of increasing concentrations of **7**, both Rist derivative peaks shift to the right. The complexation between **7** and Rist-acetyl and Rist-succinyl resulted in an increasing negative charge on the derivatives and the complexes are detected later than the uncomplexed forms. At the point of saturation, the Rist derivative peaks no longer shift. Fig. 6B is the Scatchard plot for Rist-acetyl and Rist-succinyl and ligand **7**. Table 2 summarizes the binding data for Rist and its derivatives to peptides **6** and **7**. Upon derivatizing the terminus binding to D-Ala–D-Ala terminus peptides is weakened as that found for Teic.

The decrease in the binding affinity of Teic and and Rist for the peptides upon derivatization reaffirms the importance of a charge on the N-terminus and the role of electrostatics on binding. The lower affinity of the ligands for Teic than Rist is similar to that found in previous studies [45]. These studies suggest that the lipophilic chain of Teic enhances the binding of it to target ligands by anchoring in a lipid membrane and increasing the local concentration of it on the membrane surface. The affinity of the acetylated Teic for ligands **1**, **6**, and **7** is 3–8 times weaker than that of Teic corresponding to a loss of 3 to 7 kJ/mol in the free energy of binding. The affinity of the acetylated Rist for ligands **1**, **6**, and **7** is three times weaker than that of Rist corresponding to a loss of 3 kJ/mol in the free energy of binding.

On-column derivatization coupled to ACE/PFACE has several advantages as a method for measuring biomolecular noncovalent interations. First, it requires smaller quantities of either receptor or ligand than in traditional ACE techniques. Second, purified ligand and/or receptor are not always required as long as the peak(s) of interest can be differentiated from other peaks. Third, the technique can be modifled for high-throughput analysis of bimolecular noncovalent interactions if the reaction part of the technique affords products on the timescale of CE. Fourth, the commercial availability of automated instrumentation, and the high reproducibility of data, make it experimentally convenient. Further work to demonstrate the versatility of this two-step technique is in progress.

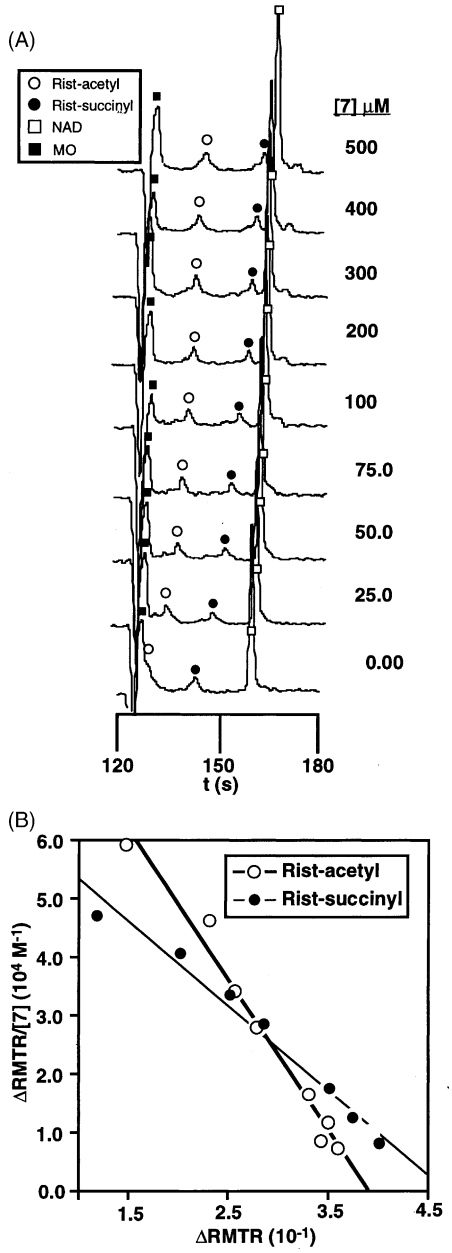

Fig. 6. (A) A representative series of electropherograms of Rist derivatives in 20 mM phosphate buffer (pH 6.9) containing various concentrations of **7**, using on-column receptor synthesis coupled to PFACE. The total analysis time in each experiment was 5.0 min. at 20 kV using a 46.5 cm (inlet to detector) 50 μm i.d. open, uncoated quartz capillary. (B) Scatchard plot of Rist derivatives with **7** using Eq. (3).

4. Conclusions

This report demonstrates the ease of using on-column ligand and receptor synthesis coupled to ACE/PFACE and how it can be utilized to determine binding constants between antibiotics and small peptides. In the present studies, we have used on-column ligand synthesis to derivatize D-Ala–D-Ala terminus peptides and examine their binding to Teic, Rist, and Van and on-column receptor synthesis to derivatize Teic and Rist and examine their binding to D-Ala–D-Ala peptides. In these studies, the capillary is filled with ligand or receptor, derivatizing reagent, and complexing molecule and electrophoresed. Derivatization occurs upon overlap of separate zones of solution yielding product which upon electrophoresis interacts with the complexing molecule existing in the capillary at varying concentrations. Binding constants are then estimated using changes in electrophoretic mobilities or the RMTR. The binding constants obtained by these techniques are in agreement with those values obtained in previous ACE techniques and traditional assay methods. This work establishes the feasibility of using on-column derivatization to probe binding between glycopeptide antibiotics and small peptides.

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References

[1] A.J.R. Heck, P.J. Bonnici, E. Breukink, D. Morris, M. Wills, Chem. Eur. J. 7 (2001) 910.
[2] K.C. Nicolaou, C.N.C. Boddy, S. Brase, N. Winssinger, Angew. Chem. 111 (1999) 2010.
[3] N.E. Allen, D.L. LeTourneau, J.N. Hobbs Jr., Antimicrob. Agents Chemother. 41 (1997) 66.
[4] H. Arimoto, K. Nishimura, T. Kinumi, I. Hayakawa, D. Uemura, Chem. Commun. (1999)1361.
[5] D.A. Beauregard, J. Maguire, D.H. Williams, P.E. Reynolds, Antimicrob. Agents Chemother. 41 (1997) 2418.
[6] G. Chiosis, I.G. Boneca, Science 293 (2001) 1484.
[7] R. Kerns, S.D. Dong, S. Fukuzawa, J. Carbeck, J. Kohler, L. Silver, D. Kahne, J. Am. Chem. Soc. 122 (2000) 12608.
[8] R.J. Dancer, A.C. Try, G.J. Sharman, D.H. Williams, J. Chem. Soc. Chem. Commun. 12 (1996) 1445.
[9] W.C. Noble, Z. Virani, R.G.A. Cree, FEMS Microbiol. Lett. 93 (1992) 195.
[10] M.P. Gasper, A. Berthod, U.B. Nair, D.W. Armstrong, Anal. Chem. 68 (1996) 2501.
[11] A.J. Pearson, J.-N. Heo, Org. Lett. 19 (2000) 2987.
[12] A.J.R. Heck, T.J.D. Jorgensen, M. O'Sullivan, M. von Raumer, P.J. Derrick, J. Am. Soc. Mass Spectrom. 9 (1998) 1255.
[13] J. Rao, I.J. Colton, G.M. Whitesides, J. Am. Chem. Soc. 119 (119) (1997) 9336.
[14] Y.-H. Chu, G.M. Whitesides, J. Org. Chem. 57 (1992) 3524.
[15] S. Handwerger, M. Pucci, K.J. Volk, J. Liu, M.S. Lee, J. Bacteriol. 176 (1994) 260.
[16] F. Lynen, Y. Zhao, Ch. Becu, F. Borremans, P. Sandra, Electrophoresis 20 (1999) 2462.
[17] E.-S. Kwak, F.A. Gomez, Chromatographia 43 (1996) 659.
[18] F.B. Erim, J.C. Kraak, J. Chromatogr. B 710 (1998) 205.
[19] J. Gao, M. Mammen, G.M. Whitesides, Science 272 (1996) 535.
[20] X.-H. Qian, K.B. Tomer, Electrophoresis 19 (1998) 415.

[21] E. Mito, F.A. Gomez, Chromatographia 50 (1999) 689.
[22] J. Heintz, M. Hernandez, F.A. Gomez, J. Chromatogr. A 840 (1999) 261.
[23] D. Amini, D. Westerlund, Anal. Chem. 70 (1998) 1425.
[24] T. Tanaka, S. Terabe, Chromatographia 44 (1997) 119.
[25] E. De Lorenzi, C. Galbusera, V. Bellotti, P. Mangione, G. Massolini, E. Tabolotti, A. Andreolim, G. Caccialanza, Electrophoresis 21 (2000) 3280.
[26] F. Progent, M. Taverna, I. Le Potier, F. Gopee, D. Ferrier, Electrophoresis 23 (2002) 938.
[27] S. Kiessig, J. Reissmann, C. Rascher, G. Kuillertz, A. Fischer, F. Thunecke, Electrophoresis 22 (2001) 1428.
[28] Y. Zhang, C. Kodama, C. Zurita, F.A. Gomez, J. Chromatogr. A 928 (2001) 233.
[29] C.F. Silverio, A. Plazas, J. Moran, F.A. Gomez, J. Liq. Chromatogr. Rel. Technol. 25 (2002) 1677.
[30] Y.-H. Chu, Y.M. Dunayevskiy, D.P. Kirby, P. Vouros, B.L. Karger, J. Am. Chem. Soc. 118 (1996) 7827.
[31] Y.M. Dunayevskiy, Y.V. Lyubarskaya, Y.-H. Chu, P. Vouros, B.L. Karger, J. Med. Chem. 41 (1998) 1201.
[32] Y.-H. Chu, L.Z. Avila, H.A. Biebuyck, G.M. Whitesides, J. Org. Chem. 58 (1993) 648.
[33] Y.-H. Chu, D.P. Kirby, B.L. Karger, J. Am. Chem. Soc. 117 (1995) 5419.
[34] F.A. Gomez, J.N. Mirkovich, V.M. Dominguez, K.W. Liu, D.M. Macias, J. Chromatogr. A 727 (1996) 291.
[35] E. Mito, Y. Zhang, S. Esquivel, F.A. Gomez, Anal. Biochem. 280 (2000) 209.
[36] M. Fang, L. Sheng, L.H. Han, X. Yu, R. Zhao, B. Zang, G. Liu, Electrophoresis 20 (1999) 1846.
[37] V.A. VanderNoot, R.E. Hileman, J.S. Dordick, R.J. Linhardt, Electrophoresis 19 (1998) 437.
[38] J.J. Golton, J.D. Carbeck, J. Rao, G.M. Whitesides, Electrophoresis 19 (1998) 367.
[39] M.H.A. Busch, L.B. Carels, H.F.M. Boelens, J.C. Kraak, H. Poppe, J. Chromatogr. A 777 (1997) 311.
[40] G.-H. Wan, X.C. Le, Anal. Chem. 72 (2000) 5583.
[41] F.A. Gomez, L.Z. Avila, Y.-H. Chu, G.M. Whitesides, Anal. Chem. 66 (1994) 1785.
[42] P.F. Vollerhaus, F.W.A. Tempels, J.J. Kettenes-van des Bosch, A.J.R. Heck, Electrophoresis 23 (2002) 868.
[43] R.M. Guijt-van Duijn, G.W. Frank, J. van Dedem, E. Baltussen, Electrophoresis 21 (2000) 3905.
[44] N.H.H. Heegaard, S. Nilsson, N.A. Guzman, J. Chromatogr. A 715 (1998) 29.
[45] P.J. Loll, P.H. Axelsen, Annu. Rev. Biophys. Biomol. Struct. 29 (2000) 265.