Affinity Capillary Electrophoresis−Mass Spectrometry for Screening Combinatorial Libraries

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Abstract: A new methodology, affinity capillary electrophoresis−mass spectrometry (ACE-MS), is introduced as a solution-based approach for screening combinatorial libraries for drug leads. The method allows on-line, one-step selection and structural identification of candidate ligands. ACE-MS is demonstrated using the binding of vancomycin to libraries of all-D-tri- and tetrapeptides as a model system. Peptide libraries of different forms of Fmoc-DDXX and Fmoc-EXX containing up to 361 compounds were successfully employed to determine interacting structural motifs. A consensus structure of the strongest interacting peptides consisted of D-Ala at the *C*-terminus and an aromatic amino acid in the penultimate position. Ligands with this structure bound more strongly to the receptor than the known ligand, D-Ala-D-Ala. A 1000 peptide library was also screened directly by ACE-MS. It was found that, for this and potentially larger libraries, incorporating an affinity solid phase extraction step prior to ACE-MS was effective in both removing a large number of non-interacting species as well as preconcentrating sample components for sequence determination by MS.

Introduction

In recent years, the strategy of drug discovery has expanded from selection of drug leads from among compounds that are individually synthesized and tested to selection of leads by screening large collections of compounds from natural[1] or synthetic[2] sources. Among the most important synthetic sources are combinatorial libraries, and screening such libraries has become a primary means of obtaining new drug leads. Originally, synthetic combinatorial libraries were based on oligomerization of amino acids and nucleotides, but the field has expanded to creating libraries from extended sets of small organic molecules.[3] Combinatorial technologies for drug discovery have been extensively reviewed.[4]

Screening a library generally involves a binding assay to determine the extent of ligand−receptor interaction. Often, either the ligand or receptor is immobilized on a solid surface (e.g., polymer bead or plate), and after detection of binding, the ligand is released and identified. For example, standard Edman sequencing has been used to identify peptides from individual support beads.[5] With the extension of combinatorial libraries to organic molecules other than peptides and oligonucleotides, and also for greater convenience, new identification schemes have been developed, e.g. tagged libraries.[6,7] Mass spectrometry has also begun to play a role in library screening for determining the bound molecule either by MALDI-TOF[8] or by electrospray ionization−mass spectrometry (ESI-MS).[9] In addition, ESI-MS has been used to study noncovalent complexes of proteins formed in the presence of mixtures of ligands.[10]

This paper presents a new, integrated, solution-based approach to drug lead discovery from combinatorial libraries using affinity capillary electrophoresis−mass spectrometry (ACE-MS). This methodology incorporates into one step the two essential parts of a screening process−compound selection and compound identification. Selection of ligands from peptide mixtures in solution using ACE[11] has previously been demonstrated with the antibiotic vancomycin as the target.[12] Also, identification of analytes with on-line CE-ESI-MS has been well established.[13] The purpose of this study was to employ ACE-ESI-MS as an on-line procedure for selection and identification of active ligands from combinatorial mixtures.

Vancomycin−Peptide Model System. Vancomycin represents the most widely recognized member of a family of

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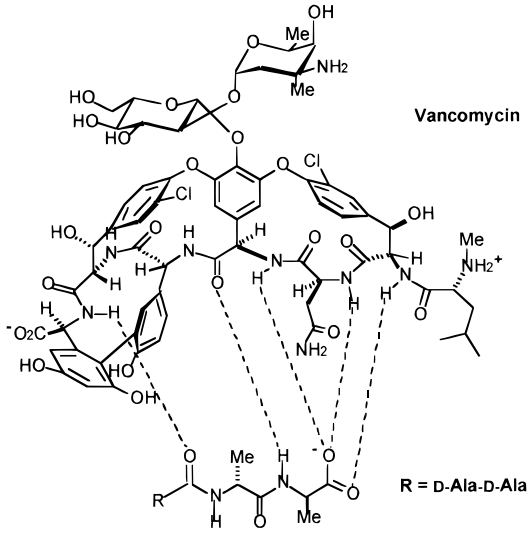
Chart 1



clinically effective glycopeptide antibiotics against bacterial infections.[14] The antibiotic action arises from inhibition of biosynthesis of the bacterial cell wall by selective binding of vancomycin to mucopeptides with the *C*-terminal sequence D-Ala-D-Ala. NMR studies of vancomycin−peptide complexes have elucidated not only the binding site of the antibiotic but also the interactions involved.[15] As a result, the binding affinity and selectivity of the antibiotic with the functional peptide and related peptides have been the subject of extensive investigation. Chart 1 shows the interaction of vancomycin with the *C*-terminal D-Ala-D-Ala structure.[15]

With vancomycin from *Streptomyces orientales* as a model receptor, we investigated the combination of ACE and ESI-MS as a one-step approach for selection and identification of active ligands in combinatorial peptide libraries. In our initial communication,[16] we used a 100-member all-D tetrapeptide library of the form Fmoc-DDXX and identified three peptides that bound more tightly than Fmoc-DDAA. This paper contains a detailed examination of the further development of ACE-MS, extending the original study to larger libraries with a wider variety of amino acid residues. The advantages and challenges of this approach are also discussed.

Experimental Section

Mass Spectrometry. All MS experiments were performed on a Finnigan TSQ 700 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) interfaced with a Finnigan atmospheric pressure ionization (API) source operated in the positive electrospray ionization (+ESI) mode. For ACE-MS experiments, the instrument was scanned over the expected mass range at a rate of 1.5−2 s/scan. ESI was performed at +3.5 to +4.5 kV with the heated capillary inlet at 150 °C. Peptide sequence determinations were performed using collisionally induced dissociation (CID) with 1−2 mTorr of argon and −15 to −30 eV collision energy. Fragment ions were detected in the range from 50 amu to the anticipated parent mass. Several runs were performed at various collision gas pressures and energies to optimize the yield of fragment ions and assist in spectral interpretation.

On-Line CE-MS. The CE instrumentation was configured in-house and was similar to that previously described.[17] Briefly, the experimental setup consisted of a 1000*R* high-voltage power supply (Spellman, Plainview, NY) operated in the constant voltage mode and connected to a platinum electrode in a vial containing the background electrolyte (BGE). The API interface was modified to achieve electrospray stability independent of experimental parameters such as liquid sheath flow rate. This modification, which will be described separately, consisted of the use of a 22-gauge liquid sheath needle to match closely the o.d. of the separation capillary and a micrometer device to adjust the position of the separation capillary with respect to the liquid sheath tube during ESI operation. The CE capillary, coated with either linear poly(acrylamide) (PA)[18] or poly(vinyl alcohol) (PVA)[19] to reduce EOF, was 360 μm o.d., 50−75 μm i.d., and 35−45 cm in length. The BGE was 20 mM Tris−acetate, pH 8.1, containing 70−120 μM vancomycin. The liquid sheath was 5−10 mM Tris−acetate, pH 8.1, in H_2O−MeOH (25−75, v/v) at a flow rate of 1.5 μL/min. Tris-acetate was added to the liquid sheath to minimize changes in the pH of the BGE due to migration of cations from the liquid sheath into the capillary during analysis.[20] Addition of Tris to the liquid sheath did not adversely affect S/N in the mass range scanned. N_2 was used as a sheath gas at 100−1000 cm^3/min to assist electrospray stability. The injection end of the capillary was held at a negative potential to produce a 400−500 V/cm potential gradient and a current of 5−8 μA. Library samples were injected hydrostatically at a height of 10 cm for 5−10 s, corresponding to an injection volume of 1.5−15 nL, depending on the internal diameter of the capillary used.

Off-Line CE-UV. A P/ACE 2100 capillary electrophoresis system (Beckman Instruments, Fullerton, CA) was employed in this study with the cathode at the injection side. Separation capillaries were coated with PVA and had an effective length of 20 cm (total length 27 cm) with an i.d. of 50 μm. The BGE was 50 mM Tris−acetate, pH 8.1, containing appropriate concentrations of vancomycin. Sample solutions containing a peptide library were introduced into the capillary by pressure injection. Electrophoresis was carried out at a constant voltage of 13 kV generating a current of approximately 16 μA. Electrophoresis was monitored on-column at 254 nm and the temperature of the capillary was maintained at 25 °C.

Library Synthesis. Synthesis of all-D peptide libraries of the forms Fmoc-DDXX (100 tetrapeptides), Fmoc-EXX (100 tripeptides), Fmoc-DDXX (361 tetrapeptides), and Fmoc-DXXX (1000 tetrapeptides) was conducted as follows: Ten (G, A, E, P, Q, L, F, H, Y, S) and nineteen (A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, Y) 2-chlorotrityl polystyrene resins, each containing 0.125 mmol of loaded D-amino acid, were selected, mixed thoroughly in dimethylformamide (DMF), and divided equally into 10 and 19 reaction vessels, respectively. Following the standard protocol for solid phase peptide synthesis, these resins were coupled to each of the protected *N*-α-Fmoc D-amino acids (0.5 mmol) using 1-hydroxybenzotriazole (HOBT) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (0.45 M, 1.0 mL) and diisopropylethylamine (DIEA) (0.174 mL) in DMF (1.0 mL). Coupling reactions proceeded to completion (∼1 h), as assayed by Kaiser's ninhydrin test. The resulting resins from each reaction vessel were then combined and the *N*-α-Fmoc group removed by piperidine (20%, v/v) in DMF. For the synthesis of the Fmoc-DXXX library, this resin mixture was evenly distributed into 10 vessels, and the synthesis cycle was repeated once more, yielding a mixture of 1000 tripeptide resins.

Synthesis of the next defined positions was carried out using solutions of those particular D-amino acids and the procedure described above. Finally, the peptide mixtures were cleaved and released from their respective resins using a solution of trifluoroacetic acid/water (90:10, v/v) at room temperature for 3 h. Isolation of each peptide library was accomplished by adding an excess volume of cold (∼20 °C) ethyl ether into mixtures for peptide precipitation. Peptides in these D-libraries were soluble and, at least, monocarboxylated in buffers having pH >7.

Affinity Column Separations. A slurry of 10 mL (10 μmoles/mL) of Affi-Gel 10 (Bio-Rad, Richmond, CA) was introduced into the

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Table 1. Data for Ligands from the Combinatorial Libraries[c]

peptide	P (μM)[a]	K_d (μM)[b]
Fmoc-DDFA	0.3	6.8
Fmoc-DDYA	0.4	7.5
Fmoc-DDEA	11	17
Fmoc-DDHA	15	26
Fmoc-DDWA		26
Fmoc-DDVA	28	44
Fmoc-DDQA	29	46
Fmoc-DDMA	30	52
Fmoc-DDAA	32	59
Fmoc-DDFG	36	N/A
Fmoc-DDYG	38	N/A
Fmoc-DDRA	52	110
Fmoc-DDSA	74	120
Fmoc-DDKA	65	140
Fmoc-DDTA	68	160
Fmoc-DDYS	90	N/A
Fmoc-DDFS	94	N/A
Fmoc-DDRG	282	N/A
Fmoc-DDKG	489	N/A

[a] P, ranking parameter of the ligand−receptor complex from on-line ACE-MS analysis of the 361-member library and calculated with eq 6. [b] K_d, Dissociation constant of the ligand−receptor complex from ACE-UV as defined in eq 3. Estimated RSD 20%. [c] Individual peptide sequences were characterized by ACE-MS-MS.

column and washed with 100 mL of water and 50 mL of 20 mM sodium phosphate buffer (pH 8.0). To the gel in the column was added 10 mL of vancomycin (250 mg) in 20 mM phosphate buffer. The chemical immobilization of vancomycin to the gel was carried out at room temperature for 8 h with continuous shaking. The resulting gel was then washed extensively with 0.1 M ammonium acetate (∼500 mL, pH 7.0) and stored and operated at room temperature. A 5-mL solution of the Fmoc-DXXX peptide library (∼50 mg) in 50 mL of Tris/acetate buffer was loaded onto the vacomycin column. The column was allowed to flow at atmospheric pressure until the liquid level reached the top of the gel. Then, 100 mL of 0.1 M ammonium acetate (pH 7.0) was applied to the column eluting unbound peptides. Tightly bound candidate peptide ligands were next eluted with 0.1 M acetic acid (pH ∼3) containing 50% (v/v) acetonitrile. This fraction was collected and lyophilized. The resulting fluffy powder was used in ACE-MS experiments.

Measurement of Binding Constants Using ACE. ACE was used to determine binding constants of vancomycin with selected ligands according to published methodology.[21] Briefly, by measuring electrophoretic mobility, μ, as a function of receptor concentration in the BGE, it was possible to determine K_d, the dissociation constant using Scatchard analysis. Peptides, identified by on-line ACE-MS-MS, were individually resynthesized and their K_d values are presented in Table 1.

Results

ACE. We first describe the principles of ACE relevant to this work. In ACE, the electrophoretic mobility, μ, of a ligand is altered upon complexation with a receptor in solution.[11,22] The receptor−ligand complex is assumed to have an electrophoretic mobility different from either of the uncomplexed components. The mobility of the complex can be approximated as:

$$\mu_c \approx C_c\left(\frac{Z \pm z}{(M+m)^\alpha}\right) \tag{1}$$

where C_c is an empirical constant characteristic for the complex and Z and z are the charges and M and m are the molecular weights of the receptor and ligand, respectively. The exponent α for globular species has been estimated to be $^2/_3$.[23] Under the experimental conditions used in this work, the pH was close to the pI of the receptor, so eq 1 can be reduced to:

$$\mu_c \approx C_c\left(\frac{z}{(M+m)^\alpha}\right) \tag{2}$$

In the ACE experiment, the running buffer containing the receptor is introduced into the CE capillary followed by the injection of the mixture of ligands. In the present system, kinetic off-rates are fast relative to analyte migration time. Therefore, the receptor−ligand complex will undergo multiple events of association−dissociation during the electrophoretic run. Consequently, complexation with the receptor shifts the mobility of the ligand toward the electrophoretic mobility of the complex. This mobility shift corresponds to the ligand's binding affinity and can be used to calculate the dissociation constant of the complex (K_d) according to eq 3[24]

$$K_d = [R]\left(\frac{\mu_t - \mu_c}{\mu_0 - \mu_t}\right) \tag{3}$$

where subscript 0 represents the free ligand in the absence of the receptor, t the ligand with receptor present in the running buffer at a concentration [R], and c the ligand complexed with receptor.

The value of K_d can be determined by measuring μ_t as a function of [R] based on one of several modifications of eq 3.[21] However, instead of performing such a tedious set of experiments for the mixture of ligands (which may even be impossible because of the library size), we introduce a parameter P to rank binding affinities of the ligands according to their mobility shift upon complexation. Using approximation 2 for the mobilities, eq 3 is transformed into:

$$K_d \cong [R]\left(\frac{1}{\mu_0 - \mu_t}\right)\left(\mu_t - \mu_0\left(\frac{C_C}{C_L}\right)\left(\frac{m}{M+m}\right)^\alpha\right) \tag{4}$$

where C_L is an empirical constant characteristic of the ligand. Assuming that constants C_L and C_C are similar for the ligand and its complex, we define an experimental ranking parameter P such that:

$$P = [R]\left(\frac{\mu_t - A\mu_0}{\mu_0 - \mu_t}\right) \tag{5}$$

where

$$A = \left(\frac{m}{M+m}\right)^\alpha \tag{6}$$

In eq 5, we assume Z is approximately zero, the case to be studied in this work using vancomycin at pH 8.1. However, a correction for charge effects on the electrophoretic mobility could be simply added, if necessary. Note also, if $M \gg m$, then eq 5 reduces to

$$P = [R]\frac{\mu_t}{\mu_0 - \mu_t} \tag{7}$$

Thus, for a fixed receptor concentration, P is related to the relative mobility shift for the specific ligand. The most active

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ligand would have the largest mobility shift resulting in the lowest *P* value.

Even though *P* was derived from eq 3 for K_d and has the same physical meaning, it is not proportional to the corresponding K_d value because *P* is determined at a given concentration of the receptor. At a high receptor concentration (i.e. about 10-fold larger than K_d of a specific ligand), no change in the ligand's mobility with [R] would be observed, since the ligand would be close to saturation with the receptor and would move most of the time in the complexed form. For such strong ligands the measured μ_t values would be very close to μ_c, resulting in *P* values approaching zero (see eq 3). If desired, a second experiment at lower receptor concentration may be conducted to discriminate further within the group of these high-affinity ligands. In summary, ACE experiments allow the qualitative ranking of library members according to their binding affinity to the receptor. When the best ligands are selected, they can be individually resynthesized and their binding constants accurately measured in separate experiments by ACE, as shown in this paper.

Design of Libraries. We first present the ACE-MS analysis of two 100-member peptide libraries of the form Fmoc-DDXX and Fmoc-EXX, where X is G, A, E, P, Q, L, F, H, Y, or S. (In these and subsequent libraries, all amino acids were D-isomers.) Following this, we explore the use of ACE-MS with a greater diversity of ligands using a 361-member library of the form Fmoc-DDXX, where X is now any one of 19 common amino acids except Cys. Then, we describe a study of ACE-MS with larger collections of compounds using a 1000-member library of the form Fmoc-DXXX, where X is again G, A, E, P, Q, L, F, H, Y, or S. Based on the results from this library, we evaluated a means of prescreening and preconcentrating the most active ligands using solid phase extraction prior to ACE-MS analysis.

Several structural features were built into these libraries to facilitate the overall analysis. First, during peptide synthesis the last deprotection step was omitted leaving the Fmoc group on the *N*-terminus to provide a strong chromophore for off-line CE-UV detection. While the possibility of interaction(s) of the Fmoc group with the receptor molecule cannot be excluded, such interactions should not affect binding of library members *relative* to one another, since only *C*-terminal amino acids interact with the carboxylate binding pocket of vancomycin.[15] A second feature was the incorporation of acidic amino acid (Glu or Asp) at the *N*-terminus so that all library members were negatively charged at the experimental pH, permitting all molecules to move in the same direction during electrophoresis. The presence of acidic residues, together with the *C*-terminal carboxylate, also aided in library solubility.

Libraries of 100 Peptides. The ACE-MS analysis of a 100-peptide library of the form Fmoc-DDXX has been described previously.[16] Briefly, the separation capillary contained a neutral, hydrophilic coating to eliminate EOF, and the pH of the BGE was adjusted to 8.1 where the receptor, vancomycin, was approximately neutral[25] and therefore had little or no mobility. The separation capillary was filled with BGE containing vancomycin, and the library was injected as a small plug. More active ligands were noted by their greater decrease in mobility due to interaction with the receptor, and several ligands with significant activity were identified by CID.

Figure 1A shows the electropherogram obtained without receptor in the BGE, where three distinct peaks (I, II, and III) from the 100-peptide library are evident. Based on MS analysis,

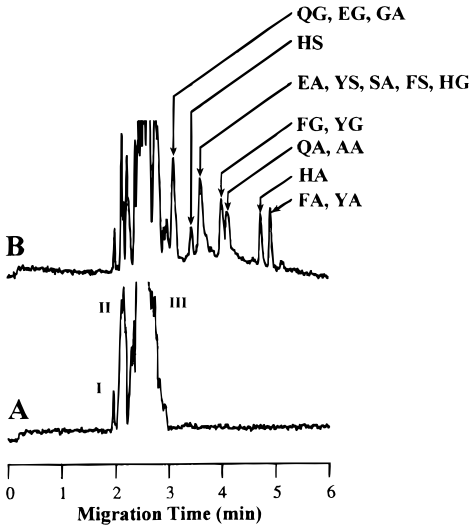

Figure 1. ACE−UV analysis of the Fmoc-DDXX library (100 peptides): (A) without vancomycin; (B) 104 μM vancomycin. Conditions: 50 mM Tris−acetate, pH 8.1, 13 kV, 16 μA; PVA coated capillary, 50 μm i.d., 20 cm effective length, 27 cm total length. Peak A-I: peptides containing two Glu residues. Peak A-II: one Glu residue. Peak A-III: no Glu residues.

these peaks contain respectively peptides with two, one, or no glutamic acid residues at increasing migration times. The three groups thus contained 5, 4, and 3 negative charges. Figure 1B, the electropherogram with receptor in the BGE, shows a similar group of three peaks followed by peaks representing active ligands with increasingly strong binding. All active ligands were identified by CID, as labeled in Figure 1, and representative on-line CID spectra are presented in Figure 2.

Mass spectra were influenced by the presence of Tris in the BGE and liquid sheath. Peptides containing His gave strong protonated molecular ions $[M + H]^+$, whereas all other peptides formed Tris adducts $[M + Tris + H]^+$ as well as $[M + H]^+$ species. Variation of the MS ion optics settings could be used to maximize $[M + Tris + H]^+$ at the expense of the corresponding $[M + H]^+$ species, and this manipulation was helpful both for distinguishing His-containing ligands and minimizing mass overlaps. Figures 2A and 2B are CID spectra originating from $[M + H]^+$ species and Figures 2C and 2D are from $[M + Tris + H]^+$ species. Both provided equivalent information, and Fmoc-peptides fragmented primarily from the *C*-terminus, yielding mostly b- and y-series ions.[26] The CID spectra, combined with other information available from the known synthetic library, such as expected sequences, molecular weights, and isobaric overlaps, readily allowed ligand identification. Note that sequences of isobaric active and inactive peptides were determined in the same ACE-MS-MS run.

The results from screening the 100-member Fmoc-DDXX library revealed that the most tightly bound ligands contained Ala at the *C*-terminus and an aromatic amino acid at the penultimate position. Other active ligands, such as Fmoc-DDEA, -HA, -QA, -FG, -YG, were found to have migration shifts and, consequently, affinities for vancomycin greater than or equal to that of Ala-Ala, the known ligand (see Table 1, to be discussed later). Our analysis confirmed that vancomycin energetically disfavors a bulky side chain on the residue at the *C*-terminus, see Figure 1. The presence of an amino acid other than Ala or Gly would require significant reorientation of the vancosamine sugar away from the larger side chains. Ala is favored over Gly because peptide binding is increased by the

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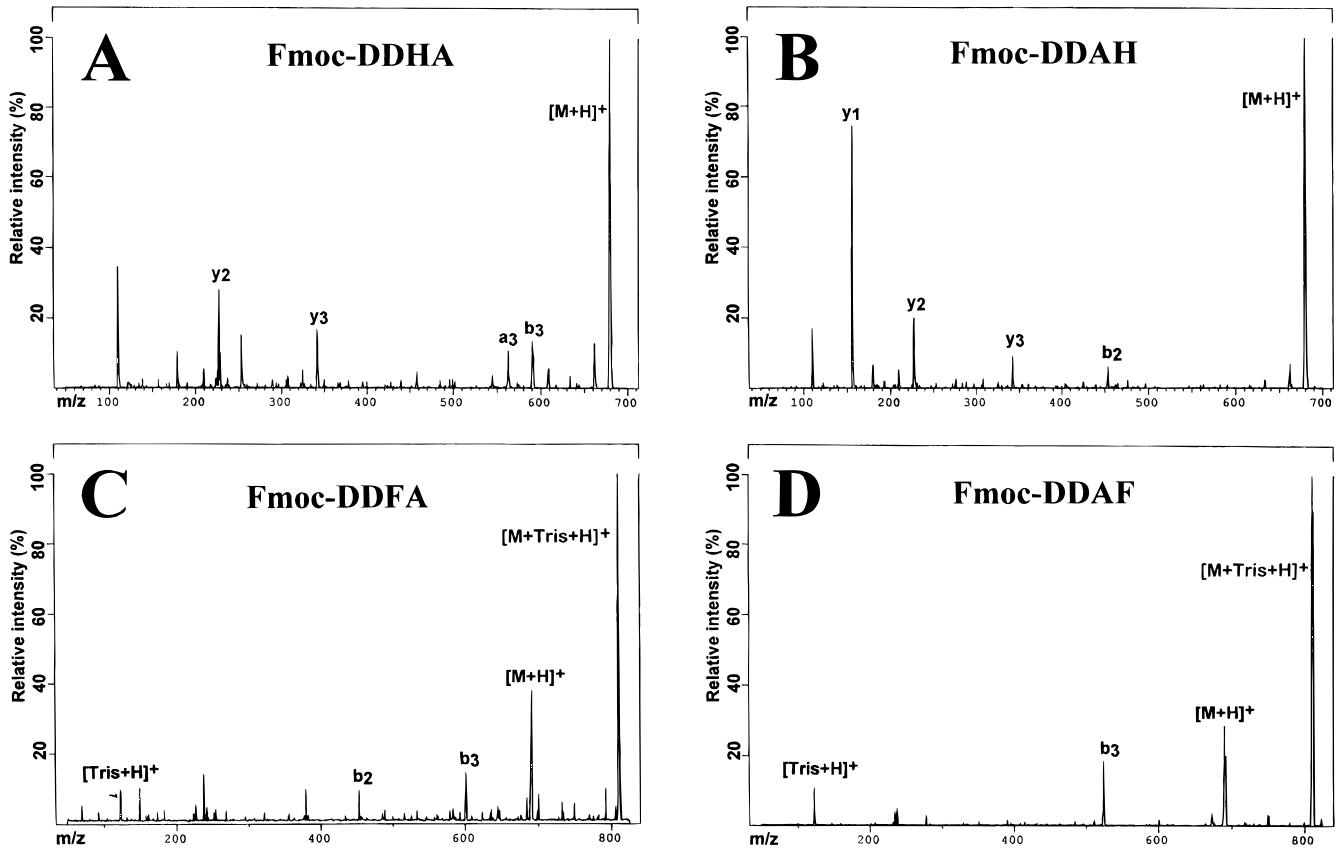

Figure 2. CID mass spectra of ligands selected by on-line ACE-MS-MS from the Fmoc-DDXX library: (A) Fmoc-DDHA, parent ion *m*/*z* 679.5; (B) Fmoc-DDAH, parent ion *m*/*z* 679.5; (C) Fmoc-DDFA, parent ion *m*/*z* 810.5; (D) Fmoc-DDAF, parent ion *m*/*z* 810.5. ACE-MS conditions: 20 mM Tris−acetate, pH 8.0, 70 μM vancomycin, 15 kV, 8 μA; PVA coated capillary, 50 μm i.d., 40 cm total length. MS-MS conditions: −50 eV collosion energy, 1.1 mTorr argon collision gas pressure.

hydrophobic interaction of the methyl group on the Ala and the 6-methyl group of the sugar.[15] The strong binding with an aromatic amino acid in the second position from the *C*-terminus is likely the result of interactions between these side chains and the aromatic rings in the receptor.

A second 100-member library was synthesized to study further the ACE-MS method while exploring the effect on binding of both the ligand length and the proximity of the Fmoc group to the receptor binding site. This library was of the form Fmoc-EXX, where X was G, A, E, P, Q, L, F, H, Y, or S, as in the previous library. The results of analysis of this library by ACE-MS are shown in Figure 3. The presence of only a single fixed negatively charged amino acid (Glu) meant that the corresponding tripeptides migrated slower than the previous tetrapeptides containing two fixed acidic residues. However, this library migrated in a narrow zone with active compounds being well separated from inactive species, and sequences of active ligands were again readily determined by CID. The data in Figure 3 confirmed the binding motif found with the first library and indicated that ligand length (tri- *vs* tetrapeptides) and proximity of the Fmoc group to the receptor are not important factors in determining *relative* binding strength.

Library of 361 Peptides. In order to test the ACE-MS methodology with a library containing more diverse members, as well as to examine further the effect on binding of the penultimate amino acid from the *C*-terminus, we synthesized and analyzed a library of the form Fmoc-DDXX, where X was now any one of 19 common amino acids except Cys (19^2 or 361 compounds). Figure 4 shows electrophoresis results with and without receptor in the BGE. The presence of positively charged amino acids in this library (Lys and Arg) complicated the electropherogram, as ligands migrated in four distinct groups

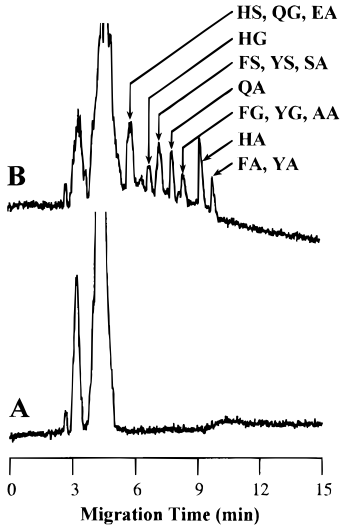

Figure 3. ACE-UV analysis of the Fmoc-EXX library (100 peptides): (A) without vancomycin; (B) 104 μM vancomycin. Conditions: same as in Figure 1, except 10 kV and 12 μA.

containing side chains with increasing cationic character. Thus, the last peak in the electropherogram of Figure 4A (∼7.4 min) contained two basic residues at -XX, for a total peptide charge of −1, the peaks between 3 and 4 min one basic residue at -XX, for a total charge of −2, and so forth.

Based on eq 5, both μ_0 and μ_t, ligand mobilities without and with receptor, respectively, must be determined experimentally in order to rank binding affinities correctly. With full scan MS data, one has the ability to extract signals of selected ions. Since we have *a priori* information on all expected ligand masses with

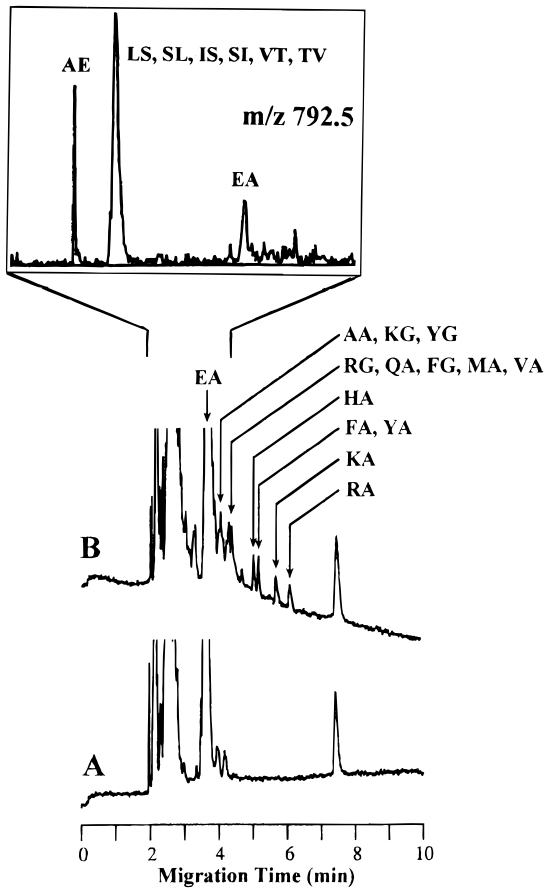

Figure 4. ACE-UV analysis of the Fmoc-DDXX library (361 peptides): (A) without vancomycin; (B) 139 μM vancomycin. Conditions: same as in Figure 1. Top inset: extracted ion electropherogram (ACE-MS) for the retained ligand EA and other isobaric peptides at *m*/*z* 792.5.

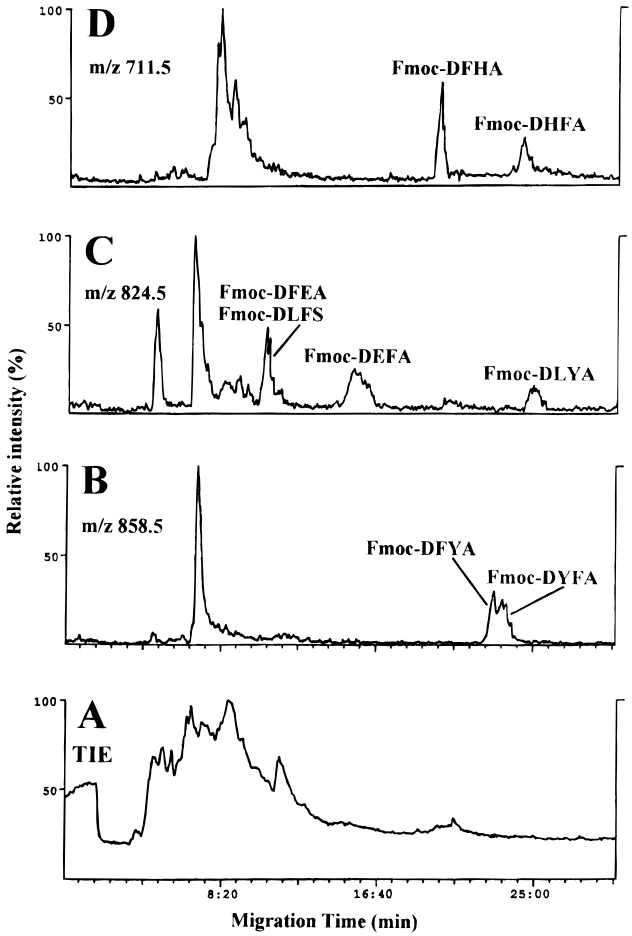

Figure 5. ACE-MS analysis of the Fmoc-DXXX library (1000 peptides): (A) total ion electropherogram; (B−D) extracted ion electropherogram, *m*/*z* 858.5 (B) *m*/*z* 824.5 (C), and *m*/*z* 711.5 (D) Conditions: same as in Figure 2, except 120 μM vancomycin.

synthetic combinatorial libraries, it is straightforward to use extracted ion electropherograms to determine μ_0 and μ_t accurately, even in the presence of multiple peak overlaps. The inset in Figure 4 shows the extracted ion electropherogram for Fmoc-DDEA, as well as its isobaric library members. It can be seen that even though the retained ligand, -EA, fell under a group of inactive ligands, its mobility could be accurately determined.

With the 361-member library, despite the increased charge diversity and concomitant migration complexity, active ligands could readily be selected, identified, and ranked (Table 1). The values of *P* for Fmoc-DDFA and -YA were very close to each other, since the concentration of vancomycin used in the ACE-MS experiment was much higher than K_d of these ligands. An ACE-MS experiment with a lower concentration of vancomycin (25 μM) showed that the difference in *P* for these ligands was more pronounced (data not shown). However, at this lower concentration of vancomycin, no other peptides changed their mobilities significantly. Thus, one can use a lower concentration of the receptor to extract the strongest ligand and a higher concentration to detect a group of active library members. After the above screening, the selected compounds were synthesized individually and their dissociation constants were determined as listed in Table 1.

Analysis of this library revealed only two additional ligands with fairly strong binding, beyond those already seen with the 100-peptide libraries: Fmoc-DDMA and -DDVA (Table 1). Surprisingly, peptides with Asp and Asn in the second position did not bind tightly to vancomycin, whereas peptides with Glu and Gln did, perhaps because of the difference in the relative sizes of the side chains involved in interaction. Since the binding motif established to this point indicated that peptides with *C*-terminal Ala followed by an aromatic amino acid bound most tightly to vancomycin, it was logical to expect that Fmoc-DDWA would also be an active ligand. However, ions corresponding to -DDWA, as well as all -DDXW (and -WX) peptides, were either absent or too weak to identify by CID. Synthesis and CE-MS analysis of individual Trp-containing ligands showed that the low intensities of these compounds were not due to poor ionization efficiencies, but rather to their low concentration in the library. Degradation of the side chains by acid treatment during peptide synthesis[27] may have diminished these peptides. The affinity of Fmoc-DDWA with vancomycin was found to be strong (K_d of 26 μM, Table 1). However, of the ligands containing aromatic amino acid in the second position at the *C*-terminus, Fmoc-DDWA had the poorest binding, possibly due to its large side chain. The ability to identify false negatives with ACE-MS and its implications for library quality determination will be discussed later.

Library of 1000 Peptides. A 1000-member library of the form Fmoc-DXXX was synthesized in order to evaluate the performance of ACE-MS with a larger library, as well as to determine the effect on binding of the third D-amino acid. Figure 5 shows typical extracted ion electropherograms and the total ion electropherogram (TIE) for this library. The analysis again confirmed the motif found with the smaller libraries: Fmoc-DXYA and Fmoc-DXFA peptides were the most retarded

(27) King, D.; Fields, C.; Fields, G. *Int. J. Pept. Protein Res.* **1990**, *36*, 255−266.

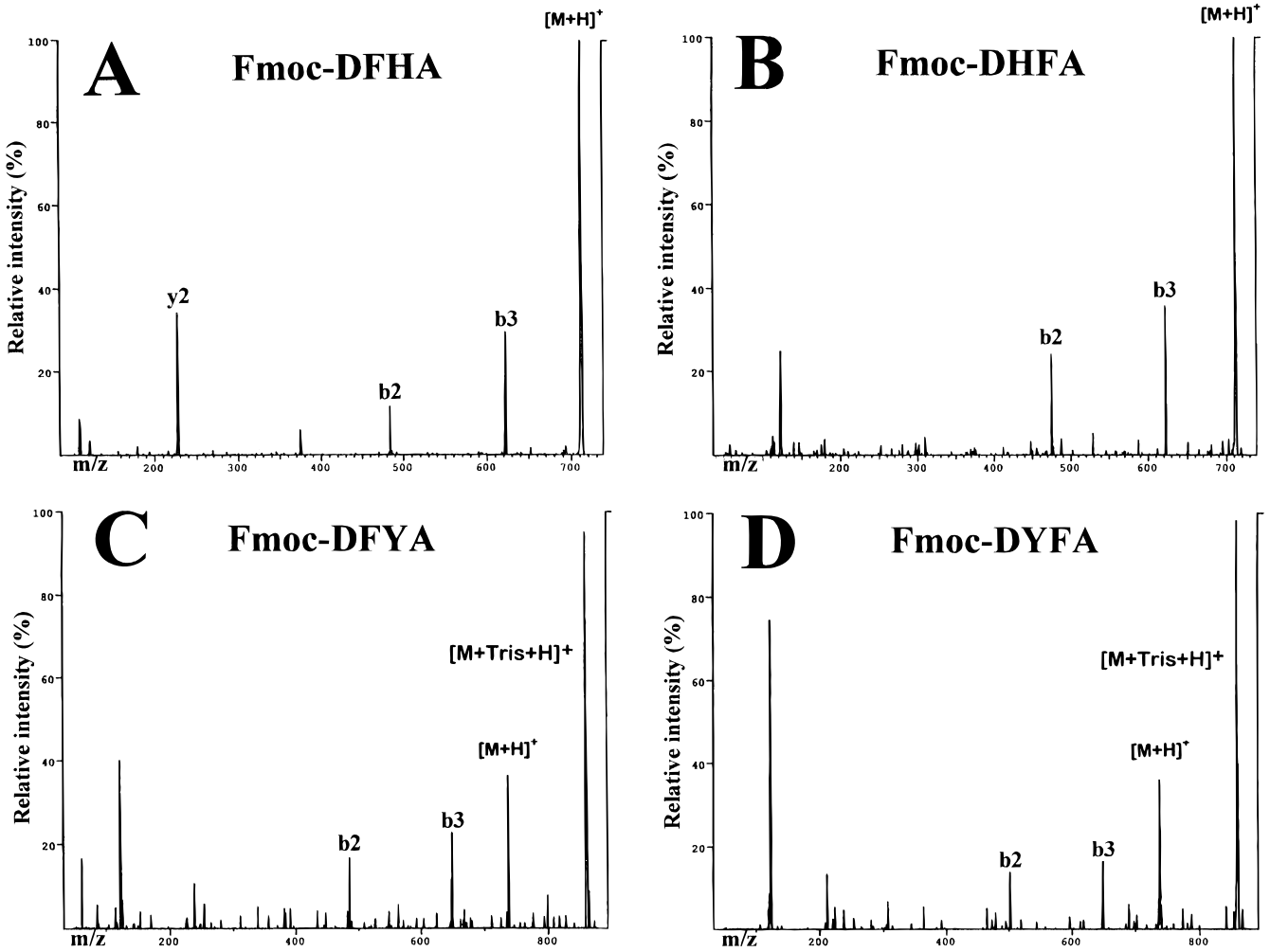

Figure 6. CID mass spectra of ligands selected by on-line ACE-MS-MS from the Fmoc-DXXX library: (A) Fmoc-DFHA, parent ion *m*/*z* 711.5 (B) Fmoc-DHFA, parent ion *m*/*z* 711.5; (C) Fmoc-DFYA, parent ion *m*/*z* 858.5; (D) Fmoc-DYFA, parent ion *m*/*z* 858.5. ACE-MS conditions: same as in Figure 5. MS-MS conditions: −50 eV collision energy, 0.8 to 1.2 mTorr argon collision gas pressure.

in the presence of vancomycin, with minor variations in mobility shifts depending on the third amino acid, indicating that the third positions is less important in binding.[15] Sequences of most of the active ligands could be obtained by CID, and representative spectra are shown in Figure 6. In a few cases, although extracted ion electropherograms indicated the presence of an active ligand, the sequence of that compound could not be unambiguously determined because of inadequate S/N in the CID spectra.

The analysis of the 1000-compound library was thus at the edge of the MS-MS detection requirements for obtaining informative daughter ion mass spectra for all selected library components. The detection limit of our MS system was approximately 5 fmol for a single, pure peptide for ACE-MS and approximately 50 fmol for ACE-MS-MS to obtain reliable fragment ions. Considering ion suppression effects in the presence of multiple analytes and the probable non-equimolarity of library members, a realistic limit for the analysis of a library by ACE-MS-MS would be approximately 100 fmol per component. Based on the relative ACE-MS-MS response for single, pure peptides compared to library members, we estimate that the sample used for ACE-MS with the 1000-member library was about 100 mM, corresponding to about 100−200 pmol (100−200 fmol per component) injected on-column in an ACE-MS-MS experiment. Considering that the 1000-member library was near its solubility limit and still had low sensitivity for some compounds, the direct analysis by this procedure of a library 10× to 100× larger would be difficult if not impossible. Other detection methods with greater sensitivity, such as ion trap mass spectrometry, may improve on this limitation. Alternatively, the sensitivity may be further increased by preselecting and preconcentrating the most active ligands from larger libraries followed by ACE-MS-MS, as discussed next.

Solid-Phase Extraction. In view of the above results with the 1000-member library, we investigated the use of affinity extraction with the receptor immobilized on a solid support as a means of both preselecting and preconcentrating the most active ligands. The ligand-loaded support was eluted with 0.1 M ammonium acetate (pH 7.0), and this fraction was found by CE-MS analysis to contain peptides with relatively low affinities (e.g., Fmoc-DXHA, -DXEA, -DXQA, -DXFG, -DXYG, -DXAA, -DXSA, -DXFS, and -DXYS). This result suggested that the peptides with the highest affinities were still bound, perhaps because of non-specific interactions of the Fmoc group or other parts of the ligand with the solid support. The support was subsequently eluted with 50:50 (v/v) 0.1 M acetic acid (pH ∼3): acetonitrile to free additional ligands. This fraction still contained a number of peptides, identified by CE/MS/MS, with potentially both high and low affinity to vancomycin. ACE-MS experiments (Figure 7) allowed us to differentiate between the most active ligands (i.e. Fmoc-DAYS, -DSYA, -DEYA, -DLYA, -DQYA) and other peptides (i.e., Fmoc-DAYS, -DYSA, -DLFS, -DYQA) present in this fraction. The largest mobility shifts (the lowest calculated *P* values) were observed for Fmoc-DXYA and DXFA peptides with some variations within this motif (K_d's are in the range 5−20 μM), confirming

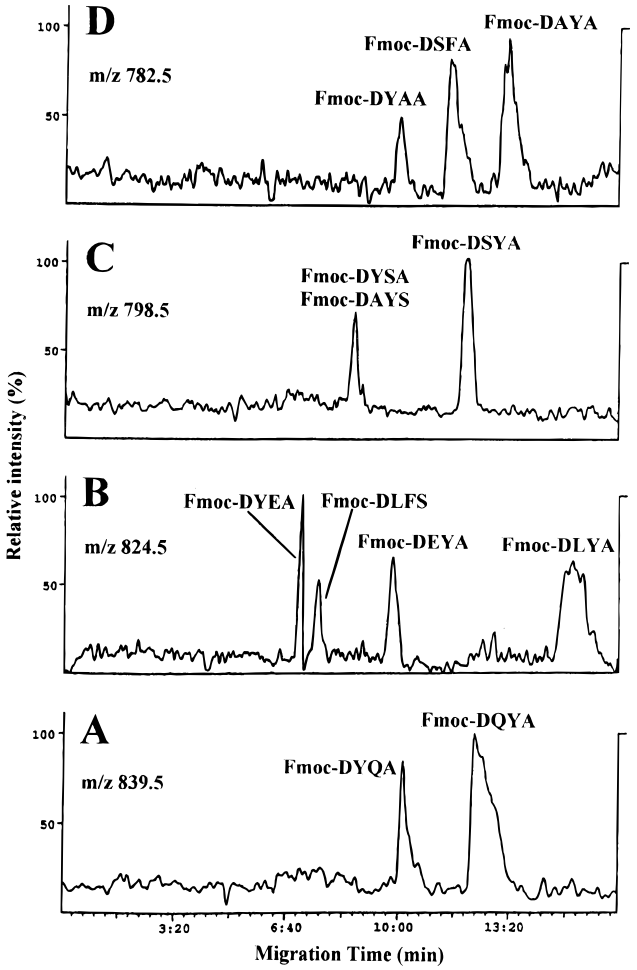

Figure 7. ACE-MS analysis of the Fmoc-DXXX library (1000 peptides). Fraction eluted with 50% acetonitrile from a vancomycin-bound affinity column. Extracted ion electropherograms: (A) *m*/*z* 782.5; (B) *m*/*z* 798.5; (C) *m*/*z* 824.5; (D) *m*/*z* 839.5. Conditions: same as in Figure 2.

the data obtained in the direct ACE-MS screening of the 100-member library. These results demonstrate the advantage of the ACE-MS free solution method over solid-phase screening, where the presence of a solid support introduced non-specific interactions. At the same time, the results of the solid-phase extraction show that preselection of the best ligands from a large pool of compounds can potentially extend the ACE-MS methodology to libraries of >10 000 species.

Discussion

In this paper we have combined the separation power of affinity capillary electrophoresis with the structural identification capability of mass spectrometry to develop a simple and rapid procedure for determining candidate ligands in drug discovery. The method has a number of features which are advantageous. First, receptor binding studies of combinatorial libraries are conducted in solution. Ambiguities arising from non-specific interactions on an adsorbent surface are removed. Second, the method is economical in that only nanoliter volumes of the library are utilized in each experiment. Moreover, the amount of receptor is also small and can be made even smaller using the plug-plug approach (see below). Third, because ACE is a separation method, there is an elimination of non-interacting compounds. Those substances that do interact can be directly sequenced by electrospray mass spectrometry.

As specifically designed, the introduced methodology has several characteristics: (1) the ligands are electrically charged and move in the same direction in the course of electrophoresis; (2) the receptor present in the electrophoretic buffer has a zero net charge or a small charge opposite to the library compounds, so that the mobility change of the ligand can be easily correlated with the extent of binding. Of course, alternative strategies can be incorporated for different libraries, e.g., neutral ligands and a charged receptor. Additionally, the kinetic off-rates must be fast compared to the time of the experiment in order to avoid peak broadening. When the off-rate is slow and stronger binding is being examined, other detection schemes can be explored to characterize the complex in the mass spectrometer.[10]

For this present model system, the one-step method appears most studied to libraries of 500−1000 compounds. This level thus represents the throughput per run. With more sensitive detection in mass spectrometry, such as would occur with ion-trap MS, larger libraries could be directly studied, leading to even higher throughput. As we have shown, an affinity extraction step can be added to remove many non-interacting compounds, as well as to preconcentrate active ligands. Employing solution-based ACE-MS after desorption would eliminate those substances that are extracted onto the solid support by non-specific interactions.

Although solid-phase synthesis is a well-established technique, equimolarity of the libraries is still an issue, giving rise to the potential for false positives and negatives. The importance of determining the quality of the synthesized libraries (presence or absence of expected compounds) has been noted in recent publications.[28] Unlike other screening procedures, ACE-MS does not require equimolar concentrations of library compounds. The concentration of the receptor in the electrophoretic zone is kept higher than the concentration of the library components. As a result, any variation in the relative concentration of two or more ligands of the same affinity is compensated for by the excess of the receptor present. Additionally, ligands of different affinity are separated due to the change in mobility upon complexing with the receptor and, thus, do not compete in the equilibrium.

Different relative amounts of library members can cause false negatives if the active ligand is present at too low a level for its identification by MS. With appropriate control of the experiment, i.e. establishment of the composition of the libraries, false negatives can be determined. As has already been noted, the use of extracted ion electropherograms of expected mixture component masses is very helpful in assessing library quality. For example, peaks corresponding to all expected masses in the 100-peptide libraries were observed in the mass electropherograms. Although we did not perform CID on all ions to confirm the presence of isobaric peptides with different sequences, the data nonetheless provided a qualitative indication of the species present. Similar data were obtained for the 361-member library, where, because of its greater size and complexity and increased frequency of overlaps, assessment of library quality was less definitive.

On the other side, false positives can arise from, for example, active byproducts formed during synthesis due to incomplete side chain deprotection, partial degradation of some amino acids (e.g. Trp) during reaction with deprotection agents, incomplete coupling of an amino acid to a growing peptide chain, etc. Although these byproducts might interfere with some screening procedures, they do not interfere in ACE-MS since only the masses of potential/expected library members are monitored.

(28) Metzger, J. W.; Wiesmuller, K.-H.; Gnau, V.; Grunges, J.; Jung, G. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 894−897. Dunayevskiy, Y.; Vouros, P.; Carell, T.; Wintner, E. A.; Rebek, J., Jr. *Anal. Chem.* **1995**, *67*, 2906−2915.

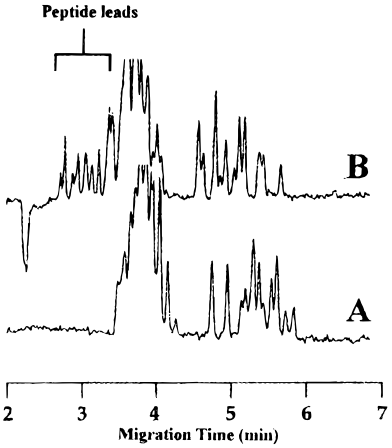

Figure 8. ACE-UV analysis of the Fmoc-DDXX library (100 peptides): (A) without vancomycin; (B) 70 μM vancomycin. Conditions: same as in Figure 1, except uncoated capillary.

If the mass of a side product should overlap with that of a potential ligand, the compounds will be distinguished by CID.

Both uncoated and coated capillaries can be used in ACE-MS and ACE-UV. Whereas the latter were employed in this work, Figure 8 shows typical results on uncoated capillaries. Here, EOF transported the sample from the anode to the cathode, and at neutral or basic pH, the EOF bulk flow was sufficiently rapid that most analytes were driven toward the cathode regardless of their charge. The presence of uncharged vancomycin in the electrophoretic buffer decreased the mobility of active ligands such that they migrated closer to the EOF velocity. Thus, the migration window for peptide leads, as illustrated in Figure 8, was small, potentially limiting the size of the library useful with uncoated capillaries. On the other hand, the use of coated capillaries, where EOF was reduced or eliminated, allowed analyzed species to migrate according to mass and charge alone. With this option, as illustrated throughout the paper, active ligands were retarded upon interaction with the receptor in the buffer. This experimental approach provided greater flexibility for the analysis of relatively large peptide libraries since, in the absence of EOF, there was no predefined window for migration of active ligands. In addition, a neutral coating effectively minimized adsorption of analytes to the capillary wall.

ACE-UV or ACE-MS experiments could also be conducted in the following manner: A BGE containing a fixed concentration of vancomycin could be introduced into the capillary as a plug of varying length by changing the time of injection. Then, a small plug of the library (sample) could be introduced. This approach, referred to as the "plug-plug" approach, is illustrated in Figure 9 for a library of 100 Fmoc-DDXX peptides. With increasing length of the vancomycin-containing BGE plug, ligands underwent increasing numbers of interaction events during migration through the capillary. With longer plugs of receptor, the most tightly bound ligands were retained for greater time compared to migration with no receptor. With the longest plugs, retention of lower affinity peptides could also be observed. Of course, the use of short plugs of receptor would mean that very small amounts of the receptor would be needed for each experiment.

The "plug-plug" approach could also be used when the receptor and ligands were of the same charge and thus migrated in the same direction. The electrophoretic conditions could be chosen so that the receptor and ligands have significantly different mobilities. In this case, the substance(s) with lowest mobility would be injected first, followed by introduction of species of higher mobilities. This approach would provide an opportunity for the interaction between receptor and ligands inside the capillary in a continuous flow mode.

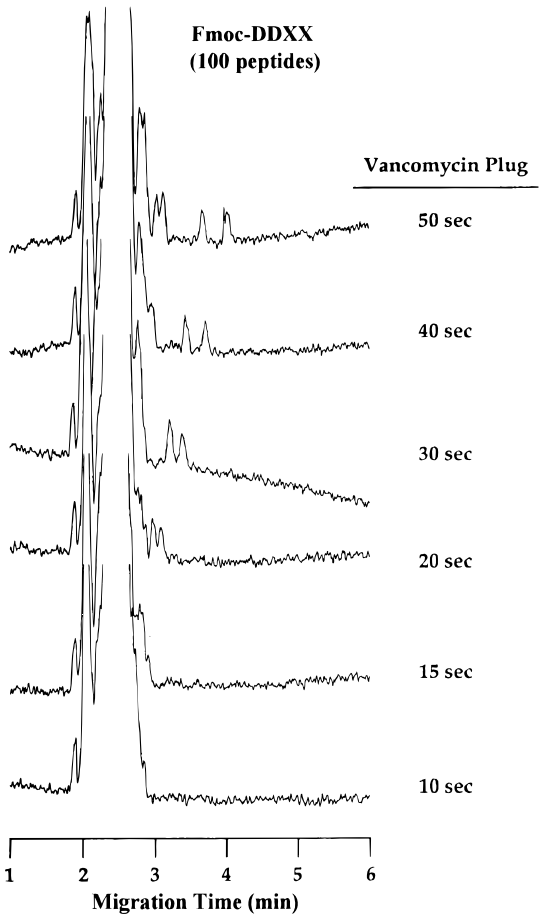

Figure 9. ACE-UV analysis of the Fmoc-DDXX library (100 peptides) with different length plugs of BGE containing 70 μM vancomycin. Conditions: same as in Figure 1.

The "plug-plug" approach was not used with on-line ACE-MS in the present work because of the inconvenience and time involved with obtaining reproducible plug lengths with a manual injection system (hydrodynamic loading by raising the capillary end). Instead, the capillary was completely filled with BGE-containing receptor, and the receptor concentration was changed to vary ligand retention. With increasing receptor concentration, binding saturation would be achieved for the most active ligands. At saturation, these ligands would show no further mobility change with increased receptor concentration, whereas less active ones would continue to vary. As a consequence, the receptor concentration was kept at the level where saturation was not achieved for any library compounds and the observed mobility changes corresponded to the strength of ligand binding.

Finally, the methodology discussed in this paper can easily be generalized to a wide variety of libraries, including diversomers and collections of small organic molecules. A great deal of information can be rapidly obtained. Moreover, the binding experiments are conducted in a homogeneous solution medium that can mimic physiological conditions, as required.

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