Free Solution Identification of Candidate Peptides from Combinatorial Libraries by Affinity Capillary Electrophoresis/Mass Spectrometry

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The purpose of this study was to develop a simple, one-step procedure using affinity capillary electrophoresis/mass spectrometry (ACE/MS) for the solution on-line separation and sequence determination of ligands from combinatorial libraries that bind most tightly to a receptor. In the ACE procedure, the receptor is present in the electrophoresis buffer as a plug, either partially or completely filling the capillary, and the library is introduced as the sample.[1] Ligands that bind tightly to the receptor are retained and thus separated from noninteracting species in the library. In the present work, the interacting ligands are subsequently detected and structurally identified by on-line mass spectrometry.[2]

Strategies for generating and screening molecular diversity have recently gained attention in the search for novel lead structures.[3,4] Among available strategies are those based on screening collections of natural compounds such as microbial fermentation broths and plant extracts, mixtures of synthetic compounds (e.g., peptoids),[5] or libraries of peptides displayed on bacteriophages or synthesized on polymer supports.[3] Successful identification of ligands from libraries requires efficient binding assays and rapid sequence determination or identification.

Current methods for selection of novel ligands from mixtures of peptides or other compounds are limited by the inability of the methods to assay in free solution and by the requirement of relatively large amounts of both receptors and libraries.[3] Capillary electrophoresis (CE) is a free solution technique that allows rapid, efficient separation of minute quantities of materials.[6] Recently, it has been demonstrated that ACE is useful for the measurement of binding constants,[1,7] estimation of kinetic rate constants,[8] and determination of binding stoichiometries of receptor–ligand interactions.[9] Prior work has shown that ACE is capable of selecting a compound from mixtures that binds most tightly to its receptor.[10] By itself, however, ACE is limited in that no structural information is provided for the selected ligands. In this Communication, we demonstrate that, upon coupling with MS,[2] ACE is capable of not only selecting but also identifying peptidyl ligands from a library of peptides.

We used vancomycin from *Streptomyces orientalis* as the model receptor because of its well-established molecular recognition of peptides containing C-terminal AA (D,D),[11] and we prepared synthetic peptide libraries using 2-chlorotrityl polystyrene resin and standard Fmoc chemistry in combination with simultaneous multiple peptide synthesis. A divide/couple/recombine protocol was employed to synthesize an all-D library of Fmoc-DDXX peptides.[12] This procedure ensured the synthesis of equimolar peptides on the resins. The aspartate residues were included both to enhance solubility of the library in aqueous solution and to permit the nonbinding peptides to migrate in a relatively compact peak.[13] The C-terminal carboxylate, which is essential for binding to vancomycin,[11] also enhanced solubility of the peptides.

For ACE/MS experiments, we utilized a neutral hydrophilic polymer coating[14] to minimize electroosmotic flow in the capillary and hence receptor flow into the MS.[15] We also chose a buffer pH where vancomycin was found not to migrate into the MS. Although plugs of receptor that only partially filled the capillary could be utilized,[16] in the example presented here, ACE/MS experiments were done with completely filled capillaries. Employing on-line ACE/MS with the 100 peptide Fmoc-DDXX library as the sample and vancomycin present in the electrophoresis buffer as the receptor, we identified three peptides that bind tightly (Figure 1). Note that, in this example, free Fmoc-peptides enter the mass spectrometer without the receptor, since the off-rate of the ligand from the receptor is rapid.[17] The reconstructed ion electropherogram in Figure 1E showed two retained peaks between 6 and 7 min (as did ACE/UV), but the selected ion electropherograms indicated that the peak at ~7.0 min actually contained two closely eluting peaks that bound most tightly to vancomycin—Fmoc-DDYA (Figure 1A) and Fmoc-DDFA (Figure 1B).[18] Thus, in contrast to UV detection, MS can readily isolate single ions in an electropherogram, even if the CE peak contains a number of species. Note that the isobaric peptides Fmoc-DDAY, Fmoc-DDAF, and Fmoc-DDAH (Figure 1A, B, and D, respectively) did not bind to vancomycin under these experimental conditions, showing

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(12) For the synthesis of the all-D, Fmoc-DDXX tetrapeptide library, 10 amino acid-loaded 2-chlorotrityl polystyrene resins (G, A, E, P, Q, L, F, H, Y, S) were used. Following the standard Fmoc chemistry and protocol for solid phase peptide synthesis, this library synthesis yields an equimolar mixture of 100 (10^2) tetrapeptides isolated as solid powder after they are released from resins (Houghten, R. A.; Pinilla, C.; Blondelle, S. E.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. *Nature* **1991**, *354*, 84–86).

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(15) We believe that our approach is generally applicable to most, if not all, binding systems of rapid kinetics, provided that peptides from the library and the receptor molecule have different electrophoretic mobilities. If both the receptor and peptides are, for example, negatively charged and the peptides migrate faster than the receptor molecule, a plug of the receptor can be introduced prior to the injection of the peptide library to the capillary column. In this case, the mass spectrometer detects the receptor first, candidate peptides next, and the rest of the library last.

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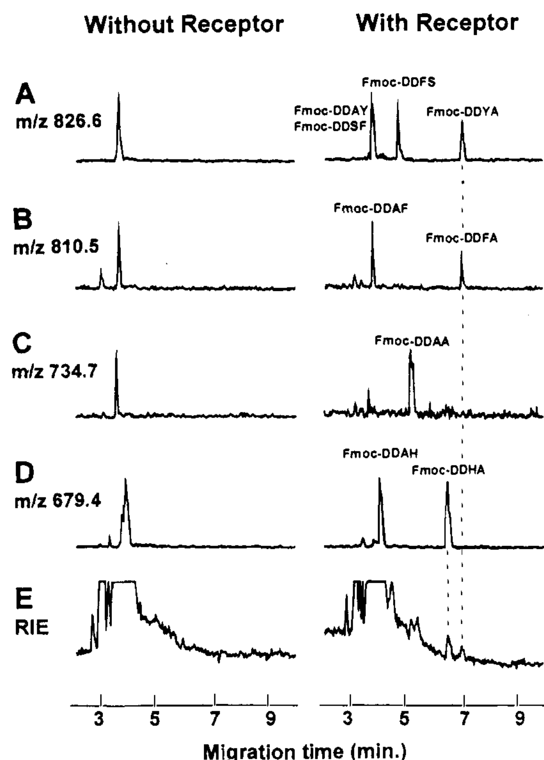

Figure 1. Affinity capillary electrophoresis/mass spectrometry (ACE/MS) of a synthetic all-D, Fmoc-DDXX library of 100 tetrapeptides using vancomycin as the receptor. A–D, Selected ion electropherograms for the masses indicated; E, reconstructed ion electropherogram for runs without (left) and with (right) vancomycin in the electrophoresis buffer. ACE conditions: capillary, 360 μm o.d. × 50 μm i.d. × 38 cm long, coated with a neutral hydrophilic polymer;[14] buffer, 20 mM Tris acetate (pH 8.1) containing no receptor (left) or 70 μM vancomycin (right); electric field, 500 V/cm, 5 μamp; sample, hydrodynamically loaded, 10 cm/8 s/~10 nL at the negative end of the capillary. MS conditions: instrument, Finnigan TSQ-700 with Finnigan API interface operated in positive electrospray ionization (ESI) mode; ESI, + 4.2 kV; gas sheath: 840 cm/min; liquid sheath (2 μL/min), 10 mM Tris acetate (pH 8.1) in H_2O/MeOH (25:75, v/v); MS, scan range, 525–925 amu scan in 2 s.

clearly that binding is sequence specific. Also, under these conditions, the known ligand, Fmoc-DDAA, was only slightly retained (Figure 1C), indicating a weaker binding.

By chemically synthesizing the ACE/MS-selected peptides and testing their binding to vancomycin, we confirmed that Fmoc-DDFA, Fmoc-DDYA, and Fmoc-DDHA bind more tightly than the known ligand, Fmoc-DDAA, to the receptor (dissociation constants of 7.2, 7.8, 26, and 59 μM at pH 8.1, respectively). It is not clear whether the selected ligands (Fmoc-DDFA, Fmoc-DDYA, and Fmoc-DDHA) mimic C-terminal AA-containing peptides in some respect or bind to vancomycin in a different way.[19] Work is under way to identify candidate ligands from a 1000 peptide all-D Fmoc-DXXX tetrapeptide library and will be reported separately.

In summary, ACE/MS has been shown to be a useful procedure for both separating and identifying the most tightly binding ligands from a library of known composition.[20,21] In addition, the technique is economical (for example, in the ACE/UV experiment in Figure 1, only 100 ng of vancomycin and 80 pg of the Fmoc-DDXX library were used, in spite of the fact that little effort was made to minimize the amount of material), rapid (~7 min in Figure 1), experimentally straightforward and versatile, and, most significantly, it is performed in homogeneous aqueous solution, so nonspecific interactions are minimized. Based on the results presented here, ACE/MS also has the potential for directly measuring binding constants of mixtures of peptide ligands in libraries to a specific receptor using selected ion electropherograms from the MS.

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Supplementary Material Available: Experimental details for the preparation of all-D Fmoc-DDXX tetrapeptide libraries, procedure and conditions for ACE/MS, and measurement of binding constants by ACE; collision-induced dissociation mass spectra of Fmoc-DDFA, -DDYA, -DDHA, -DDFS, -DDSF, -DDAF, -DDAY, and -DDAH (10 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(19) The latter possibility has been noted in the case of streptavidin-binding peptides discovered with a phage-displayed library: the peptides associate with the protein differently than does the natural ligand, biotin. Weber, P. C.; Pantoliano, M. W.; Thompson, L. D. *Biochemistry* **1992**, *31*, 9350–9354. Studies of Fmoc-DDX_2X_1 (X_2 = Y, F, H; X_1 = A) peptides binding with vancomycin using solution NMR are in progress.

(20) In conjunction with appropriate experimental procedures to enrich candidate ligands, such as the use of immobilized receptor, we believe that ACE/MS will be, in principle, applicable for ligand screening of large libraries.

(21) This work demonstrates the usefulness of ACE/MS for systems where weak to moderate binding is encountered and should, in principle, also be applicable to strong binding systems with appropriate modifications.