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Use of affinity capillary electrophoresis for the study of protein and drug interactions

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Protein–drug interactions were studied using affinity capillary electrophoresis (ACE). The initial study was performed using a model system, fibronectin–heparin interaction. Two distinct binding constants, 21 and 641 nM, were derived from the Scatchard plots. The results are consistent with reported data obtained using other analytical techniques. The ACE binding assay was applied for studying molecular interactions between kedarcidin chromophore and apoprotein. Conditions with an organic solvent as buffer component were examined to establish a suitable binding assay. It appears that the electrophoretic behavior of the protein shows little distortion in the presence of either dimethyl sulfoxide (up to 10%) or acetonitrile (ACN) (up to 30%). The binding assay was initially conducted in aqueous buffer phase. The saturation concentration of chomophore was found to be around 15 μM. A linear Scatchard plot was derived from binding data with a correlation coefficient of 0.94. The binding constant was determined as K_d = 5.6 μM. The effects of organic solvent content ranging from 0 to 30% ACN on the constant were examined. The binding constants were determined as K_d = 11, 12.5 and 16.2 μM for 5, 10 and 30% ACN, respectively. It appeared that the binding affinity between kedarcidin chromophore and apoprotein is reduced as the organic solvent content in the aqueous phase is increased.

Keywords: *Affinity capillary electrophoresis; protein–drug interactions; kedarcidin; apoprotein*

The interaction of proteins with specific ligands such as drugs and toxins is among the important aspects of biological studies in drug discovery and pharmaceutical development processes. The characterization of the binding phenomenon and the determination of binding parameters such as dissociation constant and stoichiometry are essential for the evalution of bioaffinity and the understanding of receptor–ligand interactions. Traditional biological binding assays generally require the measruement of bound or free species in an equilibrium system, or radioactively labeled ligands and relatively large amounts of sample, which is not always possible owing to insufficient labeling and sensitivity, NMR, the transition temperature in differential scanning calorimetry and chromatographic techniques, have been utilized to study molecular interactions.

The recent advances in capillary electrophoresis (CE) have generated tremendous interest in the area of biological research. As an alternative approach in the study of molecular interactions, affinity capillary electrophoresis (ACE) has been introduced for the analysis of receptor–ligand interactions and the determination of binding constants.[1–12] Although several CE-based approaches have been presented for binding studies such as the quantification of bound and free ligands after reaching an equilibrium state,[4,6] CE–fronal analysis for drug–protein interactions[10] and other similar approaches,[5] with the most recent studies dealing with receptor–ligand interactions such as protein–drug ligand, protein–sugar, DNA–peptide, peptide–drug and antibody–antigen, were accomplished using an ACE approach.[1–3] The ACE assay as an alternative screening technique for identifying a tight-binding ligand for a receptor in a small peptide library has also been described.[3] Most recently, an ACE-based binding assay has been combined with mass spectrometry (MS) for screening combinatorial libraries for drug discovery research.[12] The basic principle involves the measurement of an altered electrophoretic mobility (or migration shift) of the complexed species as compared with the free ligand. This novel affinity binding assay also inherits various advantages of CE such as high resolving power, high speed, sensitive detection and ease of automation. In addition, it requires no radiolabeling and allows the simultaneous analysis of each individual component within a sample mixture. Scatchard analysis generally can be performed by measuring the migration shift resulting from the change in charge status before and after the formation of the complex to derive the binding constant and other related interaction parameters.

Our recent efforts in studying biomolecular interactions, especially dealing with protein–drug binding, led us to utilize the ACE binding assay for the characterization of binding profiles in our drug discovery programs. One of the interesting studies involves the interaction between a chromoprotein, kedarcidin, and its chromophore. Among the enediyne class of antitumor agents, kedarcidin has been identified as a highly potent and very active chromoprotein antitumor antibiotic in both murine tumor models and in human tumor xenografts.[13] Much of the discovery and development efforts have been conducted in recent years. The structural characterization and elucidation of the molecule and related compounds and their biological relevance have been extensively studied.[14–17] The chromophore is chemically labile when free in solution. The antitumor activity of the chromoprotein is due primarily to the chromophore. The apoprotein's function is to stabilize the chromophore and appear to direct the delivery of the chromophore to the DNA of intact cells. Detailed studies on the molecular interaction between chromophore and apoprotein would provide useful information in understanding the mechanism of action. In this work, we established a CE-based method for the separation of the apoprotein and other minor species and examined the feasibility of using ACE for studying the interaction between kedarcidin chromophore and apoprotein under various conditions, including organic solvents as media additives in the background electrolyte. We demonstrate the

utility of a rapid binding assay using the highly sensitive ACE approach for the determiantion of thermodynamic association constants without radiolabeling.

Experimental

Apparatus

All ACE experiments were carried out with a Beckman (Fullerton, CA, USA) P/ACE 5010 CE equipped with either a UV or a diode-array detector. For fibronectin–heparin interaction experiments, a polymer-coated fused-silica capillary obtained from Beckman was used (360 μm od, 50 μm id, total length 27 cm). For other experiments, a sulfonic acid-coated fused-silica capillary (360 μm od, 75 μm id) with a total length of 34–56.7 cm (27–50 cm effective separation length) obtained from Scientific Resources (Eatontown, NJ, USA) were used for the separation. Pressure injection was used for sample introduction with a duration of 5–10 s. A high voltage of 29 kV set at reverse polarity was applied for electrophoretic runs. The protein was monitored at a wavelength of either 280 or 214 nm. Data were collected and analyzed with System Gold software.

Materials and methods

The protein sample fibronectin was purchased from Boehringer Mannheim (Indianapolis, IN, USA) and a low moelcular mass heparin (M_r 6000) was obtained from Sigma (St. Louis, MO, USA). The kedarcidin apoprotein and chromophore sample were obtained in-house.

The ACE binding assays were performed based on previously described methods.[1–3,7,8] Generally, an electrophoretic buffer was selected for a specific binding system to establish the separation of the protein from other sample species. A ligand molecule was subsequently added to the running buffer at various concentrations. Upon introduction of the protein sample, affinity interaction between the protein and ligand may take place during electrophoresis. In these studies, a buffer consisting of 20 mM Tricine (pH 8.0) was used for fibronectin–heparin binding. For kedarcidin chromophore and apoprotein interaction, the running buffer consisted of citrate–MES (pH 6.0) with appropriate concentrations of organic solvents, either dimethyl sulfoxide (DMSO) or acetonitrile (ACN). The concentration of the original kedarcidin apoprotein sample was 0.85–1.0 mg ml^{-1} in 50 mM TRIS–HCl buffer (pH 7.5). The kedarcidin chromophore solution was diluted to 2.91 mM. Affinity buffers were freshly prepared by adding the appropriate concentration of kedarcidin chromophore, which was stored in a refrigerator prior to injection to minimize room temperature exposure. An aliquot of the protein sample was mixed with a negatively charged small molecule (salicylic acid) as internal reference for injection.

Affinity interactions between protein and ligand molecules were examined by measuring the migration time of the internal reference and protein as a function of ligand concentrations. The analysis was duplicated and the average was taken for further calculation. Several methods have been proposed for the determination of binding constants.[1–6] to simplify the process, Scatchard analysis was performed based on a slightly modified dimensionless number, the shifting coefficient R [$R = (M_0 - M)/M_0$],[18] where M_0 is the mobility of free protein and M is the observed protein mobility during interaction. In a CE format, the determination of mobility can be converted to the measurement of migration time. A plot of R/[ligand concentration] *versus* R should yield a straight line with a slope of $-1/K_d$ for the determination of the binding constant.

Results and discussion

To validate the ACE-based binding assay for protein interaction with drug molecules, we initially evaluated a model system, protein–glycosaminoglycan (GAG) binding, studied with affinity gel retardation electrophoresis,[18] a technique similar to the ACE approach. The initial protein selected was fibronectin, a glycoprotein of two similar polypeptide chains connected by two disulfide bridges. It has been reported that fibronectin binds heparin and heparin sulfate with high affinity.[18] Analysis of the fibronectin–heparin interaction using ACE is shown in Fig. 1. The interaction was reported to be related to the structural features of GAG such as size, charge density and disaccharide unit. Therefore, a low molecular mass heparin (M_r 6000) was chosen in this case. Instead of labeling heparin as described in the literature, the molecule was mixed with the background electrolyte at various concentrations. The addition of heparin to the buffer has virtually no effect on the UV background owing to the lack of a chromophore in the molecule. The protein peak was monitored at a wavelength of 280 nm and the migration shift was determined as a function of heparin concentration in the range 0.05–100 μM.

The binding profile clearly indicated that the increased concentration of heparin resulted in a significant change in the protein mobility, similar to the retardation in gel electrophoresis, implying a high affinity interaction between heparin and fibronectin. Graphical analysis of the profile is shown in Fig. 2, resulting in two distinct linear plots. This phenomenon suggests that more than one binding site is associated with the interaction. Based on a similar method in affinity gel retardation studies, two binding constants were derived from the Scatchard plots as 21 and 641 nM. The results obtained from the ACE approach are consistent with the reported data obtained by the conventional affinity gel electrophoretic technique.[18]

It has been reported that kedarcidin is composed of a single polypeptide chain consisting of 114 amino acid residues and a cytotoxic, highly labile, non-protein chromophore.[14] The non-covalently bound apoprotein and chromophore form a 1 : 1 complex and are separable from each other. The molecular interaction between the apoprotein and the chomophore appears to be crucial for maintaining the antitumor activity, which is due primarily to the chromophore, whereas the apoprotein is believed to play a role in the stabilization and transport of the chromophore. The proposed structure of kedarcidin chromo-

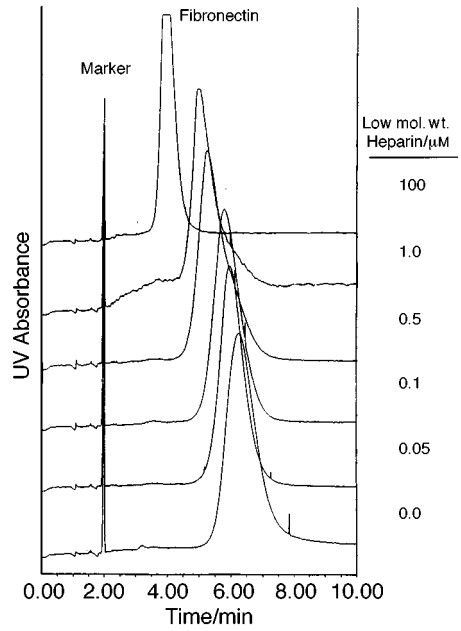


Fig. 1 Binding profiles of fibronectin–heparin interaction using ACE. Column, Beckman coated fused-silica capillary (360 μM od × 50 μM id), 27 cm in length (20.3 cm affective separation length); buffer, 20 mM Tricine (pH 8.0); pressure injection, 20 s duration; detection wavelength, 280 nm.

phore and the primary sequence structure of apoprotein are shown in Fig. 3. The apoprotein is water soluble but the chromophore is only solvent extractable and has a very limited solubility in the aqueous phase. The common aqueous buffer used in most CE applciations appears to be problematic for further ACE studies dealing with the chromophore and apoprotein interaction. The initial focus of these studies was therefore to establish appropriate conditions suitable for the apoprotein separation and the subsequent determination of the binding constant, especially in the presence of organic solvents.

With the use of a solfonic acid-coated capillary column and a buffer consisting of citrate–MES (pH 6.0), a major protein peak and two minor peaks were observed at a wavelength of 280 nm. This appears to be consistent with bands observed by SDS-PAGE. A characteristic UV spectrum was acquired for the major peak with the use of a diode-array detector. Since UV spectroscopy is generally considered not to be an ideal method for protein characterization, the apoprotein was further confirmed using electrospray ionization (ESI) MS. The CE separation and a deconvoluted (zero charge state) mass spectrum are shown in Fig. 4. The molecular mass determined (11 181) is consistent with the calculation from the sequence structure. It has been reported that the hydrophobic chromophore dissolves only in DMSO-containing buffers, so a 10% DMSO buffer solution was initially used in these studies. It was

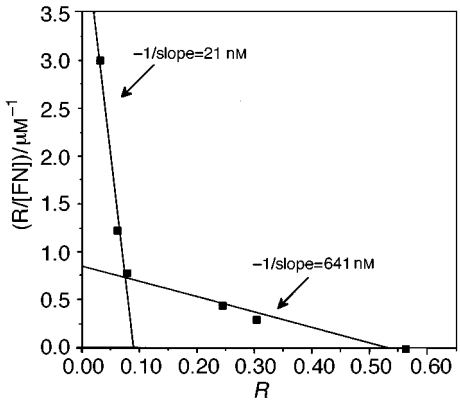
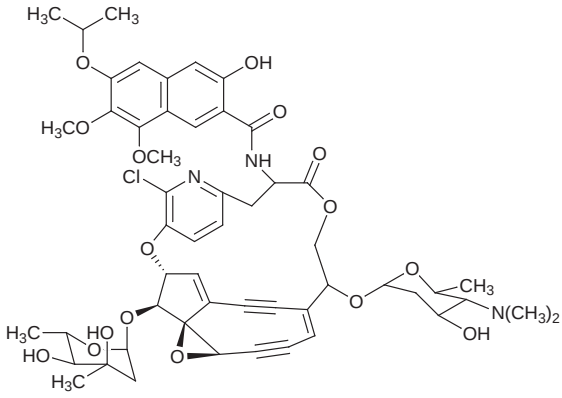


Fig. 2 Scatchard plot of bibronectin–heparin interaction derived from binding profile data using ACE. *R* is defined in the Experimental section.



ASAAVSVSPA TGLADGATVT VSASGFATST SATALQCAIL
ADGRGACNVA EFHDFSLSGG EGTTSVVVRR SFTGYVMPDG
PEVGAVDCDT APGGCEIVVG GNTEEYENAA ISFE 114

Apoprotein

Fig. 3 Proposed structure of kedarcidin chromophore and primary structure of apoprotein.

observed that the background noise increases upon addition of 5 μM of kedarcidin chromophore to the running buffer. As the concentration of chromophore reached 50 μM, the electropherogram became unacceptable for further binding studies owing to the appearance of multiple spike peaks. This phenomenon is most likely due to light scattering effects caused by chromophore particles that are perhaps not entirely dissolved in the buffer solution. Increasing the DMSO concentration in the buffer seems to reduce the spiking but causes distortion of the protein peaks, perhaps as a result of the conformational change caused by the strong organic solvent. An alternative approach is to use other moderate organic solvents. In these studies, ACN at concentrations ranging from 5 to 50% in buffer was evaluated. The electropherograms appear to be acceptable with ACN concentrations $< 35\%$.

Although the poor solubility of the kedarcidin chromophore in the aqueous phase appears to be the key factor that prevents the use of other conventional binding assays, this problem may not be that critical if a CE-based assay is utilized. The unique capability of handling small sample volumes and the high sensitivity detection offered by CE may compromise some of the experimental difficulties in other traditional methods. Based on the established experimental conditions with or without ACN as organic additive, binding interaction between kedarcidin chromophore and apoprotein was conducted. The initial ACE binding assay was performed using an aqueous buffer without an organic solvent. A representative binding profile showing the migration shift of the protein peak as a function of ligand concentration is displayed in Fig. 5. A negatively charged small molecule, salicylic acid (SA), was used as an internal reference for the measurement of relative mobility of the protein. A series of buffers containing kedarcidin chromophore at concentrations ranging from 0 to 20 μM were used for the ACE binding experiments. As the chromophore concentration in buffer solution increases, the protein peak gradually shifts away from the reference peak, indicating that a binding interaction between the chromophore and apoprotein takes place.

As displayed in Fig. 5, the internal reference has a migration time that is independent of the concentration of the chromophore. Under the above ACE conditions with a buffer of pH 6.0,

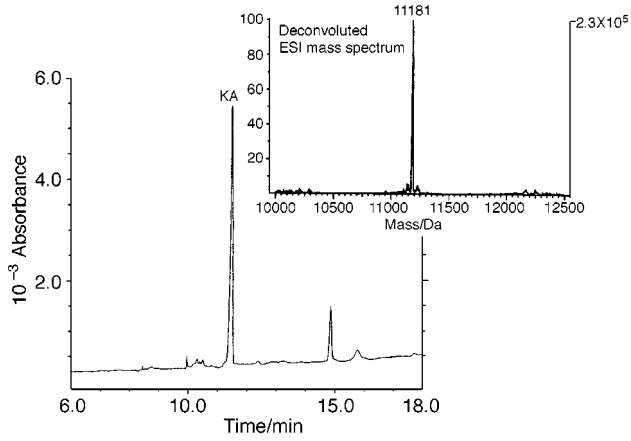


Fig. 4 Separation of kedarcidin apoprotein (KA) sample using CE and the deconvoluted (zero charge state) ESI mss spectrum of KA obtained *via* LC–ESI–MS (inset). Column, zero EOF sufonic acid-coated fused-silica capillary (360 μM od × 75 μM id), 56.7 cm in length (50 cm effective separation length); buffer, citrate–MES (pH 6.); pressure injection, 10 s duration; applied voltage, 29 kV with reverse polarity; detection wavelength, 280 nm. Mass spectra measurement of kedarcidin was performed by LC–MS using the negative ion mode. A 50 × 0.5 mm id PLRP-s 4000 A polymeric column was used at a flow rate of 20 μl min^{-1} with a gradient from 20 to 95% B in 20 min (A = H_2O–ACN–aq. NH_3 (98 + 2 + 0.1), B = H_2O–ACN–aq. NH_3 (10 + 90 + 0.1).

the ligand that most likely is positively charged has a tendency to migrate towards the cathode inlet which is opposite the detector (anode outlet). When the ligand binds to the protein, the complex has a reduced electrophoretic mobility and hence a slower migration time than the protein alone. The change in protein peak shape and the slight peak split indicate that a complex binding process occurs. This phenomenon is generally related to the affinity interaction between the two moelcules as a result of the complex formation and the on and off binding kinetic process. Attempts to increase the chromophore concentration in a non-organic buffer solution further appear to be problematic owing to its limited solubility in the aqueous phase. The correlation between relative mobility of the protein and chromophore concentration indicates that the saturation is reached at a concentration of around 15 μM. A Scatchard plot was derived from the binding data as shown in Fig. 6. The binding constant was calcualted to be K_d = 5.6 μM with a correlation coefficient of 0.94. This indicates that tight binding between kedarcidin chromophore and apoprotein is expected in the aqueous buffer phase.

Since most biological studies related to the complexation of the chromophore and apoprotein were conducted under conditions of using a certain concentration level of organic solvent owing to concerns about the limited solubility of the kedarcidin chromophore in the aqueous phase,[16,17] further investigations of the effects of organic solvent content on binding constants were performed. This approach will possibly provide further information for the understanding of the affinity interaction and the mechanism of action. ACN was selected as the additive in the binding buffer for these studies. A separation profile similar to that in aqueous buffer conditions was obtained with 5% ACN in the binding buffer. It was noted that the workable chromophore concentration increased to more than 30 μM as compared with previous studies without organic solvent. Further increases in chromophore concentration appear to cause baseline noise problems owing to its limited solubility. A correlation plot of relative mobility of the protein *versus* chromophore concentration was obtained and a saturation point seems to be reached at around 30 μM. The Scatchard plot was derived from the binding data as shown in Fig. 7(A). The binding constant was calculated to be K_d = 11 μM with a correlation coefficient of 0.90. Similar experiments were also conducted with further increased organic content. With 10% ACN in the binding buffer, the correlation between the relative mobility of the protein and chromophore concentration indicated a slightly increased chromophore concentration range of up to 40 μM. This is simply due to the increased solubility of chromophore in the aqueous buffer solution. A linear Scatchard plot was derived from the binding data as shown in Fig. 7(B). The binding constant was calculated to be K_d = 12.5 μM with a correlation coefficient of 0.86. The ACE binding assay could also be accomplished as the organic solvent content increased to 30% ACN in the buffer. There appears to be little indication of peak distortion related to protein conformational change at this level of organic solvent concentration. The workable chromophore concentration range has been extended to around 90 μM. From the linear Scatchard

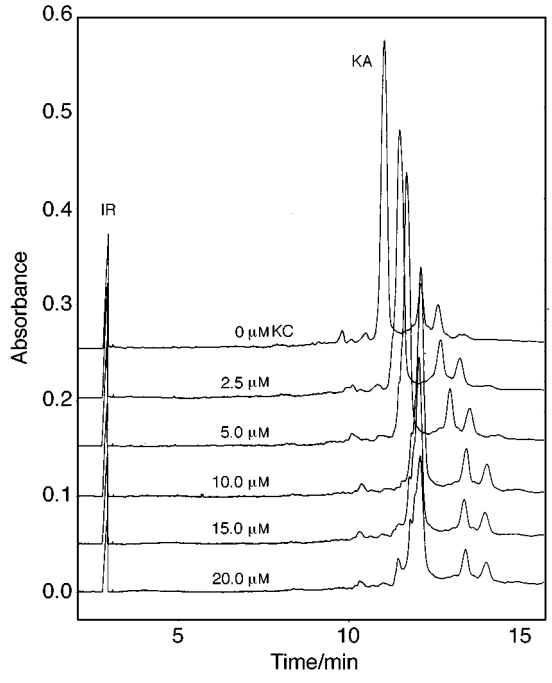


Fig. 5 ACE profiles for the interaction of kedarcidin chromophore and apoprotein in aqueous buffer phase without organic additive. IR, internal reference; KA, kedarcidin apoprotein. Concentration range of kedarcidin chromophore in the buffer, 0–20 μM; column, zero EOF sulfonic acid-coated fused-silica capillary (360 μM od × 75 μM id), 34 cm in length (27.3 cm effective separation length); buffer, citrate–MES (pH 6.0); pressure injection, 2 s duration; applied votlage, 29 kV with reverse polarity detection wavelength, 280 nm.

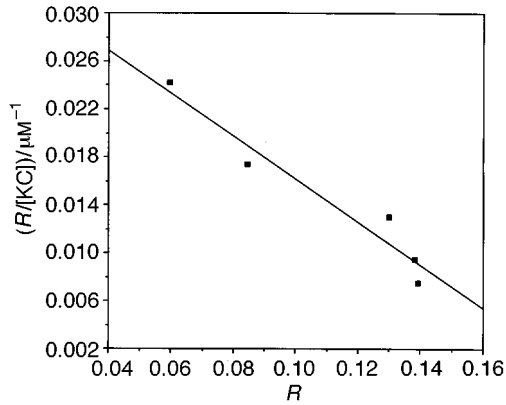


Fig. 6 Scatchard plot of kedarcidin chromophore and apoprotein binding obtained using ACE. Data derived from binding profiles under conditions of aqueous buffer without organic additive. *R* is defined in the Experimental section.

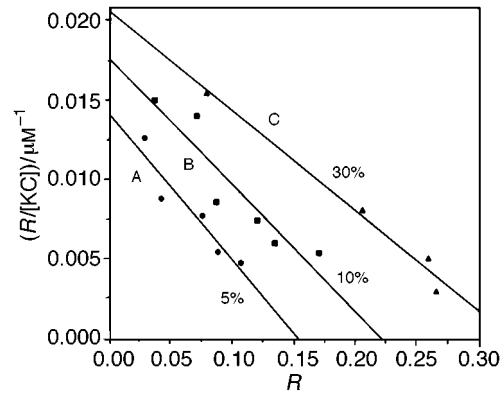


Fig. 7 Scatchard plots of kedarcidin chromophore and apoprotein binding obtained using ACE under conditions with various concentrations of ACN additive (*R* is defined in the Experimental section). Capillary column, zero EOF sulfonic acid-coated fused-silica capillary (360 μM od × 75 μM id), 46.9 cm in length (40.2 cm effective separation length); buffer, citrate–MES–5% ACN (pH 6.0); pressure injection, 5 s duration; applied voltage, 30 kV with reverse polarity; detection wavelength, 280 nm. A, 5% ACN, concentration range of kedarcidin chromophore in the buffer 0–32 μM; B, 10% ACN, chromophore concentration range 0–40 μM; and C, 30% ACN, chromophore concentration range 0–90 μM.

plot shown in Fig. 7(C) (correlation coefficient 0.981) the binding constant was determined to be K_d = 16.2 μM.

The effects of ACN concentration ranging from 0 to 30% on the binding constant were investigated and the results are summarized in Table 1. It appears that the dissociation constant (K_d) increases, or conversely there is a decrease in binding affinity between the chromophore and apoprotein, as the organic solvent content in the buffer is increased. The higher K_d values observed in the presence of organic solvent are perhaps related to several factors. Any protein conformational change could affect the specific binding pocket where the chromophore is known to have strong interactions. The addition of an organic solvent will also possibly reduce the hydrogen bond interactions between the protein and the chromophore. We generally observed a lower association constant (higher K_d) in this binding system involving the use of organic solvent, which appears to be consistent with other biological studies. The binding affinity between the chromophore and apoprotein seems not to be compromised by the increased solubility of the chromophore. The addition of an organic solvent may actually cause protein conformational changes or partial denaturation, thus reduced binding. A higher percentage of organic content (> 35%) even resulted in precipitation of buffer salts. Although the binding assay under such unconventional conditions can be developed, concerns for protein denaturation and other experimental difficulties also arise. These studies may provide a rapid approach for the determination of binding constants under normal and extreme binding conditions and provide some analytical insight into understanding the mechanism of action in the complexation process between an apoprotein and its natural ligand.

Table 1 Effects of ACN concentration on binding constants for the interaction between kedarcidin chromophore and apoprotein

Concentration of ACN (%)	Dissociation constant, K_d/μM
0	5.6
5	11.0
10	12.5
30	16.2

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