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Sensing of peptide hormones with dynamic combinatorial libraries of metal–dye complexes: the advantage of time-resolved measurements†

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A dynamic combinatorial library of metal–dye complexes was obtained by reacting aqueous solutions of the dyes Methyl Calcein Blue, Arsenazo I, and Xylenol Orange with CuCl₂ and NiCl₂. The mixture gave a characteristic UV-Vis response upon addition of the peptide hormones angiotensin I and angiotensin II. This allowed distinguishing pure samples of peptide hormones from mixtures. The discriminatory power of the sensor was enhanced when the several UV/Vis measurements were performed during the equilibration process of the library.

Introduction

Adaptive chemical networks are formed by mixing molecular building blocks, which are able to bind to each other via reversible interactions. If there is cross-reactivity between the various building blocks, the assemblies are formed in a combinatorial fashion and the resulting network is referred to as a dynamic combinatorial library (DCL). The concentrations of the different members of a DCL depend on the environment of the respective system (pH, solvent, temperature, etc.). It is possible to alter the library composition by adding molecules that interact with some members of the library. If the library composition can be transduced into a signal output, the DCL can be used as a sensor (Scheme 1).

Scheme 1 Basic principle of a DCL sensor: a dynamic mixture of colored (or fluorescent) compounds A–G undergoes an analyte-induced re-equilibration. The change in color (or fluorescence) can be used to obtain information about the identity, the quantity, or the purity of the analyte.

For a sensor of this kind, the information about the analyte is distributed over the spectrum. The spectrum therefore represents a ‘fingerprint’ of the analyte. To correlate the changes at different wavelengths with the analyte properties of interest (identity, quantity, purity), it is advantageous to use multivariate analyses. In this regard, a DCL sensor is related to sensor arrays. However, sensor arrays are analyzed by measuring the response of the independent sensor units, whereas a single UV/Vis or fluorescence measurement is sufficient for a DCL sensor.

The utilization of DCLs for sensing purposes is a relatively new concept and so far there are only few studies in this direction. Guiseppone and Lehn have investigated constitutionally dynamic polymers (“dynamers”), which were obtained by polycondensation of fluorescent and non-fluorescent diamine monomers with 2,7-fluorenebiscaraldehyde in the presence of variable amounts of Zn²⁺ ions. The polymer composition—and consequently the fluorescence—was found to depend on the concentration of Zn²⁺. The dynamic polymer can therefore be regarded as a system which is able to sense Zn²⁺ by an analyte-induced constitutional rearrangement.

The group of Anslyn has investigated DCL sensors based on two synthetic receptors for oxoanions and two dyes. Both dyes can bind to both receptors and four different receptor–dye complexes were formed. This system was used to simultaneously determine the concentration of malate and tartrate in aqueous solutions. A related approach was employed to determine the concentration of Ca²⁺ and citrate in flavored vodkas.

The Lavigne group has studied the analyte induced aggregation of carboxylic acid functionalized poly(thiophene)s. The system was used to identify low millimolar concentrations of α,ω-diamines. In a subsequent study, they have shown that a sensor of this kind can be used to quantify histamine in a fish sample.

The most recent investigation about sensing with adaptive chemical systems was published by Margulies and Hamilton. As sensors, they used dynamic libraries of fluorescent G-quadruplexes. The interaction with proteins resulted in characteristic fluorescence emission patterns, which allowed identification of the respective protein.

Our group has shown that combinatorial mixtures of transition-metal complexes and commercially available dyes can be used as powerful sensors for small peptides and nucleotides. In extension of this work, we have reported recently a sensor that is able to determine in retrospect the history of analyte variations. The study demonstrated that the adaptation process of a DCL contains useful information. This inspired us to explore whether time-resolved measurements with DCL sensors are beneficial for the resolution. In the following we will demonstrate that this is indeed the case. A DCL sensor, which is analyzed in a...
time-resolved fashion, is able to distinguish mixtures of the hormones angiotensin I and angiotensin II with a significantly improved resolution compared to a sensor that is analyzed under pseudo-equilibrium conditions.

**Results and discussion**

First, a small screening was performed in buffered aqueous solution (2-(N-cyclohexylamino)ethanesulfonic acid (CHES) buffer, 0.1 M, pH 8.4), in which the stability and solubility of fourteen commercially available dyes (50 μM) and their binding towards Ni²⁺ and Cu²⁺ (0.1 mM each) were investigated by UV/Vis spectroscopy (for details see experimental section). In order to obtain optimal signal output from a dynamic combinatorial spectroscopy (for details see experimental section), in all cases, adding sequential aliquots of aqueous metal solutions to buffered aqueous solution (CHES buffer, 0.1 M, pH 8.4, 298 K). The numbers in parentheses give the calculated error of the fitting procedure.

**Table 1** Fitted cumulative formation constants (log β_b) for the complexes [Mₓ(dye)ₓ] (M = Cu²⁺, Ni²⁺) observed during UV/Vis titrations in buffered aqueous solution (CHES buffer, 0.1 M, pH 8.4, 298 K). The numbers in parentheses give the calculated error of the fitting procedure.

<table>
<thead>
<tr>
<th>M²⁺</th>
<th>Xylenol Orange</th>
<th>Arszeno I</th>
<th>Methyl Calcein Blue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>log β₁₁</td>
<td>log β₁₂</td>
<td>log β₂₁</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>8.75(3)</td>
<td>15.98(6)</td>
<td>—</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>7.75(5)</td>
<td>13.86(5)</td>
<td>12.80(8)</td>
</tr>
</tbody>
</table>

![Scheme 2](#) Molecular structures of the three dyes used to construct the sensor.

![Fig 1](#) Changes in absorbance observed during titrations of Cu²⁺ (left) and Ni²⁺ (right) into solutions of AI ([dye]ₜₒₜ = 15 μM, CHES buffer, 0.1 M, pH 8.4, 298 K). Below the spectral overlays are corresponding plots of absorbance vs. metal/dye ratio for three selected wavelengths (the lines represent best fits to the models discussed in the text).
[Ni(AI)$_2$] suggested by the UV/Vis data (Table S2, ESI†). We note, however, the persistence of intense peaks corresponding to the free dye, even in the presence of excess metal. It is thus plausible that observation of the latter complexes is impeded by gas-phase fragmentation processes operating in the spectrometer.

Analogous titrations with MCB and Cu$^{2+}$ showed the exclusive formation of a mononuclear [Cu(MCB)] complex with one absorbance inflection occurring at a metal/dye ratio of 1.0 (Fig. S3, ESI†). Once again, however, the same titration with Ni$^{2+}$ showed biphasic behavior and the data could only be modeled satisfactorily by considering the formation of a binary complex [Ni(MCB)$_2$], in addition to the 1 : 1 complex [Ni(MCB)] (Table 1 and Fig. S4, ESI†). The mass spectra show complexes of the latter formulation in abundance for both metals (Table S3, ESI†), but as for the AI/Ni$^{2+}$ system, no binary complexes were detected in the gas phase for solutions containing e.g. a 1 : 2 ratio of Ni$^{2+}$ : MCB ([MCB]$_{tot}$ = 0.1 mM, pH 8.4).

We stress that these complex formulations are limited to the basic stoichiometries of the metal/dye complexes: no concrete conclusions regarding chemical structure, charge and/or protonation state of the various species can be drawn from the current observations. The fitted values listed in Table 1 may thus be considered effective stability constants, whose significance hold strictly within the conditions chosen for the current investigation. They are also subject to error arising from e.g. salt impurities in the dye precursors and (in some cases) high correlation between the calculated spectra of the participating species.

These considerations aside, combining all three dyes with both Cu$^{2+}$ and Ni$^{2+}$ will clearly yield a dynamic combinatorial library of complexes, metal ions and free dyes, with the metal–dye complexes being not only homometallic and homoleptic, but ideally comprising mixed species. To provide evidence of the variety of the species in this DCL, we studied an aqueous mixture (pH 8.4) of the three dyes ([MCB] = 50 mM, [AI] = 25 mM and [XO] = 12.5 mM) and Ni$^{2+}$ and Cu$^{2+}$ (0.1 mM each) by ESI mass spectrometry. Homometallic and heterometallic, as well as homo- and heteroleptic compounds (Table S4, ESI†) comprising all dyes and metal ions could indeed be identified. These results give an insight into the complexity of such a system. In fact, it is very likely that in reality this DCL contains even more species, as due to instability in the gas-phase some metal complexes might not be detectable by mass spectrometry. It should be noted that the formation of heterometallic and heteroleptic complexes is a unique feature of the DCL sensor approach. In a more classical sensor array with individual metal–dye combinations as sensor units, such species are not formed and can thus not contribute to the resolution of the sensor.

The DCL was subsequently studied for sensing of the peptide hormones angiotensin I and angiotensin II. At $t = 0$ min the metal salts were added to a solution containing the three dyes and either one or a mixture of both peptides. The data from UV/Vis measurements at different times were evaluated by multivariate analysis.

Time-resolved UV/Vis studies of the sensing ensembles showed that the major absorbance changes occurred in the first 20 min following addition of the metal ions (Fig. 2). After this initial rapid phase, the system took a further 4 days to fully equilibrate. The slow equilibration kinetics of the DCLs contrast with those characterizing the bimolecular reactions of any two of

The basic concept of the DCL sensor is illustrated in Scheme 3. MCB (50 μM), AI (25 μM) and XO (12.5 μM)$^{15}$ were mixed with either one or both peptides ([peptide]$_{tot}$ = 20 μM), followed by the addition of a 1 : 1 mixture of Ni$^{2+}$ and Cu$^{2+}$ ([M$^{2+}$] = 0.2 mM) at $t = 0$ min (CHES, 0.1 M, pH 8.4). The solutions were analyzed by UV/Vis spectroscopy at different times and the data were evaluated by multivariate analysis.

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**Scheme 3** Experimental procedure for the sensing of the peptide hormones angiotensin I and angiotensin II. At $t = 0$ min the metal salts were added to a solution containing the three dyes and either one or a mixture of both peptides. The data from UV/Vis measurements at different times were evaluated by multivariate analysis.

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**Fig. 2** UV/Vis absorbance data at different wavelengths showing the equilibration over time of DCLs containing Ni$^{2+}$, Cu$^{2+}$, Methyl Calcein Blue, Arsenazo I, Xylenol Orange and angiotensin I (filled symbols) or angiotensin II (empty symbols).
the (metal/dye) components, which are typically complete within minutes. Such effects are, however, to be expected since the true energy minimum of the complex network of reactions operating in the DCL is undoubtedly reached 

via numerous close-lying minima, each with a corresponding kinetic barrier to be overcome. In this respect, equilibration of the DCL is therefore slow enough to be followed by simple UV/Vis measurements. Furthermore, the kinetic profile of the equilibrating system was found to vary according to the analyte. This phenomenon is especially appealing for systems where different analytes give rise to the same signal when the sensor is read out at thermodynamic equilibrium.

Our DCL sensor was first implemented to differentiate between angiotensin I, angiotensin II and a 1 : 1 mixture of the two peptides. The absorbance changes (300–800 nm) were recorded at different times \( t = 2, 5, 10, 20, 40, 60 \text{ min} \) until the system reached a pseudo-equilibrium defined as \( t = 24 \text{ h} \). For each peptide, the experiment was repeated five times. The data were then evaluated by linear discriminant analysis (LDA). The results of the LDA suggested that the data from four wavelengths and at \( t \leq 10 \text{ min} \) was enough to tell apart the three sample types.

The DCL sensor was then used to distinguish between 11 mixtures (six repetitions each) of angiotensin I and II ([angiotensin I] = (20 – \( x \)) \( \mu M \); [angiotensin II] = \( x \) \( \mu M \); with \( x = 0, 2, 4, 6, \ldots, 20 \)). The sensing experiments were performed under the conditions described above and the samples were analyzed by UV/Vis spectroscopy (330–650 nm) at \( t = 2, 5, 10 \text{ min and } 24 \text{ h} \). The absorbance data at \( \lambda = 590, 560, 480 \) and 340 nm was then evaluated by a LDA. In order to determine to what extent the kinetic profiles contributed to the discriminative power of the DCL, the data was analyzed twice. First, only the data obtained from the DCL at pseudo-equilibrium \( (t = 24 \text{ h}) \) was included in the LDA (Fig. 3, top). In a succeeding analysis, all the data was used for evaluation (Fig. 3, bottom). The corresponding results are displayed as score plots.

For both analyses, most of the variance of the data was contained in score 1 (94.8 and 93.5%, respectively). Important differences were found for the resolution. Analysis (a) including only the data obtained at pseudo-equilibrium yields a score plot with considerable overlap of most of the scores of the mixtures. With a correctness of classification of 83%, the data is not sufficient to tell apart all sample types. It should be noted, however, that the scores for the pure samples (angiotensin I: dark blue crosses, angiotensin II: maroon diamonds) are well clustered and separated. Therefore, this method is still good enough to discriminate the pure samples. In analysis (b) where the entire data set, i.e. also the data from the equilibration kinetics of the DCL, is considered, the scores of all sample types are properly classified and well separated. In spite of some score clusters being in close proximity, the correctness of classification is 100%. The supplementary data used in analysis (b) led to an improvement of the classification of the scores and consequently an increase in the discriminative power of the DCL sensor.

Conclusions

A DCL of metal–dye complexes was prepared by mixing the dyes Methyl Calcein Blue, Arsenazo I and Xylenol Orange with CuCl₂ and NiCl₂. Detailed UV/Vis titration experiments and ESI mass spectrometry studies of the library and its components provided insight into the complexity of the resulting chemical network. The DCL was used as a sensor for the peptide hormones angiotensin I and angiotensin II. Despite its simplicity, the DCL sensor was found to display a remarkable discriminative power: mixtures of the two peptides were distinguished from pure samples at low micromolar concentrations (20 \( \mu M \)). The resolution of the sensor was significantly enhanced when the measurements were performed in a time-resolved fashion. It should be noted that so far, work with DCLs has primarily focused on systems under thermodynamic control. The findings described above are evidence that the kinetics of a DCL adaptation process may contain useful information, in particular if dynamic networks are used for challenging sensing problems.

Experimental

General

Alizarin Red S (Fluka), Arsenazo I (Lancaster), Azophloxine (Fluka), Galloycyanine (Acros Organics), Methyl Calcein Blue (Sigma), Methylene Blue (Sigma), Methyl Orange (Ciba), Methyl Red (Acros Organics), Mordant Blue 9 (Acros Organics), Mordant Blue 13 (Acros Organics), Naphthol Blue Black (Fluka), Orange G (Acros Organics), Pyrocatechol Violet (Acros Organic), Xylenol Red (Acros Organics), Xylenol Blue (Acros Organics), Xylenol
Orange (Fluka), NiCl₂·6H₂O (Strem Chemicals), CuCl₂·2H₂O (Fluka), CHES buffer (Fluka), and angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) and angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) (both Bachem) were used as received. Stock solutions of all dyes (1 mM, except Xylenol Orange: 0.5 mM), the metal salts (10 mM), the peptides (0.1 mM) and CHES buffer (pH 8.4, 0.2 M) were prepared in bidistilled water. All UV/Vis spectra were recorded on a Lambda 35 spectrometer (Perkin Elmer). Electrospray-ionisation MS data were acquired on a Q-ToF Ultima mass spectrometer (Waters) fitted with a standard Z-spray ion source and operated in the negative ionization mode. Experimental parameters were set as follows: capillary voltage: 3 kV, sample cone: 50 V, source temperature: 80 °C, desolvation temperature: 200 °C, acquisition window: m/z 50-1000 in 1 s. 5 µL of the sample was introduced into the mass spectrometer by infusion at a flow rate of 20 µL min⁻¹ with a solution of ACN–H₂O 50 : 50 (v/v). External calibration was carried out with a solution of phosphoric acid at 0.01%. Data were processed using the MassLynx 4.1 software.

Dye screening

For each dye, UV/Vis spectra of the free dye and the metal–dye combinations (final conc.: [dye]₀ = 50 µM; [M⁺]₀ = 0.1 mM) in buffered aqueous solution (CHES, 0.1 M, pH 8.4) were recorded after standing for 1 h at room temperature. The following dyes were studied and not considered for further investigation due to precipitation with either one of the metal ions: Azophloxine, Methylene Blue, Naphthol Blue Black. Orange G and Pyrocatechol Violet were not suitable, as they decompose in the presence of one of the metal ions. Methyl Orange and Methyl Red do not display sufficient binding affinity towards the metal ions and Alizarin Red S, Mordant Blue 9 and Mordant Blue 13 gave weak signals, when tested in a dynamic combinatorial library.

Spectrophotometric titrations

For titrations of Xylenol Orange with CuCl₂·2H₂O and NiCl₂·6H₂O, two series of measurements were performed: one in which solutions of [M⁺]₀ = 5 mM were added to solutions of [XO]₀ = 7.5 µM (3 mL, + CHES, 0.1 M, pH 8.4, 298 K) in 1 µL increments, and one in which solutions of [M⁺]₀ = 1 mM were added to solutions of [XO]₀ = 15 µM (1.5 mL, + CHES, 0.1 M, pH 8.4, 298 K) in 5 µL increments. For titrations of Arsenazo I with CuCl₂·2H₂O and NiCl₂·6H₂O, solutions of [M⁺]₀ = 10 mM were added to solutions of [Al]₀ = 15 µM (3 mL, + CHES, 0.1 M, pH 8.4, 298 K) in 1 µL increments, whereas for Methyl Calcein Blue, solutions of [M⁺]₀ = 10 mM were added to solutions of [MCB]₀ = 25 µM (3 mL, + CHES, 0.1 M, pH 8.4, 298 K) in 1 µL increments. After each addition, solutions were equilibrated for 10 min at 298 K before measuring the absorption spectra in the range 300–700 nm (XO and Al) and 300–500 nm (MCB). The speciation models described in the main text were fitted to the spectral data using an in-house built routine in MATLAB®, which implements evolving factor analysis and a Newton–Gauss multi-non-linear least squares fitting algorithm. For XO, the speciation models were fitted to the data from both series of measurements (for each metal) in a global multi non-linear least-squares fit. In all cases the fits converged with sum-of-squared residuals of < 0.005, indicating that the data was excellently modeled. Complete spectral overlays, calculated spectra and speciation diagrams for all titrations are shown in the ESI. The standard deviations for the fitted parameters (listed in parentheses in Table 1) were estimated using the sum-of-squared residuals and inverted curvature matrices as obtained from the iterative fitting processes (see ref. 13 for details).

Mass spectrometric titrations

Samples were prepared by mixing appropriate amounts of stock solutions of the dye [dye]₀ = 0.1 mM) and the metal salt (0.05 ≤ [M⁺]₀ ≤ 0.2 mM) in water. The pH was then adjusted to 8.4 (±0.2) using NaOH (0.01 M).

Mass spectrometry of the DCL

For sample preparation appropriate amounts of stock solutions of the dyes and the metal salts were mixed to give final concentrations of: [MCB]₀ = 50 µM, [Al]₀ = 25 mM and [XO]₀ = 12.5 µM; [Ni⁺]₀ = [Cu²⁺]₀ = 0.1 mM. NaOH (0.01 M) was then added to adjust the pH to 8.4 (±0.2).

Sensing experiments

The DCL sensors were prepared by mixing appropriate amounts of stock solutions containing the dyes, the metal salts and the buffer. After addition of either one peptide or a mixture of both peptides, UV/Vis spectra were recorded at t = 2, 5, 10, 20, 40, 60 min and 24 h or t = 2, 5, 10 min and 24 h. Each series of measurements was repeated five or six times. The final concentrations were: [MCB]₀ = 50 µM, [Al]₀ = 25 mM and [XO]₀ = 12.5 µM, [Ni⁺]₀ = [Cu²⁺]₀ = 0.1 mM, and [CHES]₀ = 0.1 M. The peptide concentration was varied in 0.1 eq. increments with [angiotensin I]₀ = (20 – x) µM and [angiotensin II]₀ = x µM. The data was analyzed with the commercially available statistics program SYSTAT (version 11.0) by a linear discriminant analysis algorithm.

Acknowledgements

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References

17 Different dye concentrations were chosen to account for differences in the absorption coefficient.
19 A standard jackknifed validation routine, in which one measurement at a time was randomly omitted was performed: 70% of the measurements were classified correctly.
20 82% of the measurements were classified correctly in a jackknifed validation routine.