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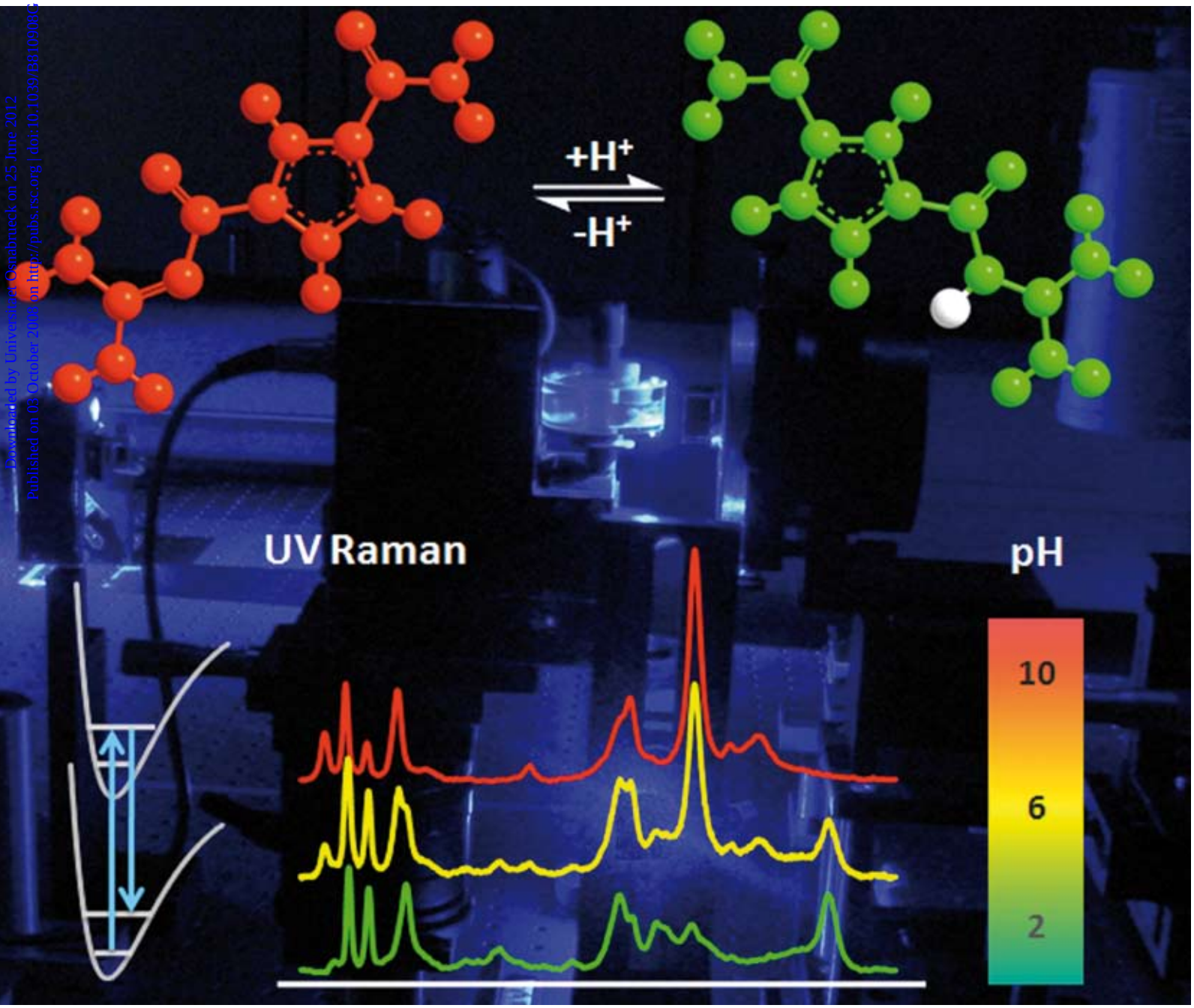

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Characterization of guanidiniocarbonyl pyrroles in water by pH-dependent UV Raman spectroscopy and component analysis†

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Guanidiniocarbonyl pyrroles are artificial receptors for the efficient complexation of carboxylates even in polar solvents such as water. Their carboxylate binding site (CBS) exhibits an electronic absorption maximum at ~298 nm and can be probed selectively by ultraviolet resonance Raman (UV RR) scattering. We present a pH-dependent UV RR spectroscopic investigation of two guanidiniocarbonyl pyrroles in water: the model receptor CBS-NH_2 and the peptide receptor CBS-Lys-Lys-Phe-NH_2. UV RR spectra of 1 mM aqueous solutions with 275 nm laser excitation were recorded between pH 6 and 7. Within this small pH range near the pK_a of 6.4, protonated and neutral CBS species are simultaneously present at similar concentrations (acid/base equilibrium). Using non-negative matrix factorization (NMF), the individual UV RR component spectra of these distinct CBS species were determined without any *a priori* knowledge. The pH-dependent UV RR spectra of the small model receptor CBS-NH_2 and the larger peptide receptor CBS-Lys-Lys-Phe-NH_2 can both be described as linear combinations of only two components. Control experiments at pH 2 and pH 10 show an excellent agreement with the derived NMF component spectra and confirm their assignment to the protonated and neutral CBS species, respectively.

Introduction

Artificial receptors that selectively bind to a given peptide sequence are ideally suited for studying the underlying principles of molecular recognition.[1] Schmuck and co-workers have developed synthetic receptors based on guanidiniocarbonyl pyrroles (Fig. 1A right), which are highly efficient in the complexation of carboxylates even in polar solvents such as water.[2] The guanidiniocarbonyl pyrrole moiety of the receptor is therefore called carboxylate binding site (CBS). In the case of protein recognition, the CBS binds to the C-terminus of the substrate and selectivity towards a given peptide sequence is achieved by a tripeptide part attached to the CBS (Fig. 1A left). The protonation state of the CBS unit in the receptor (Fig. 1B) is important for the efficient complexation of peptides. Specifically, the strong electrostatic interaction between the protonated form of the CBS moiety in the receptor (guanidinium ion, Fig. 1B left) and the negatively charged C-terminus of the tetrapeptide (carboxylate, not shown) as well as additional hydrogen bonds stabilize the receptor–peptide complex.

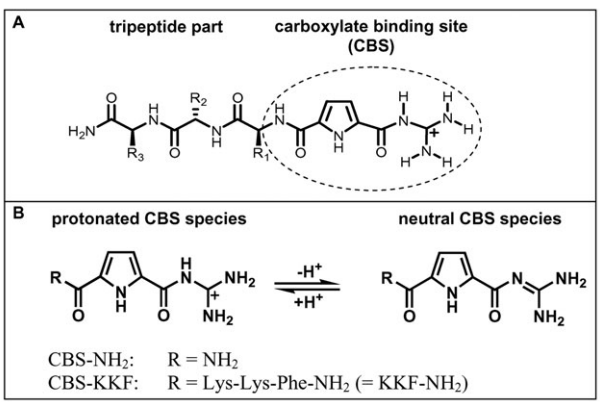

Fig. 1 (A) Guanidiniocarbonyl pyrroles are artificial peptide receptors. They contain a carboxylate binding site (CBS) and a tripeptide part for sequence selectivity; (B) acid–base equilibrium between a protonated and neutral form of the CBS subunit in the receptor.

Raman spectroscopic techniques can probe both structure and dynamics of hydrogen-bonded mixtures.[3] In particular, UV resonance Raman spectroscopy is capable of monitoring the complexation between CBS-based receptors and tetrapeptides without the need of external labels.[4] The quantitative interpretation of the spectral changes observed upon complexation requires a detailed characterization of the receptor itself. In order to determine the individual spectral contributions from the protonated and neutral form of the CBS in Fig. 1B, a pH-dependent UV Raman study is presented here. The recorded spectra contain characteristic signals from both CBS species in different proportions, depending on the corresponding pH.

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† Electronic supplementary information (ESI) available: pH titration curve for CBS–NH_2 (Fig. S1); pH titration curve for CBS–KKF–NH_2 (Fig. S2); UV Raman spectra of CBS–NH_2 (Fig. S3); NMR titration results for CBS–NH_2 (Fig. S4). See DOI: 10.1039/b810908g

Various methods for determining the distinct contributions of different chemical compounds in mixtures are available.[5–7] In the case of nonlinear least square regression,[5] the individual component spectra must be known in order to determine their relative contributions. In contrast, independent component analysis (ICA)[6,7] is a blind approach, which does not require *a priori* knowledge on the component spectra. The problem with ICA is, however, the assumption of independence among the component spectra, which in many cases is not realistic and often too constrained. A method called non-negative matrix factorization (NMF)[8–10] developed by Lee *et al.*[8] can be used for extracting the component spectra when the corresponding species are not chemically independent, *e.g.*, because they are in a dynamic chemical equilibrium. This approach treats the observed spectra as a positive linear combination of the individual signals from several components. In contrast to ICA, the non-negative constraints make the representation completely additive and more meaningful with respect to spectroscopic applications. NMF does not impose the condition of independence unlike ICA, but it is also based on matrix factorization and has been shown to be very useful for the decomposition of multivariate data.[8,11] Here we demonstrate the application of NMF to pH-dependent UV Raman data for determining the pure component spectra of the two CBS species in Fig. 1B. These component spectra are required for interpreting the experimentally detected UV Raman spectra of guanidiniocarbonyl pyrroles in water.

Experimental details

Sample preparation

The artificial receptor CBS-KKF-NH_2 and the model receptor CBS-NH_2 were synthesized according to procedures described elsewhere.[2] The compounds were used after purification by MPLC. Aqueous solutions (1 mM) of CBS-NH_2 and CBS-KKF-NH_2, respectively, were used for UV Raman spectroscopic measurements. The pK_a values of CBS-NH_2 and CBS-KKF-NH_2 were determined by pH titration. The pH of the solution was adjusted using 0.1 N HCl and 0.1 N NaOH.

Raman spectroscopy

The 275 nm line from an argon ion laser (Spectra Physics, model BeamLok 2085) was used for the UV RR measurements based on our previous studies.[4] The laser power at the sample was ~50 mW. Raman spectra were recorded employing a 90° scattering geometry, using a rotating quartz cuvette in order to minimize photochemical decomposition. The scattered light was focused on the entrance slit of a double monochromator (Spex, model 1404 with 2400 grooves mm^{-1} holographic gratings) and detected with a liquid nitrogen cooled CCD camera (Photometrics, model SDS 9000). Raman spectra were acquired with the scanning multichannel technique employing a two-fold spectral overlap. The integration time was ~3–4 min per spectral window. The Raman spectra of dimethylsulfoxide as a standard were recorded for wavenumber calibration. Sample integrity was checked before and after the UV RR experiments by absorption spectroscopy in the UV-Vis region.

Results and discussions

Two guanidiniocarbonyl pyrroles were characterized by UV resonance Raman spectroscopy: the model receptor CBS-NH_2 containing only the CBS chromophor (R = NH_2 in Fig. 1B) and the large receptor CBS-KKF-NH_2 with an additional tripeptide part (R = Lys-Lys-Phe-NH_2 in Fig. 1B). Optimal binding efficiencies to tetrapeptide substrates can be observed at pH ~6 because of the strong electrostatic interaction between the protonated CBS form (Fig. 1B left) and the carboxylate at the C-terminus of the tetrapeptide substrate. The CBS subunit in both molecules has a pK_a of ~6.4 as determined from pH titration experiments (see ESI, Fig. S1).†

Application of the Henderson–Hasselbalch equation to the chemical equilibrium in Fig. 1B leads to:

$$\mathrm{pH} \approx 6.4 + \log \frac{[\text{neutral CBS species}]}{[\text{protonated CBS species}]}. \qquad (1)$$

From eqn (1) it is evident that in the pH range ~6–7, both CBS species are present in non-negligible concentrations and therefore contribute to the corresponding Raman spectrum. Applying the NMF method to pH-dependent Raman species, however, should allow to determine the component/species spectra without *a priori* knowledge of their individual spectral contributions. Because the peptide receptor CBS-KKF-NH_2 contains additional charged amino acid side chains (R = Lys-Lys-Phe-NH_2 in Fig. 1B), we started our investigation with the small model receptor CBS-NH_2 (R = NH_2 in Fig. 1B). Our hypothesis was that the UV Raman spectrum of CBS-NH_2 near the pK_a of ~6.4 can be described as a linear combination of two component spectra.

Characterization of the model receptor CBS-NH_2

UV resonance Raman spectroscopy. UV resonance Raman experiments on the model receptor CBS-NH_2 were performed in the wavenumber region 900–1800 cm^{-1} at pH = 6.85, 6.37 and 5.89 (Fig. 2A). According to eqn (1), this corresponds to a ratio of 1 : 3, 1 : 1 and 3 : 1 between the neutral and the protonated CBS species. The spectral changes in the pH-dependent Raman spectra clearly indicate that at least two different CBS species are present in solution. The Raman bands at ~1050, 1200 and 1450 cm^{-1} show significant changes in the relative intensities upon pH change, whereas the Raman band at ~1700 cm^{-1} becomes weak in intensity and tends to disappear at high pH as the Raman band at ~1496 cm^{-1} gains intensity with increasing pH. Our explanation is that the observed spectral differences are due to the different ratio of protonated to neutral CBS species (*cf.* eqn (1)). In order to confirm this interpretation, the pH-dependent Raman spectra must be decomposed.

Non-negative matrix factorization. Non-negative matrix factorization (NMF) is a method to find non-negative matrix factors W and H for a given non-negative matrix X such that:

$$X \approx W \cdot H, \qquad (2)$$

where the matrix W contains the components and their Raman spectra, while the matrix H contains the linear coefficients of the components (see Fig. 2B). In order to determine the individual

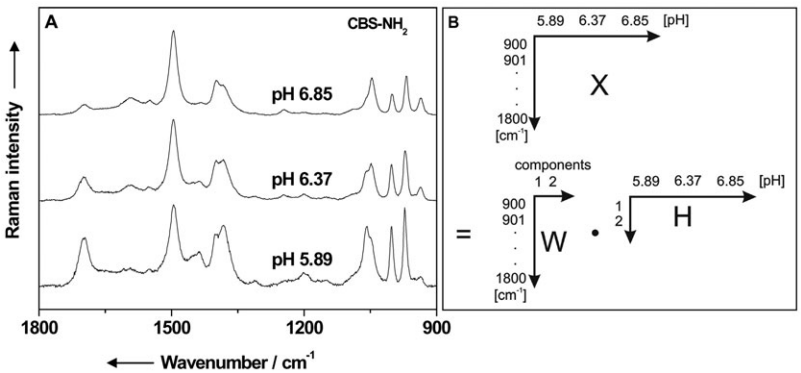

Fig. 2 (A) UV Raman spectra of CBS-NH_2 (1 mM in water) at pH 5.89, 6.37 and 6.85; (B) Decomposition of the pH-dependent Raman spectra (matrix X) into two component spectra (matrix W) and their relative contributions (matrix H) using non-negative matrix factorization (NMF).

spectral contributions of the protonated and neutral CBS species, the pH-dependent UV Raman spectra of CBS-NH_2 in Fig. 2A were analyzed by NMF using two components. The results of the NMF analysis for the Raman spectra of CBS-NH_2 at pH = 5.89, 6.37 and 6.85 are shown in Fig. 3(A)–(C). At pH 6.85 (Fig. 3A) the ratio of neutral to protonated CBS species is ~3 : 1 (eqn (1)).[12] The dominant component (hollow circles) with intense spectral contributions at 1046, 1398 and 1496 cm^{-1} is therefore assigned to the neutral CBS species. The minority component (dotted line) in Fig. 3A with spectral contributions at 1059, 1382, 1438 and 1699 cm^{-1} is due to the protonated CBS species. The characteristic Raman band at 1699 cm^{-1} is assigned to an 'amide I-like' vibration of the guanidiniocarbonyl.[4] With decreasing pH the relative contribution of the protonated species increases. At pH 6.37 (Fig. 3B) the ratio of neutral to protonated species is ~1 : 1 (eqn (1)) and both components contribute with nearly equal intensity at 1398 and 1383 cm^{-1}.[12] At pH 5.89 (Fig. 3C) the protonated species (dotted line) dominates and the ratio of neutral to protonated species is ~1 : 3 (eqn (1)).[12] The overall agreement between the sum of the component spectra (solid circle) and the experimental UV RR spectra (solid line) is very good (error of 0.02–0.10% for CBS-NH_2 and of 0.08–0.35% for CBS-KKF-NH_2).[13] This confirms the hypothesis that the pH-dependence is due to the presence of the CBS acid/base equilibrium (Fig. 1B). We have tested the validity of this two component model also for a larger pH range (see ESI, Fig. S3).†

It is important to keep the general applicability of the presented approach in mind: Raman spectra of (known or unknown) components can be determined without any *a priori* knowledge of their individual spectral contributions by the application of NMF to a minimal number of spectra recorded from mixtures. Although in our particular case we had the chance to test the validity of the two NMF component spectra by control experiments at pH 2 and 10, this is usually not possible. Many samples, for example proteins, do not tolerate extreme pH values (or extreme conditions in general) so that the corresponding component spectrum is not directly accessible. Binding studies in which increasing equivalents of substrate are added to the receptor at a certain pH, are a different example. Here, a new species—the receptor/substrate complex—is formed, but its individual spectral contributions are unknown and not directly accessible.

In order to test the reliability of the NMF approach, also UV Raman spectra at pH 2 and 10 were recorded. At pH 2 only the protonated CBS species is present, whereas at pH 10 the neutral CBS species dominates. Fig. 4 shows that the agreement between the two NMF-derived component spectra and the experimental Raman spectra at pH 2 and 10 is excellent. Thus, we have demonstrated that NMF is a reliable method for determining the Raman spectra of individual components present in a fast dynamic equilibrium in aqueous solution without any *a priori* knowledge.

In contrast to UV RR scattering, the application of pH-dependent ^{1}H-NMR spectroscopy to aqueous solutions of CBS-NH_2 was not successful: because of the relatively slow NMR time scale, the coalescence of proton shift signals is observed (see ESI, Fig. S4).† In addition to the UV RR and NMR data, also pH-dependent electronic absorption spectra of CBS-NH_2 were recorded. They exhibit only minor differences near the electronic absorption maximum at 298 nm (see ESI, Fig. S5).†

The next step after the NMF analysis of the UV RR spectra for CBS-NH_2 was then to test whether a two compound model can also be applied to the larger system CBS-KKF-NH_2 (Fig. 1A).

Characterization of the peptide receptor CBS-KKF-NH_2

UV resonance Raman spectroscopy. UV resonance Raman spectra of the receptor CBS-KKF-NH_2 in the wavenumber range 900–1800 cm^{-1} were recorded at different pH values near the pK_a of ~6.4. The UV Raman spectra obtained from 1 mM solutions of CBS-KKF-NH_2 in water at pH 7.0, 6.6 and 5.9 are shown in Fig. 5 (for pH titration, see ESI, Fig. S1B).† Similar to CBS-NH_2, significant spectral changes upon lowering the pH are observed. The intensity of the characteristic 'amide I-like' band at 1699 cm^{-1} is increasing, which we attribute to the increasing concentration of the protonated CBS species at lower pH. Also the regions ~1350–1500 cm^{-1} and ~900–1100 cm^{-1} exhibit pronounced and systematic changes in relative intensities. Because the CBS moiety is selectively monitored by using 275 nm laser excitation, we suggest that the observed spectral changes are due to the protonation state of the guanidiniocarbonyl pyrrole subunit of the entire receptor CBS-KKF-NH_2 (Fig. 1A and B).

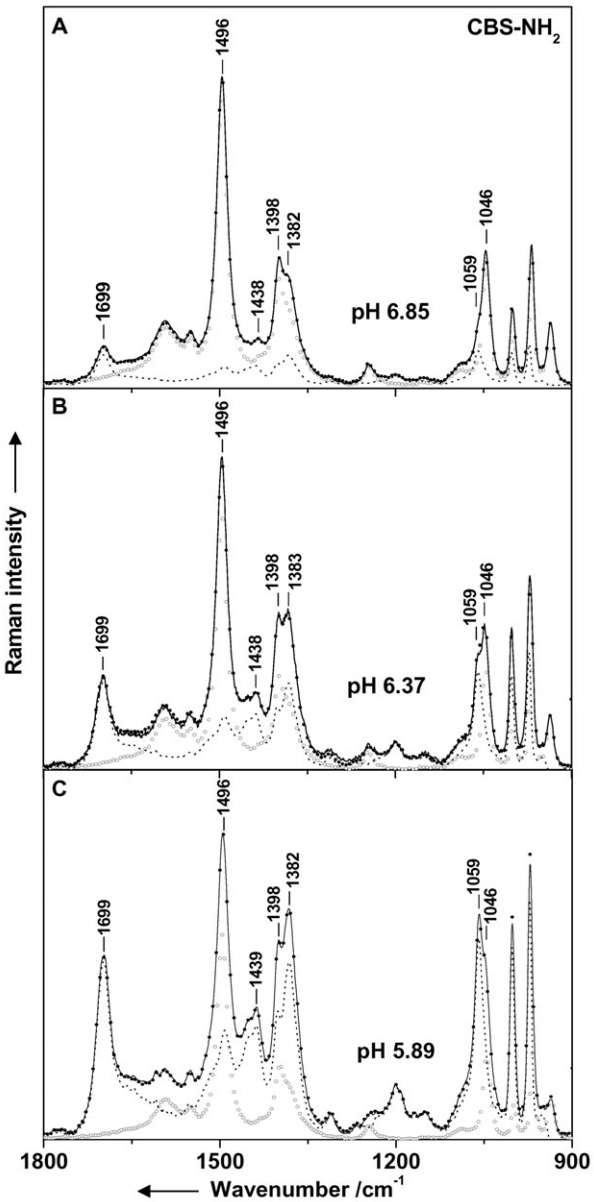

Fig. 3 NMF analysis of the pH-dependent UV Raman spectra of CBS-NH_2: (A) pH 6.85, (B) pH 6.37 and (C) pH 5.89. Component spectra from the neutral species (hollow circles), the protonated species (dotted lines) and their sum (solid circles) together with experimental spectra (solid lines) are shown.

According to eqn (1), pH 7.0, 6.6 and 5.9 correspond to ratios of 4 : 1, 3 : 2 and 1 : 3, respectively, for the neutral and protonated CBS species of CBS-KKF-NH_2. A more detailed analysis, however, requires the decomposition of the experimentally detected UV RR spectra into component contributions.

Non-negative matrix factorization. The pH-dependent UV Raman spectra of CBS-KKF-NH_2 were analyzed by NMF, assuming that also in this case, similar to CBS-NH_2, only two components contribute. The assumption of only two components for the receptor CBS-KKF-NH_2, which contains an additional tripeptide part compared with CBS-NH_2, is based

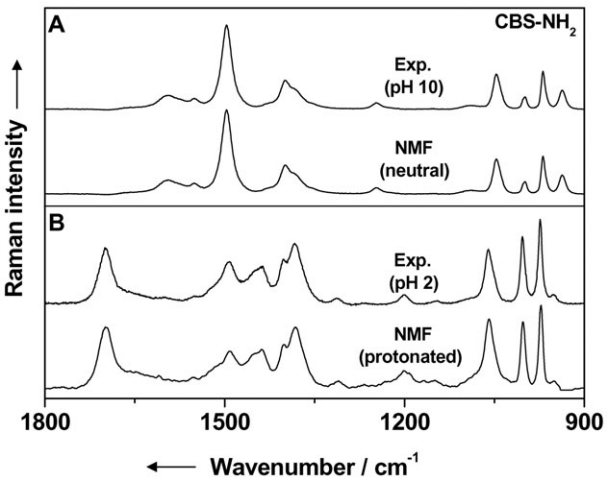

Fig. 4 (A) Experimental UV Raman spectrum of CBS-NH_2 at pH 10 and the NMF-derived component spectrum assigned to the protonated CBS species; (B) experimental UV Raman spectrum of CBS-NH_2 at pH 2 and the NMF-derived component spectrum assigned to the neutral CBS species.

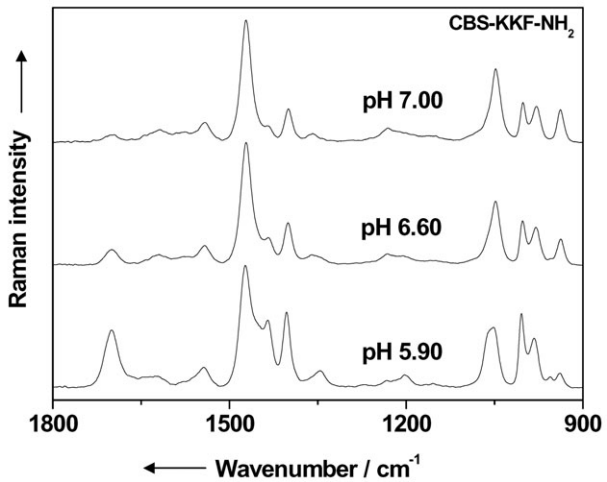

Fig. 5 UV Raman spectra of CBS-KKF-NH_2 (1 mM in water) at pH 5.90, 6.60 and 7.00.

on the selective excitation of the CBS moiety by 275 nm laser light: this leads to the resonance enhancement of Raman bands mainly from the CBS chromophore.[5] The results of the NMF analysis for CBS-KKF-NH_2 are shown in Fig. 6A–C. At pH 7.0 (Fig. 6A) the ratio of the neutral to the protonated CBS species of CBS-KKF-NH_2 is ~3 : 1 (eqn (1)). The dominant component (hollow circles) with intense spectral contributions at 1048, 1399 and 1472 cm^{-1} is therefore assigned to the neutral CBS species of CBS-KKF-NH_2. The second component (dotted line) with spectral contributions at 1060, 1403, 1434 and 1699 cm^{-1} is assigned to the protonated CBS species of CBS-KKF-NH_2. The wavenumber position of the characteristic 'amide I-like' Raman band at 1699 cm^{-1} (dotted line) is the same as for CBS-NH_2 (Fig. 3), while the other peak positions slightly differ. At pH 6.6 (Fig. 6B) the ratio of neutral to protonated species is ~3 : 2 (eqn (1)) and both components contribute with nearly equal

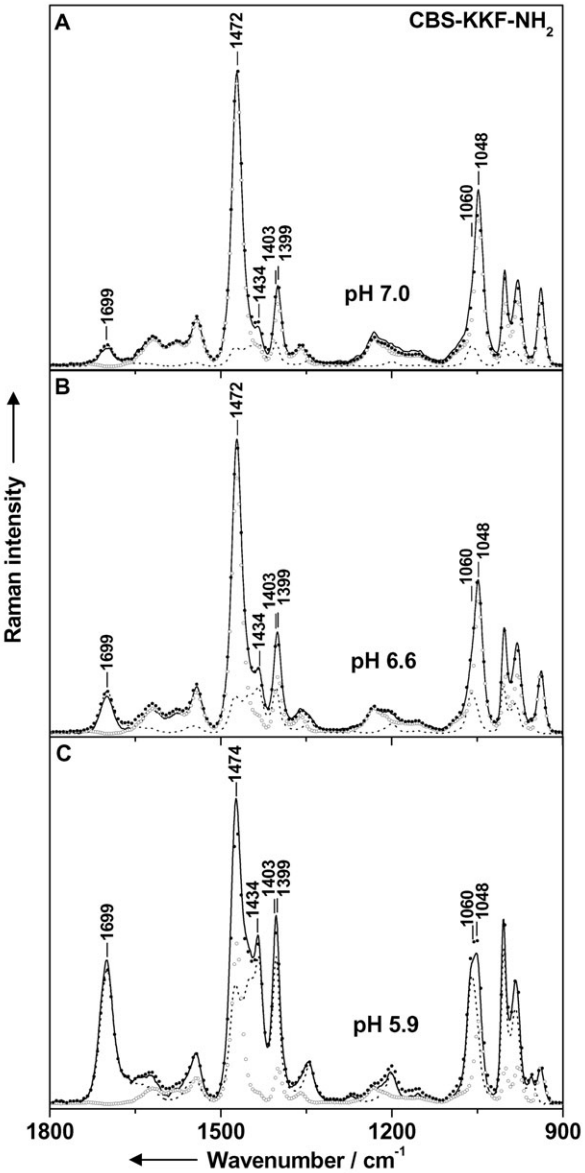

Fig. 6 NMF analysis of the pH-dependent UV Raman spectra of CBS-KKF-NH_2: (A) pH 7.0, (B) pH 6.6 and (C) pH 5.9. Component spectra from the neutral species (hollow circles), the protonated species (dotted lines) and their sum (solid circles) together with experimental spectra (solid lines) are shown.

intensities at 1399 and 1403 cm^{-1}, but they are spectrally less separated compared to CBS-NH_2. At pH 5.9 (Fig. 6C) the protonated species (dotted lines) dominates and the ratio of neutral to protonated species is $\sim$1 : 4 (eqn (1)). The agreement between the sum of the NMF-derived component spectra (solid circles) and the experimental UV RR spectra (solid lines) is also very good. This confirms that the assumption of two components is also valid for the larger system CBS-KKF-NH_2. Additionally, the NMF-derived component spectra are compared with UV RR spectra recorded at pH 2 and 10 (Fig. 7). These control experiments show that the two NMF component spectra can be assigned to the neutral and protonated CBS species of CBS-KKF-NH_2, respectively (Fig. 1B).

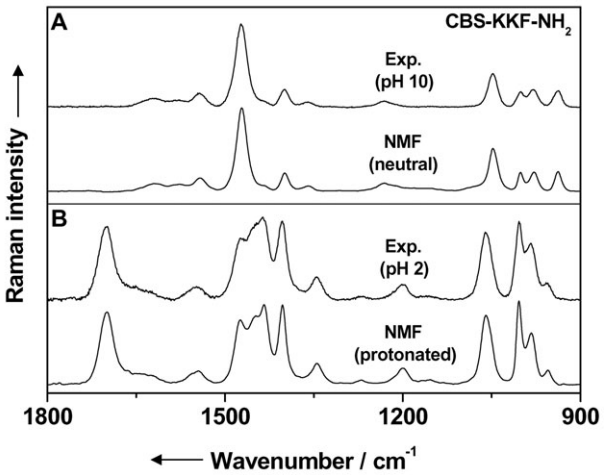

Fig. 7 (A) Experimental UV Raman spectrum of CBS-KKF-NH_2 at pH 10 and the NMF-derived component spectrum assigned to the protonated CBS species; (B) Experimental UV Raman spectrum of CBS-KKF-NH_2 at pH 2 and the NMF-derived component spectrum assigned to the neutral CBS-species.

Conclusions

UV RR spectroscopy has been used to selectively probe the CBS moiety in two guanidiniocarbonyl pyrroles at 1 mM concentrations in water. In order to determine the individual spectral contributions from the protonated and neutral CBS species, pH-dependent Raman studies on the small model receptor CBS-NH_2 and the larger peptide receptor CBS-KKF-NH_2 were performed. The corresponding component spectra were obtained using non-negative matrix factorization (NMF), *i.e.* without any *a priori* knowledge. For both CBS-NH_2 and CBS-KKF-NH_2, the assumption of two components is valid. The NMF-derived component spectra exhibit an excellent agreement with experimental UV Raman spectra obtained at pH 2 and 10. These control experiments clearly demonstrate the applicability of the NMF method in the case of a dynamic chemical equilibrium. Future studies will focus on the normal mode assignment of the NMF component spectra using quantum chemical calculations. Additionally, UV RR binding studies with tetrapeptides will be performed in order to determine the spectral contributions of the receptor–substrate complex using NMF.

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12 The ratio between the neutral and the protonated CBS species of CBS-NH_2 at pH 6.85 and 5.89 was also determined experimentally, using the UV Raman spectrum recorded at pH = pK_a (1 : 1 ratio) as a reference. From the intensities of the 1699 cm^{-1} Raman band (protonated CBS species) to the 1496 cm^{-1} Raman band (neutral CBS species), a ratio of 1 : 3.2 at pH 6.85 and 2.6 : 1 at pH 5.89 was determined. This is in good agreement with the theoretically expected values of 1 : 3 and 3 : 1, respectively, which are predicted from the Henderson–Hasselbalch equation (eqn (1)).
13 The percentage error was calculated by using the following formula:

$$\frac{\sum_{\tilde{\nu}=900\,\mathrm{cm}^{-1}}^{\tilde{\nu}=1800\,\mathrm{cm}^{-1}} [I_{\mathrm{exp}}(\tilde{\nu}) - I_{\mathrm{sum}}(\tilde{\nu})]^2}{\sum_{\tilde{\nu}=900\,\mathrm{cm}^{-1}}^{\tilde{\nu}=1800\,\mathrm{cm}^{-1}} I_{\mathrm{exp}}(\tilde{\nu})} \times 100$$