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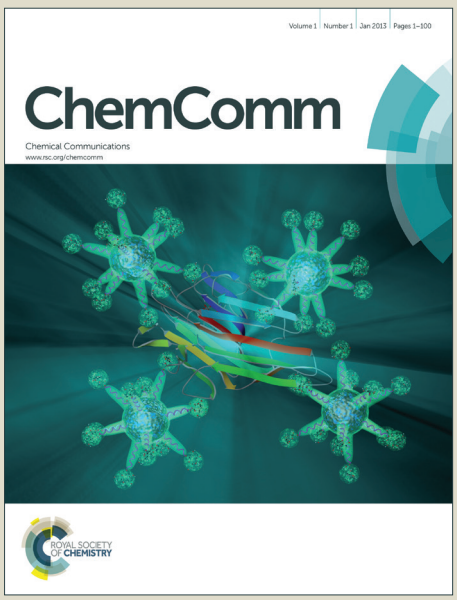

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ARTICLE TYPE

Demixing of Water and Ethanol Causes Conformational Redistribution and Gelation of the Cationic GAG Tripeptide

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The cationic tripeptide GAG undergoes three conformational changes in binary mixtures of water and ethanol. At 17 mol% of ethanol conformational sampling is shifted from pPII towards β-strands. A more pronounced shift in the same direction occurs at 40 mol%. At ca. 55 mol% of ethanol and above a peptide concentration of ca. 0.2 M the ternary peptide-water-ethanol mixture forms a hydrogel which is comprised of unusually large crystalline like non-β sheet fibrils forming a sample spanning matrix.

The random coil model suggests that amino acid residues in unfoldable peptides sample the entire sterically and electrostatically allowed space of the Ramachandran plot.[1, 2] However, results from recent examinations of short peptides in water and of coil libraries suggest that conformational distributions deviate from random coil prediction.[3-5] For alanine, they suggest a very high fraction (i.e. 0.72) of polyproline II (pPII)-like conformations in both, unblocked GAG and the canonical alanine dipeptide in aqueous solution,[6, 7] and an even higher value for trialanine (0.84-0.9).[7, 8] Multiple lines of experimental evidence suggest that pPII is enthalpically stabilized by peptide/protein–water interactions,[9-13] but the role of hydration has thus far been investigated mostly by computational means.[14-18] Here, we assess the influence of the solvent on the conformational distribution of cationic GAG by utilizing ethanol as amphiphilic co-solvent.[19-21] Such co-solvents can interact with an unfolded peptide (and proteins) indirectly by affecting backbone hydration and directly by interacting with functional backbone groups and side chains alike.[22-27] In the peptide concentration regime chosen for this study ethanol can be expected to function as a crowding agent reagent for the peptide,[28] since it produces a much larger excluded volume effect than water (the volume fraction reaches 0.8 at the highest chosen ethanol concentration). We investigated GAG in different ethanol-water mixtures by combining ^{1}H NMR, vibrational and circular dichroism (VCD) spectroscopy. IR spectra of the solvent mixture were used to obtain reorganizations of the bulk liquid and corresponding deviations from ideal behaviour. Details of our Material and Methods are given in the Supporting Information.

Figure 1 depicts the $^3J(H^NH^\alpha)$ coupling constant of the N-terminal alanine proton as a function of ethanol mole fraction, χ_{ET}, measured at the indicated temperatures. This observable is an indicator of the average φ-angle of the conformational manifold sampled by the alanine residue.[29-31] The data reveal a highly non-linear and non-monotonous relationship between $^3J(H^NH^\alpha)$ and χ_{ET}. Three regions of the plot are noteworthy. At very low χ_{ET} (0.01-0.02) a rather sharp maximum appears which grows with increasing temperature (region 1). A small but sharp increase of $^3J(H^NH^\alpha)$ by ca. 0.1 Hz occurs between χ_{ET} values of 0.12 and 0.15 (region 2). At ca. 0.4 (region 3), the data indicate a more pronounced increase of the coupling constant by ca. 0.22 Hz.

The observed changes of the $^3J(H^NH^\alpha)$ are not attributable to any type of peptide aggregation that one might suspect to occur at the rather high peptide concentration (0.1 M) chosen for our experiment to optimize the signal to noise, a necessity for measurements at higher temperatures. As shown in Figures S1 and S2, $^3J(H^NH^\alpha)$ of GAG in pure water and in ethanol/water mixtures of 0.14/0.86 mol% (region 2) and 0.48/0.52 mol% (past region 3) are within their statistical uncertainties independent on the peptide concentration between 10 and 100 mM. This suggests the absence of any structural change in this region. To ensure that peptides has not aggregated even at 10 mM, we measured the UVCD spectra of 10 mM GAG in a solution with χ_{ET}=0.48 ethanol as a function of temperature. The spectra in Figure S3 are clearly indicative of a monomeric pPII/β-strand mixture with the β-content increasing with rising temperatures.[13]

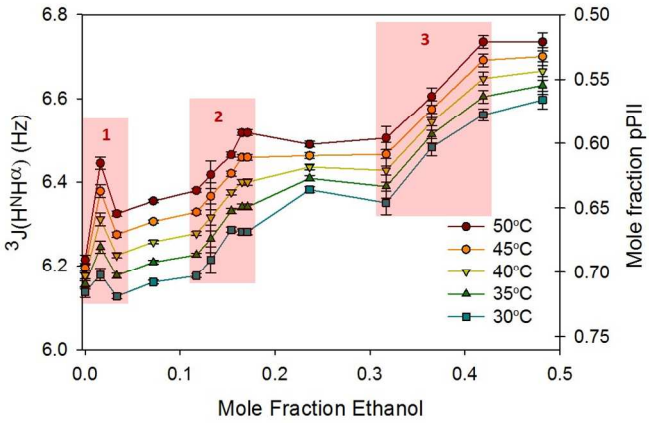

Figure 1. $^3J(H^NH^\alpha)$ of the N-terminal amide proton of cationic GAG in different water/ethanol mixtures determined from ^{1}H NMR spectra taken at the indicated temperatures. Three notable regions are highlighted and labelled accordingly.

The increases of $^3J(H^NH^\alpha)$ with χ_{ET} can be indicative of a population redistribution from pPII to β-strand or of a shift of the pPII distribution towards more negative values of φ.[6] Based on

arguments presented in the Supporting Information (Figure S4 and text) we think that the observed changes of the coupling constant are predominantly caused by conformational redistribution. Thus, we map our $^3J(H^NH^\alpha)$ onto the mole fraction of pPII as depicted in Figure 1. The data suggest that the pPII content of the alanine distribution is reduced to 78% of its value in water at χ_{ET} of ca. 0.5 and room temperature.

In order to explore whether the obtained conformational redistribution of GAG reflect changes of the solvent organization, we measured and analysed the FTIR spectra of several mixtures of ethanol/D_2O mixtures in the region between 1100 and 1500 cm^{-1}. The co-solvent system of water and ethanol is known to be not ideal and that the formation of micro-domains of ethanol can start at rather low mole fractions of this binary mixture.[25, 32] The spectra shown in Figure S5 contain several bands assignable to CH bending type modes of the co-solvent's hydrophobic tail.[33] Aggregation of ethanol in the mixture should lead to a change of their intrinsic oscillator strength and thus to a departure from Beer-Lambert's law. We decomposed all these spectra into a set of Gaussian bands by using our program Multifit.[34] The same set of spectral parameters was used for fits to all spectra. Figure 2 shows the obtained integrated intensities of selected bands plotted as a function of χ_{ET}. The concentration dependence of the intensities of the bands at 1136 and 1451 cm^{-1} start to deviate from linearity at ca. 0.17, which coincides with the increase of $^3J(H^NH^\alpha)$ in region 2 of Figure 1. Interestingly, another band at 1332 cm^{-1} appears only at an ethanol concentration of ca. 30 mol% and increases steadily with increasing ethanol concentration. Only the intensity of the 1418 cm^{-1} seems to be unaffected by any changes in region 2 in that its increase with increasing ethanol fraction remains linear. All these observation suggests that the underlying structural redistribution of the peptide probed by $^3J(H^NH^\alpha)$ in region 2 correlates with the demixing of the two co-solvents caused by the formation of ethanol clusters due to hydrophobic interactions.[35] At the relatively high peptide concentration used for our experiments, such clusters are very likely to penetrate the hydration shell of the peptide. As shown in Figure S6, these changes of peptide solvation lead to a blue-shift of the amide I' band profile in the IR spectrum. The corresponding VCD (Figure 3) signals exhibit the same behavior. The blue-shift reflects a weakening of hydrogen bonding between peptide and water and/or a partial replacement of peptide-water with peptide-ethanol hydrogen bonds.[36, 37, 38]

The data in Figure 2 do indicate further changes of the ethanol/water mixtures in region 3 of the $^3J(H^NH^\alpha)$-plot in Figure 1. The band at 1332 cm^{-1} suddenly increases its intensity relatively sharply. Another band at 1274 cm^{-1} starts to gain intensity and increases linearly with increasing ethanol concentration. On the contrary, the intensities of bands at 1274 cm^{-1} and 1392 cm^{-1} reach saturation and become nearly ethanol independent above χ_{ET} of ca. 0.5. Concomitant changes in the peptide spectrum are also noteworthy. As shown in Figure S5, the amide I' profile exhibits a further blue-shift of the low-wavenumber band (predominantly assignable to the C-terminal amide I').[39] It merges with the high-wavenumber band (N-terminal amide I') into a single broad band. The VCD blue-shifts as well (Figure 3) and becomes more negatively biased.

Taken together, our spectroscopic data provide evidence for the notion that a reorganisation (demixing) of the solvent probed by deviations from Beer-Lambert's law induces changes in the peptides hydration shell (dehydration due to enrichment with ethanol, probed by amide I'), which involve a redistribution of the peptide's backbone conformations from pPII towards β-strand. These findings underscore the notion that backbone hydration is the key stabilizing factor for pPII, in agreement with many theoretical predictions. Furthermore, they reflect that changes of the bulk solvent configuration are transduced to the peptide's hydration shell.

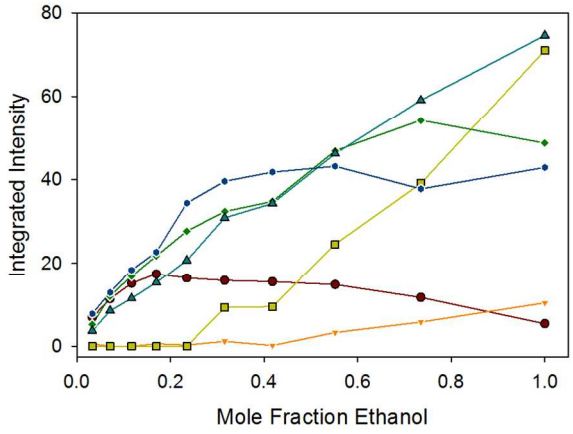

Figure 2. Integrated intensities of bands in the 1100-1500 cm^{-1} region of water/ethanol mixtures plotted as a function of ethanol mole fraction (red ●:1136 cm^{-1}, orange ▼:1274 cm^{-1}, yellow ■:1332 cm^{-1}, green ◆:1392 cm^{-1}, teal ▲:1418 cm^{-1}, blue ●: 1451 cm^{-1}). Solid lines are provided as a guide to the viewer.

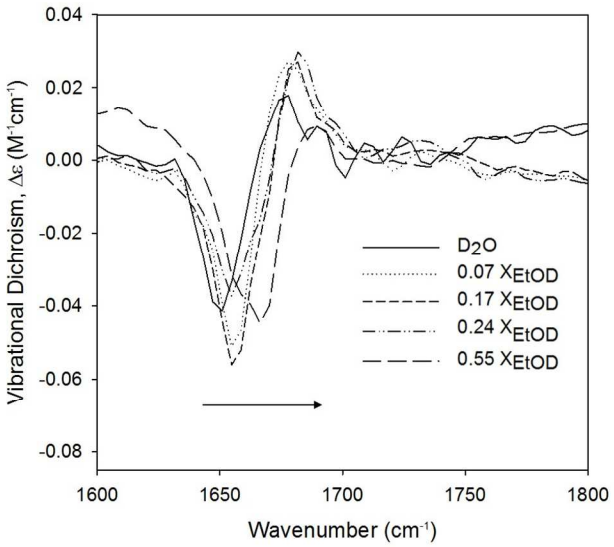

Figure 3. VCD of the Amide I' region of cationic GAG recorded for the indicated D_2O/ethan(ol)-d/ mixtures..

There is a large volume of literature on both binary alcohol/water and ternary (bio)polymer/alcohol water mixtures, which cannot be comprehensively considered in this communication. Microphase separation leading of excess molar and apparent modal volumes owing to interactions between the aliphatic groups of the alcohol co-solvent has been suggested for region 1.[25] For cationic trialanine, Toal et al. found evidence for

hydrophobic peptide-ethanol interactions at very low ethanol concentrations.[27] With regard to region 2, a combined MD/NMR study on the [val5]angiotensin peptide in χ_{ET}=0.14 ethanol/water yielded of a much higher population of β-strand like structures of the peptide's residues than one would expect from their intrinsic propensities in water.[6] Results of his MD simulations suggest a preferential binding of ethanol in the hydration shell, which involves predominantly hydrophobic interactions. [40]

In the remainder of this article we focus on more drastic changes of the investigated ternary mixture for which the changes in region 3 serve as a precursor. They are reflected by the spectral changes in the IR and VCD spectra of three only slightly different concentrations of GAG in a 55/45 mol% mixture ethan(ol-d) and water (i.e. 195.2, 208.8 and 209.5 mM GAG). The IR amide I' bands of the samples are very similar and representative of peptides in region 3. However, the corresponding VCD spectra show dramatic changes (Figure 4). At 195.2 mM we observed an abnormal enhancement of the otherwise nearly negligible rotational strength of the CH_3 symmetric bending mode of alanine. Spectra measured at 208.8 and 209.5 mM GAG, depict a dramatic increase of the amide I' signal, which now exhibits a -+- W shape (+ and - indicate the sign of maxima). The strength of the signal exceeds that observed in the spectra measured at lower χ_{ET} (Figure 3) by more than an order of magnitude. Such amide I' enhancements are assignable to peptide self-aggregation into fibrilar structures.[41, 42] A major change of the peptide's conformation and state is also indicated by the UVCD-spectra of the peptide (Figure S3).

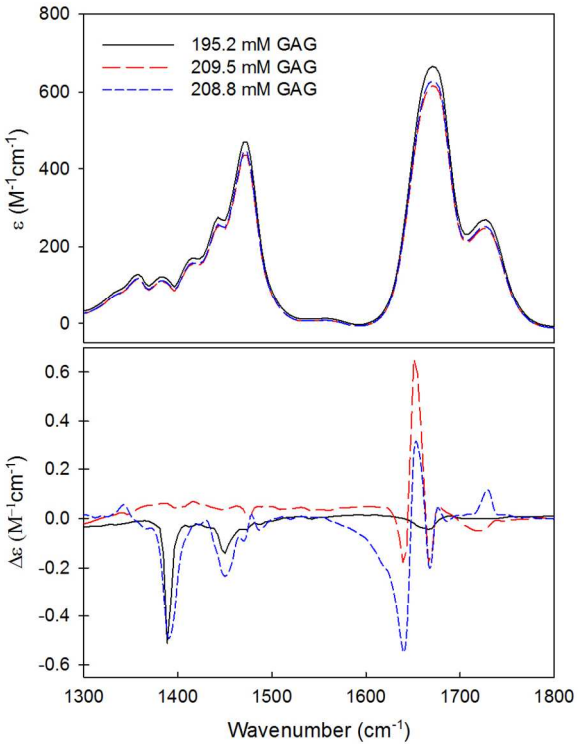

Figure 4. Amide I' IR (upper panel) and VCD (lower panel) band profile of cationic GAG disolved in a 55/45 mol% mixture of ethan(ol)-d and water. The concentrations of GAG are indicated in the figure. The spectrum of the lowest peptide concentration (195 mM, shown as the black line) has an amide I' signal intensity of the same magnitude as the spectra in Figure 3.

The above data clearly indicate peptide/solvent demixing and a self-assembly of the peptide into a supramolecular structure. Indeed, we found that the peptide/ethanol/water mixture with the 209.5 mM peptide concentration formed a hydrogel after a few minutes. The bright-field microscope image in Figure 5 exhibits a cellular structure that resembles a classical sample-spanning network of fibrils found for many hydrogels formed by organic and biological compounds.[43] However, the sub-millimeter length scale of our rather crystalline fibrils is peculiar. Normally, gel forming webs show the same structure on a sub-micrometer scale.[44-47] While the gelation of (bio)polymers in water is a well known phenomenon, the co-solvent induced gelation of such a small (low molecular weight) peptide that does not exhibit the hydrophobicity of the phenylalanine peptides[48] or peptides with other aromatic side chains or end groups,[49] has not yet been reported. The position of the amide I' band in Figure 4 (upper panel) does not suggest any formation of β-sheet-like arrangement, but the rather intense VCD signal is indicative of some long range, possibly helical order.[42] Interestingly, Region 3 corresponds to a mixing range where some solutes (e.g. poly(n-isopropylacrylamide)) are not dissolvable in ethanol/water,[50] even though they can be dissolved in either of the two co-solvents. In our case the solute forms a gel rather than precipitates.

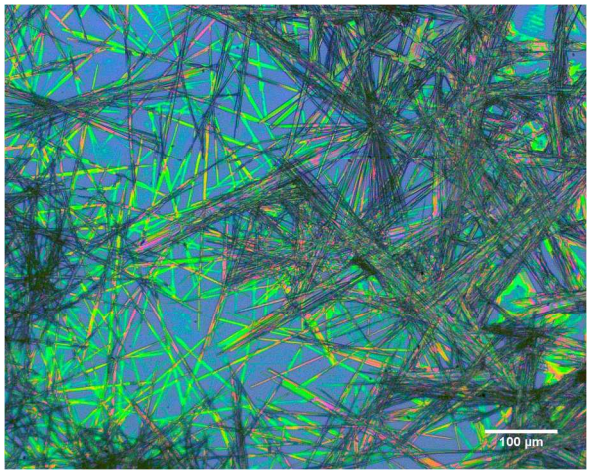

Figure 5. Bright field microscope image of the gel formed by a ternary GAG/water/ethanol mixture with a peptide concentration of 208.9 mM in 55 mol% ethanol.

It is noteworthy in this context that GAG together with long polyalanine stretches are a repeating motifs in silk proteins[51] which to a major extent determine their capability to aggregate into fibers, films and gels. The alanine rich sequences can adopt rather crystalline structures as GAG does in ethanol/water mixtures.[52]

Taken together, the present study demonstrates that unblocked GAG can be utilized as an indicator of reorganization processes in water-ethanol mixtures. This sensitivity stems firstly from the capability of ethanol to substantially increase the sampling of the extended β-strand conformation at the expense of the peptide's pPII propensity and secondly from desolvation induced demixing of the peptide from the solvent and the subsequent gel formation. The properties of the gel, its

dependence on peptide concentration and temperature and it suitability for biotechnological application will be the subject of future investigations.

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