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Hydration structure of trimethylamine *N*-oxide in aqueous solutions revealed by soft X-ray emission spectroscopy and chemometric analysis

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The hydration structure of trimethylamine *N*-oxide (TMAO) in aqueous solutions has been investigated by means of soft X-ray spectroscopy and chemometric analysis. Soft X-ray absorption spectra in the O 1s region have a concentration-dependent shoulder at 533 eV, which is assigned to the $6a_1$ resonance of TMAO. Soft X-ray emission spectra acquired at this resonance comprise both TMAO and water components, with a prominent peak at 525.6 eV which is assigned to the emission caused by the 5e to O 1s transition. An apparent inverse concentration dependence of around 523 eV suggests that the electronic structure of water is modified by the strong interaction with TMAO. Such an effect has been included in the quantitative spectral analysis, called the classical least squares regression method, to obtain information on the hydration structure of the system. The analysis indicates that nine or more water molecules interact with a TMAO molecule. The present method offers a useful technique for probing the solvation structure around the solute which interacts strongly with the solvent.

1. Introduction

Osmolytes are solutes contained in cells of water-stressed organisms to protect living cells from desiccation and shrinking.[1,2] They have different effects on the stability of proteins, causing folding or unfolding. One of such molecules is trimethylamine *N*-oxide (TMAO), which is produced as a metabolite in animals and is especially found in elasmobranchs such as sharks, skates, and rays. The TMAO concentration varies with the habitat depth of the sea animals, presumably as a response to hydrostatic pressure.[3,4] In fact, Krywka *et al.* reported that TMAO significantly stabilizes the folded state of proteins against pressure as well as against temperature-induced unfolding by means of small-angle X-ray scattering experiments.[5]

Numerous studies have been conducted to explain the folding propensity of TMAO. The mechanism of the stabilizing properties is, however, still under debate. Some have suggested that protein folding is induced by unfavorable interactions of TMAO with the protein backbone or side chains.[6–8] Because TMAO is preferentially excluded from the protein surface, the concentration gradient resulting from the depletion of TMAO from the surface may lead to favor the folded state. Other studies have proposed an indirect mechanism where TMAO alters the structure of proximate water, which in turn affects the protein stability.[9–12] Since TMAO has a large dipole moment with a partial negative charge located on the oxygen atom,[13,14] it can modify the water structure and restructure the hydrogen-bond network in its vicinity. Intermolecular interactions between TMAO and water molecules are stronger than those between pure water molecules.[11,14–16] A molecular-level understanding of the interaction of TMAO with water is thus of fundamental importance to clarify the role of TMAO in biological environments.

Many spectroscopic studies have been employed over the past few decades for describing the effects of TMAO on the water structure. They include infrared,[11,12,15,17–26] Raman,[26–29] ultraviolet photoabsorption,[30] and dielectric[17,22,31] spectroscopy as well as Brillouin light scattering experiments.[32] For example, Onori and coworkers reported a slight red shift of the O–H stretching band of water with TMAO concentration using infrared[18] and Raman[27] spectroscopy, suggesting the formation of new hydrogen bonds. Munroe *et al.*[28] measured Raman spectra under similar sample concentrations and confirmed the result. They also suggested that the oxygen atom of TMAO accepts at least three hydrogen bonds from the surrounding water molecules with the aid of density functional theory calculations. Panuszko *et al.* used isotopically labeled water, HDO, and reported that

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water interacting with TMAO forms a more ordered hydrogen-bond network than pure water.[11] Femtosecond two-dimensional infrared spectroscopy showed a significant retardation of water dynamics, correlating strongly with a reduction of the orientational mobility of water.[24] More recent results from terahertz/far-infrared spectroscopy have indicated the formation of TMAO–water aggregates involving three or four water molecules strongly bound to TMAO by hydrogen bonding, and have provided evidence for the local modification of the water structure by changing the TMAO concentration.[26] These studies demonstrate that two types of water molecules can be spectroscopically distinguished in the TMAO aqueous solutions.

There are no reports, to the best of our knowledge, employing soft X-ray spectroscopy to study the interactions of TMAO with water. Soft X-ray spectroscopy offers information on the local atomic environment of a molecular system of interest by excitation of a specific site. In particular, pre-edge absorption spectra comprise primarily dipole-allowed resonance transitions, and are thus highly sensitive to the electronic structure of the system.[33] However, as has been pointed out,[34] their application to aqueous solutions has posed many challenges, because liquids are incompatible with high vacuum and soft X-rays are attenuated strongly in materials. Recent technical developments allow us to measure soft X-ray spectra of liquids at ambient pressure by using a sample cell equipped with a silicon-nitride membrane window(s) as thin as 150 nm:[35–41] the first application to the liquid phase is water.

In this paper, we report soft X-ray absorption and emission spectra of aqueous solutions of TMAO in the O 1s region. Since X-ray spectroscopy is a unique technique for probing the local structure around specific atoms, we expect to obtain some information on the local electronic structure of TMAO interacting with water molecules. The TMAO molecule has a C_{3v} symmetry in the ground electronic state and the valence electron configuration is

$$1a_1^2 2a_1^2 1e^4 3a_1^2 2e^4 4a_1^2 3e^4 1a_2^2 4e^4 5a_1^2 5e^4 6a_1^0,$$

where 5e corresponds to the linear combinations of the outermost doubly-degenerate orbitals, n_x^{O} and n_y^{O}, with a non-bonding character localized on the oxygen atom.[30,42,43] The $5a_1$ orbital has mostly the σ_{NO} character while the unoccupied $6a_1$ orbital is the antibonding counterpart. We describe our experimental procedure in the next section followed by the results. After referring to the characteristic features of the absorption and emission spectra measured for various concentrations of TMAO, we elaborate on the analysis of the emission spectra by means of the classical least squares (CLS) regression method. We introduce and evaluate a new parameter that represents the hydration number of TMAO, and compare the result with other studies. Finally, we summarize the conclusions of this study.

2. Experimental

The experiments were performed at the A3 station on the RIKEN coherent soft X-ray spectroscopy beamline BL17SU[44] in the SPring-8 facility, Japan. Details of the experimental setup have been given elsewhere[39,45] and only a brief description is presented here. Sample solutions were introduced in a cell equipped with a gold-coated silicon carbide membrane (150 nm thickness; NTT Advanced Technology Co., Japan) separating them from the ultra-high vacuum chamber. The solutions were prepared by mixing TMAO dihydrate (with a purity > 97%; Wako Pure Chemical Industries, Ltd, Japan) with water (after purified with Millipore Direct-Q 3UV; Nihon Millipore Ltd, Japan). They were continuously flowed through the cell using a plunger pump (VSP-1050; Tokyo Rikakikai Co., Ltd, Japan) to keep them fresh to avoid measurement on radiation-damaged samples. The molar fractions measured were 0, 0.0052, 0.011, 0.022, and 0.047, corresponding to the molar concentrations of 0, 0.24, 0.51, 1.0, and 2.2 M, respectively. The sample cell was cleaned by pure water and then rinsed with the solution to be measured from run to run. It was installed at an azimuth angle of 45° relative to the direction of the incident beam.

Soft X-ray absorption spectra were measured as total fluorescence yields by using a silicon photodiode (AXUV-100; Opto Diode Corp., USA) facing the observation window of the sample cell. The photon energy scale was calibrated with 4f photoelectrons emitted from a gold foil using the first- and second-order undulator light: the photoelectron spectra were measured using a hemispherical electron-energy analyzer (SES-2002; Gammadata Scienta, Sweden) equipped at the A2 station[46] at the beginning of the experiment. Soft X-ray emission spectra were recorded by using a grazing incidence flat-field spectrometer,[45] which was mounted perpendicular to the direction of the incident soft X-ray beam. The emission energy was calibrated with elastic scattering peaks. The energy resolution for the absorption and emission measurements was set in this study at 0.05 eV and 0.4 eV, respectively.

3. Results

Both soft X-ray absorption and emission spectra of the TMAO solutions show clear concentration dependence. Fig. 1a displays the soft X-ray absorption spectra measured at various molar concentrations of TMAO. One can see two isosbestic points at 531.7 and 533.6 eV. Since the intensity at 533 eV increases with the concentration, 533 eV photons are absorbed by the solute. The transition at this photon energy is assigned to the O 1s-to-$6a_1$ resonance because no peaks or shoulders can be found below this energy. The $6a_1$ orbital of TMAO may also contain a component on the surrounding water molecules. It is worth noting that water shows absorption intensity at 533 eV as well due to a strong resonance peak at 534.7 eV. A strong absorption structure above 535 eV is also due to water, which prevents us from finding other absorption bands of TMAO.

The soft X-ray emission spectra obtained in this study have a wealth of information. Fig. 1b shows the emission spectra acquired upon the resonant excitation at 533.2 eV for a series of solution concentrations. So-called saturation artifacts or self-absorption effects[47] have been corrected with the formulation described in ref. 48. The spectra have a prominent peak at

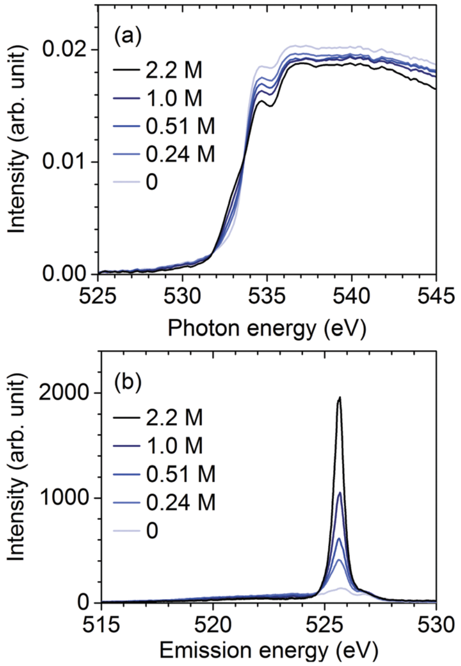

Fig. 1 Panel a: soft X-ray absorption spectra of aqueous TMAO solutions as a function of molar concentration (nominal values without taking hydration into account). Zero means pure liquid water. Panel b: soft X-ray emission spectra of the solutions acquired upon irradiation with 533.2 eV photons. The spectra have been corrected for saturation artifacts.

525.6 eV with a shoulder on the higher energy side. They contain emission from water, as can be seen in the spectrum for 0 M (*i.e.*, pure water): it has two peaks located at 525.7 and 526.7 eV which are both ascribed to the transition of a $1b_1$ electron of water to fill the O 1s hole.[37,39,49] Because the spectra show no concentration dependence at the shoulder and the higher energies, the intense peak at 525.6 eV can be attributed to the emission caused by filling the O 1s hole of the TMAO molecule with an outermost 5e electron. Apparent inverse dependence around 523 eV implies modification of the electronic structure of water through interaction with TMAO.

4. Discussion

4.1. The CLS regression analysis

The soft X-ray emission spectrum for the TMAO component is obtained by a quantitative analysis according to the CLS regression method.[50,51] The method is a natural extension of Beer's law to multi-component and -wavelength systems under the condition of the total signal being the sum of the signals for each component. The basic equation for the measured spectral intensity matrix, **A**, is expressed as

$$\mathbf{A} = \mathbf{C}\mathbf{K} + \mathbf{R},$$

where **C** and **K** are the concentration data matrix and the set of pure component spectra at unit concentration, respectively. The error matrix **R** represents spectral residuals between the model and the measured data. The above equation can be solved for **K** by applying least-squares treatment to fit the data to the model, giving

$$\mathbf{K} = (\mathbf{C}^{\mathrm{T}}\mathbf{C})^{-1}\mathbf{C}^{\mathrm{T}}\mathbf{A},$$

where $\mathbf{C}^{\mathrm{T}}$ indicates the transpose of **C** and superscript −1 denotes the inverse matrix.

In this study we make an assumption of the spectra comprising two components: solute and bulk. The modification of the electronic structure of water is thus included as the solute component. This is reasonable because the interaction between TMAO and water is strong enough to change the water structure.[26] As the concentration increases, the amount of water interacting with TMAO increases. In other words, the solute component can be regarded as the hydrated solute in a manner that the solute is accompanied with water. This makes us modify the molar concentrations of the samples for analyzing the emission spectra. Part of the solvent molecules turns to be the water of hydration, the amount of which is proportional to the solute. Emission from the hydrated water should therefore be included as the solute component and the emission intensity of the bulk water decreases accordingly. If we introduce a new parameter, w, indicating the number of hydrated water molecules per solute molecule or the hydration number of TMAO, the effective molar concentrations can now be defined by

$$C_{\text{solute}} = \frac{(w+1)n_{\text{TMAO}}}{V} \text{ and } C_{\text{bulk}} = \frac{n_{\text{H}_2\text{O}} - w \cdot n_{\text{TMAO}}}{V}$$

for the hydrated solute and bulk, respectively, where n_J and V mean the amount of J in the sample and the volume of solution in liter (dm^3), respectively. The density of the TMAO solution needed for evaluating the volume is taken from ref. 52. The optimum value of w will be determined by the CLS analysis.

As was mentioned in the Results section, inverse concentration dependence of emission is seen around 533 eV in Fig. 1b. Such behavior shows that the amount of bulk water decreases by adding TMAO, implying that part of the solvent water turns to be the water hydrated to the TMAO molecules. One can therefore determine how many water molecules are hydrated to one TMAO molecule by checking the **K** matrix elements around 533 eV. If the input w value is small, the resultant solute spectrum has a negative intensity around 533 eV, which is unreal as an emission spectrum. This means that a criterion can be set up as the analyzed solute spectrum having positive intensity at any emission energy, in particular, at around 533 eV.

The assumption that the number of constituents is two is justified by a principal component analysis (PCA). The analysis determines the number of components required to reconstruct the measured spectral data.[50] Fig. 2 depicts the principal components of the emission spectra. One can conclude that in fact two components are sufficient to reconstruct the measured spectra because the primary and secondary components contain 99.95% of the spectral information. The secondary component (PC2 in Fig. 2) describes the change with concentration relative to the primary component (PC1) which indicates the average of the emission spectra.

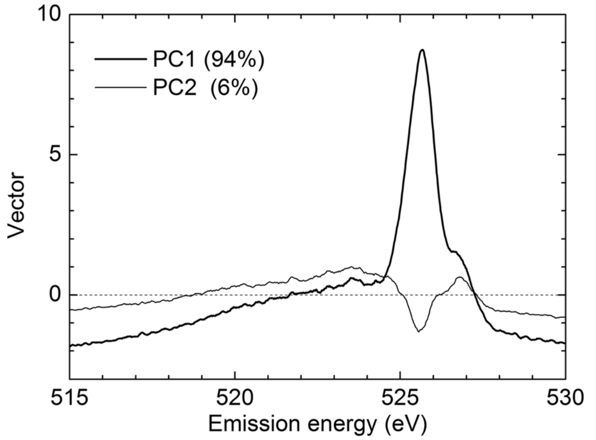

Fig. 2 Spectral components obtained by the principal component analysis (PCA) for the soft X-ray emission data presented in Fig. 1b. The primary (thick line, PC1) and secondary (thin line, PC2) principal components explain 99.95% of the spectral information.

4.2. The analyzed emission spectra

Fig. 3 shows the resultant components obtained by the CLS analysis, *i.e.*, modeled emission spectra, upon varying the value of *w*. In the present analysis one obtains a pair of spectra: the solute and the bulk components. The bulk component agrees in principle with the emission spectrum of liquid water in this study. The modeled bulk spectra in fact reproduce the 0 M data, with little dependence on the value of *w*. Thus we plot the bulk component only for *w* = 12 (labeled Bulk12) in Fig. 3. The modeled solute component, on the other hand, varies strongly with the value of *w*. The inset of Fig. 3 shows the enlargement of the modeled solute spectra in the region of 517–524 eV. The numbers next to the spectra refer to the value of *w*. The spectra for *w* = 3 and 6 are unphysical because they have negative intensity in some region. We conclude from the results that nine or more water molecules interact with, or are hydrated to, a TMAO molecule.

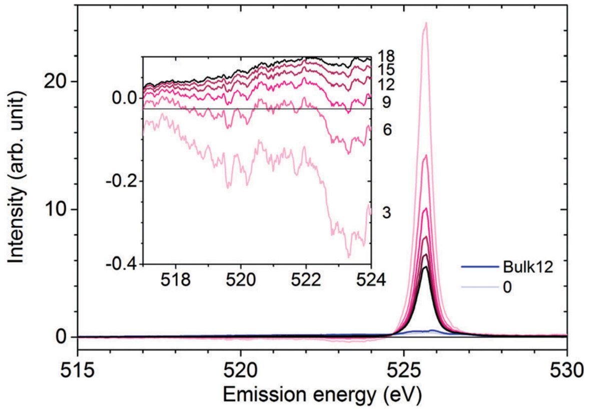

Fig. 3 Soft X-ray emission spectra of the pure components deduced from the CLS regression analysis with different values of the hydration number of TMAO, *w*. Also shown are the emission spectra measured for pure water (labeled 0). The spectrum labeled Bulk12 corresponds to the resulting bulk component with *w* = 12. The inset shows the modeled solute spectra in the region of 517–524 eV. The numbers on the right edge of the inset refer to the value of *w*.

It is informative to compare the hydration number with other studies, although the sample concentrations covered and spectroscopic techniques employed are different from study to study. Our value of nine or more is in agreement with the femtosecond mid-infrared spectroscopic study by Rezus and Bakker[20] showing that approximately 12 water molecules are strongly immobilized by TMAO. It is also comparable to dielectric measurements[22] in which a slow water fraction of 5–9 water molecules per solute is proposed at dilute concentrations: the number of the slow water relaxation mode tends to decrease at higher concentrations. Far-infrared and Raman spectroscopy indicates that at least three water molecules are strongly bound to the oxygen site of TMAO.[26,28] Usui *et al.*[53] employed *ab initio* molecular dynamics simulations to monitor reorientational dynamics of water molecules. They reported that 2.9 hydroxyl groups of water on average are hydrogen-bonded to the oxygen atom of the TMAO molecule. A very slow rotational motion of these water molecules supports the stronger hydrogen bonds. A theoretical study by means of Monte Carlo simulations coupled with an *ab initio* molecular orbital method[54] shows a complex of TMAO with 12 water molecules as a representative hydration structure. A more recent computational study[55] exhibits that the average numbers of hydrogen bonds per TMAO oxygen and of water molecules around the methyl groups are 3.1 and 17, respectively, at 1 bar. (Similar spatial distribution functions of water around the TMAO molecule are shown by another computational technique.[56]) Since our limit value of *w* is within a reasonable range, we can conclude that soft X-ray emission spectroscopy combined with CLS regression analysis offers a useful technique for probing the solvation structure around the solute.

5. Concluding remarks

Soft X-ray absorption and emission spectra have been measured for aqueous solutions of TMAO in the O 1s region. Isosbestic points are located at 531.7 and 533.6 eV. The absorption at 533 eV is assigned to the resonant promotion of an O 1s electron in TMAO to the $6a_1$ unoccupied orbital. The emission spectra acquired at 533.2 eV are composed of TMAO and water components, with a prominent peak at 525.6 eV attributable to the emission caused by the 5e to O 1s transition. Apparent inverse dependence around 523 eV implies modification of the water structure through strong interaction with TMAO. The CLS regression analysis reveals that nine or more water molecules interact with a TMAO molecule. This approach provides a useful method for probing the solvation structure around the solute which interacts strongly with the solvent. A further insight into the electronic structure of TMAO can be gained by a comparison with similar measurements in other solutions, the study on which is now in progress, and the results will be published in the near future.

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