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DOSY-NMR and Raman Investigations on the Self-Aggregation and Cyclodextrin Complexation of Vanillin

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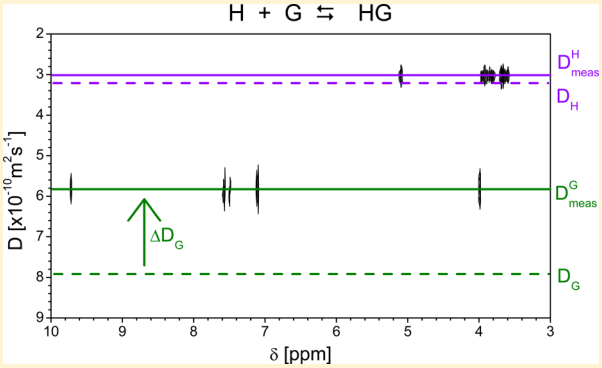
ABSTRACT: Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a phenolic aldehyde with limited solubility in water; in this work, we investigate its self-aggregation, as well as its complexation equilibria with β-cyclodextrin by using nuclear magnetic resonance (NMR) and vibrational spectroscopy. In particular, diffusion-ordered NMR (DOSY) measurements allowing to detect diffusional changes caused by aggregation/inclusion phenomena lead to a reliable estimate of the equilibrium constants of these processes, while Raman spectroscopy was used to further characterize some structural details of vanillin self-aggregates and inclusion complexes. Although the self-association binding constant of vanillin in water was found to be low (K_a ~10), dimeric species are not negligible within the investigated range of concentration (3−65 mM); on the other hand, formation of β-cyclodextrin self-aggregates was not detected by DOSY measurements on aqueous solutions of β-cyclodextrin at different concentrations (2−12 mM). Finally, the binding of vanillin with β-cyclodextrin, as measured by the DOSY technique within a narrow range of concentrations (2−15 mM) by assuming the existence of only the monomeric 1:1 vanillin/β-CD complex, was about an order of magnitude higher (K_c ~ 90) than self-aggregation. However, the value of the equilibrium constant for this complexation was found to be significantly affected by the analytical concentrations of the host and guest system, thus indicating that K_c is an "apparent" equilibrium constant.



INTRODUCTION

Noncovalent interactions are of great interest in physical chemistry, since they can play many key roles: not only can they drive molecules to form supramolecular complexes and are responsible for maintaining the three-dimensional structure of species such as nucleic acids and proteins, but they are also relevant in determining phase changes and in molecular recognition events. Depending on their different nature, noncovalent interactions are usually classified as electrostatic (which comprise hydrogen bonds and ionic forces), dispersion, and hydrophobic interactions. Whatever their origin, they can affect the chemical and physical properties of the involved species: the stronger the interaction, the larger these changes. In any case, the stabilization energy of a molecular cluster is usually small (between 1 and 100 kJ/mol) and comparable to the average thermal energy of the kinetic motion of molecules.[1] Since this is much smaller than the binding energy of covalent bonds (about 400 kJ/mol), experimental studies of these interactions can be very challenging, and only noninvasive analytical approaches can be reliably applied; basically, spectroscopic techniques can meet these demands.

When considering aromatic systems, there is no consensus about what kind of interactions may be dominant, and several hypotheses have been addressed. For example, Hunter and Sanders[2,3] introduced the "$\pi-\pi$ theory", and supposed that aromatic interactions essentially consist of van der Waals, hydrophobic, and electrostatic forces. Other authors have criticized this point of view and explained the aromatic interactions just in terms of molecular quadrupoles.[1] Even if the mechanism is still not well understood and no matter what the right interpretation may be, such interactions drive many aromatic molecules to form self-aggregates in polar solvents. Furthermore, it has been seen that several stacking geometries can occur, such as edge-to-face, face-to-face, and offset stacked, depending on the substituents at the aromatic ring and on the solvent wherein these solutes are investigated.[4]

On one hand, noncovalent interactions can cause self-aggregation processes, but on the other hand, they are also responsible for binding phenomena between different molecules as observed in host−guest complexation equilibria where two interacting species are involved: a big molecule called the "host", which usually possesses a central hole, and a smaller species called the "guest", that can form the complex by entering this hole. Among all the available hosts, cyclodextrins (Figure 1) are probably the most widely used, especially in the food, cosmetics, and pharmaceutical sectors, as slow-release and

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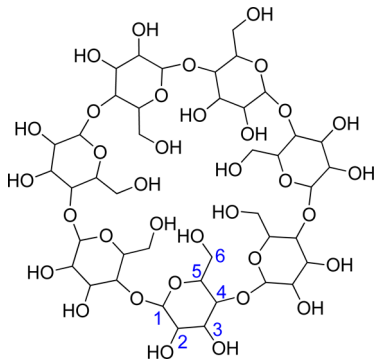

Figure 1. Chemical structure and numbering of β-cyclodextrin.

compound-delivery agents.[5] They are cyclic oligosaccharides made up of six (α-CD), seven (β-CD), or eight (γ-CD) D-glucopyranoside units linked together by 1,4-glycosidic bonds, and their peculiarity is that in water they assume a torus-like shape, with a hydrophilic outside and a hydrophobic cavity, which can therefore admit small nonpolar molecules, increasing both their aqueous solubility and, eventually, their chemical stability.[6] The process of complex formation in water can take place only if the size of the guest molecule fits well within the cavity, which mainly depends on the number of glucose units. The driving force for the guest inclusion is thought to be the release of the high-energy water molecules originally present inside the cavity, and their substitution with the hydrophobic guest.[6]

Vanillin (4-hydroxy-3-methoxybenzaldehyde, Figure 2) is a natural aromatic compound that can be found in several

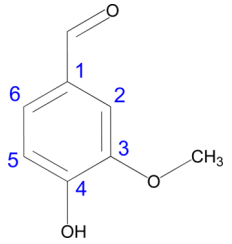

Figure 2. Chemical structure and numbering of vanillin.

essential oils and in vanilla pods, both in its free form and bound to larger molecules such as sugar and lignin. Its major commercial application is as an aroma chemical and, as such, it has been widely used in the manufacture of chocolate and ice creams, but also as fragrance in perfumes and cosmetics; it is estimated that the overall annual production of vanillin exceeds 10 500 tons.[7] From an industrial point of view, vanillin can be obtained by extraction from the pod of the vanilla orchid, but this is a very expensive operation, so that it only represents 5% of the vanillin production; the rest is artificially synthesized from raw materials such as lignin[7] and guaiacol.[8] Being an aromatic compound, vanillin is sparingly soluble in water, with a solubility limit of about 70 mM at room temperature. Recently, its aqueous self-aggregation has been investigated.[9] Also, the ability of cyclodextrins to form complexes with vanillin has already been addressed by several authors, mainly with the aim of characterizing the structural property of the resulting complex.[10−14]

Among the developing techniques that allow one to noninvasively detect aggregation and complexation phenomena in solution, diffusion-ordered NMR spectroscopy (DOSY) plays a central role, since the measure of diffusion coefficients of solute species in solution is its primary target.[15] In fact, molecular aggregations cause a change in their diffusion coefficients,[16] according to the Stokes−Einstein equation:

$$D = \frac{k_B T}{6\pi\eta R_H} \quad (1)$$

where k_B is the Boltzmann constant, T the absolute temperature, η the viscosity of the medium, and R_H the hydrodynamic radius of the species.[17] Several NMR pulse sequences are available for carrying out DOSY experiments, but all of them rely on the application of magnetic pulsed-field gradients (PFGs). Basically, in the simplest DOSY-designed pulse sequence, a magnetization ribbon is first produced by a 90° RF pulse, followed by a PFG which spatially labels the spins in the sample, generating a magnetization helix; the spins are then allowed to evolve for a definite time (called the diffusion-sensitive period). Since their motion partially disrupts this helix, faster motions lead to more significant losses of the overall magnetization. At this point, a second PFG (with opposite polarity to the first) is provided to refocus the magnetization helix back into a shortened magnetization ribbon, which generates an attenuated NMR signal. The degree of attenuation can be analytically related to the self-diffusion coefficient by the Stejskal−Tanner expression[18]

$$I_q = I_0 \exp[-D\sigma^2 q^2 \Delta'] \quad (2)$$

where I_q is the measured signal intensity, I_0 is the signal intensity that would be measured in the absence of diffusion, $q = \gamma G\delta$ is a parameter that depends on the gyromagnetic ratio (γ), the gradient strength (G), and the duration (δ), and Δ' is the effective diffusion-sensitive period. Finally, σ is the shape factor, which accounts for the shape of the applied PFG. Therefore, to measure diffusion coefficients, a DOSY sequence is repeated several times, gradually increasing G (and therefore q), and the obtained intensities are fitted to fulfill eq 2.

Among all its possible applications, the DOSY technique has also been used to detect aggregation phenomena, although so far to a limited extent, since interest was mainly focused on synthetic or natural products mixture analysis. In the present work, we report a quantitative study of self- and not self-aggregation (vanillin/β-CD complexation) of vanillin aqueous solutions. A further structural characterization of these aggregates was obtained by vibrational spectroscopy through Raman (and partially IR) measurements carried out in solutions and/or in solid state preparations.

MATERIALS AND METHODS

General Methods. Vanillin ($C_8H_8O_3$, MW = 152.15 g/mol) was purchased from Sigma-Aldrich, and β-cyclodextrin ($C_{42}H_{70}O_{35}$, MW = 1134.98 g/mol) was obtained from Fluka Chemie (Switzerland). In order to simplify the features of NMR and the Raman spectra, its exchangeable protons were replaced by deuterium atoms by repeated (four times) dissolving/evaporation cycles in deuterium oxide (D_2O, deuteration degree: 99.97%, Euriso-top, France), followed by evaporation of the solvent until dryness. Tetradeuterated ammonium formate (ND_4HCOO) was obtained by dissolving/evaporation cycles in D_2O of commercial ammonium formate (NH_4HCOO, Fluka Chemie, Switzerland). A 30 μL portion of a mother solution (1.0 M, pH 7.0) of ND_4HCOO in

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D_2O was used to prepare the buffered NMR solutions (600 μL) at the final buffer concentration (50 mM).

For the vanillin self-aggregation studies, a stock 65 mM aqueous solution was first prepared, and in order to ensure a complete solubilization, it was heated up to about 50 °C, strongly mixed, and sonicated for 15 min. Several (11) aqueous solutions with concentrations ranging from 3 to 65 mM were thereby obtained. Measurements on ammonium formate buffered solutions were carried out on five aqueous solutions with concentrations ranging from 0.5 to 35 mM. Four aqueous solutions of β-CD (unbuffered) were prepared in a similar fashion, with a concentration range from 2 to 12 mM.

For the host/guest complexation studies, a stock aqueous solution containing both vanillin and β-CD at the 15 mM concentration was first prepared following the same procedure described above. From this, another three solutions were prepared by dilution, obtaining therefore four vanillin/β-CD 1:1 solutions with concentrations ranging from 3 to 15 mM, which were used to assess the degree of aggregation and to evaluate the complexation constant.

NMR Measurements. The NMR measurements were performed with a Bruker-Avance 400 MHz spectrometer operating with a stationary magnetic field of strength 9.4 T and equipped with a 5 mm BBI probe capable of providing longitudinal pulsed-field gradients of up to 53 G cm^{-1}. The 90° proton pulse length was calibrated and established to be 9.4 μs, with a transmission power of 0 dB. In all of the measurements, the temperature was kept at 300.2 K; the chemical shift scale was referenced to the TSP resonance, which was set at 0.000 ppm. Diffusion coefficients were obtained through DOSY NMR experiments using the Bruker pulse sequence *dstegp3s* (2D ^{1}H DOSY double stimulated-echo) to compensate for possible effects of convective motions. The longitudinal eddy current delay and the gradient recovery delay were kept at fixed values of 5 and 0.2 ms, respectively. The strength of the pulsed-field gradients was incremented from 5 to 95% with respect to the maximum available in 10 increments on a quadratic scale, whereas the diffusion-sensitive period (Δ) and the gradient duration (δ) were optimized to allow the signals of interest to decrease by a factor of 10−20, in order to better characterize the signal exponential decay predicted by the Stejskal−Tanner expression (eq 2). The data from the NMR experiments were processed with the software MestReNova (Mestrelab Research S.L., Santiago de Compostela, Spain), and diffusion coefficients were then obtained by integrating several ^{1}H-signals and fitting the corresponding peaks area to fulfill the Stejskal−Tanner expression, using the software OriginPro 8 (OriginLab Corporation, Northampton MA, USA). The D value for a given solution was taken as the average of the values obtained by fitting the exponential decays of the several different resonances (five for vanillin, six for β-CD), and its uncertainty was taken as the standard deviation of the mean.

Vibrational Spectroscopy. All Raman measurements were carried out both on liquid and dried samples deposited on a glass slide in air and at room temperature. The corresponding spectra were obtained in backscattering geometry and by using two different experimental setups to better explore different spectral ranges.

The spectra in the wavenumber range between 1550 and 1700 cm^{-1} were collected by using an exciting radiation at 632.8 nm (He−Ne laser, power at the output ≈20 mW). The laser was focused onto the sample surface with a spot size of about 1 μm^2 through the 80× objective (NA = 0.9) of a microprobe setup (Horiba-Jobin-Yvon LabRam HR800) consisting of an 80 cm focal length spectrograph using a 1800 grooves/mm grating and a charge-coupled device (CCD) detector cryogenically cooled by liquid nitrogen. The elastically scattered radiation was filtered by using a notch filter. In this configuration, the resolution was about 0.28 cm^{-1}/pixel.

The band deconvolution of the Raman spectra in the 1550−1700 cm^{-1} wavenumber region was undertaken by using second derivative computations for evaluating the wavenumbers of the maxima of the different sub-bands. Multiple curve fitting into Lorentzian functions was then applied to the experimental profiles based on these wavenumber values. For each fitting session, multiple iterations were performed until a converging solution was reached by minimizing, in the meanwhile, the value of chi-square.

Low-wavenumber Raman spectra were recorded over the wavenumbers ranging from 3 to 100 cm^{-1} by using a triple-monochromator spectrometer (Horiba-Jobin Yvon, model T64000) set in double-subtractive/single configuration and equipped with holographic grating 1800 grooves/mm. Micro-Raman spectra were excited by the 647.1 nm wavelength of an argon/krypton ion laser and detected by a CCD detector cryogenically cooled by liquid nitrogen. Exciting radiation was focused onto the sample surface with a spot size of about 1 μm^2 through a 80× objective with NA = 0.75. The resolution was about 0.36 cm^{-1}/pixel.

The IR spectra were collected in an ATR geometry using an FT-IR Equinox 55 Bruker spectrometer, at room temperature with a scanning speed of 30 cm^{-1} min^{-1} and a spectral resolution of 2 cm^{-1}. A few microliters of the solutions (either vanillin or the vanillin/β-CD inclusion complex) were deposited on a ZnSe crystal and dried by nitrogen flushing. The data were analyzed with the software OPUS 2.0.5.

RESULTS AND DISCUSSION

Self-Aggregation. *NMR Measurements.* Before using the DOSY technique, we proved the existence of vanillin self-aggregation in water by recording the ^{1}H NMR spectra of several vanillin/water solutions at different concentrations. After the assignment of vanillin protons (numbers in Figure 2) for the most diluted solution [3 mM in D_2O, 3.979 (OCH_3, s), 7.112 (H-5, d, 8.0 Hz), 7.575 (H-2, d, 1.8 Hz), 7.595 (H-6, dd, 1.8 Hz, 8.0 Hz), 9.754 (COH, s)], a significant upfield effect was observed by increasing concentration, as shown in Figure 3 for H-2, H-5, and H-6. It is worth mentioning that these changes are not caused by pH changes, eventually induced by concentration-dependent acid/base species distribution by the presence of a slightly acidic phenolic group in vanillin. In fact, NMR measurements carried out on buffered vanillin solutions (50 mM, ammonium formate) at different vanillin concen-

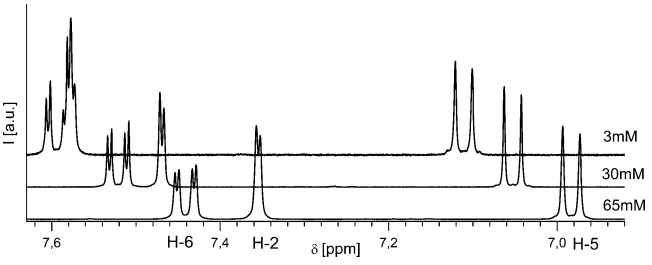

Figure 3. Intermediate δ region of ^{1}H NMR spectra of vanillin aqueous solutions taken at three different concentrations.

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trations indicated that the upfield changes of the chemical shifts of all the vanillin protons were essentially the same as those measured for the unbuffered solutions. This analysis clearly showed the salting-out effect of vanillin whose solubility was, as expected, significantly lower in the buffered than in the unbuffered solutions.

The observed effects are in agreement with anisotropic-induced ring currents in the aromatic nucleus of vanillin (π stacking) which become higher at higher concentration, thus speaking for the presence of true self-aggregation phenomena.

Since the rate of exchange between the free species and the aggregates is fast with respect to the NMR time scale, the observed resonances are found by a weighted chemical shift average of free species and aggregates:

$$\delta_{obs} = \frac{\sum_j j[V_j]\delta_j}{[V_0]} \tag{3}$$

where $[V_0]$ is the total vanillin molar concentration (analytical concentration), $[V_j]$ the concentration of the aggregate containing a "j" number of vanillin molecules, and δ_j is the chemical shift of a given resonance in this aggregate. Actually, eq 3 provides also a means for quantitatively characterizing the self-aggregation by an NMR chemical shift analysis, provided that a proper model is employed for describing the aggregation; usually, an isodesmic model is assumed,[9] which considers the aggregation process to follow subsequent dynamic binding steps:

$$V + V \rightleftarrows V_2$$
$$V_2 + V \rightleftarrows V_3$$
$$\ldots$$
$$V_j + V \rightleftarrows V_{j+1}$$

where each of them is characterized by an aggregation constant, defined as $K_{a,j} = [V_{j+1}]/([V_j][V])$. In particular, the isodesmic model assumes that $K_{a,1} = \ldots = K_{a,j} = K_a$.

By using only a chemical shift analysis, the dependence of the chemical shift on the oligomeric state has to be properly modeled. Usually it is considered to follow the relation[9]

$$\delta_j = \frac{(j-1)\delta_{min} + \delta_1}{j} \tag{4}$$

Here δ_{min} corresponds to the chemical shift of a stack made up of virtually infinite solute molecules (i.e., high concentration limit), whereas δ_1 is the chemical shift of the considered proton in the monomer (i.e., infinite dilution limit). With the above-mentioned assumptions, it is straightforward to express the observed chemical shift as a function of just K_a, $[V_0]$, δ_1, and δ_{min}. Thus, the characterization of aggregation phenomena is usually carried out by monitoring the chemical shift variations over a range of solute concentrations, and fitting the values to fulfill the resulting expression. This procedure has already been reported for the vanillin aqueous self-aggregation,[9] and thus, it will not be discussed any further.

The DOSY technique relies on a very different physical basis; in principle, an aggregate of molecules should have a lower diffusion coefficient than the free molecule itself. As predicted by eq 1, since the hydrodynamic radius is proportional to the molecular weight to the 1/3 power, the diffusion coefficient of a stack of j molecules can be related to that of the free species by the simple relation 5:

$$D_j = \frac{D_1}{j^{1/3}} \tag{5}$$

Since the stacking process is usually fast with respect to the diffusional time scale ($\Delta \approx 100$ ms), the decaying behavior of every resonance will be described by a single exponential (see eq 2), with a measured diffusion coefficient given by the weighted average

$$D_{meas} = \frac{\sum_j j[V_j]D_j}{[V_0]} = \frac{D_1}{[V_0]}\sum_j j^{2/3}[V_j] \tag{6}$$

Within the isodesmic approximation, a single expression can be obtained in which D_{meas} is related to D_1, $[V_0]$, and K_a:

$$D_{meas} = D_1\frac{[V]}{[V_0]}\sum_j j^{2/3}(K_a[V])^{j-1} \tag{7}$$

where $[V]$ can be analytically found by solving the equation

$$[V_0] = \frac{[V]}{(1 - K_a[V])^2} \tag{8}$$

which arises by imposing the mass law conservation.[9]

With these considerations in mind, we performed several concentration-dependent DOSY experiments on aqueous vanillin solutions. In order to ensure that the observed decrease of the diffusion coefficients was not caused by an increase of the medium viscosity, we also monitored the diffusion coefficient of the solvent; the latter was found constant over the whole investigated vanillin concentration range. In addition, in these solutions, we did not add buffer or TSP to avoid possible interferences with the aqueous self-aggregation process.

In Figure 4, the concentration dependence of the measured D values is shown whereby, relying on an isodesmic model, the best fitting parameters for K_a and D_1 can be evaluated.

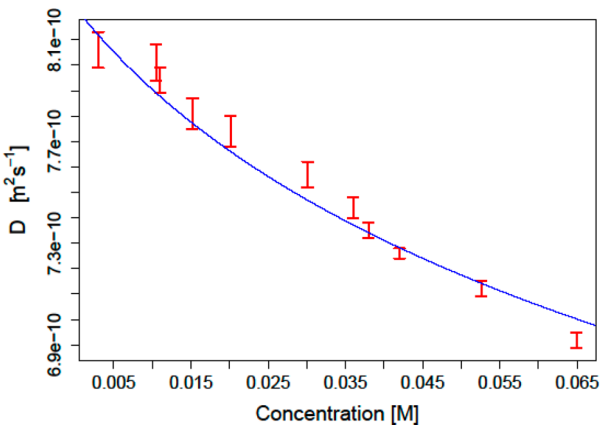

Figure 4. Measured diffusion coefficients as a function of the vanillin concentration, obtained by DOSY. The solid curve represents the result of the fitting procedure according to the isodesmic model.

The resulting parameters were $D_1 = (8.227 \pm 0.008) \times 10^{-10}$ m^2 s^{-1} and $K_a = 9.8 \pm 0.7$ M^{-1} ($\chi_r^2 = 1.8$). The value of the measured aggregation constant allows one to draw simple considerations about the distribution of vanillin among any potential aggregate species at any given concentration. First of all, since this value is low, the self-aggregation basically involves simple oligomers (dimers essentially). A simple calculation for the 15 mM solution, assuming an isodesmic model, indicates

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that the molar fraction of the free species is significantly higher (~88%) than that of dimeric species (~10%), whereas trimeric (and higher order) species are almost negligible, accounting only for about 1% molar fraction.

Things do not significantly change by following a semi-isodesmic model, where the first aggregation constant ($K_{a,1}$, responsible for the dimerization step) is allowed to differ from all the others (K_a), assumed again to all be equal. In fact, the obtained values ($K_{a,1} = 8.7 \pm 0.6$ M^{-1}, $K_a = 11.7 \pm 0.9$ M^{-1} ($\chi_r^2 = 1.4$)) are basically the same as that obtained within the isodesmic approximation. Worth of note, our results are also in good agreement with those previously obtained through the analysis of the dependence of ^{1}H chemical shifts on vanillin concentration.[9]

Finally, the measurements of the diffusion coefficients of vanillin in a buffered solution (pH 7, 50 mM aqueous ammonium formate) afforded a little bit different D values, but their dependence on the analytical concentration of vanillin (i.e., the corresponding equilibrium of self-aggregation) was found to be almost identical to that measured for the unbuffered solutions. Thus, the buffer was demonstrated to not play any relevant role on the aggregation phenomena; it eventually affects (but only marginally) the solvent viscosity.

Following the characterization of the vanillin self-aggregation, we used the DOSY technique to investigate the presence of self-aggregates also in β-CD aqueous solutions. As a matter of fact, the self-aggregation processes of cyclodextrins have already been reported.[19] In particular, dynamic and static light scattering measurements, as well as transmission electron microscopy at cryogenic temperature[20] seem to indicate that β-CD, at concentrations higher than 3 mM, is present in aqueous solutions as large-size aggregates (diameter ~100 nm). On the other hand, previously reported DOSY measurements[21] only indicate a very low decrease in the measured diffusion coefficients, possibly caused by either a slight increase of the medium viscosity at higher cyclodextrin concentration or the formation of a very low number of small aggregates. The results of our measurements on four aqueous solutions of β-CD at different concentrations, summarized in Table 1, are in agreement with this report.[21] The results do not suggest the presence of important β-CD self-aggregation processes, at least within the sensitivity of the DOSY technique. In principle, however, the reported heavy aggregates could be present in such a small amount that they do not affect the averaged measured diffusion coefficient of β-CD.

Table 1. Concentration Dependence of the Measured Diffusion Coefficients of β-CD Aqueous Solutions

[β-CD] (mM)	D_{meas} (m^2 s^{-1})
2.1	$(3.09 \pm 0.09) \times 10^{-10}$
5.2	$(3.10 \pm 0.04) \times 10^{-10}$
8.3	$(3.09 \pm 0.04) \times 10^{-10}$
12.5	$(3.01 \pm 0.03) \times 10^{-10}$

Raman/IR Measurements. In order to get insight on some structural aspects of vanillin self-aggregation phenomena, Raman spectroscopy measurements were carried out. Figure 5 shows the spectral window in the wavenumber range 1550–1700 cm^{-1} for the vanillin solid sample (Figure 5a) and for the 15 mM vanillin aqueous solution (Figure 5b). We chose to focus our attention on the vibrational spectrum of vanillin in this spectral region where the vibrational normal modes involving the stretching vibrations of the carbonyl group of aldehyde and of the aromatic double bonds of vanillin occur. On the basis of previous investigations,[22,23] these motions are also expected to be particularly sensitive to the molecular aggregation processes.

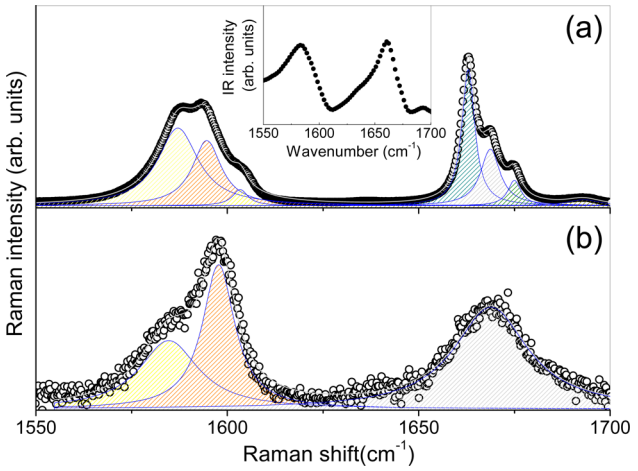

Figure 5. Raman spectra of (a) vanillin in solid state and (b) a 15 mM vanillin aqueous solution in the wavenumber range 1550–1700 cm^{-1}. The experimental data (empty circles) are reported together with the best-fit (gray line) and the deconvolution components. Inset: FTIR-ATR spectrum of solid-state vanillin in the same wavenumber range (1550–1700 cm^{-1}).

Significant differences in the spectral shape of the vibrational profile of vanillin in the solid state can be observed with respect to its spectrum in aqueous solution. For a better inspection of these spectral differences, the experimental spectra were deconvolved into their single components, by using a proper fitting procedure of the data, as shown in Figure 5 (see the Materials and Methods section for details). The spectrum of Figure 5a indicates the presence of three distinct components at 1587, 1595, and 1603 cm^{-1} which are related to vibrational modes involving the C=C bonds of the aromatic ring, while the peaks falling at 1663, 1669, and 1675 cm^{-1} can be essentially assigned to the stretching modes of the C=O group of vanillin. It is noteworthy that the same complex pattern can be recognized also in the IR spectrum of solid-state vanillin in the wavenumber range 1550–1700 cm^{-1} (see inset of Figure 5a), despite the general wider broadening observed for the IR with respect to the Raman peaks.

The Raman spectrum of the aqueous solution of vanillin (Figure 5b) shows two peaks at about 1585 and 1598 cm^{-1} (C=C stretching of the aromatic ring) and only one broad signal at 1669 cm^{-1} (C=O stretching). These assignments are derived by comparison of experimental data with the results of DFT calculations carried out on a single molecule of vanillin in the gas phase and recently reported in the literature.[24] On the other hand, previous X-ray crystallography[25] investigations performed on crystals of vanillin showed that the molecule crystallizes in a monoclinic crystal system with four vanillin in the asymmetric unit forming two pairs that lay on two parallel planes. Each pair of vanillin molecules is supposed to be held together by hydrogen bonds (HBs) established between the hydrogen atom of —OH of one molecule and the oxygen atom of the —C=O group of the other.

By comparing frequencies and relative intensities of the experimental with calculated peaks, it becomes evident that the vibrational features of the liquid solution of vanillin are very

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similar to those predicted for the monomer.[24] This finding suggests that, even if the aggregation of vanillin is taking place in water, in a 15 mM aqueous solution, the main species present is free vanillin, an outcome perfectly consistent with the results of DOSY measurements.

This conclusion is further supported by the inspection of the low-frequency region of the Raman spectrum of vanillin in solid state (Figure 6). Similarly to what has been observed on other

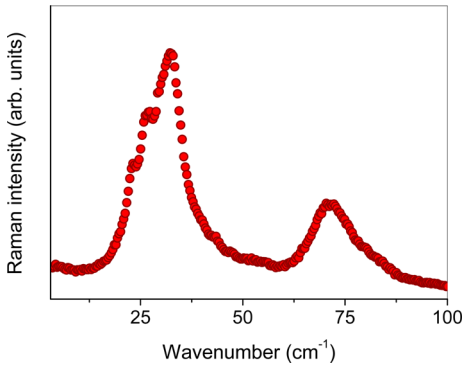

Figure 6. Experimental Raman spectrum of solid-state vanillin in the low-frequency wavenumber range 3−100 cm^{-1}.

molecules,[22,26] we find several peaks falling in the spectral range 10−45 cm^{-1} which have been assigned to bending motions of the hydrogen bond connecting the two vanillin units.[22] These peaks are not present in the computed spectrum of the monomeric species[24] where the lowest frequency mode is found at about 108 cm^{-1} attributable to vibration modes of the methyl group of vanillin. Therefore, through the inspection of the low-frequency vibrational modes representing collective motions of the system,[26,27] it is possible to gain useful information about the presence of molecular aggregation phenomena.

Vanillin/β-CD Complexation. *NMR Measurements.* Following a similar approach to the one used for studying vanillin self-aggregation processes, before subjecting the vanillin/β-CD solution system to DOSY measurements, we investigated the changes of the chemical shifts of the protons of guest (vanillin) and host (β-CD) in the vanillin/β-CD system eventually deriving from their not-self-aggregation (complexation), as compared to the shifts in the separated solutions. The details of the ^{1}H NMR assignments (in ppm) of the 15 mM solutions of free vanillin, free β-CD, and vanillin/β-CD complex are listed in Table 2, where the proton numbering is that reported in Figures 1 and 2.

Strong evidence of the host/guest complexation is clearly provided by significant changes of several resonances in the aqueous vanillin/β-CD sample as compared to those of free vanillin and β-CD. As shown in Figure 7 for the restricted ^{1}H NMR region where only resonances of the β-CD are observed (with the exception of the methoxy group of vanillin), the relevant upfield change of the β-CD resonances indicates that a host/guest complexation equilibrium is occurring. Similarly to the self-aggregation, the absence of new resonances confirms that this equilibrium is a process in fast exchange on the NMR time scale.

In order to quantitatively study the host−guest complexation, an NMR chemical shift analysis can be carried out through titration experiments where small aliquots of guest are added to a solution of host at known concentration, and the

Table 2. ^{1}H NMR Chemical Shift Assignments for Free Vanillin, Free β-CD, and Vanillin/β-CD Complex (15 mM Aqueous Solutions)

proton	vanillin (15 mM)	β-CD (15 mM)	complex (15 mM)	change (ppm)
		vanillin		
OCH_3	3.959 (s)		3.985	+0.026
H-5	7.088 (d)		7.104	+0.016
H-2	7.529 (d)		7.490	−0.039
H-6	7.562 (dd)		7.574	+0.012
COH	9.731 (s)		9.718	−0.013
		β-CD		
H-4		3.628 (t)	3.618	−0.010
H-2		3.695 (dd)	3.680	−0.015
H-5		3.902 (m)	3.814	−0.088
H-6		3.920 (m)	3.886	−0.034
H-3		4.009 (t)	3.943	−0.066
H-1		5.115 (d)	5.097	−0.018

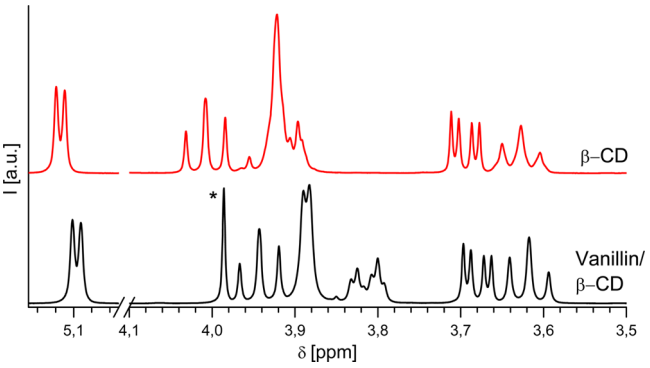

Figure 7. ^{1}H NMR spectra of the 15 mM β-CD solution (top) and of the inclusion complex (bottom); the asterisked peak is a vanillin resonance (OCH_3).

chemical shifts are plotted with respect to the host:guest ratio, in a so-called Scatchard plot.[16] In this way, three types of information are usually achieved: (a) the identification of the most affected nuclei provides a clue as to the geometry of the complex; (b) the shape of the titration curve gives quantitative information about the complexation constant; (c) the stoichiometry of the complex can be obtained by using the continuous variation method, which results in a Job plot. These techniques have already been used for the vanillin/β-CD system. In fact, the complex formation constant has been determined using the titration method ($1.11 \times 10^4 \pm 1800$ M^{-1}),[10] the continuous variation method (170.2 M^{-1}),[11] and also the fluorescence spectroscopy (270 M^{-1}).[12] Even the stoichiometry of the complexation reaction has already been determined to be 1:1.[11,13] In addition, more recently, the complexation between vanillin and β-CD has been assessed by analyzing changes in UV−visible absorption intensity with increasing β-CD concentration in excess of vanillin.[28] Although the authors did not extract a numerical value for the complexation constant, this can be guessed from the experimental data reported in their work,[28] resulting in an estimated value of about 100 M^{-1}.

The values obtained by different methods and spectroscopic techniques do not agree very well with each other, suggesting that difficulties are prone to work in these investigations. In order to understand where and how the usual assumptions

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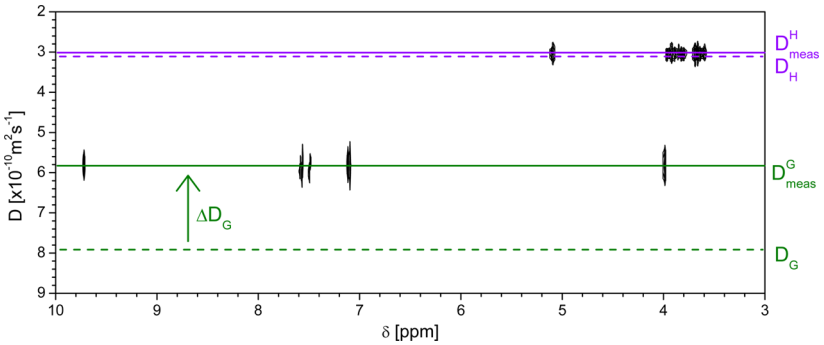

Figure 8. 2D DOSY-NMR measurements on the 15 mM aqueous solutions of vanillin, β-CD, and vanillin/β-CD inclusion complex. The horizontal axis represents chemical shifts, whereas the vertical axis diffusion coefficients; the dark spots are the resonances of the aqueous solution of the inclusion complex spread in the second dimension according to their measured diffusion coefficient.

break down, we decided to investigate this system through our optimized DOSY methodology.

The host−guest complexation is a dynamic process that can be described by

$$H + G \rightleftarrows HG \tag{9}$$

where H, G, and HG denote host (β-CD), guest (vanillin), and inclusion complex, respectively. Due to the fast exchange between the free and bound species, for each resonance of either vanillin or β-CD, there will be a single measured diffusion coefficient (D^{G}_{meas} and D^{H}_{meas}), given by a weighted average:

$$\begin{cases} D^{G}_{meas} = f_G D_G + (1 - f_G) D_{HG} \\ D^{H}_{meas} = f_H D_H + (1 - f_H) D_{HG} \end{cases} \tag{10}$$

where f_G and f_H represent the molar fractions of free vanillin and β-CD, respectively. Now, since f_G and f_H can be written in terms of the initial concentrations ($[G]_i$ and $[H]_i$) and of complex concentration at the equilibrium ($[HG]_{eq}$), provided that D_G and D_H are known, the system of eq 10 contains only two unknown parameters: the diffusion coefficient of the complex (D_{HG}) and its concentration at equilibrium. This system can therefore be analytically solved, and once $[HG]_{eq}$ is found, it is possible to determine the complex formation constant, defined as

$$K_c = \frac{[HG]_{eq}}{([H]_i - [HG]_{eq})([G]_i - [HG]_{eq})} \tag{11}$$

From eq 10, it is clear that, in order to analytically solve the problem, D_G and D_H must be known. From our above-reported experiments on β-CD aggregation, the latter can be assumed to be constant, since it does not change in a wide range of β-CD concentrations. On the other hand, this is not true for D_G (vanillin), which was demonstrated by our investigation to undergo self-aggregation at high concentrations. In estimating K_c, we therefore chose the D_G values that we have previously obtained from the vanillin solution at that definite concentration. In particular, we extracted these values from the fitted curve of Figure 4, for concentrations corresponding to those of the guest in the studied solution.

DOSY experiments were carried out on the previously prepared aqueous solutions of vanillin/β-CD. The results for the 15 mM solution are graphically depicted in the 2D DOSY plot of Figure 8, which also helps understand the basic principle allowing to estimate complexation constants by the DOSY approach.

The green and purple solid lines in Figure 8 represent D^{G}_{meas} and D^{H}_{meas}, respectively, whereas the dashed lines are the diffusion coefficients of vanillin and β-CD, both as 15 mM solutions. Basically, the extent to which the solid lines in Figure 8 are displaced from their corresponding dashed lines provides the basis for the quantitative estimate of the complex formation constant.

By taking $D_H = 3.10 \times 10^{-10}$ m^2 s^{-1} and $D_G = 7.78 \times 10^{-10}$ m^2 s^{-1}, the use of eqs 10 and 11 leads to a complexation equilibrium constant of 86 ± 7 M^{-1}, which is in good agreement with some literature data.[11,12,28] However, this value was found to significantly change when DOSY measurements were carried out in aqueous solutions of vanillin/β-CD at different concentrations (see Table 3), thus indicating that we

Table 3. Vanillin/β-CD Complexation Constant Estimated by the DOSY Technique over the Four Solutions Differing in the Solute Concentrations

[complex] (mM)	K_c (M^{-1})
6.4	106 ± 9
10.7	142 ± 9
15.0	86 ± 7

are not dealing with a true equilibrium constant of 1:1 formation of vanillin/β-CD complex but, instead, only with an apparent (conditional) equilibrium constant.

This behavior may arise from the existence of the vanillin self-aggregation phenomena but eventually also from formation of dimeric species such as (vanillin/β-CD)$_2$, still fulfilling the 1:1 stoichiometry that has been firmly established in several literature reports[11,13] for the vanillin/β-CD system. The existence of these simultaneous aggregation equilibria may also explain, by the way, the scattered results found in the literature for the value of the complexation constant of the vanillin/β-CD system where such phenomena have been completely neglected. Worth of note, the dependence of this apparent equilibrium constant by analytical concentrations of reactants cannot be explained only on the basis of the aggregation phenomena, since in this case a monotonic trend should be observed. For instance, the constant should be higher in diluted solutions where aggregations eventually leading to (vanillin)$_2$ and (vanillin/β-CD)$_2$ dimeric species must be negligible.

Raman/IR Measurements. In Figure 9 are reported the Raman spectra of the inclusion complex formed by β-CD with vanillin in solid state and in aqueous solution, in the

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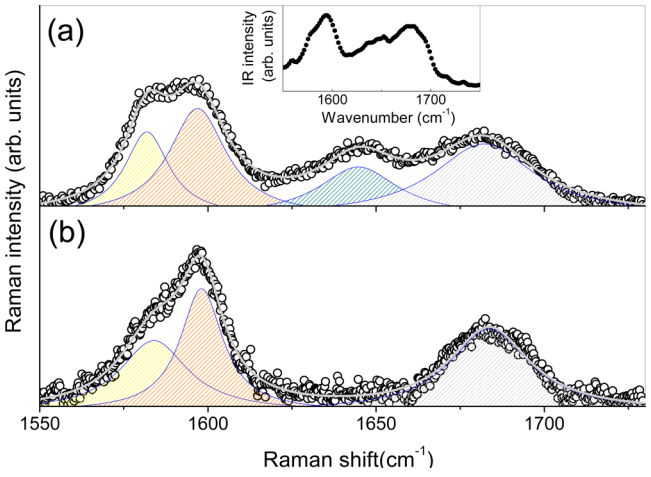

Figure 9. Raman spectra of the inclusion complex formed by β-CD with (a) vanillin in solid state and (b) in aqueous solution (15 mM), in the wavenumber range 1550−1730 cm^{-1}. The experimental data (empty circles) are reported together with the best-fit (gray line) and the deconvolution components. Inset: FTIR-ATR spectrum of solid-state inclusion complex β-CD/vanillin in the wavenumber range 1550−1730 cm^{-1}.

wavenumber range 1550−1730 cm^{-1}. Since β-cyclodextrin does not show any interfering vibrational bands,[22,23] the complexation-induced changes in the vanillin spectrum can be easily revealed.[29]

By focusing on the Raman signals associated with the stretching vibration of the C═O group of vanillin, remarkable changes in the spectral shape of these bands can be observed as a consequence of complexation of vanillin with β-CD, both in solid state and in aqueous solution. This finding suggests a great sensitivity of these vibrational modes to the structural modifications of the hydrogen bond networks in which the carbonyl groups of vanillin are involved, in turn related to the aggregation processes occurring among different vanillin molecules. The same sensitivity cannot be envisaged on the analysis of the vibrational peaks between 1550 and 1625 cm^{-1} related to the motions of the C═C bonds of the aromatic ring of vanillin, which show only a slight broadening of the vibrational signals as a consequence of complexation with β-CD, both in solid state and in aqueous solution (Figure 9).

More specifically, the Raman peak found at 1669 cm^{-1} in solid state complex (Figure 9a) and attributable to the C═O stretching mode of vanillin appears shifted at 1684 cm^{-1} in aqueous solution complex (Figure 9b). The shift to higher frequencies of the C═O stretching mode of vanillin gives evidence of a breakdown of the intra- and/or intermolecular hydrogen bond (HB) network in which the carbonyl group is surely involved. This finding is in agreement with other Raman spectroscopy investigations of CD inclusion complexes.[22,23,29]

The spectrum of the solid-state inclusion complex of vanillin with β-CD (Figure 9a) points out the appearance of an additional feature at about 1645 cm^{-1} which is well visible also in the FTIR-ATR spectrum (see inset of Figure 9a) of the complex, but it is not present in the profile of the aqueous solution (Figure 9b). This signal might be attributable to the shift to lower frequency of the intense peak observed at 1663 cm^{-1} in the solid state spectrum of free vanillin therein due to the stretching motion of the C═O group of the dimer of vanillin. This trend could be indicative of the formation, in solid state, of a wider and stronger HB network involving the carbonyl group of vanillin in the complex in comparison with vanillin alone.

CONCLUSIONS

The identification and characterization of phenomena such as self-aggregations and host−guest complexations require "soft" analytical techniques, capable of monitoring the dynamic equilibrium of the system without altering the equilibrium itself. In this work, we mainly exploited DOSY-NMR and Raman spectroscopic techniques to establish whether or not vanillin undergoes aggregation phenomena in aqueous environments, as well as to study its complexation within the β-CD hydrophobic cavity.

The DOSY technique could be reliably applied in the aqueous solutions, and allowed us to quantitatively describe the ongoing phenomena: for the vanillin, we were able to estimate its self-aggregation constant, $K_a = 9.8 \pm 0.7\ M^{-1}$, implying that the aqueous aggregates are just small oligomers; on the other hand, for β-CD aqueous solutions, DOSY did not detect any aggregation phenomenon.

Although we were able to establish a reliable value also for the vanillin/β-CD complexation constant ($K_C = 86 \pm 7\ M^{-1}$), it significantly changed by changing the host/guest initial concentrations, thus indicating that we were dealing with an apparent (conditional) constant. The existence of simultaneous aggregation equilibria is thought to significantly affect the K_c value. It is worth noting that also the vanillin distribution among its self-aggregates is, in turn, concentration-dependent.

The Raman spectroscopy was useful for comparing the solid-state samples (by definition a self-aggregate state) with the corresponding aqueous solutions (where self-aggregation may or may not take place). The spectra of vanillin showed peculiar patterns in good agreement with those expected[24] from DFT computational calculations assuming the vanillin molecules to be organized in dimers, and were in accordance with the already proposed vanillin configuration in the crystal state.[25] In the solid-state inclusion complex, the signals of vanillin (in particular the stretching of the —C═O group) resulted in being slightly shifted toward lower frequency and broader than those of pure vanillin. In addition, we observed the presence of a new signal at intermediate frequency (1644.4 cm^{-1}), presumably caused by the complexation event.

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Notes

The authors declare no competing financial interest.

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