Comparison of Enzymatic Activity and Nanostructures in Water/Ethanol/Brij 35 and Water/1-Pentanol/Brij 35 Systems

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The kinetics of enzyme catalyzed alcohol oxidation has been measured in liquid water/ethanol/Brij 35 and water/1-pentanol/Brij 35 systems, essentially in the water-rich regions. For the ethanol systems it was found that the enzymatic activity sharply decreases with increasing alcohol concentration independently of the surfactant concentration between 0 and 22 mass %. In the case of the 1-pentanol systems the enzymatic activity decreases also with increasing alcohol concentration, but this decrease can considerably be attenuated by adding increasing amounts of surfactant. To explain these results at the nanometer level, small-angle neutron scattering (SANS) experiments have been carried out on these systems. The comparison of the scattering and the kinetic measurements suggests the following interpretation. In all cases, the enzymatic activity depends on the concentration of the alcohol in the aqueous phase or in the aqueous pseudophase containing the enzyme. A certain amount of alcohol may be present in an organic pseudophase formed by direct micelles. In the case of the 1-pentanol systems the alcohol participates in the structuration of the micelles and is concentrated in the micelles, whereas in the case of the ethanol systems the alcohol remains essentially in the aqueous pseudophase and even destroys the micelles. These results suggest that in some cases enzymatic activity can be used as a probe to detect some aspects of the molecular organization of a complex liquid.

1. Introduction

Inverse microemulsions have aroused some interest as possible media for enzymatic reactions.[1−5] In these systems the enzymes are entrapped in the aqueous core of reversed micelles that are distributed in an organic bulk phase. In general, the enzyme substrate is dissolved in the organic phase and it is supposed that it does not markedly alter the nanostructure of the reaction medium. For example, the bio-oxidation of alcohols to aldehydes has often been studied without considering the possible cosurfactant role of alcohols.[6−23] The advantages of such systems are evident: the enzymatic activity can be optimized by choosing a convenient composition of the different constituents. In this way the enzymatic activity can be considerably enhanced compared to the activity in pure aqueous solutions. Even the type of reaction catalyzed by the enzyme can be altered. However, care should be taken when correlations between the molecular structure and the enzymatic activity are discussed.[24]

In contrast to these studies, enzymatic activities in direct micellar systems are rarely investigated.[19] In such media the organic substrate and eventually an organic solvent are entrapped in the micelles, whereas the enzymes are dissolved in the continuous aqueous phase. These essentially aqueous systems are more appropriate for potential industrial use of enzymatic conversion than colloidal systems of the inverse type.

The principal objective of the current paper is to show the relation between enzymatic activity and the nanostructure of the aqueous medium in the case where the substrate participates in the structuration or destructuration of the direct micellar medium. Small-angle neutron scattering is a particularly valuable tool to confirm the existence and to measure the approximate size of nanostructures.

As other authors did in several previous studies,[6−19,25−27] we have chosen alcohol dehydrogenase (HLADH) as the enzyme. This enzyme catalyzes the oxidation of different primary aliphatic alcohols with comparable activities. Owing to its hydrophilic character, HLADH is only active in aqueous phases or pseudophases.

For the media, we have chosen ternary systems water/surfactant (Brij 35)/alcohol, rich in water (direct micelle type) and with amounts of alcohol that can be considerably high.[28] This is in contrast to previous studies in which the enzymatic reaction was followed in pseudoternary systems of the inverse type where the hydrocarbon was the major constituent and only a small amount of alcohol was added.

To distinguish between different structural effects due to the presence of alcohols, we have investigated ethanol and 1-pentanol, the former being known for its effect of destructuration,[29−32] the latter being known for its structure forming or stabilizing effect.[29,33−36]

2. Materials

2.1. Materials Used for the Studies of the Enzymatic Activity. The water was freshly bidistilled, the electrical conductivity being less than 10^{-6} Sm^{-1}. The buffer contained semicarbazide chlorhydrate (purity > 99.5% (CARLO ERBA), concentration $c = 0.075$ M), glycine (SIGMA, $c = 0.1$ M), and 30% aqueous NaOH solution (PROLABO), pH = 8.7). The surfactant was poly(oxyethylene-23) lauryl ether (Brij 35) (Fluka, $CH_3(CH_2)_{11}(OCH_2CH_2)_{23}OH$, molar mass = 1199.8, purity = 99%). The alcohols were ethanol (Fluka, CH_3CH_2OH, purity > 99%) and 1- pentanol (Fluka, $CH_3CH_2CH_2CH_2CH_2OH$, purity > 99%).

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Ethanol is completely miscible with water in all proportions, whereas the maximum solubility of 1-pentanol in water is 2.2 mass % and that of water in 1-pentanol is 10 mass % at $T = 25$ °C. The enzyme was horse liver alcohol dehydrogenase HLADH (Sigma, Batch 119F-8230). Nicotinamide adenine dinucleotide (NAD^+) in its oxidized form (Sigma, Batch 87F-7230, purity 97%) acted as cofactor.

2.2. Materials Used for the Neutron Scattering Studies. The materials are deuterated water, D_2O (EURISO-TOP, degree of deuteration 99.9%), Brij 35 (Fluka), and ethanol (Fluka) and 1-pentanol (Fluka).

3. Methods

3.1. Preparation of the Reaction Mixtures. The reaction mixtures are prepared by adding 25 μL of buffer solution containing 1 g/L HLADH and then 25 μL buffer solution containing the cofactor NAD^+ (20 g/L) to 3 mL of the basic host medium.

The addition of the cofactor starts the following reaction:

$$R{-}CH_2{-}OH + NAD^+ \xrightarrow{\text{HLADH}} R{-}CHO + NADH + H^+$$

with $R = CH_3$ for ethanol and $R = CH_3{-}(CH_2)_3$ for 1-pentanol.

3.2. Determination of Enzymatic Activity. The kinetics of alcohol oxidation was followed spectrophotometrically using a Shimadzu UV 160 spectrophotometer at 25 °C. The progress of the reaction was estimated from the absorption at 340 nm of produced $NADH + H^+$, detected during the first 3 min after the addition of NAD^+. From this result the initial velocity V of the enzymatic reaction was inferred.

Since the continuous phase is water, the enzymatic activity can directly be compared to the corresponding activity in molecular aqueous solutions without micellar structures. Therefore, and in order to reduce the uncertainty of the measured result due to variations of the purity of the enzyme, the activity A was defined with respect to the initial velocity V_o of the same enzyme reaction in an aqueous ethanol solution (concentration of ethanol is 10^{-2} mol/L), $A = V/V_o$. V_o and V were determined under exactly the same experimental conditions. For each of the different reaction compositions four identical samples were prepared and the reaction velocities in them were measured independently at 25.0 ± 0.5 °C in order to verify the reproducibility of the results.

3.3. Preparation of the Samples Used for the Neutron Scattering Experiments. The mixtures studied contained deuterated water (D_2O), Brij 35, and the alcohol in the same molar proportions as those used as the reaction media for the enzymatic activity measurements. The influence of the buffer compounds, of the enzyme, and of the cofactor on the nanostructuration hence was considered to be negligible.

All mixtures were prepared by weighing the three components in 5 mL volumetric flasks closed with Teflon stoppers. As references, a Brij 35/D_2O mixture without alcohol and D_2O/alcohol mixtures without surfactant were also studied.

3.4. Small-Angle Neutron Scattering Experiments. The experiments were performed with the PACE spectrometer at the Laboratoire Léon Brillouin (CEA-CNRS), CE-Saclay, France. All neutron scattering data were collected at 25.0 ± 0.1 °C using fused silica containers of 2.00 mm path length. For each sample two runs were carried out, each run involved recording a spectrum simultaneously at 30 different scattering angles θ using neutrons of wavelengths $\lambda = 6.43$ Å and $\lambda = 9.48$ Å, respectively. The wavenumber transfer $q = (4\pi/\lambda)\sin(\theta/2)$ was in the range 0.006 51 Å$^{-1} \le q \le$ 0.0691 Å^{-1} for the higher wavelength and in the range 0.0277 Å$^{-1} \le q \le$ 0.286 Å^{-1} for the lower one.

To correct for the nonuniformity of the detector response, the data were normalized to scattering from a 1.00 mm thick water (H_2O) sample whose absolute cross section was determined independently. Corrections for background and multiple scattering and further subtraction of the incoherent scattering finally yield the q-dependent absolute values of the coherent intensity $I(q)$ scattered from the nanostructures in units of cm^{-1}. Further information about scattering data processing can be found in a paper by Cotton.[37]

4. Results

4.1. Enzymatic Activity. It should be stressed that in our experiments only the initial velocities of the enzymatic reactions were recorded. The interesting question of how the production of aldehyde alters the medium structure and how this changes the enzymatic activity will be the subject of a further study. Such a dynamic interaction between the progressive modification of the nanostructured media and enzymatic activities has already been investigated for another enzymatic reaction.[38]

In a first series of experiments the influence of the surfactant Brij 35 on the catalytic activity of HLADH was tested. For this purpose the enzymatic oxidation of ethanol has been followed in an aqueous medium at small noninhibiting substrate (ethanol) concentrations (10^{-2} M/*L*) and various surfactant concentrations. These tests showed that Brij 35 had only a small inhibition effect on the order of 10% on the enzymatic activity. This small inhibition effect remained approximately constant for surfactant concentrations between 1 and at least 22 mass %. Therefore, all further experiments were carried out for Brij 35 quantities in this concentration range.

4.2. Catalytic Activity of HLADH in Continuous Phase Systems Containing Water/Ethanol/Brij35. In Figure 1 we report the results obtained for the systems containing ethanol as the substrate for the enzymatic reaction. Figure 1a shows the phase diagram taken from ref 28 where the way it has been established is also indicated. Figure 1 also contains different points of composition for which the enzymatic activity was determined. The activity A is given in Figure 1b as a function of the ratio between the mass % of the substrate and the mass % of water, $R_{c/w}$, for different concentrations of Brij 35, P_s, also given in mass %. It can be seen that the enzymatic activity decreased nearly exponentially as the substrate concentration increased, suggesting a significant subtrate inhibition of the enzyme. Furthermore, the activity was very similar for the pure water/ethanol mixtures and the systems containing the surfactant at two different concentrations. In all three cases, for a ratio $R_{c/w} = 1$ the activity was only about 10% of the reference activity in a pure aqueous solution without Brij 35 and with a very small substrate quantity.

4.3. Catalytic Activity of HLADH in Continuous Phase Systems Containing Water/1-Pentanol/Brij 35. Figure 2 shows the corresponding results for 1-pentanol systems. The phase diagram is also taken from ref 28. Without Brij 35 ($P_s = 0$) monophasic systems were only obtained for $R_{c/w}$ ratios smaller than 0.022. This point corresponds to the maximum solubility of this alcohol in water. In this limited concentration range, the catalytic activity of HLADH strongly decreased with increasing alcohol concentration, the global activity loss ΔA being equal to about 20%. Substrate inhibition similar to that assumed for the ethanol systems is the most probable explanation.

Adding 1 mass % of Brij 35 ($P_s = 0.01$) neither increased considerably the solubility of 1-pentanol nor altered significantly

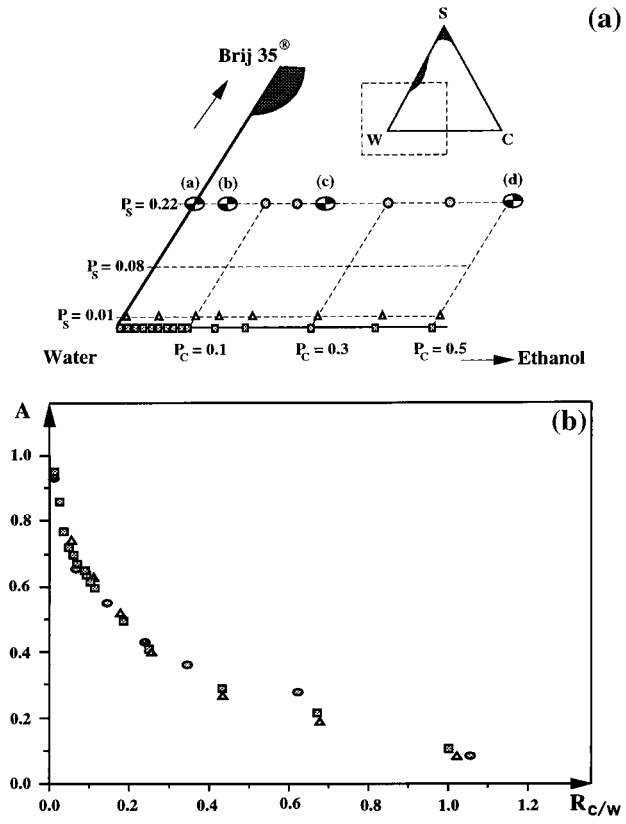

Figure 1. (a) Ternary system "aqueous buffer solution/ethanol/Brij 35" at 25 °C. The water-rich part of the whole phase diagram, shown in the insert, is given as well as the different compositions used for the enzymatic studies and the neutron-scattering measurements. The bright zones denote macroscopically homogeneous phases of low viscosity. The dark zones give the compositions of either polyphasic or crystal-like systems. P_s is the quantity of the surfactant S (Brij 35), expressed in mass % of the whole composition, and P_c is the quantity of alcohol (C) in the same units. The points symbolized by □, △, and ○ denote the compositions for which enzymatic activities were measured. The corresponding results are given in part b. The points symbolized by the partially shaded circles and labeled a, b, c, and d refer to the compositions of the systems for which scattering experiments were carried out. The corresponding results are given in Figure 3. (b) Enzymatic activity of HLADH (relative to a pure aqueous solution containing 10^{-2} mol/L of ethanol) as a function of the ratio of the mass % of ethanol to the mass % of water, $R_{c/w}$. □, △, and ○ correspond to surfactant concentrations of $P_s = 0$, 0.01, and 0.22, respectively. The different $R_{c/w}$ ratios correspond to the series of the P_c values given in part a.

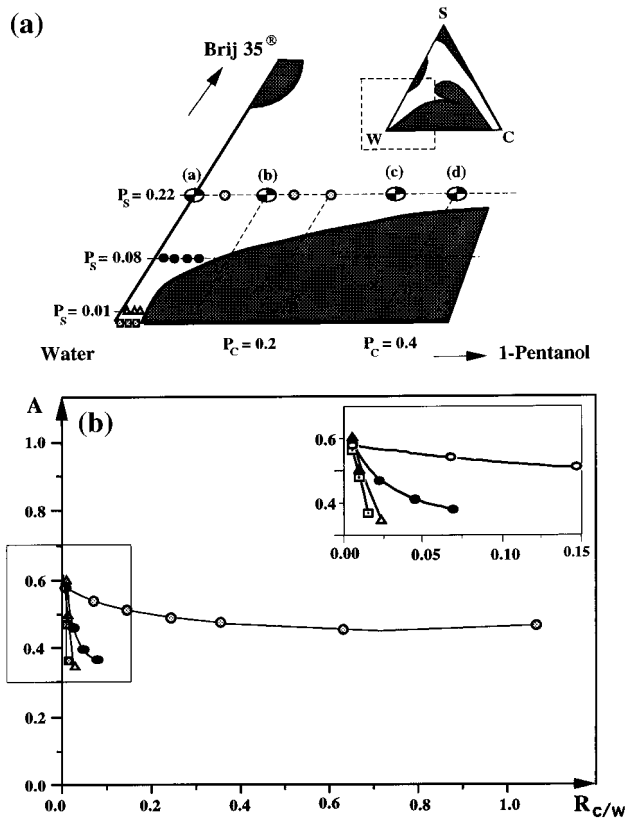

Figure 2. (a) Ternary system "aqueous buffer solution/1-pentanol/Brij 35" at 25 °C. The water-rich part of the whole phase diagram, shown in the insert, is given as well as the different compositions used for the enzymatic studies and the neutron-scattering measurements. The bright zones denote macroscopically homogeneous phases of low viscosity. The dark zones give the compositions of either polyphasic or crystal-like systems. P_s is the quantity of the surfactant S (Brij 35) expressed in mass % of the whole composition, and P_c is the quantity of alcohol (C) in the same units. The points symbolized by □, △, ●, and ○ denote the compositions for which enzymatic activities were measured. The corresponding results are given in part b. The points symbolized by the partially shaded circles and labeled a, b, c, and d refer to the compositions of the systems for which scattering experiments were carried out. The corresponding results are given in Figure 4. (b) Enzymatic activity of HLADH (relative to a pure aqueous solution containing 10^{-2} mol/L of 1-pentanol) as a function of the ratio of the mass % of 1-pentanol and the mass % of water, $R_{c/w}$. □, △, ●, and ○ correspond to surfactant concentrations of $P_s = 0$, 0.01, 0.08, and 0.22, respectively (see part a). The different $R_{c/w}$ ratios correspond to the series of the P_c values given in part a. The insert of part b is an enlargement of the activity curves at low 1-pentanol concentration.

the decrease of activity as a function of 1-pentanol concentration.

For $P_s = 0.08$ monophasic systems were obtained up to $R_{c/w} = 0.07$. ΔA, the global activity loss within this alcohol concentration range, is 21%, which is similar to those obtained for $P_s = 0.0$ and $P_s = 0.01$. Note, however, that the activity decreased less sharply with increasing pentanol concentration for $P_s = 0.08$ than in the two preceding cases. Apparently, the enhanced surfactant concentration had a positive effect on the catalytic activity.

This positive influence is clearly visible at high surfactant concentration ($P_s = 0.22$). First, this Brij 35 concentration permitted a high substrate content (40 mass % of 1-pentanol in the whole system) to be obtained corresponding to an alcohol/water weight ratio of $R_{c/w} = 1$. Second, after an initial moderate decrease of the activity in the range $0.006 < R_{c/w} < 0.15$ ($\Delta A = 7\%$) A varied only a little and remained at an enhanced level of about 0.5. The apparent activity even seemed to increase slightly at the highest $R_{c/w}$ ratio, but further experiments will be necessary to check this point.

It is interesting to compare the different average losses of activity per change in weight ratio of 1-pentanol/water, i.e., $\Delta A/\Delta R_{c/w}$. This quantity is only about 0.5 in the interval $0.006 \leq R_{c/w} < 0.022$ for $P_s = 0.22$, about 3 for $P_s = 0.08$ and about 20 for $P_s = 0.01$ and $P_s = 0$. It is thus evident that the surfactant has a positive influence on the enzymatic activity in the case of 1-pentanol oxidation in contrast to ethanol oxidation.

4.4. Neutron Diffraction. Figure 3 shows some of the spectra obtained for the binary systems D_2O/Brij 35 and the ternary systems D_2O/Brij 35/ethanol at surfactant concentration $P_s = 0.22$. Whereas for binary water/Brij systems structural studies are known,[39,40] this is not the case for the ternary systems. A detailed quantitative simulation of these spectra based on the Percus−Yevick integral equation system[41] is currently under way in our laboratory. In the present paper, it is sufficient to discuss qualitatively the neutron results in order to understand the relation between the molecular architecture of the systems and the enzymatic activities.

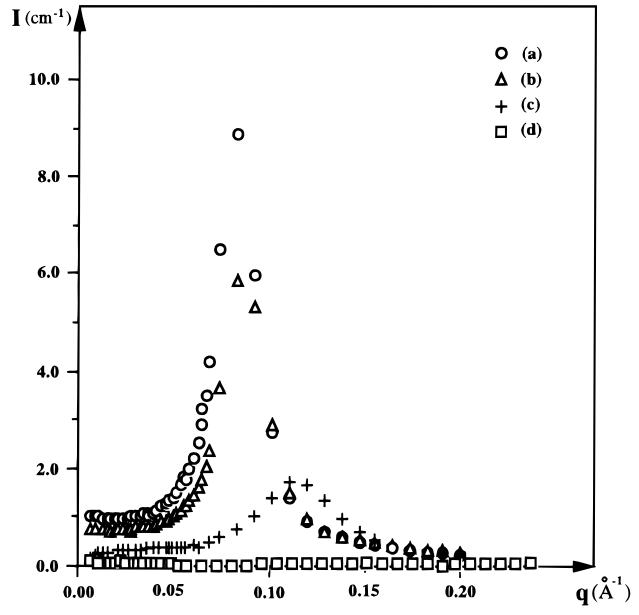

Figure 3. Absolute neutron scattering intensities *I* of different ternary systems "water/ethanol/Brij 35" at 25 °C as a function of wavenumber transfers *q*. In all cases $P_s = 0.22$ (cf. Figure 1a). The ethanol concentrations are $P_c = 0$ (a), 0.055 (b), 0.20 (c), and 0.50 (d), according to the points a−d symbolized by the partially shaded circles in Figure 1a.

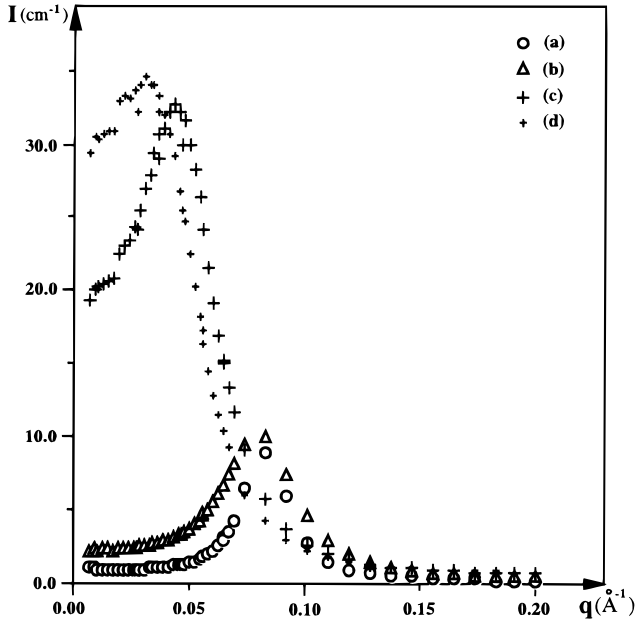

Figure 4. Absolute neutron scattering intensities *I* of different ternary systems "water/1-pentanol/Brij 35" at 25 °C as a function of wavenumber transfers *q*. In all cases $P_s = 0.22$ (cf. Figure 2a). The 1-pentanol concentrations are $P_c = 0$ (curve a, i.e., the same curve as Figure 3a), 0.106 (b), 0.30 (c), and 0.40 (d) according to the points a−d symbolized by the partially shaded circles in Figure 2a.

As can be seen, the system without alcohol (curve a) exhibits a pronounced peak at wavenumber transfers of about 0.085 Å^{-1}. This peak can be attributed to the nanostructuration of the medium, or more precisely to the presence of micelles. Preliminary Percus−Yevick results suggest that the volume fraction of these objects is very high (about 60%). Therefore, it is reasonable to assume that the peak corresponds to the average distance between the centers of two adjacent micelles in a quasi-crystalline order. If one further assumes that the micelles are spherical and that they touch each other, one can roughly infer an estimation of the size of the objects according to the formula $d = 2\pi/q$ where *d* is the micellar diameter. Such a procedure yields a value of *d* on the order of 70−80 Å.

The result is roughly the same when 5.5 mass % of ethanol is present in the system (see curve b). The micelles are still present and their average size remains nearly unchanged. The absolute scattering intensity at the peak maximum is reduced. This can be explained as follows. The alcohol molecules containing six hydrogen atoms are mainly dissolved in the bulk phase containing deuterated water. Owing to the different sign of the scattering lengths of hydrogen and deuterium atoms, such a mixture of deuterated water and nondeuterated alcohol has a lower scattering contrast than a pure deuterated continuous phase with respect to the micelles, which are essentially made up of nondeuterated material.

At a concentration of 20 mass % of ethanol, micelles were still present. However, their average size was considerably smaller (see curve c).

For very high ethanol concentrations (50 mass %) the situation is qualitatively different. As curve d confirms, the micelles disappeared and no structuration can be detected. Note that even at this elevated alcohol concentration the scattering contrast would have been sufficient to detect a micellar structuration. Obviously, with increasing ethanol concentration the micellar structure is progressively destroyed and at 50 mass % of ethanol this ternary system is a pure molecular solution.

Let us now compare these results to those obtained for the systems containing 1-pentanol (see Figure 4). Obviously, the addition of 10.62% of 1-pentanol (with respect to the total mass of the system) does not significantly alter the nanostructure of the medium: the spectra given by curves a and b of Figure 4 are similar. In particular, the position of the peak maximum is nearly the same in both cases. The absolute scattering intensity is slightly higher in the case of the ternary system. This observation suggests that the alcohol molecules, containing 11 hydrogen atoms, have preferentially replaced the D_2O molecules that hydrate the surfactant in the micelles of the binary system. The number of hydrogen atoms in the micelles increases and consequently also the contrast increases between the signal of the micelles and the mainly deuterated continuous bulk medium.

Further increase of the amount of 1-pentanol in the systems has two consequences, as shown by the curves c and d of Figure 4. First, the absolute coherent scattering intensities are considerably enhanced up to values of about 35 cm^{-1} for $R_{c/w} = 1.05$. The reason is the same as that discussed in the previous case with 10.62% pentanol: more and more alcohol enters the micelle structure, which leads to an increase in the scattering contrast. Second, the peak maxima are more and more shifted to smaller *q* values, indicating that the micelles swell more and more. For the system containing 40% of 1-pentanol, the average micellar diameter is on the order of 200 Å. Of course, such a huge increase in the micelles' average size cannot be sufficiently explained by the increase in 1-pentanol concentration. It is probable that at the same time a reorganization of the micellar structure of the medium takes place.

5. Discussion

HLADH is a water soluble enzyme, the activity of which is apparently strongly inhibited by increasing amounts of substrate (alcohol molecules), whereas it is only slightly influenced by the presence of Brij 35 surfactant molecules. Measuring the kinetics of reactions catalyzed by HLADH can therefore be a valuable tool to determine the actual alcohol concentrations in aqueous systems and in aqueous pseudophases as has been shown in the present paper for systems of the direct micellar type.

(a) Systems Containing Ethanol as the Substrate for the Enzymatic Reaction. Ethanol is completely miscible with water, as shown by the phase diagram in the insert of Figure 1a, and increasing ethanol concentration continuously decreases the enzymatic activity (Figure 1b). A nanostructuration of the aqueous system by the presence of Brij 35 molecules forming micelles does not significantly alter the enzyme activity profiles. This means that ethanol is not concentrated in the micelles but essentially remains in the continuous aqueous phase. The neutron spectra (Figure 3) confirm this result: for low ethanol concentrations (5.5%) the micelles remain approximately unchanged and the ethanol molecules are simply distributed in the aqueous bulk phase in a molecular way (see Figure 3b). In the case of very high ethanol concentrations ($R_{c/w} = 1.05$) no nanostructuring can be detected. Obviously, in this system, the ethanol molecules prevent the surfactant molecules from forming micelles, and thus water, ethanol, and Brij 35 molecules constitute a true molecular solution (Figure 3d). Consequently, the ethanol molecules are accessible to the enzyme molecules in all cases studied here.

(b) Corresponding 1-Pentanol Systems. From the results shown in Figures 2 and 4 it is clear that the increasing solubility of 1-pentanol with increasing surfactant concentration is due to the nanostructuration of the system. The 1-pentanol molecules are incorporated in the organic pseudophase. The neutron-scattering results confirm the existence of micelles for the highest surfactant concentration, and they also show that an increasing amount of 1-pentanol in the system leads to a swelling of the micelles. The enzyme activity also confirms the existence of an organic pseudophase and yields even more refined information about the partition of the alcohol concentration between the aqueous and the organic pseudophases. At constant $R_{c/w}$ values (<0.022) the enzyme inhibition by the substrate progressively decreases with increasing P_s values (see the insert of Figure 2b). This fact can only be explained by a diminution of the 1-pentanol concentration in the aqueous pseudophase in which the reaction takes place and consequently by an increasing amount of 1-pentanol in the organic pseudophase with increasing surfactant concentration.

At $P_s = 0.22$ the enzymatic activity is nearly independent of $R_{c/w}$, at least for $R_{c/w}$ values above 0.3. This behavior is probably due to a constant ratio between the concentrations of the enzyme and of the alcohol in the aqueous pseudophase, but a reorganization of the nanostructure of the whole system cannot be excluded. Further experiments will be necessary to clarify this point.

6. Conclusion

Enzymatic alcohol oxidation was studied in ternary systems water/Brij 35/alcohol. The results show that the enzymatic inhibition is very different for ethanol and 1-pentanol as far as its dependence on surfactant concentration is concerned. Neutron-scattering data show that in both systems micelles exist, and this experimental technique also permits one to follow the variation of their sizes and their possible disappearance as a function of the composition of the liquid system. In the case where the substrate participates in the structuration of the systems, the existence of nanostructures can also be inferred from the measurement of enzymatic activities. In this respect both techniques yield comparable information. However, whereas the scattering technique gives directly information about the geometry of these structures, the enzymatic activity can be a sensitive probe to detect small substrate variations in the aqueous pseudophase, provided that the substrate concentration in it is not too high, as it is the case of the ethanol system. Enzymatic activity measurements can then be used to determine the partition of alcohols between different pseudophases in macroscopically homogeneous liquids. Therefore, such measurements can be an alternative where other techniques such as gas chromatography[42] or NMR[43] are not usable. However, a quantitative verification remains to be done. Qualitatively, the enzymatic activity measurements allowed us to confirm that 1-pentanol participates in the formation of the organic pseudophase whereas ethanol does not.

In principle, the absolute scattering intensities permit quantitative modeling, for example, via the Percus−Yevick equation theory.[41,44] However, preliminary calculations showed that the underlying potential cannot be inferred without ambiguity probably because of the lack of sufficient spectra and contrast matching data.[44] Furthermore, the micellar concentration is very high in our case, nonsphericity and polydispersity of the objects is probable, and the unknown configuration of the long hydrophilic part of the surfactant molecules makes a definition of the average size of the micelles difficult. The quantitative discussion of the data will therefore be left to a following paper.

Finally, it is worth noting that such nanostructured systems can be of interest for the conception of reactional media for substrate-inhibited enzymatic reactions at an industrial level because these media allow considerable substrate concentrations to be incorporated in the system without a heavy loss of enzyme activity.

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