Colloid & Polymer Science Colloid Polym Sci 272:1098–1103 (1994)

Characterization of lecithin-based microemulsions used as media for a cholesterol oxidase-catalyzed reaction

S. Backlund, M. Rantala and O. Molander

Department of Physical Chemistry, Åbo Akademi University, Turku, Finland

Abstract: Microemulsions stabilized by soybean lecithin and ethanol have been characterized with respect to phase behavior, distribution of the ethanol cosolvent, conductivities, viscosities, and volume fractions of the different phases in Winsor III systems. The conductivities and viscosities of the surfactant-rich phase in the Winsor III system indicate that this phase exhibits a bicontinuous structure. The reaction yield at 298.2 K for the enzymatic conversion of cholesterol to cholestenone by cholesterol oxidase performed in a Winsor III system containing water, lecithin, hexadecane, and ethanol is low.

Key words: Microemulsion – soybean lecithin – partial phase equilibria – ethanol distribution – enzyme catalysis – cholesterol

Introduction

In recent years, water-in-oil microemulsions have been used as reaction media for enzyme catalysis [1–4]. Especially, enzymes acting on reactants that are insoluble or poorly soluble in water have been studied intensively. To optimize enzyme reactions in these media it is of major importance to characterize the microemulsion systems, i.e., to determine phase diagrams and to investigate their structures at different compositions. Microemulsions possess several advantages as reaction media; they provide a large interface between oil and water, the water content in the microemulsion can be varied within a large interval, and phase separation of the products is possible. The large interface, where the catalysis is supposed to take place, makes the enzymatic reaction more rapid and gives a better yield [5–7].

The aim of this work is to find a suitable Winsor III system stabilized by the natural product lecithin, which can be used as reaction medium for the catalytic oxidation of 5-cholesten-3β-ol (cholesterol) to 4-cholesten-3-one (cholestenone). In Winsor III systems the surfactant-rich phase is in equilibrium with excess oil and water phases. Due to this, the used water soluble enzyme, cholesterol oxidase, and the oil soluble reactant, cholesterol will be dissolved in a Winsor III system. Furthermore, in this type of reaction media the oil and water soluble products will separate spontaneously. The enzyme-catalyzed reaction is supposed to take place at the interfaces between oil and water microdomains in the middle phase. This means that only enzymes that are surface active can be used.

Soybean lecithin used in this work is both strongly hydrophobic, due to the two long hydrocarbon chains, and strongly lipophobic due to the zwitterionic polar head groups. At room temperature soybean lecithin and water form a lamellar liquid crystalline phase between 0.35 and 0.07 mass fraction of water [8]. In water-oil systems lecithin is slightly too lipophilic to form microemulsions spontaneously. On the other hand, Shinoda et al. [9] have shown that lecithin forms microemulsions with bicontinuous structures in a 1-propanol-water-hexadecane system. This means that lecithin forms microemulsions by adding a short-chain alcohol to the system. A surfactant phase with a bicontinuous structure consists of intertwined water and oil lamellae

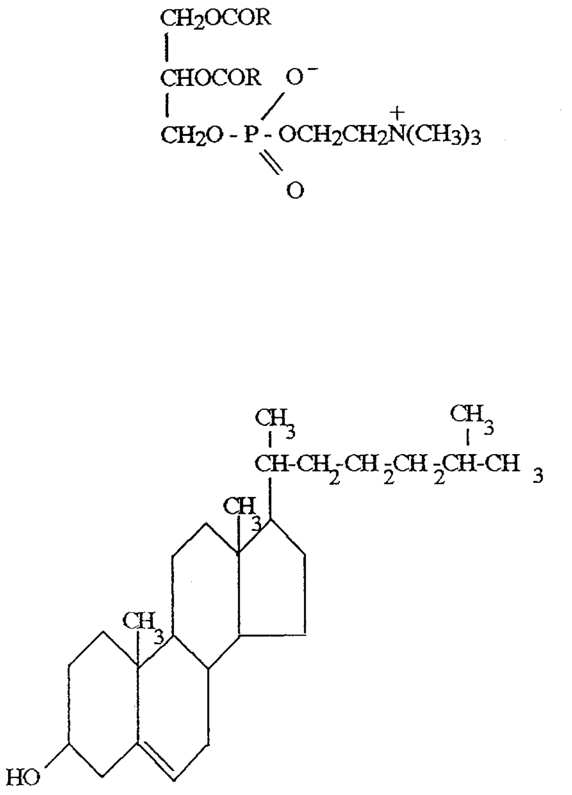

Fig. 1. Lecithin and cholesterol.

separated by a surfactant film with low or zero curvature, and hence it has the highest mutual solubility between oil and water [2, 9, 10]. For the highest possible catalytic effect of enzyme, an oil with the logarithm of the partition coefficient between octanol and water higher than 4 is preferable. This is the case when hexadecane or tetradecane is used [11]. At high alcohol contents cholesterol oxidase becomes inactive [7], so one task is also to minimize the amount of ethanol used in these systems.

Materials and methods

Chemicals

Soybean lecithin (Epikuron 200) was a gift from Lucas Meyer Co., FRG (Fig. 1). The distribution of fatty acids was, according to the producer: palmitic and stearic acid 16–20%, oleic acid 8–12%, linoleic acid 62–66%, linolenic acid 6–8%. Hexadecane and tetradecane were from Sigma Chemicals, USA. Hexane, 1-propanol and 2-propanol were obtained from Merck, FRG, and absolute ethanol from Oy Alko Ab, Finland. They all had an estimated purity > 99%. The water was distilled and deionized immediately before use. Cholesterol oxidase (EC 1.1.3.6; *Brevibacterium sp.* 4.8 U/mg) was from Beckman, USA, and cholesterol, N-(tris-(hydromethyl)-methyl-2-aminoethane-sulfonic acid (TES), 5-cholesten-3β-ol-7-one(7-ketocholesterol) and bis(trimethylsilyl)-trifluoroacetamide (TMS) for silylation were all from Sigma Chemicals.

Phase behavior

The phase behavior was determined by preparing samples with fixed compositions. The samples were thoroughly shaken and then allowed to equilibrate in a thermostated bath at 298.2 K for 2 days.

Distribution of alcohol

The distribution of ethanol between oil and water was determined gas-chromatographically. The samples were injected into a Varian 3400 instrument equipped with a Quadrex column (Quadrex Scientific 25 m × 0.53 mm with a 5.0 μm film thickness). These results were compared with results from density measurements. Densities of separated phases were measured with an Anton Paar DMA 602 Density Meter at 298.2 K.

Conductivity and viscosity

Conductivity measurements were made with a Seibold Wien Conductometer and viscosity measurements with Ostwald viscometers at 298.2 K.

Enzymatic reaction

Cholesterol (5 mM) and cholesterol oxidase (2 U cm^{-3}) were added to a Winsor III system. Samples were taken both from the oil and the microemulsion phases after given time intervals for analysis. Water and a solution of hexane and 2-propanol were added to the samples. The resulting two-phase systems were thoroughly shaken and then centrifuged for 10 min at 1500 rpm.

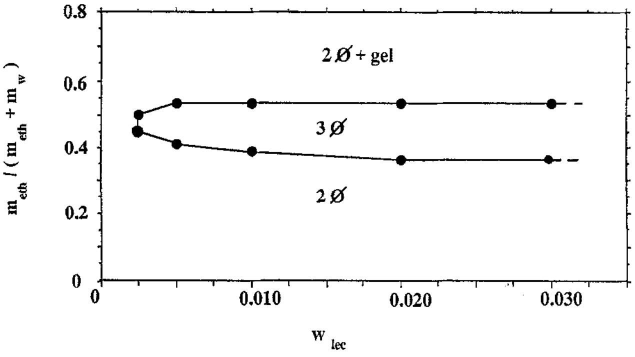

Fig. 2. Partial phase diagram at 298.2 K for the system lecithin-tetradecane-ethanol-water at $m_{oil}/(m_{water} + m_{ethanol}) = \alpha = 1$. N$\Phi$ denotes number of phases and m_i is the mass of component i.

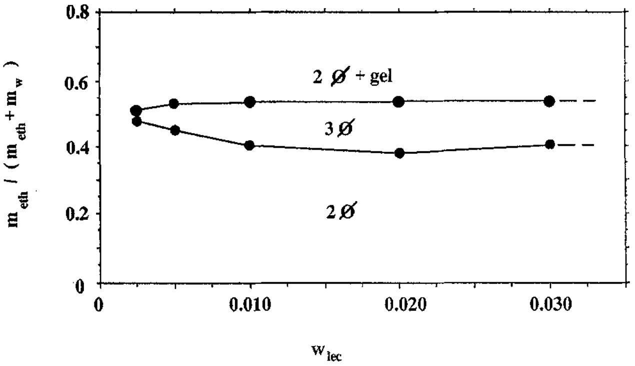

Fig. 3. Partial phase diagram at 298.2 K for the system of lecithin-hexadecane-ethanol-water at $\alpha = 1$.

From the organic phases new samples were taken and then evaporated together with an internal standard. TMS was added and the samples were kept for 1 h at 343.2 K. The samples were then analyzed gas-chromatographically. The used column was a DB-5 column (J&W Scientific; 30 m × 0.252 mm with a 0.25 μm film thickness).

Results and discussion

Figures 2 and 3 show partial phase diagrams for the tetradecane and hexadecane systems. At low ethanol contents two-phase regions are present. At almost equal ethanol and water contents the systems separate into three phases, an aqueous, a microemulsion (S), and an organic phase.

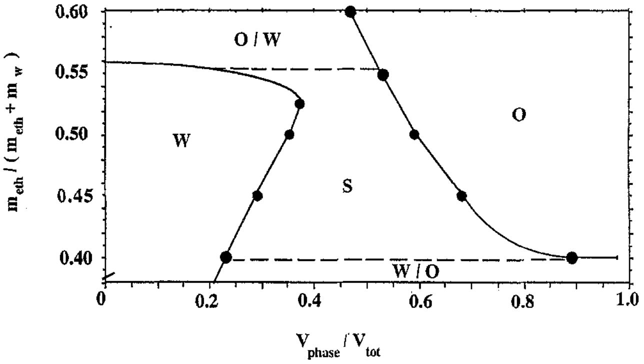

Fig. 4. Volume fractions of the phases at 298.2 K for the system in Fig. 2 at $\alpha = 1$. The mass fraction of lecithin, w_{lec}, is 0.03.

At even higher ethanol contents a two-phase region appears with a feature of gel. Preliminary investigations show that different oil to water + oil mass ratios do not significantly change the extension of the three-phase area. Therefore, only systems with the ratio 1:1 have been studied. Ahsan et al. [2] have shown that more 1-butanol per surfactant molecule is required to invert the systems from Winsor I to Winsor III containing tetradecane compared to those containing heptane or decane. Shorter alkanes penetrate into the chain regions of surfactant monolayers, at both planar and curved interfaces, more strongly than longer alkanes. They concluded that 1-butanol performs as a cosurfactant by penetrating into the surfactant region and thereby changing the curvature of the interface between oil and water. On the other hand, only somewhat more ethanol is needed to reach a Winsor III system in hexadecane systems than in tetradecane systems. The explanation is that ethanol is only marginally penetrated in the surfactant region in our systems. Thus, it works as a cosolvent rather than as a cosurfactant.

Figures 4 and 5 show how an increased alcohol content affects the volume fractions of the different phases in the Winsor systems at $w_{lec} = 0.03$. Here again, the difference between tetradecane and hexadecane is minimal. It can also be noticed that a decrease in the lecithin content decreases the volume fraction of the surfactant phase.

The distribution of ethanol between the phases in the Winsor III systems at $w_{lec} = 0.03$ is shown in Figs. 6 and 7. For the hexadecane system both

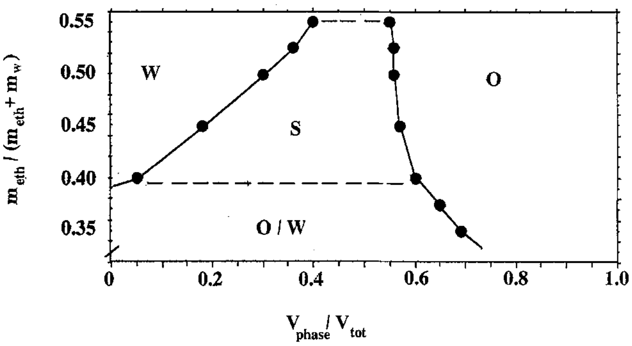

Fig. 5. Volume fractions of the phases at 298.2 K for the system in Fig. 3 at $\alpha = 1$ and $w_{lec} = 0.03$.

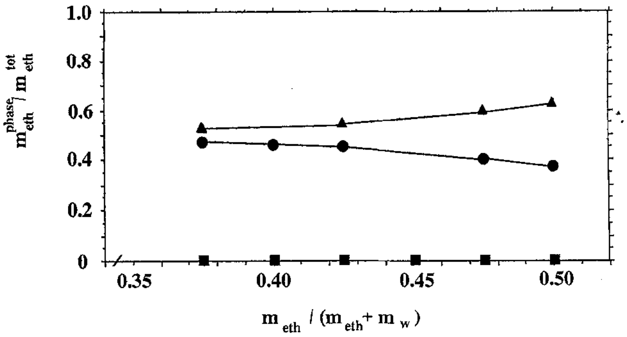

Fig. 6. Distribution of ethanol at varying ethanol contents at 298.2 K for the system in Fig. 2 at $\alpha = 1$ and $w_{lec} = 0.03$. (■) organic phase, (●) surfactant phase, (▲) water phase

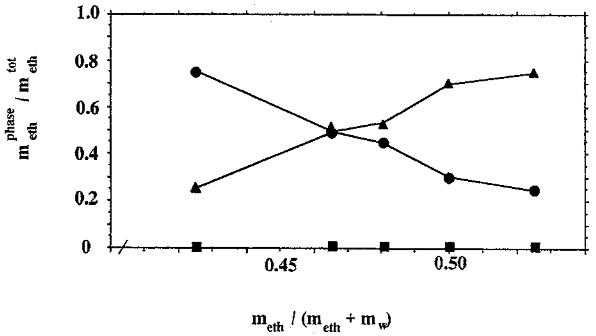

Fig. 7. Distribution of ethanol at varying ethanol contents at 298.2 K for the system in Fig. 3 at $\alpha = 1$ and $w_{lec} = 0.03$. The symbols are the same as in Fig. 6.

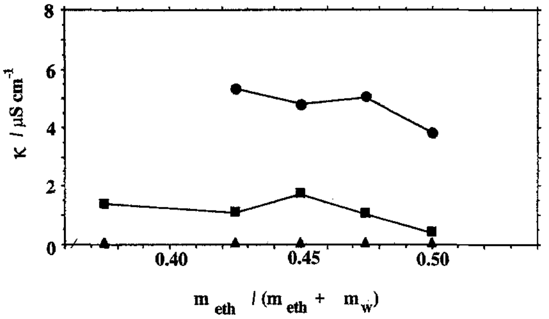

Fig. 8. Conductivities at 298.2 K at varying ethanol contents of the different phases for the system in Fig. 2 at $\alpha = 1$ and $w_{lec} = 0.03$. (▲) organic phase, (■) surfactant phase, (●) water phase

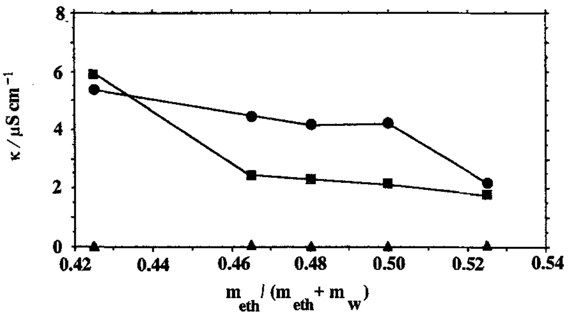

Fig. 9. Conductivities at 298.2 K at varying ethanol contents of the different phases for the system in Fig. 3 at $\alpha = 1$ and $w_{lec} = 0.03$. The symbols are the same as in Fig. 8.

the GC and density measurements gave quite similar results. However, in the tetradecane system the density measurements do not agree with the results from GC measurements. Probably some lecithin dissolves in the organic phase, thereby disturbing the density measurement. No ethanol could be detected in the organic phase and the ethanol distribution between the aqueous and surfactant phases follows the regular pattern.

Macroscopic properties like conductivity and viscosity are related to the microstructures of microemulsions. Conductivity is a measure of the mobility of ions in the aqueous domains and depends on the local viscosity. Bulk viscosity again mirrors the interactions between microstructures. In Winsor III systems the aqueous phase exhibits the highest, the organic phase the lowest, and the surfactant phase an intermediate conductivity [12]. This is also the case for these studied systems, as can be seen from Figs. 8 and 9. As expected, the conductivities in the organic phases are orders of magnitude lower than the conductivities in the other phases. The

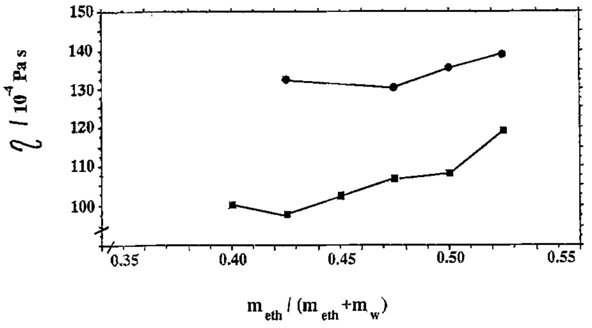

Fig. 10. Viscosities at 298.2 K of the surfactant phases at varying ethanol contents. The tetradecane system (■) and the hexadecane system (●).

conductivities in the surfactant phases are only about 0.35 times the conductivities in the aqueous phases. Shinoda et al. [9] found, depending on the alcohol content, that either discrete droplets or bicontinuous microemulsions were formed in the system H_2O-lecithin-hexadecane-1-propanol. According to Eastoe et al. [13], the conductivity is low for W/O-microemulsions composed of discrete droplets and is of the order of 10^{-9}–10^{-7} ohm^{-1} cm^{-1}. The conductivities for the surfactant phases in Figs. 8 and 9 are thus more than one decade higher than the conductivities for systems with closed structures. When sodium chloride was added to our systems the conductivities further increased. Maitra et al. [14] have shown that the conductivity at the threshold water volume fraction increases drastically and that the relative self-diffusion coefficient of water also increases at somewhat higher water volume fractions in the 0.5% brine-AOT-isooctane system as a result of the percolation process of the droplets and the formation of bicontinuous structures, respectively. Their results depict that the translational mobility of water starts increasing only when the droplets are opened up into a structure with both the oil and water as the continuous medium. The values for the conductivities of the surfactant phases in Figs. 8 and 9 and the increased conductivity upon addition of NaCl can thus be attributed to bicontinuous structure in our surfactant phases. Figure 10 presents the viscosities of the surfactant phases for the two systems as a function of increased ethanol content. There is no significant difference between the viscosities of the systems. Depending on the high surfactant content in the middle phase of a Winsor III system, this phase usually exhibits the highest viscosity of the three phases. However, the viscosities are somewhat lower than for the phases with bicontinuous structures stabilized by ionic surfactants and almost equal to viscosities for W/O-microemulsions stabilized by nonionic surfactants [5, 6].

To our knowledge, only one investigation on the use of a Winsor III system for enzymatic reactions has been reported in the literature [10]. The results show that lipase catalyzed hydrolysis of trimyristin to 2-myristoylglycerol and sodium myristate can be performed with good yield. Therefore, we examined the catalytic oxidation of cholesterol to cholestenone and H_2O_2 in a Winsor III system composed of lecithin-alkane-water-ethanol as reaction medium. This oxidation has also been studied by Hedström et al. [17] in W/O microemulsions. For performing syntheses with microemulsions as reaction media the contents of the reactants have to be much higher than usually used in enzyme-kinetic studies in these media. This again causes problems in controlling phase behaviors of microemulsions. Addition of cholesterol and cholesterol oxidase to our Winsor III system at $w_{\mathrm{lec}} = 0.03$, with tetradecane as the oil, inverted it to a Winsor II system. Therefore, we examined only reactions in media with hexadecane as the oil.

The enzyme activity is enhanced when ethanol is added to an aqueous solution of cholesterol oxidase. McGuinness et al. [7] ascribed this activation to a conformational change in the enzyme mediated through the altered structuredness of water. This enhanced activity is confined to the mass fraction range of 0.17–0.42 for ethanol, but is abolished in the presence of high surfactant contents. The mass fraction of ethanol is 0.3 in the surfactant phase in our system, i.e., the enzyme activity is apparently enhanced. However, the lecithin content in the surfactant phase is too high, which to some extent explains the low yield of cholestenone in Fig. 11. In our case, microemulsions with bicontinuous structures are present in the surfactant phase and the substrate cholesterol is distributed between the oil continuous medium and the aqueous/oil interface. Large amounts of cholesterol will also be distributed in the excess organic phase. On the other hand, the enzyme cholesterol oxidase is distributed between the water continuous medium in the surfactant phase and the excess water phase. According to Burns

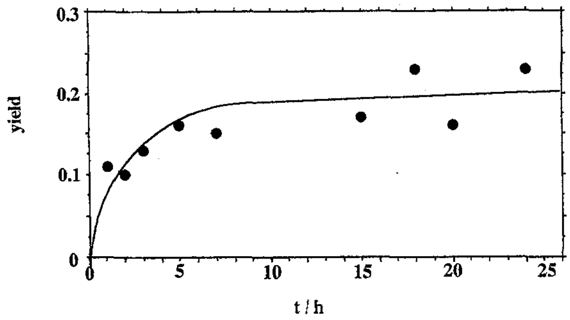

Fig. 11. The cholestenone yield for the enzymatic conversion of cholesterol at 298.2 K in the Winsor III system containing lecithin-hexadecane-ethanol-water as a function of time.

[3] cholesterol oxidase is not surface active enough to be accumulated at interfaces. This means that the enzyme distributed in the excess aqueous phase has no substrate to catalyze and the cholesterol distributed in the organic phase has no enzyme catalyzing it. All these disadvantages contribute to the low reaction yield in Fig. 11. The reaction yield was analyzed at 298.2, 308.2, and 318.2 K, respectively. The best reaction yield was obtained at the lowest temperature. This can probably be explained by a change in the distribution of enzyme and cholesterol between the phases in the Winsor III system with increased temperature. Addition of TES buffer (initial pH = 7.5) to the Winsor III systems did not increase the yield above 0.2.

Biocatalysis in Winsor III systems is of much interest and will be the subject of more detailed analysis in a subsequent report, where the effect of other cosolvents, e.g., 1-propanol, on the conversion of cholesterol to cholestenone by cholesterol oxidase will be discussed.

Acknowledgements

This research has been supported by the Technology Development Centre, Finland (TEKES).

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Received June 23, 1993;
accepted November 25, 1993

Authors' address:

Maria Rantala
Department of Physical Chemistry
Porthansgatan 3-5
FIN-20500 Åbo, Finland