Segregation of Lipid and Polymer in Emulsion Droplets Captured by Confocal Laser Scanning Microscopy

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Received: October 10, 2004; In Final Form: April 11, 2005

The phase behaviors of the subsystems of the ethyl acetate (EtAc)/monoolein/polyethylene glycol−poly(D,L-lactide-*co*-glycolide) (PLG)/water system have been determined. EtAc simultaneously solves MO and PLG in a liquid phase, denoted L. Lipid/polymer composite particles have here been formed by emulsification of such an L phase into aqueous solutions. Characterization, by means of confocal laser scanning microscopy, revealed that distinctive lipid domains appear inside the particles. In aqueous solutions, these lipid domains swell and finally leave the concentrated polymer matrix. The system exhibits a suitable phase behavior in order to form lipid/polymer composite particles. These composite particles may be interesting for drug delivery applications.

Introduction

By use of composite materials to deliver active substances, it is often possible to obtain desirable properties of a drug formulation, e.g., high efficiency of encapsulation or good protection from hostile environments. Biodegradable polymers are, together with bilayer-forming lipids, two examples of interesting components for formation of controlled-release drug delivery systems. Poly(D,L-lactide-*co*-glycolide) (PLG), a biodegradable polymer, has during the last decade been widely adopted in the medical field and some types are approved by the FDA. For instance, PLG has been used in sutures, injectable gels,[1−2] in situ forming implants,[3] and microparticles.[4] However, since PLG degrades into lactic and glycolic acids,[5] a pure PLG-based formulation is not optimal for delivery of substances sensitive to low pH.[6]

Monoolein (MO), a polar lipid, which is a metabolite during fat digestion, forms two cubic bicontinuous phases, namely, the D surface and the G surface.[7] The former is thermodynamically stable in excess water. Both cubic phases contain an interconnected bilayer, separating two water-channel systems, thus offering domains for encapsulation of hydrophilic, hydrophobic, as well as surface-active components. Moreover, MO has been reported to exhibit muco-adhesive properties.[8]

In this study, we present novel particles consisting of MO domains dispersed within a biodegradable PLG matrix, which may be useful for drug delivery of active substances, and for controlled or sustained release applications.[9] In a future application, the MO domains might offer a protective environment for sensitive active substances, while the biodegradable polymer matrix would prolong and level the release of active substance(s).[9]

In the present study, a novel approach for encapsulation of MO domains within a PLG matrix is given. The encapsulation of the lipid into the polymer matrix is a nontrivial task since lipids and polymers tend to segregate. It will be demonstrated that the phase behavior, of the system used for the encapsulation process, is of main importance, in particular when particles are created by a slow process such as emulsification. At least one other quaternary system containing a biocompatible solvent (1-methyl-2-pyrrolidinone) has been explored before.[10] The system reported in ref 10 is of general interest even though emulsification in aqueous solution is practically unworkable due to the instantaneous diffusion of NMP out to the water phase.

The approach taken in the present case is to emulsify a liquid phase, containing polymer, lipid, and a volatile solvent, which hardly is miscible with water, in an aqueous solution. Dense lipid/polymer composite particles form as a result of solvent diffusion to the outer water-rich environment. Furthermore, since MO and PLG do not mix in absence of solvent, lipid domains form within the polymer matrix as a result of the segregative phase separation in the droplets. Interestingly, a similar approach has been applied for a one-step preparation of polymer1/polymer2 multilayered microspheres by solvent evaporation.[11]

The selection of a proper solvent is of main importance in order to create lipid/polymer composite particles. The following three criteria can be used as guidance for solvent selection. The solvent should: (i) act as a solvent for the lipid and the polymer, (ii) have a very low solubility in water, and (iii) be able to leave the emulsion droplets during the particle formation process. For instance, ethyl acetate (EtAc) which is a volatile solvent with a limited solubility in water (approximately 8 wt %)[12] fulfills these criteria.[9]

By use of the EtAc/MO/PLG/water system as an example of an appropriate solvent/lipid/polymer/water system, it is here illustrated how the information, given in phase diagrams, can be used to select suitable conditions for composite particle formation and to some extent to control the rate of particle formation.

In the present study, it is possible to follow the lipid/polymer phase separation by means of a traditional non-time-resolved confocal laser scanning microscopy (CLSM). For this purpose, small amounts of a fluorescent lipid-based dye have been added to MO in order to probe the location of lipid domains. In addition, the interaction of lipid and polymer has been adjusted by using a modified PLG, with polyethylene glycol (PEG)

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10.1021/jp045373r CCC: $30.25
Published on Web 05/05/2005

chains attached to the PLG backbone. The modification (i.e., PEG segments) affects the surface energy at the lipid/polymer interface. The modified polymer is here denoted PEG-PLG.

Experimental Section

Materials. MO, i.e., Rylo MG 19 Pharma (lot no. 2119/54), was kindly provided by Danisco Cultor (Brabrand, Denmark). The monoglyceride content was 98.1%, and the rest was mainly free glycerin. The MO part of the monoglycerides was 90.3%. PLG (RG504H, MW 50 000, Mn 11 000) was obtained from Boehringer Ingelheim (Ingelheim am Rhein, Germany). The PEG-PLG, purchased from Birmingham Polymers, Inc. (Birmingham, USA), contains 70% PLG and 30% PEG1000 (lot no. D99164) with an inherent viscosity of 0.45 dL/g. EtAc (analytical reagent, Riedel-deHaën) was purchased from Sigma Aldrich. The fluorescence probes Lissamine Rhodamine B 1,2-dihexadecanoyl-*sn*-triethylammoniumsalt (rhodamine DHPE) and Bodipy disulfonate (492/515 nm) 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene-2,6-disulfonic acid disodium salt were both purchased from Molecular Probes (Leiden, The Netherlands). Glass beads (0.2 mm) were obtained from Kebo (Stockholm, Sweden). Vials for the samples prepared by the robotic liquid handler, as well as for the manually prepared samples, were bought from NTK Kemi (Uppsala, Sweden) and from Scantec Lab AB (Partille, Sweden). Silicon plugs with aluminum casings were used to seal the vials. Millipore quality water was used in all samples. All reagents were used as supplied.

Sample Preparation and Phase Characterization. The samples for the phase diagrams containing polymer were all prepared manually (0.5 g/sample). The samples for the EtAc/MO/water phase diagram were prepared by a robotic liquid handler in accordance with a recently reported method.[13] To enhance mixing and equilibration, all samples were vortexed and centrifuged. The anisotropy of the samples was studied between crossed polarizers. The phases were characterized by visual inspection. Some phases (i.e., cubic, lamellar, and liquid) of the EtAc/MO/water system were characterized by small-angle X-ray diffraction.

Particle Formation. Lipid/polymer composite particles, containing fluorescent probe molecules, were formed according to the following procedure: The lipid phase marker, rhodamine DHPE, was dissolved in 95% ethanol (0.036 mg/mL). Some of the rhodamine DHPE solution (0.59 g) was mixed with 0.063 g MO. The mixture was kept at 50 °C until all ethanol had evaporated. Polymer (0.177 g) and solvent (2.70 g) were then added until the final composition became 91.9/2.1/6.0 EtAc/MO/PEG-PLG % by weight. Particles containing the PEG-PLG polymer were formed by emulsifying 0.1 mL of the liquid mixture into 1.16 g of aqueous solution, containing 0, 4, 6, or 8% EtAc. Both phases were at room temperature, i.e., 19−22 °C, before emulsification by means of a Polytron mixer (Kinematica AG, PT300) working for 3 min at 4000 rpm.

Particles containing the nonmodified PLG polymer were created in a similar way by emulsifying the polymer-rich phase of a biphasic sample of the EtAc/MO/PLG system. The sample used for particle formation had a total composition of 97.3/0.7/2.0 EtAc/MO/PLG % by weight. Some of the polymer-rich phase (0.1 mL) was homogenized, in 1.16 g water, for 3 min at 4000 rpm.

Reference particles with both kinds of polymers, i.e., PLG or PEG-PLG, were formed from a liquid (L) phase, which contained the same amounts of DHPE probe, polymer, and EtAc as the L phase used for formation of lipid/polymer composite particles.

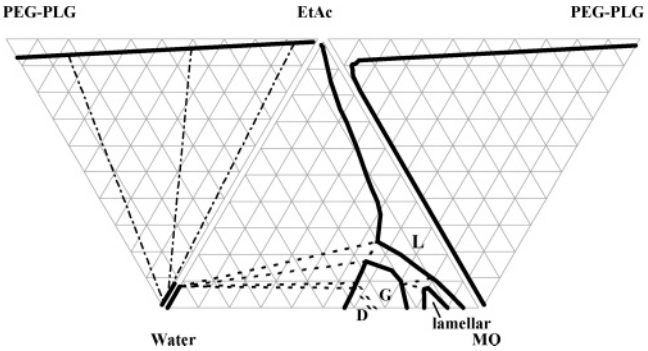

Figure 1. The phase behavior of the EtAc/MO/water, EtAc/MO/PEG-PLG, and EtAc/PEG-PLG/water ternary systems, at 20 °C. The cubic region, of the EtAc/MO/water system, consists of two different cubic phases: the D and the G surface. The liquid phase is denoted L. The SAXS data of the cubic samples will be published elsewhere.[14]

Particle Characterization. The particles were characterized by means of CLSM. The emulsion/dispersion was placed in a chamber with a 17 μm thick cover glass bottom. A hollow glass cylinder, with an inner diameter of 15 mm, was glued onto the cover glass. Before the characterization was performed by means of CLSM (Leica TCS, 4D, Leica LT, Heidelberg, Germany), the particles were immobilized by adding 0.2-mm glass beads. The 63×/1.2 water immersion objective was used, and the 568-nm line of the microscope's argon/krypton laser exitated rhodamine. The scanning was performed with an average of 8 lines, and the resolution was set to XYZ (0.12 μm/0.12 μm/0.36 μm). The pictures, showing the emission of the rhodamine DHPE probe (red) and in some cases also the reflection from the laser (green), were processed using Image Space software (Molecular Dynamics, Sunnyvale, CA) on a workstation (Silicon Graphics, Mountain View, CA).

The lipid-phase marker (rhodamine DHPE probe) was used together with the aqueous phase marker (bodipy disulfonate), the former was exited by the 568-nm line and the latter by the 488-nm line of the argon/krypton laser. The microscope's FITC-TRITC filter was used. Swollen lipid domains, consisting of MO and water, appear as yellow in the CLSM pictures when simultaneously using lipid-phase marker (red) and aqueous-phase marker (green) (see Figure 2f). The initial probe concentration of aqueous phase marker was 0.040 mg/mL in the aqueous phase.

Results and Discussion

Figure 1 shows schematic phase diagrams, at 20 °C, for the relevant ternary systems. The phase diagrams were determined with PEG-PLG. However, PLG display the same qualitative behavior. Only the most important features of the phase behavior needed to understand the formation of the lipid/polymer composite particles will be discussed. A more detailed presentation of the EtAc/MO/water phase behavior will be published separately.

EtAc/MO/PEG-PLG. According to Figure 1, MO and PEG-PLG segregate in the common solvent EtAc, which is a behavior resembling the classical "polymer incompatibility". Complete miscibility is found for EtAc concentrations larger than ca. 88 wt %. At lower EtAc contents, small amounts of MO can be solubilized in the EtAc/PEG-PLG liquid phase. At other compositions, a polymer-rich phase, containing some MO, is coexisting with a lipid-rich phase basically free from polymer.

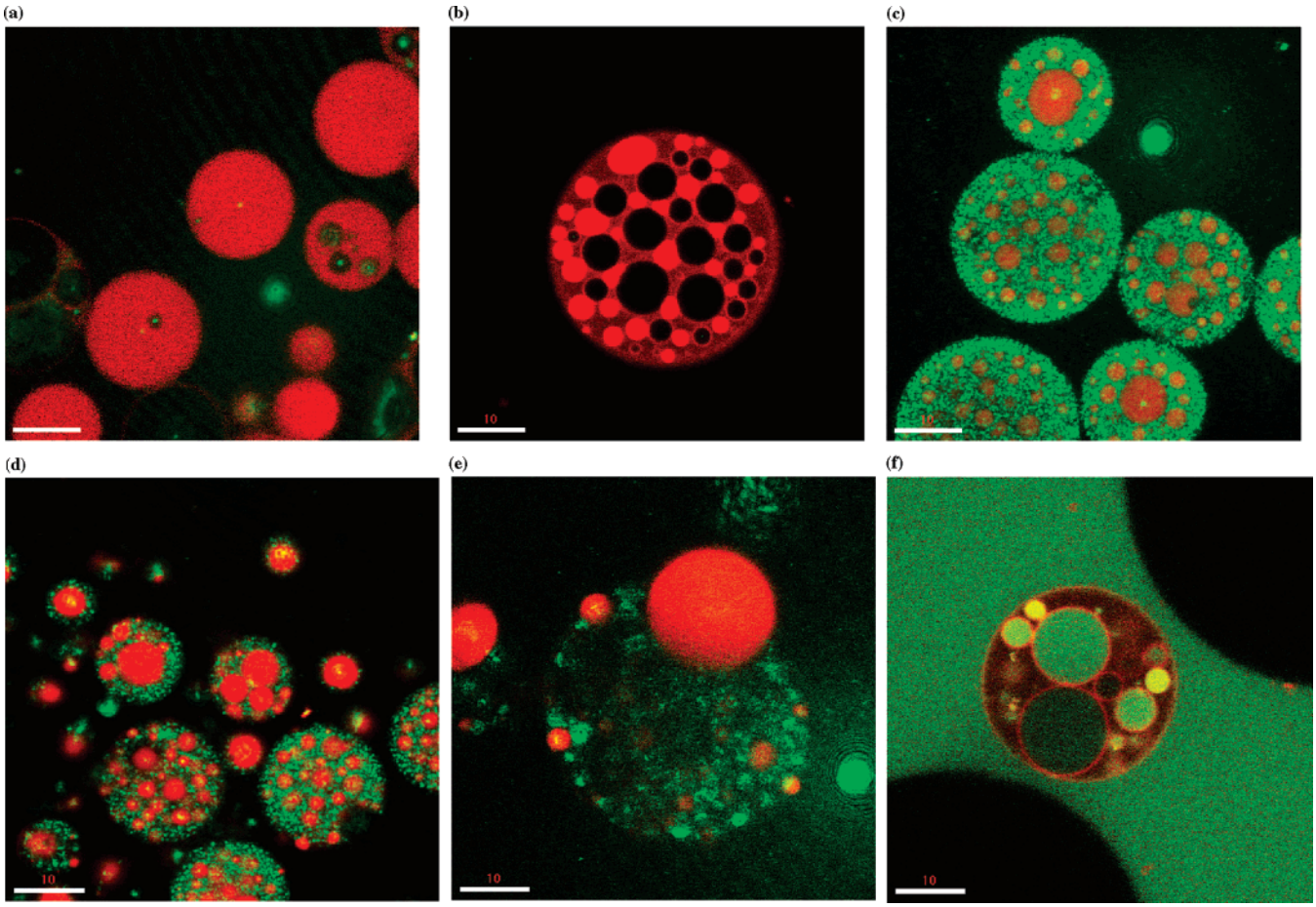

Figure 2. PEG-PLG reference particles, without MO domains (6% EtAc, 23−30 min) (a) and swelling MO domains and pores within the PEG-PLG matrix (6% EtAc, 23−26 min) (b). MO domains within the PEG-PLG matrix (0% EtAc, 10−15 min) (c). Swollen lipid domains detach from the PEG-PLG matrix (0% EtAc, 150 min) (d) and from the PLG matrix (0% EtAc, 25−45 min) (e). Swollen lipid domains (red + green = yellow) and two different kinds of pores/phases (dark and light green) present within the PEG-PLG matrix (6% EtAc, 20−23 min) (f). Scale bars indicate 10 μm. Notably, Figure 2b lacks reflection of the green laser light.

EtAc/PEG-PLG/Water. The polymer is completely soluble in EtAc but insoluble in water. Small amounts of water, which is not soluble in pure EtAc, can be incorporated into the EtAc/polymer liquid phase. However, due to the limited solubility of EtAc in water (ca. 8 wt %), the polymer-rich phase will *always* be in equilibrium with an aqueous solution. Hence, the system is suitable for formation of lipid/polymer composite particles. As indicated by the schematic tie lines, estimated from the lever rule, the polymer content of the EtAc/polymer-rich phase depends of course on EtAc content of the coexisting aqueous phase.

EtAc/MO/Water. Despite the complexity of the system, due to the lipid self-assembly, the phase behavior has features in common with the previously presented system. For instance, MO is soluble in EtAc but insoluble in water. The amount of water in the L phase is low but increases in proportion to increased MO content. At high EtAc contents, the L phase can be thought of as a solution of hydrated MO monomers; see ref 14. However, the incorporation of water appears to be facilitated at EtAc concentrations around 25%. It is possible that the increased swelling is due to aggregation of MO, which will be further discussed in ref 14. At lower EtAc contents, MO forms bilayers (as a result of the above-mentioned immiscibility between MO and water) arranged in lamellar or bicontinuous cubic phases. Similar to the case of the EtAc/PEG-PLG liquid phase, the hydration present in the EtAc/MO liquid phase is dependent on the EtAc content of the coexisting aqueous phase.

Phase Behavior and Particle Formation. In the particle experiments, liquid samples of EtAc/lipid/polymer were dispersed into an aqueous phase. Emulsion droplets were here formed. The complete phase behavior of the quaternary EtAc/MO/PEG-PLG/water system is not fully covered by the set of ternary diagrams in Figure 1. However, since water has a very low solubility in EtAc, a dilute mixture of MO and PEG-PLG in EtAc will hardly contain any water. Thus, initially when a droplet is created, the outer aqueous phase will (if not saturated with EtAc) mainly act as an acceptor of EtAc. EtAc leaves the droplet by diffusion, and the droplet is expected to go through phase transformations, according to the phase diagram of the EtAc/MO/PEG-PLG system. Both polymer and lipid are water insoluble and will thus remain in the droplet. Accordingly, when enough EtAc has left the droplet, MO and PEG-PLG will segregate and separate domains will form. The MO-rich domains, which basically are free from polymer, are expected to display the same phase behavior as EtAc/MO/water in Figure 1. Likewise, even though the polymer-rich domain should contain some lipid, its phase behavior should be similar to that of EtAc/PEG-PLG/water. These aspects simplify the interpretation of observations made at different times, i.e., different stages of the development from droplets to particles. The development from emulsion droplets into particles is further discussed below.

Notable is also that wetting of the polymer may occur. However, since wetting phenomenon is present both in samples used for the phase study as well as during the particle

formation process, no further discussion on wetting will here be made.

Development from Droplets to Particles. A series of experiments were carried out to investigate how the gradual removal of EtAc transformed EtAc-rich droplets into dense lipid/polymer composite particles. The experiments were performed in two steps. First, emulsions were formed by dispersing an EtAc/MO/polymer liquid phase in aqueous solutions containing 0, 2, 4, 6, or 8 wt % EtAc. Note that the polymer is present at considerably larger amounts than the lipid. This promotes the formation of discrete lipid domains surrounded by polymer in the droplets. In the second step, emulsion droplets were studied by means of CLSM where the lipid/polymer segregation was captured by scanning selected droplets at different levels at different times, see Figure 2. During the microscopy study, EtAc leaves the system by evaporation. Probably, this is the rate-limiting process of the observed lipid/polymer segregation. The EtAc/water exchange, between droplets/particles and an outer aqueous phase, is expected to be fast. Thus, as the concentration of EtAc in the aqueous phase decreases, the droplets quickly adjust their composition to re-establish equilibrium with the aqueous phase. Therefore, the experiments give no information about the *kinetics* of phase transitions. Rather a sequence of quasi-equilibrium states is monitored. The compositions, of phases in the droplets, are believed to follow the phase behavior presented in Figure 1, i.e., should be close to the compositions of the phases coexisting with excess aqueous solution.

The particle formation process can be described in the following way: When emulsion droplets have been formed, they can be assumed to have reached their first equilibrium state, where they become arrested as long as the EtAc concentration is constant. The time needed to reach the equilibrium state is therefore affected by the EtAc concentration in the outer aqueous phase. Since EtAc leaves the system by evaporation, the initial EtAc concentration of the aqueous phase determines when segregation in the droplets begins. The formation of lipid and polymer domains occurs when the EtAc content gets low enough. The compositions of the domains follow the phase boundaries in the direction away from the EtAc corners in EtAc/MO/water and EtAc/PEG-PLG/water phase diagrams. As time goes by, EtAc leaves the system by evaporation and the emulsion droplets will reach new equilibration states, i.e., the polymer-rich phase will concentrates as the lipid-rich phase increases its water/EtAc ratio.

Representative pictures of droplets/particles formed under various conditions are shown in parts a−f of Figure 2. Figure 2a shows reference droplets, 10−15 μm in diameters, made in 6 wt % EtAc in absence of MO. The picture was taken 23−26 min after preparation. The lipid-phase marker (red) is evenly distributed in the droplets and therefore the reflection from the laser (green) is hardly visible since the excitation of the lipid probe (red) dominates. The dark "voids" present in some of them are probably a dispersed aqueous phase (double emulsion) since these voids were generally observed shortly after preparation of the emulsions. However, it is also possible that the dark voids are air. Figure 2b shows a droplet made in the same way but with MO present (23−30 min). Note the presence of voids also in this case. Lipid-rich domains (red) are present throughout the droplet. In this picture there is no reflection from the laser. Our interpretation is that liquid−liquid-phase separation, between lipid-rich and polymer-rich domains, has occurred (see Figure 2b).

Figure 2c shows a picture taken 10−15 min after preparation in pure water. In this case, the polydisperse droplets have transformed into lipid/polymer composite particles, probably containing only small amounts of EtAc. The polymer matrix is green in Figure 2c, indicating that the matrix is dense. The lipid domains enclosed in the matrix are very bright. The interplay between the kinetics of phase separation and final structure of the particles can be seen in Figure 2c, where the distribution of the lipid domains is different in the smaller particles compared to in the larger particles.

At low EtAc content, lipid and water form specific microstructures, i.e., phases, in accordance with the EtAc/MO/water phase diagram. Interestingly, the distinctive lipid domains separate from the polymer matrix but maintain a shell of polymer, as time goes by. This can be seen in Figure 2d, showing particles from the same experiment as illustrated in Figure 2c, after 150 min. Possibly, an increased swelling of the lipid domains, as expected from the phase diagram, ruptures the polymer matrix. In this respect, a clear difference in behavior was seen between PLG and PEG-PLG. While in the latter case the separated lipid domains always carried a polymer shell (see Figure 2d). In the former case pure lipid domains were ejected from the polymer matrix (see Figure 2e). This difference must be ascribed to the PEG modifications, reducing the surface energy between polymer and lipid phases. Probably the favorable interaction between PEG and the hydrophilic parts of the lipid bilayers helps keep the phases together. The structure and composition of the lipid domains is not a topic of the present study, but recall that a D-cubic phase can be in equilibrium with excess water.

To obtain information of the contents of the voids, an experiment was performed with simultaneous use of water and lipid markers. The CLSM picture, presented in Figure 2f, reveals that two different phases are present: one is water rich (light green), and the other is most likely EtAc rich (dark). (The dark objects in the corners of Figure 2f are glass spheres present to reduce convection). The former is probably an example of the above-mentioned double emulsion, and the latter should be the liquid EtAc/MO phase. The yellow domains contain both probes and should be water-swollen domains of lipid bilayers. The lipid domains have probably swelled by absorbing water from either the aqueous voids or from the outer aqueous solution. This would explain the absence of voids at late stages. However, water is also expected to be transported through the polymer matrix at a reasonably high rate.

To sum up, in all experiments with MO present, lipid domains were observed at different times after mixing, depending on the initial concentration of EtAc in the aqueous phase. In the case with pure water as outer phase, MO domains formed in less than 10 min. With 4 and 6 wt % EtAc in the outer phase, MO domains were formed after 10 and 16 min, respectively. In the case of 8 wt % EtAc, i.e., the solubility limit, no domains had formed after 90 min. However, after 2 h, ejected swollen domains were observed. Therefore, *the final state* reached when EtAc had evaporated was qualitatively the same irrespective of the initial amount of EtAc in the aqueous phase. However, before reaching the final state several alternatives seem possible. For instance, recall the different behavior of the large and small particles, presented in Figure 2c. From this figure it seems not impossible that the observed segregative behavior will be dependent upon kinetics (e.g., homogeneous or heterogeneous nucleation). However, the water transport through the polymer matrix and the possibility for initial presence of water droplets may be other explanations. Since the finding was general (in

the entire sample volume) the former explanation, i.e., water transport vs EtAc evaporation, seems to be the most probable one.

The time scales of the different processes are important. Immediately after a droplet has been created in, for instance, pure water, EtAc will start to diffuse out. The distribution of EtAc (and water) between the droplet and the aqueous layer just outside will equilibrate very quickly. (As can be inferred from Figure 1, the concentration of EtAc in the aqueous layer will be close to the saturation since the concentration of polymer and lipid is small.) The difference in concentration between the bulk aqueous phase and the solution just outside the droplet causes a transport of EtAc to the bulk at a rate depending on the concentration difference and the liquid flow rate in the aqueous phase. The time-scale for this process, which can be estimated by considering, e.g., "stagnant layer" diffusion, will be considerably longer than the time needed to maintain the equilibrium between the droplet and its immediate vicinity. If we, for simplicity, consider a droplet containing polymer but no lipid, the composition of the droplet will change continuously along the phase boundary, given in the ternary EtAc/PEG-PLG/water phase diagram, in the direction away from the EtAc corner. Importantly, during the process the droplet will be in contact with an EtAc/water mixture. The gradual change should prevent dense polymer layers to develop on the surface of the droplets. As already mentioned, the processes here discussed are expected to be completed during the first step, i.e., before the emulsions are studied by CLSM, and cannot be followed in real time with the present experimental setup. However, by carefully controlling the concentration of EtAc in the water phase, a system can be "frozen" at different stages.

Despite the fact that the emulsions are complex four-component, nonequilibrium systems, with many processes and transitions taking place at different levels at the same time, the set of ternary phase diagrams, given in Figure 1, contain information sufficient to account for the observed lipid/polymer segregation.

It should also be noted that the droplets/particles observed in the microscope were polydisperse. The observed droplets/particles were about 1−100 μm in diameter. The particle size affects the kinetics of phase separation, i.e., different surface/volume ratio. Hence, MO tends to form domains more quickly in smaller particles in comparison to larger (as briefly were mentioned above). The particles examined by means of CLSM were, due to practical reasons (i.e., immobilization of the particles), 15−35 μm in diameter.

Conclusions

The phase behavior of the quaternary EtAc/MO/PLG/water system is suitable for formation of MO/PLG composite particles. The following properties of the system are important for particle formation by emulsion techniques: (i) MO and PEG-PLG segregate but can coexist in an EtAc-rich liquid phase, denoted the L phase. The L phase is suitable for particle formation by emulsion methods and as a consequence of solvent diffusion and evaporation, segregation between the lipid and the polymer occur. (ii) The EtAc/polymer phase can coexist with a water-rich phase. Hence, it is possible to create a rather stable emulsion. (iii) The solvent, EtAc, is volatile and not completely miscible with water. Therefore, it is easy to concentrate the polymer-rich phase by removing the solvent. (iv) EtAc/MO forms a liquid phase that, as a result of incorporation of water, can transform into interesting cubic phase(s).

Microparticles, with distinctive water-swelling MO domains that finally detach themselves from the particles, have been created with the route given by the phase behavior. As EtAc is somewhat soluble in water it is possible to adjust the kinetics of the particle formation and the corresponding segregation of lipid and polymer. For this reason, the system seems also suitable for phase separation studies in general.

When the kinetics of phase separation is slow, i.e., at 6−8% EtAc in the outer water-rich phase, some pores/voids are present. The formation of the pores is not yet fully understood but most likely a double emulsion, stabilized by MO, is formed during the homogenization process. From the experiment performed with simultaneous use of lipid and water markers, it seems as if the lipid/polymer segregation starts with formation of a water-poor phase (dark-green pores in Figure 2c) which most likely is the EtAc/MO L phase that, according to the phase behavior, should transform into the cubic phase(s).

Acknowledgment. Dr. Göran Ocklind is acknowledged for technical assistance as well as for fruitful discussions (CLSM). Dr. Harald Liedkte, Boehringer-Ingelheim, is acknowledged for contributing with some polymers. The "A8-students", Henrik Gold, Pär Matsson, Johan Unga, and Per Wessman, are acknowledged for technical assistance with some sample preparation.

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