Spherulites: A new vesicular system with promising applications. An example: Enzyme microencapsulation

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Spherulites: A new vesicular system with promising applications. An example: Enzyme microencapsulation

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A new technology is tested for enzyme encapsulation. The capsules are small multilamellar vesicles of surfactant called spherulites which are produced by shearing a lamellar phase under well-controlled conditions. Encapsulation of alkaline phosphatase into spherulites is studied here as an example. Once encapsulated, the enzyme is shown to be unable to develop any enzymatic activity on its substrate, the *p*-nitrophenylphosphate. This is due to the absence of contact between the enzyme and the substrate. Interestingly, the whole enzymatic activity is recovered after destruction of the vesicles. Encapsulation efficiency ranges between 70% and 95% depending upon the enzyme over phospholipids ratio. Beyond the example of alkaline phosphatase, many applications of spherulites in the medical or in the biotechnology fields seem now at hand. © *2000 American Institute of Physics.* [S0021-9606(00)70407-7]

I. INTRODUCTION

Microencapsulation has been a rapidly expanding technology, giving birth to a wide variety of systems. Among the most frequently used systems, one finds liposomes, which are vesicles made of natural lipids,[1,2] and nanospheres, which are polymeric particles in which the drug is dispersed throughout the particles.[3] In the medical domain, such systems are used as ''vectors,'' that is to say, to convey a load (e.g., a drug, a hormone, or a gene) and deliver it in the interior of living cells.[4] Although the role of such vectors in the stage of transfer through the cell membrane is not yet well known, they indisputably play an important role in protecting their load from the attacks of the environment.[5] Besides, if sensors are grafted onto the liposome membrane or onto the polymer, they can specifically recognize target cells and deliver their load to them.[4] Thus, the use of an encapsulated drug can decrease the adverse side effects on organs not targeted for treatment and therefore lower both toxicity and cost.

Another major feature of liposomes is their ability to slowly release their load.[6–8] The development of encapsulated vaccines is presently creating great interest.[9,10] The slow release of a vaccine out of capsules allows a reduced immune response with fewer bothersome side effects. The use of biopolymers like poly(lactide-co-glycolic acid) is also of interest because the active material is slowly released during the erosion of the microcapsules.

Encapsulated enzymes also have many industrial applications. For instance, proteolytic enzymes entrapped in liposomes are used to increase the ripening of cheese.[12,13] It avoids a premature attack of the milk proteins by the enzyme and allows a uniform distribution of enzyme in the cheese curd. Another example is the encapsulation of proteases in liquid detergent in order to protect the other enzymes (i.e., lipases or cellulases) from proteolysis during storage. During the cleaning process microcapsules are disrupted and the enzymes are released.[14]

The following criteria play a major role in choosing a microencapsulation process. First, one has to consider the capsules size and their chemical and mechanical resistance.[15,16] In the medical domain, the capsules—if used for intravenous injections—should be both small in size and resistant in the plasma to ensure either slow release or targeting. In the industrial domain, their recycling is necessary as they serve as micro-bioreactors in batch and packed-bed reactors. Thus the main requirement is their mechanical strength. For encapsulation of costly materials or applications to the industrial scale, high encapsulation ratios[17,18]—i.e., a high proportion of encapsulated material compared to the total amount put in the encapsulation process—are required to limit the cost of lost material and if possible avoid running a stage of separation of the encapsulated material from the nonencapsulated one. For the encapsulation of some fragile biomaterial such as enzymes that can undergo denaturation, gentle encapsulation processes have to be applied and, in particular, the use of organic solvents[11] or of violent mechanical constraints should be avoided.

Despite the outstanding efforts to develop high-performance processes of encapsulation, none of them fulfill all the above-mentioned requirements. Numerous ways of preparing liposomes have been developed,[1] but in most of them the rate of the encapsulated drug is below 50% and costly separation processes have to be applied in order to separate the free drug from the encapsulated one. The mechanical strength of liposomes is also a critical condition that has led to the development of polymerized vesicles which in return are often too impermeable and too solid to release their load. In general, the preparation of polymeric microspheres requires the presence of an organic polymer solution to emulsify the water solution of proteins (this is the so-called ''double-emulsion-solvent evaporation technique'').[11] Multiple emulsions are also a promising method of microen-

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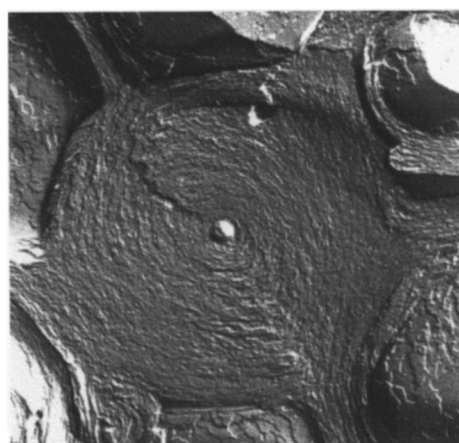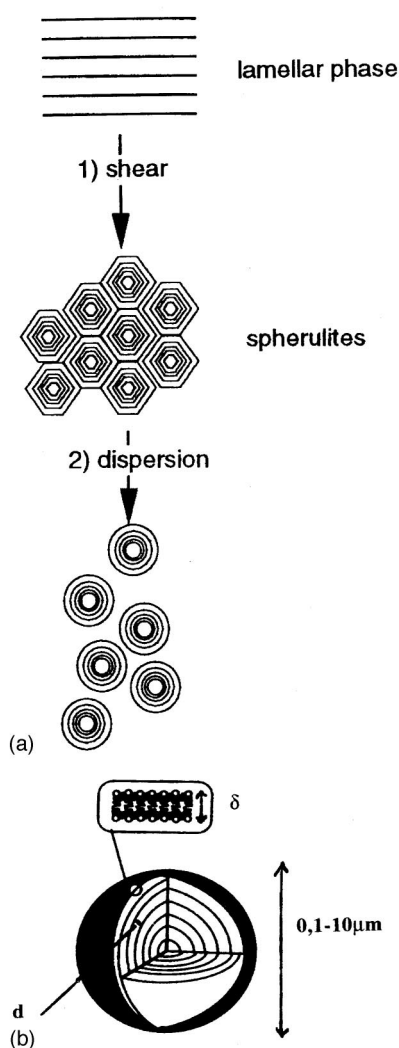
(c)

FIG. 1. (a) Process of preparation of a suspension of spherulites, (b) schematic representation, and (c) electronic microscopic picture (freeze fracture) of a spherulite.

capsulation of drugs, proteins, or vaccines. But coalescence and diffusion processes of the dispersed phase must be controlled in order to obtain a stable delivery system during storage and within the application.[19]

Here, we describe a new process for the microencapsulation of enzymes in well-controlled and small-sized multilamellar vesicles of surfactants. This process is based on the recent discovery of the formation of a new type of multilamellar vesicles when a lamellar phase of surfactant is sheared at an appropriated shear rate.[20–22] This was shown to result from the development of an hydrodynamical instability within the lamellar phase appearing when the lamellar phase is sheared.[22] The multilamellar vesicles so formed have been called spherulites®. Their size is comprised between 0.1 and 10 μm and they present a high entrapment efficiency.

Our present purpose is to show through a very simple example that spherulites can be a high-performing system for encapsulation and immobilization of enzymes. For a better understanding we will first present briefly the physical principles of spherulites formation, even though it was previously described in detail elsewhere.[20–22] Then we will see that this technique can be used to control enzymatic activity. For this, we have encapsulated the alkaline phosphatase (AP) and investigated its activity on the p-nitrophenylphosphate

(p-NPP). The encapsulated enzyme is found to have no measurable activity. However, a residual activity is observed in the encapsulated system because of a small fraction of free enzyme. This result allows us to easily determine the encapsulation efficiency, which reaches extremely high levels. Moreover, the activity of the encapsulated enzyme can be recovered at will be destruction of the capsules, indicating that denaturation does not occur.

II. SPHERULITES TECHNOLOGY

Spherulite technology was born from the study of lamellar phases of surfactant under shear. A lamellar phase [Fig. 1(a)] is composed of a stack of surfactant bilayers separated with water layers: It is a liquid crystalline phase. Sometimes an additional component, an oil, swells the surfactant bilayers. As they are states at thermodynamical equilibrium, lamellar phases form spontaneously after simple mixing of the components in an appropriate composition. In surfactant/water/oil phase diagrams, lamellar phases are most often found for relatively high concentration of the surfactant.[23]

Upon dilution with extra water, a lamellar phase generally undergoes one of the two following transitions.[24] The dilution of the lamellar phase either leads to a phase transition with an isotropic liquid phase (micellar phase or sponge
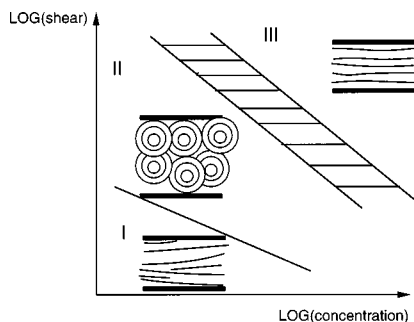
FIG. 2. Representation of the shear diagram of a typical lamellar phase (in this case made of water, dodecan, pentanol, and sodium dodecyl sulfate). The organization of the lamellae is represented as a function of the concentration of dodecan and of the shear rate.

phase), or the dilution of the lamellar phase leads to a phase transition with another liquid crystalline phase (e.g., hexagonal phase). However, in a limited number of interesting cases the lamellar phase separates with excess water when it reaches a maximum of uptake. For instance, this is the case when phospholipids are used as a surfactant.

When submitted to shear, lamellar phases undergo a succession of interesting transformations. Using a number of structural techniques under shear such as scattering techniques (light scattering,[22] neutron,[25] x-ray[26]) or dielectric measurements,[27] it is possible to study these transformations, and the effect of shear can be described using a so-called ''shear diagram.'' This diagram, which can be considered as a generalization for out-of-equilibrium systems of phase diagrams, describes the effect of shear as a succession of stationary states of orientation separated by dynamic transitions. Indeed, while always remaining a thermodynamically stable lamellar phase, the phase experiences a series of transitions modifying the orientation of the lamellae in space respecting the direction of the shear. These different orientations correspond to an organization of the defects in space which is different from one orientation to the other. Consequently, it is not a phase transition but has to be considered as an instability. It is however different from the classical hydrodynamic instabilities observed when a fluid is submitted to shear because it does not involve length sales directly related to the size of the shear cell but rather some microscopic length scale related to the intrinsic structure of the fluid. Figure 2 is a schematic representation of the shear diagram obtained in the case of a lamellar phase made of water, dodecan, pentanol, and sodium dodecyl sulfate.[22] At very low shear rate the phase is more or less oriented with the membrane parallel to the velocity, however defects remain in the velocity direction as well as in the vorticity direction. At high shear rate the orientation is basically very similar but the defects in the velocity direction have disappeared. In the intermediate regime, a new and interesting orientation appears. The membranes are broken into pieces by the flow and the phase organizes itself into a phase of multilamellar vesicles all of the same size. Those vesicles are called spherulites and are the subject of our interest here.

The spherulite phase is a state of organization under shear. However, if one stops the shear cell very quickly it is

possible to quench the structure. In this case, all the observations indicating the existence of spherulites can be done on the system at rest. Certain systems can be prepared that way and are stable for several months. Freeze-fracture pictures of spherulites can be obtained [Fig. 1(c)].[21] It is important to notice that in this state spherulites fill up space. Thus, except for the small water layer between adjacent spherulites, all the lamellar phase components are now part of one spherulite or another. This is responsible for the exceptionally high encapsulation efficiency obtained with spherulite technology. Indeed, if an active molecule is dissolved in the lamellar phase prior to applying the shear, it will be inserted either in the membrane or in the water layers of the spherulites depending upon its hydrophobicity or hydrophily.

Many surfactant systems have now been studied and exhibit the same kind of behavior.[28] In most cases, the spherulites' size is inversely proportional to the square root of the shear rate. This is an easy way to experimentally control the size of the vesicles. Depending upon the formulation (i.e., the surfactant choice) and the shear rate, sizes ranging from 0.1 to more than 50 μm have been found. After stopping the shear, one can disperse the lamellar phase prepared under shear in an excess solvent. Depending upon the phase transition that the lamellar phase experiences upon dilution, two scenarios may occur. If the lamellar phase changes phases under dilution, the spherulites are slowly dissolved into the excess solvent to give the corresponding equilibrium phase. However, in the interesting case where the lamellar phase is in equilibrium with excess water (e.g., no micelles formed), the spherulite phase remains in suspension in the water. One consequently obtains a monodisperse dispersion of spherulites. If an active molecule (a drug, a perfume, a protein, or any type of other molecule) is dissolved in the lamellar phase prior to shearing and then the obtained spherulites are dispersed into excess water, one obtains a suspension of encapsulated molecules. The size of the microcapsules can easily be chosen by applying the appropriated shear rate. Just before dilution, the active molecules are encapsulated inside the multilamellar structure of the spherulites. However, depending upon thermodynamics and kinetics, the encapsulated molecules will either stay inside the spherulites or leak out and equilibrate with the excess water.

Like liposomes, spherulites have rapidly found many applications as encapsulating systems. The formulation can be adapted depending on the type of use: For biological purposes the spherulites can be made of phospholipids and for cosmetic or food applications less expensive surfactants can be used. This is a patented process and products using spherulites are currently on the market.[29] Recently we have been using spherulites to demonstrate the potential interest of using such structures as chemical microreactors.[30–32]

III. STUDY OF ENCAPSULATED ALKALINE PHOSPHATASE (AP)

Our purpose in this section is to demonstrate, that spherulites technology can be successfully applied to enzyme encapsulation, using the very well known enzyme alkaline phosphatase. For this purpose spherulites containing AP were prepared and dispersed in a buffer. We then compare

the activity for such a preparation to the activity of a nonencapsulated enzyme over the enzyme substrate: the p-nitrophenylphosphate (p-NPP). The formation of p-nitrophenol (p-NP), which is the product of the reaction, is monitored by UV–visible absorbance measurements. The reaction of AP over its substrate p-NPP is described by the classical Michaelis–Menten equations of enzymatic kinetics. When the enzyme reacts with a large excess of substrate, a stationary state is reached almost instantaneously. Under these conditions, the initial rate of product formation is written as follows:

$$V = \frac{d[p-\text{NP}]}{dt} = \frac{k[p-\text{NPP}]}{K_m + [p-\text{NPP}]} \cdot [\text{AP}_t], \qquad (1)$$

where the notation [ ] expresses the concentrations, $[\text{AP}]_t$ the total amount of enzyme, k and K_m are kinetics constants. In the presence of a large excess of substrate, $[p\text{-NPP}]$ is assumed to be constant. According to Eq. (1) the initial rate is then proportional to the total enzyme concentration $[\text{AP}]_t$.

All the following experiments are run with large excess of the substrate so that Eq. (1) can be applied to describe the initial reaction rates.

A. PA encapsulation

AP was encapsulated into spherulites according to the previously explained technique. Since most of the applications of enzyme encapsulation concern the pharmaceutical industry, we chose to use pharmaceutical grade phosphatidylcholine purified from soybean (P90) as the main surfactant. The composition of the lamellar phase is typically as follows (weight percentages): P90 (40%), sodium oleate as a cosurfactant (10%), and an aqueous enzymatic solution of AP (50%) usually at the concentration 100 units/ml. First the phosphatidylcholine is mixed with sodium oleate, then the aqueous enzymatic solution is added. The mixture is stirred and left at rest until a birefringent texture typical of a lamellar phase can be observed under a microscope equipped with polarizers. The lamellar phase is then placed in a homemade Couette cell[11,12] and sheared at a constant shear rate (typically 20 s^{-1}) for 2–3 h. One obtains, at this stage, the concentrated phase of multilayered vesicles. This phase can then be dispersed in an excess volume of buffer depending upon the enzyme concentration required.

B. Kinetic study of the encapsulated system

First, we compare the activity of the free enzyme to the activity of the same quantity of enzyme previously encapsulated in spherulites and then dispersed into a buffer solution. In the latter case, we consider the total amount of enzyme laid out in the encapsulation process, although a fraction of this amount might not be encapsulated into the spherulites. This fraction of nonencapsulated enzyme will then be free in the buffer solution. The kinetics is measured in both cases with the same concentration of substrate and all other conditions being equal. Figure 3 shows the kinetics of formation of the product of the reaction as followed by UV–visible absorbance measurements. It is clear that a very important decrease in the activity is caused by encapsulation. Quanti-
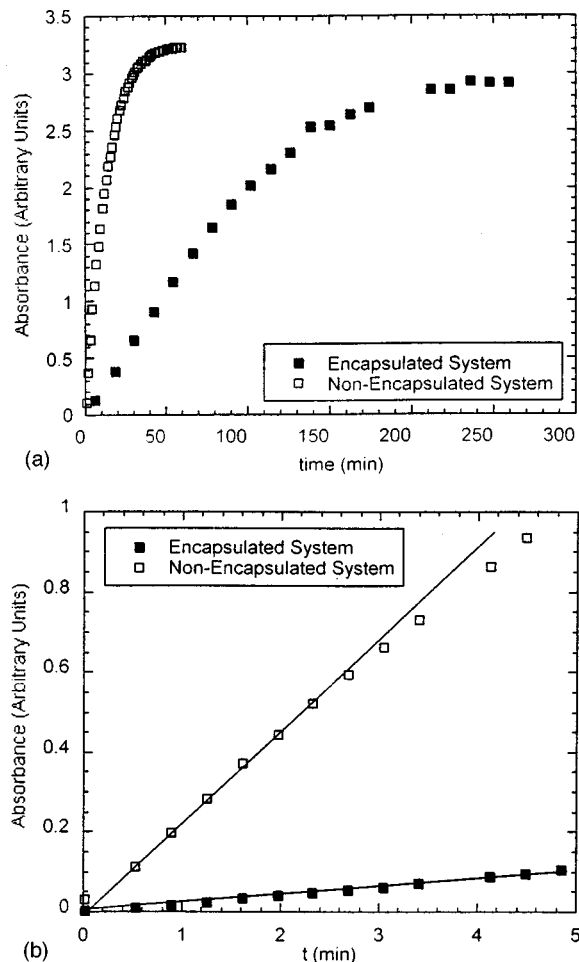

FIG. 3. (a) Comparison of the enzymatic activity for the encapsulated (closed squares) and the nonencapsulated systems (open squares). p-NP formation is followed by absorbance measurements. (b) Focus on the short times. The slopes are representative of the initial rates.

tatively, the initial reaction rate decreases from 5 to 60 times for different enzyme/phospholipid ratios (see Sec. III D). With the experimental conditions corresponding to Fig. 3 the initial reaction rate is decreased 9 times [Fig. 3(b)]. In all cases, the same final plateau is eventually reached [Fig. 3(a)], indicating that for both the encapsulated and the nonencapsulated systems the same amount of p-NPP is cleaved into p-NP.

Although it is much lower than for the free system, the encapsulated system still exhibits some activity over p-NPP. The enzymatic activity observed with the encapsulated system may have two origins. The first may be that a fraction of the enzyme is not encapsulated and the activity of this free enzyme fraction is measured. The second may be a slow penetration of the substrate inside the capsules or a slow release of the enzyme. To investigate the reason for this residual activity, the following experiment was carried out. First, some AP was encapsulated into spherulites and the spherulites were dispersed into a buffer solution. This dispersion was divided into two parts. One part was used to measure the global activity and was used as a reference. Then, the other part of the dispersion was centrifuged to separate the spherulites (in the pellet) from the bulk (the supernatant
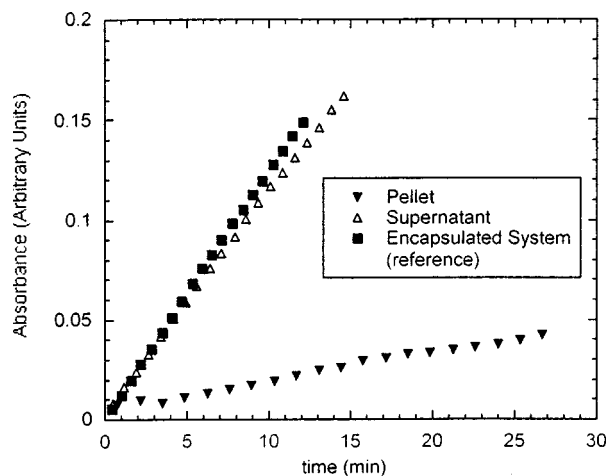
FIG. 4. Activities of the three systems: supernatant, pellet, and encapsulated enzyme. The pellet has a very small activity (possibly due to nonperfect separation) whereas the supernatant activity is very close to that of the encapsulated system. $[p\text{-NPP}] = 10^{-3}$ M, $[\text{AP}] = 2.5 \times 10^{-2}$ units/ml, $[\text{spherulites}] = 0.4$ g/ℓ, $T = 37$ C, pH$=10$.
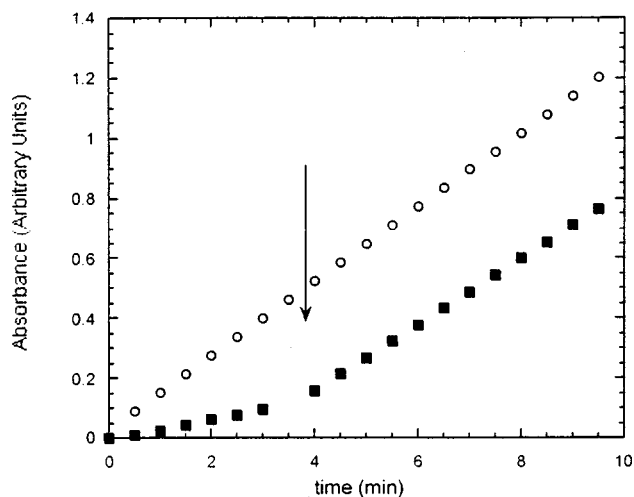

FIG. 5. Recovery of the enzymatic activity after destruction of the spherulites. Absorbance vs time of the pellet before and after addition of deoxycholic acid. $[p\text{-NPP}] = 10^{-3}$ M, $[\text{AP}] = 2.5 \times 10^{-2}$ units/ml, $[\text{spherulites}] = 0.4$ g/ℓ, $[\text{deoxycholic acid}] = 0.06$ g/ℓ, $T = 37$ C, pH$=10$.

phase). The activities of the pellet and of the supernatant were measured separately (after dilution of the pellet in an equivalent amount of buffer) and compared to the activity of the global system (reference). The results are displayed in Fig. 4. The pellet has a very low activity (probably due to a nonperfect separation) whereas the supernatant activity is very close to that of the reference system. We also checked that if the substrate is encapsulated into spherulites it does not leak. This was done by dispersing some (p-NPP)-containing spherulites in a buffer solution of PA: such a system behaves as if no p-NPP was present, even after several hours. Therefore, in the typical experiment described here (encapsulated enzyme dispersed in a substrate solution) we can reasonably assume that substrate molecules would not enter into the spherulites. These two experiments show that the residual activity measured in the encapsulated system is due to a fraction of nonencapsulated enzyme.

As the residual activity results from the sole nonencapsulated enzyme, Eq. (1) allows us to determine the amount of free enzyme in the supernatant. This will be used later on to measure the encapsulation ratio of AP.

C. Recovery of full activity after destruction of the spherulites

As seen previously, the microencapsulation into spherulites causes a tremendous decrease in activity. This could lead to many important applications if the activity can be recovered, fully or at least partially, after destruction of the capsules. Spherulites could then be used as reservoirs which are easy to manipulate and can release their contents at a chosen moment. The destruction of the spherulites can be performed using deoxycholic acid, which is known to solubilize lecithin into micelles. Upon the addition of deoxycholic acid the spherulites solubilize, which causes the suspension to turn from turbid to clear. One can check under the microscope to see that no spherulites are visible.

To investigate the recovery of enzymatic activity after destruction of the capsules we ran the following experiment

(Fig. 5). A dispersion of encapsulated enzyme is prepared and mixed with the substrate. After 4 min of monitoring the kinetics, deoxycholic acid is added to destroy the spherulites. An increase of the kinetics is observed, and the speed of substrate conversion becomes equivalent to the one with the free enzyme. Since in this type of experiment the full enzymatic activity is recovered, we conclude that the encapsulation process into spherulites does not alter the enzyme.

D. Influence of the enzyme phospholipid ratio

Taking into account that the activity which is measured in a dispersion of encapsulated enzyme corresponds to the fraction of ''free'' enzyme, it is easy to measure the encapsulation ratio. We recall that we define the encapsulation ratio as the amount of encapsulated enzyme over the total amount of enzyme put in the encapsulation process, it then characterizes the efficiency of the encapsulation process. According to Eq. (1). the initial rate of p-NP formation is proportional to the concentration of free enzyme. The encapsulation ratio is then given by

$$\tau = 1 - \frac{[\text{PA}]_{\text{free}}}{[\text{PA}]_t} = 1 - \frac{V_{\text{enc}}}{V_{\text{ref}}},$$

where $[\text{AP}]_{\text{free}}$ and $[\text{AP}]_t$ are the concentration of the residual free enzyme and the total amount of enzyme in the dispersion of spherulites, respectively, and V_{enc} and V_{ref} are the initial rates of p-NP formation in the encapsulated system and in an equivalent nonencapsulated system containing the same global concentration of enzyme, all other parameters being identical.

Since we believe that an important parameter controlling the encapsulation ratio is the enzyme/surfactant ratio, we have determined the encapsulation ratio for several values of enzyme/surfactant (Fig. 6). The enzyme concentration in all the experiments we performed is the same, only the phospholipid quantity changes. In terms of weight percentage (enzyme/surfactant) the enzyme concentration is approximately varied from 0.15% to 0.006%. The encapsulation ra-
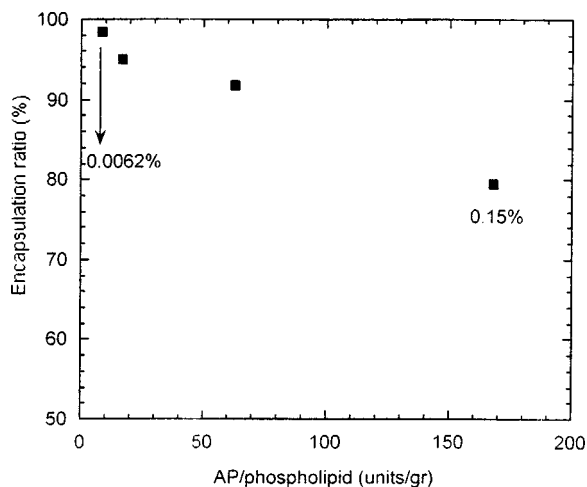
FIG. 6. Encapsulation ratio vs enzyme/surfactant ratio. The weight percentages of enzyme over surfactant ranges approximately from 0.006% to 0.15%.

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tio slightly decreases when the proportion of enzyme is increased, however in the studied range the encapsulation ratio remains very high, between 80% and 98%. Prior to this experiment it was checked that varying the dilution of spherulites (That is to say the spherulites over bulk buffer ratio) does not affect the measured encapsulation ratio.

IV. MATERIALS AND METHODS

Alkaline phosphatase (AP) (EC 3.1.3.1) from porcine kidney was purchased from Sigma Chemical (approximately 90% protein). All experiments were run with 0.025 units/ml of AP in a 1 M buffer solution made of 2-amino-2-methyl-1-propanol (95% purity, Aldrich) adjusted to pH 10 with HCl. The substrate, disodium p-nitrophenylphosphate, was purchased from Sigma (Crystalline Grade) and used at the concentration 10^{-2} M together with 2 mM of $MgCl_2$ in the same buffer. Phosphatidylcholine from Soybean Lecithin (Phospholipon 90) was obtained from Rhône–Poulenc Rorer and used without further purification, as was sodium oleate (Witco). The enzymatic solution was prepared by dissolving the whole content of the commercial enzyme container in 1 ml of buffer. Then, 100 units of the dissolved enzyme were measured and buffer solution ($pH=10$) added up to 1 ml. To obtain the lamellar phases, the surfactants were mixed with the buffered solution of enzyme containing 100 units/ml. The compositions of the lamellar phases were typically as follows (weight percentages): Phospholipon 90 (40%), sodium oleate (10%), aqueous enzymatic solution of AP (50%). Note that for the last series of experiments (Sec. III D) the amount of AP varies: this was done by using enzyme solutions of various concentrations. All the components of the lamellar phases were manually stirred together using a needle and the evolution of the samples was followed by observation under a microscope equipped with polarizers. After 5–6 h we checked that the samples looked homogeneous under a microscope, and that the microscopic texture between crossed polarizers was the one of a lamellar phase. Spherulites were

then formed by shearing the lamellar phases using a home-made Couette cell designed for small samples (0.3 ml). This device is made of a rotor with a conical extremity adapted to fit in an Ependorf tube. The lamellar phase is placed in the Ependorf tube, and the tube is inserted into a metallic holder that can be thermoregulated. The rotor is then inserted in the tube. Under rotation of the rotor, the sample is sheared in the gap in between the conical extremity of the rotor and the Ependorf tube. In the present experiments, shearing was processed at approximately 80 s^{-1} for 20 min at 20 °C. The shearing rate is not perfectly well controlled because of the discrepancies in the size and shape from one Ependorf tube to another. Thus, the gap between the rotor and the tube can be different for different samples. However, a good control of the size of the spherulites is not necessary in the experiments described here, so the shearing rate did not need to be exactly adjusted. After stopping the shear, we checked that the microscopic texture between crossed polarizers was typical of spherulites. 100 mg of AP-containing spherulites were then delicately removed from the Ependorf tube and dispersed into 10 ml of the buffer solution. Then 0.5 ml of this spherulite suspension was placed into a spectrophotometric cell and 0.5 ml of a substrate solution 2×10^{-2} M in buffer was added. The formation of p-nitrophenol was monitored by absorbance measurements at 410 nm using a PYE Unicam PU 8800 UV–vis. spectrophotometer. In all cases the experiment temperature was set up at 37 °C. For the recovery of enzymatic activity, spherulites were destroyed using deoxycholic acid (Sigma) in an aqueous solution: 0.15 mmol of deoxycholic acid was added to clear off 1 ml of dispersion containing 0.5 mg of spherulites. Experiments of separation of the spherulites from the bulk were performed by centrifugation at 40 000 rpm for 2 h.

V. CONCLUSION

This work is the first attempt to apply spherulite technology to enzyme microencapsulation. It is very interesting that this new encapsulation process does not alter the enzyme whose activity can be recovered at will. Besides, in addition to their well-defined small size and mechanical strength (due to the multilamellar structure), the very high encapsulation ratios that are obtained and the ease with which spherulites can be prepared and handled, also make these vesicles very attractive for medical and industrial applications.

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