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Nanocapsules of biodegradable polymers: preparation and characterization by direct high resolution electron microscopy

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Abstract

In the field of pharmaceutical applications relying on encapsulation of drug by polymer coating, capsules based on biodegradable polymers with mean size of about 500 nm have been obtained by a patented emulsion–diffusion method. The morphology, size and structure of the nanocapsules (NC) control their pharmaceutical properties, especially release of the drug. Here is reported a decisive contribution of transmission electron microscopy (TEM) in revealing the controlled form and size of the NC: mean size values measured by TEM do agree with data obtained by laser granulometry. Moreover, the TEM magnification allows an estimation of the membrane thickness.

Keywords: Nanocapsules; Emulsion–diffusion; Size distribution; TEM

1. Introduction

Nowadays, a considerable range of industrial applications relies on the microencapsulation of solid or liquid by polymer coating. This general technique is widely used in the pharmaceutical field, as numerous studies in the last 20 years have shown that a good way to enhance drug action is to associate the active molecule with a carrier system. The encapsulation allows enhancing the drug stability by protecting it towards its environment (storage conditions) and reducing adverse or toxic effects. Another objective of these delivery systems is to control the further release of the drug. In the pharmaceutical-sustained release preparations, the nanoparticles can be considered as a successful alternative to other forms for carrying active molecules [1,2]. The main interest of these colloidal vectors is to enhance the therapeutic effect by targeting the active molecule to its site of action and by creating a high local concentration. The pharmacokinetic profile of the drug is then modified; the efficiency is increased while the amount of drug administered and the risks of side effects are decreased [3].

Several microencapsulation methods are available for the preparation of colloidal drug delivery systems [4]. A part of them is using natural macromolecules or synthetic polymers; for the others, the polymer is created by chemical reaction simultaneously to the encapsulation step. The choice of a method depends primarily on the drug properties (especially its solubility) and on the type of particle searched (protection and liberation).The methods using preformed polymers are essentially based on rapid diffusion and mass transfer leading to the insolubilization of the polymer in the form of very small particles. The particles can be classified according to the nature of materials (amphiphilic macromolecules, monomers, or hydrophobic polymers) used for a preparation process or by their internal structure type: capsules (core shell) or spheres (matrix).

The emulsion–diffusion technique, patented by Quintanar-Guerrero et al. [5] is used to produce nanocapsules (NC) based on biodegradable polymers. It is a two-step process based on the production of an emulsion, followed by a dilution leading to the deposition of the polymer around the droplets, thus, the formation of NC. The emulsification involves a partially water-soluble solvent previously saturated with water in order to ensure the initial thermodynamic equilibrium between the two liquids (water and solvent). Polymer, oil and drug are dissolved into the saturated solvent producing the organic phase. The aqueous phase is

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previously saturated with the solvent and contains a stabilizer. The obtained emulsion presents size values between 0.4 and 1.3 μm and is stable at room temperature for at least 5 months (no coalescence or breakdown detected). The subsequent addition of water to the system causes the solvent to diffuse into the external phase: this should result in the interfacial deposition of polymer to form the NC.

In order to contribute to a better knowledge of the NC formation by emulsion–diffusion, we have studied the effect of the experimental parameters of this method on the final properties of the NC such as morphology and particle size distribution.

The size of the particles is a very important parameter, because it is one of the factors controlling the kinetics of drug release. The structure of the particle and the thickness of the membrane are also key parameters, due to their major role played in the protection of the drug and in the kinetics of drug release. The particle sizes were measured using the technique of laser granulometry where data interpretation relies on the utilization of models based on the optical properties of the dispersions. Thus, a great contribution to the granulometric and morphological study of the NC was brought about by transmission electron microscopy (TEM) which provides information from direct observation of the particles.

The aim of the present work is to describe the characterization of the NC morphology and structure by TEM achieved up to high spatial resolution adapted to the nanometer-scale size of the NC.

2. Materials and methods

2.1. Materials

The polymer used for the NC formation was poly-ε-caprolactone (PCL) (Aldrich Chemical, USA). Its average molecular weight given by Aldrich was close to 80 000 Da. It is often involved in the production of NC, because of its properties of biocompatibility and biodegradability. It was used for the controlled delivery of several low molecular weight drug [6].

The core of the NC was made of an oil, Miglyol® 812, a mixture of capric and caprylic triglycerides from Condea Chemie. The solvent was pure ethyl acetate from Laurylab. For the tests of drug encapsulation, indomethacin (1-[*p*-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid) from Sigma was chosen as the active principle in solution in the oil.

The nonionic and hydrophilic stabilizer commonly used was polyvinyl alcohol (PVA) (Mowiol® 40-88, 88% hydrolyzed, M_w ca. 127 000 Da, from Aldrich Chemical). For the tests of drug encapsulation, it was replaced by a nonionic stabilizer, a poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) triblocks copolymer (Pluronic F68®, M_w 8400 Da from Sigma, Cell Culture, USA).

Distilled water saturated with solvent was used as a nonsolvent and distilled water as a diluent for the emulsion.

2.2. Methods

2.2.1. Preparation of nanocapsules

The preparation of nanocapsules follows different steps described below:

1) solvent and water mutual saturation
2) preparation of the aqueous phase
3) preparation of the organic phase
4) emulsification (oil in water)
5) dilution of the emulsion
6) purification and concentration.

2.2.2. Experimental conditions

Most of the experiments were carried out in the following conditions.

2.2.2.1. Saturation. Mixing of water (100 ml) and solvent (50 ml). Rest during several hours. The saturated solvent contains 2–3% of water. The saturated water contains 8–10% of solvent.

2.2.2.2. Organic phase (10 ml). Ethyl acetate saturated with water and containing the polymer (PCL) at the concentration of 20 g l^{-1} and the oil (drug) at the concentration of 50 g l^{-1}. This phase is prepared under mechanical stirring at 40 °C for 20 min.

2.2.2.3. Aqueous phase (40 ml). Water saturated with solvent and containing the stabilizer (PVA) at the concentration of 25 g l^{-1}. This phase is prepared at 50 °C under mechanical stirring for 2 h (PVA) or at ambient temperature for a few minutes (Pluronic F68®).

The saturated water contains 8–10% of solvent (ethyl acetate).

2.2.2.4. Emulsion. The resultant organic solution was poured into saturated water containing the stabilizer and stirred with a rotor–stator device (Ultraturrax® T25) at 8000 rpm during 5 min in a cylindrical vessel. The emulsion O/W is formed at 20 ± 5 °C.

2.2.2.5. Dilution. Addition on the emulsion of a large volume of distilled water (four times the emulsion volume), under moderate stirring (300 rpm), with subsequent formation of NC.

2.2.2.6. Purification and concentration. Elimination of the solvent and of part of the water by evaporation under reduced pressure to get a purified and concentrated suspension.

The final suspensions have a solid content of about 1%, which is increased at 5% after the concentration step and can easily reach 15% by further evaporation.

2.2.3. Nanocapsules characterization

2.2.3.1. Laser granulometry size analysis. The particle size distribution was determined by a diffusion method using a light-scattering particle size analyzer Coulter LS 230 (Beckman Coulter, Coultronics France). The LS 230 measures the size distribution using the diffraction of laser light by particles (diffraction pattern).

Information about particles smaller than 0.4 μm is limited in diffraction pattern, so another technique is used. Thus, the LS 230 includes another measurement assembly, called Polarization Intensity Differential Scattering (PIDS). The PIDS assembly consists of an incandescent light source and polarizing filters, a PIDS sample cell and an additional seven photodiode detectors (six to measure scattered light plus one to monitor the beam strength).

To perform the measurement, the sample is placed into the suspension fluid at a high dilution level. Our emulsions were analyzed using the water saturated with solvent as suspension fluid. This procedure avoids the solvent diffusion (and therefore, the production of NC) inside the analyzer itself. Otherwise, the NC sizes were analyzed in distilled water. The theory of light diffusion (Mie) [7] requires the refractive index of the media and the particles, which are approximated by models of O/W emulsion, i.e. water as refractive index for continuous phase and oil Miglyol®812 as refractive index for our particles.

2.2.3.2. TEM studies. Morphology and structure of the NC were studied in a TOPCON 002B microscope operating at 200 kV and of a 0.18 nm capable point-to-point resolution. Combination of bright field (BF) imaging at increasing magnification and of diffraction modes was used to reveal the form and size of the NC and to determine the amorphous or crystalline character of their components.

In order to perform the TEM observations, the NC-concentrated suspension was first diluted in water, after this, two different preparation methods were used. In the first method, known as negative fixation [8], the diluted suspension was added with 2% sodium phosphotungstic acid solution; a drop of the mixture was deposited on a standard copper grid covered by a holey carbon film and dried at ambient temperature before observation. After evaporation of the liquids, the NC, i.e. the oil/drug core inside the PCL membrane, are fixed on the carbon film of the grid by the stabilizer polymer, which is filled with heavy W atoms producing a high TEM absorption contrast. Thus, the NC will appear as white particles in the TEM image, whereas the surrounding areas are dark (see Fig. 3 for instance). Such samples are sensitive to the electron beam both from the irradiation effects and thermal effect of the electron beam. Our estimation was that maximal current densities of about 1 A/cm^2 could be used for stable imaging. This limited the high magnification imaging due to the exposure time which became too long.

In the second method of TEM specimen preparation without negativation, a drop of the diluted NC suspension was directly deposited on the holey film grid and observed after drying. In this case, the NC are fixed on the carbon film of the grid by the stabiliser polymer. The NC appear dark and the surroundings are bright, i.e. a "positive" image is seen. The contrast is lower than in the negativated specimens, but the specimen thickness is smaller and more details are visible. The direct observation also enabled us to perform selected area electron diffraction (SAED) to check the crystallinity of the polymer.

Confrontation of the two methods of preparation allows us to get complementary data about the NC.

3. Results and discussion

All the experiments carried out resulted in the formation of spherical individual particles, the mean size of which is ranging between 0.2 and 0.6 μm. The concentrated suspensions of NC are stable for at least 6 months (storage at room temperature). Fig. 1 gives an example of the size distribution of the droplets of the emulsion and of the corresponding NC. The comparison of the emulsion and NC suspension size distributions showed that the solvent diffusion occurs only after the emulsion formation, while the dilution water is added. However, some NC can also be formed at the step of emulsion due to partial solvent evaporation, which could explain the first peak observed in the size distribution for the emulsion.

Indeed, the NC mean size is always smaller than that of emulsion droplets. The size reduction between particles and droplets depends on the experimental conditions (solvent volume, dilution). A 500-nm reduction of size is obtained in the standard conditions (see above). These observations bring to the fore the fact that the formation of NC by emulsion–diffusion of solvent is a dynamic process associated with the diffusion of the solvent from the droplet to the external phase. This transfer is induced by the addition of water on the emulsion and results in the transformation of each droplet in a particle of smaller size [9].

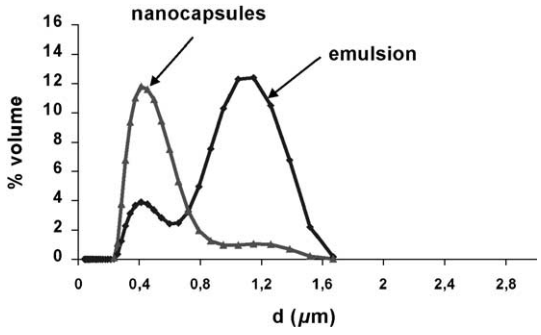

Fig. 1. Size distributions of emulsion droplets and of NC.

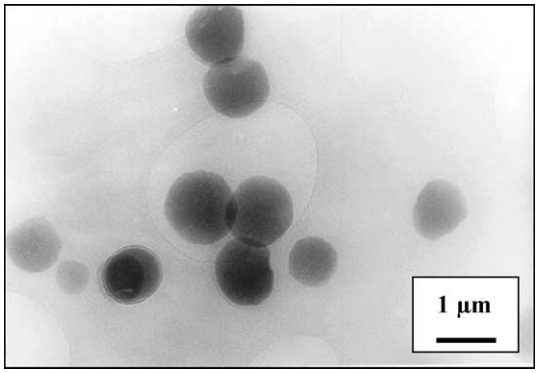

Fig. 2. NC of amorphous PCL observed after direct deposition on the TEM grid.

3.1. TEM observations

Low to medium magnification BF images obtained from specimens prepared by direct deposition (Fig. 2) allowed us to visualize the form of the NC and to check the distribution of their size.

For comparison, Fig. 3 reproduces a higher magnification BF image of a small NC, which appears brighter than the dark surrounding negativated matrix in which it is practically embedded.

All these images show that the NC morphology is spherical. In some cases, two NC were observed in close contact like in Fig. 4. In this case, they deform and the adjacent capsule membranes become parallel and planar, showing that the spherical shape is not rigid in fact (due to the liquid content inside). Such a high resolution image allowed us to visualize and estimate the thickness of the polymer membrane of the NC. In Fig. 4b, enlarged details from the image in Fig. 4a are seen, revealing the double PCL membrane of the two adjacent NC. We consider that the negativation salt has not penetrated in the NC or between the adjacent membranes of the two NC since a white contrast of about 1 nm extent separates the two walls. From this image, we can estimate the PCL membrane thickness to be between 1.5 and 2 nm.

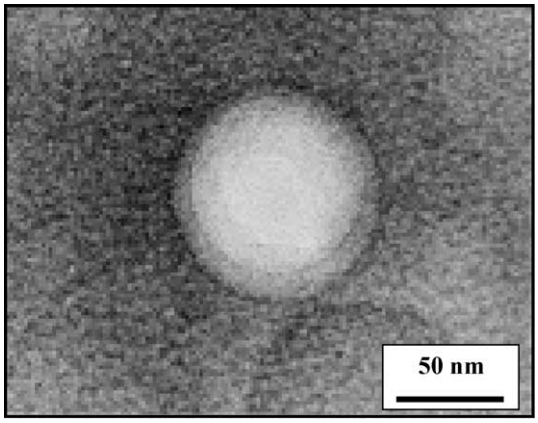

Fig. 3. High magnification TEM image of a spherical NC observed after negative fixation.

In Fig. 5 is reproduced a BF image of a small NC from a specimen prepared by direct deposition (without negativation). In this case, the membrane is discerned in the projection contrast, but somewhat blurred by a dark halo due to the stabilizer polymer which fix the capsule on the carbon film. From such images, the thickness of the NC membrane can be estimated to be less than 4 nm. The direct observation established that the membranes of all the NC exhibit a similar aspect and that the NC content density appears lower than that of the PCL polymer membrane.

The direct observation of the non-negativated specimens allowed us to use electron diffraction for crystallography. The PCL polymer is generally amorphous. In few cases however, we found crystallized polymer with dendritic

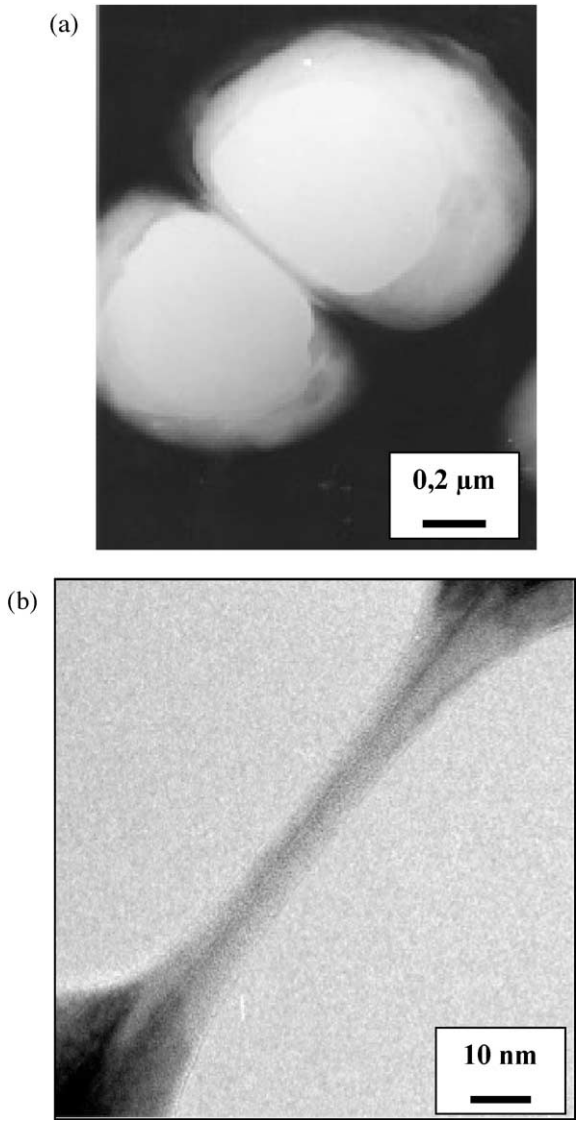

Fig. 4. TEM observations of adjacent NC after negative fixation: (a) medium-sized magnification image; (b) high magnification image of the area of contact between the two NC, revealing their membranes observed at high resolution. The membrane thickness for one NC is estimated to be 1–2 nm.

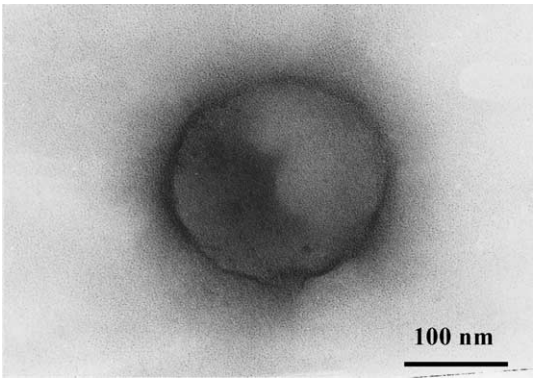

Fig. 5. High magnification TEM image of a rather small NC observed after direct deposition. The membrane is visible, surrounded by a dark halo due to the stabilizer polymer.

morphology [10]. This crystallization is probably due to the aging of the initially semicrystalline polymer.

The specimens obtained by direct deposition are also sensitive to the large intensity of the electron beam. Some of the NC broke down during the TEM observations and released their liquid content in the surrounding areas: a somewhat bright halo is then seen around the NC (Fig. 6a). Some NC can experience a collapse, which modifies their morphology, like in Fig. 6b. Obviously, this collapse is limited, which suggests that there may be a spongy structure of polymer filled with liquid inside the NC. This structure

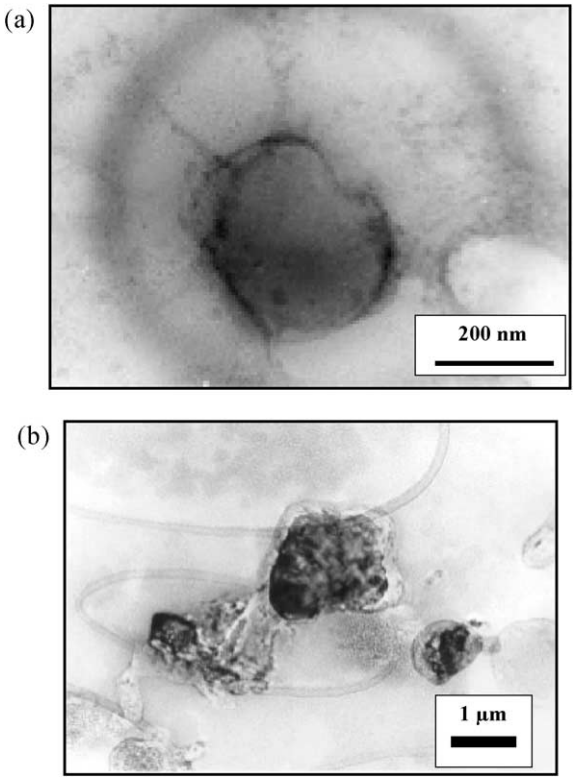

Fig. 6. TEM visualization of the content of the NC: (a) image of an NC (the ratio PCL/oil being 2:5) whose membrane is broken; the NC is seen surrounded by a halo of liquid; (b) oil content spilled out of broken NC.

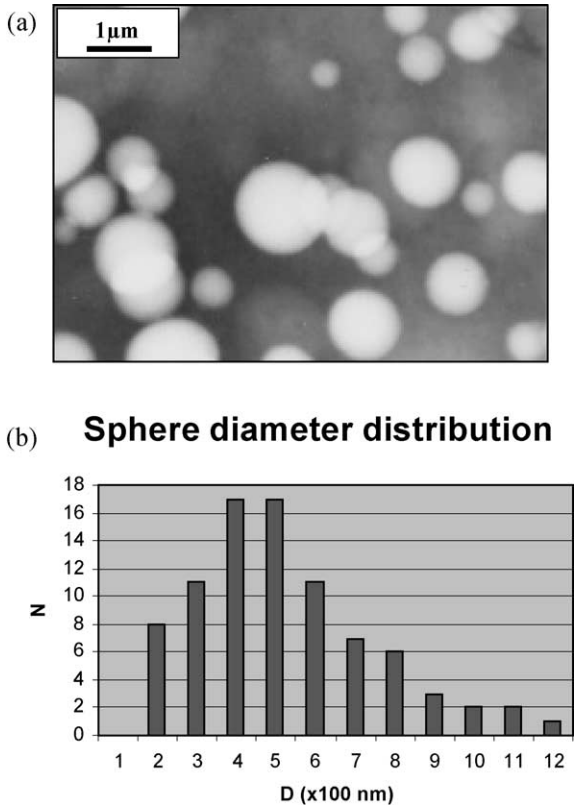

Fig. 7. Example of size distribution measured by TEM: to the left is the image of NC population (the PCL/oil ratio being 0.4:0.5) observed after negative fixation; to the right is the histogram of sizes measured over about 100 NC; the mean size is determined as 520 nm.

relaxes when the NC is broken, but such a relaxation is limited. It must be pointed out that at the same time, the stabilizer polymer that fixes the NC on the grid carbon film also maintains the shape of the NC when it breaks.

An example of NC size distribution measured by TEM is shown in Fig. 7. As it can be observed, the size of the NC varies from about 200 to 1200 nm, and the average diameter is determined as 520 nm. Table 1 displays comparison between size distributions determined by laser granulometry and TEM: the agreement is satisfactory, if taking into account the poorer statistics of TEM measurements and the fact that laser granulometry detects and includes some NC agglomerates; thus, granulometry mean sizes tend to shift to larger values.

Table 1
Laser granulometry and TEM measurements of NC mean size depending on the PCL concentration, the oil percentage being fixed as 2.5%

Mass ratio of PCL/oil	Percentage of PCL in organic phase (g/l)	Mean size measured by granulometry (nm)	Mean size measured by TEM (nm)
2:5	20	500	470
4:5	20	500	520
10:5	50	741	585
25:5	125	1325	694

4. Conclusion

The emulsion–diffusion is an original and efficient method to produce colloidal encapsulation systems. TEM studies proved that spherical NC are elaborated by the emulsion–diffusion route with the expected mean size. This mean size can be controlled by the formulation and process parameters such as the polymer content and the agitation during the emulsion step [9,11]. Basically, there is a good agreement between the mean size values measured by TEM and laser granulometry. The slight differences which are observed can be explained by the fact that the measurements by laser granulometry are done in diluted suspensions where agglomeration of particles can occur (despite agitation) and thus, a larger mean size.

The vesicular structure of the NC was brought to the fore by the TEM observations. The PCL membrane and the oil content of the NC were visualized and owing to the high resolution of the TEM images, the membrane thickness could be evaluated at 1–2 nm. This characterization of the NC morphology is very important to quantify the capacity of storage of a drug inside the core of the NC and the further release of this drug. These results are in agreement with previous studies of the NC structure by centrifugation on sugar gradient density [12,13].

Moreover, combination of TEM observations by direct deposition and after negativation enables us to gather data about all the components of the NC.

Further investigations on the vesicular or spongy structure of the particles will be achieved by studying the influence of the quantity of polymer in the NC. For this study, the basis of comparison will be matrix systems (involving nanospheres) without oil.

Furthermore, different solvents can be used for the preparation of the NC and study of the influence of the solvent diffusion rate on the structure of the NC is under way.

References

[1] N. Ammoury, Etude physico-chimique et biologique de vecteurs colloïdaux vésiculaires d'indométacine-acide polylactique, Thèse de Doctorat, Université Paris Sud, mention Sciences Pharmaceutiques no. 167, 1990.
[2] S. Stainmesse, Etude galénique d'un nouveau procédé d'obtention de vecteurs colloïdaux submicroniques à partir d'une protéine ou d'un polymère synthétique, Thèse de Doctorat, Université Paris Sud, mention Sciences Pharmaceutiques no. 129, 1990.
[3] S. Benita, in: S. Benita (Ed.), Microencapsulation—Methods and Industrial Applications, Marcel Dekker, New York, 1996.
[4] C. Vauthier-Holtzscherer, S. Benabbou, G. Spenlehauer, M. Veillard, P. Couvreur, Methodology for the preparation of ultra-dispersed polymer systems, STP Pharma Sciences 1 (2) (1991) 109–116.
[5] D. Quintanar-Guerrero, H. Fessi, E. Doelker, E. Allemann, Methods for preparing vesicular nanocapsules, PCT Patent no. W09904766A, 1999.
[6] Y. Cha, C.G. Pitt, The biodegradability of polyesters blends, Biomaterials 11 (2) (1990) 108–112.
[7] F. Guimberteau, F. Leal Calderon, Granulométrie des émulsions de bitume, Bulletin des Laboratoires des Ponts et Chaussées (1999) 13–22.
[8] F. Leria, R. Marco, F.J. Medina, in: University of Barcelona (Eds.), Microscopy, 2001, p. 145.
[9] A.P. Colombo, S. Brianon, H. Fessi, J. Lieto, Project, design and use of a pilot plant for nanocapsules production, Drug Development and Industrial Pharmacy 27 (2001) 10.
[10] V.S. Teodorescu, M.G. Blanchin, S. Guinebretière, S. Brianon, H. Fessi, HREM Structural study of nanocapsules in view of drug release, Nature Materials, 2002, submitted.
[11] D. Quintanar-Guerrero, E. Allemann, E. Doelker, H. Fessi, A mechanistic study of the formation of polymer nanoparticles by the emulsification–diffusion technique, Colloid and Polymer Science 275 (1997) 640–647.
[12] S. Guinebretière, S. Brianon, J. Lieto, C. Mayer, H. Fessi, Study of the emulsion–diffusion of solvent: preparation and characterization of nanocapsules, Drug Development Research, 2002, in press.
[13] S. Guinebretière, Nanocapsules par émulsion–diffusion de solvant: obtention, caractérisation et mécanisme de formation, Thèse de Doctorat, Université Claude Bernard Lyon 1 (2001).