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Methodology

Drug Delivery

Microscopy characterisation of micro- and nanosystems for pharmaceutical use

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Abstract

Introduction

This review provides an overview of the use of microscopy as a tool to characterise shape and dimension of micro and nanoparticulate systems.

In the pharmaceutical field, the use of microscopy has been exerted an important role since the advent of micro and nanotechnology. Indeed, the morphology of particles and their inner structure does influence the modalities of administration and release of encapsulated drugs.

Scanning electron microscopy can be employed to study the morphology of dry powders. In particular microparticles made of polymers can be well visualised and their diameters can be measured. By cutting the powder during sample preparation, it is possible to obtain important information about the inner morphology of the microstructures, discriminating either the capsule or the sphere microstructure.

Cryo transmission electron microscopy is a precious tool for characterising colloidal systems. In particular, external as well as internal shape of nanoparticulate systems such as solid lipid nanoparticles or lyotropic mesophases can be well identified. Moreover, size of disperse phase and the overall structure of the dispersion can be monitored.

We provide an overview about the use of electron microscopy as technique for characterising microparticles and nanosystems recently developed by our research group. In particular, polyester or acrylic polymer microparticles for fenretinide administration are presented here. Moreover, with regard to nanosystems, solid lipid nanoparticles, nanostructured lipid carriers for prednisone and clotrimazole administration and monooleine aqueous dispersion are discussed.

Methodology

Microparticles were produced by the 'solvent evaporation method'. Solid lipid nanoparticlesand nanostructured lipid carriers were prepared by stirring, followed by ultrasonication. Monooleine aqueous dispersionswere produced by the hydrotrope or by the hot homogenization methods. For scanning electron microscopyanalysis, microparticles were metallized by gold coating (Edwards Sputter coat-ing S150) and visualized at 15 – 20 kV with a 360 Stereoscan (Cambridge Instruments, Cambridge, UK). For Cryo-TEM analysis samples were vitrified and transferred to a Zeiss EM922Omega (Zeiss SMT, Oberkochen, Germany). Images were recorded by a CCD digital camera (Ultrascan 1000, Gatan) and analyzed using a GMS 1.8 software (Gatan).

Conclusion

Microscopy is to be considered as an indispensable tool to study drug delivery systems.

In particular, scanning electron microscopy is helpful in giving information about micro-sized powders, allowing to identify microspheres and microcapsules, as well as to obtain size distribution of the observed particles.

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Introduction

Micro- and nanoparticles have attracted pharmaceutical interest in the last decades since they offer a number of advantages with respect to other delivery systems such as: (a) the ability to maintain unaltered physicochemical characteristics for long periods allowing long-term storage; (b) the possible administration through different ways (oral, intramuscular or subcutaneous) depending on their composition and (c) their suitability for industrial production[1].

Concerning nanosystems, lipid dispersions possess a potential use as matrixes able to dissolve and deliver active molecules in a controlled fashion, thereby improving their bioavailability and reducing side-effects[2].

In particular, solid lipid nanoparticles (SLN) are delivery systems in which the nano-dispersed phase has a matrix of crystalline solid lipids. SLN are able to protect encapsulated molecules from degradation and modulate their release[3,4]. Another generation of SLN is represented by nanostructured lipid carriers (NLC) whose matrix is composed of a mixture of solid–liquid lipids able to better solubilise lipophilic drugs[5,6].

Another type of lipid dispersion able to provide matrices for sustained drug release is typified by monooleine aqueous dispersions (MAD).

MAD are heterogeneous systems generated by the dispersion of an amphiphilic lipid, such as monoolein, in water. They are constituted of complex lyotropic liquid crystalline nanostructures like micellar, lamellar, hexagonal and cubic phases, the predominance of one species over

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the other mainly depending on temperature and water content of the system[7,9].

Electron microscopy is a precious mean to investigate micro and nanosized pharmaceutical forms. It gives information about shape, inner structure and dimensions of powders and dispersions that couldn't otherwise be identified.

Scanning electron microscopy (SEM) can be employed to analyse shape, external surface and dimensional distribution of dry microparticles[10]. It can also be used to visualise the inner structure of sectioned microparticles, giving information about the mutual distribution of components, such as polymers and drugs, within the particle. Thanks to this technique, it is possible to define the analysed micro-object as 'microsphere' or as 'microcapsule'[11].

For instance, the use of ideal perfectly round polymeric microspheres with exactly the same size circumvents all the disadvantages encountered using powders and granulates. Microspheres are free-flowing and roll without friction, thus no abrasion is present, guaranteeing a dust-free environment. Drugs embedded in the microsphere matrix are released continuously and at a constant rate[12].

Cryogenic transmission electron microscopy (Cryo-TEM) can be used to analyse liquid dispersions very close to their native state, due to the possibility of vitrifying samples[13]. Nanosized disperse phases that appear as milky liquids by eye, can be accurately identified by cryo-TEM. Uni- or oligo-lamellar vesicles, lamellar phases, cubic phases, cubosomes, exosomes, etc. can be specified and measured one by one[14].

This article provides an overview about the use of electron microscopy technique as means for characterising microparticles and nanosystems, as recently demonstrated by our research group. In particular with respect to microsystems, polyester- and acrylic polymer-based microparticles are presented here. On the other hand, concerning nanosystems, nanoparticulate dispersions such as SLN, NLC and MAD are illustrated.

Methodology

The authors have referenced some of their own studies in this methodology. The protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed.

Production of microparticles

Microparticles were produced by the 'solvent evaporation method'[15]. Briefly, 250 mg of a polymer and cationic agent mixture were dissolved in 7.5 ml of CH_2Cl_2 or ethyl acetate. The mixture was emulsified with 200 ml of an aqueous phase containing 2% (w:v) of 88% hydrolysed polyvinyl alcohol (PVA) (Airvol 205, Air Products, PA, USA) as the dispersing agent. The obtained emulsion was maintained under continuous stirring with a three-blade turbine impeller Eurostar Digital (Ika Labortechnick, Germany) at 700 rpm and by heating (hot plate RCT basic, Ika Labortechnick, Germany) at a temperature higher than the solvent evaporation temperature (40°C in the case of methylene chloride and 77°C for ethyl acetate). At different time intervals, samples were observed microscopically up to complete evaporation of solvent, usually occurring in 3–5 h. Microparticles were then isolated by filtration.

Production of nanoparticles

SLN and NLC were prepared by stirring followed by ultrasonication[16]. Briefly, 1 g of lipidic mixture was melted at 75°C. The lipidic mixture was constituted of tristearin (in the case of SLN) or tristearin/caprylic/capric triglycerides (Miglyol 812) 2:1 w/w (for NLC). The fused lipid phase was dispersed in 19 ml of an aqueous poloxamer 188 solution (2.5% w/w). The emulsion was subjected to ultrasonication (Microson TM, Ultrasonic cell Disruptor) at 6.75 kHz for 15 min and then cooled down to room temperature by placing it in a water bath at 22°C. NLC dispersions were stored at room temperature.

In the case of drug containing nanoparticles, the drugs were added to the molten lipidic mixture and dissolved before addition to the aqueous solution.

Production of MAD

MAD were alternatively produced by the hydrotrope or by the hot homogenisation methods.

The hydrotrope method is based on the solubilisation of monooleine in ethanol and its subsequent dilution in a surfactant solution[17]. In particular, 5 ml of an ethanolic solution of monooleine (10% w/w, with respect to total weight of the dispersion), was slowly added to an aqueous solution of Poloxamer 407 (10% w/w) under stirring at 1500 rpm for 90 min, until complete ethanol evaporation.

The hot homogenisation method is based on the emulsification of monooleine (4.5% w/w) and Poloxamer 407 (0.5% w/w) in water (90%, w/w), as described by Esposito et al.[18]. After emulsification, the dispersion was subjected to homogenisation at 15 000 rev/min (Ultra Turrax, Janke & Kunkel, Ika-Werk, Sardo, Italy) at 60°C for 1 min; afterwards, it was cooled and maintained at room temperature in glass vials.

SEM analysis

For the electronic analysis, microparticles were metallised by gold coating (Edwards Sputter coating S150) and analysed at 15–20 kV by a SEM 360 Stereoscan (Cambridge Instruments, Cambridge, UK)[19].

Cryo-TEM

Samples were vitrified as described in a previous study by Esposito et al.[20]

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The vitrified specimen was transferred to a Zeiss EM922Omega (Zeiss SMT, Oberkochen, Germany) TEM using a cryoholder (CT3500, Gatan, Munich, Germany). Sample temperature was kept below 100 K throughout the examination. Specimens were examined with doses of about 1000–2000 e/nm^2 at 200 kV. Images were recorded by a CCD digital camera (Ultrascan 1000, Gatan, Germany) and analysed using GMS 1.8 software (Gatan, Germany).

Discussion

SEM can help in giving information about the influence of technological parameters on the shape of microspheres.

The morphology of microsystems can influence the release behaviour of the encapsulated drug. In particular SEM enables to discriminate the surface aspect of microspheres focusing on the presence or the absence of pores, indeed the porosity is a factor that can promote drug release[21]. Moreover, by cutting the sample before the visualisation, it is possible to obtain cross-sectioned microparticles and to observe their inner structure, allowing to discern between a microsphere and a microcapsule[22]. It is well known that a microsphere is a solid matrix type particle whereas a microcapsule has an inner core (reservoir) and an outer shell. The active ingredient in a matrix particle is typically dissolved or melted. Whereas the microcapsule core, either liquid or solid, usually contains the active ingredient[1].

It is well known that the release of the active constituent is an important parameter depending on the nature of both the polymer used in the preparation as well as the active drug.

Particularly, the release of drug from microparticles is influenced by structure or micro-morphology of the carrier and the properties of the polymer itself[23–25].

Drug release from the microparticles can be understood by considering the geometry of the carrier. The geometry of the carrier (i.e. whether it is reservoir type where the drug is present as core, or matrix type in which drug is dispersed throughout the carrier) governs overall release profile of the drug or active ingredients[26].

Depending on the type of polymer and on the method of production, different type of microparticles can be achieved[27].

Figure 1 reports SEM images of DL-PLGA 50:50 microparticles obtained by the solvent evaporation method. One can observe the round shape and the smooth surface of the particles; moreover from Figure 1A it is possible to obtain a dimensional distribution by measuring the mean diameters of the particles.

In Figure 2 are reported images showing external and internal morphology of empty DL-PLGA 50:50 microparticles produced by the solvent evaporation method, using as solvent methylene chloride (Figure 2A and B) or ethyl acetate (Figure 2C and D). It is interesting to note that methylene chloride induces the formation of irregular shaped microparticles with different dimensions, whereas the use of ethyl acetate results in round microparticles with smooth surface and homogeneous dimensions. Moreover, analysing the cross-section, it can be noted that the

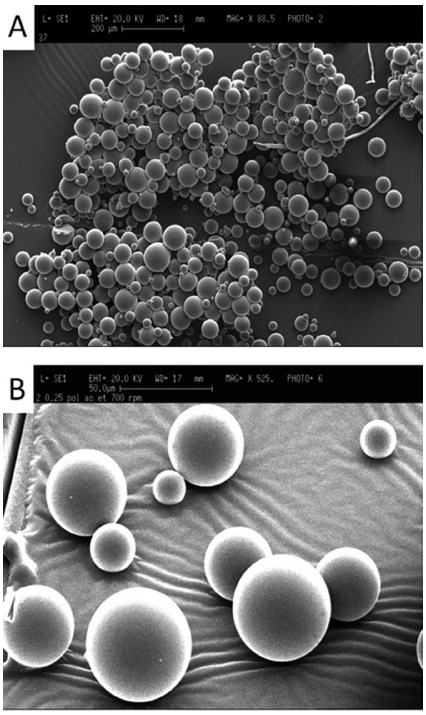

Figure 1: SEM images of plain DL-PLGA 50:50 microparticles.

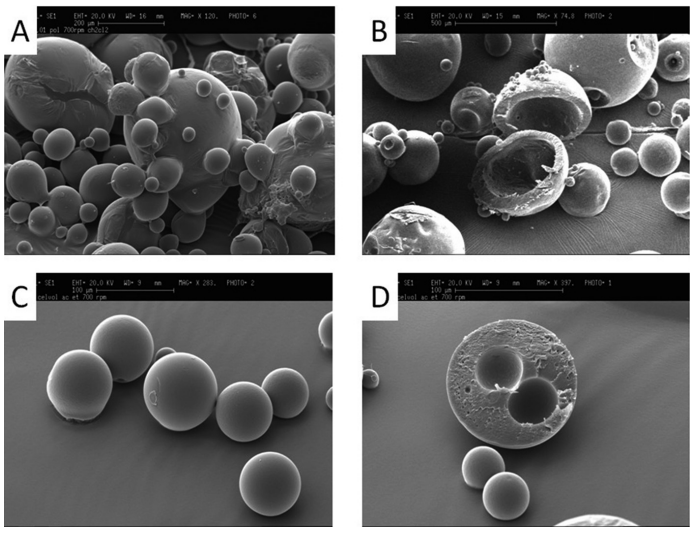

Figure 2: SEM images showing external and internal morphology of plain DL-PLGA 50:50 microparticles produced by the solvent evaporation method, using as solvent methylene chloride (A and B) or ethyl acetate (C and D).

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use of methylene chloride allows the production of mononucleate microcapsules with a classic reservoir structure, while the particles by ethyl acetate show a multinucleate structure. This result can be attributed to the different evaporation rates of the solvents; in fact, methylene chloride (boiling point 40°C) evaporates more rapidly than ethyl acetate (boiling point 77°C).

Figure 3 reports external and internal morphology of fenretinide containing DL-PLGA 50:50 microparticles produced by the solvent evaporation method using as solvent ethyl acetate. One can observe that the presence of fenretinide doesn't modify the external morphology and surface, being similar to those reported in Figure 2C.

On the contrary the cross-section, reported in Figure 4B reveals that in the presence of fenretinide the inner morphology of the microparticles is more compact with respect to the microcapsular reservoir structure of the plain microparticles (Figure 2D).

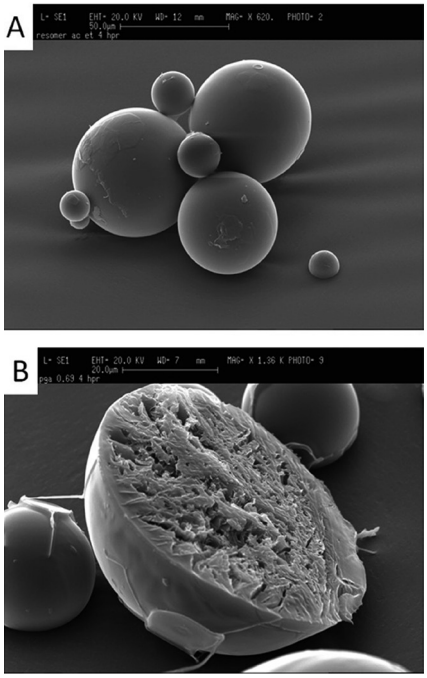

Figure 3: SEM images showing external and internal morphology of fenretinide containing DL-PLGA 50:50 microparticles.

Figure 4 shows external morphology of empty Eudragit RS 100 microparticles produced by the solvent evaporation method using as solvent ethyl acetate. From Figure 4A it is possible to obtain the dimensional distribution of microparticles, ranging from 5 to 40 µm, with a mean diameter of 27 µm. Figure 4B evidences the round shape and the porous surface of microparticles, differently from DL-PLGA ones.

In the case of nanosized liquid systems, cryo-TEM is an optimal method to obtain structural information, revealing morphology and inner details of the samples studied[13]. Figure 5 shows cryo-TEM images of SLN and NLC dispersions constituted of tristearin and tristearin-Miglyol 812 (2:1), respectively. In particular, Figure 5A shows an image of plain SLN, Figure 5B refers to plain NLC, while Figure 5C and D, respectively show prednisone and bromocriptine containing NLC.

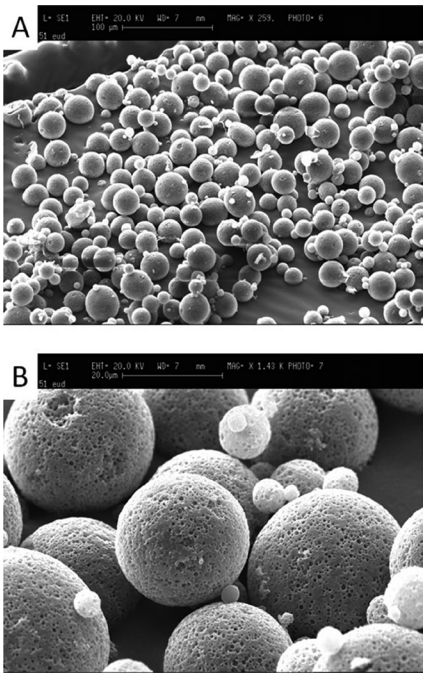

Figure 4: SEM images of Eudragit RS microparticles.

The three-dimensional particles are projected in a two-dimensional (2D) way. In Figure 5A–D, depending on the angle of observation in the vitrified samples, the particles appear as deformed hexagonal, elongated circular platelet-like crystalline (SLN/NLC top views) or 'needle'-like structures edge-on viewed (SLN/NLC side views). The measured thickness of nanoparticles is between ca. 5 and 40 nm, according to the number of layers in the platelets. However, the exact thickness is difficult to measure since the tilt of the particles can't exactly be determined. Figure 5B, C and D, besides the presence of hexagonal and circular NLC top views, the 'needles' are more elliptically shaped, with respect to the SLN side views (Figure 5A). This may be attributed to the presence of Miglyol 812, which is an oil, forming compartments sticking to the surfaces of the nanoparticles solid matrix[28,29]. This behaviour is clearly noticeable in the presence of prednisone and bromocriptine (Figure 5C and D).

Figure 6 shows cryo-TEM images of NLC dispersions constituted of tristearin-Miglyol 812 in the presence (Figure 6A) and in the absence of clotrimazole (Figure 6B). One can observe elongated ovoidal and circular platelet-like crystalline particles in both Figure 6A and B. The nanoparticles edge-on viewed appear as rectangles with stacked tristearin lamellas in Figure 6A, while in Figure 6B the presence of clotrimazole affects nanoparticles aspect, resulting in 'UFO' profile, as shown in the insets.

Figure 7 shows two aqueous dispersions of monooleine produced in the presence of P oloxamer 407 obtained by the hydrotrope (Figure 7A) or by the hot homogenisation method (Figure 7B). Some authors have reported that the method resulted in the production of cubosome dispersions. Nonetheless in this case only differently shaped vesicular structures mainly unilamellar or with some invaginations were obtained[17,30].

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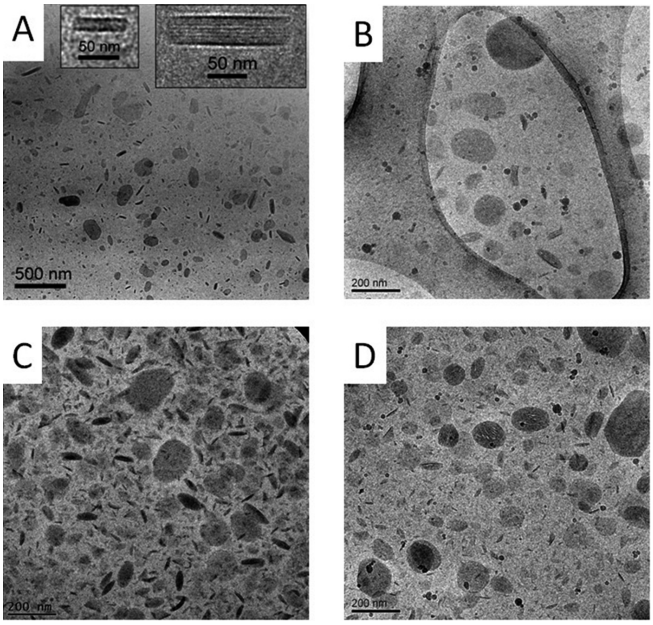

Figure 5: Cryo-TEM images of empty SLN (A), empty NLC (B), prednisone containing NLC (C) and bromocriptine containing NLC (D).

Whereas the dispersion reported in Figure 7B displays the coexistence of vesicles and spherical particles with a homogeneous ordered inner structure typical of cubosome.

It is interesting to note the presence of vesicular structures attached on the surface of cubosomes, as found in many cryo-TEM and X-ray studies, suggesting that through time, a transformation may take place from conglomerates of partially fused vesicles to well-ordered particles[18,31].

Considering a number of pictures taken from cryo-TEM observation, dimensions can be measured by a scale bar, finding that two different populations are often present: one constituted of large cubosomes (dimensions over 200 nm) and another of smaller cubosomes and vesicles with dimensions about 100 nm and below.

Figure 8 reports two cryo-TEM images of a monooleine/Poloxamer 407 dispersion, evidencing the presence of vesicles and ordered structures.

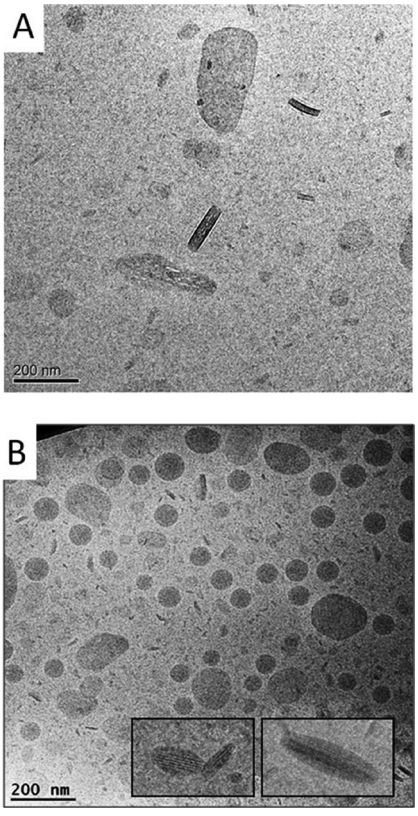

Figure 6: Cryo-TEM images of empty NLC (A) and clotrimazole containing NLC (B).

Fast Fourier transform (FFT) easily enables to obtain an optical diffractogram similar to an electron diffraction pattern. In this way, periodic or repeatable distances in the mesophase structure could be detected, together with the symmetry of the motif. FFT is, therefore, a precious means to identify the different type of liquid crystalline structures[13,32]. For instance, according to the different internal morphologies shown in Figure 8, FFT allowed to evidence two different patterns. In Figure 8A, the first corresponds to a rectangular symmetry with 2D lattice parameters of 7.1 × 10.2 nm (labelled a) while the second corresponds to a 2D hexagonal symmetry with a 7.1 nm lattice parameter (labelled b).

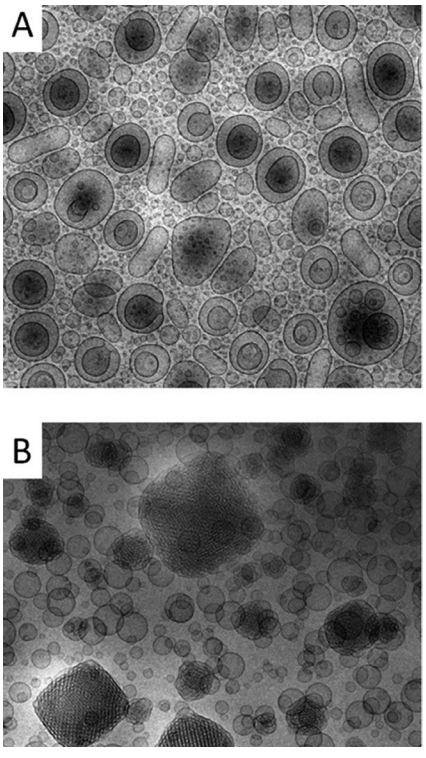

Figure 7: Cryo-TEM images of monoolein–Poloxamer 407 dispersions obtained by the hydrotrope (A) and by the hot homogenization (B) methods.

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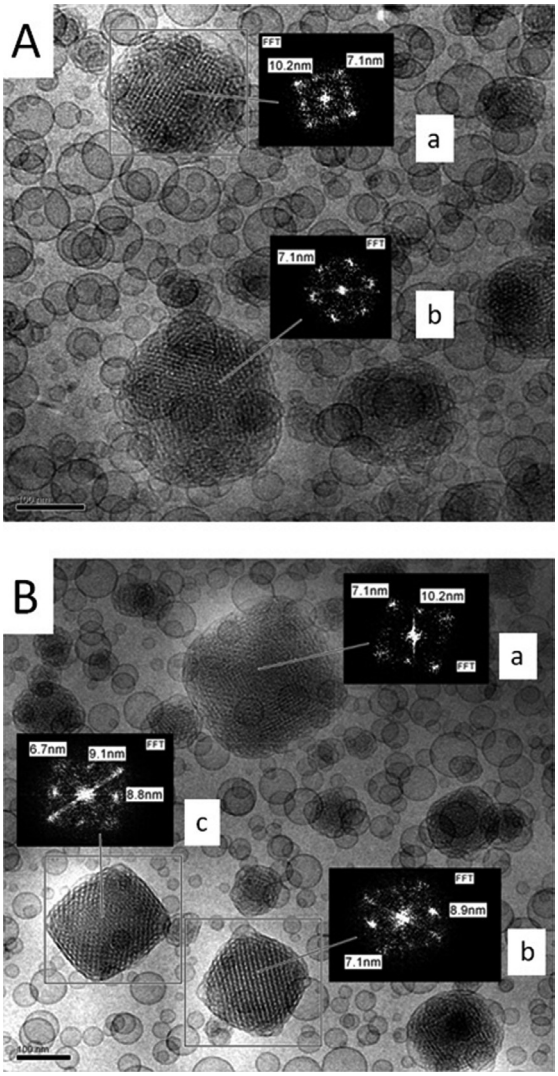

Figure 8: Cryo-TEM images of cubosomes dispersions. The insets show the FFT lattice parameters of the indicated nanostructures.

In Figure 8B, patterns with rectangular symmetry with differently sized 2D lattice parameters are present. In particular, lattice cubic particles with parameters of 7.1 × 10.2 nm (labelled a) and 7.1 × 8.9 nm (labelled b) can be observed. Moreover, also the diagonal dimension can be measured (labelled c).

Conclusion

Microscopy is to be considered as an indispensable tool to study drug delivery systems.

In particular SEM is helpful in giving information about micro-sized powders, allowing to identify microspheres and microcapsules, as well as to obtain size distribution of the observed particles.

Colloidal drug delivery systems, filling the most part of pharmaceutical panorama in nanotechnology field, can be unambiguously detected by the use of cryo-TEM. Charming images can reveal the external and inner structure of nanosystems, giving information about the drug release modality of the included molecules and suggesting their suitability for the chosen type of administration.

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