Engineering Materials

Analytical Imaging Techniques for Soft Matter Characterization

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Chapter 2
Visualization of Organic–Inorganic Nanostructures in Liquid

2.1 Introduction

The characterization of colloidal systems like pharmaceutical or hydrated chemical formulations by microscopic techniques is essential to obtain reliable data about the actual morphology of the system. Since the size range of colloidal drug delivery systems has long ago reached the lower end of the nanometer scale, classical light microscopy has been replaced by electron microscopy techniques which provide sufficient resolution for the visualisation of nano-sized structures. Indeed, the superior resolution and methodological versatility of electron microscopy has rendered this technique an indispensable tool for the analysis of nanoemulsions. Microscopic analysis of these lipid-based drug delivery systems with particle sizes in the lower submicron range provides critical information about the size, shape and internal structure of the emulsion droplets. Moreover, surfactant aggregates such as liposomes or multilamellar structures which remain unnoticed during particle size measurements can be detected in this fashion. This chapter provides a brief overview about both transmission electron microscopy (TEM) and scanning electron microscopy (SEM) techniques which have been employed to characterise colloidal solutions. Of special interest are sophisticated cryo techniques of sample preparation for both TEM and SEM which deliver high-quality images of pharmaceutical formulations in their natural state. An overview about the instrumentation and sample preparation for all presented methods is given. Important practical aspects, sources of error and common artefacts as well as recent methodological advances are discussed. Selected examples of electron microscopic studies of nanoemulsions are presented to illustrate the potential of this technique to reveal detailed and specific information.

The colloidal solution structure can be viewed in an electron microscope without further preparation and certain conclusions can be drawn from the obtained images if the circumstances are favourable. However, far more representative information
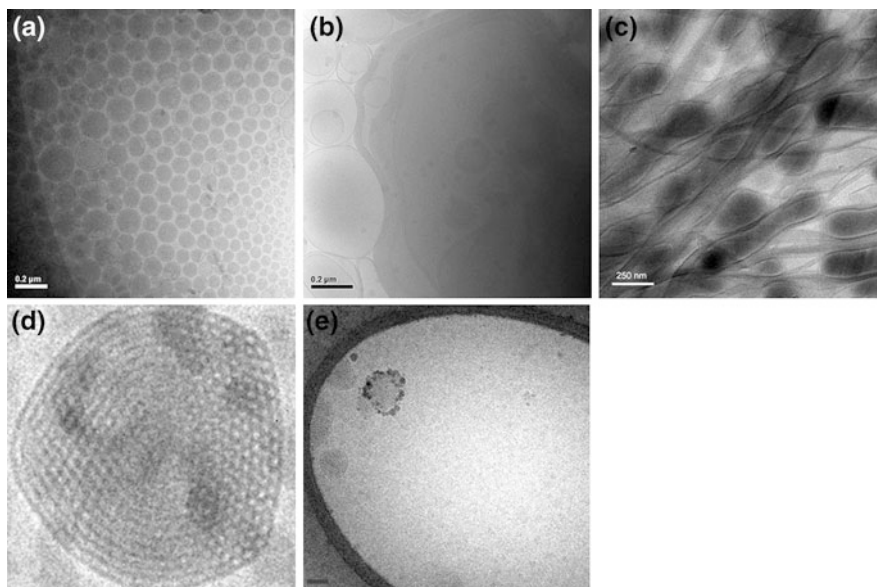
Fig. 2.1 Cryo TEM of different hydrated systems. **a** nanoemulsion, **b** liposomes-proteins solution, **c** solid lipid nanocaring system, **d** liquid crystal structure (Scale bar 20 nm), **e** self-assembled oil in water system functionalised with silica particles (Scale bar 50 nm)

can be gained upon observation of the sample in its native state after cryo-preparation in thin vitrified layers or at a fracture plane using both transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Fig. 2.1) [1]. Although some of the described techniques are rarely employed for nanoemulsion characterisation due to the time-consuming and complex sample preparation and high costs, all methods reported in the literature are briefly described here. Since TEM in combination with cryogenic techniques of sample preparation is among the most suitable methods for the investigation of nanoemulsions in their original state, a focus is laid on these and related techniques. The innovative techniques of electron energy-loss spectroscopy (EELS) and energy dispersive X-ray spectroscopy (EDX) are likewise presented in this context. Other microscopic techniques such as atomic force microscopy (AFM) have been employed for the investigation of colloidal solution. However, the output of this technique is more essential for the investigation of high pressure frozen and freeze substituted biological systems than for colloidal systems (see Chap 4, 5, 6, and 7). The electron microscopic techniques that are most frequently employed for the analysis of nanoemulsions, nanosuspensions, polymer solutions etc. today as well as recent methodological advances are elucidated in the following sections.

2.2 Analytical Techniques Employed for the Characterisation of Colloidal Systems

The characterisation of nano-sized colloidal systems requires diverse techniques and a certain experience. Basic formulation characteristics such as visual appearance, pH, mean particle size, particle surface charge, chemical stability of employed excipients and the localisation of incorporated drugs provide useful information [2]. Especially, the mean particle size and the particle size distribution are frequently employed to characterise the long-term stability of novel formulations. These parameters can be determined by optical light scattering techniques. More specifically, dynamic light scattering (DLS, photon correlation spectroscopy) is frequently employed for determination of nano-sized oil droplets within emulsions. Among the obtained results, the mean particle size as intensity-weighted mean of the hydrodynamic diameter and the polydispersity index (PDI) are mostly presented [3, 4]. The latter characterises the width of the particle size distribution and thus the homogeneity of the formulation. A small PDI below 0.2 indicates a narrow droplet size distribution and thus better stability against destabilisation phenomena such as Ostwald ripening [2]. The characterisation and stability assessment of nanoemulsions is strongly associated with their droplet size and PDI. If both parameters remain largely unchanged during a prolonged observation period, a formulation is usually considered physically stable [5].

2.3 Transmission Electron Microscopy: Experimental Setup, Sample Preparation and Potential Artefacts

For hydrated samples such as nanoemulsions, the factors affecting the preservation of the structural integrity are identical in both TEM and SEM [6]. Shrinkage of the colloidal system due to complete dehydration and drying usually causes strong structural changes. As a result, the final electron microscopic images may describe completely modified structures which have nothing in common with the original formulation morphology. Techniques based on cryofixation and low temperature electron microscopy help to overcome these major problems.

If cryo TEM is not available, a conventional negative staining analysis with or without dilution can be performed on nanoemulsions. Staining techniques are frequently employed for imaging with TEM since they are easy, fast and universally applicable. The most common staining agents are salts of heavy metals such as molybdenum, tungsten or uranium which possess atomic numbers between 42 and 92. These agents must be benign to the wet specimens, form a thin glassy layer upon drying and must resist electron beam radiation damage to a satisfying extent. During sample preparation, a droplet of the nanoemulsions is placed on a carbon coated grid onto which it is rapidly adsorbed. Subsequently, an aqueous solution of a heavy metal salt is applied for staining. The sample is then left to dry

and finally observed by TEM at room temperature. This technique allows for the identification of the dehydrated shells of the nanoemulsion droplets which are stabilized by surfactant. The strongly scattering metal ions form an amorphous shield enveloping the weakly scattering structural features to enhance the electron microscopy contrast. A high reverse contrast is thus seen in bright field TEM images, with light droplets against a darker background. Negative staining can be used to visualise the size, shape and internal structure of the sample. The negative stain not only provides contrast for weakly scattering specimens, but also physical support against collapse of the sample structure during drying and protection against electron beam damage. Such sample can be also use for the topographical EFTEM characterisation (see below).

However, it should be kept in mind that conventional electron microscopy is prone to artefacts in case of surfactant solutions, i.e., hydrated colloidal dispersions. Both drying and staining techniques can affect the structure and morphology of the sample; thus, great care should be taken during interpretation of the obtained images. Conventional negative staining on continuous carbon support films bears the risk of sample distortion due to adsorption and flattening during the drying of the thin aqueous film of negative stain or evaporation in the TEM. Apart from adsorption artefacts, variable spreading and incomplete specimen coverage by the stain solution can lead to non-uniform staining results. Specimen distortion from surface tension forces during evaporative drying or the formation of a saturated salt solution before the final drying may occur as well. Ice crystal formation may occur during investigation of the sample, which may lead to misinterpretations. Even the modified technique of cryo negative staining bears the risk of selective particle orientation due to interfacial forces and flattening of fragile structures despite the maintained sample hydration and protection against electron beam damage. However, not all TEM laboratories are equipped with cryo electron microscopy facilities. Therefore, the application of air-dried negative staining techniques for biological specimens or other aqueous systems such as colloidal dispersions remains justified.

2.4 Cryogenic Transmission Electron Microscopy (Cryo TEM): Experimental Setup, Sample Preparation and Potential Artefacts

Generally speaking, the electron microscopic technique of choice for an artefact-free visualisation of nanodispersions in water is cryo TEM. Figure 2.1 represents a few examples of the effective usage of cryo TEM for the detailed investigations of nanoemulsions, liposome containing systems, liquid crystals, self assembled structures functionalised with silica particle etc. By means of a complex sample preparation, the formulation microstructure is displayed in its original state and a clear differentiation between nano-sized oil droplets and other structures can be obtained. An equally suitable alternative is freeze-fracture TEM which will be discussed later.

Although water is the most abundant component of biological material, it is inevitably excluded from conventional electron microscopy since it evaporates rapidly under the vacuum conditions of an electron microscope. The development of cryo-electron microscopy of vitrified specimens has radically changed the situation over the last decades [7, 8]. The discovery of vitrification opened the door to a number of further developments which greatly facilitated cryo-electron microscopy studies. Simple methods were found for preparing a thin vitrified layer of aqueous specimens [9, 10]. The cutting of vitrified sections, thin enough for high resolution observation, was also found possible. Among the various kinds of cryo specimen holders and plunge freezing devices, those which were of a simple nature and easy to handle turned out to give the best results. It was also observed that an optimal use of phase contrast could compensate to a large extent for the inherently low contrast of unstained vitrified specimens. Furthermore, a rapid drop in temperature during freezing provides the possibility to capture hydrated specimens in their momentary movement without changing their structure and helps to reduce the effect of electron beam damage. One of the basic requirements for TEM is that the specimen be very thin. This can be achieved by the technique of cryo-sectioning using high pressure freezing devices after the bulk sample has been vitrified [11, 12]. Alternatively, liquid suspensions can be prepared in the form of a thin film which is subsequently vitrified as a thin layer. The first method can generally be applied for various tasks, but is rather complex and demanding in nature. The second method is simple and rapid, but is limited to the analysis of liquid suspensions of moderate viscosity with particle diameters of less than 200 nm.

The sample preparation for cryo TEM involves three main steps: A small aliquot (approximately 3 μl) of a fluid suspension containing the sample is applied to the surface of a supporting substrate such as a holey or continuous carbon film that is attached to the surface of a standard TEM specimen grid. Subsequently, the droplet is carefully blotted with filter paper until most of the supernatant liquid is removed and only a thin layer of approximately 100 nm thickness is left on the support substrate. On perforated carbon support films, the thin sample film is left stretched over the holes.

The thin fluid layer is rapidly immersed into a suitable cryogen of high heat capacity, which leads to instantaneous and contaminant-free freezing. This vitrification or shock-freezing by propelling the grids into a cryogen is ideally performed by means of a plunging device and a humidity- and temperature-controlled environmental vitrification system. The vitrified samples then need to be transferred into the TEM cryo holder of the microscope under liquid nitrogen and are examined at temperatures around 100 K.

There are two key factors regarding specimen preparation that are critical to obtaining high quality cryo TEM data, assuming of course that biochemical integrity of the specimen is given: the proper preparation of the support substrate and the considerate blotting of the sample droplet to a thin fluid layer on the substrate prior to freezing. Once the thin film containing the specimen is produced, it is immediately plunged into a suitable cryogen. A cryogen that is commonly employed for this task is liquid ethane. Liquid nitrogen is used to maintain the

temperature of the ethane near its melting point of −183 °C. With a freezing rate around 1,000,000 K/sec, the fluid surrounding the specimen does not have time to form crystalline ice, which would damage the fragile sample; it is vitrified instead [8]. Embedded within this layer of vitreous ice, the specimen is essentially preserved in its native state at near atomic resolution.

However, there are certain limitations to the applicability of this technique. Artifacts may emerge during the freezing process, such as ice crystal formation or modifications due to humidity or temperature changes. For an excellent overview about the variety of potential artifacts in cryo TEM, the reader is referred to the recent review by Kuntsche et al. In addition, the maximum specimen thickness that can be observed is limited to a few hundred nanometers. Thus, cryo electron micrographs of polydisperse systems may be biased towards small particles due to the preparation technique. The specimen preparation involves application of the liquid sample on the microscopic grid and removal of the surplus liquid with filter paper until an ultra-thin sample film remains in the holes of the grid, particularly in their centre. Structures which exceed the thickness of this film are either removed or relocated to thicker areas of the film during this procedure. Unfortunately, areas of increased thickness are often too sensitive towards the electron beam to deliver reliable results upon investigation. As a consequence, aggregates or droplets with large dimensions may remain undetected. Thus, a direct comparison of droplet sizes as observed in cryo TEM with the results of particle size measurements by DLS or laser diffraction should be performed with great caution. The data obtained by cryo TEM should be regarded as complementary qualitative information about the shape and size of the observed particles. A quantitative evaluation of cryo electron micrographs of a certain formulation aiming at an accurate size distribution of the observed droplets would require evaluation of large amounts of individual images and specific programmes.

Apart from these projection issues, the contrast of electron microscopy is comparatively poor and may require additional staining techniques. Despite a certain protection due to the cryo-fixation of the samples, severe electron beam damage of frozen materials may occur as well, resulting in "bubbling" of the sample and out-of-focus images.

Nevertheless, cryo TEM of frozen-hydrated unstained specimens is presently among the preferred approaches for high-resolution studies because it provides data on the fully native structure and, as indicated, some protection of the specimens against electron radiation damage. Cryo TEM is particularly useful to investigate structural details of colloidal nano-sized systems, e.g., to detect the presence of vesicles among nanoemulsion droplets (Fig. 2.1). The fine ultrastructure of both ordered and non-ordered multilamellar structures can be resolved (Fig. 2.2). A lot of questions concerning the membrane organisation of the lipid-drug containing systems also can successfully addressed by cryo TEM study (Fig. 2.3). Overall, oil droplets are comparatively simple to be distinguished in cryo TEM images. They always appear as spherical dark droplets while solid lipid nanoparticles, liposomes or other related lipid structures may appear as needle- or rod-like structures when viewed edge-on. The systems can be investigated with or
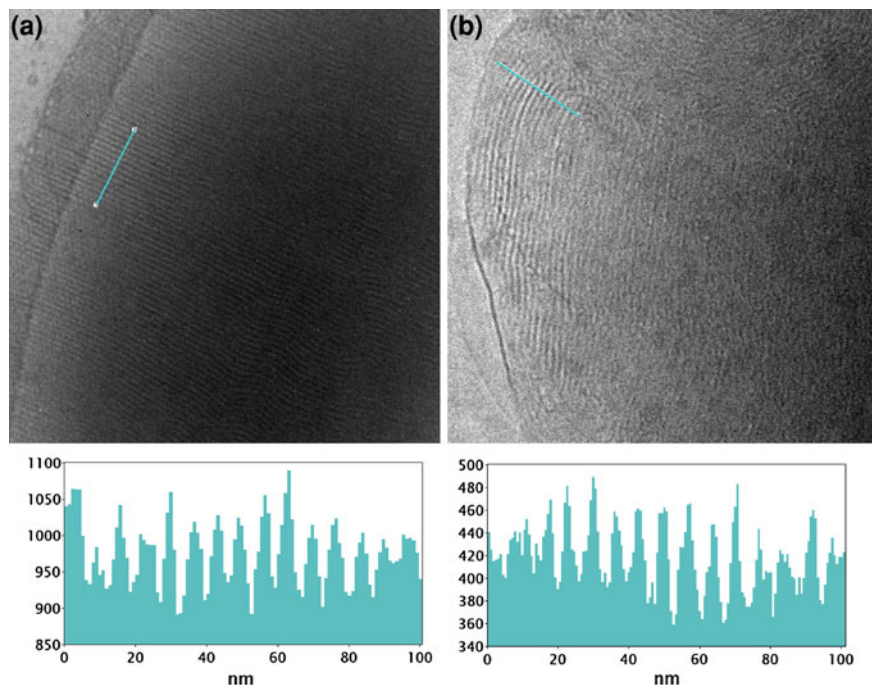
Fig. 2.2 Self assembled oil in water system (lamellar phase): cryo TEM images and TEM intensity profiles

without dilution. In general, dilution of the investigated nanoemulsions is advisable since examination of undiluted samples frequently leads to crowded images where individual structures cannot be clearly characterised.

Cryo TEM study in correlation with conventional negative staining can bring up new aspects of sample characterisation. For example, astonishingly interesting results could be obtained for conventional nano-sized oil-in-water emulsions by investigation with TEM at room temperature after negative staining with uranyl acetate (Fig. 2.4). A direct comparison with images of the same samples obtained by cryo TEM suggests that the morphology of the systems is comparatively well preserved despite the unfavourable surroundings within the TEM. Summarising our experiences with TEM analysis of nanoemulsions at room temperature, it may be assumed that it is not primarily the nature of the oil which is decisive for the quality of the obtained images, but the efficacy of the employed surfactant to stabilise the oil droplets. In Fig. 2.4, a conventional cosmetic oil of a molecular weight around 300 g/mol was emulsified using different amounts of a sucrose ester surfactant. Perfectly clear phase boundaries of the droplet shells after evaporation in the TEM were found for systems of an ideal surfactant concentration of 2.5 % w/w and high physical stability. A surplus of surfactant did not improve the formulation's general properties, but rather destabilised the system by introducing aggregates and leading to droplet deformation. The obtained images of both
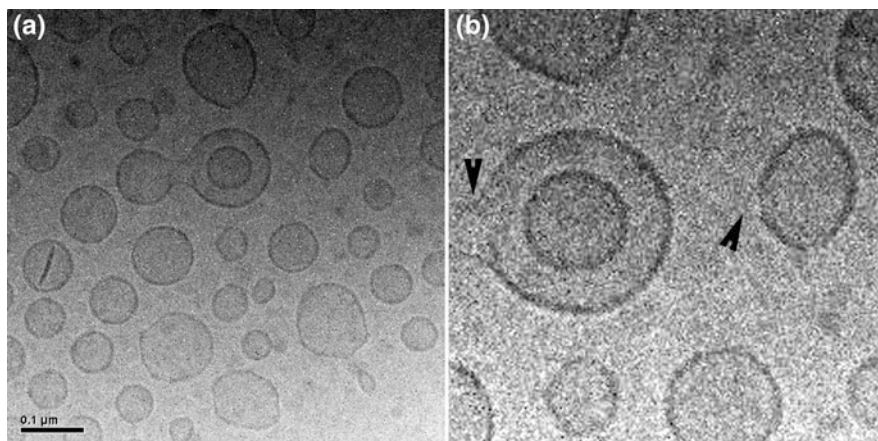
Fig. 2.3 Cryo TEM images of liposomes including membrane incorporated drug molecules

formulations corresponded very well to the native structures as observed by cryo TEM. It may thus be concluded that TEM images obtained at room temperature provide reliable information about the quality of the investigated nanoemulsion, i.e., the stability of the emulsified oil droplets, even though an exact visualisation of the droplet shape remains confined to cryo TEM.

2.5 Laser Light Scattering Versus Electron Microscopy

DLS exhibits certain limitations and may provide incomplete information. Firstly, it may fail to recognise the presence of a small population of large droplets present in nanoemulsions. Likewise, other surfactant aggregates (Fig. 2.5) such as liposomal (Fig. 2.6) vesicles or lamellar structures are not detected; the exact composition of the colloidal system thus remains unknown. However, such structures are frequent by-products of high-pressure homogenisation and should be accounted for [4]. Moreover, the shape of the analysed oil droplets is usually assumed to be a perfect sphere for calculation of the DLS results, which is not always the case. Thus, determined particle sizes for droplets of variable shape may not be entirely representative. Furthermore, most samples have to be diluted prior to DLS measurements to ensure sufficient transparency for accurate droplet size determination. As a consequence, reversible destabilisation phenomena such as flocculation or the appearance of larger aggregates may remain unnoticed. In order to account for these issues, additional techniques of analysis are highly recommendable. Sophisticated methods such as sedimentation, field flow fractionation, nuclear magnetic resonance spectroscopy or Fourier transform infrared spectroscopy have been proposed in this context. However, the microscopic visualisation
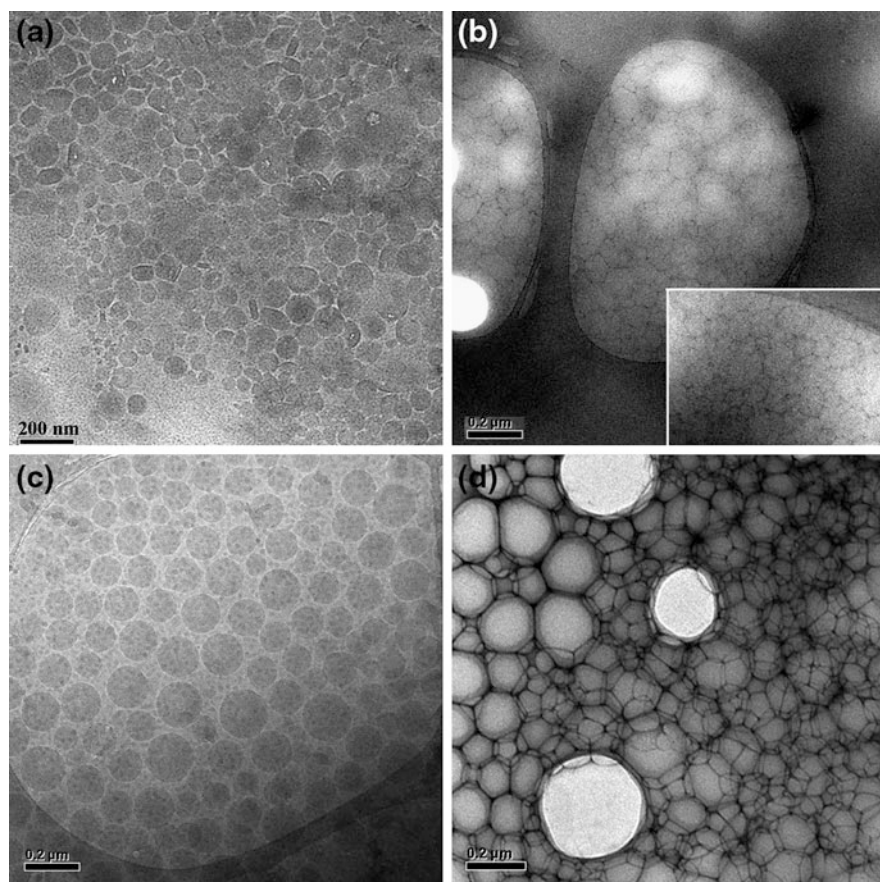
Fig. 2.4 Comparison of cryo TEM and conventional TEM after negative staining with uranyl acetate: nanoemulsions stabilised by either 5 % (**a** and **b**) or 2.5 % (w/w) of sucrose stearate (**c** and **d**) were investigated with both methods. On the *left hand side* (**a**, **c**), the cryo TEM images are given. On the *right hand side* (**b**, **d**), the corresponding images obtained by conventional TEM at room temperature are given

of the investigated nanoemulsions might represent the most reliable and informative method for formulation characterisation.

When employing microscopic techniques for nanoemulsion characterisation, the presence of larger droplets is not an entirely uncommon observation [13, 14], albeit a rarely reported one. Experience has shown that it is possible to obtain excellent DLS data for nanoemulsions over months of stability monitoring while a microscopic analysis of the same sample reveals a definite change of the internal structure. Recently, Preetz and co-workers [14] demonstrated the importance of microscopic analysis for the characterisation of nanoemulsions and nanocapsules. It was found that the mean droplet size determined by DLS was around 150 nm for all investigated systems. In contrast, freeze-fracture TEM revealed variable droplet
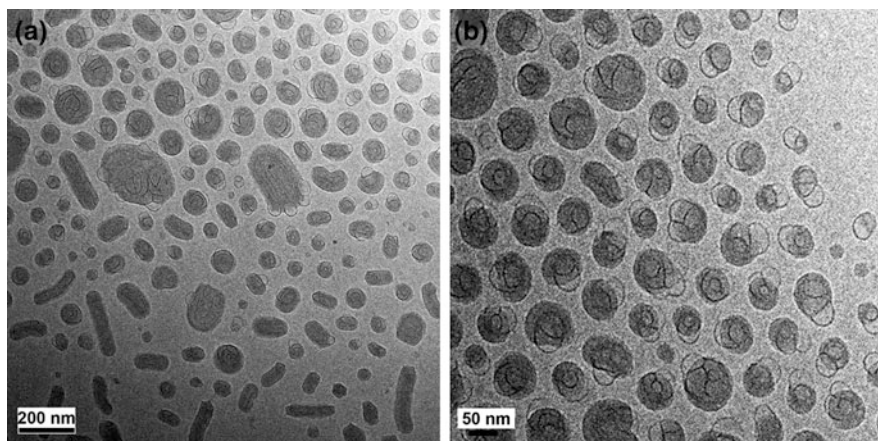
Fig. 2.5 Cryo TEM image of self-assembled oil in water system (hexagonal phase). The oil droplets are surrounded with attached surfactant bobbles
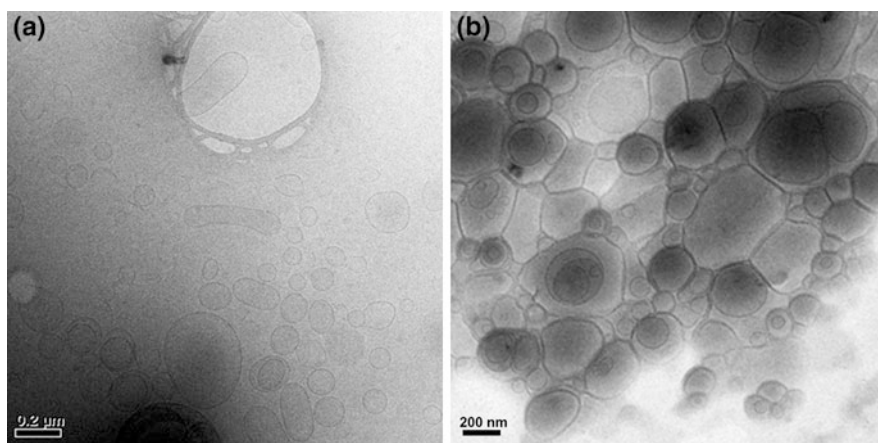

Fig. 2.6 Cryo TEM images of the liposomes-protein complex. **a** The area of the sample where liposomes are not aggregating, **b** the area containing dense aggregates

sizes between 50 and 500 nm with the highest frequency around 100 nm, which was additionally confirmed by atomic force microscopy.

Thus, the importance of microscopic techniques for the analysis of nano-emulsion droplet size and overall morphology needs to be emphasized. Cryo TEM is certainly among the most useful techniques for this task since it delivers detailed information about the internal structure of the observed colloidal systems in their native state. However, it is important to note that the studied images have to be representative of the whole sample. Image analysis software should be employed only for systems with a suitable contrast and composition. Several rounds of

analysis are highly recommendable. A good overview of the investigated systems can be obtained by combination of DLS or static laser diffraction and cryo TEM.

2.6 Freeze-Etching and Freeze-Fracturing of Nanoemulsions for Transmission Electron Microscopy (FF–TEM): Experimental Setup, Sample Preparation and Potential Artefacts

Freeze-etching, freeze-fracturing and cryo electron microscopy of frozen fluids are complementary techniques mainly because the respectively obtained information is based on different mechanisms [8].

Freeze-fracture electron microscopy techniques have emerged during the 1950 to 1960s and have been successfully employed for the analysis of hydrated specimens for well over 30 years [15, 16]. The freeze-fracture technique consists of physically fracturing a frozen biological sample. The structural detail exposed by the fracture plane is visualised by vacuum-deposition of platinum–carbon to make the replica for examination in a TEM.

The key steps involved in this procedure are rapid freezing of the sample, fracturing, replication and replica cleaning. The rapid freezing, i.e., cryofixation, of the nano-sized suspension is usually performed by swiftly immersing the sample into a liquid coolant such as subcooled liquid nitrogen. In this context, pre-treatment with cryoprotectants such as glycerol is sometimes necessary to avoid ice crystal damage. Chemical fixation with glutaraldehyde beforehand serves to avoid artefacts induced by the cryoprotectant. In many cases, successful freezing of hydrated samples requires ultrarapid freezing techniques, such as optimised plunge freezing, jet freezing, spray freezing, high-pressure freezing or freezing by impact against a cold metal block [16]. Subsequently, the fracturing of the sample is carried out under vacuum at liquid nitrogen temperature by breaking the sample in a hinged device or by using a liquid nitrogen-cooled microtome blade. If deemed necessary, an additional etching step may be performed which consists of vacuum sublimation of ice after fracturing. In other words, the ice can be removed from the surface of the fractured specimen by freeze-drying by increasing the temperature to about $-100\ °C$ for several minutes to let ice sublime.

The replicas are then prepared by shadowing and backing of the specimen. The surface of the sample is usually shadowed with platinum to achieve a good topographic contrast and then covered with a strengthening layer of electron-lucent carbon to stabilise the ultra-thin metal film. More specifically, the cold fractured surface, possibly "etched", is shadowed with evaporated platinum or gold at an average angle of 45° in a high vacuum evaporator. A second coat of carbon, evaporated perpendicular to the average surface plane, is often performed to improve stability of the replica coating. The topographical features of the frozen,
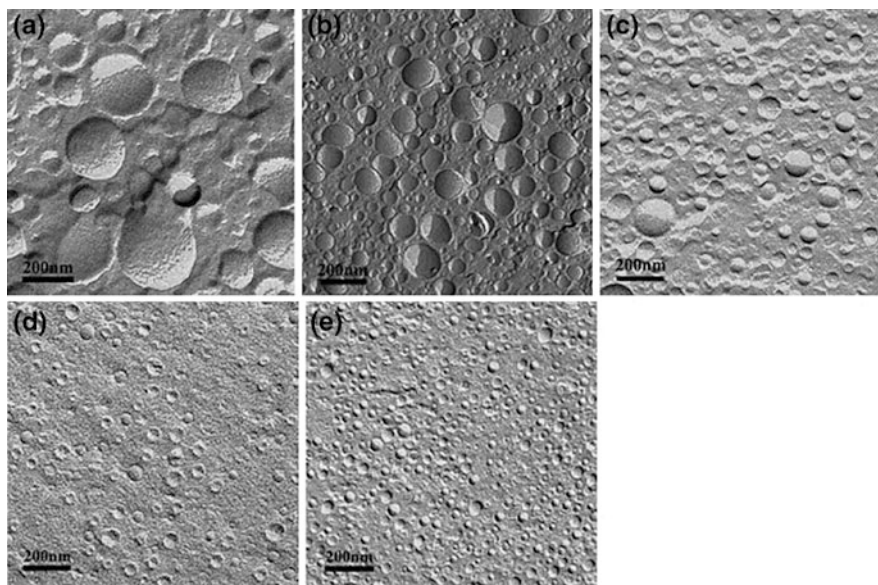
Fig. 2.7 Freeze-fracture TEM micrographs of different lecithin-based nanoemulsions. Images reprinted from [19] with kind permission from Springer Science and Business Media. The influence of increasing contents of glycerol within the formulation is demonstrated by decreasing particle sizes and a more homogeneous droplet size distribution. Coalescence phenomena can be detected in image **a**

fractured surface are thus transformed into variations in the thickness of the deposited platinum layer of the replica.

The specimen is then returned to ambient temperature and pressure and the extremely fragile "pre-shadowed" metal replica of the fracture surface is released from the underlying biological material by careful chemical digestion with acid solutions or detergents. The still-floating replica is thoroughly washed free from residual chemicals, carefully placed on fine grids, dried and then investigated in the TEM. Further details and practical advice on freeze-fracture electron microscopy can be found in the literature [16]. Overall, freeze-fracture electron microscopy can be employed for the analysis of a large spectrum of different materials, including liquids and dispersions, at intermediate to low resolution. Freeze-fracture TEM (FF–TEM) is well adapted to study lipid-containing colloidal suspensions, such as liposomes, nanoemulsions and nanoparticles despite the relatively low signal to noise ratio of the replicas. Polymer solutions, microemulsions and biological systems can be investigated as well. The most important feature of this technique is the tendency of the fracture plane to follow a plane through the central hydrophobic core of frozen membranes, thus splitting them in half. As a result, planar views of the internal structure of the samples are obtained. As for all microscopic techniques, care must be taken to avoid misinterpretation due to artefacts. Freeze-fracturing techniques are complex in nature and the different steps of sample preparation, such
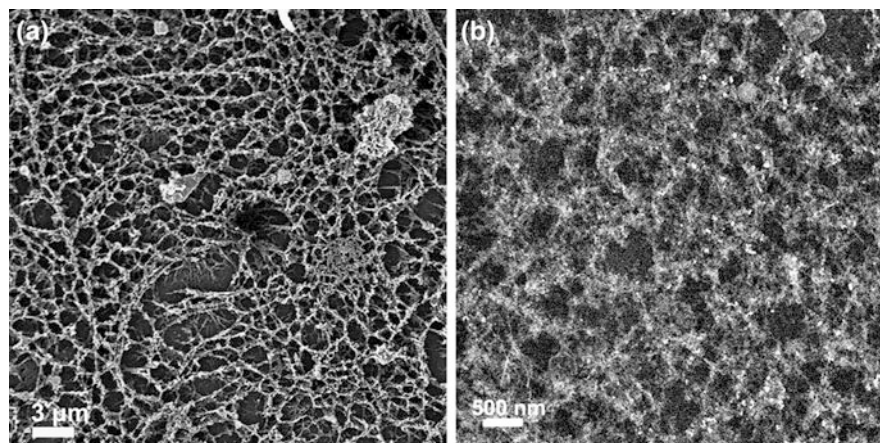
Fig. 2.8 Cryo SEM image of hydrated protein system. The sample has been plunge frozen in liquid nitrogen, partially freeze dried, Pt coated and investigated at low temperature by high resolution SEM

as chemical fixation, cryoprotective pre-treatment, cryofixation, freeze-fracturing, etching and replication, may significantly influence the appearance of the investigated sample. Therefore, considerate specimen preparation is essential to ensure reproducible and reliable microscopic data.

The morphology of a lecithin-based nanoemulsion for topical application was investigated by FF–TEM following a standard protocol of sample cryofixation, freeze-fracturing, freeze-etching and covering with platinum/carbon. The influence of increasing amounts of glycerol within the systems was clearly shown (Fig. 2.7). The droplet size, shape and size distribution could be monitored very well by this technique. Likewise, instability phenomena such as the agglomeration of droplets could be detected.

2.7 Scanning Electron Microscopy, Cryo SEM and Freeze-Fracture SEM: Experimental Setup, Sample Preparation and Potential Artefacts

Indeed, cryo SEM of freeze fracture-freeze dried samples is the best solution for hydrated systems, which are highly viscous and/or have a strong tendency to aggregation. Cryo SEM in combination with freeze drying will be also an only solution when colloidal hydrated systems are used as a coating layer for improving the adhesion of cells etc. Figure 2.8 represents a freeze dried cover glass which was coated with dense protein layer. SEM micrographs clearly show the protein layer morphology. On the one hand, an ultrathin layer of such a
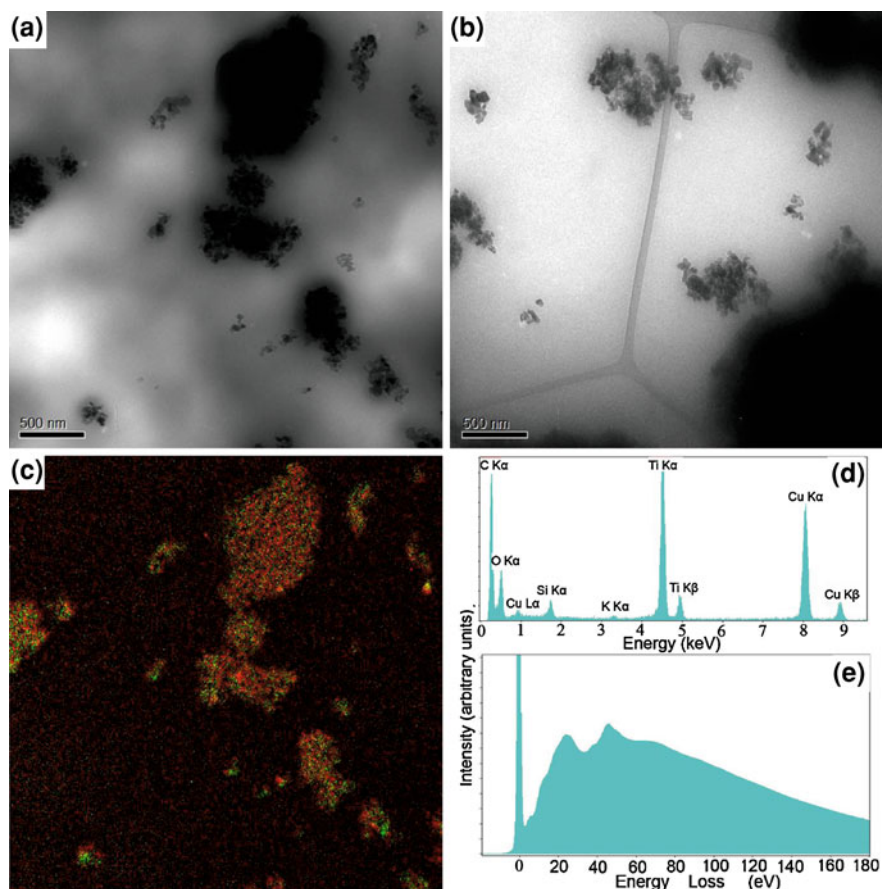
Fig. 2.9 ATEM characterisation of the nano- **a** and macro **b** emulsions containing titanium dioxide particles. **c** represents EFTEM 2D map of titanium (*green*) and oxygen (*red*) destitution, **d** show a EDX spectra of nanoemulsion, and **e** low loss region of EELS spectra obtained from the titanium dioxide aggregate [17]

solution for cryo TEM investigation can hardly be obtained. Viscous protein network are adsorbed firmly onto the carbon coated grid and are not readily removed by filter paper. The resulting layer may not be thin enough to be transparent for the electron beam. Thus, cryo SEM provides a more adequate impression of the overall morphology of inhomogeneous and/or viscous hydrated biological and pharmaceutical systems.
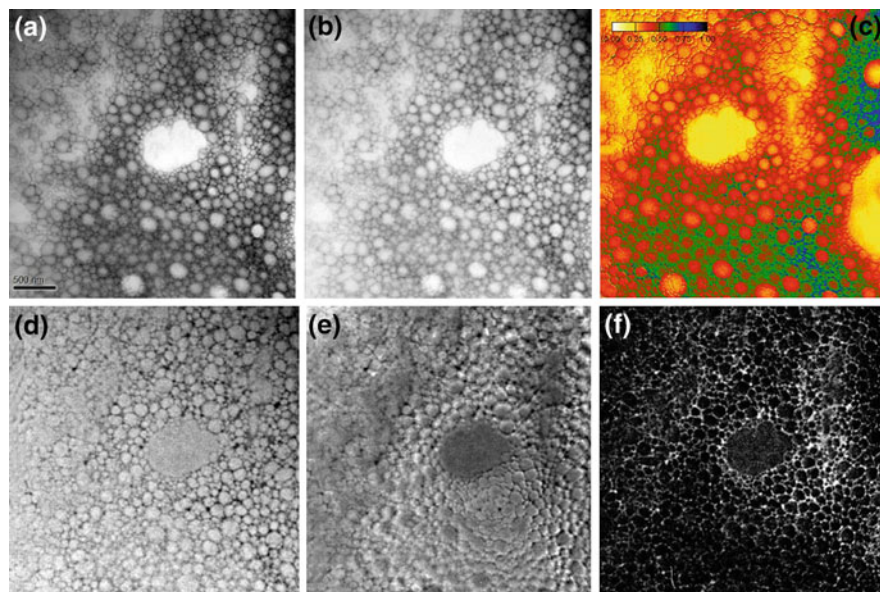
Fig. 2.10 TEM and EFTEM of negatively stained nanoemulsion: **a** elastic filtered TEM image, **b** EFTEM image, which was obtained at plasmon region (20–30 eV), **c** relative thickness map (t/λ), **d** carbon K map, **e** CPR (carbon map/bulk Plasmon) image, **f** EFTEM uranium map (U)

2.8 Recent Advances: Cryo Analytical TEM (cryo ATEM)

It has to be mentioned that ATEM including EELS, EFTEM and EDXS has not yet found broader application for pharmaceutical system characterisation. However, EELS and cryo EELS can be extremely useful for nanoemulsions containing additives such as pharmaceutical substances or functionalised mineral particles. The localisation of the additives can be determined without damaging the native structure of the system. Work is in progress in this area. Figure 2.9 elucidates the importance of ATEM for the identification of metal particles within a nano- or emulsion solution. Usage of EFTEM allows one to obtain a precise 2D map of the titanium dioxide particle distribution. EDX spectra usually gives an impression about all chemical elements which are present in the irradiated area of the sample in a concentration higher than 0.2–0.5 w/w. EELS spectra from the low loss region clearly shows a pronounced ELNES features of titanium dioxide. It has to be mentioned that in most of the cases an accurate comparison between obtained and reference spectra from the EELS database may give a clear impression about the chemical state of a different component of the colloidal system. For example silicon, which is very often used in pharmaceutical systems, can appear as a hydrated silica or silicon oxide. Both forms have pronounced ELNES features which can be easily identified using a reference spectra from EELS atlas incorporated in Digital Micrograph software [17].

The most astonishing usage of EFTEM can be assigned to the determination of a topographical profile of negatively stained organic materials (Fig. 2.10) [18]. The correlative surface profile of polymer and biological materials and its volume projection along with chemical composition with nanometer special resolution have important implications for friction, lubrication, adhesion, macromolecular interaction and any application involving (bio-) polymer surface modification by way of coatings, temperature or chemical treatments etc. So far, such correlative analysis was not achievable due to absence of a high resolution imaging technique which could provide such correlative study. Here, an analytical TEM method is presented which allows one to extract a surface profile of negatively stained organic materials. The fine metal replica formed by negative staining can be mathematically extracted by dividing the images acquired in the bulk plasmon energy region (which contain information mostly about carbon and uranium for stained organic materials) and on the carbon K edge which shows carbon content solely. The resulting ratio map (CPR) shows a fine distribution of uranium atoms, which replicate the sample surface topography with nanometer precision. The proposed CPR method allows one to detect a complementary transmission, chemical and topographical information of the same area of 10–1000 nm thick samples. The proposed method is easy to perform, does not require an additional instrumentation set up and has a great potential for comprehensive investigation of any kind of organic materials.

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