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Microscopies at the Nanoscale for Nano-Scale Drug Delivery Systems

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Abstract: One of the frontier of nanoscience is undoubtedly represented by the use of nanotechnologies in the pharmaceutical research. During the last decades a big family of nanostructures that have a surface-acting action, such as NanoParticles (NPs), lipid nanocarriers and many more, have been developed to be used as Drug Delivery Systems (DDSs). However, these nanocarriers opened also new frontiers in nanometrology, requiring an accurate morphological characterization, near atomic resolution, before they are really available to clinicians to ascertain their elemental composition, to exclude the presence of contaminants introduced during the synthesis procedure and to ensure biocompatibility. Classical Transmission (TEM) and Scanning Electron Microscopy (SEM) techniques frequently have to be adapted for an accurate analysis of formulation morphology, especially in case of hydrated colloidal systems. Specific techniques such as environmental scanning microscopy and/or cryo preparation are required for their investigation. Analytical Electron Microscopy (AEM) techniques such as Electron Energy-Loss Spectroscopy (EELS) or Energy-Dispersive X-ray Spectroscopy (EDXS) are additional assets to determine the elemental composition of the systems. Here we will discuss the importance of Electron Microscopy (EM) as a reliable tool in the pharmaceutical research of the 21st century, focalizing our attention on advantages and limitations of different kind of NPs (in particular silver and carbon NPs, cubosomes) and vesicles (liposomes and niosomes).

Keywords: Drug delivery systems, electron microscopy, energy-dispersive X-ray spectroscopy, nanomedicine, nanotechnology, nanotoxicology, pharmaceutical research.

1. INTRODUCTION

The impact of nanoscience in many fields connected to health and well-being is nowadays very strong and the applications of nanotechnologies are expected to bring large benefits. Nanomedicine, which aims is the monitoring, repairing and improvement of all human biological systems, includes powerful molecular technologies such as: i) nanomaterials-derived biosensors and devices for advanced diagnosis, smart drugs and targeted drug delivery and immunoisolation therapies; ii) molecular medicine *via* genomics, proteomics, and artificial engineered microbes; iii) molecular machine systems and medical nanorobots [1]. One area of the nanomedicine that is currently under extensive research is the development of new generation of nanocarriers that can combine diagnosis and therapeutic applications [2]. The problems to overcame are the production of non-toxic and biocompatible nanocarriers without any side effects for humans and environment [3]. However, even if these nanoproducts have a potential broad spectrum of applications, there is still little (and sometimes also controversial) knowledge of their interaction with cells and organisms and on the possible risks connected to their use. Athorough understanding of biological responses to nanomaterials is needed in order to develop and apply safe nanomedicines in the immediate future [4]. To achieve the major diagnostic and/or therapeutic benefit of nanocarriers, *i.e.,* the potential deliver of drugs to site-specific targets without harming the neighbouring healthy tissues [5], a deep and extensive physico-chemical characterization of the carrier is fundamental [6].

As a result of the research in pharmaceutical technology, steadily shifted towards the development and optimisation of nano-scale Drug Delivery Systems (DDSs), Electron Microscopy (EM)-based techniques represent a powerful tool to characterize pharmaceutical nanosystems such as NanoParticles (NPs) and microparticles, nanoemulsions, microemulsions, solid lipid NPs, different types of vesicles, nanofibers and many more. The wide range of applications of EM, utilized in different scientific and industrial fields, allows to yield information of surface features, shape, size and structure. The new-generation increasingly powerful microscopes allows to obtain accurate information about morphology of these systems near the atomic resolution and, when equipped with spectroscopic components the chemical composition of nano-scale DDSs can be investigated as well. The elemental composition of nano-scale DDSs is very important in order to: i) prevent erroneous interpretation; ii) exclude the presence of contaminants introduced during the synthesis proce-

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dures and iii) confirm the successful design of the nanocarriers [7].

This review provides an overview about the EM-based techniques utilised for characterization of the most broadly used nanocarriers for drug delivery (*i.e.*, solid and liquid NPs, uncharged-single-chain and (un)charged-double-chain liposomes, microemulsions). Advantages and limitations of nanocarries will also be discussed.

2. NANO-SCALE DDSs: ADVANTAGES AND LIMITATIONS

A comprehensive list of communally investigated nanoscale DDSs, used in drug delivery and characterized by different materials, sizes, shapes and various chemical and surface properties is shown in Table **1**. The pharmaceutical field of nanotechnology is under constant and rapid growth and new additions are continuous supplementing this list. Nanoscale DDSs, conversely to the conventional pharmaceutical drugs, that have the disadvantage to be released relatively quickly, are able at the same time to transport drugs and to control the release rate, prolonging the duration of the therapeutic activity and/or directing the release of the drug to a specific tissue [8]. As a general recommendation, nanocarriers for therapeutic purposes have to be completely biocompatible (able to integrate with a biological system without eliciting immune response or any side effect) and non-toxic (harmless to a given biological system) [9]. The main undesirable effects are due to their hydrodynamic size, shape, amount, surface chemistry, route of administration, immune system reaction (in particular the uptake by macrophages and granulocytes) and residence time in the bloodstream [9]. It derives that, before the effective clinical application of a nanocarrier, it is necessary to gain a deep knowledge of it. Among the major nano-scale DDSs used, in this review we will deal with NPs, liposomes, cubosomes and microemulsions.

2.1. Nanomaterials and NPs

According to the definition of National Nanotechnology Initiative, natural or synthetic nanomaterials or NPs are 'structures of sizes ranging from 1 to 100 nm in at least one dimension'. However, the prefix 'nano' is commonly used for particles that are up to several hundred nm in size. NPs can be classified as: i) 0-Dimensional (0D), when all the three dimensions are below 100 nm (*e.g.,* NPs, quantum dots); ii) 1-Dimensional (1D), with two dimensions below 100 nm (*e.g.,* nanotubes, nanowires, nanorods); iii) 2-Dimensional (2D), with only one dimension below 100 nm (*e.g.,* ultrathin films); iv) 3-Dimensional (3D), when are not nanometer sized materials and are constituted by the assembly of nanometer elementary structures (*e.g.,* materials with nanocrystalline grains, clusters of nanomaterials) or by filling a host matrix (*e.g.,* nanocomposites) [10].

The conscious synthesis, manipulation, assembly and use of nano-sized objects, leading to novel materials with properties tailored to specific scientific and technological purposes, is called nanotechnology. Indeed, a great variety of NPs can be synthesized and they display properties significantly different from those observed in fine particles or bulk materials [11], that are exploited in nanomedicine to design novel therapeutic and diagnostic procedures engineered nanoscaled materials. In fact, exploiting the characteristic physicochemical properties of nanomaterials, such as ultra small size, large surface area to mass ratio, and high reactivity, allows overcoming the limitations of traditional therapeutic and diagnostic agents.

Compared to the naked drug, the key advantages of NPs-conjugated drug are: i) improved bioavailability by enhancing aqueous solubility; ii) increased retention time in the body by increasing half-life for clearance and specificity for its cognate receptors and iii) specific targeting to site of action. These properties allow to reduce the drug amount required, enable the safe delivery of toxic therapeutic drugs and prevent side effects in non-target tissues and cells [12].

2.1.1. NPs

In 1985 the discovery of buckyballs (or fullerenes) [13] has opened entirely new perspectives about the carbon chemistry and physics, leading in 1991 to the discovery of Carbon NanoTubes (CNTs) [14]. The fullerenes (C60, C70, and others) are polyhedral objects formed by a monolayer of carbon atoms forming a network of hexagons and pentagons. CNTs are rolled-up graphene sheets of cylindrical shape with Single (SWCNT) or Multiple layer Walls (MWCNT), exhibiting outstanding properties, very different from those of bulk carbon or graphite. Nano-carbon, due to its unique properties is employed in many applications that span the most different areas of application, including nano-electronics, composite materials, energy research, and biomedicine [15-18]. In particular, the outstanding properties of SWCNTs and the peculiar nano-properties of carbon depending significantly on size, shape and dimensionality, represented by diamond particles with sizes in the range 3-5 nm (UNCD) [19] make these materials ideal component for the development of new technologies. The extremely attractive properties of the C-based nanomaterials allowed the realization of innovative Polymer NanoComposites (PNCs) [20, 21].

Actually, the most commonly used carbon nanocarriers as controlled DDSs are CNTs and NanoHorns (NH) in which drugs are immobilized by encapsulation, nanoprecipitation, chemical adsorption or attached to their surface previously functionalized [9]. However, not only C-based NPs have been investigated in several pharmaceutical and medical fields. Different types of NPs have been tested to improve drug delivery and effect in target tissue, to solubilize drugs for intra-vascular delivery and to protect therapeutic agents against enzymatic degradation. NPs formulations consisting of spherical amorphous particles without cosolvent avoid aggregation, allow safe administration *via* intravenous route and decrease the overall toxicity of drug, representing a very promising carrier system for the delivering of anti-cancer agents and for the targeting of drugs in the brain. Also, NPs can specificially target inflamed areas and deliver drugs by a number of possible administration routes and are currently under investigation for the cosmetic applications. The ideal properties of NPs for drug delivery are: natural or synthetic material, inexpensive, non-toxic, biodegradable, non thrombogenic, non immunogenic, particle diameter <100 nm, no platelet aggregation, non inflammatory and prolonged circulation time.

Table 1. Nanocarriers for the drug delivery.

Nano-scale DDS type	Description	Composition
Lipid-based nano-scale DDSs	Nanocochleates	Lipid layer sheet rolled up in spiral fashion
	Nanoliposomes/Archaeosomes	Bilayer lipid vesicles
	Micelles	Single layer lipid vesicles
	Niosomes	Self-assembled bilayer non-ionic surfactants vesicles
	Cubosomes	Dispersed nanostructured particles of cubic phase liquid crystal
Solid NPs	Complex non-spherical particles	Compositionally homogenous
	Compositionally heterogeneous particles	Compositional variation core *vs.* surface
		Distributed compositional variation
	Heterogeneous aggregates/agglomerates	Consisting of a diverse particles types
		Consisting of a single particles class
	Nanofibers	Compositionally homogenous
	Nanoplates	Compositionally homogenous
	Quantum Dots	Material embedded in semiconductor or other matrices
	Nanowires	Quasi-one dimensional semiconductor or metallic structure
	Nanorods	Single crystalline structure of different materials
	Nanotubes	Compositionally homogenous
	Spherical or compact particles	Compositionally homogenous
Polimer-based nano-scale DDSs	Nanocapsule/Polymersome	Polymer membrane surrounding a central cavity:
		- nanocapsule: oily liquid cavity, single layer membrane
		- polymersome: aqueous cavity, bilayer membrane (similar to nanoliposome)
	Nanospheres	Aggregated copolymers generating a solid central core
	Micelles	Aggregated copolymers

The most used NPs for drug delivery are: gold, carbon, silver, *etc.*-based NPs, Quantum Dots, Nanocrystalline Silicon, Crystals, Gliadin NPs, Polymeric NPs, Solid Lipid Quantum NPs (SLN), Cubic Liquid Crystalline (Cubosomes).

2.1.2. Gold and Silver NPs

Due to the high stability and easy synthesis, gold NPs (AuNPs) have been intensely studied in recent years. Their physical, chemical, mechanical, optical and electronic properties make them suitable to design nanobiomaterials exhibiting high selectivity, specificity, and sensitivity in the early detection, diagnosis, and treatment of diseases. In addition, in recent years gold has been used in manufacturing drug and gene carrier devices because it is adapt to stably bind therapeutic agents and biomolecules and to create biocompatible structures [22].

Due to its antimicrobial properties, nano-silver (AgNPs) is increasingly used in manifacturing a wide kinds of products, including medical devices [23]. In medicine, AgNPs are used both in diagnosis, such as molecular imaging, and therapy, such as drug delivery, treatment of vascular diseases and wound healing [24].

Au-, Ag- or C- NPs have a wide range of applications continuously increasing.

2.1.3. Quantum Dot

A Quantum Dot belongs to semiconductor nanostructures with a dimension ranging between 2 to 10 nm, corresponding to 10 to 50 atoms in diameter and a total of 100 to 100,000 atoms within the quantum dot volume. It has a discrete quantized energy spectrum and confines the motion of conduction band electrons, valence band holes, or excitons (pairs of conduction band electrons and valence band holes) in all three spatial directions. A cutting edge application of quantum dots is its use as fluorophore for intra-operative detection of tumours.

2.1.4. Nanocrystalline Silicon

Nanocrystalline silicon (nc-Si) can be considered for some aspects an allotropic form of silicon. It is similar to amorphous silicon (a-Si) for the presence of an amorphous phase. However, it differs from a-Si for the small crystallites dispersed within the amorphous phase, making them also different to polycrystalline silicon (poly-Si) which consists solely of randomly oriented crystallites, separated by grain boundaries without an amorphous phase.

Nc-Si has key advantages compared with other semiconductor NCs due to silicon's high natural abundance, low toxicity and strong biocompatibility, optical properties, and photostability. These features make nc-Si highly attractive material for a wide range of future applications, from optoelectronic devices to medical imaging.

2.1.5. Gliadin NPs

Gliadin is a protein rich in neutral and lipophilic residues able to interact with the mucosa. Neutral amino acids allow hydrogen bonding interaction with the mucosa, whereas the lipophilic components allow interaction within biological tissue by hydrophilic interaction. The gliadin possessing amino and disulphide groups on the side chain has a good probability to bind mucin gel. In order to improve bioavailability of antibiotics with anti-*H. pylori* effects at the sites of infection, Gliadin-based NPs (GNPs) have been prepared [25]. *In vivo* gastric mucoadhesive studies suggested that GNPs have a higher tropism for the gastrointestinal regions than for other intestinal regions and confirmed the strong mucoadhesive propensity and specificity of GNPs towards stomach [26].

2.1.6. Polymeric NPs

Polymeric NPs, exhibiting a long shelf life and a good stability on storage, have been proposed by Spicer *et al.* [27] as DDSs, representing an interesting alternative to liposomes (see paragraph below). In addition, by adsorbing and coating their surface with different substances, they are able to specifically target specific organs or tissues much more liposomes.

2.1.7. Solid Lipid NPs

Solid Lipid NPs (SLNs), by combining of polymeric NPs, fat emulsions and liposomes, have been suggested and developed as alternative biodegradable, biocompatible and non-toxic delivery system to conventional polymeric NPs. SLNs are sub-micron colloidal carriers (50-1000 nm) composed of physiological lipid, such as triglycerides, partial glycerides, PEGylated lipids, fatty acids steroids and waxes dispersed in water or in an aqueous surfactant solution. Emulsifiers such as poloxamer, polysorbates, lecithin and bile acids have been used to stabilize the lipid dispersion. They are very much like nanoemulsions, differing in lipid nature.

2.1.8. Cubic Liquid Crystalline: Cubosomes

Cubosomes (diameter size 10-500 nm) are self-assembled liquid crystalline particles prepared from certain surfactants with a proper ratio of water and with a microstructure ensuring unique properties of practical interest [28]. They exhibit a bicontinuous cubic liquid optically clear, very viscous crystalline phase with an unique structure at nanometer scale. The bicontinuous phase is formed by two continuous but non-intersecting aqueous regions by lipid bilayer twisted into space filling structure. Simple emulsification of monoglyceride and a polymer, accompanied by sonication and homogenization represent the method of preparation. In particular, hydrating a surfactant or polar lipid that forms cubic phase and then dispersing a solid like phase into smaller particles usually forms cubosomes [29].

They appear like spherical or square shaped dots that exhibit different internal cubic structure and composition with different drug-loading modalities. Overall, cubosomes have great potential in drug nanoformulations for cancer therapy, especially to treat melanoma, owing to high drug payloads due to high internal surface area and cubic crystalline structures, relatively simple preparation method, biodegradability of lipids, the ability of encapsulating hydrophobic, hydrophilic and amphiphilic substances, targeting and controlled release of bioactive agents [30].

2.2. Liposomes

A liposome is a spherical uni- or multi- lamellar vesicle with a membrane composed of a phospholipid bilayer used to deliver drugs or genetic material into a cell. Indeed, liposomes are the pioneers among drug carriers. They are nano/micro-particular or colloidal carriers, usually with 80-300 nm size range [31]. Liposomes can be composed of naturally-derived phospholipids with mixed lipid chains (like egg, phosphatidylethanolamine) and steroids (*e.g.,* cholesterol), or of pure components like DOPE (DiOleoylPhosphatidylEthanolamine). They can be created by sonicating phospholipids in water [32]. Liposomes, by fusing with other bilayers (*e.g.,* the cell membrane), increase the solubility of drugs. Moreover, they improve drugs pharmacokinetic properties, such as the therapeutic index of chemotherapeutic agents, rapid metabolism, reduction of harmful side effects and increase of *in vitro* and *in vivo* anticancer activity [33].

By making liposomes in a solution of DNA or drugs (which would normally be unable to diffuse through the membrane), they can be (indiscriminately) delivered past the lipid bilayer.

The technique that use liposomes for introduce DNA into a host cell is known as lipofection. Some representative examples of liposomal drugs, delivered with higher efficacy and lower toxicity than their nonliposomal analogues, on the market are showed in Table **2**. Unfortunately, liposomes are also subject to some limitations. To data, the unresolved problems of using nano-scale DDSs based on liposomes remain: i) their accumulation in cells outside the target tissues (liver macrophages); ii) the unpredictable effects of the active agents that they carry; iii) the cellular death [34]; iv) low encapsulation efficiency; v) fast burst release of drugs; vi) poor storage stability and vii) lack of tunable triggers for drug release [35].

2.2.1. Niosomes

The acronym niosome stands for Non-IOnic lipoSOME. They are microscopic lamellar structures formed upon com-

Table 2. Representative examples of liposomal drugs on the market (modified by Zhang *et al.*, 2008).

Active ingredient	Trade name	Supplier	Indication	Administration
Amphotericin B	Abelcet®	Enzon	Fungal infections	IV
	AmBisome®	Gilead Sciences	Fungal and protozoal infections	IV
Cytarabine	DepoCyt®	SkyePharma	Malignant lymphomatous meningitis	IT
Daunorubicin	DaunoXome®	Gilead Sciences	HIV-related Kaposi's sarcoma	IV
Doxorubicin	Doxil/Caelyx®	Ortho Biotech, Schering-Plough	HIV-related Kaposi's sarcoma, metastatic, breast cancer, metastatic ovarian cancer	IM
	Myocet®	Zeneus	Combination therapy with cyclophosphamide in metastatic breast cancer	IV
Estradiol	Estrasorb®	Novavax	Menopausal therapy	Topical
IRIV vaccine	Epaxal®	Berna Biotech	Hepatitis A	IM
	Inflexal V®	Berna Biotech	Influenza	IM
Morphine	DepoDur®	SkyePharma, Endo	Postsurgical analgesia	Epidural
Verteporfin	Visudyne®	QLT, Novartis	Age-related macular degeneration, pathologic myopia, ocular histoplasmosis	IV

HIV = Human Immunodeficiency Virus; IRIV = Immunopotentiating Reconstituted Influenza Virosom; IV = IntraVenous; IM = IntraMuscular; IT = IntraThecal

bining non-ionic surfactant of the alkyl or dialkyl polyglycerol ether class with cholesterol. Niosomes are an evolution of the liposomes; in fact, liposomes are prepared from double-chain phospholipids (neutral or charged) whereas niosomes contain uncharged single-chain surfactant and cholesterol [36]. By using energy derived from heat or physical agitation, the non-ionic surfactants form a closed bilayer vesicle in aqueous media based on its amphiphilic nature. In the bilayer structure, hydrophobic parts are oriented away from the aqueous solvent, whereas the hydrophilic heads remain in contact with the aqueous solvent [37]. A lot of drugs have been injected in animal through different routes by using the niosomal delivery, such as anti-AIDS, anti-cancer, and anti-viral drugs [38]. The advantages that derive from the use of niosomes are: i) the properties of the vesicles can be changed by varying the composition of the vesicles, size, lamellarity, tapped volume, surface charge and concentration; ii) due to presence of hydrophilic, amphiphilic and lipophilic moieties in the structure, these can accommodate drug molecules with a wide range of solubility [39]; in addition, niosomal vesicles: iii) are usually non-toxic; iv) require less production costs and v) are stable over a longer period of time in different conditions, so overcoming some drawbacks of liposomes [40]. Whereas problems of niosome regard their physical stability, *i.e.*, aggregation, fusion and leaking [39]. The stability of niosomes is affected by: i) type of surfactant; ii) nature of encapsulated drug; iii) storage temperature; iv) detergents; v) use of membrane spanning lipids; vi) the interfacial polymerisation of surfactant monomers *in situ*; vii) inclusion of charged molecule [37].

2.3. Microemulsions

Microemulsions (size usually in the range of 20-200 nm) are isotropic, thermodynamically stable transparent (or translucent) systems of oil, water, surfactant and cosurfactant, characterized by ultra low interfacial tension between oil and water phases. Depending on their structure, they can be classified as oil-in-water (o/w), water-in-oil (w/o) or bicontinuous systems. These versatile systems are currently of great technological and scientific interest. Depending on the presence of both lipophilic and hydrophilic domains microemulsions can incorporate a wide range of both hydrophilic and hydrophobic drug molecules. They protect the drugs against oxidation and enzymatic hydrolysis and improve the solubilisation of lipophilic drugs enhancing their bioavailability. Finally, they are amenable for sustained and targeted delivery through ophthalmic, dental, pulmonary, vaginal and topical routes [41]. Unfortunately, microemulsions have also several concerns, such as sensitivity to temperature changes that can introduce artefacts, high vapour pressure, lack of contrast between the sample and its environment and potential chemical reactions and structural changes caused by the electron beam [42]. However, crystal formation leading to artefacts can be avoid through the use of controlled environmental chambers, improvements in thermal fixation and fast sample cooling rates [43].

3. ANALYSIS OF NANO-SCALE DDSs BY USING EM: GENERAL AND SPECIFIC ASPECTS, ADVANTAGES AND LIMITATIONS

Besides the size and the chemical composition, the unique properties of nanostructured materials depend on a number of physico-chemical parameters, such as shape, surface properties, crystalline phase, density, porosity, solubility or (photo)catalytic activity. Size information is of course the most critical, since it is size that makes a particle a NP. As far as size and size distribution is concerned, EM remains a reference method, with TEM providing accurate measure-

ment of particles down to the low nanorange besides giving morphological information.

Surface effects are produced by the increase in the surface-to-volume ratio; the larger surface-to-volume ratio represents an advantage for the surface functionalization of nanomaterials compared to their bulk counterparts, which is of the utmost importance in the technological applications of the drug delivery. It is, thus, essential an adequate characterization in order to relate the behaviour and functional properties of nano-scale DDSs to an unequivocally established identity. This in turn will help to design new materials with definite features for specific drug delivery. The characterization of nano-scale DDSs before being actually used by clinicians is very important. When the sizes of interest are beyond the diffraction limit, optical microscopy and related techniques cannot be used, even if the Nobel Laureates in Chemistry 2014 (E. Betzig, S. W. Hell and W. E. Moerner) ingeniously have bypassed the limit of optical microscopy through the development of super-resolution fluorescence microscopy. Due to their achievements the optical microscope can now peer into the nanodimension.

In order to characterize the nano-scale DDSs, EM-based and Scanning Probe Microscopy (SPM) techniques are becoming an avoidable for the analysis of nano-scale DDSs. Table **3** is listing the principal EM methods with their advantages and limitations. The availability of a variety of different microscopies offers many possibilities for the identification and characterization of specific nano-scale DDSs. However, the length of time required for sample processing limits the high throughput use of SPM and EM methods.

Microscopy analysis is an indispensable tool for the characterization of nanoscale objects: visualization, determination of size, aggregation state, structure and shape of NPs can be obtained by EM methods (SEM, TEM).

TEM requires very thin specimens for the electrons to pass through, vacuum conditions incompatible with liquid samples. To overcome this, cryo-TEM, by freezing samples, has been used, while the use of Wet-SEM and SPM tools, such as AFM, allows characterization of NPs in liquid samples [44, 45].

3.1. EM of Nanocarriers: TEM and SEM

3.1.1. TEM

The electron microscope uses electrons to produce magnified images of the objects. The electrons, by possessing wavelengths about 100,000 times shorter than the photons of visible light, allow a resolution better than 50 pm [78] *versus* the resolution of around 200 nm of light microscopes. Today the resolution of multi-purpose TEMs is generally around 0.2 nm [79, 80].

The different working principles, such as mode of interaction of the electron beam with the sample and the principles of image formation, classify two major types of electron microscopes: the Transmission Electron Microscope (TEM) and the Scanning Electron Microscope (SEM). Among the many other different types of electron microscopes here we mention: cryo-EM, Atomic Force Microscopy (AFM) and analytical electron microscope or combinations thereof.

Conventional TEM [81] is an electron optical instrument analogous to light microscope. It uses as light source the electrons, generated by a tungsten filament cathode, whose much lower wavelength allows to get a resolution a thousand times better than light microscope one. The electrons are emitted by an electron gun while system of electromagnetic lenses focuses the electron beam on the sample. The inner structure of sample at magnifications up to one million times and more can be analyzed with a spatial resolution that is typically of the order of 0.2 nm by a TEM [65, 82]. Spherical and chromatic aberration represent the limitation in the resolution of TEM; though a new generation of aberration correctors are able to overcome or limit these anomalies.

The state-of-art TEMs equipped with hardware and software systems for the correction of the aberrations can achieve a resolution of about 50 pm. The Fig. (**1**) evidences the capability of an aberration corrected TEM to image at an extremely high resolution the inner crystalline structure of nanodiamonds, that are becoming extremely attractive, together their derivatives, for single particle imaging in cells, drug delivery, protein separation, biosensing and other applications [83].

Since gas molecules may scatter the electrons and bias the analysis, the observation is performed at room temperature under high-vacuum. These conditions allow accurate study of only for pharmaceutical nanocarriers such as powders or NPs, whereas hydrated pharmaceutical systems, such as emulsions, microemulsions or liposomes, are strongly affected during analysis. In fact, high vacuum destructs their native morphology and forms artefacts, that can be circumvent by using different approaches. In particular, successful analysis can be achieved by using cryo preparation methods in combination with cryo-EM analysis [84, 85].

To obtain a proper image formation, the specimen thickness in TEM is generally limited to a few hundred nanometres and a sufficiently large number of electrons have to pass through the specimen. The interaction of the electrons with the sample is the main limitation in EM. In fact, the electrons can ionise the specimen, which results in the breaking of atomic bonds and the generation of free radicals. The diffusion of free radicals and the loss of mass may cause physical damage to the sample [86-88]. The extent of damage depends on the electron dose required for imaging, which in turn depends on the employed instrument. Again, the exposure to the electron beam can induce heating, mass loss and other changes which alter the structure and make worse the resolution. These problems remain almost difficult to overcome [89], though, at the moment, low-dose exposure and cryo protection are the major techniques utilized to limit this damage due to electron radiation [90]. Low contrast and radiation sensitivity are other obstacles to overcome in order to obtain high resolved structural information of biological or organic supramolecular architectures. In fact, the use of contrast agents, which aims to embed objects in an electron dense matrix of heavy metal salts, permits to increase contrast and is a simple and fast preparation method, but prone to generate artefacts [91]. This is due to a structural chemical interference between dye and sample. Another problem is the air drying process preceding the transfer of the sample in the

Table 3. Electron analytical modalities, their strengths and limitations, for evaluation of the physicochemical properties of different nano-scale DDSs types (adapted from Lin *et al.*, 2013).

Characterization technique	Detectable properties	Strengths	Limitations	References
Scanning Electron Microscopy (SEM)	Size and size distribution Shape Aggregation Dispersion	Direct measurement of the size/size distribution and shape. High resolution (down to sub-nanometer)	Conducting sample or coating conductive materials required Dry samples required Sample analysis in non-physiological conditions Biased statistics of size distribution in heterogeneous samples Expensive equipment Cryogenic method required for most NP-bioconjugates	[47-53]
Environmental SEM (ESEM)	Size and size distribution Shape Aggregation Dispersion	Direct measurement of the size/size distribution and shape. High resolution (down to sub-nanometer) in atural state	Reduced resolution	[54, 55]
Transmission Electron Microscopy (TEM)	Size and size distribution Shape heterogeneity Aggregation Dispersion	Direct measurement of the size/size distribution and shape with higher spatial resolution than SEM. EELS and EDXS analytical methods can be coupled to investigate electronic structure, phase state, chemical composition and concentration of the elements present in the analyzed area.	Ultrathin samples required Samples in nonphysiological condition Sample damage/alteration Poor sampling Expensive equipment Cryogenic method required to overcome heating, mass loss and other changes which alter the structure	[50, 55-65]
Scanning Tunneling Microscopy (STM)	Size and size distribution Shape Structure Dispersion Aggregation	Direct measurement High spatial resolution at atomic scale	Conductive surface required Surface electronic structure and surface topography unnecessarily having a simple connection	[66-71]
Atomic Force Microscopy (AFM)	Size and size distribution Shape Structure Sorption Dispersion Aggregation Surface properties by modifying the tip of the AFM probe.	3D sample surface mapping Sub-nanoscaled topographic resolution Direct measurement of samples in dry, aqueous or ambient environment Magnetic properties of the investigated surface upon magnetic coatings of the tip of probe	Overestimation of lateral dimensions Poor sampling and time consuming Analysis in general limited to the exterior of nanomaterials	[54, 55, 57, 72-77]

high vacuum of the microscope. Again cryo-techniques, so as cryo-fixation, permit to overcome these limitations of contrast agents and/or sample drying, increasing even the tolerance for electron irradiation [92, 93]. Thus, the most used approach, which delivers direct structural information of the samples in almost native state of aggregation (most importantly in aqueous solution, but preparation in selected organic solvents such as toluene is also possible) is the vitrification of ultrathin sample layers (200-300 nm) by a fast transferring into a cryogen such as liquid ethane or propane, a procedure widely known as the cryo-TEM method. Since in cryo-TEM the sample is directly visualized in the frozen-

hydrated state, it allows to observe structures formed by amphiphilic molecule in aqueous environment. For this purpose, cryo-TEM requires additional features to the conventional TEM [85]. Unluckily, the main limitation of cryo-TEM is the short time period of sample viewing. In fact, 'bubbling' of the structures is observed as most samples are sensitive to radiation damages [85, 94]. Indeed, as in all EM methods, different artefacts may hinder cryo-TEM analysis. For example, the sample can contain cryogen residues that an increased energy input could remove. However, the high content of evaporate water in the TEM column [95] could induce a phase transition of the vitrified ice into cubic or hexagonal ice [85, 95]. In addition to the formation of artefacts, cryo-TEM technique has other limitations, such as getting adequate film thickness. The maximum specimen thickness that can be observed is limited to a hundred or less nm [94].

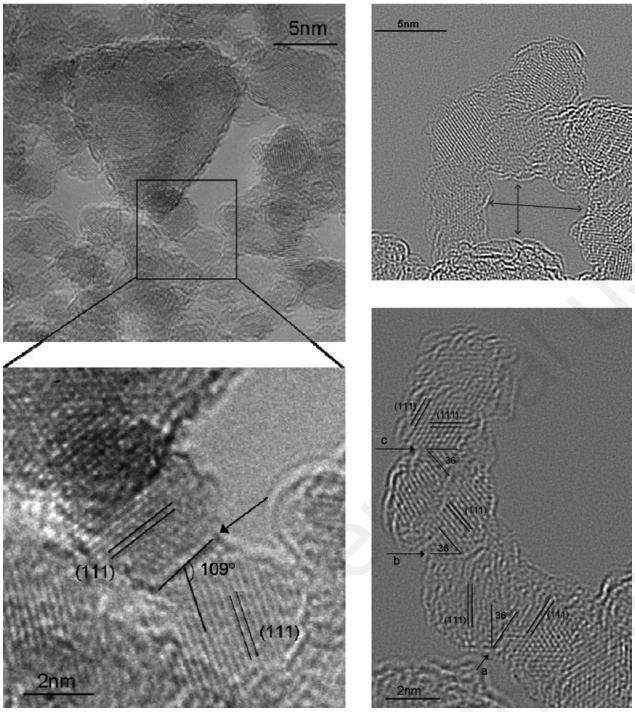

Fig. (1). High-Resolution TEM (HRTEM) micrographs of nanodiamonds. Left side: the arrow indicates (111)|(111) interface between two 4 nm sized nanodiamonds. The orientation and the separation of the (111) lattice fringes are also shown. Right side (above): a 3D loop of nanodiamonds. The size of the individual nanodiamond from 4-5 nm, and the size of void within the loop is 4 nm (vertical width) by 6 nm (horizontal width). Due to the 3D configuration, the locations of the interface are obscured. Right side (below): a linear chain of four ~4.5 nm sized nanodiamonds. Their interface are determined to be (111)|(220) type (labeled as a, b, c), with one interface that can not be uniquely determined. Reprinted with permission from *Chang LY, Osawa E, Barnard AS. Confirmation of the electrostatic self-assembly of nanodiamonds. Nanoscale 2011; 3: 958-962*, copyright (2011) Royal Society of Chemistry.

Cryo-TEM is the most suitable technique to visualize the NPs covered with lipid bilayers coating [96, 97]. Cryo-TEM is a widely used technique to investigate a variety of self-assembled nanostructures [44, 98], such as liposome, cubosomes, hexosomes and microemulsiones, either to characterize their ultrastructure that to monitor their different stages of transformation during synthetizing [27, 44, 99-104]. It should be taken into account that in case of NPs in a dispersed state, artefacts may be formed by aggregated NPs or formation of crystals from the surrounding media [105], thus the final image may not be the real representation of NPs suspension; the use of a commercially available substrate to avoid image modification is recommended [106].

3.1.2. Freeze-Fracture Preparation for TEM

A technique that has revolutionised our understanding of membrane structure from the 1970s onwards is the freeze-fracture and freeze-etching preparation for EM. Combinations of freezing and etching, *i.e.,* sublimation of ice to visualise surface structures [107, 108], soon followed by additional fracturing of the frozen specimens were proposed [109]. This technique raised big interest for the possibility to observe hydrated systems like cells in a *quasi*-3D ultrastructural views. The four key steps in making a freeze-fracture involve the rapid frozen of a biological sample by using liquid nitrogen (cryo-fixation), a physically breaking apart (fracturing), by simply breaking or by using a microtome, replication by shadowing under vacuum-deposition of platinum-carbon the cold fractured surface and replica cleaning. In routine protocols, a pretreatment step is carried out before freezing, typically comprising fixation in glutaraldehyde followed by cryoprotection with glycerol. An optional etching step, involving vacuum sublimation of ice, may be carried out after fracturing [110].

Nano-sized suspension have to be rapid cryo-fixed by swiftly immersing the sample into a subcooled liquid nitrogen.

To avoid ice crystal damage, pre-treatment with cryoprotectants, *e.g.,* glycerol, is sometimes necessary. Artefacts formed by cryoprotectant can be avoid by pre-fixing the sample with glutaraldehyde.

Regarding the investigation of pharmaceutical nanocarriers, freeze fracture EM, by exploiting fracture course within the hydrophobic zones, is especially useful to characterise the lipid structures morphology. In fact, the plane of fracturing favours the path of low resistance represented by the bimolecular lipid layer of plasma membranes. Thus, more or less extended areas of membranes are not cut but split into two leaflets. Unlike water-containing material, hydrophobic fats and oils are non etchable and can therefore easily be recognised in freeze-fracture specimens after etching.

Compared to thin sections, replicas of freeze-fractured specimens have some disadvantages. For example, the resolution is limited by the grain size of the evaporated material; the course of the plane of fracturing cannot be controlled; it can be difficult or impossible to ascribe the exposed structures, for instance within a plasma membrane, to a specific cell [111].

3.1.3. SEM

In SEM (the term 'scan' comes from the fact that the electron beam is not fixed but is forced to scan the sample through a rectangular area, line by line), the image is formed point by point by scanning a focused electron beam across

the surface of a solid specimen. The primary electrons penetrate the solid specimen and different (both elastic and inelastic) scattering processes are generated, and the related different signals are collected by different detector systems to made an image [45], that give specific information. There are two main types of contrast:

i) secondary electrons with exit energies below 50 eV provide the surface information of sample, recording the *quasi*-3D surface topography. The specimen must have a thickness of a few nanometers. The image contrast is obtained by selecting angular range of the electrons collected [112];

ii) back-scattered electrons with energies between 50 eV and the primary energy passing through the specimen produce an increasing intensity with high atomic number mean, that results in another contrast [113].

TEM is most frequently used for morphological analysis, although SEM can likewise be employed [114]. SEM is one of the most used tools in the nanotechnologic field. In fact, the SEM technique is adopted for the ultrastructural investigation of different kinds of materials, including nanomaterials. It is used as radiation source a beam of electrons. The sample is struck by the electron beam and emits consequently numerous particles including the secondary electrons, suitably detected and converted into electrical impulses. Images are obtained analogously to those of the cameras but of extremely small solid specimens, which can reach the size of 1 nm.

A first advantage of SEM analysis is the high depth of focus in combination with the particular method of image formation: areas in relief appear like shadows, while hollow areas appear dark. In contrast, there is a further difficulty in interpreting 2D TEM micrographs. SEM has also the advantage that no specimen preparation is needed for solid samples and a high depth focus allows to investigate large areas. Since SEM can be operated with low voltage, it can provide useful information about beam-sensitive materials [80]. As other electron microscopes, also SEM is not free from disadvantages. The main one is that images are obtained pixel by pixel, so the sample is exposed to a longer exposure time compared to the TEM. However, a solution to this problem may be to use a high-brightness field-emission guns [80]. In addition, there are other limitations that include a lack of internal details, a resolving power rather limited and the risk of electron beam damage [113]. Cryogenic-temperature-using SEM (cryo-SEM) was first suggested already in the 1970s [115]. Cryo-SEM is very suitable to investigate nanometric details in liquid and semi-liquid materials of high vapour pressure, with high viscosity or containing large (> 0.5 μm) aggregates. The main advantage of this technique than conventional SEM is certainly a convenient and rapid sample preparation, while the major disadvantages are the low resolution and the formation of ice (during transfer of the samples), that may lead to misinterpretation of native nanostructure because cover structures of interest or may resemble actual microemulsion structures. The comparatively low resolution can be improved (close to 2-3 nm) by using field emission with improved immersion lenses, cold emission electron guns and finer metal coating [116], while sublimation of ice or rapid freezing rates for propane can help to avoid the ice or at least to keep the size of ice crystals below the resolution of the EM [117].

TEM instruments usually work with electron energies well above 100 keV and allow a higher lateral resolution than SEM instruments, which employ lower electron energies. Accordingly, TEM has significantly higher purchase and operation costs than SEM. In general, however, it is possible to obtain nanometer resolution with moderate electron energies of some 10 keV as those used for SEM.

A way to reduce the costs of TEM is to use a SEM with a transmission electron detector (Transmission Scanning Electron Microscopy, TSEM), as on the other hand TEMs can be equipped with a scanner unit (Scanning Transmission Electron Microscopy, STEM).

Recently, the Focused Ion Beam (FIB) technique, widely used in the semiconductor industry and in materials science, is beginning exploited in the biological and pharmaceutical fields for site-specific analysis, deposition and ablation of materials. It has the same setup of a SEM, but it use for imaging a ions focused beam instead of a electrons focused beam of electrons as in the SEM. The FIB-methods have a particular application in the 3D imaging technique known as 'volume EM' or 'Focused Ion Beam Scanning Electron Microscopy (FIB/SEM)', where a dual beam FIB/SEM integrates the two techniques within one device [118]. It consists of a SEM equipped with a focused gallium ion beam and a Back Scattered Electrons (BSE) detector that scan the sample surface and image the scanned surfaces, respectively. Thus, it provides a series of sequential images which can be combined into a 3D image of the sample.

This technique also allows to obtain structural information regarding volumes of tens of thousands of cubic micrometres, revealing complex microanatomy with subcellular resolution. It could have wide application also in pharmaceutical applications [118].

The necessity for high-vacuum conditions during both FIB and SEM/TEM analyses for soft matter characterisation, that inevitably leads to a loss of sample mass, is the main limitation of the FIB/SEM or STEM approach. In particular, frozen-hydrated systems show this effect [119].

3.1.4. Environmental Scanning Microscopy

Environmental Scanning Electron Microscopy (ESEM) allows the observation of hydrated samples in their native state. This technique allows do not coat the sample with a conducting layer as for conventional SEM technique. It is therefore useful to analyze colloidal pharmaceutical dispersions and their aggregation or film formation [120-122]. The main disavantage of ESEM is the lower resolution compared to TEM and SEM and its limit to observing the top layer of sample.

Water is an integral part of nanoemulsions or liposomes and its removal leads to significant morphological damage. Few techniques can combine the possibility of wet-imaging with good resolution and depth of field. Conventional EM analysis is not able to give high resolution imaging of colloidal systems in their native since it was previously limited to performing experiments under high vacuum of the instrument. Thus, many sample preparation steps (for example the

freeze-drying) were necessary before analysis. Sometimes ice crystals formation or redistribution of compounds lead to artefacts. The introduction of ESEM in the 1990s and its variations at the low vacuum end, near 10-200 Pa, (known as leaky SEM, low vacuum SEM, variable pressure SEM, *etc.*) was an important implement to get artefact-free imaging of hydrated colloidal samples without cryo preparation processes [120-121]. Even if a cooled stage is used, the sample can dry, because of water evaporation, and it can change in structure.

The possibility to use ESEM for dynamic experiment makes it one of the main technique used to characterize hydrated systems. The main disadvange of ESEM is that it is not possible to analyse structures below a significant layer of water. Thus, some particles in hydrated colloidal dispersions should be sufficiently close to the surface for imaging and the sample behaviour at the interface between air and water must be typical of the bulk. Otherwise, not representative information can be obtained.

3.1.5. Characterization of Nano-Scale DDSs by Using EM

The following sections provide a non-exhaustive overview on selected nano sized DDSs in the focus of the role of EM for their investigation, highligthing the advantages and pitfalls.

The characterisation of NPs is of importance both in formulation development and nanotoxicological studies (see paragraph **4**) [106].

Morphological analyses are most frequently performed by TEM, although SEM can likewise be employed [114]. Many detailed informations about nanocrystals and their surface and planar defects can be investigated with High-resolution TEM [123]. A successful analysis depends on different conditions such as an appropriate choice of grids, preparative technique and TEM imaging conditions. Many care is needed because of artefacts that can lead to misinterpretation (*e.g.,* crystallisation of nanoclusters in the electron beam) [124]. In (Figs. **2**-**3**) examples of NPs are reported.

Nanoparticulate samples can be deposited on the grid as a drop that is left to dry before analysis and negative staining can be used to improve contrast [125]. The characterisation of nanoparticulate DDSs concerns their morphology, size distribution and shape; thus aggregation and cluster formation have to be prevented. Alternative deposition methods, such as aerosol or the use of stabilisers, should be employed in case of samples particularly prone to aggregation. SEM was used to investigate the positive effect of the stabilisers on the agglomeration tendencies of zinc oxide and titanium dioxide NPs in aqueous dispersion by fixing single isolated NPs on poly-L-lysine coated substrates [106]. The size and the agglomeration size together with the wetting properties of nanoparticulate systems, monitored by TEM, are pivotal information since these parameters may affect their uptake through biological membranes and thus their biological effect. In this respect, it is important to mention that NP size may influence the distribution within the organism after interaction [126].

To ensure that changes regarding sample preparation, sample application, electron beam voltage or exposure time does not bias the outcome control experiments should always be performed. This is particularly important for the FF-TEM, in which sample preparation is rather invasive and only a replica is investigated under the microscope and artefacts are likely to occur [117]. For example, spherical shapes in the replica represent imprints of NPs rather than fractured particles.

Other aspects likewise of importance for NPs application that can be characterised by TEM are: i) the internal structure consisting of shells (nanospheres) or a core/shell structure (nanocapsules) [127]; ii) particle shape dictating the interaction with target sites. Likewise, TEM and Energy Filtering-Transmission Electron Microscopy (EF-TEM) were successfully used in investigating the interaction between NPs shape and bacterial cells in terms of biological effect [128]. Conversely, SEM allows to correlate particle morphology with the employed compounds and methods used during the synthesis. For example, colloidal silver NPs have been extensively studied with respect to the size, morphology, composition and crystallographic properties by TEM in combination with EDS and High Energy Electron Diffraction (HEED) [129]. Various forms of AgNPs, such as cubes, triangles, wires or alignments of wires were investigated for their antibacterial activity [130, 131] and for their effect on bacterial growth using TEM and a High Angle Annular Dark Field (HAADF) STEM technique [132, 133], in addition to the elemental mapping by EDS to define the localization of the NPs. SEM and high-resolution SEM [134] were extensively used to characterise the dehydrated state of antibody CD54-conjugated composite organic inorganic NPs for treatment of leukaemia, while for the study of their native, hydrated state wet SEM was used. TEM and scanning AES can be used to study at high resolution the binding of NPs to cell surface antigens [134].

SEM, as already said, is pivotal for monitoring the preparation of nanosized carriers, it successfully allowed to establish the distorting effect of increasing chitosan concentrations on alginate/chitosan microparticles, consisting of a scratchy microparticle surface and numerous invaginations representing the drug internalization under particle surface [135]. However, the modifications in microparticle morphology upon loading with daunorubicin and doxorubicin have been visualized also by using TEM.

As already said, cryo preparation is the elective method for the microscopic characterisation of lipid NPs [85], but satisfying results can be also provided with ESEM [136] or with conventional TEM after negative staining [137-142]. On the other hand, by applying immunolocalization methods to the microscopical observation of liposome the presence of the loaded proteins can be detected by using immune gold [143]. Cryo preparation is useful in determining particle shape, that has a great influence on the cell viability as analysed for mouse fibroblasts [144]. Cryo-TEM is elective microscopy for characterising the different particles, shapes of supercooled liquid particles had a droplet shape, crystalline particles displayed platelet-like anisometric structures or regular platelet formed shapes and supercooled smectic formulations exhibited a cylindrical, barrel-like shape. Interestingly, cell viability is lower in presence of less spherical and more angular particle shapes. Lipid matrix correlated closely

to particle shape might have a pivotal role likely to lead to these results. Both cryo-TEM and FF-TEM are suitable for the shape characterization of SLN and NLC [143, 145]. SEM is suitable to obtain information not only about SLN shape, but also for surface properties and stability after coating [146].

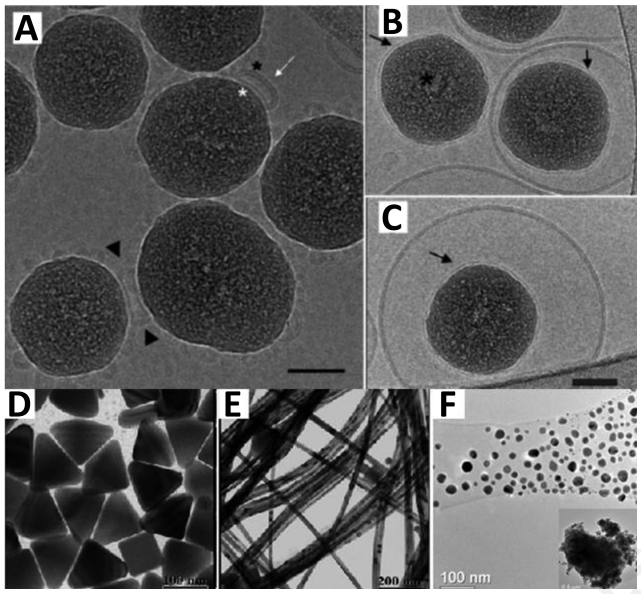

Fig. (2). NPs in EM. (**A**) Cryo-TEM: silica NPs covered by small unilamellar vesicles. Deformation of vesicles is seen (double dark arrowheads, white arrow). Two lipid leaflets of the adsorbed vesicles are visible in the nonadhesive areas (black asterisk); only one leaflet is visible in the adhesive areas (white asterisk). Scale bar: 50 nm. Reprinted with permission from *Mornet S, Lambert O, Duguet E, Brisson A. The formation of supported lipid bilayers on silica nanoparticles revealed by cryoelectron microscopy. Nano Letters 2005; 5: 281-285*, copyright (2005) American Chemical Society. (**B** and **C**) Cryo-TEM: images of liposomes containing engulfed silica NPs (dense grainy spheres) that are covered by a supported lipid bilayer (black arrows). NPs covered only with a supported lipid bilayer are also visible (asterisk). Scale bars 50 nm. Reprinted with permission from *Le Bihan O, Bonnafous P, Marak L, Bickel T, Trepout S, Mornet S, De Haas F, Talbot H, Taveau JC, Lambert O. Cryo-electron tomography of nanoparticle transmigration into liposome. Journal of Structural Biology 2009; 168: 419-425*, copyright (2009) Elsevier. (**D-E**) TEM: images of (**D**) triangles and (**E**) wires of silver nanoparticles. Reprinted with permission from *Yu D, Yam VW. Hydrothermal-induced assembly of colloidal silver spheres into various nanoparticles on the basis of HTAB-modified silver mirror reaction. Journal of Physical Chemistry B 2005; 109: 5497-5503*, copyright (2005) American Chemical Society. (**F**) TEM: silver nanoparticles released from a carbon matrix. The inset corresponds to a higher magnification image. Reprinted with permission from *Morones JR, Elechiguerra JL, Camacho A, Holt K, Kouri JB, Ramirez JT, Yacaman MJ. The bactericidal effect of silver nanoparticles. Nanotechnology 2005; 16: 2346-2353*, copyright (2005) IOP publishing.

Recently SLN have proposed as carriers for SuperParamagnetic Iron Oxide Nanoparticles (SPIONs) and employed for magnetic resonance imaging or site-specific drug delivery [147]. However, for *in vivo* application, coating these nanocarriers with biocompatible material such as polymers or lipids is needed. TEM was used to characterize these negative stained hybrid SLN containing magnetic NPs and EDS was employed for characterization of NPs within the lipid vectors. Liquid crystals are states that present properties of both liquids and solids. Their shape, such as lamellar, hexagonal or cubic structures, may influence the nature of the surfactant molecules in different concentration in the solvent [148, 149]. These liquid crystalline cubic phases by forming in aqueous phases a thermodynamically stable, curved, bicontinuous lipid bilayer separating two congruent networks of water channels are very interesting as DDSs [150].

Cryo-TEM is the technique adopted to analyse the crystallographic structure of single vesicles, like cubosomes or hexosomes [85, 103] in combination with FF-TEM, that is indeed suitable to visualise liquid crystalline structures without prior etching [148]. It is, thus possible, to distinguish various shape like layered lamellar phases, rod-like micelles hexagonal arranged, closely packed spherical micelles, *etc.*

The comparatively new DDSs consisted of non-lamellar Liquid CNPs (LCNPs) of lipid liquid crystalline phases [151] possessing large surface areas, show fascinating spherical, hexagonal or deformed cubic structures when visualized by cryo-TEM [152]. Cubic phases are a particular type of carriers called cubosomes. [153]. The characterization of complex multicomponent liquid crystalline lipid carriers that may form sponge-like nano-assemblies fit for internalization of peptides, proteins or nucleic acids has been recently analysed with the use of FF-TEM [154]. The concept of nanopockets as vehicles for biomolecules, such as proteins, that may be located at the interfaces between the fragmented cubosome particles or associated with the surface of the connected cubosomal units have been confirmed by FF-TEM [154]. Lipid cubic phases can be fragmented and dispersed into LCNPs with an internal structure and cryo-TEM was employed to study populations of vesicles merging with cubosome particles or aggregates with a dense internal bilayer organisation [154, 155].

In spite of their borderline morphology, microemulsions are usually considered to be colloidal DDSs. Visual inspection, optical light microscopy and polarisation microscopy allow to guarantee that the systems are isotropic and do not contain birefringent structures [117]. As for all colloidal systems, for analysis of microemulsions, both cryo-TEM and FF-TEM have been successfully employed [156]. In particular, cryo preparation is essential to observe the microstructure in the native state.

EM techniques are used also to characterize another class of elastic vesicles named niosomes, containing non-ionic surfactants instead of phospholipids [157], the vesicular carriers consisting of phospholipids, ethanol and water termed ethosomes [158] and the carriers termed invasomes, composed of phosphatidylcholine, ethanol and terpenes as additional permeation enhancers, [159, 160].

In (Fig. **3**) examples of nano-scale carriers, *i.e.,* liposomes, niosomes, cubosomes, characterized by TEM, FF-TEM and by immunogold labelling are reported.

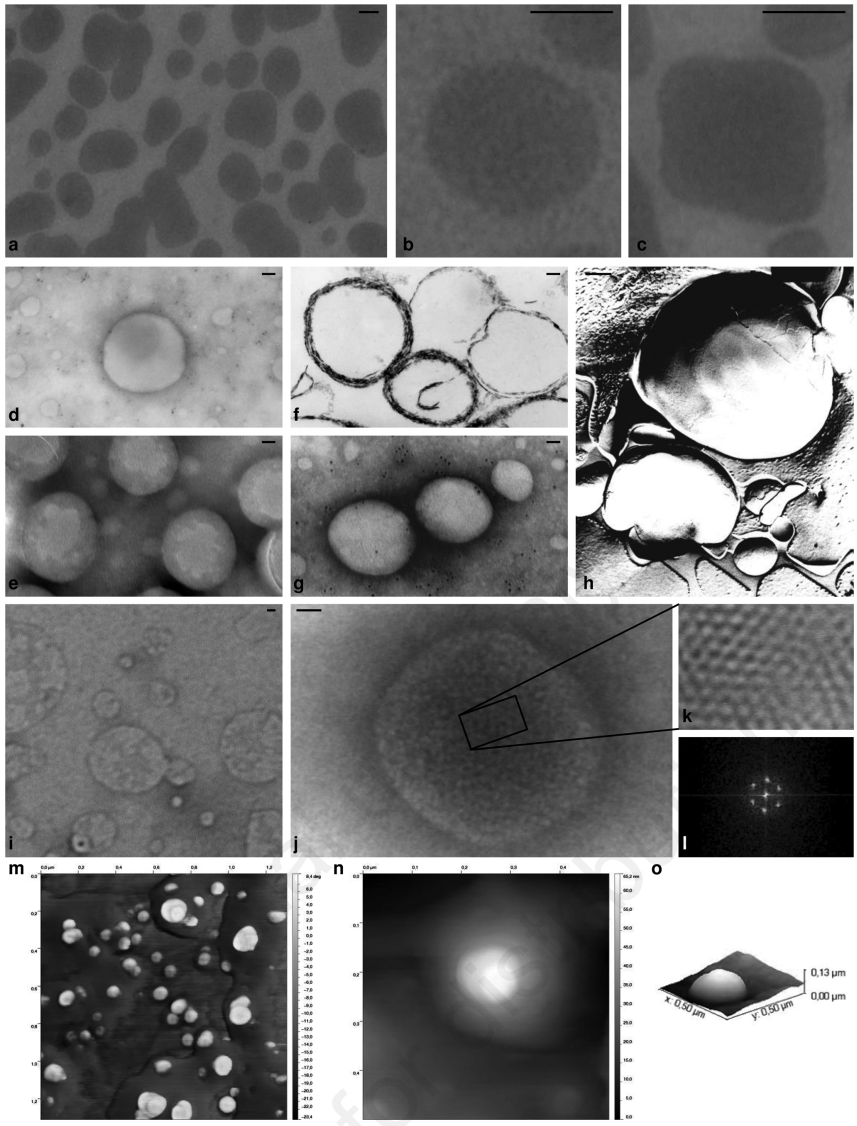

Fig. (3). Examples of nano-scale carriers, *i.e.,* liposomes, niosomes, cubosomes, characterized by TEM, FF-TEM and by immunogold labelling. TEM (**a-l**) and AFM (**m-o**) micrographs of different NDDSs. **a-c**: TEM micrographs of different sized and shaped cubosomes (**a**), exhibiting almost a spherical (**b**) or a square (**c**) shape. **d-h**: TEM micrographs of empty (**d**), ascorbate-oxidase-and-beads-loaded (**e**), ascorbate-oxidase-and-agar-loaded (**f**), immulabelled ascorbate-oxidase-loaded (**g**), and freeze-fractured ascorbate-oxidase-loaded (**h**) liposomes. **i-l**: TEM micrographs of different sized and shaped empty niosomes (**i**), showing almost a spherical shape (**j**) and an ordered crystalline nature (**k**), as shown by the corresponding electron diffraction pattern (**l**). **m-o**: AFM micrographs of different sized and shaped temozolomide-loaded niosomes (**m**), almost showing a spherical shape (**n**) and sized as shown in figure (**o**). Niosomes Tween 20, Tween 20-glycine and cholesterol in a molar ratio of 1:3:2 (**i-l**) or Tween 20, Tween 21 and cholesterol in a molar ratio of 0.5:0.5:1 (**m-o**). Bars = 10 nm.

3.1.6. Structural Properties Investigation by Diffraction Analyses

It has to be noted that all the functional properties of nanomaterials depend not only on shape and particle nanosize but also on the crystal structures. The crystalline structure of a material is then one of the principal keys for understanding its properties and therefore is the most desired piece of information.

So, the structural analysis, including phase identification and unit cell determination, become one of the fundamental problems during the characterization and quality control of nanomaterials. At this purpose diffraction techniques operating in the reciprocal space must be used. Diffraction analyses may be performed using either light of very short wavelength like X-rays or matter waves like electrons (and neutrons) whose wavelength is on the order of (or much smaller than) the atomic spacing [161].

X-ray powder diffraction is the most used technique to analyze the crystal structure in bulk materials, but is less used in the characterization of nanomaterials for its intrinsic limits, due to X-ray is only scattered by the electrons of scattering atoms, that means that relatively large volume of material and long times of data acquisition are required, with the consequent incapability to perform structural analyses at the nanoscale.

These limits are not present in the case of electron diffraction, being electron cross sections 10^4 to 10^5 times larger than that of X-ray due to the strong interactions between electrons with nucleus of the scattering centers.

As a result, also very tiny diffracted volume upon short exposure time (*e.g.,* a few seconds) exhibits electron diffraction pattern and it is possible to perform sophisticated and reliable structural analysis at the nanoscale [162], making also possible the characterization of single crystallites [163]. Electron diffraction can probe volumes down to 20-30 nm in diameter, delivering 3D sub-Ångstrom structural information with good signal-to-noise ratio. The capabilities and performances of the large variety of ED techniques today available [164] represent powerful, and sometimes unique, tools for the structural analysis and identification of the nanomaterials. But the rapidly developing nanotechnology constantly needs new analytical tools to characterize increasingly smaller nano-volumes. In such a context, one of the most advanced tool for nanocrystal structure analysis can be considered the method called Automated Diffraction Tomography (ADT) [165, 166], based on a collection of electron diffraction patterns at different specimen tilt angles, similar to how it is done in real space tomography. ADT makes possible the collection of 3D diffraction data sets from crystals down to a size of only few nanometers.

3.2. New Emergent EM Systems to Investigate Nano-Scale DDSs: I AFM

In Atomic Force Microscope (AFM) the surface of the investigated sample is scanned through a sharp probe tip placed at the end of a cantilever a few hundreds of microns long, some tens of microns wide and a few microns thick. Changes in tip-sample interaction force - which can be as low as a few piconewtons - are monitored by detecting the deflection of the cantilever through an optical lever technique, *i.e.,* a laser beam focused on the back of the cantilever and reflected into a four sectors photodetector. By raster-scanning the tip across the surface, in standard AFM contact mode a map of surface topography is generated by maintaining the cantilever deflection (and thus the tip-sample interaction force) at a constant value (set point) through the use of a feedback circuit acting on piezoelectric actuator that modifies the actual tip-sample distance. The topography of sample can be reconstructed from displacements of the piezoelectric actuator. To avoid the continuous tip-sample contact which may modify the surface of soft samples (*e.g.,* polymers or biological specimens), AFM intermittent contact mode - also referred to as tapping mode - is generally employed. In tapping mode the cantilever is oscillating at its first resonance frequency, its oscillation amplitude is measured through a lock-in amplifier, used as the feedback signals maintained at a constant set point value. In tapping mode, the tip-sample interaction is limited to a fraction of the oscillation period which makes this operation mode particularly suitable for studying compliant samples. A further advantage of AFM is represented by the possibility of operating in air, different gases, vacuum, and liquid environment. Taking advantage of the close proximity to the sample surface of the AFM tip, different physical and chemical properties of the sample can be probed with nanometrical lateral resolution at selected locations and mapped [167-169]. Advanced AFM-based techniques have been developed in combination with ultrasound methods to map mechanical properties of samples at the nanoscale [170]. AFM force curves can be used to measure physical properties including elasticity, hardness, Hamaker constant, adhesion and friction [167]. The latter two are involved in many systems at nanoscale, where adhesion plays a pivotal role [171]. Thus, AFM, in addition to the imaging of small biological samples such as biomolecules (*e.g.,* proteins and DNA) [172, 173] and small organisms (*e.g.,* viruses and bacteria) [174, 175], it is also useful to characterize the morphology of the drug-loaded nanocarriers [176, 177]. Electric forces and electric current flowing between the tip and the sample can be monitored, which enables the study of surface charges distribution, surface potential, dielectric constant, resistivity. Finally, equipping the AFM with magnetically coated tips, magnetic properties of the sample can be investigated using the so-called Magnetic Force Microscopy (MFM). Among other applications, MFM has been recently proposed to study nanocarriers constituted by vesicular systems loaded with superparamagnetic NPs [178].

Like all microscopic techniques, also the AFM has advantages and limitations. Advantages: i) AFM produces high-resolution images with a lateral resolution in the range of few nm and vertical resolution less than 1 nm; ii) AFM can be used to produce images from the surface of non-conductors (polymers) without any special preparation to make the sample conducting as required in SEM and TEM; iii) simple modification of the AFM allows other properties to be detected. If the sample tip is ferromagnetic, then we can detect the stray magnetic field thus showing the magnetic domain structure of the sample; iv) AFM give direct 3D images of surface structure of polymers. By using lateral force, phase contrast modes, or nanomechanical imaging, it is possible to differentiate the type of materials at the surface of polymer blends; v) AFM is suitable for the characterization of nanocomposite materials; vi) radiation damage of the sample as observed in SEM and TEM is avoided; vii) the best resolution obtained is 0.3 nm which is better than SEM. Limitations: i) AFM is complex and susceptible to outside influences like contamination of surfaces and instrumentation control; ii) in contact mode of AFM imaging, there is a possibility that the soft polymer samples may be damaged [179].

3.3. New Emergent EM Systems to Investigate Nano-Scale DDSs: II Analytical Electron Microscopy (AEM)

The need to obtain more information about nanostructures led to use other electron microscopies integrated systems, indicated with the term of AEM. This big field refers to the spectroscopic data obtained by using EM, *i.e.*, from interaction of the incident electron beam with the sample, that are complementary to the conventional microscopic observation. These signals allow to identify morphology, phase state, composition and concentration of the elements present in the analyzed area, at a spatial resolution of 1 nm or better. Thus, today, EM instruments are rather equipped with a system based on the measurement of the energy lost by the incident electrons, called Electron Energy-Loss Spectroscopy (EELS), or with a system based on the detection of X-ray signals generated in the sample by the primary incident electrons, called Energy-Dispersive X-ray Spectroscopy (EDXS)

[82]. EDXS makes use of the X-ray spectrum emitted by a solid sample bombarded with a focused beam of electrons to obtain a localized chemical analysis [65, 123]. The spectra generated by EDXS analysis show peaks corresponding to all the elements forming the true composition of the sample being analysed. EDXS systems are attachments to electron microscopies, SEM as well as TEM. EDS is based on the counting of X-rays emitted from the specimen region affected by beam as a function of the photon energy, and it probably allows to carry out the most precise microanalysis in TEM [123]. However, SEM, closely related to the electron probe, can realize primarily electron images, but can also used for element mapping, and even point analysis, by adding an X-ray spectrometer allowing to overlap the functions of two instruments. In EELS the primary process of electron excitation results in the fast electron losing of a certain amount of energy. When the sample is affected by electron beam, some of the electrons are inelastically scattered and lose a part of their energy; so, this technique analyzes the intensity distribution of the transmitted electrons as a function of their decrease in kinetics energy. It can give not only the chemical information about the specimen but also its electronic structure [82, 123]. EELS allows to detect and quantify all elements present in the periodic table. Since electron lenses that direct the electrons into an electron spectrometer are already present in a TEM, it is possible to couple EELS to identify the structure of the analyzed material [82]. In fact, conventional TEM is not adequate for the ultrastructural analysis of NPs, because of artifacts due to specimen preparation (*e.g.,* heavy metals used for contrast enhancement) [180]. EELS associated to TEM can also be employed to achieve chemical mapping, working in a different mode that involve electronic properties of the chemical phase in which the elements are engaged and not the atomic number or the density [181]. The technique of EELS-TEM is of great interest for the analysis of lipid nanocarriers. In a study, it was observed the existence and the site of AgNPs inside the dipalmitoylphosphatidylcholine bilayer by TEM, but only by using EELS it was also confirmed that AgNPs were entrapped in the liposome [182]. In addition, a cryo-preparation can be combined to the conventional EELS technique (cryo-EELS). In order to determine the biocompatibility of NPs, it is pivotal to obtain information about the composition of organic and inorganic materials in NPs [183]. Thus, other complementary new-generation techniques are used to get more information about NPs morphology and chemical composition. For example, analytical TEM as EF-TEM, combining the two microanalytical techniques of Electron Spectroscopic Imaging (ESI) and EELS, allows to investigate the interaction of NPs with cells at the ultrastructural level through the resolution of the chemical composition of structures and serves as an important tool [180].

3.4. Safety Assessment: The Role of EMs

A comprehensive characterization of nanostructured materials is essential to understand the biological properties of nanomaterials and assess their safety. Indeed, the increased application of nano-scale DDSs is not paralleled by a systematic investigation about their putative detrimental effects on humans and environment [184-186]. A new branch of toxicology, called nanotoxicology, is developing with the aim to bridge the gap between the powerful of nano-scale DDSs and their safe use. Indeed, nanotoxicology addresses the toxicology of NPs which appear to have toxicity effects that are unusual and not seen with larger particles. With this respect microscopies are still invaluable tools. They allow to gain information on the morphological changes induced by the nanocarries to the cells. It is worth to mention that morphological changes are sensors of the changes in the biochemical and molecular pathways. In addition, microscopies are fundamental in deciphering the route of entry of the nanocarries, and in defining their organ, cellular and subcellular bioaccumulation. In order to promote nanotechnology for biomedical applications (NPs-based imaging, drug delivery and other therapeutic applications of nano-scale DDSs), quantitative and qualitative studies on the cellular internalization of NPs, with respect to their size and shape, are required. NPs size and shape are important information to evaluate, because they may influence the distribution within target after application [126, 187]. In addition, it is important to evaluate not only the effect of NPs on human health, but also their toxicity and the mechanism/s of penetration in the human body. Thus, several studies dealing with NPs for cancer therapy use both TEM and SEM for their characterization [175, 188]. TEM analysis is used to obtain more information about the internalization and localization of NPs in different types of cells, normal as well as cancer cells. For example, internalization of iron oxide NPs in growing neurons [189], as well as the internalization mechanism of hydroxyapatite particle agglomerations into hepatocellular carcinoma cells [190], can be investigated by TEM. In addition, TEM permits a quantitative analysis of NPs internalization. In fact, in a recent study, it was possible to find the endocytotic vesicles inside the cytoplasm of mammalian cells containing clusters of many NPs [191].

It should be also considered that nanocarriers can be released into the environment (in particular into the water) and thus could be potentially toxic for plants and animals. Their concentration is increasingly growing in water and soil that are therefore very significant routes of exposure to environmental risk. The AgNPs, for example, seem to be responsible for the death of fishes, causing changes in gene expression and embryo toxicity [24].

To this purpose it is therefore essential to identify the possible toxicity of different nanoparticles though the exploitation of attractive animal models. In a work of 2013 we studied the embryotoxic potential of the Ag- and C- NPs, by integrating microscopic analyses with UV-visible spectroscopy investigations, in sea urchin *P. lividus* development, from fertilization until the larva stage. The embryotoxicity tests revealed that both NPs types were embryotoxic since they caused embryo malformations and alteration of the normal progression through the development stages. Embryonic development was slowed down by AgNPs and sped up by CNPs. Interestingly, AgNPs delayed embryo development, caused malformations leading to embryos death in a concentration dependent way; while embryos bearing CNPs-induced malformations survived for a longer time [185].

Recently, by using an another cell model, *i.e.*, human lymphocytes isolated from peripheral blood, we demonstrated that AgNPs are absorbed/taken up by these cells and

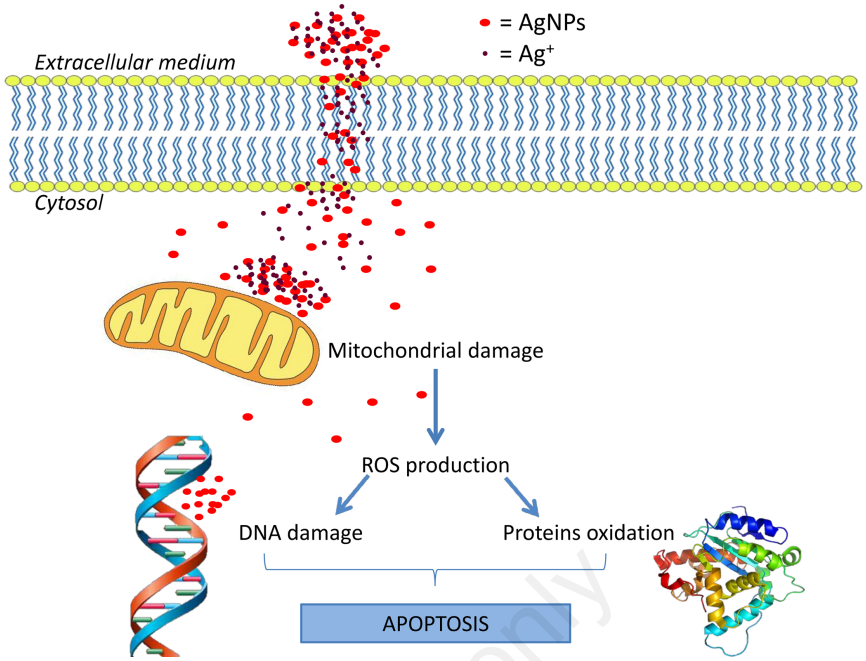

Fig. (4). Schematic representation of a putative AgNPs-induced cytotoxicity mechanism in accord to [46].

cytotoxicity and morphology changes are amount and time-dependent. AgNPs entered into the cells when they were still viable, with a maximum absorption after 2h of incubation, and determined a decrease of their viability and a gradual releasing of silver into the culture medium [186, 192]. However, the mechanism(s) by which the AgNPs are internalized and provoke cell death is(are) unknown. As shown in (Fig. **4**), it seems that the accumulation and dissolution of AgNPs at plasma membrane causes cell leakage [24]. Once these NPs are entered within the cell, they cause DNA damage and the induction of apoptosis-related genes and the activation of mechanisms leading to apoptosis [193-195].

CONCLUSIONS

Commercialization of nanotechnology in pharmaceutical and medical science has made great progress. Taking USA alone as an example, at least 15 new pharmaceuticals approved since 1990 have utilized nanotechnology in their design and nano-scale DDSs [196]. To achieve efficient cytosolic delivery of therapeutics or nuclear targeting, different DDSs have been developed (macromolecular drug conjugates, chemically or genetically modified proteins, and particulate drug carriers) capable of subcellular internalization overcoming the biological barriers, by passive targeting and especially by active targeting (receptor-targeted delivery). The success depends on the physicochemical nature of DDS, intracellular barriers that these systems need to overcome, the bioavailability of the bioactive drug, biodistribution, the intracellular pharmacokinetics and its influence on the pharmacodynamic effect [197-200]. However, as discussed in this review, any nano-scale DDSs, as well as any EM techniques used for their analysis, it is not free from undesirable effects. So much has been done already, but just as there is yet to be done: as challenges for the researchers of the 21st century there are the development and the application of most appropriate and sophisticated microscopic investigation techniques in order to ensure the safety of all these new-generation nano-scale DDSs, *i.e.*, to exclude any risks either for humans that for the environment. There is no doubt that if researchers will able to address all points of concern, the benefits that will accrue to the medicine will be considerable, *e.g.*, extending the expectancy of life for humans.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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