Electron microscopy to characterise nanoparticles in the biological environment

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SUMMARY

Nowadays the use of nanomaterials has led a growing interest in biomedical application as drug delivery systems for the treatment and the diagnosis of different pathologies. Different analytical techniques have been applied to characterise nanoparticles in the biological environment. However, in the attempt to describe in detail the interaction of NPs with the living systems and to detect the possible occurrence of cell damage or death, electron microscopies proved to be especially suitable and actually are irreplaceable techniques thanks to their image resolution at the nanoscale. In this review article, the attention will first be focused on the influence of nanoparticles features on their interaction with tissues and cells; then, the advantages and limits of transmission and scanning electron microscopy to evaluate the suitability of nanovectors as drug-delivery systems will be discussed.

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Introduction

Nowadays the use of nanomaterials has led a growing interest in biomedical application as drug delivery systems for the treatment and the diagnosis of different pathologies. The delivery of therapeutic agents through nanoparticles represents an attractive option in the attempt i) to protect the therapeutics from enzymatic degradation, ii) to tune the biodistribution and targeting after systemic administration, iii) to prolong the drug circulation, and iv) to reduce the systemic toxicity of therapeutics.

According to the European Commission, "nanomaterial" means "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm" (http://ec .europa.eu/environment/chemicals/nanotech/faq/definition_en.htm).

Due to their small size, nanoparticles (NPs) should -at least in principle- easily enter the tissues and cells, but the biomedical impact they have on *in vitro* and *in vivo* systems are closely related to their physicochemical properties (Lin *et al*., 2014). When nanomaterials, particularly NPs, are injected into a live organism, they come into contact with a complex physiological environment (the biological fluids, tissues, intercellular matrix and cells) (Kettiger *et al*., 2013), the interaction with the biological components depending on the nature of the NPs such as their chemical composition, size/size distribution, shape and surface properties. To enter a cell, NPs must contact with the plasma membrane, and the mode of uptake and intracellular interaction with specific organelles is crucial to make a drug-delivery system biocompatible and effective. On the other hand, the structural and molecular features of the cells also affect the efficiency of NP uptake.

Different analytical techniques have been applied to study NPs especially as drug-delivery systems (Figure 1). However, in the attempt to describe in detail the interaction of NPs with the living systems and to detect the possible occurrence of cell damage or death, electron microscopies proved to be especially suitable and actually are irreplaceable techniques thanks to their image resolution at the nanoscale (Reifarth *et al*., 2018).

In this review article, the attention will first be focused on the geometrical and surface properties of nanovectors that are especially important for their capability to interact with tissues and cells; then, the advantages and limits of transmission and scanning electron microscopy (TEM and SEM, respectively) to evaluate the suitability of nanovectors as drug-delivery systems will be discussed.

Influence of nanoparticle size, shape and surface properties on their interaction with the biological environment

The size strongly influences the impact NPs have on living organisms. Half-time circulation, tissue biodistribution, interactions/uptake at the cellular level, and intracellular trafficking/removal are all parameters that are affected by the particle size (Duan and Li, 2013; Kettiger *et al*., 2013).

Regarding NP delivery in the whole organism, previous studies have demonstrated that after systemic administration small NPs (10-20 nm) are able to easily pass through the thin endothelial junction reaching different organs and tissues (Duan and Li, 2013). Small NPs are also characterized by a faster renal clearance in comparison to the bigger ones; for very large NPs (>1 μm) the clearance should also be fast, but they tend to more easily aggregate inside the blood vessel causing a mechanical retention by capillaries with a low distribution into tissue. Based on these considerations, NPs having a size between 20 and 100 nm are considered as the more suitable in terms of circulation time, biodistribution and clearance (Choi *et al*., 2007).

At the cellular level, the NPs size has a great impact on the mechanisms of uptake and cell internalization. Normally, only lipid-soluble NPs presenting a size lower than 30 nm are able to directly cross the cellular membrane (Kettler *et al*., 2014), whereas the uptake of bigger NPs often occurs through active, energy-dependent processes. The main mechanisms of uptake in eukaryotic cells are endocytosis (often receptor-mediated), pinocytosis and phagocytosis. In general, NPs having a size <100 nm can enter by pinocytotic pathways, whereas NPs with a size range between 120-150 nm principally enter by receptor-mediated endocytosis; NPs ranging 250 nm to 3 μm have been shown to mainly enter by phagocytosis (Foroozandeh and Aziz, 2018). The size of NPs not only influences the uptake mechanism but also affects its efficiency. Works in the literature demonstrated that the concentration of saturation as well as the uptake efficiency of NPs having a diameter around 50 nm are higher than the ones of larger NPs (Soppimath *et al*., 2001; Mailänder and Landfester, 2009; Kumari and Yadav, 2011). It is however worth recalling that the uptake of NPs is also largely dependent on the cell type (Adjei *et al*., 2014).

From the biological point of view, NP shape may affect the circulation time, biodistribution, targeting efficiency, cell internalization and intracellular fate (Champion and Mitragotri, 2006; Geng *et al*., 2007; Gratton *et al*., 2008; Muro *et al*., 2008). Regarding the circulation life, NP shape may significantly influence the phagocytotic process by macrophagic cells: it has already been shown that NPs with one elongated axis have a longer circulation time being less

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prone to be phagocytosed by macrophages (Duan and Li, 2013). Moreover, concerning the circulation profile, non-spherical shapes influence NPs *in vivo* distribution in target organs such as spleen (Devarajan *et al*., 2010), lung (Decuzzi *et al*., 2010) and tumour tissues (Christian *et al*., 2009). On the other hand, *in vitro* studies demonstrated that round NPs more easily enter the cells compared to the rod-shaped ones (Wilhelm *et al*., 2003; Limbach *et al*., 2005).

Several studies *in vitro* and *in vivo* have also underlined the importance of the NP surface properties regarding the interaction with the anionic cell membrane, the cellular uptake and the intracellular behaviour.

Following cellular uptake, NPs interact with the intracellular milieu (cytosolic component and organelles), and their intracellular distribution and location (in the cytoplasm or the nucleus) are crucial for their functional effects. Depending on their chemical composition, NPs may target cytoplasmic organelles (such as the mitochondria, the Golgi complex or the endoplasmic reticulum) or enter the nucleus. For instance, the physicochemical characteristics of the NP surface influence the penetration through the mitochondrial membrane (Adjei *et al*., 2014). An intranuclear localization is required when the drug or genetic material the nanocarrier is loaded with must interact with the nuclear chromatin, but the penetration into the nucleus is strongly dependent on the dimension of the drug-loaded NPs (it is to be taken into account that molecular complexes smaller than 45 kDa are able to easily penetrate the nuclear envelope whereas the nuclear pore complexes are responsible for the transport of larger macromolecular complexes) (Adam *et al*., 1990; Hagstrom *et al*., 1997; Hillaireau and Couvreur, 2009).

Electron microscopy is the most adequate approach for visualizing NP-cell interactions

To visualize the uptake, intracellular distribution and degradation/release of NPs, appropriate microscopy techniques are used (Figure 1). Both light and electron microscopy may be exploited to characterize the cellular fate and performance of NPs (Costanzo *et al*., 2017).

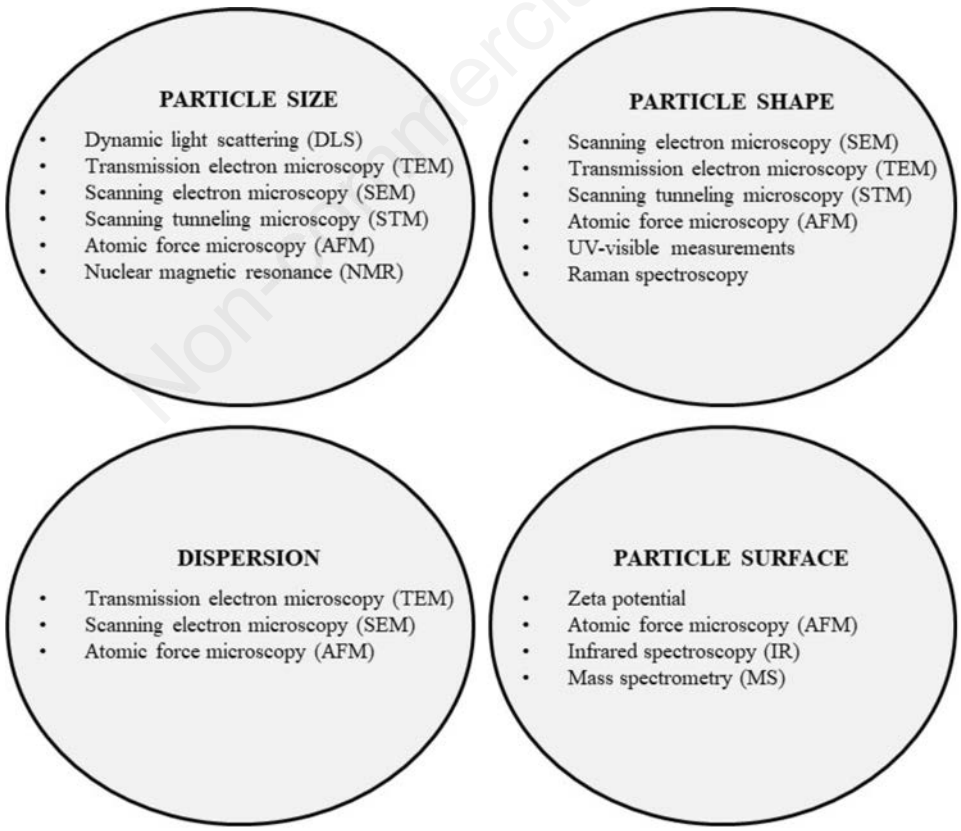

Figure 1. Techniques commonly used for the characterization of nanomaterials.

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Conventional and confocal fluorescence microscopy allow to locate properly labelled nanoconstructs at the surface or inside the cells (Costanzo *et al*., 2016), and super-resolution microscopy promises to be adequate for tracking them at the subcellular level (Jin *et al*., 2018; Shang *et al*., 2018); however, TEM and SEM, thanks to their much higher resolution, are the techniques of choice to investigate nanostructured materials inside the cells at the nanoscale.

SEM, as a surface imaging technique, is a powerful and popular approach to acquire information on the size, shape and surface morphology of nanomaterials (Lin *et al*., 2014), as well as to elucidate the interaction of NPs with the plasma membrane: as a consequence of its great depth of focus, SEM provides detailed three-dimensional topographic images of the cell surface, thus being particularly appropriate to get insight into the mechanisms of NP internalization. Besides the morphological evidence, SEM may also allow to collect information on the chemical composition through the analysis of the element-specific X-ray emission (Hall *et al*., 2007; Wang and Lee, 2008): this would especially be useful to discriminate the simultaneous presence at the cell surface of NPs with different metal components.

Analyzing the cell surface by SEM usually needs to dehydrate the biological samples and make the surface conductive, by coating with a thin metallic layer: as a negative aspect of this procedure, alteration of the plasmalemmal structures (such as microvilli or caveolae) or shrinkage of the NPs may occur (Bootz *et al*., 2004). It is however worth recalling that the possibility exists to operate at low voltage with environmental SEM (ESEM): thanks to a partial vacuum, high humidity level and a lower energetic beam, this instrument allows obtaining reasonably good images of still partially hydrated biological samples in the absence of conductive coating (Perez-Arantegui and Mulvey, 2005). Nonetheless, this method engenders a lower resolution imaging (Oatley *et al*., 1966). Another drawback of SEM for NP investigation is that image pixels are collected one by one by scanning the sample surface, which leads to a long exposure time to the electron beam that may cause degradation of beam-sensitive NPs (Klang *et al*., 2013).

TEM is the most exploited tool for characterizing nanomaterials as it provides images at higher spatial resolution than SEM, from the micrometer level up to the sub-nanometric or atomic level.

First, TEM can be used to characterize newly synthetized nanomaterials: observing dispersed NPs on formvar-coated grids it is possible to acquire information on the size, size distribution and shape of the nanoconstructs as well as on the interparticle interaction (aggregation or dispersion). When coupled with the appropriate analytical techniques through the electron interaction with the sample, TEM can also supply data on the chemical composition of nanomaterials (Kettiger *et al*., 2013).

In thin sections of resin-embedded samples the internal NP morphology may be investigated (Kola-Mustapha, 2019), while freeze-fracture techniques are suitable to elucidate the inner organization, crystallinity and granularity of NPs (in fact, the sample is vitrified by rapid freezing and then fractured, thus adequately preserving the native state) (Kuntsche *et al*., 2011). This latter technique is also appropriate to observe the physico-chemical modifications of the nanovectors upon drug incorporation.

However, the most significant application of TEM in the field of nanobiology is for investigating the behavior of NPs while interacting with cells. By SEM, the NP interaction with the plasma membrane can be observed, but the cellular uptake and the intracellular dynamics of the nanoconstructs may be more accurately described using TEM on thin sections, by taking static snapshots of the events occurring within a tissue or a cell at different times upon NP administration (Figure 2).

These investigations at the cellular level are crucial, once a new synthetized nanomaterial is characterized physico-chemically, in order to understand its effects and possible toxicity (Klang *et al*., 2013).

Indeed, TEM provides images *in situ* of the biological mechanisms responsible for the internalization and eventual interaction of NPs with specific organelles.

The chemical nature of the nanosystems is responsible for their uptake as single particles or multi-particulate aggregates; furthermore, in the literature detailed ultrastructural reports were published demonstrating how NPs of different composition enter the cells, either associated with intracellular vesicles following the endocytic or phagocytic process or occurring free in the cytosol (Park *et al*., 2006; Grant and Donaldson, 2009; Martens *et al*., 2014; Venkatachalam *et al*., 2015; Costanzo *et al*., 2016; Wong *et al*., 2017; Guglielmi *et al*., 2019). When endocytosed, NPs generally follow the lysosomal pathway, while when free in the cytosol, they may interact with (and eventually penetrate) organelles such as the mitochondria or the nucleus. In the former case, NP degradation by the lysosomal enzymes is likely to occur, although ultrastructural evidence has sometimes been provided for the mechanism of endosomal escape (Hillaireau and Couvreur, 2009; Martens *et al*., 2014; Wong *et al*., 2017; Foroozandeh and Aziz, 2018; Guglielmi *et al*., 2019), by which a membrane-bounded NP may exit the organelle to be released in the cytosol. On the other hand, cytosolic NP may re-enter the endo-lysosomal pathway through autophagic processes (Costanzo *et al*., 2016).

Thus, depending on their chemical and physical properties NPs may interact with intracellular organelles and

undergo rupture and degradation by completely different and often peculiar ways that obviously affect the intracellular release (and the action) of the loaded drugs (Malatesta, 2016). In addition, it is worth reminding that the same nanoconstruct may differently behave (as for the uptake and degradation) in cells of different origin.

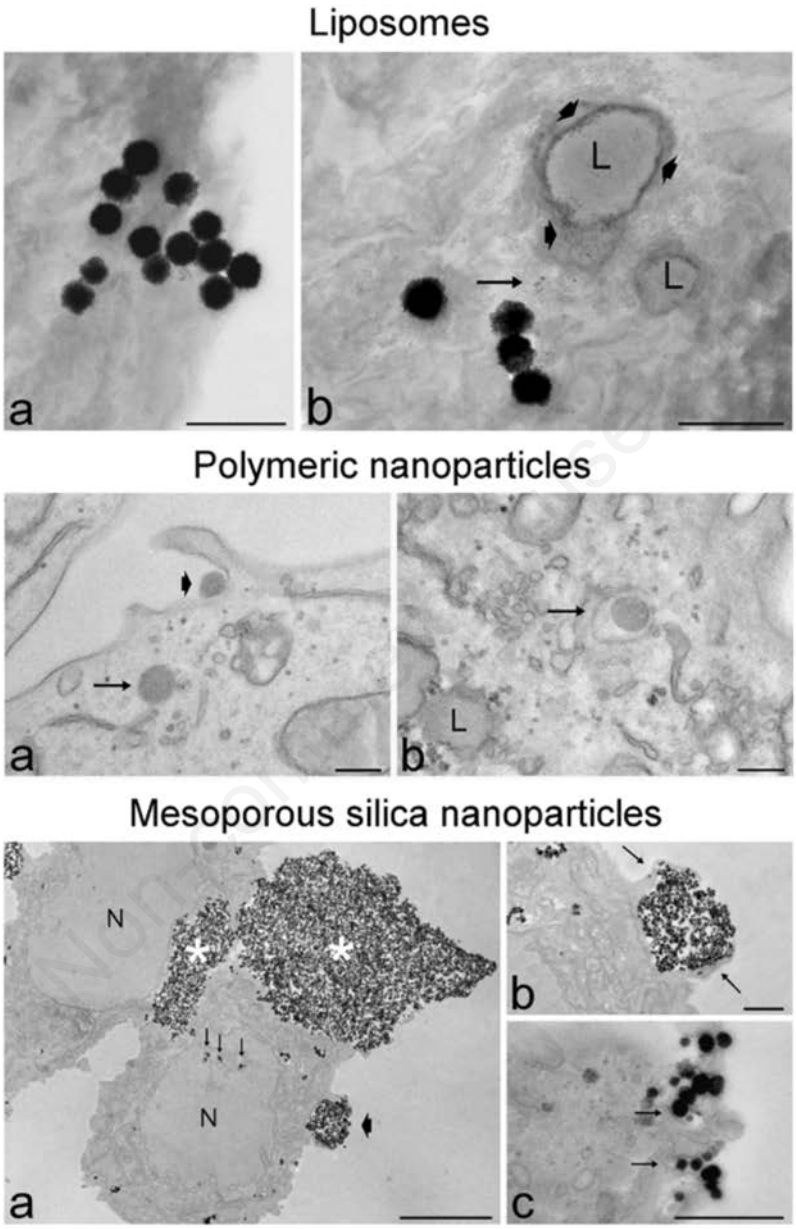

Figure 2. Interactions between different NPs and cultured cells analysed at TEM. Liposomes: a) Several liposomes enter the cell apparently without endocytotic process and occur free in the cytoplasm; note their loose filamentous periphery. b) Electron dense fine granular material (arrow) occur in the cytosol in close proximity to liposomes and lipid droplets (L); scale bars: 500 nm. Polymeric nanoparticles: a) Two NPs occurs at the cell periphery: one adhering to the cell surface (arrowhead), the other freely distributed in the cytosol (arrow). b) A NP is enclosed in an endosome (arrow). Scale bars: 200 nm. Mesoporous silica nanoparticles: a) Large aggregates of NPs occur at the cell surface (asterisks); small clusters of NPs are visible inside the cytoplasm, even inside nuclear invaginations (arrows); the arrowhead indicates the detail showed in b. b) A NP cluster is internalised *via* phagocytosis by the extrusion of pseudopodia (arrows). c) Small clusters of NPs enter the cells by endocytosis (arrows). Scale bars: a) 5 µm; b,c) 500 nm. Adapted from Costanzo *et al.*, Eur J Histochem 2016;60:2640.

Concluding remarks

Nanoconstructs for biomedical applications are synthesized starting from biocompatible compounds and tested to confirm their low cytotoxicity. This is obviously correct but the ever growing experimental evidence demonstrates that to validate a nanovector of whatever nature as an efficient drug-delivery system it is necessary to exhaustively know how it behaves once inside the target cell.

To track NP presence, dynamic relocation and ultimate disposal, imaging techniques are needed: among them, electron microscopies are undoubtedly the most appropriate and versatile tools.

They surely have important drawbacks: the procedures for sample preparation are often complicated and time-consuming, the application of histochemical methods to chemically characterize the specimens are much trickier than in light microscopy, and the need to operate in the vacuum makes the artefactual dehydration and coating/embedding mandatory. However, despite these limits, SEM and especially TEM will remains unreplaceable for nanomedical research also in the years to come.

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