Microscopy techniques in nanomedical research

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Summary
In recent years, the application of nanotechnology to biomedicine has been exponentially increasing. The physical and chemical properties, quality and safety of nanomaterials designed for biomedical application need to be accurately evaluated by means of reliable and robust techniques. Among the methods used, microscopy techniques play a primary role. This paper presents a brief overview of the contribution of different microscopy techniques to the study of the structural and functional aspects of nanoconstructs and their relationships with the biological milieu, demonstrating the great impact that microscopy sciences have in nanomedical research and applications.

Key words: electron microscopy, fluorescence microscopy, nanomedicine, nanoparticles.

Introduction

In recent years, the application of nanotechnology to biomedicine for the development of e.g. new drug delivery systems, diagnostic tools, sorting systems, scaffold components (Lim *et al.*, 2010; Bobo *et al.*, 2016; Fernandes *et al.*, 2016; Soica *et al.*, 2016) has been exponentially increasing. Understanding the structure of nanocomposites is crucial to elucidate their physical and chemical properties, quality and safety, as well as their distribution and behavior *in vivo*. All these features, in fact, strongly affect the efficiency of nanoconstructs in the living organism, from the molecular to the systemic level. It is therefore essential to perform accurate studies by means of reliable and robust techniques. Among the methods used for evaluating the structural and functional aspects of nanoconstructs and their relationships with the biological milieu, microscopy techniques play a primary role. This paper presents a brief overview of the contribution of different microscopy techniques to the development of nanomedicine.

Microscopy to characterize nanoconstructs for biomedical application

A wide variety of analytical methods have been used for evaluating the physico-chemical characteristics of manufactured nanomaterials (for a review, see Lin *et al.*, 2014): these include chromatography, electrophoresis, magnetic resonance, X-ray scattering and spectroscopy, mass spectrometry, circular dichroism spectroscopy, zeta-potential measurements, as well as techniques of microscopy on which the present article will especially be focused.

In fact, transmission electron microscopy (TEM) is one of the most efficient tools for the characterization of nanomaterials. TEM provides high resolution of minute structural details, which is essential, for instance, to obtain information about the crystalline structure and granularity of the nanoparticles (Williams and Carter, 2009). Through TEM it is also possible to detect alterations in nanoparticle morphology due to the incorporation of drugs at different concentrations, thus representing an indispensable technique for the development of drug delivery systems (Govender *et al.*, 2000). To be suitable for observation at TEM, nanomaterials usually need to be dehydrated, but it is also possible to freeze them (cryo-TEM), thus better preserving their original morphology (Williams and Carter, 2009). Although TEM provide 2D images, the technique of electron tomography can be used to create 3D images using a sequence of micrographs taken at different tilts (Williams and Carter, 2009).

Scanning electron microscopy (SEM) uses electrons for high resolution imaging of the sample surface (Reimer, 2000), and represents a valid tool to investigate some nanomaterials (Bogner *et al.*, 2005). The topography of the nanostructured samples can be preserved using special techniques that avoid any manipulation (environmental or wet SEM) or pre-

serve their morphology by rapid freezing (cryo-SEM). The environmental SEM, allowing analyses on hydrated materials without fixing, drying, freezing or coating the specimen (Bogner *et al.*, 2005), is especially suitable to characterize microspheres and microcapsules (Xiong *et al.* 2012). Cryo-SEM method has been applied for the characterization of microspheres (Allan-Wojtas *et al.*, 2008) and nanoemulsions (Hoesli *et al.*, 2012).

Polarized light microscopy (PLM) may be used for the preliminary identification of many liquid-crystalline structures (Gaisin *et al.*, 2010). The anisotropic systems cause a deviation in the plane of polarized light and show typical black and white or colored textures. Based on this texture, liquid-crystalline structures can be classified in: (i) lamellar liquid crystalline phase which reveals oily streaks with inserted "maltese crosses" in the micrograph; (ii) hexagonal liquid-crystalline structure which is indicated by a fanlike texture (Müller-Goymann, 2004; Carvalho *et al.*, 2010; Rissi *et al.*, 2014). However, PLM can be applied to particles whose size approaches the wavelength of visible light (400 to 700 nm); for liquid crystal particles presenting smaller dimensions, TEM is necessary to resolve them (Müller-Goymann, 2004).

Atomic force microscopy (AFM) is one of the most popular scanning probe microscopy methods (Binning *et al.*, 1986) and the interaction of nanoparticles with the AFM probe has been extensively studied from different experimental points of view (AFM tip modification, nanoparticle manipulation, substrate influence) (Theil Hansen *et al.* 1998; Lee *et al.*, 1998; Klapetek *et al.*, 2011; Henry, 2005). AFM allows detection and imaging of nanoparticles from 0.5 nm in diameter and, although it has been mostly applied to inorganic nanoconstructs, it is also suitable to characterize hydrated nanomaterials.

Microscopy for visualizing nanoconstructs in living organisms

To be used in nanobiology and nanomedicine, nanoconstructs need to be tested in living organisms. Cells cultured *in vitro*, which ensures simple and controlled conditions, represent the experimental model of choice. The preliminary utilization of *in vitro* systems also allows short experimental times and reduction of the number of laboratory animals for the following *in vivo* studies, thus implying a significant decrease of the research costs.

Light microscopy has been largely applied for the safety assessment of nanomaterials and for designing efficient administration strategies for biomedical use. Microscopy techniques proved to be useful to study the interaction of nanoparticles with the cells and to visualize their intracellular fate, while allowing to simultaneously evaluate signs of cell damage or death (for a review, see Ostrowski *et al.*, 2015).

By definition, nanoparticle are less than 100 nm in size and cannot be resolved as single entities even at the highest magnification in conventional light microscopy. Thus, only nanoparticulates that form clusters or aggregates of more than 200 nm in size can directly be visualized in single cells or tissues. Depending on their chemical composition, some nanoconstructs (e.g., carbon nanotubes, iron oxide or titanium dioxide nanoparticles) can be directly observed as naturally colored deposits (Porter *et al.*, 2010; van Landeghem *et al.*, 2009; Adachi *et al.*, 2010). Enhanced darkfield microscopy has also been used to detect metal oxide nanoparticles in histological samples (Roth *et al.*, 2015). In addition, some histochemical techniques are suitable to stain either inorganic or organic nanoparticles; Prussian blue may be used to stain iron containing nanoconstructs (Bumb *et al.*, 2011 and Figure 1a) while nanoparticles con-

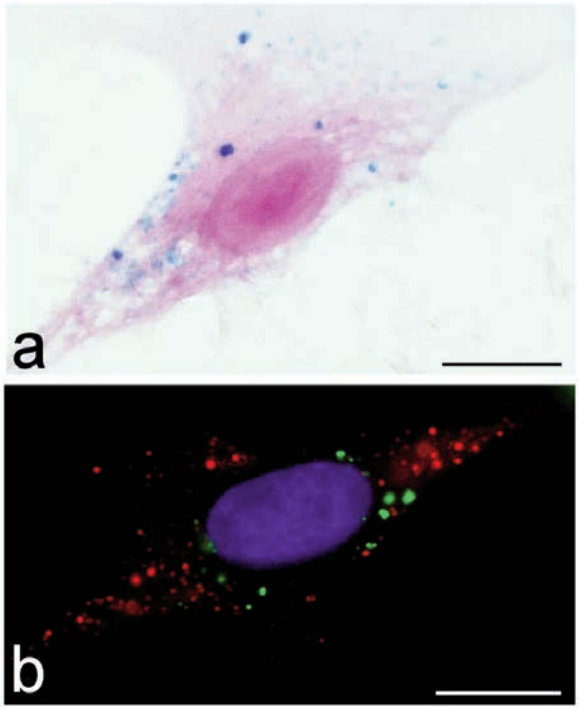

Figure 1. a) Brightfield microscopy. Iron oxide nanoparticles (Prussian blue staining) inside a murine fibroblast (hematoxylin countestaining). b) Fluorescence microscopy. Chitosan nanoparticles (green) inside a human epithelial cell; lysosomes (red) are visualized by specific immunostaining; DNA (blue) is stained with Hoechst 33258. Bars: 20 µm.

taining polysaccharide with negatively charged sulfate groups have been successfully visualized by Alcian blue staining (Holzhausen *et al.*, 2013).

No doubt, fluorescence microscopy is the most widely used approach to investigate the biodistribution and the intracellular localization of nanoconstructs at light microscopy. To this purpose, nanoparticles are usually labelled with fluorochromes (Figure 1b), which must be selected for their structure, molecular weight and charge not to alter the physicochemical characteristics of nanoconstructs.

The interaction of fluorochrome-conjugated nanoparticles with specific cells or intracellular organelles may be visualized by the simultaneous immunofluorescence labelling of marker proteins (Cho *et al.*, 2009; Malatesta *et al.*, 2015 and Figure 1b). A more precise spatial localization of nanoparticles in their interactions with cells may be obtained by confocal laser scanning microscopy: by this technique, serial optical sectioning of the sample are obtained, which allows 3D reconstructions of single cells or tissues sections. However, confocal microscopy is diffraction-limited as much as conventional fluorescence microscopy, so that the X-Y resolution is restricted to about 200 nm, substantially larger than the <100 nm size of nanoparticles. Techniques of super-resolution light microscopy may overcome this limitation, allowing to significantly increase X-Y resolution up to about 30 nm (Willig *et al.*, 2006; Sonnefraud *et al.*, 2014; Guggenheim *et al.*, 2016).

TEM, thanks to its higher resolution, is however the most appropriate approach to obtain detailed and unequivocal information on each step of nanoconstruct interactions with the cell components, from their uptake at the cell surface to their intracellular degradation (Figure 2). A clear analysis of nanoparticle internalization mechanism(s) can be

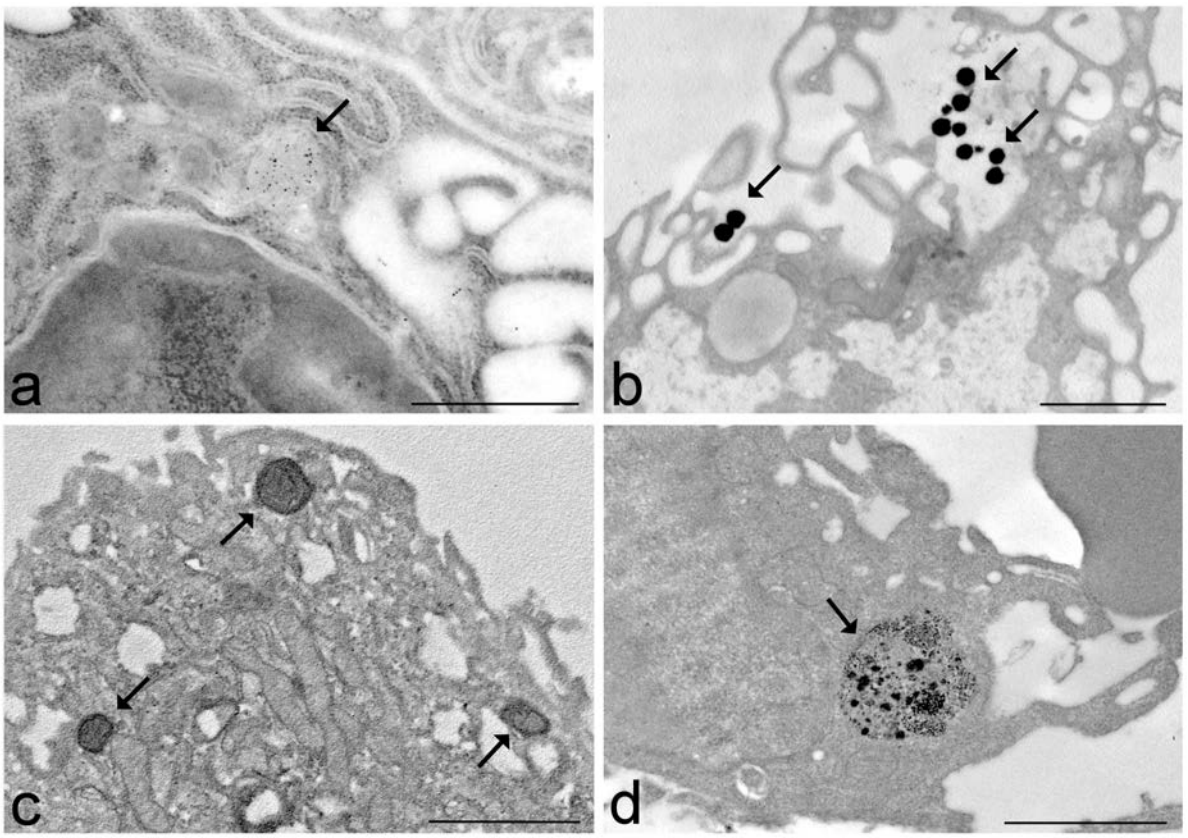

Figure 2. Transmission electron microscopy. a) Gold nanoparticles (arrow) internalized in a human macrophage. b) Lipid nanoparticles (arrows) entering a human epithelial cell. c) Polymeric nanoparticles (arrows) in a human myoblast. d) Quantum dots (arrow) inside a murine macrophage. Bars: a,c 200 nm; b,d 1000 nm.

obtained, visualizing the contact with the plasma membrane and the passage into the cell by endocytosis, phagocytosis or membrane fusion (as in the case of nanoconstructs of lipid nature) (e.g., Zhang *et al.*, 2011; Malatesta *et al.*, 2012; Costanzo *et al.*, 2016a,b; Boyles *et al.*, 2015; Poussard *et al.*, 2015; Lopes *et al.*, 2016; Messerschmidt *et al.*, 2016; Zielinska *et al.*, 2016). The distribution of the nanoparticulates in the cellular compartments provides information on their fate: the entrapment into endosomes or phagosomes prefigures their rapid degradation in the lysosomal compartment, while their free (organelle-unbound) occurrence in the cytosol indicates their ability to escape endosomes and, consequently, the enzymatic lysis (Panyam *et al.*, 2002; Varkouhi *et al.* 2011). However, TEM observations revealed that these free nanoparticles may re-enter the lytic pathway by autophagosomal processes (Costanzo *et al.*, 2016a,b). Importantly, TEM allows to distinguish the presence of intact nanoparticles or their remnants after enzymatic lysis, thus providing unequivocal information on their biodegradability. TEM can also provide clear evidence for the distribution of nanoparticulates inside the cell nucleus: some nanoparticles may, in fact, enter the nucleus by passing through the nuclear pores or being entrapped therein at the end of mitosis (Nabiev *et al.*, 2007; Colonna *et al.*, 2011; Guan *et al.*, 2012; Malatesta *et al.*, 2013, 2015; Zhang *et al.*, 2015). This is a crucial information for evaluating the safety of nanoconstructs, since the persistence of exogenous materials in close proximity of nucleic acids may have unpredictable consequences on whole cell activity.

An important contribution to nanomedical research has been given also by correlative microscopy. Light (especially fluorescence) microscopy was combined with advanced TEM methods (conventional, immuno and energy-filtered electron microscopy, and electron tomography) to analyze the biodistribution of different types of nanoparticles (Mühlfeld *et al.*, 2007). Quantum dots were identified in *in vitro* and *ex vivo* samples by combining fluorescence microscopy, TEM and scanning transmission electron microscopy (STEM) (Dukes *et al.*, 2010; Killingsworth and Bobryshev, 2016), and the combination of TEM and Serial Block Face SEM allowed to quantify their intracellular uptake (Hondow *et al.*, 2016). The intracellular distribution of gold nanoparticles was investigated by using interferometric photo-activated localization microscopy and electron microscopy (Shtengel *et al.*, 2014), while their identification inside tumor masses was performed by combining optical microscopy and SEM (Kempen *et al.*, 2015). The uptake and intracellular fate of ZnO-based nanoparticles were analyzed combining dynamic confocal imaging, low resolution bright field TEM and dark field STEM (Othman *et al.*, 2016). Cryo-soft X-ray tomography was used to obtain three-dimensional information on the interaction of super-paramagnetic iron oxide nanoparticles with cancer cells (Chiappi *et al.*, 2016). Fluorescence microscopy and SEM were combined to investigate macrophage uptake of cylindrical nanoparticles (Tscheka *et al.*, 2015).

Concluding remarks

In the last 15 years, more than 190,000 articles have been published in qualified journals on nanoparticles, (source: Scopus database, https://www.scopus.com), and in about 57,000 papers of these, microscopy techniques were used among the experimental methods. This clearly indicates the great impact that microscopy sciences have in nanomedical research and applications. It is easy to foresee that this will even increase in the years to come, thanks to the continuous progress in microscopy technology and instrumentation. TEM still is the most informative approach for investigating the interaction of nanoconstructs with cells and intracellular organelles, but super-resolution light microscopy may be envisaged as the future in the field: multicolor histochemical techniques will allow to simultaneously detect the interactions of nanoparticles with several subcellular components at the nanodimension of super-resolved fluorescence microscopy.

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