Microscopy techniques in nanomedical research

Manuela Costanzo, Flavia Carton, Manuela Malatesta

Department of Neurosciences, Biomedicine and Movement Sciences, Section of Anatomy and Histology, University of Verona, Italy

Corresponding author: Manuela Malatesta
Department of Neurosciences, Biomedicine and Movement Sciences, Section of Anatomy and Histology, University of Verona
Strada Le Grazie 8, I-37134 Verona, Italy
Tel. +39.045.8027569
E-mail: manuela.malatesta@univr.it

Introduction

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.

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.

Microscopy techniques in nanomedical research

Manuela Costanzo, Flavia Carton, Manuela Malatesta

Department of Neurosciences, Biomedicine and Movement Sciences, Section of Anatomy and Histology, University of Verona, Italy

Corresponding author: Manuela Malatesta
Department of Neurosciences, Biomedicine and Movement Sciences, Section of Anatomy and Histology, University of Verona
Strada Le Grazie 8, I-37134 Verona, Italy
Tel. +39.045.8027569
E-mail: manuela.malatesta@univr.it

Introduction

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.

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.
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-
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 nanoparticles 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 nanoparticles 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 (Tscheke 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.

**Acknowledgements**

We thank Prof. A. Sbarbati for kindly providing us with an image of iron oxide nanoparticles. F.C. is a PhD student in receipt of fellowship from the Doctoral Program in “Nanoscience and advanced technologies” of the University of Verona.
CONTRIBUTI SCIENTIFICI

References

Lopes VR, Lottito V, Audinot JN, Bayat N, Gutleb AC, Cristobal...


Müller-Goymann CC. Physicochemical characterization of colloidal drug delivery systems such as reverse micelles, vesicles, liquid crystals and nanoparticles for topical administration. Eur J Pharm Biopharm 2004;58:343-56.


Rissi NC, Guglielmi DAS, Corrêa MA, Chiavacci LA. Relationship between composition and organizational levels of nanostructured systems formed by Oleth 10 and PPG-5-Ceteth-20 for potential drug delivery. BJPS 2014;50:653-61.


