Imaging techniques in nanomedical research

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ABSTRACT

About twenty years ago, nanotechnology began to be applied to biomedical issues giving rise to the research field called nanomedicine. Thus, the study of the interactions between nanomaterials and the biological environment became of primary importance in order to design safe and effective nanoconstructs suitable for diagnostic and/or therapeutic purposes. Consequently, imaging techniques have increasingly been used in the production, characterisation and preclinical/clinical application of nanomedical tools. This work aims at making an overview of the microscopy and imaging techniques *in vivo* and *in vitro* in their application to nanomedical investigation, and to stress their contribution to this developing research field.

Key words: Electron microscopy; histochemistry; light microscopy; magnetic resonance imaging; nanoparticles; optical imaging.

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Introduction

Since the Nineties of the last Century, nanotechnology began to be applied to biomedical issues, giving rise to a new research field called nanomedicine.[1] Until that time, the development of nanomaterials had been almost exclusively the prerogative of chemists and physicists who obviously mostly used physico-chemical methods to characterise new nanoproducts. With the advent of nanomedicine, the interactions between nanomaterials and the biological environment -from the single cell to the whole organism- became of paramount importance in order to set up safe and effective nanoconstructs suitable for diagnostic and/or therapeutic purposes. Consequently, imaging techniques have been increasingly used in the production, characterisation and preclinical/clinical application of nanomedical tools,[2-5] taking advantage from the great improvement and evolution imaging techniques have experienced in biomedical research and clinical applications, especially in the years 2000.

In vivo imaging techniques (*e.g.*, magnetic resonance imaging, optical imaging) have been applied in studies aimed at investigating the targeting, biodistribution and clearance of the nanoconstructs in the whole organism with a view in the short, medium and long time. Light and electron microscopy have mostly been used to evaluate the impact of new nanoparticles (NPs) in single cells, in order to understand the internalisation efficacy and mechanisms, intracellular fate and relationships with cell organelles; moreover, microscopy proved to be useful to track NPs in tissues and organs. Both *in vitro* and *in vivo* studies have been combined in many researches (*e.g.*,[6-14]) and proved to be crucial to characterise novel nanocarriers and design proficient and safe strategies for their use in nanomedicine.

The present paper aims at browsing the scientific literature of the last decade to get an overview of the microscopy and imaging techniques in their application to nanomedical investigation, and to evaluate their contribution to this recent and developing research field.

It is worth noting that the number of articles in qualified journals on the application of nanoconstructs in biology and medicine has dramatically increased since 2000, but the percentage of papers where imaging and microscopy techniques were used has constantly ranged between 20-25% (Figure 1).

Imaging techniques applied to *in vivo* models

In the last decades, *in vivo* imaging devices have become fundamental tools in basic sciences, in preclinical research and in modern drug development to visualize nanocomposites. The most suitable and commonly used techniques are magnetic resonance imaging (MRI), optical imaging (OI), positron emission tomography (PET),[15] computed tomography and ultrasonography,[16] and a number of recent articles focused on the visualization of nanoconstructs by these approaches in a biological environment.

Madru *et al.*[17] proposed new hybrid superparamagnetic iron oxide NPs labelled with ^{64}Cu for PET/MRI *in vivo* imaging, to be detected and located in sentinel lymph nodes where the presence of metastases is an important marker for cancer staging and treatment: through a biodistribution study, the authors demonstrated the stability of radiolabelling up to 24 h and NPs accumulation in the sentinel lymph nodes.

Magnetic NPs with an iron core have been used in MRI for more than twenty years as contrast agents with a particular affinity toward specific organs and tissues,[18] and more recently they have also been applied as effective agents in hyperthermic therapy mainly in tumour pathology.[19-22] Quantum dots are both fluorescent and magnetic NPs, thus being suitable tools for protocols of both OI and MRI *in vivo*. Through OI acquisitions, very small quantity of quantum dots can be detected and located, while MRI allows extrapolating a detailed morphological information of the anatomical sites where they accumulate.[13] New-generation quantum dots have specifically been used for fluorescent imaging in the field of drug discovery[23,24] and in the functional imaging (*e.g.*, detailed three-dimensional quantitative flow maps of the brain vasculature were obtained using these quantum dots[25]).

Fluorescent imaging techniques proved to be also suitable for studying *in vivo* solid lipid NPs that are very advantageous nanoconstructs for their biocompatibility and low toxicity,[12,14] and can be used as nanocarriers being easily targeted and able to cross the blood brain barrier.[26]

Nanoscale highly echogenic agents for imaging and ultrasound-mediated drug delivery were developed by Perera *et al.*[27] who demonstrated by *in vivo* ultrasound analysis and fluorescence-mediated tomography that these innovative NPs exhibit greater tumour extravasation and accumulation than classical microbubbles, thus having great potential for diagnostics and drug delivery.

More than one imaging technique has often been simultaneously used in multimodal imaging protocols *in vivo*.[13,28-31] In this approach, different techniques are selected according to the chemical and physical characteristics of the nanocompounds under study. For instance, radiolabelled molecules such as ^{18}F-flurodeoxyglucose (^{18}F-FDG) may be used as bimodal tracers for PET and for OI based on the Cerenkov radiation emission: this was demonstrated by Boschi and colleagues[32] in an experimental mouse model of mammary carcinoma, where similar images of the ^{18}F-FDG biodistribution (indicative of the glucose metabolism) in different organs were obtained using PET scanner and Cerenkov OI.

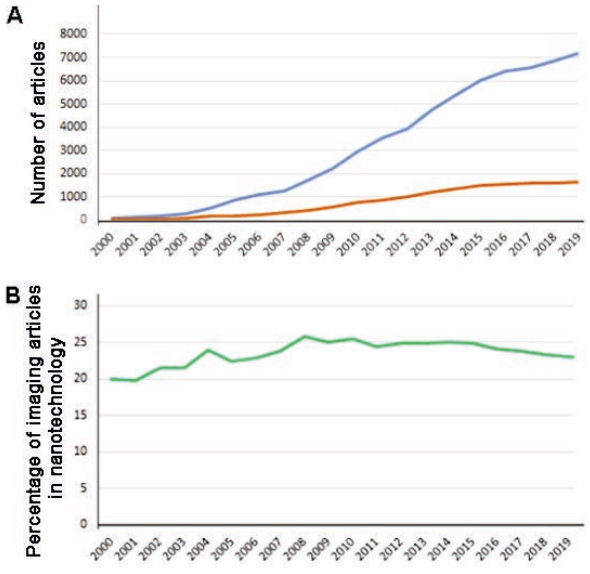

Figure 1. A) Total number of published articles on nanotechnology applied to the biomedical field (blue line) and articles on nanotechnology where imaging or microscopy techniques were used (red line), in the years 2000-2019; B) percentage of articles where imaging techniques were used. Data were taken from the Web of Science database.

The interesting study by Sulheim and colleagues is another example of multimodal imaging:[33] the authors investigated the organization and density of functional blood vessels that newly developed in a tumour tissue by intense angiogenesis. Actually, angiogenesis is crucial to understand how tumour heterogeneity affects the uptake and accumulation of therapeutic agents (among which NPs in nanomedical therapy). In this investigation, three different *in vivo* imaging techniques were used (*i.e.*, micro-computed tomography, contrast-enhanced ultrasound, and diffusion-weighted and dynamic contrast-enhanced MRI) and the authors demonstrated that NP accumulation depends on the extent of the tumour vasculature as wells as on the morphology and perfusion of the vessels.

The *in vivo* imaging techniques are also powerful and irreplaceable tools for tracking and monitoring the so-called theranostic NPs, *i.e.*, the multifunctional nanosystems where the diagnostic and therapeutic capabilities are combined into one single biocompatible nanoconstruct.[34]

Qiu and coauthors[35] developed multifunctional theranostic NPs based on gold nanocages (AuNCs) modified with hyaluronic acid and functionalized with anti-Glypican-1 antibody, oridonin, gadolinium, and Cy7 dye for accurate diagnosis and effective treatment of pancreatic cancer. With an imaging longitudinal study *in vivo*, the authors monitored the biodistribution of the nanoconstructs and, at the same time, the evolution of a nano-mediated therapy as well as the morphological and functional modifications of the diseased tissues and organs. Kwak and colleagues[36] studied a specific dodecapeptide probe as a promising candidate for both colon tumour diagnosis and targeted drug delivery: fluorescently labelled NPs loaded with this peptide conjugated to a photosensitizer showed a significantly enhanced cellular uptake and high photodynamic effect to kill tumour cells in tumour-bearing mice.

Gawne and collegues[37] set up the encapsulation of glucocorticoids into long-circulating liposomes to reduce the side effects of glucocorticoids and improve the treatment of inflammatory diseases; these nanoconstructs were radiolabelled thus allowing the characterization and tracking in an *in vivo* model using PET imaging for a theranostic approach.

Imaging techniques applied to *in vitro* models

The most common imaging technique applied to detect NPs inside cultured cells and tissues is fluorescence microscopy (FM). In particular, confocal FM (CFM) has widely been used in parallel with physico-chemical analyses, to demonstrate the efficacy of novel nanoconstructs in cell targeting and drug delivery, frequently using established cancer cell lines.

The capability of polyamidoamine dendrimers,[38] or nanosized polyethylenimine complexes[39] to deliver antisense oligonucleotides as well as of polyethylenimine-hexametaphosphate NPs to carry nucleic-acid-based therapeutics[40] to tumour cells was evaluated by CFM. The same technique was also used to test the uptake efficacy of solid lipid NPs aimed at HIV prevention,[41] silica NPs for tumour targeting,[42,43] or avidin-conjugated calcium phosphate NPs[10] and AuNCs for harnessing imaging and hyperthermia therapy of cancer.[44] CFM gave information also on the functionalization efficacy in increasing quantum dots uptake by cancer cells.[45] The internalization mechanisms of gold nanoclusters, intended as fluorescent nanoprobes for bio-imaging and related applications in cancer treatment, were investigated by CFM in cell culture models of tumour and non-tumour cells.[46] CFM allowed testing the efficacy of paclitaxel-loaded expansile NPs in a mesothelioma spheroid model,[47] the uptake and distribution of nanodiamonds in different cell lines and organ slices,[48] and the ability of Pullulan acetate NPs to pass the placental barrier in *in vitro* cell monolayers.[49]

Furthermore, CFM was frequently used in combination with other imaging/microscopy techniques.

CFM and flow cytometry have been associated to investigate the mechanism of dendrimers uptake,[50] as well as the internalization efficacy of zein/carboxymethyl chitosan NPs as delivery vehicles for drugs or nutrients.[51] The same approach was used to test PEGylated NPs[52] and cyclodextrin-based NPs[6] for enhanced tumour cell internalization and cytotoxicity, or gold nanoclusters for fluorescence imaging and enhanced drug transport,[53] or poly(lactide-co-glycolide) NPs for protein delivery to macrophages.[54] Combination of CFM and flow cytometry also allowed understanding the effect of functionalization on the uptake of dense-silica NPs by gastric cancer cells,[8] or the influence of anaesthetics on the internalization efficacy of dendrimers by microglial cells.[55]

The uptake efficacy of poly (lactic-co-glycolic acid)-poly(ethylene-glycol)-folate NPs was studied in cancer cell culture combining CFM, flow cytometry and MRI,[9] while superparamagnetic iron oxide NPs were visualised inside the cells with CFM and MRI.[56]

The endocytosis pathways, intracellular fate and release of polystyrene NPs[57] and multifunctional NP-EpCAM aptamer bioconjugates[58] were investigated by combining CFM and spectrofluorometric/spectrophotometric analyses, and the internalisation of carboxyl-coated quantum dots was studied by CFM and steady-state fluorescence spectroscopy.[59] By using CFM in combination with traction force microscopy, the capacity of cultured cells of internalising NPs was related to the mechanical stress.[60]

To better elucidate the internalisation mechanisms and intracellular pathway of NPs and their impact on cell organelles, a higher resolution is needed than the one of light microscopy. Consequently, many studies on NPs were performed by transmission electron microscopy (TEM) or scanning transmission electron microscopy (STEM). TEM was used to investigate the uptake by a human cell line of gold NPs prepared in aqueous biocompatible solution,[61] while the internalization of chitosan-functionalized gold NPs was assessed by combining TEM and electron energy loss spectroscopy.[62] TEM and inductively coupled plasma mass spectrometry were employed to visualize and quantify the internalization and distribution of gold NPs for drug delivery and imaging diagnostics in isolated endothelial cells and whole vessels.[63] STEM and electron tomography were crucial to demonstrate that the particle size affects the ability of functionalized platinum NPs to escape the endo-lysosomal pathway.[64]

High-resolving electron microscopies (TEM and SEM) have increasingly been associated with other imaging/microscopy techniques in nanomedical research to analyse the intracellular fate of NPs.

FM, CFM and TEM were used to investigate the tumour cell uptake of different biocompatible NPs,[65-67] drug-gold NP conjugates incorporated into liposomes,[68] phospholipidic manganese-based NPs,[69] nickel nanowires,[70] and magnetic NPs.[71] CFM and TEM were also combined to investigate the lipolytic potential of superparamagnetic iron oxide hyperthermic NPs,[72] the capability of cycling and non-cycling muscle cells to internalise different biocompatible NPs,[73,74] and the ability of drug-loaded gold NPs to target macrophages and fibroblasts to treat lung fibrosis.[75] The influence of a static magnetic field on the delivery of magnetic NPs was investigated by combining CFM, TEM and SEM.[76] The uptake efficiency of surface-active maghemite NPs was assesses in mesenchymal stromal cell by using bright-filed microscopy, flow cytometry, SEM and atomic force microscopy (AFM).[77] Sphero magnetic NPs were detected in a multicellular neural model by using time-lapse phase contrast microscopy, CFM, TEM and SEM.[78] The intracellular fate of

superparamagnetic NPs intended for nanothermal ablation and MRI contrasting was investigated by phase contrast, CFM and TEM.[79] Bright-field microscopy, FM and TEM allowed describing the cell and tissue distribution of solid lipid NPs.[14]

The study of the NP uptake and intracellular fate in cultured cells has sometimes been performed by specially designed imaging techniques. As an example, penetration of gold nanoshells into 3D cell culture was evaluated using hyperspectral imaging with dark field microscopy.[80] Moreover, radiolabelled superparamagnetic NPs intended as a new contrast agent for multimodal imaging were detected in mouse mesenchymal stem cells by *in vitro* PET/MRI.[11]

The detection of NPs in the cell or tissue milieu may be sometimes problematic, especially when the nanoconstructs are made of organic material. To overcome this limit, specific histochemical techniques proved to be suitable. The iron-specific Prussian blue staining has been the method of choice to visualize superparamagnetic iron oxide NPs at bright-field microscopy,[81] sometimes in association with MRI,[39,82] CFM,[83] TEM,[84] or SEM and AFM.[77] Gold NPs were detected and quantified at bright-field microscopy by silver-enhancement staining.[80] Diaminobenzidine photo-oxidation was appropriate to correlate FM and TEM with the aim of precisely tracking the NPs intracellular fate.[65,66,69,85] Recently, the Alcian blue staining has been used to detect hyaluronic-acid based NPs[86] at both bright-field microscopy and TEM.[87] Immunocytochemistry allowed detecting chitosan NPs loaded with a synthetic opioid at both FM and TEM,[88] and this technique may simultaneously be performed with photo-oxidation.[89]

Conclusions

The great development of scientific research in nanomedicine resulted, especially in the last decade, in the extensive use of several imaging techniques to visualise the nanoconstructs in cells, tissues, organs or the whole organism. *In vivo* imaging techniques have the big advantage to make longitudinal studies possible and to allow monitoring the administration, biodistribution, accumulation and clearances of different kinds of nanocompounds. They have, however, different sensitivity and resolution: for example, MRI gives high resolution (in the order of μm), anatomical information, and good soft-tissue contrast but has low sensitivity (in the order of mM) compared to the nuclear-medicine imaging techniques (PET), that are highly sensitive (in the range of pM) and quantitative, but suffer from a poor resolution (in the order of mm). In the attempt to finely describe the interaction of the nanoconstructs with the cells' compartments, from the plasma membrane to the cytoplasmic organelles, the nucleus and the sub-nuclear domains, microscopy techniques and histochemistry proved to be crucial. In fact, the wide use of techniques at light and electron microscopy inspired a sort of *Renaissance* for many long-established morphological methods that, in the "omics era", had long been seen as merely descriptive.[2,90,91] When applied to nanotechnology, these methods proved to be essential not only to understand the spatial relationships between the nanoconstructs and the biological environment, but also provided functional information, and were central to design efficient diagnostic or therapeutic strategies. In turn, the application of imaging techniques to the nanotechnological issues has led to adapt standard methods to special purposes and to originally develop new technical tools.[92-94]

Undeniably, imaging techniques have significantly contributed to the development of nanotechnology in the biomedical field, thanks to the integration of apparently distant methodological approaches that enabled to get a comprehensive anatomical, histological and functional picture of the complex interactions the nanoconstructs exert with the living systems.

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