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Assessing the interactions between nanoparticles and biological barriers *in vitro*: A new challenge for microscopy techniques in nanomedicine

Flavia Carton,[1] Manuela Malatesta[2]

[1]Department of Health Sciences, University of Piemonte Orientale "A. Avogadro", Novara
[2]Department of Neurosciences, Biomedicine and Movement Sciences, Anatomy and Histology Section, University of Verona, Italy

ABSTRACT

Nanoconstructs intended to be used as biomedical tool must be assessed for their capability to cross biological barriers. However, studying *in vivo* the permeability of biological barriers to nanoparticles is quite difficult due to the many structural and functional factors involved. Therefore, the *in vitro* modeling of biological barriers - 2D cell monocultures, 2D/3D cell co-cultures, microfluidic devices- is gaining more and more relevance in nanomedical research. Microscopy techniques play a crucial role in these studies, as they allow both visualizing nanoparticles inside the biological barrier and evaluating their impact on the barrier components. This paper provides an overview of the various microscopical approaches used to investigate nanoparticle translocation through *in vitro* biological barrier models. The high number of scientific articles reported highlights the great contribution of the morphological and histochemical approach to the knowledge of the dynamic interactions between nanoconstructs and the living environment.

Key words: nanoconstructs; cell culture; organoid, microfluidics; bioreactor; fluorescence microscopy; electron microscopy.

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

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Introduction

One of the main issues in the development of nanoconstructs intended for biomedical purposes is the assessment of their capability to cross biological barriers. In fact, to reach their target and play therapeutic or diagnostic functions, nanoparticles (NPs) must face various barriers, not only the plasmalemma that surrounds any cells but first the more complex histological structures , which regulate the molecular traffic in specific organs or anatomical regions of the body: the skin, the endothelial, intestinal, lung or placental barriers, the blood brain barrier (BBB), or the tumor microenvironment barrier.

Studying the permeability of biological barriers to NPs is a quite difficult task to perform *in vivo*, due to the numerous structural and functional factors influencing the process. For this reason, *in vitro* modeling of biological barriers -from the simple 2D cell monocultures to 2D and 3D cell co-cultures, to the high technologic microfluidic devices- is becoming more and more popular in nanomedical research (recent review in[1]). By using these *in vitro* models, it is possible to track the NPs during their passage across the barrier, to elucidate the mechanisms involved in their dynamic interactions, and to identify the NPs' physico-chemical features necessary to guarantee their translocation.

Microscopy techniques are crucial in these studies, as they allow both visualizing the NPs through the barrier and evaluating their impact on the barrier components. Depending on the microscopy technique used (bright-field or phase contrast microscopy, fluorescence microscopy or transmission electron microscopy), NPs can be made detectable by either linking/loading appropriate dyes during the synthetic process or by labelling with specific histochemical staining after their administration.[2-16] Moreover, some nanoconstructs such as quantum dots or nanodiamonds, are characterized by an intrinsic photoluminescence that can be easily detected at fluorescence microscopy in the absence of an additional staining.[17,18]

This paper aims at providing an overview of the various microscopical approaches used to investigate *in vitro* the translocation of NPs through biological barriers, thus highlighting the great contribution of microscopy techniques to the knowledge of the interactions between the living environment and the nanoconstructs.

Tracking nanoparticles across biological barriers

Fluorescence microscopy is the most widely used technique to analyse NPs translocation through biological barrier models. Among the different fluorescent-related microscopy techniques, laser scanning confocal microscopy (LSCM) is extensively applied because, compared to the conventional epifluorescence microscopy, it provides an increase in the effective signal-to-noise ratio and allows visualizing the 3D distribution of NPs by the collection of serial optical sections from the entire sample thickness.[19]

Numerous works have exploited the potential of LSCM to detect the uptake, diffusion and localization of several kind of NPs through different biological barriers (blood vessel, skin, intestine, mucus, tumor) simulated *in vitro* under static or dynamic conditions.[20-33] George and collaborators investigated the translocation of silica NPs in a transwell model of the human bronchial epithelial barrier, obtaining also quantitative information on the NP distribution in the apical and basolateral compartments.[34] Similarly, Schimpel and colleagues[35] performed a z-stack scan to demonstrate a highest particle uptake in the double culture model of Caco-2 cells (immortalized cell line of human colorectal adenocarcinoma) and Microfold (M) cells, compared to Caco-2 monolayer and Caco-2/HT29-MTX triple culture as *in vitro* models of the intestinal barrier.

LSCM was also used to visualize the adhesion, accumulation and dynamic relocation of NPs in function of their geometry, size and cell type in *in vitro* 3D models of dermis or cerebral endothelium.[36,37] Through LSCM it was possible to perform a semi-quantitative analysis of the kinetics mechanisms of penetration through tumor barriers and accumulation of fluorescent NPs, by directly scanning 3D models or measuring the fluorescence intensity of cryosections taken either from a mid-penetration depth or from the entire thickness of the 3D matrix.[38-42]

Moreover, LSCM offers the possibility of optically reconstructing the barrier in 3D with extremely low out-of-focus noise and improved spatial resolution. In this context, many researchers have been using the 3D modelling, obtained from z-stacks acquisition, to visualize the spatial distribution of NPs. Moreira and collaborators investigated in 2D and 3D tumor models the distribution and effects of a novel pH- and thermo-responsive carrier composed of doxorubicin-loaded gold-core silica shell nanorods and salicylic acid loaded poly(lactic-co-glycolic acid)-based microparticles,[43] while Hu and collaborators[44] evaluated the penetration capability of NPs into an artificial skin model generated by the 3D bioprinting technology. Papademetriou and colleagues[45] used LSCM to obtain 3D images for evaluating the internalization of Angiopep-2-coupled (Ang2)-liposomes through the brain endothelial cells using a microfluidic model of the BBB. Similarly, a 3D intensity map of a human BBB microvasculature and a tumor-on-a-chip model was obtained to quantify the gradient formation and space/time-dependent distribution of NPs.[46,47]

Besides LSCM, also the simpler epifluorescence microscopy proved to be a suitable technique to visualize the translocation of fluorescent NPs into different *in vitro* barrier models.[48-53] For example, Jia and collaborators[54] obtained information about the mobility of NPs in the mucus barrier. In detail, fluorescent polystyrene NPs were injected at a constant flow speed in the "lumen channel" of a mucus-on-chip device, and their transport within the mucus from the mucus-liquid interface was visualized over time using conventional fluorescence microscopy. A time-lapse configuration was introduced in the microscopy system to track fluorescent NPs as they move through simulated biological barriers such as microfluidic devices of the endothelial barrier or 3D human lung-on-a-chip models.[55-59] As demonstrated by Kiew and collaborators,[56] conventional fluorescence microscopy also allows semi-quantitative evaluation of NPs permeability in an *in vitro* biomimetic microfluidic model of blood vessel.

Other light microscopy techniques have been applied for the dynamic observation of NPs through biological barriers or to determine the efficiency of NPs as suitable shuttles for pharmaceutical applications. For instance, Brancato and collaborators[60] used bright-field microscopy to investigate the efficacy of polymeric NPs in reducing the diameter of 3D tumor tissue models. Optical microscopy was also used by Albanese and colleagues[39] to confirm the presence of polyethylene glycol NPs inside tumor-like spheroids in a microfluidic system, by measuring the intensity of silver staining in each spheroid section. Live-cell imaging videos acquired through phase contrast microscopy allowed monitoring the passage of silica NPs across a microfluidic *in vitro* model of endothelium.[61] The relevance of the light microscopy techniques for studying the dynamic interaction of NPs is confirmed in a study by Hudecz and collaborators,[62] who obtained high-resolution imaging of the NP interactions with endothelial cells and the capture of rare NP translocation events in an *in vitro* BBB model using a specially designed bioreactor with ultrathin silicon membranes.

Despite the numerous advantages of light microscopy to track NPs in 2D and 3D biological barrier models, a limitation is repre-

sented by the achievable spatial resolution. In this regard, transmission electron microscopy (TEM) proved to be especially adequate to study the penetration and distribution of NPs at the sub-cellular level in tumor or atherosclerotic vessel or in epithelial airway models.[39,50,51,58,63] Stereological analysis of TEM micrographs allowed the evaluation of the number of intracellular particles in a human epithelial airway model,[64] while electron spectroscopic imaging was used by Raemy and collaborators[65] to localize at TEM cerium oxide NPs in the intercellular milieu of an *in vitro* lung barrier.

Evaluating the effects of nanoparticles on biological barriers

Microscopy is also applied to determine the effects of NPs on 2D and 3D *in vitro* biological barriers in order to evaluate the efficacy and potential risks associated with the administration of nanoconstructs. To this aim, fluorescence microscopy is again the most widely used technique. In several studies, the cell viability was investigated by various assays based on fluorescent dyes after NPs exposure in tumor and dermal barrier models.[44,66,67] The impact on cell viability of titanium dioxide NPs in an *in vitro* gut epithelial model was evaluated by staining with a mixture of acridine orange and ethidium bromide.[63] Moreover, the changes in cell morphology and activities were measured through fluorescence microscopy to evaluate the effect of ZnS NPs on fibroblasts cultured in a 3D wound healing model.[68] To further characterize the effect of NPs penetration across biological barriers, immunofluorescence staining of specific biomarkers was also performed. As an example, the expression of junctional proteins was assessed to prove the cell integrity after NPs exposure in models of BBB,[70] bronchial epithelial barrier[34] and skin.[69] Fluorescence microscopy was also used to evaluate the morphological changes of 3D multicellular spheroids mimicking the solid tumor barrier, after administration of doxorubicin-loaded NPs.[49] Similarly, Moreira and collaborators[43] investigated the capability of gold-core silica shell nanorods with salicylic acid loaded poly(lactic-co-glycolic acid) based microparticles to promote tumor cells death and spheroid disassembly; to do this, the authors monitored by light microscopy the variation in time of the spheroids size.

The morphological integrity of tissues after NPs administration was evaluated using histological staining techniques. For example, the classic hematoxylin and eosin staining was used to visualize the skin structure,[53,69,71,72] the integrity of the BBB,[48] and the effect of photodynamic therapy in an *in vitro* 3D microfluidic breast cancer tissue model.[66] The effect of retinol-lipid NPs on collagen in an *in vitro* model of human skin was assessed by the Masson's trichrome staining.[73]

Electron microscopy is also gaining relevance in determining the effectiveness of NPs in biological barrier models. In fact, Wang and colleagues[74] used scanning electron microscopy images to demonstrate the antibacterial efficacy of Carbopol nanogel particles in reducing the biofilm attached on a 3D co-culture model of biofilm/human keratinocyte clusteroid.

Concluding remarks

Nanomedicine is a rapidly developing research area and represents a stimulating challenge for many scientific and technological disciplines.

The novel nanoconstructs must be characterized for their chemical nature, electric charge, size and shape, then carefully tested for biocompatibility: in this context, microscopy not only plays an irreplaceable role for the structural characterization of NPs, but is also crucial to elucidate their spatial interactions and functional effects on living organisms.

To reach their organ, tissue or cell targets, NPs must cross different and complex biological barriers, and light and electron microscopy techniques proved to be especially appropriate to visualize their dynamic behavior. The modelling of *in vitro* systems that mimic the physiological complexity of living structures is becoming increasingly frequent in science, to ensure controlled experimental conditions while meeting the ethic and economic issues aimed at reducing the number of the animals to be used in the research practice. The growing number of scientific papers that deal with the application of a variety of microscopy techniques to assess NPs' crossing of *in vitro* biological barriers testifies the importance of the morphological and histochemical approach in this advanced research field.

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