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Dynamics of nanoparticle diffusion and uptake in three-dimensional cell cultures

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ABSTRACT

This study aims at elucidating the effect of three-dimensional (3D) extracellular matrix on cell behaviour and nanoparticle (NP) diffusion and its consequences on NP cellular uptake mechansims. For this purpose, human dermal fibroblasts (HDF) and human fibrosarcoma (HT1080) cell lines were grown within a 3D collagen gel and exposed to model polystyrene (PS) NPs of controlled size (44 and 100 nm). Results indicate that, in 3D, cell morphology dramatically changes compared to standard 2D cultures and NP diffusion within the matrix is hampered by the interaction with the collagen fibres. As a consequence, NP cellular uptake, modeled with equations describing the stoichiometric exchange between NPs and cell membrane, is significantly slowed down in 3D and in the case of 100 nm NPs, in part due to the hampered diffusion of NPs in collagen gel compared to their transport in standard cell culture medium. Furthermore, our outcomes point at a significant contribution of the cytoskeleton assembly, in particular actin microfilaments, in governing the uptake of PS NPs in a 3D environment, and also that the macropinocytosis process is preserved and is mainly involved in the internalization of PS NPs in a 3D environment. However, depending on cell type and nanoparticle size, other endocytic pathways are also implicated when moving from 2D to 3D culture systems. This work highlights the importance of studying the nano-bio interaction in experimental models that resembles in vivo conditions in order to better predict the therapeutic efficacy of drug delivery systems.

1. Introduction

Antitumor drugs cannot adequately discriminate normal and tumor cells and, therefore, there exists an extremely labile border between their curative and toxic effects. Consequently, at present there is a flourishing pursuit in the development of nanoparticles (NPs) for the targeted release of antitumor drugs, which promise to be helpful in enhancing the efficacy of drugs, tune their biodistribution and limit their systemic side effects [1,2]. Understanding the mechanisms of endocytosis that promote the internalization of NPs in the destination cells is essential for a targeted drug release. Importantly, the therapeutic efficacy of NPs directly depends on their uptake, which is influenced by a number of parameters [3], such as NP size/shape, their surface chemistry/charge, the scalability of the production process, as well as the identification and optimization of the biophysicochemical features of the targeted NPs. All these features determine the particular endocytic pathway used by the cells (e.g. phagocytosis, pinocytosis, or receptor-mediated endocytosis), along with cell viability and NP-cell interactions [4–7].

Currently, the *in vitro* cytotoxicity, efficacy and targetability of NP cargos are generally assayed using standard two-dimensional (2D) cell cultures. 2D adherent systems poorly mimic the complexity of the three dimensional (3D) *in vivo* environment, in which the effects of extracellular matrix (ECM), cell-matrix interactions, transport hindrances and external stimuli are highly combined [8–10]. In fact, the 3D microenvironment provides cells with physical and biological support, as well as with a dynamic modulation of cell behavior. For example, integrin surface receptors get anchored to the ECM, and can mediate the transduction of biochemical signals, which in turn dictate cell differentiation, apoptosis, proliferation and invasion [11,12]. Consequently, the cells cultured in 2D dramatically differ from those grown *in vivo* in terms of

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gene expression [13], matrix biosynthesis [14], components of the cytoskeleton [15] and proliferation rate [16,17]. All these characteristics are lacking in 2D cultures and, as a consequence, the results of cell experiments in 2D generally fail to predict 3D and *in vivo* results [18].

For these reasons, there is an indubitable urge to improve the correlation between *in vitro* and *in vivo* results and, therefore, physiologically significant 3D cell culture systems *in vitro* are gaining increasing attention [19]. In particular, 3D cell cultures for cancer studies can be basically referred to spheroids [20–22] or 3D scaffolds [23–26]. Specifically, spheroids are microtissues mimicking *in vitro* the architecture, transport obstacles, cell-cell interactions, pH gradients and necrotic regions of tumors [27], while 3D scaffolds are constructs which allow to improve the understanding of disease mechanisms [13].

Type I collagen is the main component of ECM in tissues, such as skin, tendons and blood vessels [28]. Collagen-based 3D scaffolds have been widely investigated for tissue engineering applications [28–30] by virtue of their biocompatibility, biodegradability and non-toxicity; moreover, such scaffolds possess a porous structure that facilitates the infiltration of cells and the transport of nutrients [31]. Therefore, in this study we aimed to investigate how NPs of controlled size are shuttled through the cell membrane in standard 2D cultures and in a 3D collagen constructs. In a previous work, we had studied the cytotoxic effect of doxorubicin (Dox)-loaded NPs, made up of a biodegradable poly(d,l-lactic-co-glycolic acid) (PLGA)–*block*–PEG copolymer (PELGA), with various sizes against tumor cells in 3D collagen scaffolds, and showed that their uptake/cytotoxicity result from the interplay of device size/diffusion in gel, along with drug release kinetics [32]. In another investigation we produced NPs made of PELGA blended with a tumor-activated prodrug composed of a matrix metalloprotease 2 (MMP2)-sensitive peptide [33] bound to Dox and PLGA. The NPs could release the drug upon enzymatic cleavage, and could also promote drug penetration within 3D tumor spheroids [34]. Furthermore, we have recently shown that 44 nm-diameter polystyrene (PS) NPs enter renal cells taking advantage of both energy-dependent endocytosis and energy-independent mechanism [35]. Inspired by these results, here we studied the mechanisms regulating the uptake of model PS NPs (44 and 100 nm diameter) in a 3D collagen matrix, so as to define the relations among cell architecture, cytoskeleton assembly and NP uptake. Here, uptake kinetics and mechanisms were determined on human dermal fibroblasts (HDF) and human fibrosarcoma (HT1080) cell lines, and traditional 2D cultures systems were used as a control. To this aim, NP diffusion within collagen matrix and their interaction with collagen fibres were investigated, and NP uptake kinetics and mechanisms were studied as a function of size, cell type and cytoskeleton assembly through spectrophotometric analyses and confocal microscopy. Internalization kinetics of NPs in 2D and 3D models was also modeled using a system of equations based on single cell membrane adsorption/desorption. These equations were used in combination with the internalization data obtained with HDF and HT1080 cells.

2. Materials and methods

2.1. Polystyrene NPs

Green dye-loaded (fluorescence: $\lambda_{ex} \sim 468$ nm; $\lambda_{em} \sim 508$, Duke Scientific Corporation) NPs of 44 and 100 nm (namely, 44 and 100 NPs) were used without further modification or purification. NP dispersions were prepared by diluting the concentrated stock solutions into the serum medium. For cell culture, Eagle's minimal essential medium (EMEM, Lonza) was used at room temperature (RT), by applying identical time delays between its dilution and administration to cells in all experiments. Before experiments, NPs were vigorously mixed by vortexing, as recommended by the manufacturing company. The mean size and ζ-potential of NPs in different experimental conditions, including water, serum free and complete medium, were determine using ZetaSizer Nano ZS (Malvern Instruments, Malvern, UK; Table S1). The NPs used in this work do not suffer leakage of free dye in cell culture conditions, as previously demonstrated [36] thus preventing artifacts in cell uptake results [37].

2.2. Three-dimensional (3D) collagen matrix preparation

The collagen gels were prepared by diluting collagen stock solutions (Sigma Aldrich, USA) with 10× Dulbecco's phosphate saline buffer (D-PBS, Gibco; 8:1 vol ratio) at a final concentration of 2.4 mg ml^{-1} and adjusting the pH to 7.2 by dropwise addition of NaOH and HCl. Afterwards, cells were dispersed in collagen solution, that was then poured in appropriate cell culture dishes, depending on the type of the experiment, and incubated at 37 °C for 1 h to allow collagen gel to form and to obtain collagen embedded cells. After incubation, fresh cell culture medium was added on the top of the gel. The 3D collagen gels were also used for NP permeability measurements by Transwell permeable inserts (Corning Incorporated, Corning, NY) and, in this case, cell-free collagen solution was poured on filter inserts and allowed to fibrillate as previously described.

2.3. Scanning electron microscopy (SEM)

Cell-free 3D collagen matrices were incubated at 37 °C with 44 and 100 NPs for 24 h at final concentrations of 4.2×10^{11} and 3.6×10^{10} NP ml^{-1}, respectively. After incubation, the matrices were rinsed twice with PBS, fixed with 2.5% glutaraldehyde + 0.1 M cacodylate for 3 h, dehydrated through a series of increasing ethanol concentrations (70%, 80%, 90% and 100%) and, finally, by critical point drier (LEICA EM CPD300). The samples were platinum/palladium–sputtered and analyzed by SEM (Leica S400).

2.4. NP diffusion in collagen matrix

The permeability of NPs across collagen matrix was measured using Transwell permeable inserts (6.5 mm diameter, 0.4 μm pore size). For 2D experiments, 0.1 ml of NP suspension in cell culture medium w/o phenol red were added to the donor chamber of the Transwell system, while the receiver compartment was loaded with 0.4 ml of fresh cell culture medium. For 3D experiments, the medium with NPs was added after collagen fibrillation. Aliquots of the samples were collected from the receiver compartments at scheduled time points (1, 3, 6, 9, 15 and 24 h) and the sample volume was replaced with fresh pre-warmed cell culture medium. The fluorescence tracer concentration in the samples was determined by a spectrophotometric analysis (Enspire 2300, Perkin-Elmer, λ_{ex} 488 nm). The results are reported as the percentage of transported NPs.

2.5. Cell culture

To test the biological response to NPs in a 3D environment, primary Human Dermal Fibroblast (HDF) and Human Fibrosarcoma cell line (HT1080) were used as models of healthy and tumor tissue, respectively. The latter were cultured with complete medium, composed of Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, 100 mg ml^{-1} streptomycin. HDF cells were