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Research paper

Tunable mechanical properties of stent-like microscaffolds for studying cancer cell recognition of stiffness gradients

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ABSTRACT

Physical and mechanical properties of extracellular matrix (ECM) have been proved to be crucial in the metastatic process. However, currently available studies on the interplay between ECM stiffness and cancer cell invasive behaviour are performed on planar assays, while the in vivo interaction takes place in three-dimensions. To take into consideration the ECM structural and mechanical complexity in the cell/structure interactions, we fabricated 3D microscaffolds through two-photon lithography (2PL) and tested how they are invaded by human colorectal adenocarcinoma (LS-174T) tumor cells, showing that it is possible to detect significant differences in cells/structure interaction when structural parameters are modified. In particular, both scaffold geometry and 2PL fabrication parameters were optimized to obtain 3D polymeric cylindrical structures with controlled Young's modulus and with linear stiffness gradients. The ability of LS-174T to migrate in the scaffolds was tested in different experimental conditions, including scaffolds functionalization and under β-catenin downregulation. It was observed that high Young's modulus scaffolds are always less invaded than softer ones, confirming the role of the 3D micro-environmental stiffness in mediating cells migration, including when specific functionalization or pharmacological treatments are performed.

1. Introduction

Cancer cell mechanobiology is an emerging field of study combining biology and physics to analyse cell behaviour, aiming at understanding the role of microenvironmental mechanical properties in regulating cancer cells response to mechanical signals, especially during carcinogenesis [1]. In particular, tumor phenotype can be driven by both biomechanical and biochemical properties of the surrounding environment, where extracellular matrix (ECM) components play a crucial role in synergistically stimulating cell migration, invasion, proliferation and survival [2].

Mechanical interactions between cells and the ECM have been shown to play a key role in metastases formation [3–6], with the stiffness of the substrate on which cells grow being a key factor controlling cell migration rates [7]. In this context, the possibility to create three-dimensional (3D) microenvironments for cell culturing provided a significant improvement in the attempt to monitor mechanical cell behaviours driving the invasive process [8,9]. Two-photon lithography (2PL) represented a breakthrough technique in the development of controlled 3D microstructures for a variety of biological applications, including cell mechanosensing [10–12], cell discrimination [13,14], and tissue engineering scaffolds [15–18]. One of the major advantages of 2PL structures is the possibility to finely control architectures and mechanical properties [19] during fabrication: briefly, two-photon absorption, i.e. a nonlinear process generated by a high spatiotemporal photon density of a focused laser beam, induces polymerization of a photosensitive material within an ellipsoid-shaped volume (called voxel) [20,21]. The relative movement of the substrate with respect to the laser focal point allows realization of complex structures. Moreover, tuning the laser beam power allows obtaining photopolymerized structures with different mechanical properties.

In this work, we engineered 3D cylindric stent-like structures realized through 2PL with different mechanical properties to investigate the invasive behaviour of a human colorectal adenocarcinoma cell line (namely, LS-174T). Writing parameters were optimized in order to create 3D cylindric microscaffolds with open ends and tailored mechanical properties. In particular, we fabricated scaffolds having different values of Young's modulus (constant stiffness structures, CSSs) and structures with axial stiffness gradients (stiffness gradient structures, SGSs) to

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verify whether cells capability to invade the structures was correlated to the mechanical properties of the stent-like structures. SGSs were then further engineered with the introduction of partition walls, which were realized to verify cells ability to discriminate and preferentially invade structures presenting a stiffness gradient from their softer or stiffer open end.

Results show that stiffer CSSs are always less invaded than softer CSSs, and that SGSs present intermediate invasion rates, suggesting that the average stiffness values of 3D microscaffolds may influence the behaviour of the investigated cell lines.

In addition, since biochemical and physical cues deriving from the external microenvironment can influence intracellular machinery responses and promote epithelial cells malignancy, we also tested the invasive behaviour of LS-174T cells in presence of two factors aimed at influencing cell/structure interactions and cell proliferation. Scaffolds were (i) functionalized with fibronectin (FN), an ECM protein widely diffused in physiological conditions that acts as a positive stimulus for cells [22], and (ii) treated with doxycycline (DOX), a widely used molecule capable of inducing the knockdown of β-catenin, a cell-cell junction protein involved in the Wnt pathway (also acting as co-transcriptional activator or repressor of different genes controlling cell growth and survival, [23–27]). Interestingly, even in presence of these biochemical factors, cell migration in the microscaffolds is mostly driven by structures stiffness, although invasion rates result generally higher in the case of FN functionalization, while β-catenin knockdown reduces invasive rates.

These findings lead us to hypothesize that also in physiological microenvironments both the overall ECM stiffness, as well as the presence of soft inlets, may play a role in regulating tumor cell behaviours like extravasation or migration in confining tissues.

2. Results

2.1. Scaffold design and fabrication

The working principle of 2PL is sketched in Fig. 1a. Briefly, photopolymerization of an acrylate-based resist is achieved in correspondence to the focal spot of a femtosecond pulsed 780 nm laser, and the relative movement between the laser and the substrate allows the realization of a 3D structure. In the case of functional cylindric microscaffolds, structures were designed taking into account three main features: (i) open ends with size allowing invasion by cancer cells; (ii) porosity of the cylinder lateral surface allowing efficient exchange of cell culture medium between the inner and the outer part of the scaffold but preventing cell invasion; (iii) easiness of fabrication and time efficiency, i.e. possibility to reproduce several structures in a few-hour process. These criteria were fulfilled through a modular structure, based on adjacent one-voxel wide repetitive units (Fig. 1b), which allowed to obtain 80 μm-long cylinder-like scaffolds (Fig. 1c), open ends area of $\approx 90\mu m^2$ and porosity of the lateral surface $\approx 2.2\mu m^2$, which does not allow cells to cross the scaffolds from the lateral surface (see supplementary Fig. S1). Design was realized through DeScribe software (from Nanoscribe GmbH), which allowed for simulating the actual dimensions of voxels during fabrication, i.e. ≈0.45 μm along the minor axis and ≈1.5 μm along the major axis [28], and for programming trajectories of the laser to build the repetitive units composing the scaffolds. Microfabrication of scaffolds was performed on a glass substrate through a commercial 2PL system (Photonic Professional GT from Nanoscribe GmbH) and lasted less than five minutes per each cylinder. CSSs were obtained realizing repetitive structure basic units along the y-axis (see definitions in Fig. 1b–d) with constant laser power, i.e. 45%, 65% and 85% of the maximum available power (namely 20 mW), resulting in structures having Young's modulus of 2.7 GPa, 3.7 GPa and 4.7 GPa [19]. SGSs were instead obtained increasing the laser power of 0.5% for each repeating unit along the y-axis, starting from 45% of the maximum available power (Fig. 1d). Since a quasi-linear relationship between laser power and resulting material stiffness was recently proposed [19], a linear gradient along the cylinder axis was obtained, with Young's modulus ranging from 2.7 GPa to 4.7 GPa. It is important to notice that using different laser powers to polymerize different parts of the scaffolds affects voxel dimensions [29], with higher laser powers resulting in increased voxel dimensions. In the specific case of the experiments reported in this work, however, this effect does not influence neither lateral pores size nor open ends area, as shown by the data reported in Fig. 1e. Moreover, the stiffness variation obtained even for slight increases of the laser power used was verified through atomic force microscopy (AFM) measurements. Indeed, indentation measurements allowed for detecting a stiffness increase of ≈3% with a spatial resolution of ≈250 nm on purpose-fabricated patterned structures (see Fig. 1f and supplementary Fig. S2 for details). After polymerization and removal of unexposed resist, correspondence of actual scaffold dimensions to design was verified through scanning electron microscopy (SEM) inspection of sample structures (Fig. 1g).

2.2. Invasive behaviour of cancer cells

LS-174T adenocarcinoma cells were tested for their ability to invade stent-like microscaffolds. After sterilization of samples, LS-174T cells were cultured until 70–80% of confluency was reached, i.e. the majority of the sample glass substrate was covered with cells, and then fixed with 4% paraformaldehyde. Structures presenting at least one cell nucleus completely inside the structure were considered as “invaded” (Fig. 2a). It was thus possible to identify the percentage of invaded CSSs and SGSs over the total number of fabricated scaffolds, with results displayed in Fig. 2b. It is clearly detectable an increase of the invasion rate as the stiffness of the structures decreases, suggesting that softer scaffolds, and in general softer ECM-like environments, may provide a more favourable environment for cell migration structure invasion. As shown in Fig. 2b, SGSs present an invasion rate which is comparable to CSSs of intermediate stiffness, suggesting that SGSs are “sensed” from cells as overall CSSs having average stiffness between the soft and the stiff ends. In order to verify that the interaction of cells with the microscaffolds was not the result of cell overpopulation when 70–80% of confluency was reached, a confocal microscopy timelapse was performed. Samples were inspected after 4 h from seeding and cells approaching one of the cylinder open ends and in some cases entering the microscaffolds (Supplementary Video 1) were detected. Moreover, in proximity of the structures, cells were observed to move directionally toward the cylindric open end, as they sensed the presence of the structure, with this behaviour being more evident for the softer side of the scaffolds. Such behaviour suggests that cell invasion of structures occurs as a consequence of spontaneous cell/structure interactions and that it is not due to cell overpopulation in quasi-confluency conditions.

2.3. Sensitivity of stiffness gradient

In order to better investigate the invasion behaviour of cells in relation to SGSs, previously designed structures were modified to introduce separating walls in correspondence of: (i) the softer open end; (ii) one third and (iii) two thirds of the total length of the microscaffold (Fig. 3a–b). Engineered microstructures were intended to assess whether cells could be driven inside SGSs not only by the presence of a stiffness gradient but also by the possibility to preferentially interact with the softer or the stiffer inlet. Interestingly, scaffolds resulted to be more invaded by cells entering from the softer end, independently on where the separating wall is located (with the obvious exception of walls blocking the soft end), as shown in Fig. 3c, thus suggesting that the direction of invasion is an important parameter regulating cells behaviour. Moreover, in preliminary measurements of cells invading the scaffolds from both sides of the separating walls, i.e. from both softer and stiffer ends, the number of nuclei found inside the softer portion resulted always higher than the stiffer part of the cylinder (see supplementary Fig. S3).