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VA-MWCNT micropillars

Silicon micropillars

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Vertically Aligned Carbon Nanotube Micropillars Induce Unidirectional Chondrocyte Orientation

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ABSTRACT

Articular cartilage is a highly organized tissue with very limited regenerative capacities. One limitation to mimic cartilage structure in tissue engineering is due to specific orientation of chondrocytes. Here, we use vertically aligned multi-wall carbon nanotubes (VA-MWCNT) micropillars to achieve unidirectional orientation of chondrocytes. We demonstrate that the attachment, proliferation and extracellular matrix (ECM) production by the chondrocytes is enhanced on VA-MWCNT micropillars compared to controls. The nanostructures offered by the VA-MWCNT allow the chondrocytes to anchor at cellular structure level, while mechanical flexibility of the VA-MWCNT micropillars mimics the cartilage's natural ECM Young's modulus. We exploit these features to extrapolate the contractile forces exerted by the chondrocytes on the micropillars. Our findings will guide the design of VA-MWCNT templates to model cell's contractile forces. Furthermore, the capability of VA-MWCNTs to induce unidirectional chondrocytes orientation open new perspectives in cartilage tissue engineering.

ABBREVIATIONS

AC: Articular Cartilage; ECM : Extracellular Matrix; VA-MWCNT: Vertically AlignedCN Multi-Walled Carbon Nanotube; SWCNT: Single-Walled Carbon Nanotube; TGF β 1 : Transforming Growth Factor Beta 1; IL-1 β : Interleukin 1 beta; ROI : Region of Interest; CVD : Chemical Vapor Deposition.

1. INTRODUCTION

Articular cartilage (AC) is a highly organized, avascular, aneural and alymphatic connective tissue lining the ends of bones in load-bearing diarthrodial joints. It provides a near frictionless surface in the joint, enabling the redistribution of shear and compressive forces during load-bearing and locomotion. In order to provide these structural and mechanical properties, AC is composed of a dense extracellular matrix (ECM), consisting mainly of collagen type II and proteoglycans. The ECM is produced and maintained by a single cell type, the articular chondrocyte. AC is divided into three layers (superficial, transitional and deep zone), each with specific properties, such as ECM content, chondrocyte orientation and morphology[1,2]. In contrast to other tissues, AC has very limited regenerative capacity, which accounts for the rising burden of osteoarticular diseases and several challenges in AC tissue engineering. One of the major challenges in tissue engineering is to reproduce and biologically recapitulate the required chondrocyte orientation and morphology in the different layers of the cartilage scaffold as well as the ideal ECM chemical-physical properties, allowing optimal biomechanical, mechanotransductive and metabolic responses by the cells in a biomimetic microenvironment.

Over the years, different strategies[2] have been tried using polymeric-based hydrogels[3–5] or scaffolds[6] in order to obtain the layered structure of cartilage with the specific properties of each layer. The most significant drawbacks of these approaches are the lack of attachment sites and the lack of mechanical strength to maintain the structural shape required for tissue regeneration. When chondrocytes are grown in 2D templates, the topography and stiffness of the

support influences the behavior of the cells[7,8], also the differentiation of stem cells into adipocytes, osteoblasts or chondrocytes is influenced by the stiffness of the support[9,10]. Furthermore, studies have shown that the response of chondrocytes to chemical and biological cues such as TGF- β 1 or IL-1 β depends on the stiffness of the substrate on which they are grown[11] and they lose their chondrogenic phenotype when cultured on hard culture dishes with a stiffness around 1 GPa[12].

Seeking to develop a widely applicable template to induce cell orientation, we focused on the tailoring of vertically aligned multi-walled carbon nanotubes (VA-MWCNT) by creating micropatterned templates with optimal dimensions and different geometries in order to achieve cell unidirectional orientation[13]. In the past decade, MWCNTs have been recognized as a potential functional material to enhance cell growth either as a reinforcement component into polymeric-based matrices or as 2D matrices for cell behavior modeling [13]. MWCNTs have a high surface area and related chemical absorption. This particular characteristic reflects the MWCNTs ability to structurally mimic collagen fibers, which is one of the main components of the ECM in articular cartilage[14]. Previous studies showed that the use of single wall carbon nanotubes (SWCNTs) incorporated on electrospun polycaprolactone (PCL) lead to enhanced human mesenchymal stem cell (hMSC) growth as well as chondrogenic differentiation *in vitro*[15] and that superhydrophilic VA-MWCNT films provide support to maintain chondrogenic phenotype[16]. We demonstrated that the nanostructures offered by our templates allows the cells to anchor to the pillars at nanoscale level. Using the mechanical flexibility of the VA-MWCNT pillars, we were able to estimate the cellular traction forces exerted by chondrocytes adhesion points. The VA-MWCNT micropatterned templates we fabricated promoting unprecedented unidirectional chondrocyte orientation and chondrocyte specific ECM

production. The ability of control unidirectional growth of chondrocytes using VA-MWCNT open new perspectives to biologically mimic the architecture of mature articular cartilage.

2. MATERIALS AND METHODS

2.1. VA-MWCNT micropatterned templates preparation

Templates of 10 μm high vertically aligned multiple wall CNT (VA-MWCNT) micropatterns organized in different geometries templates were fabricated by lift-off lithograph over Si/SiO₂ surfaces via chemical vapor-phase deposition. The photoresist was spin-coated on 4" Si wafers, followed by illumination and development. (Microchemicals AZ[®] 1512HS positive photoresist, Suss RC8 Spinner, Compugraphics 5" Cr-quartz photomask, Suss MA8 Mask Aligner, Microchemicals AZ[®] 726MIF developer). After development an Al₂O₃ buffer layer (thickness of 10 nm, PRF=165 W, p=2.4 mTorr, deposition rate=0.1 Å/s) and Fe catalyst (thickness of ~1.5 nm, PDC=30 W, p=2.4 mTorr, deposition rate=0.1 Å/s) were sputtered on the wafers (Torr International Physical Vapor Deposition System). After stripping the photoresist, the wafers were diced into discs of 9.5 mm in diameter by a pulsed laser (LPKF ProtoLaser U3, Nd:YVO₄, $\lambda=355$ nm, P_{avg}=6 W, f=40 kHz, $\tau\sim 20$ ns, focal point radius of ~20 μm) and cleaned in acetone and isopropanol. VA-MWCNTs were synthesized by chemical vapor deposition (CVD) in a cold-walled low-pressure reactor equipped with a 2" graphite heater (Aixtron Black Magic). The catalyst was first reduced for 5 minutes at 700°C in H₂ (700 sccm), after which the temperature was lowered to 670°C and C₂H₂ (20 sccm) was introduced to the chamber for 28 seconds to grow the MWCNT micropillars (10 μm in height). The pressure was kept at 25 mbar during CVD the process.

Several different VA-MWCNT micropatterns designs were produced including (i) squared arrays of pillars (with pillar footprint sizes of $5 \times 5 \mu\text{m}^2$ and $20 \times 20 \mu\text{m}^2$, with centre-to-centre spacings of $10 \mu\text{m}$, $45 \mu\text{m}$, $70 \mu\text{m}$ and $220 \mu\text{m}$); (ii) pillars along spiral patterns (pillar footprint size of $5 \times 5 \mu\text{m}^2$, with centre-to-centre spacings of $10 \mu\text{m}$ and $15 \mu\text{m}$, and with spacings between successive turns of the spiral of $20 \mu\text{m}$ and $50 \mu\text{m}$; (iii) pillars arranged in stripes (pillar footprint size of $5 \times 5 \mu\text{m}^2$, with centre-to-centre spacings of $10 \mu\text{m}$), with stripes spacing of 25 , 50 , 100 and $200 \mu\text{m}$). All micropatterned templates were sterilized by UV-C exposition for 30 minutes.

2.2. Silicon micropatterned templates preparation

Si micropillar arrays with pillars arranged in stripes (pillar footprint size of $5 \times 5 \mu\text{m}^2$, with centre-to-centre spacings of $10 \mu\text{m}$, and with line spacings of 5 - $150 \mu\text{m}$), similar to pattern (iii) of the VA-MWCNT micropatterned templates, were prepared on 4" silicon wafers by reactive ion etching (RIE, Oxford Instruments Plasmalab 80 Plus, $P=200 \text{ W}$, $p=50 \text{ mTorr}$, 6 sccm SF_6 flow, 2 sccm O_2 flow, temperature $20 \text{ }^\circ\text{C}$ with liquid N_2 cooling of the sample holder, 50 min process time). The wafers were lithographically defined same way as the wafers for CNT synthesis but instead of a catalyst, a Cr layer (thickness of 200 nm) was sputtered on the wafers as a hard mask. After the Si etching process, the Cr layer and natural surface oxides of Si were removed by wet etching in aqueous solutions of $9\% \text{ HClO}_4$ and $20\% (\text{NH}_4)_2\text{Ce}(\text{NO}_3)_6$ as well as in aqueous HF (5%) buffered in NH_4F (36%). Finally, the wafers were diced into chips of 9.5 mm in diameter by pulsed laser, and subsequently cleaned with acetone and isopropanol. All micropatterned templates were sterilized by UV-C exposition for 30 minutes.

2.3. Atomic Force Microscopy

All measurements were performed on a Bruker Multimode 8 atomic force microscope. The Young's Modulus of pillars was evaluated by atomic force microscopy, using 10.0 μm borosilicate glass sphere probes (Novascan Silicon AFM probe), with Young's modulus around 14 N/m. Individual cantilevers were calibrated using touch-calibration of the Bruker NanoScope 9.2 software. Force measurements were conducted in air for a threshold of 50 nm absolute indentation at 3 $\mu\text{m/s}$ with a ramp size of 500 nm, velocity of 502 m/s, rate of 0.5 Hz with 1024 sample points/ramp. The stiffness of single $5 \times 5 \mu\text{m}^2$ micropillars were evaluated using the spiral design template with centre-to-centre spacings of 10 μm and spacings between successive turns of the spiral of 50 μm . The Young's modulus was extracted from 54 pillars by using the Hertzian (spherical) indentation model[17–20] measuring the slope on the initial 30% of the unloading (retract) load-displacement curve with a linearized model of each ramp on the Bruker NanoScope 9.2 software. The indentation depth of this model resulted in an indentation diameter lower than the size of the pillars, ensuring that the Hertz spherical model is suitable for this analysis[21]. Young's modulus of the continuous VA-MWCNT film was calculated with the same method as for the VA-MWCNT pillars in 100 points. Lorentz curves were fitted on the histograms representing the distribution of the Young's modulus of both samples.

2.4. Transmission Electron Microscopy

VA-MWCNTs from micropatterned pillared ($5 \times 5 \times 10 \mu\text{m}$) templates and from continuous VA-MWCNT films were prepared by carefully scrapping out the VA-MWCNT pillar and continuous film and suspending each of them in ethanol. A drop of each suspensions was dried at room temperature on separate Copper mesh covered with Holey Carbon Film (HC300-Cu, Electron Microscopy Science). All the images were taken from JEOL JEM-2200FS transmission electron

microscope. The carbon nanotube outer diameter was evaluated using EM measure software Beta 0.85 (Teitz Video and Image Processing Systems GmbH) by measuring the distance between the outer dark lines at the both edges of the tube walls. Gaussian curves were fitted on the histograms representing the distribution of the diameters of the VA-MWCNT pillars, while a Lorentz curve was fitted for the continuous VA-MWCNT film results.

2.5. Chondrocyte seeding

Adult human primary chondrocytes were purchased from Sigma-Aldrich and were seeded on the VA-MWCNT and silicon micropatterned templates at passage 2 after purchase at a density of 12 000 cells/cm² by seeding a 100 µL drop on top of the micropatterned surface (1 cm diameter), and letting the cells attach for 30 min at standard culture conditions (37 °C, 5% CO₂), before adding 1 mL growth media/well in a 24 well-plate. Cells were grown on the templates for 1, 3 and 7 days in Chondrocyte Growth Medium (Cell Applications Inc. – 411-500), containing 100 u/mL Penicillin, 100 µg/mL Streptomycin Sulfate, 250 ng/mL Amphotericin B. Medium was changed ever 2-3 days. The test was repeated three separate times with 6 samples per pattern (including the silicon control pillars).

2.6. Live/Dead Staining

Life/Dead staining was performed on duplicate samples for days 1, 3 and 7 using Cellstain double staining kit (Sigma-Aldrich 04511) according to the manufacturer's instructions. Briefly Live cells were stained with Calcein-AM, while nuclei the dead cells were stained by propidium iodide. Cells were stained for 30 minutes at room temperature after which they were fixed in 4% PFA until observation.

2.7. Immunostaining

Immunohistochemical staining was performed to visualize chondrocyte specific collagen II/aggrecan production and to evaluate the actin/vinculin cytoskeleton of the cells on the various templates. Cells were first fixed for 15 minutes in 4% PFA after which they were permeabilized for 5 minutes with 0,5% Triton X-100 (VWR Chemicals). Samples were then blocked in 1% BSA (PanReac AppliChem) for 1 hour and stained with the primary antibodies overnight. Collagen II was stained with mouse anti-collagen II antibody (5B2.5) (Novus, NB600-844) at 5 ng/mL; aggrecan with goat anti-human aggrecan G1-IGD-G2 Domains antibody (Novus, AF1220) at 5 ng/mL; vinculin with mouse anti-vinculin monoclonal antibody, purified clone 7F9 (Part No. 90227 from kit FAK100, Merck-Millipore) at 2 ng/mL. The samples were then incubated in secondary antibodies at 1/200 for 1 hour with respectively rabbit anti-mouse IgG (H+L) secondary antibody – FITC (Novus, NB7543), donkey anti-goat IgG NorthernLights NL557-conjugated antibody (Novus, NL001) and goat anti-mouse IgG (H+L) secondary antibody – FITC (Novus, NB7538). The samples stained for vinculin were co-stained with TRITC-conjugated Phalloidin for actin staining during the secondary antibody staining (kit FAK100, Merck-Millipore). Nuclei were then stained with DAPI (Novus, NBP2-31156) at 1/1000 dilution for 10 minutes and samples were stored in PBS at 4°C until observation.

2.8. Fluorescence imaging

All fluorescence imaging was performed on a Leica DM4B with a CoolLED pE300W lightsource combined with system DAPI ET, k; GFP ET, k and Y3 ET, k filters. Images were

acquired with LasX Software (Leica) with 10 x water and 40 x water dipping objectives, using a Leica DFC3000 G camera.

2.9. Image analysis for cell orientation evaluation

Image analysis was performed with ImageJ software. The DAPI images (cell nucleus staining) were exported and converted into binary images, after which each nucleus was fitted to an ellipse to evaluate the cell orientation using the “Analyze Particles” tool with an authorized size of 0-infinity and a circularity of 0.00-1.00. Ellipse areas smaller than the equivalent of the size of the pillars were discarded to avoid unspecific staining of the pillars. The angle between the long diagonal of each ellipse versus the X axis of the image (corresponding to the orientation of the lines in pattern 3) was extracted as $[0^\circ, 180^\circ]$, corresponding to the alignment direction of the cells. Data were then represented as compass graphs using OriginPro2019b.

2.10. Scanning electron microscopy

Imaging of VA-MWCTN micropillars and continuous film is achieved without any fixation or coating, while imaging after fluorescence observation of the live/dead staining required sample fixation and coating as follow. Fixation was performed with 5% glutaraldehyde (Sigma Aldrich) in PBS at room temperature for 1 h. The samples were dehydrated by incubation in increasing ethanol concentrations (10, 20, 40, 60 and 80%) for 10 min each, followed by a 30 min incubation in absolute ethanol (99.5%) and dried overnight at room temperature. In order to avoid charging artefacts, a ~5nm layer of Pt was sputtered coating the samples (Agar High Resolution Sputter Coated 208HR). The acquisition of electron micrographs was carried out using a field emission scanning electron microscope (FESEM, Zeiss Ultra Plus). It must be noted

that the cross-section of the pillars after cell plating, dehydration and drying, is reduced due to van der Waals forces that collapse the porous structure. After drying, the cross-section of the $5 \times 5 \mu\text{m}^2$ and $20 \times 20 \mu\text{m}^2$ pillars decrease respectively to $\sim 2.2 \times 2.2 \mu\text{m}^2$ and $\sim 13.8 \times 13.8 \mu\text{m}^2$.

2.11. Finite Element Method analysis

The CNT-pillars were simulated using COMSOL Multiphysics 5.2 software. The geometry of the model was a $5 \times 5 \times 10 \mu\text{m}$ 3D block. The bottom of the pillar was fixed and the traction force actuated the pillar perpendicularly to the pillar surface. The traction area was $5 \times 0.5 \mu\text{m}^2$ on the side of the pillar in which the traction force was distributed evenly. The simulation was carried out as a static parametric simulation (using triangular mesh and quadratic elements) where the force and its acting point on the pillar was varied. The pillar was simulated as an isotropic material with Young's modulus of 8 MPa and Poisson's ratio of 0.3.

3. RESULTS

3.1. Topography and mechanical cues of VA-MWCNT micropillars

VA-MWCNT micropatterned templates with various pillar size, spacing and patterns, including grids, stripes and spirals were designed and manufactured by lift-off lithography followed by chemical vapour deposition (CVD) on Si/SiO₂ substrates (Supplementary Figure S1a-b). VA-MWCNT pillars were grown to height of $10 \mu\text{m}$ for all patterns designs (Figure 1a). The average outer nanotube diameter, as calculate from transmission electron microscopy (TEM), is $8.8 \pm 0.4 \text{ nm}$ and $6.0 \pm 0.2 \text{ nm}$ for VA-MWCNT grown as pillars and continuous film, respectively (Figure 1b). The Young's modulus of VA-MWCNT was evaluated by atomic force microscopy (AFM)[22,23] using $10 \mu\text{m}$ -diameter borosilical sphere probes with a spring constant of 12 N/m.

The mean of the fit of a Lorentz curve resulted in a Young's Modulus of 7.5 ± 0.1 MPa and 17.1 ± 0.6 MPa for VA-MWCNT grow as $5 \times 5 \mu\text{m}^2$ pillars and continuous film, respectively (Figure 1c). This difference is evidenced by the wide range of mechanical properties founded in the literature for VA-MWCNT, such as variations in modulus from MPa to GPa levels[19,22]. These variations are attributed to the different microstructures of the VA-MWCNT, which is strongly dependent of synthesis method, height, density and diameter of CNTs[19]. Nevertheless, the Young's Modulus found for VA-MWCNT is compatible with the Young's Modulus reported for cartilage's ECM which is in the range of 5-20 MPa[24], indicating that our micropatterned VA-MWCNT templates mimic the mechanical cues offered by the natural ECM. In addition, Ge et al.[25] have reported an adhesive strength of 36 N cm^{-2} (i.e. 3.6 MPa) for grown VA-MWCNT using the same substrate and iron as catalyst.

Figure 1. Micropatterned VA-MWCNT pillars and continuous VA-MWCNT films nanostructure. a. SEM images (sample tilted in 45°) of VA-MWCNT pillars and VA-MWCNT

film, Scale bar: 10 μm (low magnification) and 1 μm (high magnification). **b.** TEM image showing individual MWCNTs extracted from VA-MWCNT pillars samples and VA-MWCNT films. Scale bar: 20 nm. **c.** Distribution histogram and Lorentz fit of the Young's Modulus of VA-MWCNT pillars and VA-MWCNT films obtained by AFM. The number of samples is respectively 54 and 154, binning is 2.5 MPa for both samples. Top right is a schematic representation of the AFM measurement with a 10 μm hemisphere cantilever for the pillars and films.

3.2. Inducing cell orientation by optimized VA-MWCNT micropillars

To evaluate the influence of cues provided by the VA-MWCNT pillars, similar Si/SiO₂ micropatterned templates were fabricated via reactive ion etching and used as reference templates (Supplementary Figure S1b). Primary chondrocytes were grown for 1, 3 and 7 days on both templates and evaluated via immunostaining and scanning electron microscopy (SEM) imaging. The cytoskeleton of the cells was stained with vinculin and phalloidin (actin staining) allowing the evaluation of the distribution and orientation of the cells over the micropatterned templates. The orientation of the cells on the templates was defined by fitting an ellipse to the nucleus (DAPI staining) of each cell and calculating the angle between the long axis of the ellipse and the horizontal axis of the imaging defined as 0-180°. This analysis was performed in a defined region of interest (ROI) and the result shown as compass graphs. In the VA-MWCNT grid design, the orientation of the cells (either parallel or perpendicular to the horizontal axis) was visible in the 5x5 μm^2 micropillars with a spacing of 10 μm (Figure 2a), but no preferable orientation was observed with the 20x20 μm^2 pillars with spacing >45 μm , most likely due to the large spacing between pillars (supplementary Figure S2). This result indicates that in the

templates with large spacing the cells sense the surface as flat and the micropillars do not influence cell growth.

Figure 2. Orientation of the chondrocytes on the VA-MWCNT pillars in grid design. a. Immunostaining of the cytoskeleton of chondrocytes at 1, 3 and 7 days on grid micropatterned design ($5 \times 5 \mu\text{m}^2$ pillars with $5 \mu\text{m}$ spacing) showing strong horizontal and vertical orientation of the cells. Green: anti-vinculin staining, Red: phalloidin staining (actin), Blue: cell nucleus staining (DAPI). Scale bar: $100 \mu\text{m}$ (low magnification) and $30 \mu\text{m}$ (high magnification). **B.** Compass graph, showing the quantification of the orientation of the cells (based on the nucleus orientation) at the different days in the grid micropatterned template. $0/180^\circ$ corresponds to horizontally aligned cells and 90° corresponds to vertically aligned cells.

The micropatterned stripes were designed using the optimal spacing and pillar size found with the grid pattern to induce unidirectional cell orientation. Indeed, the micropatterned stripes clearly induced to chondrocytes orientation in only one direction of the chondrocytes independent of the spacing between the stripes (Figure 3a). Although the unidirectional orientation was observed for both VA-MWCNT and Si/SiO₂ templates, it is evident that the number of cells on VA-MWCNT templates are higher. At day 1, similar number of cells are present both in the reference (i.e. Si/SiO₂) and the VA-MWCNT stripe micropatterns (Figure 3b D1), as expected since the same cell suspension was used to seed both templates. At day 3 and day 7, cell proliferation is increased in the VA-MWCNT stripe micropatterned template compared to the reference template, and the cells are mainly aligned along the stripes (angle ~ 0° or 180° in Figure 3b D3, D7). By using the optimal pillar size and spacing between pillars, chondrocytes can be also induced to follow non-straight geometries such as spirals (Figure 4).

Figure 3. Orientation of the chondrocytes on the VA-MWCNT and Si/SiO₂ pillars in stripe design. *a.* Merge of immunostaining for the actin cytoskeleton and brightfield images for chondrocytes grown for 3 days on Si/SiO₂ pillars compared to VA-MWCNT pillars with the stripe micropatterned template composed of 3 lines 5x5 μm² pillars with 10 μm center to center spacing with an inter-stripe spacing of 25 μm, 50 μm, 100 μm and 200 μm. Green: anti-vinculin

staining, Red: phalloidin staining (actin), Blue: cell nucleus staining (DAPI). Scale bar: 100 μ m.

b. Compass graph quantifying the orientation of the chondrocytes (based on the nucleus orientation) on the stripes (ROI) micropatterned templates at day 1, 3 and 7, for VA-MWCNT in red and Si/SiO₂ templates in blue. 0/180° corresponds to horizontally aligned cells. These reveals an increased cell number on VA-MWCNT pillars compared to Si/SiO₂ pillars, as well as cell unidirectional orientation following the pattern. Furthermore, the number of cells is proportional to the number of stripes (ROI) in the complete image, as expected (resp. 11, 7, 4 and 2 stripes for the inter stripe spacings of 25, 50, 100 and 200 μ m).

Figure 4. Extracellular matrix production by the chondrocytes in spiral micropatterned templates. Immunostaining of chondrocytes grown for 1, 3 and 7 days on VA-MWCNT pillars. Green: anti-collagen type II staining, Red: anti-aggrecan staining, Blue: cell nucleus staining (DAPI), Merge: brightfield, collagen II, aggrecan and DAPI. Scale bar: 100 μ m.

3.3. Extracellular matrix production and chondrocyte attachment

ECM production by chondrocytes was evaluated by anti-collagen II and anti-aggrecan (the main proteoglycan in the ECM) staining, showing a positive ECM production in all micropatterned templates (Figure 4 for spirals). The immunofluorescence micrographs also showed good attachment and survival of the cells for all the investigated templates as the chondrocytes continue to proliferate up to 7 days, especially on the VA-MWCNT micropillars (Figures 2-4). Interestingly, SEM images showed that chondrocytes tend to attach on the micropillars and do not rest on the Si/SiO₂ flat surface (Figure 5). Furthermore, while chondrocytes attached to Si/SiO₂ pillars via bundle-like structures wrapping the whole diameter of the pillar, the cells anchored to individual VA-MWCNT pillars using much thinner structures with size at the scale of the CNTs (Figure 5). This is a clear evidence that the MWCNTs provide an environment with structures with similar size to cell's structures, facilitating the interaction and interface between cells and materials.

Figure 5. SEM images of chondrocytes grown on VA-MWCNT and Si/SiO₂ pillars in stripe micropatterned templates. This shows the bending of the VA-MWCNT pillars due to the force

exerted by the cells, as well as different anchorage patterns of the cells on the VA-MWCNT pillars vs Si/SiO₂ pillars. Scale bar: 10 μ m (black with white border) and 1 μ m (white).

3.4. Chondrocytes traction forces

Bending of the VA-MWCNT micropillars surrounded by chondrocytes was observed in the SEM images (Figure 6a). Although sample preparation for SEM might cause some cell shrinking, the observed bending can be attributed to cellular traction forces[26]. According to the SEM images, the displacement (δ) of the center node on the top of the surface of the VA-MWCNT pillar was ranged from 2 to 7.5 μ m (Figure 6a). Finite-element method (FEM) analysis was performed to determine the displacement owing to horizontal traction force (F), indicating that traction force applied by the chondrocytes on top of the pillars is in the range of 2.5 to 5 nN (Figure 6b). Higher horizontal traction forces are needed to cause the same displacement when the force is applied in the middle-height of the VA-MWCNT pillar. Furthermore, when the cellular traction force is applied on the top of the micropillar, the stress (σ) in the bottom of the pillar increases (Figure 6c). Indeed, SEM images revealed that the VA-MWCNT micropillars detached from the Si/SiO₂ substrate (Figure 6d).

Figure 6. Bending of the VA-MWCNT pillars. *a.* SEM image from a top view of the VA-MWCNT and Si/SiO₂ pillars in stripe micropatterned templates, showing the bending of VA-MWCNT due to the traction force from the chondrocytes. Scale bar: 20 μ m. *b.* Graphical representation of FEM simulation of the displacement resulting from the application of a specific force on the middle of and the top of the pillars in the VA-MWCNT and Si/SiO₂ pillars. *c.* Representation of the von Mises stress of the VA-MWCNT pillar with 20 nN force acting at the middle and top of the pillar. *d.* SEM image (sample tilted in 45°) showing the detachment of the VA-MWCNT pillar due to the force exerted by the chondrocytes (white arrow). Scale bar: 5 μ m. *e.* Schematic representation of the measurement of the displacement (δ) of the pillars after bending.

4. DISCUSSION AND CONCLUSION

Here we demonstrate that VA-MWCNT micropatterned with optimal dimensions provided ideal topographical and mechanical environment for unidirectional orientation of chondrocytes. Cell attachment interplayed with VA-MWCNT at nanoscale level. It is well established that topography cues play a major role on cell morphology and proliferation[8,10,11,27]. By optimizing the pillar size and spacing of VA-MWCNT micropillars, we were able to control chondrocyte proliferation achieving unidirectional orientation either in straight lines or in circular patterns. Considering the cartilage tissue structure, our results using the grid, stripe and spiral patterns provide a wide overview of what is achievable in terms of cell orientation in pseudo-3D templates.

In contrast to our novel work described here, most of the previously published studies have been applying protein pattern to control focal adhesion points and cell morphology[28,29] or creating polymer-based micropillars[26,27]. In the first case, the templates lack essential three-dimensional (3D) features, while the second is limited to microscale topography. The nanostructure offered by our VA-MWCNT micropillars provided anchorage points mimicking protein or collagen structures but as well created a pseudo-3D environment. These key features increased the complexity of the template, leading to chondrocyte attachment and proliferation while they are hanging on to the micropillars instead of resting in the flat substrate.

It has been reported that cell signalling, proliferation, differentiation and migration are also governed by the mechanical properties of the ECM[23,30,31]. Our VA-MWCNT offered Young's Modulus in the range of the natural articular cartilage ECM, providing optimal mechanical cues for cell attachment and proliferation. The cell response to substrate rigidity at

microscale (e.g. sensing the rigidity between adhesion sites) and/or at nanoscale (e.g. sensing receptor-ligand binding sites) is unclear. Recent reports[26] suggest that rigidity sensing occurs at micrometre scale based on the non-observed changes of the focal adhesions. This particular work employed polymer-based micropost arrays, which do not have nanoscale features similarly to our Si/SiO₂ templates. Furthermore, current micropillar tensile strength arrays[32–37] use micropillars with very small inter pillar distances leading the cells to grow on a microrough “flat” surface, while the approach in our model provides a pseudo-3D environment closer to what cells encounter *in vivo*, enabling the cells to interact with the nanostructures of the micropillars mimicking ECM proteins. We have shown here that the cell adhesion can also change from microscale to nanoscale accordingly to the pillar structure, i.e. microstructure only (Si/SiO₂) or nano/microstructure (VA-MWCNT). By using FEM analysis, we were able to demonstrate that the chondrocytes traction force on one pillar is in the range of 2.5 to 5 nN. This finding is consistent with the observation of human mesenchymal stem cells traction force per focal adhesion reported to be in the range of 2-8 nN[26].

One of the main concerns when working with CNT for tissue engineering is the cytotoxicity of the CNTs. It was shown that, when CNT are freely in solution with the cells, the intracellular uptake of MWCNT is decreased when the MWCNT have adsorbed some proteins[38]. VA-MWCNT micropillar templates supported cell proliferation and survival better than the reference templates to 7-day period experiment. Moreover, significant ECM production (i.e. collagen II and aggrecan) was observed on VA-MWCNT micropillars templates. It is important to mention that in contrast to co-culture studies, the CNTs are currently attached on a rigid surface and do not detach as individual nanotubes during our experiments. Thus, no signs of cytotoxicity were observed during our studies.

In the future development of our approach, VA-MWCNT templates could be employed to investigate the influence of mechanical cues on cell regulation at micro- and nano- levels in pseudo-3D conditions, using the nanostructure of the VA-MWCNT as well as the Young modulus of the micropillars compatible with ECM in various tissues. In addition to fundamental understanding of cell regulation, these templates could be used to create models to predict diseases where cellular traction force is relevant (e.g. scleroderma, fibrosis, connective tissue disorders,...). Furthermore, the ability of VA-MWCNT to control unidirectional orientation growth in our pseudo-3D model could be exploited in complete 3D scaffolds to address several challenges currently faced by investigators in the field of tissue engineering.

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AUTHOR CONTRIBUTION

The study was designed by L.J. and G.S.L. Growth template were made by O.P and M.S., cell culture and immunostaining were performed by L.J. and M.S., AFM measurements were conducted by G.S.L. and L.J., FEM analysis were performed by M.L. Data-analysis and image treatment was performed by L.J. The results were discussed and interpreted by L.J., G.S.L. and A.M. The manuscript was written by L.J. and G.S.L. with the kind assistance of each co-author.

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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AUTHOR CONTRIBUTION

The study was designed by L.J. and G.S.L. Growth template were made by O.P and M.S., cell culture and immunostaining were performed by L.J. and M.S., AFM measurements were conducted by G.S.L. and L.J., FEM analysis were performed by M.L. Data-analysis and image treatment was performed by L.J. The results were discussed and interpreted by L.J., G.S.L. and A.M. The manuscript was written by L.J. and G.S.L. with the kind assistance of each co-author.

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: