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Endothelial cell alignment on cyclically-stretched silicone surfaces

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Endothelial cells at the interface between the bloodstream and the vessel wall are continuously subjected to mechanical stimulation *in vivo*, and it widely recognised that such stimulation plays an important role in cardiovascular physiology. Cell deformation is induced by mechanical forces such as cyclic stretch, fluid shear stress, and transmural pressure. Although much of the work in this field has dealt with the effect of fluid shear stress, very little is known about how cyclic forces modulate and alter the morphology of single endothelial cells, and thereafter, how they effect the confluent layer of endothelial cells lining the vessel wall. The aim of this study is to investigate the response of endothelial cells when subjected to substrate deformation of similar magnitude to those experienced *in vivo*. Human umbilical vein endothelial cells (HUVEC) were cultured on plasma-treated silicone strips and uni-axially cyclically stretched using a custom made mechanical device. Results showed that endothelial cells subject to 10% deformation for as little as 4 h reoriented perpendicular to the stretch direction. In addition, although no integrin coating was applied to the substrate, it was found that plasma-treated silicone provided a cell adhesion substrate comparable to the commonly used collagen type I. Thus the results show that the stretch stimulus alone affects the morphology of endothelial cells. Further studies are required to establish the relative importance of substrate strain vs. fluid flow stimuli.
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1. Introduction

In the last decade many studies have considered the behavioural response of endothelial cells when subjected to mechanical stimulation. Researchers have attempted to use devices to apply the mechanical stimulation [1–6]. Studies have recognised that the arterial pulsatile flow acts in combination with the cyclic stretching and the relaxation of the vessel wall [7]. It was Ives *et al.* [4] who first reported a longitudinal stretching system. In this work, they reported that over a period of 66 h stretching, at 1 Hz and 10% strain, both human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial (BAECs) cells were almost perpendicularly aligned to the direction of the membrane cyclic deformation. In a more recent study, Wang *et al.* [5] also found reorientation and alignment of human aortic endothelial cells when cultured on silicone membranes and subjected to 5–10% strain at 0.25–1 Hz frequency range for 3 h.

Although these two studies showed the reorientation and alignment contribution induced by the mechanical forces to the endothelial cells during *in vitro* experiments, the repeatability of these tests is partly compromised by the large number of parameters involved. In fact, in Ives *et al.* [4] and Wang *et al.* [5], and others studies [8–12], factors such as the source of the endothelial cells, the culturing conditions, and the substrates and the experimental conditions adopted may be providing non-physiological results. In particular, the extracellular matrix (ECM) mimicked by coated biomaterial substrates on which the endothelial cells were placed might profoundly influence the measured cell spreading and migration.

Therefore, in this paper we engineered the surface properties of a silicone substrate to enhance and promote cell adhesion, spreading and alignment. This can provide a basic understanding of the endothelial cell adhesion response and behaviour when these are not biased by the presence of adhesion promoters. Specifically we test the hypothesis that plasma-treated silicone surfaces can be used for the analysis of endothelial cell reorientation when subject to cyclic stretching without the use of ligand coatings. We aimed to investigate whether or not a few hours of cyclic stretching at ''*in vivo*'' magnitudes would succeed in evoking or inducing reorientation in these cells in the absence of a ligand coating to the substrate.

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2. Materials and methods

2.1. Substrate treatment

The material used in this study is a commonly available silicone polymer, polydimethylsiloxane (PDMS). PDMS is a widely used biomedical material for its biocompatibility, sterilisability, thermal, mechanical, and chemical properties [13]. Therefore, a commercially available silicone elastomer film based on PDMS (Goodfellow, UK) was chosen. Plasma treatment was employed to increase the hydrophylicity of the PDMS prior to cell culture analysis. Treatments were carried out in air using a power of 150 W (Europlasma Junior Plasma System, Europlasma NV, Oudenaarde, Belgium), under a pressure of 220 mtorr for 10 min. The ideal plasma treatment conditions were determined using a range of techniques to examine the wettability and the roughness of the PDMS surface. Water contact angle analysis was carried out on a FTÅ Contact Angle Instrument, using a sessile drop technique, over an average measurement of 10 drops per treated substrate (error $= \pm 1°$). In order to control and maximise the substrate hydrophilic property the hydrophobic recovery was measured at different time intervals after the plasma treatment, by contact angle. In addition, the influence of the alcohol on the plasma treated surface was also examined, since the silicone substrate was sterilised with alcohol before cell seeding. Thereafter, the surface morphology of the modified silicone substrates was investigated using white light scanning interferometry and scanning electron microscopy.

2.2. Cell culture

The endothelial cells used in this work were HUVECs, obtained from American Type Culture Collection (CRL-1730, VA, USA). These cells were cultured in Dulbecco's modified eagle medium (DMEM, GIBCO, MD, USA) in a humidified incubator (37 °C and 5% CO_2) until they were subconfluent. They were then serially passaged using a solution of 0.25% Trypsin-0.03% EDTA (Trypsin/EDTA, GIBCO, MD, USA) at a subcultivation ratio of 1 : 2 to 1 : 3. During cell culturing the medium was changed every second day. Cells were then seeded on hydrophilised silicone membranes placed in plastic Petri dishes and cultured until sub-confluent, between three and four days. Cell seeding was carried out immediately after membrane treatment. For the full cell culture length on the silicone substrate there was no evidence in change in cell morphology or growth rate compared to the standard endothelial culture on a plastic flask.

2.3. Stretching apparatus

A mechanical apparatus was designed to deliver a controlled cyclic uniaxial stretch (Fig. 1). This employed a 12 V d.c. geared motor (#225-9605, Radionics, UK) to drive a cam to deliver the cyclic stretching to a linear slide assembly to which the silicone strip was firmly attached. The slide assembly had a 26-mm stroke (#BWU25-45, Ondrives Ltd, UK). Different cams could, in principle, be used to deliver different loading profiles; in this study an eccentric circular cam was used

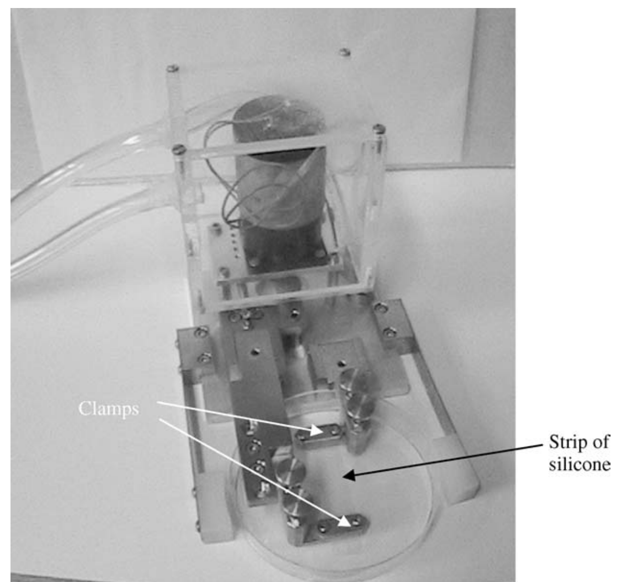

Figure 1 Photograph of the stretching device. The strip of silicone is held between the two clamps within the dish. The strip is placed under an optical microscope for observation of the cells.

to deliver 4 mm displacement. Since the dimensions of the strip of silicone used were 10 mm wide, 40 mm long and 100 μm thick, this resulted in 10% strain. Particular emphasis was placed on the accuracy of the design features and manufacturing to ensure that the clamping and stretching of the silicone membrane resulted an even force distribution on the silicone strip. Moreover, to ensure the video-capturing of the stretching endothelial cells, the rig had to be designed to fit under an inverted microscope. Finally, to allow sterilisation and reduce the possibility of contamination the rig was manufactured in stainless steel.

2.4. Experimental setup

The silicone strips were rinsed with medium to remove unattached cells to the surface. Testing and control samples were then submerged into a fresh medium solution in two different petri dishes. The silicone membrane to be tested was carefully secured into the rig-clamps and then placed over the microscope table stage for time lapse analysis (Nikon Eclipse TE300). The rig was then calibrated in conjunction with the digital camera attached to the microscope, in order to reduce image distortion and then, images of different fields were taken from both testing and control sample. After that the cells were stretched by 10% (1 Hz) for 4 h and a sequence of images was captured with a capturing frequency of 1 h. For the duration of the experiment the temperature of the medium was set at 37 ± 0.1 °C.

2.5. Substrate

Surface roughness and morphology was qualitatively analysed using a scanning electron microscope (SEM) (Hitachi S-4300 Field Emission, Hitachi High Technologies, CA, USA). Quantitative analysis was performed using a white light scanning interferometer (WLSI) (Zygo New View 100, Zygo Corp., CT, USA) to measure the apparent roughness R_a values pre and post

plasma treatment and to assess the amount of micro-roughness and macro-roughness (waviness).

2.6. Cell orientation analysis

Morphometric investigation of cells was measured on different parameters using Scion image (Scion Corporation, MD, USA). Parameters such as perimeter, alignment angle and occupied area were quantified for each cell $(n = 405)$ in 13 separate experimental tests. From the 2-D image, unattached cells could be distinguished because of the spherical shape whereas cells attached to the surface start immediately to spread, stretch, flatten and successively polarise. Since the perimeter of the cell is the shortest line maximising the contained area, any morphological change in cells trying to adhere to the silicone substrate is immediately reflected by a change in the perimeter length. Thus the perimeter value was chosen as a threshold to distinguish between adherent and non-adherent cells to include in the analysis. A preliminary tests on cell adhesion and attachment were carried out on a smaller sample of cells $(n = 21)$ in order to distinguish between attached and non-attached cells. From the morphometric analysis the perimeter of each cell was easily calculated and an average perimeter value of $66.46 \pm 10.60\,\mu\text{m}$ was found for the adherent cells.

In order to assess the orientation of the cells along the stretching substrate, before and after the cyclic test on the silicone substrate, the angle of alignment relative to the stretching direction was measured for each cell from the acquired images by tracing the contour perimeter of each cell inside the field of view. Using Scion image integrated functions the major and minor axis of the best-fit ellipse was determined. This allowed calculation of the orientation angle relative to the stretching direction, represented by the stretching direction, before and after testing. In order to equally evaluate cell positive (0–180°) or negative (180–360°) orientation, with respect to the perpendicular to the baseline, the calculated angles were converted into a 0–90° range, with 90° corresponding to the perpendicular direction to the stretching direction. Then, a statistical analysis was performed using a two-sample *t*-test assuming unequal variances to estimate the statistical significance in cell orientation distribution with no stretching, or after the application of a 10% strain for 4 h.

3. Results

The hydrophilic superficial properties of silicone substrates were enhanced by the air plasma treatment. Although the treatment was subjected to a rapid decay of hydrophilicity over time, the surface tension of the cured substrate promoted cell adhesion. This was mainly associated with the strong interface bond between substrate and cell culture, dispersed in medium solution. Under the same experimental procedure a comparative analysis was also carried out to quantify the loss of hydrophilicity (water contact angle measurements) between the two standard storage procedures used in cell culture: desiccated air and alcohol preservation. It was found that the desiccated air storage had a less

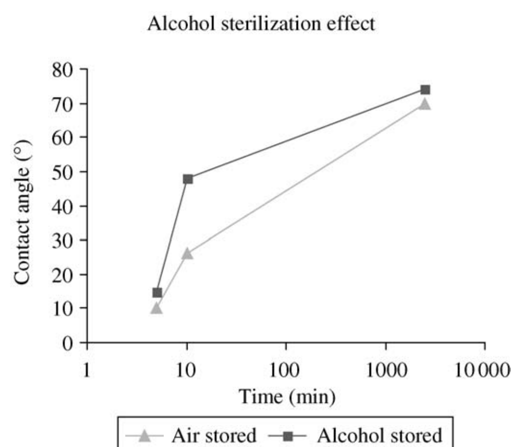

Figure 2 Water contact angle measurements of plasma treated silicone samples.

aggressive oxidation on top of the substrate compared to the alcohol solution, although the end result was a drastic loss of hydrophilicity as shown by Fig. 2. Since the hydrophilic surface was important for cell adhesion, these results seemed to indicate that in order to maximise the usage of the enhanced hydrophilic condition, it was necessary to expose the cells to the sterilised surface immediately after plasma treatment.

Another substrate property that was characterised in this study was the superficial roughness of the treated substrate (apparent roughness). By carrying out SEM investigation it was shown that air plasma treatment modified the surface morphology of PDMS in two different ways and at two different scale levels. At the beginning of the process the plasma treatment uniformly etched the surface of the silicone substrate and then, after 10 min, an irregular distribution of channels began to appear, as shown in Figs. 3 and 4. This was also confirmed by a further quantitative analysis of the surface, by using WLSI that showed a correlation between exposure treatment time and surface apparent roughness. In fact it was found that the apparent roughness (R_a) significantly $(p < 0.0001)$ increased from $0.259 \pm 0.184\,\mu\text{m}$ for untreated silicone to $0.677 \pm 0.098\,\mu\text{m}$ for the air plasma treated substrates $(n = 18)$. Micro and macro roughness where then measured and analysed by WLSI (Fig. 5) for the

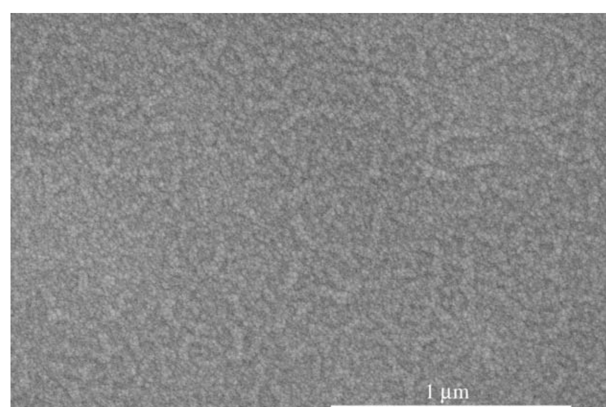

Figure 3 SEM image of untreated PDMS at a magnification of $5 * 10^4$. The grainy-like surface is clearly visible, probably due to polymer processing.

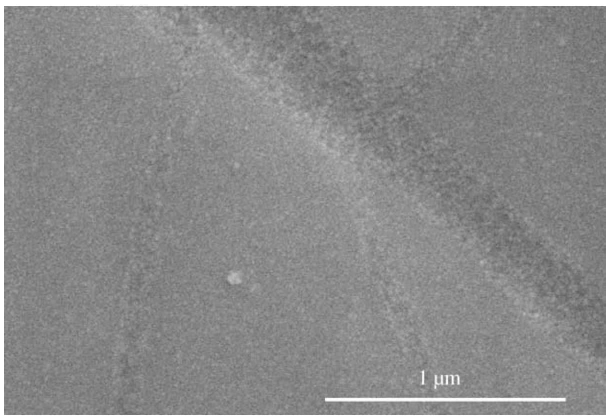

Figure 4 SEM image of plasma treated PDMS at a magnification of $5 * 10^4$. Channels are starting to form on the surface of the polymer, suggesting that an etching process is going on at microscopic level.

untreated and treated substrate used in this study, see Table I for quantification.

Despite the fact that the plasma treated substrates were not coated with cell-adhesion promoters, subconfluent

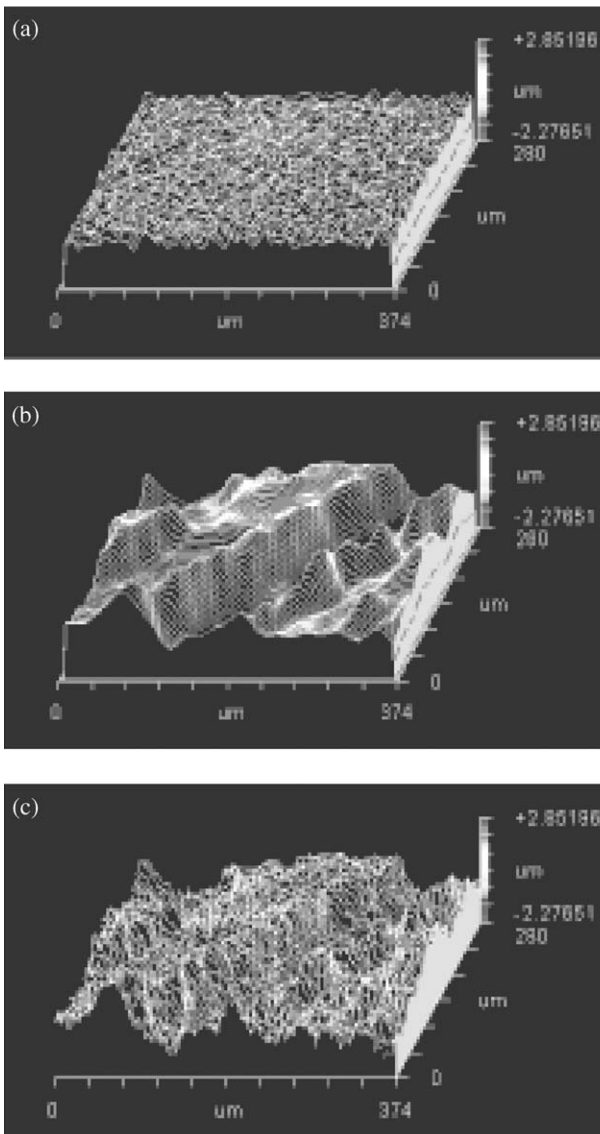

Figure 5 WLSI software elaboration of PDMS plasma treated surface, (a) micro roughness, (b) macro roughness (waviness), and (c) overall roughness.

TABLE I Micro and macro surface roughness measurements of plasma treated silicone samples

Sample	Macro-roughness (μm)	Micro-roughness (μm)
Untreated	0.387 ± 0.117	0.056 ± 0.009
Plasma treated	0.654 ± 0.028	0.119 ± 0.001

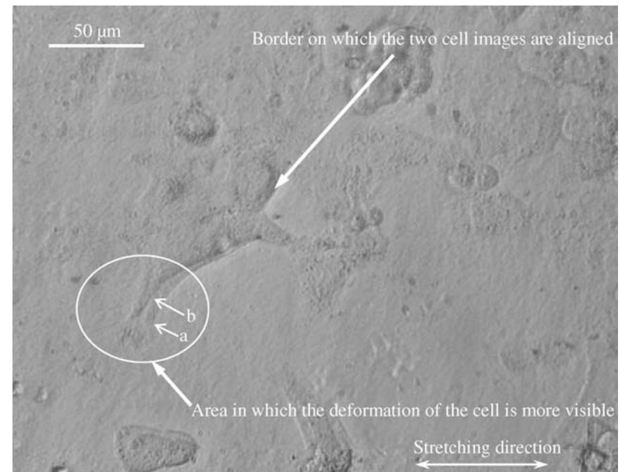

Figure 6 The superposition of two images shows how the cell deforms following the substrate deformation. The two small arrows show the two different contours of the cell in its original (a) and deformed (b) status. Magnification is 400×.

cultured HUVECs adhered to the substrate for the full length of the experiment. Clear evidence of cell adhesion was found by superimposing the adhesion process of a single cell to the surface, as shown in Fig. 6. This highlighted the partial deformation to which the cell was subjected due to the stretching of the substrate.

Quantitative analysis of the orientation of cells pre-stretching showed an isotropic distribution of the cells on the substrate (Fig. 7(a)). After that, by stretch-elongation of the silicone membrane for 4 h at 1 Hz, cell reorientation response was promoted. HUVEC cells started to align perpendicularly to the stretching direction. This aligning behaviour emerged more clearly from the overall analysis of all the cells measured (Fig. 7(b)). Thus, after only 4 h at 10% stretching, cells had a significantly different orientation compared to the un-stretched cells ($p < 0.0001$). An example of HUVEC cells before and after testing are shown in Figs. 8 and 9. From the quantitative analysis of the data acquired it was also interesting to note that the perimeter measurements of cells before testing were 201 ± 54 μm, where after were 203 ± 53 μm. This suggested that there was no correlation between the perimeter parameter and cell orientation and confirms that the perimeter length was a reliable threshold for cell alignment analysis.

4. Discussion

The importance of a better understanding of endothelial cell response in its biophysical environment lies in the fact that a constant loss of morphological heterogeneity occurs in the human arterial endothelium with ageing [2], and events involving endothelial cell perturbation are believed to be related to atherogenesis [3]. From the results of earlier studies suggestions were made that cells

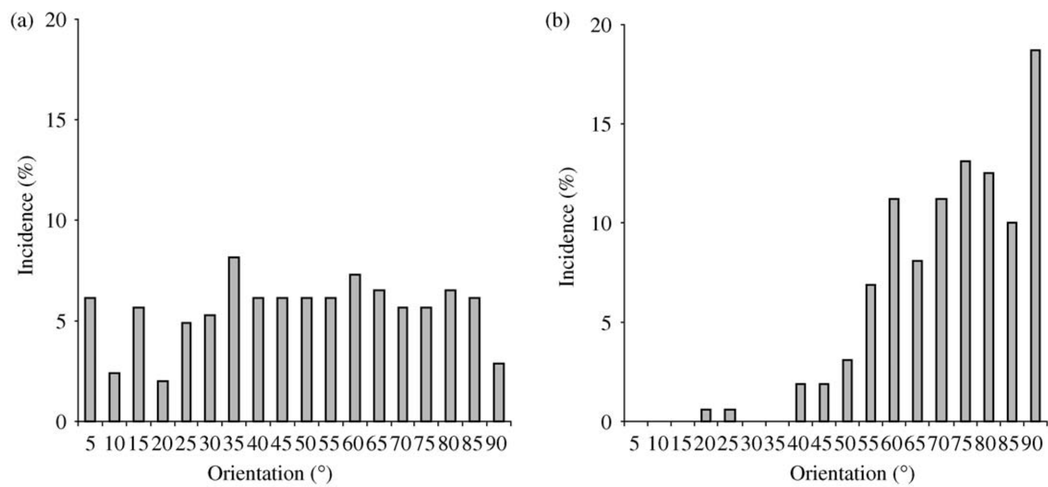

Figure 7 Distribution of cell orientation: before (a) and after (b) stretching. Note that cell orientation has been grouped between 0° and 90°, where 90° represents the perpendicular to the applied stretching direction. The two distributions ($n = 405$) are significantly different ($p < 0.001$).

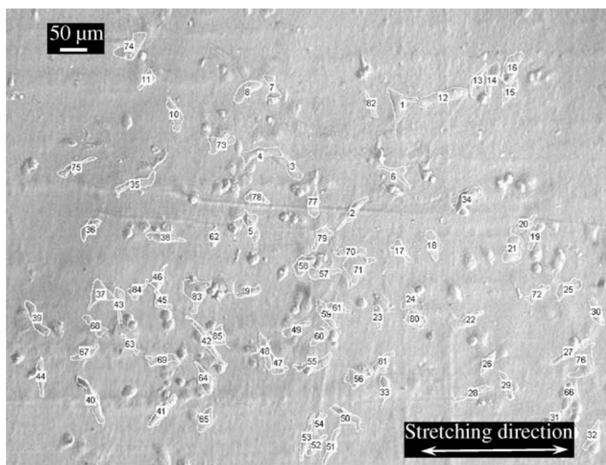

Figure 8 High-resolution photograph of unstretched cells. Note the random orientation of the cells.

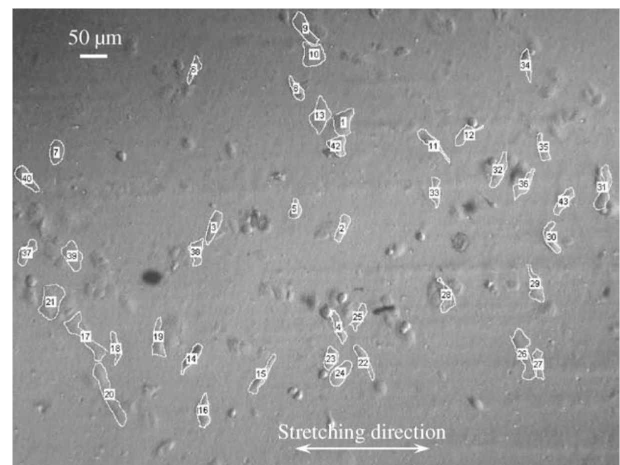

Figure 9 High resolution photograph of cells after 4 h cyclic stretching. Cells start reorienting towards the perpendicular to the stretching direction.

could either orient to avoid stretching or to reduce stress or strain acting on them, or possibly maximise the stretch on the microfilaments. The orientation of the HUVEC cells has been investigated with respect to the direction of stretch and the stretch magnitude [5, 10, 11].

In our experiment, we minimised the influence of the environmental and experimental conditions on cell behaviour by using a non-coated but air plasma treated substrate; plasma-treatment allowed direct cell-adhesion to the surface. Furthermore, the cell population density was kept very low to avoid any cell-cell interactions, and the stretching time was limited to a few hours to avoid any long-term experimental complications such as cell proliferation, CO_2 control or medium impoverishment.

In most of the studies examined the cell culture was carried out on a layer mimicking the ECM (e.g. fibronectin or collagen) in order to promote and trigger cell attachment. Furthermore, when the substrate used to carry the experiments was polymer based, a suitable coating was used to enhance endothelial cell adhesion [14–17], and in particular when using silicone substrate surfaces. Some studies have also reported changes in cell behaviour and morphology as a consequence of the growing of endothelial cells by using different coatings [18] and this indirectly affected the cell morphological response. In our study we cultured cell on substrates which were not precoated, in order to promote the direct attachment of the cells to the surface of the PDMS used as base polymer layer for our experimental analysis. To accomplish this, plasma treatment of the silicone substrates was performed to increase the wettability of the surfaces, as also recently shown by Liao *et al.* [19].

Another well-known problem when working with silicone elastomer substrates is the hydrophylicity time-dependence of plasma treated surfaces [20, 21]. In this study we found that our contact angles and recovery time measurements of silicone after plasma treatment are similar to those found by Hillborg *et al.* [20]. Although this was in contradiction with the results presented by Owen and Smith [22], where they used a thicker PDMS substrate (0.5 mm); these differences highlighted the need of accurate material characterisations of each batch used prior to the experimental testing. In this work, to avoid batch differences, PDMS from a single batch was employed and treated under the same experimental conditions. Differences in surface roughness values were found when compared to Hillborg's study [20]. These differences were already highlighted in the untreated

samples, and could have been increased by the different plasma treatment dose given.

In summary, we showed that plasma treated silicone surfaces provided a suitable environment for cell attachment, allowing cells to remain adherent during and after cyclic stretching of the substrate. This is in agreement with another study [23] where there was no difference in cell adhesion on plasma treated, or protein coated, silicone surfaces, after morphometrical analysis. Also, plasma surface treatment enabled endothelial cells to attach directly on the surface without any kind of interposition between them and the substrate and allowing a direct interaction and response to the mechanical stimulus. This has the advantage of achieving results more dependent on the intrinsic nature of the cells and less influenced by the environment used in investigation.

The reorientation of cells subjected to cyclic stretching has already been shown in different studies. However, in all those early studies [4, 7, 24, 25], cyclic stretching was imposed for periods of time ranging from one to several days. One of the most extensive studies was recently reported by Wang *et al.* [5] who used a bioengineered cell-attachment promoter ProNectin-F to coat a silicone substrate. They found endothelial cell reorientations within 3 h. Our results are in agreement with this, but additionally show that the result can be achieved without the cell-attachment promoter. Our findings show that HUVECs adhere and reorient towards the perpendicular to the stretching direction after only 4 h of stretching at 10% strain. The orientation of cells proves to be significantly different ($p < 0.001$) from the un-stretched control specimen. Wang *et al.* [5] found that human endothelial cells aligned ($p < 0.05$) almost completely in a 3-h time frame; in contrast with the long testing time reported in all early studies.

Differences in the results of various studies can be found, such as degree of orientation or alignment time. To trace the origin of these inconsistencies many different parameters used for the experimental setups should be considered and monitored. In fact, Ives *et al.* [4] suggested that the alignment rate was not dependent on initial cell shape, but was influenced by the degree of confluence, whereby the cells in less confluent areas aligned more rapidly. Iba *et al.* [7] noted that cells from different species and from different vascular beds align differently; besides, the use of different stretching apparatuses, culture and stretching conditions should be considered. Also the use of different surface coatings or treatments could suggest that cells might sense mechanical stimuli from the substrate in a different way.

In conclusion, in this study it was shown that HUVEC's subjected to 10% stretching for short period of time (less than 4 h) significantly reorient perpendicularly towards the stretching direction as a response to the cyclical mechanical stimulus, and this result is independent of any cell-adhesion promoter on the substrate surface.

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