Electrophoretic coating of amphiphilic chitosan colloids on regulating cellular behaviour

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In this communication, we report a facile nanotopographical control over a stainless steel surface via an electrophoretic deposition of colloidal amphiphilic chitosan for preferential growth, proliferation or migration of vascular smooth muscle cells (VSMCs) and human umbilical vein endothelial cells (HUVECs). Atomic force microscopy revealed that the colloidal surface exhibited a deposition time-dependent nanotopographical evolution, wherein two different nanotopographic textures indexed by ‘kurtosis’ ($R_{kur}$) value were easily designed, which were termed as ‘sharp’ (i.e. high peak-to-valley texture) surface and ‘flat’ (i.e. low peak-to-valley texture) surface. Cellular behaviour of VSMCs and HUVECs on both surfaces demonstrated topographically dependent morphogenesis, adherent responses and biochemical properties in comparison with bare stainless steel. The formation of a biofunctionalized surface upon a facile colloidal chitosan deposition envisions the potential application towards numerous biomedical devices, and this is especially promising for cardiovascular stents wherein a new surface with optimized texture can be designed and is expected to create an advantageous environment to stimulate HUVEC growth for improved healing performance.

1. Introduction

Cell-to-cell and cell-to-extracellular matrix (ECM) contacts are biologically critical for $in$ vivo formation of tissues and organs [1]. Numerous molecular mechanisms by which cells find out and respond to their surrounding environments have been extensively studied, including integrin–ligand interactions [2,3], influences of surface wettability [4] and topographic interactions of matrices [5–7]. Concerning the significance of cell adhesive surfaces in biomedicine and biotechnology, the advancement of $in$ vivo biomimetic scaffold materials enabling cell attachment and proliferation with an appropriate topographic texture has been widely studied [8]. Generally, these scaffolding materials are composed of biological and synthetic components that have been shown to regulate cellular responses $in$ vitro. The $in$ vitro control of the cellular environment is a major problem for managing both cell evolution and scaffold function in the field of bioengineering. The majority of $in$ vitro studies have used 316L stainless steel during cell culture, whose topographies are the most widely used substrate materials in implantation. Notably, three-dimensional condition supplying a circumstance assist for cells $in$ vivo had been shown conclusive for various pathological and physiological processes in numerous studies [9].

Nanotopographically engineered surfaces can manipulate biological behaviour ranging from protein adsorption, cytoskeletal arrangement and cell adhesion as well as spreading [10,11]. Therefore, to mimic cell adhesion and subsequent behaviours in an $in$ vitro condition, the surfaces of cell-carrying scaffolding materials are required to present biocompatible touching sites in a periodic nanoscale dimension. Nonetheless, the interactions between cells and their surfaces in controlling the specific cell behaviour remain unclear, in regards to their physical and/or chemical nature.
As one of the most well-known examples, with increasing attention towards the inflammatory process in injured arterial wall over recent decades, it has been well recognized that a great extent of migration and exaggerated proliferation of vascular smooth muscle cells (VSMCs) in the lumen predominates the narrowing of the injured artery [12]. In local anti-proliferative strategies, inhibition of VSMC proliferation has been demonstrated from in vitro to clinical trials by using drug stent coatings (paclitaxel, actinomycin-D, rapa-my-cin, etc.), decreased neointimal thickening in animal models of restenosis and produced prospective results in tentative human studies [13,14]. However, after stent implantation, some side effects including late endothelialization process caused by high local toxicity and late thrombosis were observed. Therefore, a new strategy to minimize or reduce restenosis by arresting VSMC growth and decrease collagen production without altering and/or improving reendothelialization has been proposed, in order to overcome the challenges associated with their use. Drug eluting stent technologies were composed of sustained drug release systems encapsulating anti-proliferative drugs to inhibit the proliferation of smooth muscle cells and they have been discovered to reduce the rate of in-stent restenosis [15,16]. Nevertheless, the anti-proliferative role of the drugs may impede endothelialization and blood is uncovered to the stent struts and/or to the surface coating, obviously increasing the proclivity of thrombosis [17,18]. Delayed or impaired endothelialization also limits the long-term success against restenosis [17,19].

A significant number of VSMCs undergo apoptosis (in humans, 20–30%; in a rat carotid model, 60–70%) in the injured artery within 1 h after angioplasty has been studied [20]. To prevent restenosis, drugs need to be delivered at sufficient concentrations for a prolonged period of time. However, recent studies demonstrated that the delivery efficiency and intramural deposition of drugs remain rather low and advantageous solutions remain to be explored [21,22]. The need for such a graft is evident as peripheral vascular disease affects millions of people worldwide [23]. The VSMC plasticity can be viewed in response to vascular injury when VSMCs significantly increase their proliferation, migration and molecular regulation playing an important role in vascular repair [24,25]. Improvements in drug interaction of these devices might be attained through the use of coating surfaces with specific nanotopography.

Surface modification to gain biocompatibility has been a key aspect in biology and biotechnology, including cell expansion, biomaterials development and preparation of substrates for regenerative medicine [26–28]. Thus, surface engineering is of utmost significance for devices with improved biological performance [29,30]. Many topographical characteristics, including pores, wells, steps, grooves, ridges and nodes in micro- or nanoscale [31,32] have been evaluated to a wide variety of cells: endothelial cells and smooth muscle cells [33,34]. Since the arterial wall tissue possesses a high degree of nanometre surface roughness, it is surprising that current efforts to simultaneously control human umbilical vein endothelial cell (HUVEC)/VSMC behaviours with modified surface topography, for the most part, have not yet focused on this approach.

Various surface topographies may alter different cell responses such as advancement of cell movement, cytoskeletal reorganization and changes in gene expression [35]. The cellular response to topographic surface patterning can be regulated by the size and topography of the patterns and their regularity [36]. The response of cells to random features was found to be cell type-dependent [37]. To investigate cell behaviours in biocompatible and biomimetic conditions in a nanoscale manner, we developed a highly biocompatible coating onto stainless steel using colloidal amphiphilically modified chitosan (termed as CHC (carboxymethyl-hexanoyl chitosan)) colloids that were developed in this laboratory [38]. Apart from existing periodic surface patterning reported in the past, here we employ a simple, cost-effective colloidal coating via an electrophoretic deposition approach to form a surface with controlled nanotopologial texture. The resulting CHC coating provides endothelial cells with selective advantages in adhesion, spreading, viability and proliferation; but for smooth muscle cells, showing disadvantages in adhesion, spreading, viability and proliferation. The coating design is composed of one basic component that is equally critical for optimal functionalization of the metal surface in order to decrease VSMC proliferation; and in this study, VSMCs were employed as a model cell for demonstration and HUVECs as a comparative group. Our results suggested that after a simple but delicate control of the nanometre-sized topographical modification, the resulting coated surface exhibited a completely distinct in vitro environment compared with the bare surface, and thereby facilitated a better manipulation and understanding of cell behaviours that resemble those that occur in an in vivo environment.

2. Material and methods

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4’,6-diamidino-2-phenylindole (DAPI), phosphate-buffered saline (PBS), absolute ethanol, and rhodamine-phalloidin were purchased from Sigma. Amphiphilically modified CHC, having a molecular weight of 120 000–150 000 g mol⁻¹, was purchased from Advanced Delivery Technologies Inc. (Hsinchu, Taiwan). All other chemical reagents in the study were of analytical grade and used as received without further purification.

2.1. Electrophoretic deposition

The stainless steel plate was coated with amphiphilically modified chitosan nanoparticles (CHC) with an average size of 23.5 ± 0.7 nm in diameter, being successfully developed in this laboratory [38], by electrophoretic deposition (EPD). EPD was achieved via the forced motion of charged particles under an applied voltage towards an electrode of the opposite charge, followed by the coagulation of the particles to form a deposit. The electrodes used in the present work were a platinum chip (100 × 30 × 1 mm) and a stainless steel plate (316L, 50 × 10 × 1 mm). The metal substrates were carefully cleaned by sonication in acetone, ethanol and deionized water sequentially at room temperature for 30 min and were arranged in a 15 mm apart parallel configuration. Both electrodes were immersed in a container with a liquid medium containing CHC, prepared by a mixture of ethanol and deionized water. The portion of ethanol in the mixture of ethanol and deionized water was optimally selected after preliminary experiments. The prepared CHC solution was dissolved and diluted using the deionized water (containing 10% ethanol) to a concentration of 0.5 mg ml⁻¹. EPD was carried out under various time intervals. A constant DC voltage of 3 V with a 1 min EPD time was applied to generate a sharp (i.e. high peak-to-valley texture) surface (termed as CHC-1) and with a 5 min deposition to form a flat (i.e. low peak-to-valley texture) surface (termed as CHC-5). The stainless steel plate and platinum chip were used as an anode and a cathode,
respectively. The EPD was carried out in an ice bath in order to diminish the heat generated during the process.

2.2. Electron spectroscopy for chemical analysis
The surface chemistry of the CHC coating was characterized by electron spectroscopy for chemical analysis (ESCA, Ulvac-PHI PHI 1600) with 15 kV Al/Mg X-ray source. In this study, all the ESCA measurements were performed by using Mg Kα emission at 1253.6 eV. A single survey scan spectrum (0–1100 eV) and narrow scans for C 1s (280–300 eV) were recorded for each sample with a pass energy of 1 and 0.1 eV, respectively. Background was subtracted by using the Shirley formula. The deconvolution was done by fitting the spectra to multiple peaks comprising a Gaussian function. The charge effect was compensated by setting the binding energy of alkyl carbons, which do not link to oxygen or nitrogen, at 284.5 eV.

2.3. Surface characteristics
To visualize nanotopography of stainless steel by EPD, scanning electron microscopy (SEM; S6500, JEOL, Japan) was used. SEM samples were prepared on cleaned stainless steel surface with various time intervals by EPD. The samples were dried under vacuum for 24 h and coated with a gold layer for SEM examination. The surface hydrophilicity of the substrates was characterized using contact angle measurement. A droplet of deionized water (volume = 2 μl) was first placed on the substrates at room temperature, and then the sessile contact angle was determined to derive the hydrophilicity after the droplet was stabilized. Atomic force microscopy (AFM) apparatus was employed (Digital Instruments, Veeco, Plainview, NY, USA) for surface texture examination by scanning at least three different locations of CHC-coated stainless steel surface over an area of 5 × 5 μm in tapping mode. The spring constant was 37 N m⁻¹ with a resonant frequency of 175 kHz.

2.4. Cell culture
The VSMCs from Food Industry Research and Development Institute (Hsinchu, Taiwan) were cultured in Dulbecco’s modified Eagle medium supplemented with 10 per cent foetal bovine serum and 1 per cent antibiotic antitoxycotic solution (Gibco, USA) in a humidified atmosphere containing 5 per cent CO₂ in air at 37°C. HUVECs were grown in M200 medium and low serum growth supplement (Invitrogen Molecular Probes, Carlsbad, CA, USA). Culture flasks were coated with 1 per cent gelatin (Sigma, USA). Cells were cultured at 37°C in a humidified 5 per cent CO₂ incubator. Only passages 4–7 were used.

2.5. Adhesion, spreading and cytoskeleton analysis
Early adhesion of VSMCs was analysed on CHC-coated stainless steel surfaces for various time intervals (i.e. 0.5, 1, 2 and 4 h for short periods). Prior to adhesion experiments, cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) and re-plated at 4 × 10⁵ cells per well on the aforementioned substrates present in 24-well culture plates (37°C and 5% CO₂). After various time intervals, the non-adherent cells were washed with PBS, and the remaining adherent cells were stained with 1 mM Calcein AM (Invitrogen). Relative cell adhesions were quantified by counting the number of cells on different locations (at least six different locations per surface) of the surface using a fluorescent microscope. Spreading and cytoskeletal organization of VSMCs were examined on CHC-coated stainless steel surface at 2 h. Preparation of the sample to be examined at 2 h incubation was the same as the sample for cell adhesion experiments. After various time intervals of incubation, cells were fixed with 3.7 per cent formaldehyde and permeabilized in 0.1 per cent Triton X-100. Cells were subsequently washed twice with PBS and incubated at room temperature with rhodamine-phalloidin overnight. After washing with PBS, cells were stained with DAPI for 1 h. The samples were observed by fluorescence confocal microscopy with a Nikon C1 Plus confocal system. The interactions of VSMCs with matrix were confirmed using SEM. VSMCs were cultured on CHC-coated stainless steel surface in the same way as described above. Following various time intervals of incubation, VSMCs were fixed with 2.5 per cent glutaraldehyde and stained with 1 per cent osmium tetroxide solution at room temperature for 0.5 h each, sequentially. The cells were washed twice with PBS, dehydrated in a series of ethanol-containing solutions with increasing ethanol concentration (10 min for each gradient), and finally dried with a critical point dryer (HCP-2, Hitachi). The samples were coated with a gold layer for SEM examination.

2.6. Viability and proliferation of cells on CHC-coated stainless steel
Viability of VSMCs was analysed on CHC-coated stainless steel surface for various adhesion time intervals (i.e. 0.5, 1, 2 and 4 h for short time periods; and 24, 48 and 72 h for long time periods). Viability of HUVECs was demonstrated on CHC-coated stainless steel surface for various adhesion time intervals (24, 48 and 72 h). The investigation of cell viability is a common method to evaluate the biocompatibility of biomaterials. The cell viability of CHC-coated surface was evaluated using HUVECs and VSMCs by counting the number of live cells by staining dead cells with trypan blue. Cells were seeded onto CHC-coated surface at densities of 4 × 10⁵ cells per well in a 24-well culture plate (Corning, USA). At predetermined time durations, the medium was aspirated and the wells were washed twice using PBS solution to remove non-adherent cells. Then, the adhered cells were collected with trypsin-EDTA and counted with a hemocytometer. Proliferation of cells was evaluated from a standard curve obtained for different densities of HUVECs and VSMCs on 24-well culture plate at 72 h, and the optical density values were assessed with MTT assay. Purple formazan was solubilized with isopropanol and measured in a microplate reader (GDV model DV 990 BV4, Italy) at 955 nm. Data are normalized with respect to the cultured VSMCs at the time period of 24 h of each group.

2.7. Western blotting
Cell lysates were collected after the VSMCs were replated for 24 h on the aforementioned substrates. Equal amounts of whole cell lysates were subjected to sodium dodecysulfate polyacrylamide gel electrophoresis, followed by western blot analyses using anti-Pi3K and anti-actin antibodies.

3. Results
3.1. Surface characteristics
The chemical structure of the CHC-coated surfaces was analysed using C 1s spectra from the ESCA analysis. The deconvolution of C 1s indicated four split peaks, namely C–C (284.6 eV), C–N (285.2 eV), C–O–C (286.5 eV) and C=O (288.2 eV) [39]. The elemental compositions calculated from the wide scan spectra are given in table 1, where the C–N percentage is lowest for 0.05 min deposition (9.53%) but achieves stable values (around 13–14%) for samples with deposition time more than 5 min. The percentages of other bonds did not show significant changes for all samples. For longer time of deposition, the chemical proportions of each bonding showed relatively stable distribution. It indicates that
in the very beginning stage of deposition the C–N group (–NH₂ and –NH–) plays a decisive role in CHC consolidation behaviours on the substrate.

The surface morphology and contact angle measurement were employed to determine the surface hydrophilicity of the aforementioned substrates, and the results (table 2) show that the contact angles are 30.5 ± 1°, 71.4 ± 3° and 75.8 ± 1° for bare stainless steel substrate, high peak-to-valley surfaces (termed CHC-1) and low peak-to-valley surfaces (termed CHC-5), respectively. It is reasonable to consider a shift towards hydrophobic surface compared with the bare stainless steel upon CHC modification, although a contact angle less than 90° has been considered to indicate a certain degree of wettability [40]. As expected, the nanotopographic feature of the surfaces decreased the wetting ability emanating from the surface structure. Figure 1 shows the SEM and AFM images of the resulting surface texture of bare stainless steel and CHC-1 and CHC-5 coatings on stainless steel. Both coating surfaces demonstrated similar average roughness of 24 and 27 nm, respectively (table 2). However, ‘average roughness’ of a given surface appeared relatively featureless in better understanding of the topographic-dependent cellular behaviour. We therefore carried out a profound analysis of nanotopographic characteristic based on the parameter ‘kurtosis’ (Rkur), which represents a key feature of the height distribution, i.e. the ‘peakness’ of the profile [41]. In principle, a surface with a Gaussian height distribution has a kurtosis value of 3; a surface with a narrow height distribution has a kurtosis value greater than 3; while a surface that has a well spread out height distribution has a kurtosis value of less than 3 [42]. It should be noted that Rkur of the surfaces of CHC-1 was distinctly different from that of CHC-5 (p < 0.05). In particular, the larger values of Rkur for CHC-1 are a signature of a more pronounced peakedness of the surface profile in that condition (table 2). Along with the peakedness profile, the surface morphology of CHC-1 was then defined as a sharper surface (i.e. a narrower height distribution) than that for CHC-5 surface.

### Table 1. Surface elemental compositions from ESCA.

<table>
<thead>
<tr>
<th>samples (s)</th>
<th>C–N</th>
<th>C–C</th>
<th>C–O</th>
<th>C=O</th>
</tr>
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<tbody>
<tr>
<td>3</td>
<td>9.53</td>
<td>7.77</td>
<td>59.14</td>
<td>23.56</td>
</tr>
<tr>
<td>6</td>
<td>14.90</td>
<td>6.09</td>
<td>54.97</td>
<td>24.03</td>
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<td>10</td>
<td>14.94</td>
<td>7.78</td>
<td>54.16</td>
<td>23.11</td>
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<tr>
<td>60</td>
<td>13.38</td>
<td>6.14</td>
<td>59.61</td>
<td>20.87</td>
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<tr>
<td>300</td>
<td>13.83</td>
<td>6.75</td>
<td>57.75</td>
<td>21.67</td>
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</table>

3.3. Cell adhesion and viability

To investigate the time-dependent characteristic of the foregoing substrates on cell adhesion, cell adhesion evaluations were performed by re-plating VSMCs at various time intervals (i.e. 0.5, 1, 2 and 4 h). Cell adhesion was measured by counting the retained cells on the substrates. Statistical calculation derived from three repeated experiments (as given in figure 3a) illustrated that cell adhesion increases in association with a prolonged re-plating period up to 4 h. The enhancement of cell adhesion was obviously observed in a protruding increment on the CHC-1 comparing with the CHC-5. These results confirmed that the nanotopographical modification via a simple CHC colloidal deposition on the stainless steel substrates is a predominant factor in the stimulation of cell adhesion. Consistently, the CHC-1 rather than the CHC-5 demonstrated a similar consequence of cell attachment in cell viability of average adhered VSMCs by counting the adhered cell number at various incubation time periods (figure 3b), suggesting a predominant effect of the nanotopographical modification on cellular and physiological functions.

In specific extracellular environments, the shape and adhering nature of cells are related to the effects of nanotopography on cytoskeleton orientation and focal adhesion formation [43]. In the light of the inimitable morphologies, as aforementioned, we investigated the actin cytoskeleton organization of VSMCs that had adhered to the foregoing substrates through rhodamine-conjugated phalloidin immunofluorescent staining. In figure 4a, the distribution of actin filaments (stress fibres) is mainly across the centre of the cell cultured on the bare stainless steel, similar to that seen on glass coverslips (data not shown). However, the VSMCs cultured on the CHC-5 demonstrated F-actin that was predominantly clustered in a punctuate manner, which might express the contact sites of the cell–extracellular nanotopography, as revealed by the arrows in figure 4c. Cortical actin bundles primarily arise at the edges of the cells, identical with less-spread and rounded-up morphology. The delimitation of actin aggregation in one orientation, contrary to the nanoscale contact sites, was not affected by the surface chemistry, since it remained obviously illustrated in the cells that had adhered to the stainless steel coated with colloidal CHC nanoparticles (figure 4b,c) compared with the bare stainless steel (figure 4d). Taken together, it should be noted that the observations of cell interaction through the nanoscale contact sites of the CHC-coated surface offered new insights into mimicking in vivo cell–ECM interactions, which vary extremely from the ordinary cell planar surface interactions currently used in cell culture conditions in vitro.

To estimate how VSMCs interact with the foregoing substrates, the cells were cultured on 1 cm² stainless steel substrates and those coated with CHC nanoparticles (CHC-1 and CHC-5). After re-plating for 2 h, the morphological features of the adhered...
Figure 1. SEM images and AFM images of functionalized substrates: (a) bare stainless steel; (b) sharp surfaces (high peak-to-valley texture, termed CHC-1); (c) flat surfaces (low peak-to-valley surfaces, termed CHC-5). (Scale bar: 1 µm.) (Online version in colour.)

Table 2. An overview of the effects of VSMCs cultured on the various CHC-coated stainless steel substrates.

<table>
<thead>
<tr>
<th></th>
<th>stainless steel</th>
<th>CHC-1 (sharp surfaces)</th>
<th>CHC-5 (flat surfaces)</th>
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<tr>
<td>hydrophilicity (contact angle)</td>
<td>30.5° ± 1°</td>
<td>71.4° ± 3°</td>
<td>75.8° ± 1°</td>
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<tr>
<td>cell morphology</td>
<td>flattened</td>
<td>flattened</td>
<td>rounded</td>
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<tr>
<td>AFM</td>
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<tr>
<td>roughness (nm)</td>
<td>1.09 ± 0.25</td>
<td>23.93 ± 1.31</td>
<td>27.30 ± 1.22</td>
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<tr>
<td>$R_{kur}$</td>
<td>3.30 ± 0.15</td>
<td>2.81 ± 0.15</td>
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<tr>
<td>cytoskeleton</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>central stress fibre</td>
<td>++</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>cortical actin fibre</td>
<td>–</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>protrusions</td>
<td>–</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>punches</td>
<td>–</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>cell viability (VSMCs, 2 h)</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>cell viability (VSMCs, 72 h)</td>
<td>+</td>
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VSMCs were imaged by SEM (figure 4d–f). The VSMCs on the stainless steel substrates appeared similar to those on the ordinary culture dishes (figure 4d). Intriguingly, for the case of CHC-1 (sharp surfaces, figure 4e), the VSMCs stretched out and extended radial nanospikes from the cell peripheries, even though they were visualized as having a rounded-up shape. By contrast, in figure 4f, while VSMCs were cultured on the CHC-5 (flat surfaces), nanospikes are no longer visualized in spite of the cells having a rounded-up shape and being comparatively smaller in whole size.

Biocompatibility of surface coating in a long-term period is an important parameter to evaluate the potential benefit of such a coating in practical considerations. The endothelium is critical in preventing thrombosis, adjusting mass transportation and regulating arterial smooth muscle cell growth and function [44]. The viability of HUVECs at 24 h was found to be comparable on CHC-1, CHC-5 and bare stainless steel surfaces (figure 5a). After analysing early adhesion and spreading of cells on CHC-coated surfaces, a longer term evaluation of the morphology, viability and proliferation of the VSMCs on the substrates was carried out. Surprisingly, the viability of VSMCs was found to decrease sharply on CHC-5 with respect to bare stainless steel surface. Viability of VSMCs decreased to 39.3 ± 9.0% on CHC-5 with respect to bare stainless steel surface (figure 5b). It seems, however, that viability of VSMCs was not altered on the CHC-1 as cells behaved similarly to that on bare stainless steel surface at 24 h period of incubation. Overall, this trend
of decreased viability of VSMCs on CHC-5 strengthened the idea that this coating condition provides an unfavourable environment for this cell type. The adaptive and viable microenvironment provided by CHC-coated surface imparted a selective advantage for HUVECs to proliferate at a higher rate over a longer period of time (figure 5c). The increase in proliferation of HUVECs might be due to the adaptive microenvironment provided by the nanoscale matrix through surface topography, and structure that mimics natural matrix. In addition, CHC-5 demonstrated a coating with considerable decrease in hydrophilicity (table 2), which may dramatically influence the growth of VSMCs on this surface. The growth of HUVECs was previously shown to increase on nanoscale-roughened surfaces [45]. Proliferation rates of VSMCs were also in a similar trend with viability results. The proliferation of VSMCs on CHC-1 was found to be 95.4 ± 5.6% and reduced to 63.8 ± 3.5% on CHC-5 compared with that of cells cultivated on bare stainless steel surface (figure 5d). Despite the fact that VSMCs attached at comparable rates and spread in a similar morphology on CHC-coated and bare stainless steel surfaces, the viability and proliferation of these cells remarkably decreased at 24 and 72 h. This observation gives a strong indication that the deterioration of proliferation rates of VSMCs was a result of the unfavourable surface texture upon a subtle CHC colloidal deposition.

3.4. Biochemical insights

Phosphatidylinositol 3-kinase (PI3K) is an 85 kDa cytoplasmic tyrosine kinase that plays an important role as mediator in integrin-mediated signal transduction pathways in response to cell adhesion, migration, proliferation and so on [46,47]. VSMC proliferation was reported to depend on PI3K signalling [48]. To further explore the effect of the interaction between VSMCs and the aforementioned substrates, we conducted biochemical analyses. A representative outcome is shown in figure 6 for PI3K, a key signalling protein tyrosine kinase which is known to be an important protein of the PI3K/AKT pathway in mediating cell migration and/or proliferation, respectively. The results showed that the PI3K exerted no effect on the CHC-1 and bare stainless steel surface, but a moderate effect on the CHC-5. For HUVEC analysis, there is no difference between bare stainless steel and CHC-coated surfaces (data not shown). This finding suggests that the CHC-5 is unable to promote cell proliferation but retains an efficient survival rate for cells.

4. Discussion

Biomimetic environment cues direct many cell functions, such as adhesion, growth, proliferation, migration and cell death. Initially, the cell adhesion is enhanced on rougher surfaces [49] but more recent studies suggest that the influence of topography on cell behaviour is more complicated and critical. Through physical interactions (i.e. upon physical contact) between cells and surrounding cues/surfaces, some changes in morphology, biochemistry and physiology occur so that the cells can adapt to an appropriate environment and behave in response towards specific extracellular conditions. In the present study, we developed a biomimetic CHC colloidal coating onto bare stainless steel substrate and demonstrated the characteristics of the cell response towards the modified substrate. By analysing the cell morphological appearance and biochemical changes, and by performing functional characterizations of the CHC-coated substrate, we depicted the merit of the CHC-coated substrates for understanding the responses of cells in contact with nanostructured surfaces and for biomimicking the in vivo nanoscale environment for biomedical engineering applications. A previous study indicated that the cellular interactions of HUVECs were affected by wettability and functional groups of artificial materials surfaces [4]. In this study, the results showed that the HUVEC adhesion on CHC-coated surfaces was better than that of VSMCs and was functional group (–NH2) dependent (table 1).

Filopodia are slender, actin-rich plasma-membrane protrusions that function as antennae for cells to probe their environment [50]. Consequently, the filopodia explore from the fringe of the cells, linking onto extracellular surfaces through the formation of adherent structures. These filopodia-like structures are primarily visualized when VSMCs were cultured on the bare stainless steel substrate and CHC-1 (figure 4a,b). However, they were not seen on the CHC-5 (figure 4c). Indeed, a number of studies have examined some properties of scaffold materials including surface wettability and charge influence on cell adhesion, shape and spreading [4,51]. suggesting that improved cell spreading happens on hydrophilic surfaces rather than on hydrophobic surfaces. To the development of tissue scaffolds, numerous natural and synthetic polymers were used [52–54]. According to the literature, a bioactive scaffold would both enhance the rate of endothelialization and specifically inhibit the migration of smooth muscle cells to the graft lumen [55]. However, these scaffolds need more additives to accomplish these effects. In ESCA, the results showed that the CHC-coated surface is rich in hydrophilic
**Figure 4.** Laser scanning confocal microscope images of the cytoskeleton and nuclei of VSMCs cultured on functionalized substrates: (a) bare stainless steel, (b) CHC-1 (sharp surfaces), (c) CHC-5 (flat surfaces). SEM morphologies of VSMCs cultured on functionalized substrates after re-plating cells for 2 h on the substrates: (d) bare stainless steel substrate, (e) CHC-1 (sharp surfaces), (f) CHC-5 (flat surfaces). The arrows in (c) indicate F-actin clustering into small punches. (Online version in colour.)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control</th>
<th>Bare</th>
<th>CHC-1</th>
<th>CHC-5</th>
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<td>24</td>
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**Figure 5.** The relationship between the viability of (a) HUVECs and (b) VSMCs; the proliferation of (c) HUVECs and (d) VSMCs, at the long period of cultured time (24, 48 and 72 h) on the bare stainless steel, CHC-1 and CHC-5. The mean ± s.d. from at least three experiments is shown. *p < 0.05; **p < 0.01; ***p < 0.001; n.s., not significant. All of the p values were compared between the bare stainless steel and the CHC-coated substrates at the same time points. Control is the cells cultured on dish. (Online version in colour.)
expressed as the mean for 24 h, and lysates were collected for western blotting using antibodies to PI3K and beta-actin. (Figure 6.)

- **References**


5. Conclusions

The newly developed nanotopographic CHC-coated stainless steel provided more biological flexibility in manipulating cell morphogenesis and understanding the biochemical, physiological and functional responses of a cell in a biomimicking environment *in vitro*. In summary, the close relationships for cell adhesion and hydrophilicity, cell morphological adjustment and nanotopography of CHC surfaces were investigated. These nanotopographic textures could control VSMC behaviour via a facile colloidal CHC nanoparticle deposition. This understanding may be advantageous in cardiovascular therapies that can be used in conjunction with modified nanotopographic structures to reduce the incidence of restenosis, which is currently under evaluation *in vivo* and will be reported separately. Accordingly, the utilization of nanotopographic CHC-coated stainless steel substrates in biomedical engineering is expected to be beneficial to complex tissue manipulations.

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**Figure 6.** Biochemical analyses of PI3K on the various functionalized substrates. (a) VSMCs were re-plated on the various functionalized substrates for 24 h, and lysates were collected for western blotting using antibodies as indicated. (b) Densitometric analyses of the western blots. The data are expressed as the mean ± s.d. (*n* = 3). *p < 0.05 compared between CHC-1 and CHC-5; ***p < 0.001 compared between bare stainless steel and CHC-5; n.s., not significant. (Online version in colour.)

Groups, but the contact angle measurements showed decreased hydrophilic effect. It was determined that the surface hydrophilicity of CHC-coated substrates was decreased by nanotopographic development of the surfaces. The evidences in correcting the interaction between cell spreading and the nanotopography of the coating were relatively pronounced (figure 4). However, the variation of surface nanotopography after CHC coating influenced the cell behaviour to a considerable extent (figure 4b,c). Experimental data clearly indicated that nanostructured surface played a dominant role in directing cellular behaviours, such as cell adhesion. Substratum topography is a non-incursive and non-biological method of adjusting cell function, because textured surfaces serve simply as an extracellular physical circumstance without incorporating biomolecules. Because the topography of base membranes is composed of complex mixtures of nanometre-sized (5–200 nm) pits, pores, protrusions, striations, particulates and fibres [32,56], nanotopographical surface in controlling cellular behaviour is then expected to be more influential than microtopographical feature. On the other hand, the scale of topography is one of the most important factors determining whether such specific cell reactions occur. Remarkably, current experimental observation strongly indicated that the VSMCs exhibited different propensity of attachment between the CHC-1 and CHC-5 surface, exhibiting a distinct preference for the CHC-1 surface (figure 5). The cellular characteristics of VSMCs were correlated with nanotopographic surface characteristics. As a result, the nanostructured feature of the CHC coating may act as biomimicking surface for a wide variety of biomedical uses.

The distinct responses of VSMCs on the CHC coating with different nanotopological features are compared in table 2. Cell adhesion is mainly determined by the surface hydrophilicity, because the cells may tend to adhere to hydrophilic surfaces rather than to hydrophobic ones. Therefore, the results confirmed that nanotopographic modification of CHC coating decreases the surface hydrophilicity to the extreme by physical domain (surface topographic variation). A comparison of the CHC surface featuring different nanotopographic texture, i.e. sharp versus flat, proved its preferential response to the cells, on which central stress fibres was detected on the CHC-1 surface while a large number of cortical stress fibres was observed on the CHC-5 surface (figure 4b,c). Cortical actin bundles emerged at the edge of the cells, coherent with less-spreading and rounded-up cell behaviours. This work confirms the uses of colloidal CHC coating via a facile electrophoretic assembly to form surfaces preferentially viable for specific cellular behaviours between HUVECs and VSMCs, which gives potential control over those surface features in the design of implantable devices with improved biomedical performance.


