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Stem cell attachment to layer-by-layer assembled TiO_2 nanoparticle thin films

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Abstract

Surface topography is one of the most important factors influencing the attachment and spreading of cells. In the present study, layer-by-layer assembled titanium dioxide (TiO_2) nanoparticle thin films were chosen for attachment, proliferation and spreading studies on mouse mesenchymal stem cells (MSC). Increasing surface roughness was observed with increasing number of layer-by-layer assembled TiO_2 thin films. Four layer TiO_2 thin film showed higher number of attached cells than a one layer thin film and control surfaces. MSCs experienced no cytotoxic effects after culture on the TiO_2 coated substrates as observed from the cytotoxicity tests. Cell spreading, visualized with scanning electron microscopy, showed a faster rate of spreading on a rougher surface. Cells on a four-layer substrate, at 12 h showed complete spreading, where as most of the cells on a control surface and a one-layer surface, at 24 h, retained a rounded morphology. In conclusion, TiO_2 nanoparticle thin films were successfully assembled in alternation with polyelectrolytes and in-vitro studies with MSC showed an increase in the attachment and faster spreading of cells on rougher surfaces.
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Keywords: Layer-by-layer assembly; Stem cells; Nanoparticles; Surface roughness; Surface modification; Titanium dioxide

1. Introduction

Initial attachment and spreading of the cells determines the long-term viability of cells on substrates designed for various orthopedic and dental applications [1,2]. Nano-structured materials offer many possibilities for the modification of various materials for optimal attachment, proliferation and spreading of different kinds of cells. Previously, various research groups have shown increased attachment of cells in the presence of nano-structured materials [3–5]. Different aspects, such as surface wettability and free energy [6], surface roughness and material composition [7,8], method of preparation [9], etc. of various materials have been studied for the attachment of cells and were determined to be major factors influencing the behavior of cells in-vitro. A variety of nanostructured materials such as, ceramics [10], nanoparticles [11], polymers [12] and extracellular matrix proteins [13], when coated or present, have been shown to be effective in increasing the proliferation of different kinds of cells. A range of different techniques have been employed for surface modification for cell attachment including self assembled monolayers [14], micro-contact printing [15], microfluidic patterning [16], etc.

A relatively new technique used for surface modification is layer-by-layer nanoassembly (LbL) [17,18], which works on the principle of electrostatic attraction between oppositely charged species such as polymers [19], nanoparticles [20,21], enzymes/proteins [22] and so on. LbL technique has been employed in various applications such as surface modification with polymeric patterns for selective attachment of cells [23], hydrophilic [20] and hydrophobic coatings [24], control of corrosion [25] and sensors [26]. LbL assembly offers a greater advantage over other

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methods of surface modification, in that the adsorption of material can be controlled with nanometer precision and the architecture of the assembled films can be tuned to include multiple components (internal multilayered organization) with specific purposes for each component without compromising the integrity of the assembled multilayer film or the material being coated.

Recently, for the first time, we have shown LbL assembly of nanoparticles for the modification of various surfaces for improved attachment of cells [20]. Increased attachment of cells on an otherwise cell repellent surface was observed after coating the surfaces with TiO_2 nanoparticles. In a related study, TiO_2 thin films were shown to be the optimal surface for the faster attachment and spreading of cells compared with other kinds of nanoparticle thin films [21]. Based on these initial studies, TiO_2 nanoparticles were chosen to study the effects of surface roughness of LbL assembled thin films on the attachment, proliferation and spreading of mouse mesenchymal stem cells (MSCs).

2. Materials and methods

Glass substrates (corning brand microscope slides, cut into required sizes using a diamond cutter) were used for the preparation of nanoparticle thin films via LbL assembly. Unless otherwise mentioned, the size of the glass substrates is $(2 \times 2)\,cm^2$. The substrates were ultrasonicated in 35% isopropyl alcohol for 15 min and rinsed in deionized (DI) water for 2 min and were used immediately for the LbL assembly of nanoparticles. For control experiments, the glass slides without any coating were used after ultrasonication and rinsing with DI water.

2.1. QCM studies

Poly(dimethyldiallyl ammonium chloride) (PDDA, 100-200KDa, Sigma) and poly(styrene sulfonate) sodium salt (PSS, 70 KDa, Sigma) were used as received. For thickness measurements, polyelectrolytes and nanoparticles were assembled on 9 MHz quartz crystal resonators. Three precursor bilayers of PDDA/PSS were assembled on the resonator surface followed by the alternate adsorption of polyelectrolytes and nanoparticles. TiO_2 nanoparticles (diameter 21 nm, Aeroxide TiO_2, P25 Degussa AG, Germany), were dispersed in DI water, ultrasonicated for 30 min and used for LbL assembly. Polyelectrolytes were used at a concentration of 3 mg/mL in DI water and TiO_2 nanoparticles were used at a concentration of 6 mg/mL in DI water. For LbL assembly of nanoparticles, PSS (anionic polyelectrolyte) was used for alternation with cationic TiO_2. The surface charge on the nanoparticles was determined using a Brookhaven Zeta Plus micro-electrophoretic instrument. The change in frequency after deposition of each layer was monitored using a USI-system, Japan and was correlated with the film thickness by $\delta d = -0.016\delta F$, where δd is the change in the thickness of the film and δF is the change in frequency of the resonator. The resonator was rinsed in DI water after the deposition of each layer, to wash off excess or loosely attached material.

2.2. Thin film formation via LbL assembly

The entire assembly on the glass substrates was carried out using a dipping machine manufactured by Riegler & Kirstein Gmbh, Germany. The glass substrates after sonication and rinsing in DI water were mounted on the dipping machine and were sequentially dipped for 10 min in respective solutions or dispersions. Three precursor bilayers of PDDA/PSS were assembled on the substrates followed by the assembly of TiO_2 nanoparticles. The final layer was nanoparticles in all cases.

2.3. Surface roughness

A non-contact optical profiler (Wyko surface profiler, Veeco instruments, USA) was used for the measurement for the average surface roughness (R_a) of the substrates coated with TiO_2. Six R_a measurements per sample and three samples per layer number were measured and were plotted as averages against the number of layers of TiO_2 deposited in alternation with PSS. Each substrate had TiO_2 as the top most layer. The quantity R_a is given by $R_a = (1/M \times N)\sum_{i=1}^{M}\sum_{i=1}^{N}|Z_{ij}|$ where M and N are the number of data points in the X and Y direction of the array and Z is the surface height relative to the reference plane.

2.4. Cell culture

MSC were maintained in Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic-antimycotic (complete DMEM). Culture media was replaced with fresh complete DMEM every other day and cells were allowed to grow until sufficient cell density was reached, cells were then trypsinized, collected by centrifugation at 1000 rpm for 5 min, and resuspended in complete DMEM prior to plating on the nanoparticle coated substrates. Substrates coated with the nanoparticles were immersed in a 75% ethanol solution and rinsed in Hank's balanced salt solution (HBSS) before plating with cells. The substrates were removed from HBSS and placed in 60 mm dishes and MSCs were seeded onto the nanoparticle coated substrates. The dishes containing the nanoparticle-coated substrates were then incubated at 37 °C and 5% CO_2 and 95% air within a humidified environment.

2.5. Cytotoxicity

A qualitative two-color cytotoxicity assay was performed to determine the cytotoxicity of the substrates coated with TiO_2. The substrates were incubated with the cells for 48 h and rinsed with sterile PBS and were incubated with viability/cytoxicity reagents (LIVE/DEAD assay, Molecular Probes, USA) for 30 min. The cells were then visualized using a confocal fluorescence microscope.

2.6. Cell number

MTT based assay was performed on the cells attached on nanoparticle thin films to determine cell numbers. A calibration curve correlating the absorbance with the number of cells was first obtained. Briefly, the cells were seeded on the substrates at a density of 6000 cells/cm^2 and were incubated for 72 h with media changes every other day. At the end of 72 h, the media in the culture dishes was drained and the substrates were removed from the culture dishes and put in fresh dishes. The cells were then incubated with 10% MTT solution in the media for 4 h. The culture dishes were drained of the media and the formazan crystals formed due to the interaction of the MTT solution with the cells was dissolved in acidified isopropyl alcohol and the absorbance of the resulting solution was read spectrophotometrically at 570 nm with background subtraction at 690 nm, using an Agilent 8453 UV-vis spectrometer, and correlated with the calibration curve.

2.7. Cell attachment

For the cell attachment studies, the substrates (three substrates for every time period, for every layer, substrate size $1 \times 1\,cm^2$) were seeded with 3000 cells/cm^2 and the cells were allowed to attach to the substrate for 4, 12 and 24 h. The substrates were then taken out at the end of each time period, rinsed in PBS and then incubated with the cytotoxicity reagents for 30 min. Images of the cells on the substrates were then taken using a Nikon digital camera mounted on a Nikon Eclipse inverted fluorescence microscope and the cells in each image was counted to determine the number of cells on the substrates.

2.8. Scanning electron microscopy

Scanning electron microscopy was used to visualize the manner of cell attachment and the degree of cell spreading on the substrates. Briefly, the cells were incubated on the substrates for 1, 4, 12 and 24 h. The substrates were then taken out of the dishes and were rinsed with sterile PBS. The cells on the substrates were then fixed with 2.5% gluturaldehyde in PBS for 1 h, washed in copious amounts of PBS and incubated with osmium tetraoxide for 1 h and rinsed with PBS. The cells were then dehydrated by sequential dipping in increasing concentrations of alcohol. The cells on the substrates were finally critical point dried, 6 nm of gold/palladium alloy sputter coated on the substrates and were visualized using an AMRAY scanning electron microscope.

2.9. Statistical analysis

Means and standard deviations (SD) were calculated for descriptive statistical documentation. The student's *t*-test was applied for analytical statistics.

3. Results

3.1. QCM studies

The thickness of the assembled nanoparticle layers was determined by the deposition of the precursor and nanoparticle layers on quartz crystal microbalances. The increase in the thickness was plotted against the number layers of material deposited and is depicted in Fig. 1. The average bilayer thickness of the TiO_2/PSS was calculated to be around 11 nm. The bilayer thickness increased linearly with increasing number of bilayers of the material deposited (inset in Fig. 1). The thickness of the precursor layers was around 5 nm.

3.2. Surface roughness

Surface roughness of the deposited TiO_2 nanoparticle layers was determined using a non-contact optical profiler which gives the average roughness of the surfaces. The average roughness in an area of about 0.07 mm^2 was measured for different number of layers of TiO_2 deposited. The R_a values plotted against the number of layers of TiO_2 deposited shows a linear increase in the roughness with increasing number of layers of TiO_2 (Fig. 2(a)), which is consistent with the findings about the increase in surface roughness with increasing number of LbL assembled layers shown by other researchers [27]. Also shown is the representative data sheet obtained from the optical profiler measurements (Fig. 2(b)).

3.3. Cytotoxicity

The cytotoxicity of the substrates studied using commercially available fluorescent dyes showed very little cell death on the substrates. Fig. 3 shows the overlaid confocal fluorescence image of the cytotoxicity test performed on a glass substrate coated with four layers of TiO_2 in alternation with PSS. One can see that all the cells in this image are alive (green). These results show that the TiO_2 coated substrates support cell attachment and growth and that these substrates do not have a toxic effect on the attached cells. Quantitative cytotoxicity experiments were not conducted for this report.

3.4. Cell proliferation on TiO_2

Different number of TiO_2 layers was coated on glass substrates and MSC were seeded at equal densities (6000 cells/cm^2) on these substrates. Fig. 4 shows the results of the MTT assay performed on these substrates 72 h after seeding. It was observed that coating the substrates with TiO_2 increases the attachment of cells. One layer of TiO_2 increases the number of cells by approximately 20% from the control surface. Obvious

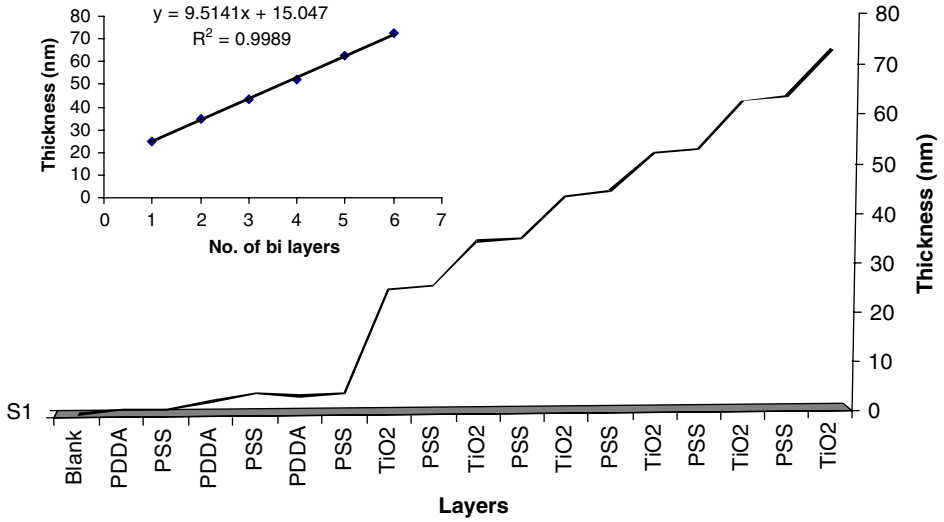
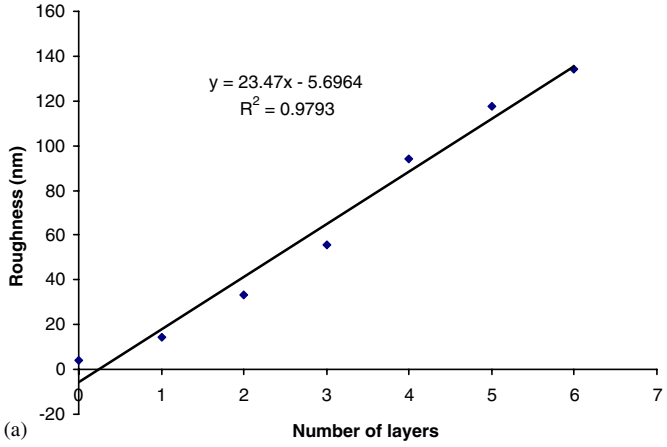
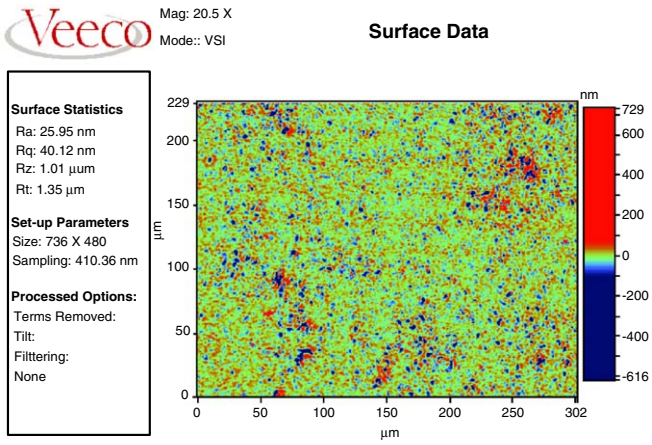


Fig. 1. Change in thickness of TiO_2 nanoparticles monitored using quartz crystal microbalance. Inset shows the linear increase in the thickness of the TiO_2/PSS bilayers.



(a)



(b)

Fig. 2. (a) Surface roughness of deposited TiO_2 layers and (b) representative optical profiler data of a substrate coated with two layers of TiO_2.

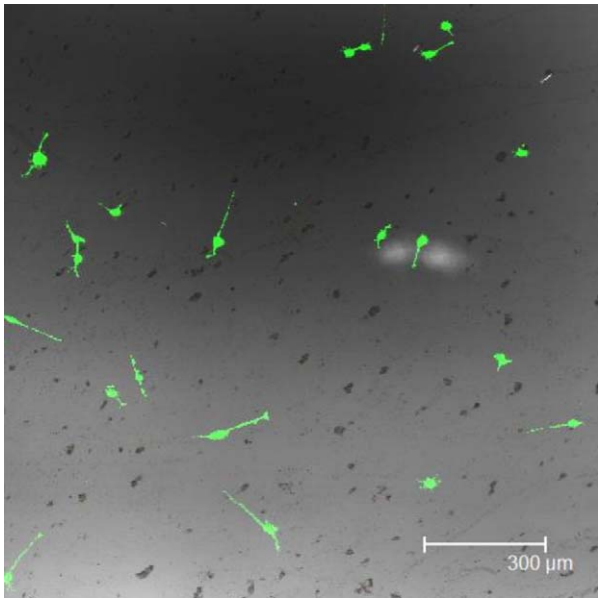


Fig. 3. Cytotoxicity assay performed on a glass substrate coated with four layers of TiO_2 showing live cells (green fluorescence). The image is the overlay of the excitations at 488 nm, 543 nm and transmission modes.

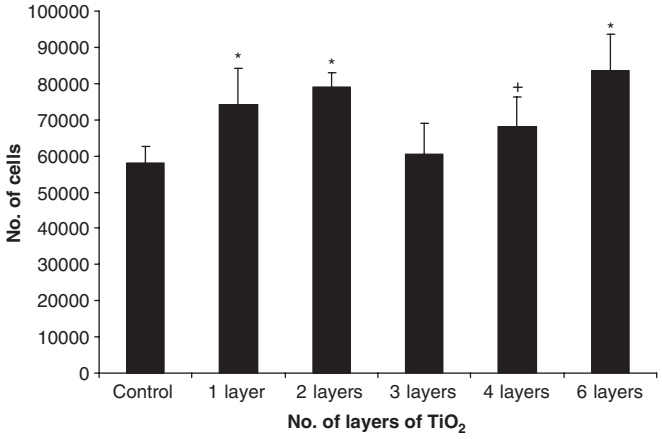


Fig. 4. Cell numbers on various layers of TiO_2 coated on glass. (*$p<0.01$, +$p<0.05$ compared with the control, $n=3$).

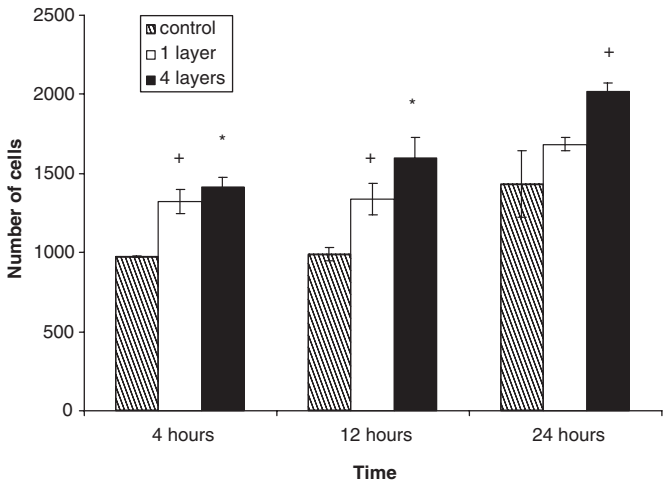


Fig. 5. Number of cells attached on control, one-layer and four-layer substrates (*$p<0.01$, +$p<0.05$ compared with control).

trend in the attachment of MSC was not observed with increasing number of layers of TiO_2 (increasing surface roughness), but coating the substrates with TiO_2 clearly shows an increase in the number of cells attached after 72 h. Statistically significant differences were observed with 1, 2 and 6 layers ($p<0.01$, compared with the control surface) and 4 ($p<0.05$, compared with the control surface) while no significant difference was observed with three layers of TiO_2.

3.5. Cell attachment

MSC attachment was studied on the control, one-layer and four-layer substrates and the results are shown in Fig. 5. The number of cells on the substrate coated with four layers of TiO_2 shows an increase from the control substrate and the substrate coated with one layer of TiO_2, and at all time periods. The number of attached cells on each substrate increased with increasing time. A statistically significant increase (compared with control) in

attachment of cells to the substrate coated with four layers and one layer was observed.

3.6. Scanning electron microscopy

Initial cell attachment and spreading was visualized using scanning electron microscopy. Four different time periods (1, 4, 12 and 24 h) were chosen for control (uncoated glass slide), 1 layer and 4 layers of TiO_2 coated substrates. The cells on all substrates after 1 h of seeding had a rounded morphology (data not shown). Fig. 6 shows the scanning electron micrographs of the cells on the substrates after 4 h of seeding. The cells on control (a) and one layer (b) show rounded morphology; where as some cells (black arrows) on four layers (c) show a partially spread morphology on the substrate surface, it is to be noted that the difference in cell morphology is not significant at this time period on different substrates. Fig. 7 shows the SEM images of the cells on the substrates after 12 h of incubation. Most of the cells in control (a) show rounded morphology after 12 h of incubation. On the substrates coated with one layer of TiO_2, most of the cells have spread, but some cells still retain their rounded morphology (white arrow), however the number of cells with such morphology is far less than the number of cells that have spread. On the substrate coated with one layers

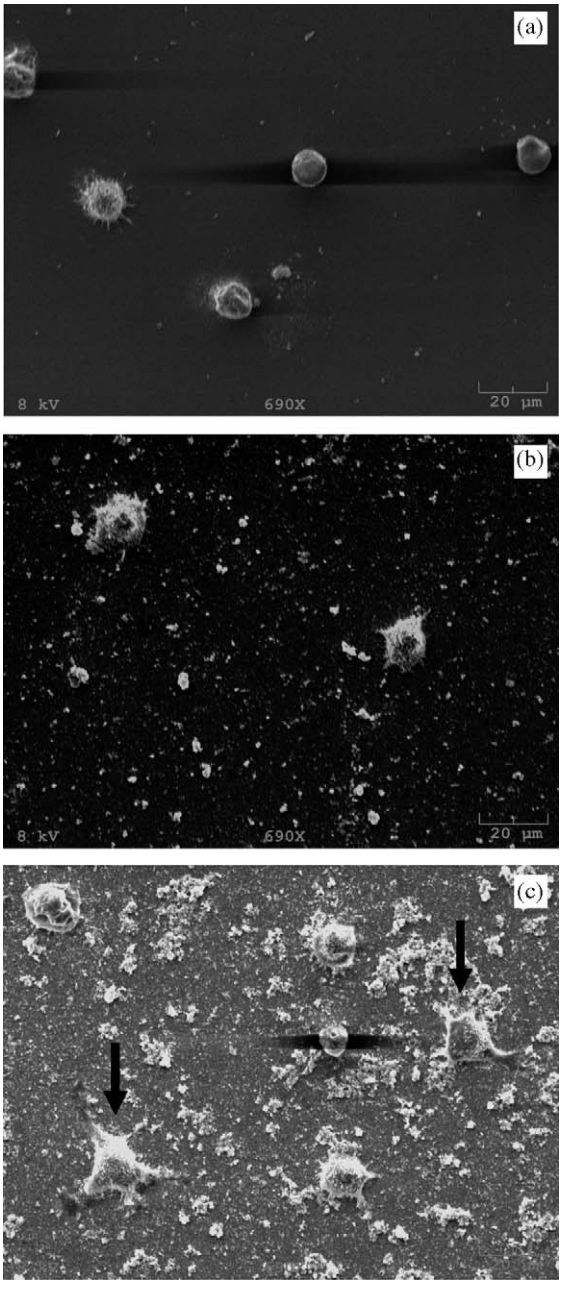


Fig. 6. Cells attached on (a) control and substrates coated with one layer (b) and four layers (c) of TiO_2 at 4 h after seeding on the substrates.

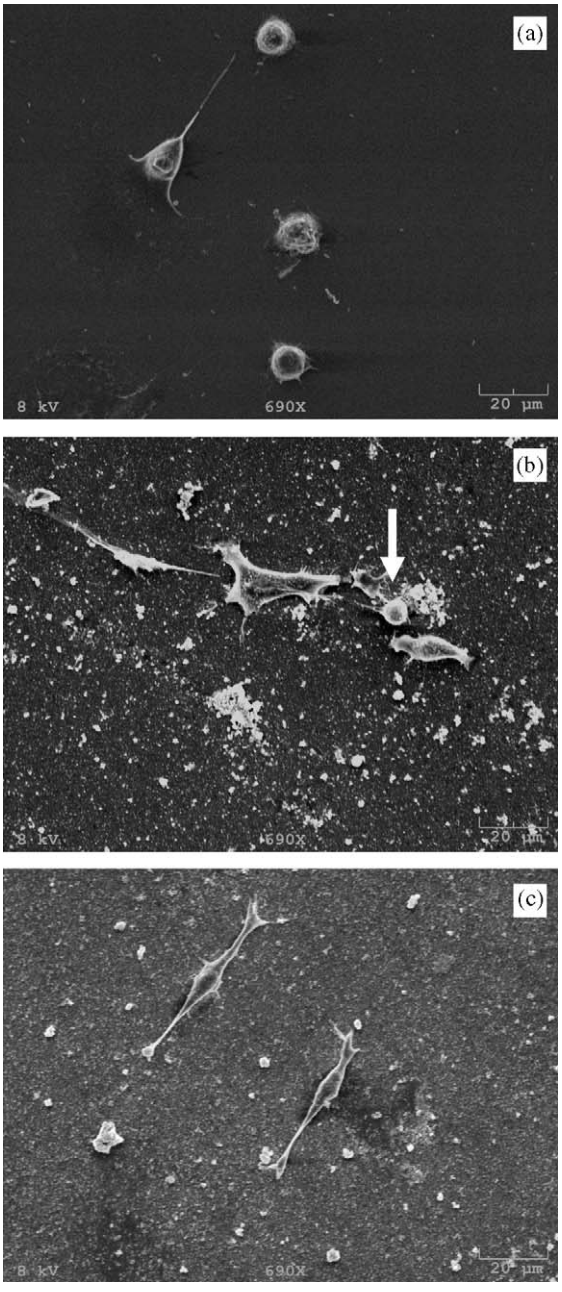


Fig. 7. Substrates at 12 h after incubation with cells: (a) control and substrate coated with (a) one layer and (c) four layers of TiO_2.

of TiO_2 (c) the cells have long pseudopodia after 12 h of incubation, suggesting that the cells have begun to spread across the substrate surface. The morphology of the cells after 24 h on different substrates is shown in Fig. 8. One can see that some of the cells on the control substrate (a) still have a rounded morphology after 24 h of incubation. On the substrate with one layer of TiO_2 (b), almost all the cells show spread morphology, but some of the cells have partially spread (white arrow) suggesting that the process of cell spreading is still continuing. The morphology of the cells on the substrate coated with 4 layers of TiO_2 (c) after 24 h shows that the cells have spread completely and have adapted well to this surface.

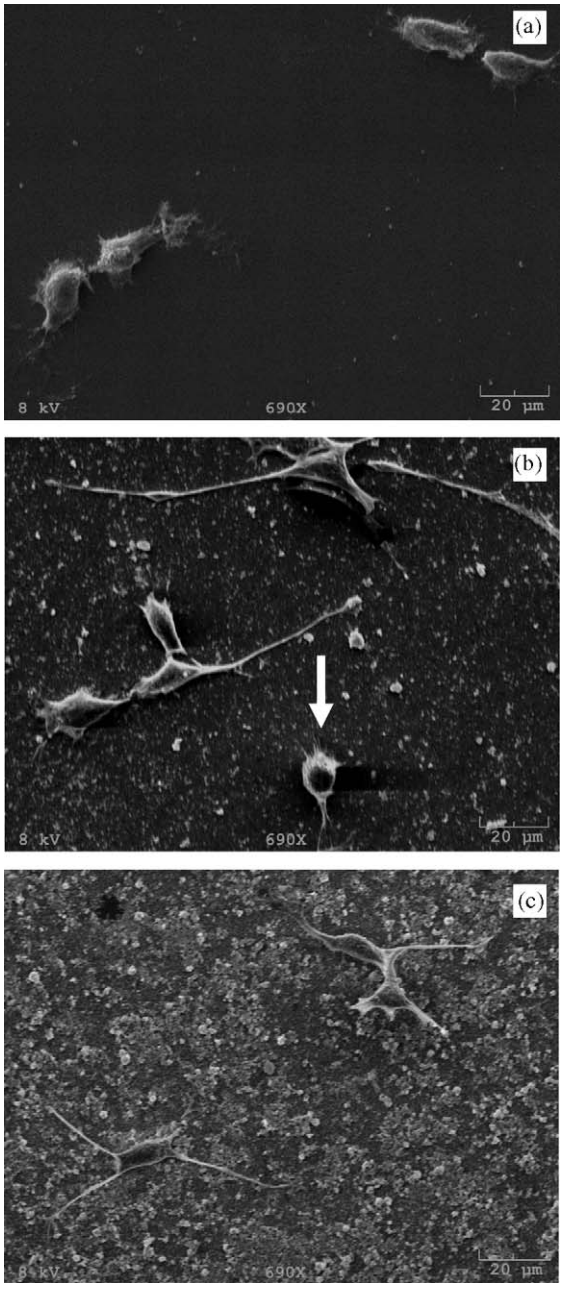


Fig. 8. Substrates at 24 h after incubation with cells. (a) control and substrate coated with (a) one layer and (c) four layers of TiO_2.

4. Discussion

Implant modification for cell attachment has been an interesting area of study for a long time and various methods have been proposed and implemented for the same. The initial attachment of cells to these surfaces is critical for the functioning and longevity of these materials. Nanostructured materials have been targeted for the purpose of surface modification of implantable materials and these materials have been shown to increase the cell attachment considerably. Attachment of cells for both soft and hard tissue replacement have also been shown to increase in the presence of nanomaterials [28,29].

LbL nanoassembly has been a method for surface modification with applications in many areas of scientific research and recently, LbL assembly of macromolecules has been applied for surface treatment and coating for implants [30]. LbL also offers the possibility to incorporate various components (drugs, enzymes), in the nanophase multilayer coatings, imparting a unique composition for the increased attachment of cells. Sinani et al. [31] were the first to show the attachment of cells to nanoparticles thin films, by coating collagen on the nanoparticle thin films. To the best of our knowledge, there have been only two previous studies on the attachment of cells directly on nanoparticle thin films, which were demonstrated by our group [20,21]. In a previous study on the attachment of human dermal fibroblasts on nanoparticle thin films, we observed higher and faster attachment of cells on TiO_2 nanoparticle thin films compared with three other kinds of nanoparticle thin films tested. In this study, we selected TiO_2 nanoparticle thin films for surface modification and various studies were performed.

Mesenchymal stem cells are a versatile cell population since their numbers can be expanded significantly and differentiated into multiple cell types with diverse applications for tissue engineering. Our studies with MSC on nanoparticle thin films showed that a greater number of cells attached on TiO_2 coated substrates (compared with the control). One explanation may be that an initial adsorption of a thin layer of negatively charged extracellular matrix proteins [32] from the cell culture media on the positively charged TiO_2 surface encouraged initial cell attachment and may explain the increased attachment of MSC on the TiO_2 nanoparticle thin film. Moreover, TiO_2 is a known biocompatible material and the chemistry of cell adhesion on TiO_2 promotes this increased attachment. Increased cell attachment of MSC on a rougher TiO_2 surface (higher number of layers, four layers of TiO_2) than a smoother TiO_2 surface or the control surface was observed. This is probably due to the increased surface area available for the cells to interact initially with the material [33], on a rougher surface as opposed to lower surface area available on a lower surface roughness

material of the same composition and chemistry of cell attachment behavior. One more factor promoting increased protein adsorption and hence the cell attachment is the surface roughness of the thin films and it has been shown that the total protein adsorbed on rougher surfaces is grater than that of the smooth surfaces [34]. The results from the attachment studies are corroborated by the scanning electron micrographs of the cells. The electron micrographs demonstrate that the cells attached and spread faster and had good adaptation to surfaces with higher number of TiO_2 layers (greater surface roughness/surface area) than a surface with lower number of TiO_2 layers (lower surface roughness/surface area). At 24 h cells retained rounded morphology on the control surface, where as at 4 h on a four layer surface, the cells show signs of spreading across the substrate and at 12 h, they have spread and have long pseudopodia, a sign of good adaptation to a rougher surface. One layer TiO_2 surface shows a trend that is intermediate between control and 4 layer surfaces. We conclude that a combination of cell adhesion biochemistry and TiO_2 nanoparticle thin film surface properties make this material a good candidate for further studies with MSCs.

5. Conclusion

In this work, LbL assembly was used for the successful deposition of TiO_2 nanoparticles and the surface roughness associated with these thin films was characterized and was found to increase with increasing number of layers of TiO_2 nanoparticle layers. The proliferation and attachment of MSC increased after deposition of TiO_2 nanoparticle layers and a higher number of cells attached on increasing number of layers of TiO_2 nanoparticle thin films. The spreading of cells was found to be faster on surfaces with increasing number of layers of TiO_2 nanoparticles. In conclusion, LbL assembly of TiO_2 nanoparticles offers a promising tool for the modification of implantable surfaces for increased cell attachment. Our future work is directed towards promoting osteogenesis and chondrogenesis of MSC on TiO_2 nanoparticle thin film surfaces for possible applications for soft and hard tissue repair and reconstruction using the LbL nanoassembly technique.

Acknowledgements

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