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Nanoporous Titania Coating of Microwell Chips for Stem Cell Culture and Analysis*

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Abstract

Stem cell research is today an active and promising field of research. To learn more about the biology of stem cells, technical improvements are needed such as tools to study stem cells in order to characterize them further and to gain insights to the molecular regulations of their maintenance, differentiation and identification. Common procedure when studying stem cells is to coat the surface where the stem cells are to be cultured with organic materials like matri-gel, poly-L-lysine and fibronectin. The resulting coating is usually relatively fragile and it is difficult to know if the coating is evenly distributed. In addition, these forms of coatings cannot be sterilized and re-used, but must be added as an initial, time-consuming step in the daily protocol. A microwell chip with hundreds of 500 nl wells has recently been shown to be a useful tool for stem cell culturing. This platform is here modified to facilitate and improve the coating conditions for adherent cell culture. A robust and highly porous film of TiO_2 is deposited in the wells prior cell seeding. TiO_2 is known to be biocompatible and provides a surface that is even and well characterized, simple to produce and re-usable. Furthermore it enables the microwell chips to be stored pre-coated for longer periods of time before use. We investigated the growth of rat mesenchymal stem cells on nanoporous titania films and found that they proliferated much faster than on conventional coatings. The combination of the robust TiO_2 coating of the microwell chip enables thousands of individually separated single, or clones of, stem cells to be studied simultaneously and opens up the possibility for more user-friendly cell culturing.

Key words: Stem Cells, Titania, Biocompatibility, Microwells, Surface Modifications

1. Introduction

Stem cells are of great interest to researchers due to their potential for use in tissue engineering and for fundamental biological studies [1-2]. Areas where there is

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hope for great scientific advances using stem cell research include; neurodegenerative diseases, spinal cord injury and transplantation. The screening of many different compounds for their effects on cell proliferation rate, health and state of differentiation is an important part of many stem cell based studies [3]. Employing conventional bulk scale methods for the widespread screening of different factors is impractical, since such techniques tend to be labour intensive, time consuming and also wastes scarce cells and expensive reagents. Microfluidic devices are thus excellent platforms for the study of stem cell proliferation and differentiation, since they can be less time and labour intensive and require far smaller quantities of cells and reagents [4]. Furthermore, greater control over local cell environment is achievable with microsystems and they also show potential of mimicking *in vivo* conditions [5].

Some chip substrate materials are not readily compatible with biological systems resulting in problematic cell adhesion and proliferation. Substrates made from materials such as PDMS have been used successfully to improve compatibility, although this in some cases has also introduced problems due to its high permeability [6]. Glass remains a popular device substrate material because of its chemical and thermal stability, its optical properties and the comparative ease with which it can be microfabricated. The biocompatibility issues associated with glass are usually overcome by employing relatively fragile surface coatings that may degrade over time [7]. For stem cell research, liquid coatings such as matri-gel, poly-L-lysine and fibronectin are commonly used, alone or in combinations of several coating layers dependent on cell type. Care must be taken so that the cell adhesive function of the coating, *i.e.* the extra-cellular matrix (ECM) containing sensitive proteins, are not damaged during i) storage before an experiment (freeze/thawing), ii) the course of an experiment (washing, background fluorescence, etc), or iii) that they do not lead to the contamination of the cells under study (sterile handling). Above all, such coatings are used one time only wherefore iv) the coating step (approximately one or two hours) must be carried out at the start of each experiment. Previously, we demonstrated a simple technique for depositing a robust and highly porous film of titania nanoparticles on the inside walls of microphotoreactors [8]. Titania is well known for its biocompatibility [9] and thus is a promising coating material for microdevices.

Recently, we have developed a microwell chip (672 wells of 500 nl) consisting of glass and silicon, with a spacing between wells that is compatible with flow cytometric cell sorting (often referred to as FACS), *i.e.* automatic sorting of single cells into individual wells [10]. In the previous study, single leukemic non-adherent cancer cells were investigated for their heterogeneity in cell proliferation, in order to show the potential of finding the few proliferating cancer cells among the many non-proliferating healthy cells. The chip has subsequently also shown potential in protein analysis [11], mutation analysis by PCR [12], microfluidic integration [13] and recently also for stem cell research [14]. The combination of i) the hundreds of experiments that can be run simultaneously in the chip and ii) the small volume per well saving reagent cost in molecule screens, makes the microwell chip a perfect match to stem cell research where high-throughput analysis is of utmost interest and the cellular effects of expensive molecules often are to be studied.

However, due to time-consuming and uncontrolled parameters (*e.g.* even surface, thickness of coating) when pre-coating the microwell chip with conventional liquid coating matrices for stem cell culture, an alternative coating procedure was desirable. This paper presents coating of the microwells with nanoporous titania films, whereby a robust, biocompatible and storage-friendly coating with good surface characteristics (flatness, controlled thickness) was developed. Hence an improved and more robust microwell platform for large screens of single stem cells was developed.

2. Materials and methods

Titania coated substrates were prepared by treating glass slides with 2M sodium hydroxide before dip coating in colloidal anatase TiO_2 suspension (Ishihara STS-01, Osaka, Japan). These coated slides were left to dry, before being consolidated at 400°C for 6 hours. This produced a mechanically stable, highly nanoporous film approximately 1 micron thick (Figure 1). Collagen coated substrates were produced using conventional preparation techniques [15] by dropping an aqueous solution of type I collagen onto glass slides and leaving them to dry for one hour. The collagen was prepared from porcine tendon by pepsin treatment in HCl (3 mg/ml, pH 3.0) and was supplied by Nitta Gelatin Inc., Osaka, Japan. Mesenchymal stem cells (MSC) were isolated from the femurs of 3-week-old male Wistar rats [16]. The bone marrow was flushed out using Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 15% fetal calf serum (FCS) and 50 U/ml penicillin and streptomycin. The cell suspensions were placed into T-25 flasks and cultured in 5% CO_2 at 37°C. Cells were sub-cultured at a density of 2×10^4 cells/cm^2. The cells of second-passage at 70% sub-confluence were used for all experiments. MSC were seeded on the titania coated substrates at a density of 1×10^6 cells/ml and incubated until a confluent cellular monolayer was formed at 5% CO_2 at 37°C. As controls, culture medium containing MSC (1×10^6 cells/ml) was also seeded on uncoated polystyrene tissue culture plates, plain glass slides and collagen coated slides. Proliferation rates and cell morphologies were observed using optical microscopy. Microwell chips with silicon walls (h=500 µm) and glass bottoms (h=175 µm) were fabricated as described earlier [10] having an outer format of 26 x 76 mm. Each microwell has the dimensions of 650 x 650 µm. Such chips were diced into smaller pieces (10 x 10 mm) in order to maximize the number of experiments. TiO_2 coating of the microwell chips were performed as described above and the chips were kept dry at room temperature until use. For cell culture in the microwells, a transparent and gas-permeable membrane was placed on top of the microwells to prevent evaporation and facilitate gas exchange, as described elsewhere [10]. The human epithelial carcinoma cell line A-431 (DSMZ German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was used to verify cell adhesion and the ability to detect fluorescent cells on the titania coated microwells. The cell line was maintained in McCoy's 5a medium with 1.5 mM L-glutamine (Lonza Biowhittaker, Basel, Switzerland) supplemented with 10% fetal bovine serum and 1% antibiotic/antimyotic solution (both from Invitrogen, Carlsbad, CA) in 5% CO_2 at 37°C. The cell lines were maintained, in parallel with the chip-experiments, in 100 mm cell culture dishes (BD Falcon, BD Biosciences, San Diego, CA) and split sub-confluence using a 0.25% Trypsin/0.53 mM EDTA solution (Invitrogen). Cell seeding into the microwells were performed in a manual fashion, using a pipette as described earlier [14]. For fluorescence staining of cells, calcein AM (Molecular Probes) was applied to A-431 cells. The cells were incubated with 2 mL calcein AM (0.5 mM)/mL cell solution for 30 min without rinsing. Fluorescence imaging was performed with a Zeiss LSM 510 Meta confocal microscope, 10x objective.

3. Results and discussion

A mechanically stable, highly nanoporous titania film of approximately 1 µm thickness was successfully produced on glass slides (Figure 1).

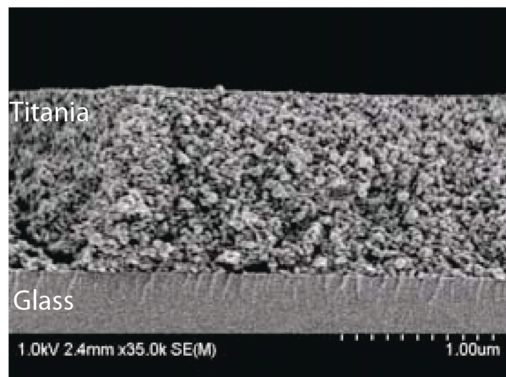

Figure 1. Cross sectional SEM picture of a nanoporous film of anatase titania deposited onto glass.

In addition to their biocompatible suitability, the titania films are robust, they do not degrade significantly over time and they can be sterilized by heating or by exposure to UV light and have also provided promising results for re-use (data not shown).

As can be observed from Figure 2, after seven days, far greater stem cell proliferation occurred on the titania coated substrates than on uncoated culture plates or on glass slides. These experiments were repeated several times and the mean proliferation rates and standard deviations for growth on bare glass, collagen coated glass and titania coated glass are plotted in Figure 3. As can be seen, there was a greater initial adhesion of cells to the titania coated substrates in addition to the higher overall proliferation rate. The cells were found to be very strongly bound to the titania coated substrates. This is of particular importance for cell investigations in microfluidic devices, with a constant risk of cells being removed from the surface by the hydrodynamic pumping of solutions.

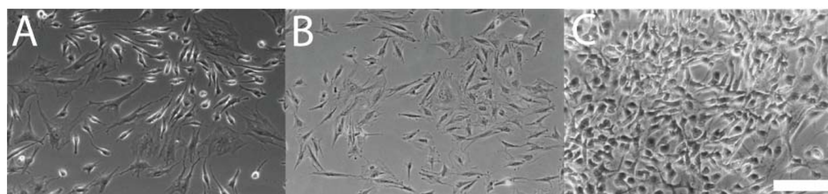

Figure 2. Bone marrow stem cells seven days after subculture on (A) an uncoated glass slide, (B) an uncoated polystyrene tissue culture dish, (C) on a glass slide coated with nano-porous titania. Scale bar 50 µm.

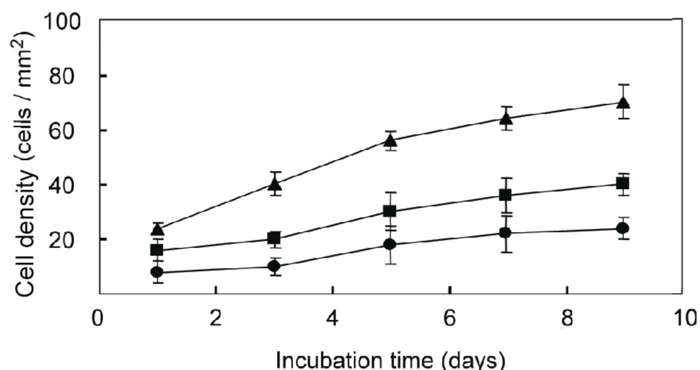

Figure 3. Time course of MSC growth on glass slides coated with: TiO2 (▲), collagen type I (■) and without any substance (●).

The biocompatibility of titania is well known, although the reasons for it having a higher compatibility than collagen are not clear. Interface/Cell compatibility is a complex issue that depends on the interplay of a large number of factors. These factors include: cell type, culture media type, surface chemical composition, crystallographic structure, wettability, morphology and porosity [17-20]. The titania films used in this work were hydrophilic, composed of small particles (around 12 nm) and were highly porous (67 % porosity).

Studies have shown that nanophase materials can enhance the adhesion of certain cell types to surfaces with subsequent enhancements in proliferation rates through the increased adsorption of matrix proteins [21-24]. In those studies titania was shown to outperform alumina, demonstrating the great potential of titania as a material for biological studies. The method of deposition may also have a significant effect on biocompatibility. Oxide coated titania films deposited by evaporation showed no significant change in protein adsorption and cell proliferation with differences in surface morphology. Furthermore, these films did not perform any better than polystyrene culture plates [25]. Previously, nanophase titania was produced from mixtures of rutile and anatase polymorphs [21-24]. In the presented work, we used 100 % anatase titania. However, it has been reported that it is preferable to use rutile titania rather than anatase for implanted materials since it is less likely to leach titanium into solution [26]. Hence, for future studies it will be interesting to compare the effectiveness of anatase, rutile and amorphous titania for biocompatibility and how this changes with particle size and film porosity.

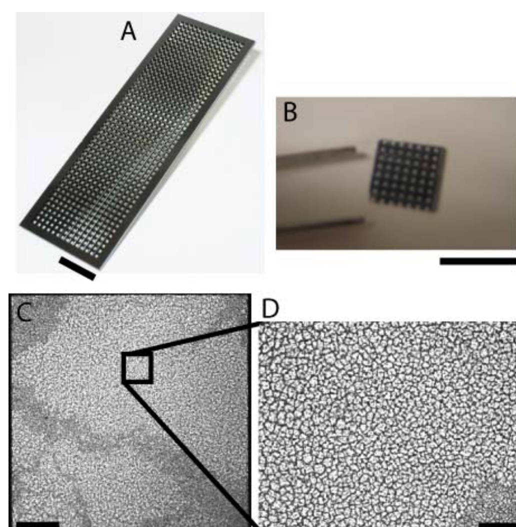

Figure 4. (A) Photograph of a microwell chip with outer dimensions of 26 x 76 mm, holding 672 microwells. (B) Smaller chips (10 x 10 mm) holding approximately 50 wells. (C) Micrograph of one well (650 x 650 μm) that has been coated with TiO_2. (D) Enlargement of a smaller area of the coated microwell. Images (C-D) obtained using a light microscope. Scale bars: A-B: 1 cm, C: 100 μm, D: 10 μm.

The titania film was then applied to microwell chips that have previously been shown to be suitable for culture and analysis of mouse embryonic, adult neural and human embryonic stem cells [14]. In the previous study, conventional coatings like poly-L-lysine, fibronectin etc were used as a first step in the daily protocol. However, a way to be able to produce pre-coated chips for long-term storage and timesaving culture protocols was desired, wherefore the titania coating was an interesting alternative. The chip consists of a bottom glass slide (cover slip thickness for optimal imaging properties) anodically bonded to a silicon grid, using standard microfabrication techniques (Figure 4a). The outer format of the microwell chip is 26 x 76 mm, the same as a standard microscopic glass slide to match conventional holders in varying microscopes, flow cytometers, etc. The chip consists of 672 microwells with a volume of 500 nl per well. The original chip was diced into smaller pieces to maximize the number of experiments, each with an area of 10 x 10 mm holding approximately 50

wells each (7 rows x 7 columns) as the photograph demonstrates in Figure 4b. The smaller pieces were coated with titania films, resulting in a smooth surface in the microwells (Figure 4c-d). The bottom area of an entire microwell (650 x 650 μm^2), is shown in Figure 4c. Figure 4d is an enlargement on a smaller area of the coating, visualizing in detail the structure of the film.

The microwell chip is commonly used both for light- and fluorescence microscopy due to its thin glass bottom and excellent imaging properties. Therefore, it was of uttermost interest to verify that adherent cells could be easily detected by light microscopy in the titania coated microwells. As visualized in Figure 5a, such experiments produced good results (A-431 cells used) and the titania film did not prevent conventional light microscopy from detecting cell growth.

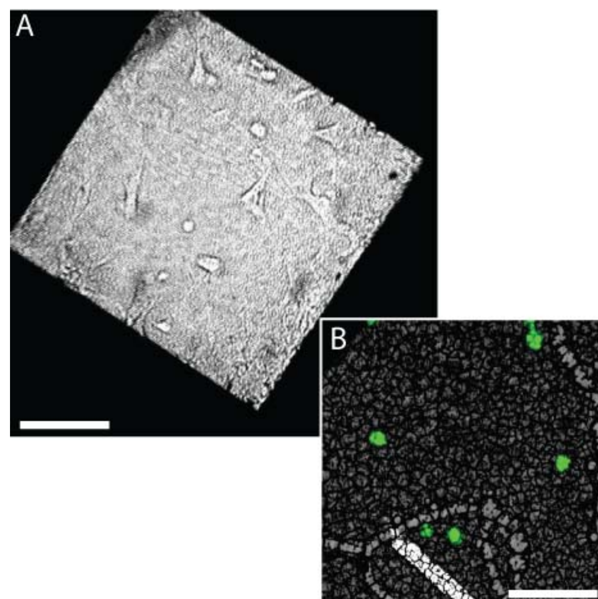

Figure 5. (A) Micrographs on adherent epithelial A-431 cells growing on titania coated microwells. (B) Calcein-AM labeled live cells (green) in suspension (A-431 cells) on titania coated microwell. Enlargement from a microwell. Scale bars: A: 200 µm, B:100 µm.

Furthermore, fluorescence imaging is highly suitable for screening many microwells for a given molecular marker, a “live/dead” cell tracker, a fluorescent dye, etc routinely used in proteomics and molecular biology. Different coating materials might have different characteristics in fluorescent detection, and sometimes background fluorescence from the coating material prevents or disturbs the objects of interest, *i.e.* the cells. To test the fluorescence characteristics of the titania films in the microwells, A-431 cells were stained with calcein AM, commonly used for staining living cells (dead cells are excluded) before seeded into the microwells. As Figure 5b demonstrates, no disturbing background fluorescence from the titania film could be detected. Hence, the material should be suitable also for immunocytochemistry with fluorescent detection, a widely applied methodology in various cell based research.

Future studies will involve further optimization of the titania film in the microwells since we did notice some irregularities during the experiments. Such an irregularity is visualized in Figure 5b (light grey/white pattern) but since this did not affect the results it was not further investigated in this study. The wells coated with titania had been stored on the shelf in room temperature for 1-4 weeks, with no apparent change in material properties. Storage and material change over time is however interesting and could be further investigated in the future along with experiments on re-using the coated microwells after cell culture. Further testing on material characterization and more extensive proliferation studies will clarify the potential of TiO_2 as a general coating for future advances in stem cell research, such as differentiation potential etc.

4. Conclusions

We investigated the growth of rat mesenchymal stem cells on nanoporous titania films and found that they proliferated much faster on such surfaces than on collagen coated substrates. These titania films were simple to produce and were applied onto microwell chips, previously shown useful in controlled and miniaturized yet highly parallel stem cell culture. Standard detection methods such as light- and fluorescence microscopy were successfully verified in the coated microwells, along with cell adhesion and growth. The combination of TiO_2 coating in the microwells has been shown to be promising and has significant potential to become a useful tool in high-throughput stem cell culture and research.

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