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Full Length Article

Particle aggregates formed during furfuryl methacrylate plasma polymerization affect human mesenchymal stem cell behaviour

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ABSTRACT

Human Mesenchymal Stem cells (hMSCs) are becoming a major focus in biomedical fields. Application of *in vitro* expanded hMSCs to treat numerous ailments has led to a commercial emphasis on improving hMSC growth ex vivo. Production of substrate independent, novel thin films is one potential tool for production of commercial viable hMSC expansion. Plasma polymerization allow controlled chemical optimisation of large scale surface areas in a substrate independent manner. Previous study shown that plasma polymerized Furfuryl Methacrylate (ppFMA) surfaces allowed primary fibroblast cells adhesion and proliferation. However, under some deposition conditions, particle aggregates formation was observed. These aggregates had the effect of disrupting cell attachment, despite being chemically indistinguishable from the underlying surface. Herein, hMSCs were cultured on ppFMA surfaces to determine their suitability for stem cell culture and observe the effect of particle aggregates on hMSC attachment and growth. Both metabolic and DNA quantification assays showed that surfaces with particle aggregates had lower numbers of attached cells and slower growth. Uniform surfaces without aggregates showed higher cell attachment and growth levels, which were comparable to Thermanox. Phenotypic analysis showed that there was no change to hMSCs phenotype after 7 & 14 days of culture on uniform ppFMA surface. Further investigation using time-lapse image analysis indicated that particle aggregates reduced cell attachment by presenting a physically weak boundary layer, which was damaged by intracellular tension during cell spreading. ppFMA surface can provide a stable substrate independent hMSCs expansion interface that could be applied to larger scale bioreactors, beads or scaffolds.

1. Introduction

Cell therapies are viewed as a potential cure for a range of human diseases and health conditions, where damaged cells can be replaced by healthy functional cells that have been grown *ex vivo* [1,2]. Efficient expansion of patient derived stem cells, within a laboratory, is a major focus for the cell therapies fields. Mesenchymal stem cells are of particular interest as they can be both expanded *ex vivo* and differentiated into specific mesodermal lineages by osteogenesis, adipogenesis or chondrogenesis [3–7].

To provide commercial quantities of stem cells for medical applications, a culture surface which provides good adhesion, proliferation together with suitable release and maintenance properties needs to be utilised. Previous studies, using traditional polymerisation techniques, have identified key chemical functional groups suitable for application in the tissue engineering and biomedical fields [8,9]. Furfuryl methacrylate (FMA) has been identified as a hit monomer, and its use for coatings and biomedical applications is the subject of current investigations [10]. FMA has received particular attention as it is a stable molecule that is not polymerized at room temperature, exhibits low shrinkage and has been used in biomedical and biomaterial applications as coating and adhesives [10–15].

It has been shown that poly(FMA) surfaces are suitable for human embryonic stem cell adhesion, proliferation and maintenance [8]. However, polymer based coatings of FMA have distinct limitations when scaling the technology into commercially viable settings. Thus, alternative surface modification methods are being explored in order to facilitate industrial uptake. One of the surface

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modification methods is plasma polymerization, a well-developed technique that has been widely used for the last 20 years [16–22]. Plasma polymerization has been shown to be a suitable way to produce thin film coatings on various planar or textured substrates with retention of desired functional properties. Plasma polymerization does not require surface preparation or any initiator in the process and is a single step, fast, easy, dry and scalable process [16,20,23–26].

Previous work has shown that plasma polymerized FMA (ppFMA) surfaces, produced under suitable conditions, promoted fibroblast cell adhesion and proliferation, but the conditions needed to be carefully controlled to avoid the confounding effect of particle aggregate formation on cell attachment [27]. Mesenchymal stem cells are particularly sensitive to environmental biological cues which can result in differentiation. As such, cell therapy applications using ppFMA coatings must both be sufficient to grow large numbers of cells while simultaneously maintaining pluripotent phenotype.

In this study, the interaction of human mesenchymal stem cells (hMSCs) with ppFMA surfaces is investigated during the attachment and proliferation stages. Metabolic and DNA quantification assays were performed to assess the expansion of hMSCs. Concurrent experiments using confocal time-lapse imaging was performed to explore the dynamics of cell attachment and growth in the presence of ppFMA particle aggregates. In addition, flow cytometry was performed on hit ppFMA surfaces to assess hMSCs phenotype after 7 & 14 days of culture.

2. Experimental

2.1. Materials

FMA 97%, containing 200 ppm monomethyl ether hydroquinone as inhibitor, Sigma–Aldrich was used as received. Thermanox coverslips (13 mm dia) (ProSciTech), silicon wafers (M.M.R.C), 35 mm optical petri dishes (Ibidi) and 24 well plates (Corning Costar) were used as substrates for deposited plasma polymer coatings. Silicon wafers were cut into 1 cm × 1 cm squares and cleaned with acetone before being rinsed with MilliQ water and dried with nitrogen gas.

2.2. FMA plasma polymerization

A steel capacitively coupled reactor, 25 cm long and 30 cm in diameter that encloses an RF electrode of 28 cm in diameter, was used for plasma polymer coating deposition. RF power at 13.56 MHz was applied to the internal electrode through an impedance matching network (AMN 150R). A rotary pump was used to evacuate the plasma chamber, to a base pressure of $<3 \times 10^{-3}$ mbar [28]. After degassing the monomer with freeze-pump-thaw cycling, it was allowed to flow into the reactor and controlled with a 13 mm ball valve. The glass coverslips and 24- well plates were placed on the bottom (earthed) electrode. During the plasma polymerisation experiment the working gas pressure was either 2.3×10^{-2} mbar or 4.5×10^{-2} mbar and the flow rate was either 2.6 sccm or 4.9 sccm. After setting the pressure and flow rate, the RF power of 2 and 4 W were used to deposit the plasma polymers for a duration of 1 and 10 min.

2.3. Surface Characterizations: XPS, ToF-SIMS and SEM

The ppFMA surfaces were characterized with X-ray Photoelectron Spectroscopy (XPS) and Time-of-Flight Secondary Mass Spectrometry (ToF-SIMS) for their chemistry and Scanning Electron Microscopy (SEM) to assess morphology. In a previous study on ppFMA, the surfaces were analysed and discussed in detail [27]. In brief, for XPS, a SPECS SAGE instrument was used to record high resolution (20 eV pass energy) spectra for C1 s and O1 s peaks where all binding energies were referenced to the aliphatic C1 s carbon peak at 285 eV [29]. Casa XPS software was used to process and analyse the spectra with typical full-width-at-half-maximum (FWHM) of 1.6 eV.

For ToF-SIMS analysis a PHI TRIFT V nanoTOF was used. The system was operated at 30 kV energy under vacuum of 5×10^{-6} and armed with a pulsed liquid metal $^{79+}$Au primary ion gun (LMIG). Positive SIMS spectra were collected for duration of 1 min using 100×100 μm raster with bunched Au_1 beam for better mass resolution [27].

SEM micrographs were obtained with a ZEISS Gemini 2 (crossbeam 540). The images were taken with working distance of 1–2 mm and magnification of 1–10 μm with the choice of in-lens secondary electron imaging. The resolution was 1024 × 768 with a scan speed of zero for image focusing and a scan speed of 4 or 5 for capturing the image.

2.4. Human mesenchymal stem cells (hMSCs) and ppFMA surfaces

Human Mesenchymal stem cells (Roosterbio) were cultured in αMEM (Lonza) supplemented with 10% foetal calf serum (FCS, Lifetech (Gibco), Glutamax (1X, Invitrogen) and Pen/Strep (10000 U/ml, Invitrogen)). Cells were incubated in a humid atmosphere with 5% CO_2 at 37 °C. Three sets of plasma polymer samples were seeded with three different human mesenchymal stem cell populations (p4-7) to investigate the surface attachment and proliferation properties.

24-well plates coated with ppFMA surfaces were seeded at 3×10^4 cells/well for 1 h attachment assays and 1×10^4 cells in 500 μL for proliferation and growth assays (over 5 days). To measure the hMSCs attachment and proliferation, resazurin assays were conducted. A Nikon Eclipse TE 2000-U Microscope with plan fluor 4x/0.3 objective lens was used to observe and image the attachment and proliferation of hMSCs.

2.5. Resazurin assay

A FLUOstar OPTIMA plate reader from BMG LABTECH was used to perform the resazurin assay. After 1 h from initial seeding, the 30×10^3 seeded well plates were washed twice with PBS to remove any unattached cells and 500 μL of 10% resazurin medium added to each well and incubated for another hour together with bare Thermanox as the control. After 1 h, 100 μL of the solution was transferred to a fresh 96-well plate for reading at an excitation and emission wavelength of 544 and 590 nm respectively. The same procedure was followed with 10×10^3 seeded well plates for the proliferation assays after 1st and 5th day. The data from three biological repeats were analysed and compared to bare thermanox as a control.

2.6. Confocal microscopy live cell imaging

Nikon's A1R$^+$ confocal laser scanning microscope- (Coherent Scientific, Australia), was used to capture images of live hMSCs. The cells were stained with Cell Tracer, Oregon Green 488 (Invitrogen), 24 h prior to seeding. The stain solution was prepared by adding a mixture of 1 vial of cell tracer plus 20 μL of DMSO into 5 mL PBS. Cells were washed thrice with PBS and cell tracer solution added to them. After adding the stain solution, cells were incubated for 20 min. Subsequently, cells were washed with PBS to remove excess stain and incubated with fresh complete media overnight. Stained cells were counted the following day and seeded at 50 K density onto 35 mm ibidi optical dishes which coated with ppFMA