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Osteocyte Characterization on Polydimethylsiloxane Substrates for Microsystems Applications

Spencer L York^{1,a}, Ahmad R Arida^{1,b}, Karan S Shah^{1,c}, Palaniappan Sethu^{2,d}, and Marnie M Saunders^{1,e}

¹Department of Biomedical Engineering, The University of Akron, 260 S Forge St, Akron, OH 44325, USA

²Department of Bioengineering, University of Louisville, 2100 S Brook St, SRB 357, Louisville, KY, 40292, USA

Abstract

In the body, osteocytes reside in lacunae, lenticular shaped cavities within mineralized bone. These cells are linked to each other and surface-residing osteoblasts via physical channels known as gap junctions. It has been suggested that osteocytes sense mechanical load applied to bone and relay that signal to osteoclasts and osteoblasts. Current *in vitro* and *in vivo* models of mechanotransduction face temporal and spatial barriers. Recent advances in polydimethylsiloxane (PDMS) based microfabrication techniques may be able to overcome some of these hurdles. However, before the bone research field can effectively utilize microsystems techniques, fundamental groundwork must be completed. This study characterized the behaviour of osteocytes on PDMS coated with collagen type I (CTI) and provides the framework for bone cell mechanotransduction studies using microsystems. The goal was to determine whether osteocytes were adversely affected by the substrate material by comparing their behaviour to a standard glass substrate. In addition, optimal culture conditions and time points for growing osteocytes on PDMS substrates were determined. Results of this study suggested that use of PDMS does not adversely affect osteocyte behaviour. Furthermore, the results demonstrated that osteocytes should be cultured for no less than 72 hours prior to experimentation to allow the establishment and maintenance of phenotypic characteristics. These results completed essential groundwork necessary for further studies regarding osteocytes in microsystems modelling utilizing PDMS.

Keywords

Osteocytes; PDMS; gap junctions; sclerostin

^asly15@zips.uakron.edu

^bara2@zips.uakron.edu

^ckss32@zips.uakron.edu

^dp.sethu@louisville.edu

^emms129@uakron.edu

Introduction

Significant advances in microfabricated systems have been made in recent years. While many fields, such as cardiovascular research, have taken advantage of these advances, the bone field has not yet begun to utilize these technologies. With the advent of microfluidics and micro total analysis systems, studies of small isolated groups or individual cells will become more feasible. The cellular environment may overcome several limitations of current *in vivo* models such as temporal and spatial variations [1]. These smaller scale studies are more easily achieved due to a significantly larger degree of control over the cellular environment, such as precise control over chemical gradients or the use of a 3D environment for cell culture [1,2]. With the use of the microscale tools and technologies as a long-term goal, the short-term goal is to begin to lay the foundation for the use of these devices in the bone field. In the current work, osteocytic behaviour on PDMS was characterized. PDMS is a common substrate for use in microsystems research and the focus of this work was to determine if the use of the PDMS as a substrate material affected the viability, growth, and functional behaviour of the osteocytic cells. While previous microsystems work with osteocytes has utilized fibronectin as a coating to enhance cell adhesion, collagen type I (CTI) was the recommended substrate for this osteocyte cell line [3,4,5]. CTI has been shown to enhance osteocyte markers, such as high levels of osteocalcin, connexin 43 (cx43), the antigen E11, and the development of gap junctions [3,6]. While PDMS has been well characterized for cell culture experiments, no previous experiments have involved the usage of CTI as a coating for cell adhesion [1,2].

Osteocytes and the MLO-Y4 Osteocytic Cell line

In 1997, Kato et al. published an article on the development of an immortalized osteocytic bone cell, the MLO-Y4 cell line [3]. This cell line has been critical to advances made in bone biology in the last decade and is still widely used [4,7,8,9]. The MLO-Y4 cell line was derived from the long bones of transgenic mice overexpressing the T-antigen driven by the osteocalcin promoter [3]. Early work with these cells led to some general observations and recommendations for their proper use and maintenance. Rat tail CTI was recommended as a substrate to maintain cellular phenotype [3]. Cells in medium supplemented with calf serum (CS) grew rapidly in contrast to cells in medium supplemented with fetal bovine serum (FBS). Furthermore, cells grown without FBS lost the characteristic dendritic morphology of gap junctions [3]. As a result, optimal supplementation of equal volumes of CS and FBS was recommended [3].

Sclerostin

Sclerostin was selected for study because of its importance to bone formation and resorption and its emerging links to a variety of bone associated disorders. Sclerostin is associated with Wnt signalling, which regulates bone mass [10,11]. More specifically, decreased levels of sclerostin results in high bone mass, and this is accomplished through the regulation of osteoblasts [12]. An increased level of sclerostin leads to decreased osteoblast activity, and sclerostin appears to affect osteoblast and osteocyte viability [13]. Studies conducted have also linked sclerostin activity to a variety of bone diseases such as sclerosteosis and Van

Buchem disease [14]. Animal models have shown that mechanical loading alters sclerostin levels and strain is purported to be the key mechanical regulator [13,15].

Gap Junctions

A key characteristic of mature osteocytes is that they are capable of forming gap junction channels with nearby cells. These channels, called connexons, are made up of protein subunits, called connexins. While many types of connexins exist, the most common in osteocytes is cx43 [6,10]. Gap junctions are found connecting both osteocytes and osteoblasts and serve as a critical component of intercellular communication and signalling by allowing the diffusion of small molecules between cells [6]. Previous studies have demonstrated that gap junctions are also involved in the cellular response to fluid flow, and thus mechanotransduction, as the gap junctions are an essential component in the protein production following fluid flow [6,16]. In addition, gap junctions may play a role in the differentiation of osteoblasts, indicating a role in the actual formation of bone [6].

An effective model for mechanotransduction studies must incorporate a wide variety of components, due to the many environmental factors that can impact bone. Before proceeding with the development of a model, the cells must be characterized using the material in question, to insure they behave physiologically. To simplify this process, a single cell type, osteocytes, was studied in order to determine how the cell responds to a commonly used material, PDMS. It must be demonstrated that osteocytes can live, grow, and communicate with each other on PDMS.

Methods

Culture of MLO-Y4 Cells

MLO-Y4 cells (a generous gift from Dr. Lynda Bonewald) were cultured using a previously established technique [7]. Briefly, the cells were cultured in minimum essential alpha medium (α -MEM, Invitrogen) supplemented with 2.5% FBS (Hyclone), 2.5% CS (Hyclone) and 1% penicillin/streptomycin (P/S, Invitrogen). The cells were maintained at 5% CO₂ and 37°C. Cells were grown in 100 mm polystyrene tissue culture dishes. For experimentation, cells were seeded on 25 mm diameter PDMS circles, 25 mm diameter cover glass, or 35 mm diameter MatTEK glass bottom dishes. Cell seeding density was chosen by data obtained in pilot studies (data not included) to ensure that 100% confluence was not achieved prior to 120 hours. Cell seeding density for both the PDMS and cover glass ranged between $1-2 \times 10^4$ cells/cm² for all studies. All substrates were coated overnight (18 hours) with CTI. Cells were fed once every 4 days and used between passages 3 and 13 after thawing. Collagenase in α -MEM was used to release osteocytes for plating on the PDMS or glass substrates for experimentation.

PDMS Fabrication

PDMS was fabricated using a commercial kit (Sylgard 184, Dow Corning) by mixing the PDMS elastomer with cross-linking agent at a ratio of 10:1. To develop a consistent substrate thin enough to visualize with standard microscopy, 16.5 grams of solution (15:1.5) was placed on a 100 mm silicon wafer (Silicon Quest, Int) and attached to a standard hand

drill, used as a rudimentary spin coater to spin down the PDMS to a uniform thickness. Wafers were initially run at low speed to spread the PDMS and then increased to maximum speed over 1 minute and were then spun for 1 minute at maximum speed (1500 RPM). Once spun the PDMS was baked for 15 min at 150°C. Using this mixture volume the thicknesses of the PDMS averaged 200 microns ($\pm 2\%$) for successive batches and resulting substrates were readily visible with standard microscopy techniques.

Prior to making the PDMS the silicon wafer was exposed to the commercial releasing agent chlorotrimethylsilane (Acros Organics) under vacuum for 30 min to ease removal of the PDMS from the silicon wafer. Once the PDMS sheet was released it was transferred to a biological safety cabinet. 25 mm diameter circles were cut from the PDMS and placed in a 100 mm Petri dish for surface sterilization. The PDMS was soaked in 70% alcohol for 5 min then flipped and this process was repeated for the other side. Following the alcohol wash, the PDMS was rinsed in sterile water and allowed to dry. To ease manipulation and removal from the culture plates the PDMS circles were adhered to cover glass prior to placement in 6-well culture plates.

Collagen Type I Coating on Substrates

Rat tail CTI (#354236, BD Bioscience) in 0.02 M acetic acid was coated on PDMS and standard cover glass at a concentration of 5 $\mu\text{g}/\text{cm}^2$. The collagen was applied and sat overnight before removal, and the remaining solution was allowed to evaporate. The surface was then rinsed with PBS 3 times for neutralization and then rinsed with distilled water before drying in the tissue culture hood prior to cell seeding.

MLO-Y4 Cell Viability

To assess cell viability, staining for lactate dehydrogenase (LDH) activity was completed at 4, 24, 48, 72, 96, and 120 hours. The cells were washed with Hank's buffered saline solution (HBSS) and then incubated in the reaction solution, which contained 5% Polypep (Sigma-Aldrich) base solution, 2 mM gly-gly (Sigma-Aldrich), 1.75 mg/ml nicotinamide adenine dinucleotide (NAD) (Fluka), 60 mM lactic acid (Sigma-Aldrich), and 3 mg/ml nitroblue tetrazolium (Sigma-Aldrich), and HBSS at a pH of 8.0. Following 1 hour of incubation, the cells were washed with distilled water and fixed in 4% paraformaldehyde overnight. Following fixation the cells were washed in water and cover slipped with an aqueous mounting solution (Vectashield) for imaging. The viability studies were repeated a minimum of three times in triplicate.

MLO-Y4 Cell Proliferation

To assess cell proliferation the osteocytes were seeded on CTI-coated PDMS or cover glass and quantified at 4, 24, 48, 72, 96, and 120 hours. The rate of cell growth was tracked on sequential days by using a glass alphanumeric gridded dish (P35G-2-14-CGRD, MatTEK). Control MLO-Y4 cells were plated directly onto the glass grid; for the MLO-Y4 cells grown on PDMS the cells were seeded onto the PDMS which was laid over top of the glass grid. Therefore, cells plated on glass and the grid were in the same focal plane. For osteocytes on PDMS, the PDMS was secured to the grid, so the cells and grid were not in the same focal

plane. The letters/numbers on the grid were used to quantify growth in the identical regions over the culture period. Each alphanumeric grid was 600×600 microns.

A minimum of 9 images per sample area were photographed repeatedly for each of the 6 time points from 4 to 120 hours. The images were collected from the stage of a Nikon Eclipse TS100 microscope with a Nikon CoolPix5400 digital camera. The same enlargement factor was established each day with the use of a stage micrometer. The collected images were viewed in Photoshop Extended and manual counts completed. Using standard cell counting techniques, cells crossing either the top or right side of the grid were counted, while cells crossing either the bottom or left side of the grid were not counted. In addition, only those cells with a visible nucleus were included in the counts. A baseline count was established during the first 4 hours of culture and percentage growth was established relative to that baseline. The proliferation studies were repeated a minimum of three times in triplicate.

Sclerostin and Cx43 Identification

Detection of sclerostin was accomplished with the use of the avidin-biotin complex method (Santa Cruz Biotechnology). The cells were fixed with methanol at -10°C for 5 min and air dried. The cells were processed according to manufacturer's recommendations using $5\text{ }\mu\text{g/ml}$ of polyclonal IgG sclerostin antibody (N-22, Santa Cruz Biotechnology). At the end of the processing the cells were not dehydrated through a series of alcohols (as recommended in manufacturer's instructions) but washed in distilled water and cover slipped with aqueous mounting media for microscopic examination. This was done so as to prevent curling of the PDMS and separation of the CTI. A glass bottom dish was used to enable the removal of the substrate from the bottom of the dish and the substrates were inverted on a slide for viewing with an upright microscope.

For sclerostin quantification 12 fields of images were randomly acquired from the PDMS and glass samples at each of the 6 time points. This was repeated with 3 separate experimental runs. The images were acquired with the 60x PlanFluor objective on the Nikon Eclipse microscope with the background subtracted and identical settings for all acquisitions. Within Photoshop Bridge these RGB images were transformed into grayscale images. Intensity ranged from 0 (black) to 255 (white), with the lower intensity corresponding to a greater sclerostin level. The perimeter of the cell soma was traced, and the pixel intensity values between 0–160 gray scale level were identified in the traced region, representing the area of interest. This gray level intensity was converted to optical density (OD) and calibrated using MetaMorph such that a higher level of OD corresponded to a greater level of sclerostin. The OD ranged from 0.05 to 3.39 and was recorded in each pixel in the relevant area before being averaged within each cell. These average values per cell ranged from 0.162 to 1.94, and were used for further calculations such as determining the average optical density in expressing cells. Several cells produced a minimal sclerostin level that did not fall within the measured range or had no detectable sclerostin level. These cells were labelled 'non-responders'.

Quantifiable Cell-Cell Communication

Functional cell communication assays were completed using epifluorescence microscopy and a double labelling technique utilized previously [16,17,18]. In this technique, cells were labelled with the fluorescent dyes, calcein AM (Molecular Probes, Eugene, OR) and 1,1'-diiododecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes).

Donor osteocytes labelled with a fluorescent dye mixture containing 10 μ M of calcein AM (Molecular Probes, Eugene, OR) and 14 μ M of DiI in 20 mg/ml of bovine serum albumin (BSA) enriched PBS with 10 μ M pluronic acid (Molecular Probes) were dropped onto confluent of unlabelled osteocytes after 72 hours in culture at a density of 7×10^3 cells/cm². The cells were then incubated for 2 hours at 37°C on surfaces of CTI-coated glass or PDMS. Cells were visualized using a Nikon fluorescence microscope (80i) with FITC and TRITC filters to locate the calcein and DiI labelled cells, respectively. Following incubation, a field image was captured with the Plan Apo 10x objective on a Nikon Eclipse microscope attached to a DN 100 camera. On the same field, one image was captured with a TRITC filter (540 λ) and another with the FITC filter (488 λ) and an overlay created in Photoshop for quantification of cell communication.

Communication assays were repeated for the addition of the gap junction inhibitor, 18 α -glycyrrhetic acid (AGA), or the sham dimethyl sulfoxide (DMSO). For these studies, 30 min prior to dropping the double labelled cells onto the unlabelled monolayer, 30 μ M AGA or an equal volume of DMSO was added to the stock calcein and DiI cocktail.

Results

As the effectiveness of PDMS as a substrate for osteocytes was under study, the viability, growth, and functional behaviour of the osteocytic cells must be examined. Specifically, viability and growth were quantified out to 120 hours in culture and the effect of PDMS on two critical functions of osteocytic cells, the ability to express sclerostin and the ability to communicate with neighbouring osteocytes via gap junctions was examined. For comparison purposes, osteocytic cell behaviour on cover glass was also quantified. Therefore, all comparisons were completed with osteocytes on CTI-coated PDMS or glass, as opposed to the fibronectin used in previous experiments with PDMS and osteocytes [3,4,5]. The CTI and media conditions were chosen so as to maintain the cells under culture conditions previously recommended by the researchers responsible for the establishment of the MLO-Y4 cell line. Finally, the optimal culture conditions and time points for growing osteocytes on PDMS substrates for microsystems work were addressed.

MLO-Y4 Cell Viability

In order to determine the suitability of the MLO-Y4 cells for adaptation to microsystems approaches, the impact of the PDMS, on cell viability was first determined, through the use of an LDH assay. In this assay, cells that were viable prior to fixing stain blue, while dead cells do not. The viability results are shown in Figure 1 for osteocytes at 72 hours of culture on CTI-coated PDMS. Indicative of live cells, the osteocytes stained heavily for LDH activity. This result was not affected by time in culture as cells were viable throughout the

120 hour culture period (data not shown). Therefore, MLO-Y4 cell viability was not significantly affected by culture on CTI-coated PDMS.

MLO-Y4 Cell Proliferation

The ability of MLO-Y4 cells to maintain normal proliferation is another essential component for their adaptation to microsystems work. MLO-Y4 cells were grown on either glass or PDMS that were both coated with CTI. The results of the proliferation studies are shown in Figures 2 and 3. Figure 2 illustrates typical osteocyte proliferation on cover glass and PDMS for the various time points. Given that osteocytes on cover glass were directly seeded on the CTI-coated glass grid, the cells and grid were focused in the same focal plane. For osteocytes on PDMS, the PDMS was secured to the grid and the cells and grid were not well focused in the same focal plane. Osteocyte growth increased 257% on PDMS and increased 222% on glass during the 120 hour culture period as shown in Figure 3. Osteocyte growth on PDMS slightly lagged the growth on glass at 24 hours ($p < 0.05$) but was not significantly different at 48, 72, 96, or 120 hours.

Sclerostin Identification

Mature osteocytes are marked by the production of sclerostin and their ability to communicate with neighbouring osteocytes via gap junction channels. For this reason, immunochemical studies were completed for sclerostin and the most abundant connexin protein in bone, cx43 [6,10,11]. As shown in Figure 4, sclerostin was abundantly expressed in osteocytes plated from 4 to 120 hours in culture. Sclerostin levels in each cell were measured as the OD (from light intensity) with time in culture for the randomly selected fields of view, as Table 1 displays. For PDMS the average OD for all cells at 4 hours was 0.198. The intensity dropped at 24 hours to 0.112 thereafter increasing to a maximum 0.451 at 120 hours. Based upon these results, sclerostin levels fluctuated during the first 48 hours with levels at 4 and 24 hours statistically less than levels at 48 hours ($p < 0.05$). The level of sclerostin expression was relatively stable from 48–96 hours before significantly increasing at 120 hours ($p < 0.05$) in comparison to all other time points. For glass the average OD at 4 hours was 0.174. The OD dropped at 24 hours to 0.098 and then increased to a maximum of 0.430 at 120 hours. Thus, sclerostin levels again fluctuated during the first 48 hours with levels at 24 hours statistically less than levels at 48 hours ($p < 0.05$). By 72 hours on cover glass, the sclerostin levels had increased significantly ($p < 0.05$) in comparison to 4 and 48 hours. The expression level then increased slowly from 72 to 120 hours. Thus, the level of sclerostin on the PDMS and the glass are comparable, as indicated by comparable maximum levels of optical density.

As shown in Table 1, the percentage of osteocytes expressing sclerostin decreased significantly at 24 hours for cells grown on either PDMS or glass suggesting a substrate-independent event. The sclerostin levels recovered at 48 hours for both samples. After this initial drop and recovery the level of sclerostin expression on PDMS levelled off after 72 hours, and similar behaviour was observed on glass. On both substrates the percentage of cells expressing the signal increased over time with an average of 86.9% of cells expressing on PDMS and 84.8% of cells expressing on glass after 72, 96, and 120 hours. This also

displays that the PDMS did not significantly impact the amount of sclerostin observed in osteocytes.

In addition to quantifying OD of the field sample the sclerostin levels were further analysed by quantifying the average optical intensity of the cells meeting the threshold (intensity level 160), which were labelled as responders, as shown by Figure 4 and Table 1. For the osteocytes seeded on PDMS, when the average OD of the responders was considered there was a relatively constant OD observed from 4–48 hours. Interestingly, the percentage of cells responding at 4, 24, and 48 hours (55.3%, 30.6%, and 92.8%, respectively) was quite variable. By 72 hours, there was a significant increase in the OD of the responding cells which was again significantly increased to a maximum at 120 hours. At the later culture time points, the percentage of cells responding was more robust with an average of 88.4% responding between 48 and 120 hours. While trends were relatively similar between osteocytes on PDMS and cover glass, maximum sclerostin levels for PDMS were 10% lower than maximum sclerostin levels for cover glass. While this difference was statistically significant ($p < .05$), there was not a significant difference in sclerostin levels for osteocytes on PDMS and cover glass at 4 and 72 hours. Glass and PDMS displayed similar trends, with a significant increase between 48 and 72 hours, and a maximum level of expression at 120 hours. Although the level of maximum expression was lower on PDMS, the increased level of sclerostin at 120 hours does indicate the necessary production of sclerostin.

Connexin 43 Identification and Quantifiable Cell-Cell Communication

Due to the significant role of intercellular communication in osteocytes, the level of Cx43, a common component of gap junctions, was also examined. As shown in Figure 5, the level of cx43 was abundant in osteocytes seeded from 4 to 120 hours in culture. At 4 hours, the osteocytes lacked intercellular connections, but by 24 hours the dendritic processes were highly visible and extended between the cells. With the increased time in culture (48, 72, 96, and 120 hours) extensive branching of the dendritic processes was observed. This was not unexpected given the abundance of cx43 in this cell line, as previously noted [3]. Cx43 fluorescence intensity was present and similar in the osteocyte population, regardless of substrate. Cx43 was present within the cytoplasm and by 72 hours its presence was observed in a punctate pattern as it paralleled the dendritic process development of the cell. From 72 to 120 hours, regardless of substrate, the punctate pattern was present. Not unexpectedly, this development parallels the increasing communication observed at 72 hours in culture for both substrates.

As communication is best displayed by the active transfer of small molecules and ions, and not only by the presence of gap junction proteins, functional cell communication was quantified using cells labelled with Calcein AM and DiI. Calcein AM, once in the cell was cleaved of its AM group and was no longer able to leave the cell through the cellular membrane. As a result of its small molecular size, less than 1 kDa, calcein was gap junction permeable and able to transfer to neighbouring cells if functional (open) gap junctions were established. DiI was of a larger molecular size, intercalated within cell membranes and was not able to transfer to neighbouring cells via gap junctions. Therefore, DiI was used as an identifying tag to visually separate the labelled donor cells (calcein AM and DiI) from the

unlabelled acceptor (calcein transfer) cells. Labelled cells were then dropped onto unlabelled cells in monolayer and dye transfer between cells was quantified. If functional gap junctions were established the calcein transferred to neighbouring cells which then fluoresced green. Table 2 and Figure 6 display that osteocytes were highly communicative in culture regardless of substrate, with over 60% of the cells communicating on both substrates, as expected. On PDMS, 63.5% of osteocytes were in communication while 64.1% of osteocytes seeded on glass were in communication.

AGA, an inhibitor specific to gap junctions, has previously been used to inhibit functional communication on glass, for functional studies [19]. These results must be verified for the PDMS, insuring that the substrate has no impact on the behaviour. DMSO was used to verify that the loss of communication in the presence of AGA was specific to AGA and not a result of a reaction to a generic topical additive. Specifically, 12.5% of osteocytes seeded on PDMS were communicating in the presence of AGA, while 14.4% of osteocytes seeded on glass were communicating in the presence of AGA. In addition, sham samples did not significantly inhibit the number of cells communicating on either substrate suggesting the communication was specifically inhibited by the presence of the AGA. Since the natural extent of communication was not significantly different between the PDMS and cover glass substrates and communication was equally inhibited in the presence of AGA, the use of PDMS did not seem to affect osteocytic communication.

Discussion

In this work, the behaviour of the MLO-Y4 osteocytic cell line on PDMS was examined to determine the feasibility of utilizing these cells in microsystems studies. The goal was to not only address the feasibility of their use by qualifying and quantifying their behaviour on PDMS in comparison to cover glass, but to address these issues while maintaining the cell line in conditions previously recommended for the MLO-Y4 line [7]. Furthermore the goal was to determine optimal conditions for use of these cells on PDMS for microsystems research applications and to provide insight into issues that surfaced in conducting this work that may prove useful to future studies.

Regardless of substrate, the MLO-Y4 cells exhibited dendritic characteristics, viability, proliferation, cx43 production, and sclerostin production to a similar extent. The cell plating was an important sequence of events in this study. Care was taken to ensure that the cells were evenly dispersed on the CTI-coating. Cell clumps were separated by gentle pipetting to allow for individual cells to be seeded as evenly as possible. For PDMS, additional issues were observed. During pilot studies with the PDMS the cells were seeded onto the PDMS and the PDMS was then adhered to glass cover slips and placed inside 6-well tissue culture plates; this was done to minimize PDMS sticking to the culture dish and ease removal from the plates. Initially, the dispersed cells attached to the cover glass when the PDMS substrate was cut smaller than the cover glass. For subsequent studies the PDMS was cut to extend to the sides of the 6-well culture dish and completely cover the cover glass.

The rudimentary spin coating technique used to fabricate the PDMS sheets was sufficient, but certainly not ideal. The preparation of the PDMS by spinning it on the silicon disk

produced a substance of even thickness and clarity. The PDMS readily picked up dirt in this fabrication process, thus necessitating the sterilization in alcohol. The optical quality was less than glass but this additional care in handling greatly improved the quality of the PDMS and enabled tissue culture on PDMS without contamination. In addition to the sterility issues, standard manufacturer's instructions for CTI coating (minimum of 1 hour coating) were modified. Instead, the collagen coating was left on the culture dish overnight. As prepared with this approach, the dishes could be used immediately or wrapped with aluminium foil and refrigerated for later use. The CTI was also prone to tearing, thus necessitating additional care in preparation and use throughout the experiment, such as rinsing with water and utilizing aqueous mounting media instead of dehydrating with alcohol.

Over the time course utilized in this study, we observed extensive branching of the dendritic processes. It was our impression that the dendritic appearance of the cells grown on PDMS was not as pronounced as it was for MLO-Y4 cells grown on cover glass. Furthermore, the dendritic appearance was largely absent when dense, over-confluent areas developed at 96 to 120 hours regardless of substrate. Confluent cells seeded on PDMS quickly detached, while confluent cells on glass, while losing their dendritic morphology remained adhered to the cover glass for up to one additional week in culture. However, the relevance to osteocytes in this overly confluent environment may be questionable, as the MLO-Y4 cells may not have the high level of expression for osteocalcin, cx43, and E11, low level of expression of alkaline phosphate, or the dendritic morphology found in osteocytes [4,5,7].

The location of sclerostin within each cell was first observed at the border of the nuclear membrane, with subsequent accumulation in the cytoplasm. The cells maintained their dendritic morphology while the sclerostin accumulated in the cytoplasm, and eventually became more proximal to the dendrites. Interestingly, by 120 hours many osteocytes were observed with rounded soma and multiple dendrites coinciding with peak accumulation of sclerostin. This development parallels the increasing intercellular communication observed at 72 hours in culture for both substrates as shown by the increasing Cx43 fluorescence intensity over time. The punctuate pattern, first seen by 72 hours parallels the dendritic process development of the cell. Functional cells were described as cells properly seeded on CTI-coated substrates, viable, and dendritic in appearance with punctuate cx43 visible along the dendrites and the presence of sclerostin. Thus, functional cells were first seen by 72 hours.

Cell culture conditions previously described for growing the MLO-Y4 cells on PDMS and cover glass to maintain an osteocyte-like phenotype were used [3]. The culture conditions employed here combined with a minimum of 72 hours of culturing on substrate were critical for obtaining functional osteocytes for microsystems applications on PDMS. While little research is available, others have tested the expression of sclerostin at both the mRNA and protein level between 1 and 24 hours with mixed results which could, in part, be attributed to non-fully functional osteocytes at these time points [8,9]. Additionally, researchers have used fibronectin to plate MLO-Y4 cells in contrast to the recommended CTI-coated substrate. The CTI substrate is recommended for optimal osteocyte morphological maintenance and functionality such as the development of gap junctions [3,4,5]. While the

effect of the short-term culture and different coating selection on the behaviour of the osteocytic cell line is not clear, our results suggest that post-plating osteocyte behaviour was affected, but recovery was evident by 48–72 hours. This further demonstrates the necessity of culturing osteocyte cells for 72 hours to observe full functionality.

These results suggest that microsystems work using osteocytes may be successfully completed using the osteocytic MLO-Y4 cell line and that the importance of attention to culture conditions should not be underestimated. This initial groundwork, although limited in scope, demonstrates an important characterization of MLO-Y4 cells on PDMS. Important further work remains, such as studying the impact of a plasma treatment, and moving to a 3D environment. Because the plasma coating commonly used for microfluidic devices sterilizes and changes the surface from hydrophobic to hydrophilic, the impact of a plasma treatment and the CTI coating on the MLO-Y4 cells must be studied [20]. In addition, 3D environment studies remain essential as they would improve the quality and biomimicry of the model, by better simulating the natural environment of the osteocytes [1,4].

Conclusions

Technology development in the microfabricated systems field has been highly productive in recent years. To begin to lay the foundation for microsystems in bone research, this study was conducted to determine what effect a common microsystem substrate, PDMS, has on the behaviour and survival of the osteocyte. Specifically, the MLO-Y4 osteocytic cell line was grown on CTI-coated PDMS, and some cellular characteristics as a result of the substrate were assessed qualitatively and quantitatively by comparison to MLO-Y4 cells seeded on cover glass. Regardless of substrate, the cells exhibited the characteristic dendritic morphology [3]. Equally important, quantitative studies determined that cell viability, proliferation, sclerostin levels and functional communication were not adversely affected by the PDMS. These results demonstrate the usefulness of the PDMS based scaffold and support the possibility of utilizing microsystems tools and technologies for advancing bone cell research. An effective microsystem model for bone cells may revolutionize the field, allowing improved studies into the signalling pathways involved in physiologic and disease state bone.

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References

1. Bauer M, Su G, Beebe D, Friedl A. 3D microchannel co-culture: method and biological validation. *Integr Biol (Camb)*. 2010; 2(7–8):371–8. [PubMed: 20577680]
2. Paguirigian A, Beebe D. Microfluidics meet cell biology: bridging the gap by validation and application of microscale techniques for cell biological assays. *Bioessays*. 2008; 30(9):811–21. [PubMed: 18693260]
3. Kato Y, Windle JJ, Koop BA, Mundy GR, Bonewald LF. Establishment of an osteocyte-like cell line, MLO-Y4. *J Bone Miner Res*. 1997; 12(12):2014–23. [PubMed: 9421234]

4. You L, Temiyasathit S, Coyer SR, Tao E, Prinz F, Jacobs CR. 3D microfluidic approach to mechanical stimulation of osteocyte processes. *Cell Mol Bioeng*. 2008; 1(1):103–7.
5. You L, Temiyasathit S, Coyer SR, Garcia AJ, Jacobs CR. Bone cells grown on micropatterned surfaces are more sensitive to fluid shear stress. *Cell Mol Bioeng*. 2008; 1(2–3):182–8.
6. Donahue HH. Gap junctions and biophysical regulation of bone cell differentiation. *Bone*. 2000; 26(5):417–22. [PubMed: 10773579]
7. Rosser J, Bonewald LF. Studying osteocyte function using the cell lines MLO-Y4 and MLO-A5. *Methods Mol Biol*. 2012; 816:67–81. [PubMed: 22130923]
8. Papanicolaou SE, Phipps RJ, Fyhrie DP, Genetos DC. Modulation of sclerostin expression by mechanical loading and bone morphogenetic proteins in osteogenic cells. *Biorheology*. 2009; 46(5): 389–99. [PubMed: 19940355]
9. Santos A, Bakker AD, Zandieh-Doulabi B, Semeins CM, Klein-Nulend J. Pulsating fluid flow modulates gene expression of proteins involved in Wnt signalling pathways in osteocytes. *J Orthop Res*. 2009; 27(10):1280–87. [PubMed: 19353691]
10. Galli C, Passeri G, Macaluso GM. Osteocyte and WNT: the mechanical control of bone formation. *J Dent Res*. 2010; 89(4):331–43. [PubMed: 20200416]
11. Li X, Zhang Y, Kang H, Liu W, Zhang J, Harris SE, Wu D. Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signalling. *J Biol Chem*. 2005; 280(20):19883–87. [PubMed: 15778503]
12. van Bezooijen RL, ten Dijke P, Papapoulos SE, Lowik CW. SOST/sclerostin, an osteocyte-derived negative regulator of bone formation. *Cytokine Growth Factor Rev*. 2005; 16(3):319–27. [PubMed: 15869900]
13. Lin C, Jiang X, Dai Z, Guo X, Weng T, Wang J, Li Y, Feng G, Gao X, He L. Sclerostin mediates bone response to mechanical unloading through antagonizing Wnt/beta-catenin signalling. *J Bone Miner Res*. 2009; 24(10):1651–61. [PubMed: 19419300]
14. ten Dijke P, Krause C, de Gorter DJ, Lowik CW, van Beezooijen RL. Osteocyte-derived sclerostin inhibits bone formation: its role in bone morphogenetic protein and Wnt signalling. *J Bone Joint Surg Am*. 2008; 90(sup 1):31–35.
15. Robling AG, Niziolek PJ, Baldridge LA, Condon KW, Allen MR, Alam I, Mantila SM, Gluhak-Heinrich J, Bellido TM, Harris SE, Turner CH. Mechanical stimulation of bone in vivo reduces Osteocyte expression of Sost/sclerostin. *J Biol Chem*. 2008; 283(9):5866–75. [PubMed: 18089564]
16. Saunders MM, You J, Trosko JE, Yamasaki H, Donahue HJ, Jacobs CR. Gap junctions and fluid flow response in MC3T3-E1 cells. *Am J Physiol Cell Physiol*. 2001; (6):281–25. C1917–25.
17. Saunders MM, You J, Zhou Z, Li Z, Yellowley CE, Kunze EL, Jacobs CR, Donahue HJ. Fluid-flow induced prostaglandin E2 response of osteoblastic ROS 17/2.8 cells is gap junction-mediated and independent of cytosolic calcium. *Bone*. 2003; 32(4):350–56. [PubMed: 12689677]
18. Saunders MM, Seraj MJ, Li Z, Zhou Z, Winter CR, Welch DR, Donahue HJ. Breast cancer metastatic potential correlates with a breakdown in homospecific and heterospecific gap junctional intercellular communication. *Cancer Res*. 2001; 61(5):1765–67. [PubMed: 11280719]
19. Davidson JS, Baumgarten IM, Harley EH. Reversible inhibition of intercellular junctional communication by glycyrrhetic acid. *Biochem Biophys Res Commun*. 1986; 134(1):29–36. [PubMed: 3947327]
20. McDonald JC, Metallo SJ, Whitesides GM. Fabrication of a configurable, single-use microfluidics device. *Anal Chem*. 2001; 73(23):5645–50. [PubMed: 11774902]

Figure 1. LDH staining of osteocytes seeded for 72 hours on CTI-coated PDMS. While cells displayed different activity levels, as evidenced by the range of staining intensity, cell viability was not adversely affected by the PDMS substrate in comparison to the cover glass. Scale bar represents 50 microns.

Figure 2.

Osteocyte proliferation on PDMS and glass was completed for 4, 24, 48, 72, 96 and 120 hours in culture. The figure shows cell proliferation and morphology for osteocytes seeded on PDMS and cover glass at 24, 72, and 120 hours. The cell proliferation and morphology for osteocytes on PDMS and cover glass were comparable. Given that the osteocytes seeded on glass were grown directly on the glass grid while the osteocytes seeded on PDMS were grown on the PDMS placed on top of the glass grid, the focal plane to visualize the grid and the osteocytes on the PDMS were not the same. However, images for all time points were cropped to an equivalently sized field of view for quantification. Scale bar represents 20 microns.

Figure 3.

Osteocyte proliferation quantified over the 120 hours culture period. As shown for both the PDMS and the cover glass substrates, proliferation steadily increased throughout the 120 hours culture period. From 4 hours baseline levels, osteocyte proliferation on PDMS increased 17.4, 58.0, 116.8, 207.0, and 257.3% at 24, 48, 72, 96 and 120 hours in culture, respectively. All comparisons were statistically significant ($p < 0.05$). From 4 hours baseline levels, osteocyte proliferation on glass increased 46.9, 83.5, 150.2, 191.3, and 222.4% at 24, 48, 72, 96, and 120 hours in culture, respectively. All comparisons were statistically significant ($p < 0.05$). As illustrated, proliferation was not significantly different between 72 and 120 hours for the PDMS and glass substrates. Results are plotted as mean $\pm$ SEM. Above each graph is a list of the other data points from which it is statistically different.

Figure 4.

Quantification of sclerostin optical density for ‘responders’ in randomly selected fields of view. Above each graph is a list of the other data points from which it is statistically different. Inset. Immunochromatological detection of sclerostin seeded on PDMS and maintained for 72 hours. Results are plotted as mean $\pm$ SEM

Figure 5.

Immunofluorescence demonstrating the presence of sclerostin and connexin 43 in osteocytes plated on PDMS or glass. As illustrated, sclerostin and connexin 43 were continuously expressed throughout the 120 hours culture period.

Figure 6.

Left) Typical gap junction fluorescence transfers with osteocytes plated for 72 hours on PDMS. As illustrated, osteocytes were capable of communication to an equal extent, regardless of substrate. Scale bar represents 100 microns. Right) Fluorescent detection of connexin 43 in osteocytes seeded on PDMS and maintained for 72 hours. Higher magnification reveals extensive dendritic processes, characteristic osteocyte morphology and intense cellular staining for cx43. Scale bar represents 10 microns.

Optical Density for Sclerostin Data

Table 1

Optical density results for sclerostin quantification. In addition to the number of cells expressing sclerostin, the percentage of the cell area demonstrating a presence of sclerostin was quantified. Results are shown as mean $\pm$ SEM.

	Total cells	Optical density in all cells	Expressing cells	Percent cells expressing	Optical density in expressing cells	Average area of expression
PDMS 4 hours	217	0.198 $\pm$ 0.012	120	55.3%	0.357 $\pm$ 0.004	2.4 $\pm$ 0.3%
PDMS 24 hours	206	0.112 $\pm$ 0.012	63	30.6%	0.368 $\pm$ 0.009	0.4 $\pm$ 0.1%
PDMS 48 hours	209	0.354 $\pm$ 0.008	194	92.8%	0.381 $\pm$ 0.004	5.7 $\pm$ 0.6%
PDMS 72 hours	214	0.357 $\pm$ 0.014	174	81.3%	0.439 $\pm$ 0.010	7.6 $\pm$ 0.7%
PDMS 96 hours	167	0.342 $\pm$ 0.012	140	83.8%	0.408 $\pm$ 0.004	17.4 $\pm$ 1.6%
PDMS 120 hours	223	0.451 $\pm$ 0.010	213	95.5%	0.472 $\pm$ 0.008	20.6 $\pm$ 1.4%
Glass 4 hours	179	0.174 $\pm$ 0.013	88	49.2%	0.353 $\pm$ 0.005	7.3 $\pm$ 1.1%
Glass 24 hours	113	0.098 $\pm$ 0.015	32	28.3%	0.347 $\pm$ 0.011	4.2 $\pm$ 0.7%
Glass 48 hours	209	0.214 $\pm$ 0.013	129	61.7%	0.346 $\pm$ 0.010	4.7 $\pm$ 0.8%
Glass 72 hours	133	0.398 $\pm$ 0.024	112	84.2%	0.472 $\pm$ 0.022	4.3 $\pm$ 0.5%
Glass 96 hours	90	0.415 $\pm$ 0.025	79	87.8%	0.473 $\pm$ 0.021	6.6 $\pm$ 1.3%
Glass 120 hours	223	0.430 $\pm$ 0.015	184	82.5%	0.522 $\pm$ 0.009	12.3 $\pm$ 1.0%

Table 2
Communication for Substrate Data

Percentage of osteocytes communicating on PDMS and cover glass in the presence and absence of AGA and DMSO. Communication was significantly reduced with the addition of the topical gap junction inhibitor, AGA. DMSO, used as a control further confirmed that the communication inhibition with the addition of AGA was specific to AGA and not a result of a generic topical additive.

	Total Cells	Percent Communicating	Statistical Significance	
PDMS	3009	63.5	PDMS vs. PDMS + AGA PDMS vs. PDMS + DMSO PDMS + DMSO vs. PDMS + AGA	***
PDMS + AGA	1975	12.5		NS
PDMS + DMSO	319	53.1		***
GLASS	1903	64.1	GLASS vs. GLASS + AGA GLASS vs. GLASS + DMSO GLASS + DMSO vs. GLASS + AGA	***
GLASS + AGA	1806	14.4		NS
GLASS + DMSO	403	59.3		***
			PDMS vs. GLASS PDMS + AGA vs. GLASS + AGA PDMS + DMSO vs. GLASS + DMSO	NS
				NS
				NS