Published in final edited form as:

Med Eng Phys. 2016 October ; 38(10): 1115–1122. doi:10.1016/j.medengphy.2016.06.002.

In Vitro Osteocytic Microdamage and Viability Quantification Using a Microloading Platform

S.L. York^{a,*}, P. Sethu^b, and M.M. Saunders^a

^aDepartment of Biomedical Engineering, The University of Akron, Akron, OH, 44325, USA

^bDepartment of Medicine, University of Alabama at Birmingham (UAB), Birmingham, AL 35294, USA

Abstract

Bone remodeling is a process in which bone is resorbed by osteoclasts and formed by osteoblasts. This is normally a paired process, although it can be disrupted by changes in mechanical load. One theory is that osteocytes play a key role in the cellular regulation of this process. Mechanotransduction studies, which investigate how cells convert mechanical stimuli into biophysical effects and cellular activity, offer one way to investigate this theory. Mechanotransduction work is commonly done by applying an isolated mechanical load to cells grown *in vitro*, and quantifying the response. While *in vitro* work does not fully replicate the natural environment, it does allow the study of isolated factors. In this study, a mechanical loading platform was designed, fabricated, and characterized for bone mechanotransduction studies. This platform was designed to tent cell-seeded substrates from below, loading using out of plane distension. This introduced a nonuniform strain profile, enabling the study of cells cultured under identical conditions and variable strains as a function of substrate location. An alphanumerically gridded polydimethylsiloxane well substrate was designed and fabricated for cellular loading experiments. Following initial characterization, a study was run to quantify the cellular activity of osteocyte-like MLO-Y4 cells as a function of strain field. The results indicated that regions with lower strains led to an increase in cellular activity while higher strains led to a reduction in cellular activity. This demonstrated that cells could be exposed to mechanically-induced microdamage using the microloading platform.

Keywords

Osteocyte; Mechanotransduction; Bone Remodeling

*Corresponding author. Tel.: (262) 719-9153. sly15@uakron.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest Statement:

The authors have no conflicts to declare.

1. Introduction

Bone is continually remodeling, as osteoclasts resorb old bone and osteoblasts lay down new bone. This is normally a paired process used to maintain bone at equilibrium [1]. Bone remodeling also allows for bone to adapt to changes in its mechanical environment by altering the balance between resorption and formation. Previous research has indicated that bone gains mass and becomes stronger when heavily used, such as the dominant arm of tennis players, and that decreases in use lead to loss of bone mass, such as in hospital patients undergoing extended bed rest [2; 3, 4]. The cellular regulation of bone remodeling is not clear.

One theory is that osteocytes, residing inside of bone, play a role in the bone remodeling process [5; 6]. As osteoblasts lay down new bone matrix, some become trapped in the matrix and differentiate into osteocytes [7]. Their location inside of bone allows them to detect changes in the mechanical environment and the strains placed on bone [8; 9]. Osteocytes also possess narrow channels, gap junctions, which extend between nearby cells [10]. These channels have been shown to allow communication through the passage of small molecules such as calcium ions and phosphates [11; 12; 13]. Finally, osteocytes have been shown to express factors known to have important roles in bone remodeling such as sclerostin and receptor activator of nuclear factor kappa-B ligand (RANKL) [14; 15]. These factors suggest osteocytes may play a role in controlling bone remodeling on a cellular level [5]. Osteocytes undergoing apoptosis are thought to play a role in controlling bone remodeling on a cellular level, and mechanotransduction studies offer one way to investigate these theories [16; 17].

Mechanotransduction studies aim to elucidate the mechanisms by which cells convert mechanical stimuli into chemical signals. Tissues in the body are exposed to a variety of mechanical forces, including gravity, movement, and pressure [18]. Mechanotransduction studies commonly subject cells cultured *in vitro* to an isolated mechanical load. Methods for loading in mechanotransduction studies include fluid shear stresses, stretching a substrate seeded with cells, and ultrasound [19; 20; 21]. To increase the level of biomimicry of an experiment, *in vitro* studies often aim to replicate the *in vivo* environment. To accomplish this, devices are often built in house [19].

The goal of this study was to design a low cost mechanical loading platform for mechanotransduction studies, and quantify the impact of mechanically induced microdamage on MLO-Y4 viability. This loading platform was designed to be easy to fabricate, easy to use, and adaptable to later lab on a chip studies. The platform was fabricated, assembled, characterized, and a polydimethylsiloxane (PDMS) well substrate for *in vitro* cell culture was designed and fabricated. PDMS was chosen due to its optical clarity, ease of fabrication, and previous research indicating that the osteocytic cell line used in this work grew, proliferated, and exhibited the same characteristic protein expression on PDMS and polystyrene [22]. The fabricated platform was then used to study the lactate dehydrogenase (LDH) levels of cells at a range of strains due to mechanical displacement and substrate location. It was anticipated that mechanical damage, via cell viability, could be quantified and that higher levels of strain would induce microdamage.

2. Materials and methods

The platform consisted of an aluminum base plate with leveling screws connected to a Plexiglass® top plate by aluminum corner rods. An additional aluminum mounting plate was suspended from the top plate by corner rods and was used to mount a Zaber linear actuator with a resolution of .48 μm (Zaber Technologies, T-NA08A25). The Plexiglass® top plate had a center hole cut out to allow sample tenting from below using the linear actuator. A Sensotec 250 gram load cell was attached to the actuator to detect contact with the substrate, while a stainless steel cylindrical platen was used to tent substrates from below (Honeywell). As the platen was raised, the force reported by the load cell was tracked and used to determine contact with the sample above, which was exposed to out of plane distension as it was tented by the platen. This exposed the sample substrate to a complex stress and strain profile.

A polycarbonate alignment block and cell culture holding system were designed and fabricated for the top of the platform and microscope. The alignment block positioned substrates during loading directly over the cylindrical platen, using aluminum centering legs that extended over the edge of the Plexiglass® top plate, and a slot for a polycarbonate cell culture holding system during loading. This prevented movement of substrates during loading and generated a more consistent load. The slot was designed to fit a cell culture holding system fabricated out of polycarbonate that eliminated preloading of cells. Additionally, a polycarbonate alignment block was designed for the microscope (Zeiss). This allowed the use of stage controls during imaging, and reduced handling of samples following experimentation.

The microactuator behavior was also characterized by assembling the platform and aligning the platen with a fixed surface. The displacement of the microactuator was decreased until a gauge block of known thickness fit between the surface and the platen. Measurements were taken in triplicate and repeated on 3 separate days. The displacement reported by the microactuator controller was compared to the thickness of the gauge blocks, characterizing the accuracy and repeatability of the microactuator.

Mechanical characterization of PDMS membranes with thicknesses from 100 to 2000 μm was performed. PDMS membranes were assembled by thoroughly mixing PDMS base and curing agent at a 10:1 ratio (Dow Corning, Sylgard 184). This solution was degassed in a vacuum desiccator before being poured onto a silicon disc mounted to a spin coater. This was run at speeds between 200 and 3000 RPM for 30 seconds, generating membranes at a range of thicknesses. The PDMS solution was cured in an oven at 70° C for 3 hours, and samples were cut to shape.

Narrow strips of PDMS were 6.35×25.4 mm with a uniform thickness and loaded in a small scale loading machine augmented with a 44.48 N load cell and 25 mm displacement sensor. PDMS strips were mounted to the loading machine using serrated friction clamps. Strips were exposed to tensile testing until failure, at a rate of .3 mm second⁻¹. Load displacement data was recorded in real time, normalized for geometry to generate stress-strain curves, and used to study the material properties of the PDMS. Additionally, the

overall strain of the PDMS strip was calculated by measuring the movement of the loading machine.

A PDMS well was designed to allow cells to grow *in vitro* prior to experimentation. Three layers of PDMS were glued together using uncured PDMS. The bottom layer was 1.9 mm thick, with a center hole removed using a core borer for cylindrical platen clearance. The center layer was .2 mm thick and had a positional reference grid. This allowed the same area to be studied before and after experimentation, improve repeatability between experiments, and allowing for detailed quantification of cellular behavior. The top layer was approximately 3.5 mm thick and had a center hole removed using a core borer, generating a well used for *in vitro* cell culture.

A digital image correlation algorithm was used to estimate the surface strain on the PDMS well substrate during loading. A PDMS substrate was spray painted to enhance contrast and images were taken as the substrate was loaded using the microloading platform. An algorithm in MATLAB was used to track the movement of pixels between images and estimate the surface strain.

Osteocyte-like MLO-Y4 cells, a generous gift from Dr. Lynda Bonewald, were cultured using a previously established protocol [23]. Minimum essential alpha medium (α -MEM, Gibco) supplemented with 5% fetal bovine serum (Hyclone), 5% fetal calf serum (Hyclone), and 1% penicillin/streptomycin (Pen/Strep, Invitrogen) was used for cell culture. The cells were maintained at 5% CO₂ and 37° C in 25 cm² flasks coated with rat tail collagen type I (BD Bioscience) at a concentration of 5 μ g/cm² in .02 M acetic acid (Sigma). Cells were passaged between 70 and 80% confluency using .25% Trypsin-EDTA.

MLO-Y4 cells were seeded onto PDMS substrates at a density of 2×10^4 cells/cm² for experimentation. These cells were maintained in .5 mL of medium, seeded 4 days prior to experimentation for reaching their characteristic protein expression and dendritic morphology, and fed every other day [22]. Two Kimwipes (Kimtech) were dipped in sterile water and wrapped around the perimeter of the plate to generate a humidified chamber. PDMS well substrates were maintained in the cell culture holding system to eliminate preloading due to PDMS sticking to the petri dish.

PDMS well substrates in a cell culture holding system were removed from the incubator after four days in culture and positioned using the alignment block. The microactuator displacement was increased until the load cell detected contact and then further increased to load the PDMS substrate. All loading was done for 15 minutes prior to 90 minutes of incubation. This short loading period was designed to study the initial cellular response due to microdamage. Initial results showed that displacements of 3.84 mm, 5.76 mm, and 7.2 mm induced a range of cellular responses and were used for further study. These displacements corresponded to actuator controller values of 8 mm, 12 mm, and 15 mm due to a scale factor of .48. Each loading condition was studied in triplicate and repeated on 3 separate days.

A LDH assay was used to measure cellular activity. Cells were washed with Hank's buffered saline solution prior to a 2 hour incubation in a solution containing 5% polypep (Sigma-

Aldrich), 2mM of gly-gly (Sigma Aldrich), 1.75 mg/ml of nicotinamide adenine dinucleotide (Sigma-Aldrich), 60 mM lactic acid, and 3mg/mL of nitroblue tetrazolium. This allowed cells with LDH activity, an enzyme associated with cellular activity, to stain purple. Higher levels of activity led to dark purple staining, while cells that were dead or not undergoing energy conversion did not stain.

PDMS well substrates were imaged using a microscope following the LDH stain. The pixel intensity in cells was measured using FIJI, a distribution of ImageJ. On each PDMS substrate, 40 cells were randomly measured in the positional reference grid in the center, 40 in each of the 4 squares at the edge of the platen in the 4 cardinal directions from the center, and 40 in each of the 4 squares halfway in-between, for a total of 360 cells in each PDMS well. Adjustments were made for the background in the square the cell was measured in, and compared to the LDH staining in control cells that were cultured on PDMS well substrates but not loaded. For statistical analysis, a D'Agostino & Pearson normality test was run. After the results indicated that not all of the data followed a normal distribution, all statistical analysis was completed using a Mann Whitney U Test for nonparametric statistics, with a p value of .05.

3. Results

Figure 1 displays the components of the microloading platform, including the microactuator and load cell used. Part D of the figure shows a PDMS substrate in position for loading, over the center hole and the platen connected to the microactuator and load cell. Increasing the actuator displacement tented the sample from below using the platen. The behavior of the actuator was characterized using gauge blocks of known thicknesses. The results, not shown, found a slope of .9994 when plotting the actuator controller displacement vs the gauge block thickness, and an R^2 value of 1.

A PDMS well substrate was designed for this experiment, figure 2. Part B of the figure shows the positional reference grid system, allowing the comparison of the same area between experiments. Mechanical characterization of PDMS strips indicated an elastic modulus of 1.7 MPa, figure 3A. The results of the parametric study of platen diameter and PDMS well thickness indicated significant differences in strain values, table 1. The results indicated that a PDMS well thickness of .5 mm and a platen diameter of 6 mm would be used in further experiments. The digital image correlation algorithm estimated the surface strains on the PDMS well substrates, results not shown. A polycarbonate cell culture holding system and alignment blocks were fabricated for this study, figure 3B and C. The PDMS well substrate, in the polycarbonate cell culture holding system, was positioned and held in place using the alignment block. Part B of showed the details of the out of plane distension loading, as the white tipped platen began to tent the PDMS well substrate.

Initial characterization of the LDH assay and experimental technique were also completed prior to experimentation, figure 4. The LDH stain was completed on unloaded cells on PDMS at a range of time points. The results in part A indicated that the cells were undergoing a color change, and that two hours was sufficient for all loading studies. Initial loading results, part B, indicated that the pixel intensity, measuring LDH activity, varied

with position in the PDMS well substrate. Thus, areas of the well were chosen to study between different experiments to achieve repeatable and quantifiable results at a range of strains. Cell adhesion after loading and staining was also verified, part C.

Following loading, an LDH assay was used to analyze cell activity around the PDMS well, Figure 5 A. This was used as an analog of cell viability, with a decrease in LDH activity and energy conversion corresponding to microdamage in the MLO-Y4 cells. The surface strain estimates were obtained from the digital image correlation algorithm. The lowest surface strain (5%) was observed in the middle of the PDMS well substrate at the lowest displacement, 3.84 mm. The highest surface strain (60%) was observed at the edge of the platen, 6 squares away from the center, at a displacement of 7.2 mm. Surface strains were found to increase as the location moved away from the center of the substrate, and at higher displacements. The LDH pixel intensities indicated significant changes in LDH activity at the varying displacements, figure 5. The results indicated an increase in LDH activity and energy conversion at the lowest displacement, 3.84 mm and a decrease at the highest displacement, 7.2 mm. The data was tested for normality using a D'Agostino & Pearson omnibus normality test, and the results indicated the data did not have a normal distribution. Non parametric statistics were run comparing each of the areas and the control, and the results indicated each strain was significantly different from the control, table 2. Thus, the mechanical loading platform significantly altered LDH expression, and cellular activity. Additionally, figure 5 indicates an increase in activity at surface strains of 5, 8, and 15%, and to a decrease at 10, 19, 34, 35, 54, and 60%.

4. Discussion

The microactuator characterization data indicated that the known thickness of the gauge blocks corresponded closely to the theoretical thickness reported by the microactuator, indicating precise and repeatable measurements. The mechanical characterization of narrow sheets of PDMS indicated values which agreed with literature values, indicating that narrow sheets could be used for the cellular experiments [24; 25]. A digital image correlation algorithm was used to estimate strains across the PDMS well substrate, and the results indicated that the strains peaked at the edge of the platen, and had a minimum directly over the center of the platen. To study a range of strains at a constant displacement, the reference grid square directly over the middle of the platen, 4 reference grid squares at the edge of the platen, and the 4 squares halfway between these two extremes were used for cellular studies.

PDMS well substrates were loaded and cellular activity was measured using a LDH assay. This study aimed to study the initial impact of cellular microdamage, so 15 minutes of loading was used. Additionally, the loading ranges in this study exceeded physiologic levels, as fluid shear stresses were not used and the initial response to damage was under study. The results indicated that loading using this platform could alter cellular activity. There was a significant increase in activity at a displacement of 3.84 mm, values were near the control at a displacement of 5.76 mm, and a displacement of 7.2 mm led to a significant decrease in activity, figure 5. Results were obtained by measuring the LDH staining intensity of individual cells, in contrast to many cellular studies that obtain a result averaged across cells. The results indicated that the platform and the out of plane distension could be used to

induce microdamage in the MLO-Y4 cells. Microdamage was determined to occur at a surface strain between 15 and 19%, as all conditions above 19% showed a decrease in cellular activity. This trend of low strains leading to increases in viability prior to a decrease at higher strains, is consistent with previous research in which bones were exposed to mechanical strains [26]

This study also demonstrated a method for studying cellular behavior at a range of strains in one experiment, through the use of a nonuniform strain profile and a positional reference grid, fabricated from PDMS. This experiment used this grid to track position relative to the center of the mechanical loading platen, and thus estimate local surface strains. A positional reference grid could also be used for cellular adhesion studies, as it would allow the study of the same area before and after applying a treatment. The use of PDMS for Mechanotransduction studies may also allow the development of lab on a chip devices. These devices could be designed to provide a platform for cell culture and loading, and then performing analysis on the same substrate.

The use of PDMS also serves as an important step in further mechanotransduction studies. This work demonstrated that the microloading platform could be used for inducing microdamage in osteocyte-like MLO-Y4 cells. Inducing microdamage was the first step in the development of a multicellular model for bone remodeling, incorporating osteocytes, osteoblasts, and osteoclasts. This device would allow the study of the interactions between bone cells as part of the cellular regulation of bone remodeling. Additionally, by culturing osteoblasts and osteoclasts on bone wafers, the impact of osteocyte microdamage on the rates of bone remodeling and bone resorption can also be quantified. This provides a method for further elucidating the cellular regulation of bone remodeling.

5. Conclusion

In this work, a mechanical loading platform was designed, fabricated, and characterized for mechanotransduction studies. The results indicated that the microactuator was accurate and repeatable. This study, analyzing LDH activity, indicated that loading on the platform led to an increase in cellular activity and energy conversion at a low displacement. The results also indicated that the cells had a decrease in LDH activity at the highest displacement studied, indicative of cells undergoing microdamage. This demonstrated that the mechanical loading platform could be used for mechanotransduction studies of MLO-Y4 osteocyte-like cells by loading substrates with a range of strains and generating varying degrees of cellular activity. This LDH study indicated that the mechanical loading platform could induce damage to MLO-Y4 cells, allowing further mechanotransduction studies using a lab on a chip approach. This ongoing work aims to further characterize the strain values of the PDMS well substrate, further investigate the impact of mechanically-induced microdamage on MLO-Y4 behavior and protein expression, and examine the impact of conditioned medium from microdamaged cells on the bone remodeling process.

Acknowledgments

The authors would like to thank Dale Ertley for his help with machining components for the mechanical loading platform, the NSF for funding through a CAREER award (1060990), and the NIH for funding through an NIH NIDCR AREA (R15) award (DE022664).

References

1. Parfitt AM. The cellular basis of bone remodeling: the quantum concept reexamined in light of recent advances in the cell biology of bone Calcified. Tissue Int. 1984; 36:S37–S45.
2. Haapasalo H, Kontulainen S, Sievanen H, Kannus P, Jarvinen M, Vuori I. Exercise-induced bone gain is due to enlargement in bone size without a change in volumetric bone density: a peripheral quantitative computed tomography study of the upper arms of male tennis players. Bone. 2000; 27:351–357. [PubMed: 10962345]
3. Huddleston AL, Rockwell D, Kulund DN, Harrison B. Bone mass in lifetime tennis athletes. J Am Med Assoc. 1980; 244:1107–1109.
4. Leblanc AD, Schneider VS, Evans HJ, Engelbretson DA, Krebs JM. Bone mineral loss and recovery after 17 weeks of bed rest. J Bone Miner Res. 1990; 5:843–850. [PubMed: 2239368]
5. Bonewald LF. The amazing osteocyte. J Bone Miner Res. 2011; 26:229–238. [PubMed: 21254230]
6. Burger EH, Klein-Nulend J. Mechanotransduction in bone- role of the lacuna-canalicular network. FASEB (Fed Am Soc Exp Biol) J. 1999; 13:S101–S112.
7. Noble BS. The osteocyte lineage. Arch Biochem Biophys. 2008; 473:106–111. [PubMed: 18424256]
8. Han Y, Cowin SC, Schaffler MB, Weinbaum S. Mechanotransduction and strain amplification in osteocyte cell processes. Proc Nat Acad Sci USA. 2004; 101:16689–16694. [PubMed: 15539460]
9. Rath AL, Bonewald LF, Nicoletta DP. Tissue strain amplification at the osteocyte lacuna: a microstructural finite element analysis. J Biomech. 2007; 40:2199–2206. [PubMed: 17196968]
10. Jones SJ, Gray C, Sakamki H, Arora M, Boyde A, Gouride R, et al. The incidence and size of gap junctions between the bone cells in rat calvaria. Anat Embryol. 1993; 187:343–352. [PubMed: 8390141]
11. Donahue HJ. Gap junctions and biophysical regulation of bone cell differentiation. Bone. 2000; 26:417–422. [PubMed: 10773579]
12. Henriksen K, Neutsky-Wulff AV, Bonewald LF, Karsdal MA. Local communication on and within bone controls bone remodeling. Bone. 2009; 44:1026–1033. [PubMed: 19345750]
13. Taylor AF, Saunders MM, Shingle DL, Cimbala JM, Zhou Z, Donahue HJ. Mechanically stimulated osteocytes regulate osteoblastic activity via gap junctions. Am J Physiol-Cell Ph. 2005; 292:C633–C643.
14. Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-hora M, Feng JQ, et al. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. Nat Med. 2011; 17:1231–1234. [PubMed: 21909105]
15. Winkler DG, Sutherland MK, Geoghegan JC, Yu C, Skonier JE, Shpektor D, et al. Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. Eur Mo Biol Organ J. 2003; 22:6267–6276.
16. Cardoso L, Herman BC, Verborgt O, Laudier D, Maeska RJ, Schaffler MB. Osteocyte apoptosis controls activation of intracortical resorption in response to bone fatigue. J Bone Miner Res. 2009; 24:597–605. [PubMed: 19049324]
17. Verborgt O, Gibson GJ, Schaffler MB. Loss of osteocyte integrity in association with microdamage and bone remodeling after fatigue in vivo. Biochem Biophys Res Commun. 2000; 348:1082–1088.
18. Ingber DE. Tensegrity: the architectural basis of cellular mechanotransduction. Annu Rev Physiol. 1997; 59:575–599. [PubMed: 9074778]
19. Brown T. Techniques for mechanical stimulation of cells in vitro: a review. J Biomech. 2000; 33:3–14. [PubMed: 10609513]

20. Liu, D.; Ou, Z. Proceedings of the 54th Annual Meeting of the Orthopaedic Research Society. San Francisco, CA: 2008. Low intensity pulsed ultrasound synergistically modulates sost and OPG/RANKL ratio in MLO-Y4 osteocytes in vitro. 2008
21. Tan SD, Vries TJ, Kuijpers-Jagtman AM, Semeins CM, Everts V, Klein-Nulend J. Osteocytes subjected to fluid flow inhibit osteoclast formation and bone resorption. *Bone*. 2007; 41:745–751. [PubMed: 17855178]
22. York SL, Arida AR, Shah KS, Sethu P, Saunders MM. Osteocyte characterization on polydimethylsiloxane substrates for microsystems applications. *J of Biomim, Biomater, and Tissue Eng*. 2012; 16:27–42.
23. Rosser J, Bonewald LF. Studying osteocyte function using the cell lines MLO-Y4 and MLO-A5. *Bone Res Protoc*. 2012; 816:67–81.
24. Mata A, Fleischman AJ, Roy S. Characterization of polydimethylsiloxane (PDMS) properties for biomedical micro/nanosystems. *J Mech Behav Biomed*. 2005; 28:183–194. 2005.
25. Schneider F, Draheim J, Kamberger R, Wallrabe U. Process and material properties of polydimethylsiloxane (PDMS) for optical MEMS. *Sensor Actuat A-Phys*. 2009; 151:95–99.
26. Noble BS, Peet N, Stevens HY, Brabbs A, Mosley JR, Reilly GC, Reeve J, Skerry TM, Lanyon LE. Mechanical loading: biphasic osteocyte survival and targeting of osteoclasts for bone destruction in rat cortical bone. *Am J Physiol Cell Physiol*. 2003; 284:C934–C943. [PubMed: 12477665]

Figure 1.

A) Drawing of microloading platform. The platform was designed to have the actuator displacement increase and test samples from below. Designed without specific fixtures, this platform could be adapted for samples of a variety of shapes and sizes. B) Zaber microactuator, Zaber microactuator controller, and Sensotec load cell. The microactuator was shown attached to the actuator base plate from part A, and the microactuator controller was used to set the displacement of the actuator. The load cell was attached to the microactuator and used to detect contact during loading. C) Assembled microloading platform frame. Three adjustable screws were attached to the bottom of the aluminum base plate to level the platform. Corner rods attached the Plexiglass top plate and aluminum base plate. D) Assembled Microloading Platform. Samples for loading were placed on top of the center hole, allowing loading from below using the platen connected to the microactuator.

Figure 2.

A) Drawing of PDMS well substrate. The well substrate was designed to allow *in vitro* cell culture for experimentation, and had an alphanumeric positional reference system. This system enabled study of the same area before and after loading, as well as comparing the same area between experiments. B) Fabricated PDMS well substrate in polycarbonate cell culture holding system. The PDMS substrate had a section removed from the top to generate a well for cell culture, and a section removed from the bottom to allow tenting of the PDMS substrate from below. Inset displays the positional reference system.

Figure 3.

A) Setup and results of PDMS mechanical characterization. Tensile testing was completed to confirm the properties of strips of PDMS. Results indicated an elastic modulus of 1.7 MPa, agreeing with literature values [Mata et al., 2005; Schneider et al., 2009] B) Detail view of sample loading. Samples were centered on top of the mechanical loading platform using the alignment block. The microactuator loaded samples by tenting from below, applying an out of plane distortion load. This introduced a nonuniform strain on the sample, allowing the study of a range of loads. The platen tip was colored white to allow the tip to be more easily visualized in images. C) Setup of mechanical loading platform for experimentation. Increasing the displacement of the microactuator raised the attached platen and led to substrate loading. D) Detailed view of out of plane distortion in PDMS substrate, caused by the white tipped cylindrical platen.

Figure 4.

A) Time comparison of LDH activity on PDMS. Results indicated an increase in staining intensity in 30 minute time increments from no staining up to two hours. This indicated that future studies would need two hours of LDH staining. B) Position comparison of LDH activity on PDMS. The LDH activity was analyzed on a loaded sample at a variety of locations. As the location moved away from the center of the well substrate, the LDH activity decreased. This indicated that loading of the PDMS well substrate resulted in a change in LDH activity, allowing further experimentation. C) Analysis of cell adhesion

following loading. Using the positional reference grid, the same area was examined before and after loading. By counting the number of cells present after loading, the percentage of cells remaining adhered to the PDMS could be quantified. Results indicated that 97% of cells remained adhered following loading.

Figure 5).

A) Areas used for quantification following loading. The mosaic displays areas where LDH activity was quantified. Areas chosen were the middle square of the well, colored in white, 3 positional reference squares away in the four cardinal directions, colored in black and white, and 6 positional reference squares away, colored in black. 6 squares away from the center corresponded to the edge of the platen used for loading, and thus the highest stresses and strains as shown in digital image correlation data, not shown. B) LDH activity in middle Square. Estimated surface strains observed in the middle square were 5, 15, and 35%, as determined by a digital image correlation algorithm. Results indicated that in the middle square at the two lower displacements, LDH intensity increased significantly, prior to a decrease at the highest surface strain. Each bar indicates the average measurement from 360

cells. C) LDH activity 3 squares away. A significant increase in intensity was observed at 8% surface strain, prior to a return to values near the control at 19% and a decrease at 54%. Each bar indicated the measurement from 1440 cells. D) LDH activity 6 squares away. The data indicates a statistically significant decrease in LDH expression near the edge of the platen. E) LDH activity by displacement. The data from the first three graphs was combined, with surface strains of 5, 8, and 10% (corresponding to 3.84 mm), 15, 19, and 34% (corresponding to 5.76 mm), and 35, 54, and 60% (corresponding to 7.2 mm). The significant increase in activity at 3.84 mm corresponded to an increase in cellular activity, while the decrease at 7.2 mm was indicative of cellular damage. Each bar represented the average measurement of 3240 cells. F) LDH activity by region. The left bar contains coupled data from the middle square of the PDMS well substrate (Figure 5 Part A), the middle bar corresponds to three squares away from the center of the well (Figure 5 Part B), and the final bar corresponded to 6 squares away (Figure 5 Part C). Results indicated a significant decrease in expression as moving from areas of low to high strain, indicative of cellular damage at high strains in the 6 squares away region.

Parametric study of PDMS well substrate at a range of well thicknesses, platen diameters, and load applied. Results indicated failure at high loads at well thicknesses below .5 mm, and this value was chosen for the PDMS well design. Additionally, the parametric results indicated that a platen diameter of 4 or 6 mm would allow the study at a wide range of strains. 6 mm was chosen as the wider diameter would allow the study at several locations that were loaded throughout the well.

Table 1

-	Max Stress (vonMises) (MPa)	Max Displacement (mm)	Max Strain (mm/mm)	Max Principal Stress (MPa)	Minimum Principal Stress (MPa)
0.25 mm well thickness					
2, LC, 0.25	3.3	1.86E+00	1.14E-01	3.6	1.2
4, LC, 0.25	4.9	3.423	2.77E-01	6.5	3.2
6, LC, 0.25	4.7	3.23E+00	1.65E-01	5.3	2.6
2, LC, 0.50	6.6	3.716	2.28E-01	7.1	2.5
4, LC, 0.50	9.9	6.85E+00	5.54E-01	12.9	6.5
6, LC, 0.50	9.5	6.463	3.30E-01	10.6	5.3
2, LC, 0.75	10	5.574	3.43E-01	10.7	3.7
4, LC, 0.75	14.8	1.03E+01	8.29E-01	19.4	9.8
6, LC, 0.75	14.2	9.696	4.95E-01	15.9	7.9
0.38 mm well thickness					
2, LC, 0.25	1.5	5.68E-01	5.20E-02	1.6	0.5
4, LC, 0.25	3.9	1.039	2.28E-01	8.4	4.9
6, LC, 0.25	2.1	9.93E-01	7.53E-02	2.2	1
2, LC, 0.50	3	1.136	1.04E-01	3.1	0.9
4, LC, 0.50	7.7	2.08E+00	4.56E-01	16.8	9.8
6, LC, 0.50	4.3	1.985	1.51E-01	4.4	2
2, LC, 0.75	4.5	1.704	1.56E-01	4.7	1.4
4, LC, 0.75	11.5	3.119	6.96E-01	25.2	14.8
6, LC, 0.75	6.4	2.977	2.26E-01	6.6	3
0.5 mm well thickness					
2, LC, 0.25	0.7	2.65E-01	3.06E-02	0.9	0.3

-	Max Stress (vonMises) (MPa)	Max Displacement (mm)	Max Strain (mm/mm)	Max Principal Stress (MPa)	Minimum Principals Stress (MPa)
4, LC, 0.25	1.3	4.78E-01	8.15E-02	2.9	1.7
6, LC, 0.25	1.2	4.66E-01	4.44E-02	1.3	0.5
2, LC, 0.50	1.7	5.30E-01	6.12E-02	1.9	0.5
4, LC, 0.50	2.5	9.56E-01	1.60E-01	5.7	3.4
6, LC, 0.50	2.5	9.31E-01	8.87E-02	2.6	1.1
2, LC, 0.75	2.5	7.95E-1	9.18E-02	2.8	0.8
4, LC, 0.75	3.8	1.434	2.45E-01	8.6	5.1
6, LC, 0.75	3.7	1.397	1.33E-01	4	1.6

Notes:
2, 4 and 6: refers to the diameter of the platen used in mm
LC: refers to load controlled test setup
0.25, 0.50 and 0.75: refers to the load applied by the platen in N
Highlighted cells indicate material failure as vonMises stress exceed the yield stress

Table 2

Statistical analysis of LDH data.

	Control	5%	8%	10%	15%	19%	34%	35%	54%	60%
Control		*	*	*	*	*	*	*	*	*
5%	*		NS	*	*	*	*	*	*	*
8%	*	NS		*	*	*	*	*	*	*
10%	*	*	*		*	NS	*	NS	*	*
15%	*	*	*	*		*	*	*	*	*
19%	*	*	*	NS	*		*	NS	*	*
34%	*	*	*	*	*	*		*	*	NS
35%	*	*	*	NS	*	NS	*		*	*
54%	*	*	*	*	*	*	*	*		*
60%	*	*	*	*	*	*	NS	*	*	

*'s indicate statistical significance with a p value of .05, while NS stands for not significant. Results indicated that the LDH activity was significantly altered by the mechanical load, as each surface strain was different from the control value of no load. The statist results, in conjunction with data from figure 5, indicated that damage occurred at a surface strain between 15 and 19%. Values below 15% led to an increase in LDH staining intensity, by up to 58%. Surface strains from 19 to 60% led to significant decreases in LDH staining intensity and thus cellular activity by up to 16%, indicative of damage.