Stem Cell Research & Therapeutics
SCRT, 5(1): 146-163
www.scitcentral.com

ISSN: 2474-4646

Review Article: Open Access

Curvature-Defined Culturing Technology – A New Tool for the Studies of Stem Cell Mechanobiology: A Perspective

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Received July 02, 2019; Revised July 24, 2019; Accepted July 26, 2019

ABSTRACT

Increasing evidences have shown that substrate geometries have profound influences on stem cell behaviors. Motivated by the necessity to study cellular behaviors on curved surfaces, a few methods have been developed to fabricate convex and concave microstructures for cell culturing, but these methods cannot precisely control the surface shapes of the fabricated microstructures, and it is also very problematic to use these methods to fabricate microstructures at larger or millimeter scales. We have recently developed a curvature-defined (C-D) cell culturing technology; a class of micro glass ball (MGB) embedded polyacrylamide (PA) gels, which virtually has no limits on the range of the presented surface curvatures for cell culturing. This cell culturing technology provides a unique and effective tool and opens up a systematic paradigm for the studies of cell mechanobiological responses to substrate curvatures (i.e., the surface curvatures of the substrates) and their related applications. We have cultured human mesenchymal stem cells (hMSCs) on the embedded MGBs, and we found that, the substrate curvatures restricted the spreading and induced the differentiation of the hMSCs, which justifies the necessity to carry out the substrate curvature-related systematic experimental and theoretical studies for the development of stem cell mechanobiology. Independent from substrate matrix elasticity and substrate rigidity, substrate curvature presents another substrate mechanical parameter to modulate cell contractility, which may motivate researchers to fabricate new curved substrates and conduct new related experiments to study the underlying biophysical mechanisms and biomolecular signaling pathways for the observed mechanobiological responses of stem cells to the mechanical factors including substrate geometries, substrate matrix elasticity, and substrate rigidity.

The perspectives of this C-D culturing technology and its potential applications in the studies of stem cell mechanobiology are discussed. Since polydimethylsiloxane (PDMS) materials are much more rigid than PA gels, to make this C-D culturing technology much more effective and efficient, PDMS materials may be used to immobilize the MGBs to make MGB embedded PDMS substrates and C-D concave and convex spherical PDMS surfaces may then be obtained. Arrays of C-D convex or concave spherical PA gel surfaces may be obtained by coating the arrays of the obtained C-D MGB surfaces or convex or concave spherical PDMS surfaces with a PA gel of a uniform thickness, and then the effects of surface curvatures on cellular traction forces, focal adhesions, stress fibers and contractile actomyosin apparatus may be studied. Substrates with simple varying surface curvatures may also be designed and fabricated to study the abilities of stem cells to sense and to react to variations in substrate curvatures. The relevant substrate curvature-dependent mechanics and energetics of stem cells at both the continuum and molecular levels may be developed. The quantitative equivalencies between the decreased cell contractility of hMSCs growing on C-D substrates with larger surface curvatures and that of hMSCs growing on softer PA gels and softer PDMS micropost arrays may be sought. Culturing hMSCs on convex and concave spherical PA gel surfaces will reveal the combined effects of surface curvature and matrix elasticity on stem cell differentiation. This C-D culturing technology may also be used as an effective tool to deform the nucleus of a stem cell to study the roles of the induced-nuclear deformations in inducing the observed stem cell differentiations.

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Citation: Yang S. (2020) Curvature-Defined Culturing Technology – A New Tool for the Studies of Stem Cell Mechanobiology: A Perspective. Stem Cell Res Th, 5(1): 146-163.

Keywords: Stem cell, Curvature-defined culturing, Mechanobiology, Differentiation, Micro glass ball embedded gel, PDMS

Abbreviations: 3D: Three-Dimensional; AFM: Atomic Force Microscopy; C-D: Curvature-Defined; hMSCs: Human Mesenchymal Stem Cells; MGB: Micro Glass Ball; PA: Polyacrylamide; PDMS: Polydimethylsiloxane; RT-PCR: Real-Time Polymerase Chain Reaction; SS: Spherical Surface

INTRODUCTION

Increasing evidences have shown that mechanical factors have profound influences on cellular biochemical and biological behaviors, but the relevant studies in cell mechanobiology are far from being systematic or well-documented. Substrate geometries, which belong to mechanical factors, have been shown to influence and induce stem cell differentiation. These substrate geometries mainly include the planar geometrically defined micro-patterns [1-6] and the non- planar nanotopographies [7-11]. Motivated by the necessity to study the behavioral responses of cells growing on curved surfaces [12], a few methods, including sucking a thin polydimethylsiloxane (PDMS) membrane through a shadow mask [13], thermal reflow of photoresist [14] and stereolithography and the after slowly-shrinking [15], have been developed to fabricate convex and concave microstructures to culture cells. However, these methods cannot precisely control the geometrical shapes of the surfaces of the fabricated convex and concave microstructures and the shapes of the surfaces of these fabricated convex and concave microstructures are not necessarily spherical and the curvatures of the surfaces of these fabricated microstructures normally cannot be precisely known or defined. It is also very problematic to use these methods to fabricate convex and concave microstructures at larger or millimeter scales for cellular studies.

THE CURVATURE-DEFINED (C-D) CULTURING TECHNOLOGY AND STEM CELL EXPERIMENTAL RESULTS

We have recently developed a class of C-D substrates, micro glass ball (MGB) embedded polyacrylamide (PA) gels, for cell culturing [16,17]. In such a substrate, the PA gel is used to immobilize the MGBs. Before the polymerization of the PA solution, the MGBs with diameters for the desired studies were pressed into the surface of the PA solution. After the polymerization of the PA solution, the MGBs were immobilized or embedded on the surface of the formed PA gel and the exposed parts of the embedded MGBs from the surface of the PA gel were used as the convex microstructures to culture cells. The three-dimensional (3D) shape of the surface of a MGB is spherical and is well-defined, and the principal curvature at any point on the surface of a ball is the same and calculated as the inverse of the radius of this ball's spherical surface (SS) (i.e., the inverse of the radius of this ball). For this situation of curved- surfaces with well-defined shapes, the principal curvatures at any point on such a surface can be readily obtained by using the results in differential geometry, and therefore, we say the curved-surfaces with well-defined shapes are C-D, and then MGB embedded PA gels have C-D surfaces. Here, for cell culturing, substrates having C-D surfaces are called C-D substrates and MGB embedded PA gels are C-D substrates.

This method of making C-D substrates by immobilizing the microstructures, which in the current case are the MGBs, with well-defined surface shapes and surface curvatures in polymerizing gels, ingeniously avoids the difficulty of fabricating microstructures with C-D surfaces of the other developed methods that directly fabricate on the surfaces of the substrates. The other vital advantage of this method is that it virtually has no limits on the sizes of the microstructures that it immobilizes, i.e., this method virtually has no limits on the range of the generated surface curvatures for cell studies. To date, the diameters of the glass balls that we have used to make MGB embedded PA gels were from 5 μm to 6 mm [17], the ball diameters were from 5 μm to 4 mm and recently we used 5 mm and 6 mm diameter balls and the experimental results have not been published). While the surface of a glass ball with a diameter of several millimeters is virtually flat with respect to the size of a cell, the small surface curvatures of the glass balls with diameters of several millimeters can have profound effects on stem cell behaviors, as shown in our experimental results on human mesenchymal stem cells (hMSCs) [17]. The effects of substrate curvatures (i.e., the surface curvatures of the substrates) at this small scale, or the effects of curved substrates with diameters at the millimeter scale, on cellular behaviors have also not been investigated before. Therefore, due to its C-D nature and wide-range-of-curvature-coverage nature, we say that this class of substrates, MGB embedded PA gels, provides a unique and effective tool and opens up a systematic paradigm for the studies of cell mechanobiological responses to substrate curvatures and their related applications.

We have cultured NIH-3T3 fibroblasts and hMSCs on these MGB embedded PA gels [16,17]. We found that, as expected, overall both the cell attachment rate and mean cell spread area of both cell types decreased with the decrease of the substrate ball diameter. But, the sensitivities of the attachment and spreading morphology of an hMSC to substrate curvature were very different from those of a fibroblast. Specifically, (1) Among the used diameters, the minimum diameter of a glass ball on which an NIH-3T3 fibroblast can attach and spread, without wrapping over the ball, was 58 μm, whereas the minimum diameter of a glass ball on which an hMSC can attach and spread was 500 μm.

This indicates that the attachment of an hMSC is much more sensitive to the large surface curvatures of the small substrate balls than that of a fibroblast. (2) The spreading morphologies of the fibroblasts on the 2 mm-balls were almost indistinguishable from those of the fibroblasts on the flat glass plates, but the hMSCs on the 4 mm-balls were majorly spindle-shaped with only two lamellipodia while the hMSCs on flat plates were well-spread with randomly multiple lamellipodia. This indicates that the spreading morphology of an hMSC is much more sensitive to the small surface curvatures of the large substrate balls than that of a fibroblast. (3) The hMSCs on the balls with diameters from 4 mm to 500 μm were always majorly spindle-shaped with only two lamellipodia, whereas the morphologies of the fibroblasts varied from the well-spread shapes on the 2 mm-balls to the round-shapes on the 500 μm-balls. This indicates that the spreading morphology of a fibroblast is much more sensitive to the intermediate surface curvatures of the intermediately-sized substrate balls than that of an hMSC.

Due to the abrupt change in spreading morphology, from the well-spread shapes on the flat plates to the spindle shapes on the MGBs, of the hMSCs and due to the decreased mean cell spread area of the hMSCs with the decrease of the substrate ball diameter, we say that, the curvatures of the substrates restricted the spreading of the hMSCs and this restriction was larger when the substrate curvature was larger. Based on the related reports on substrate geometries [1-6] and substrate matrix elasticity [18-20] and substrate rigidity [21] influencing and inducing stem cell differentiation, it is very reasonable for us to hypothesize that substrate curvatures influence and induce stem cell differentiation. Therefore, we conducted the real-time polymerase chain reaction (RT-PCR) analysis to quantify the relative osteogenic and adipogenic gene expressions of the hMSCs growing on the MGBs. Without the corresponding differentiation induction media, with respect to the corresponding gene expressions of the hMSCs growing on the flat plates, we did not find any significant osteogenic gene expression for all the hMSCs growing on the MGBs, but we found that there was significant adipogenesis for the hMSCs growing on the 1.1 mm, 900 μm, 750 μm and 500 μm balls and the hMSCs growing on the 2 mm, 3 mm and 4 mm-balls and on the flat plates had negligible adipogenesis. Thus, adipogenesis of hMSCs can be induced purely by appropriate substrate curvatures, i.e., substrate curvatures alone can induce stem cell differentiation. We also found that, the variation of the relative adipogenic gene expression of the hMSCs with the diameter of the substrate ball was not monotonic, and there was no obvious trend of this variation with the diameter of the substrate ball. **Figure 1** schematically illustrates the MGB embedded PA gels and our experimental findings on the hMSCs growing on the MGBs.

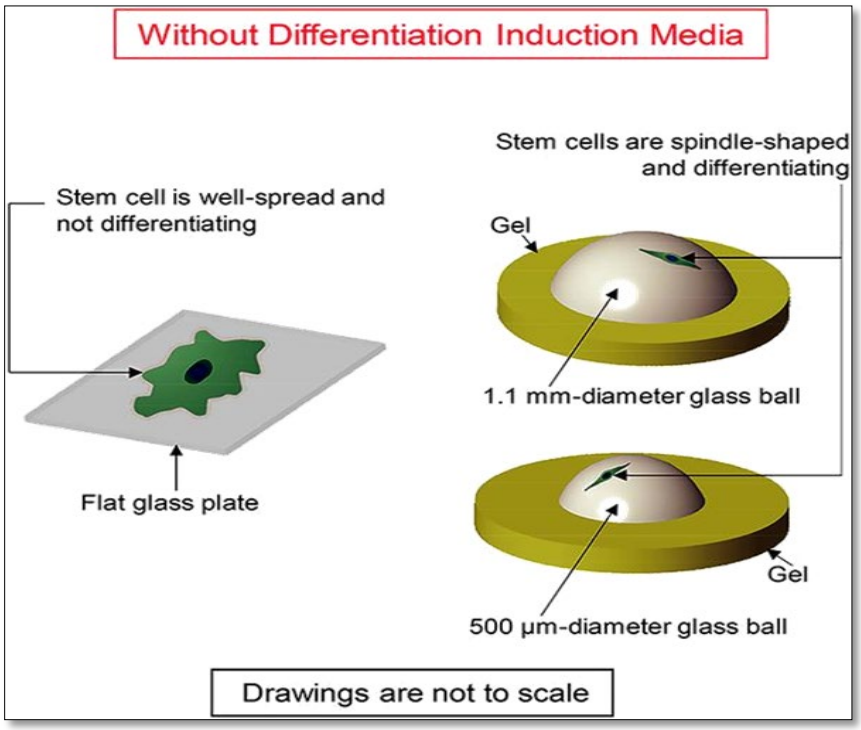

Figure 1. Schematic drawing to illustrate the class of curvature-defined substrates, micro glass ball (MGB) embedded gels and to illustrate that (1) substrate curvatures restrict the spreading of stem cells: the stem cells growing on the glass balls are almost uniformly spindle-shaped and substrate curvatures alone can induce differentiation of the stem cells. Figure reprinted with permission from Lee and Yang [17].

PERSPECTIVE OF THIS C-D CULTURING TECHNOLOGY IN STEM CELL MECHANOBIOLOGY

Because of the above-mentioned significant experimental findings on the hMSCs growing on the curved substrates (i.e., the MGBs) [17] and its online supporting information, it is necessary to carry out the substrate curvature-related systematic experimental and theoretical studies for the development of stem cell mechanobiology which has vast applications in tissue engineering and regenerative medicine [20,22]. This C-D culturing technology, i.e., the class of MGB embedded PA gels, provides a quantitative experimental tool for these systematic studies. The following discusses the perspectives of this C-D culturing technology and its potential applications in the studies of stem cell mechanobiology.

MGB embedded PDMS substrates

Due to its ideal properties such as non-toxicity, biocompatibility, blood compatibility, elasticity, transparency and durability [23], the elastomer material, PDMS, is widely used to fabricate microstructures for cellular studies [13,14,21,24-26]. By using the same idea of making MGB embedded PA gels; C-D MGB embedded PDMS substrates for cell culturing may also be made through utilizing the polymerization process of forming PDMS microstructures to immobilize the MGBs **(Figures 2a and 2b)**. Because of the much larger Young's moduli of PDMS materials compared with those of PA gels, PDMS substrates are much more rigid and their geometrical sizes are much less temperature-sensitive than those of PA gels and then embedding and holding a MGB on the surface of a PDMS substrate should be much easier than on the surface of a PA gel and an embedded MGB should be much less likely to roll on and detach from the surface of a PDMS substrate than to roll on and detach from the surface of a PA gel. Therefore, overall to make and use MGB embedded PDMS substrates should be much easier than to make and use MGB embedded PA gels for cell culturing (as discussed in the online supporting information [17], it is very challenging to make and use MGB embedded PA gels for cell culturing, majorly due to the significant rolling and detaching of the embedded MGBs on and from the surfaces of the PA gels during the entire experimental process). Thus, developing MGB embedded PDMS substrates for cellular studies and their biomedical applications may be the next effort to make this C-D culturing technology much more effective and efficient.

Also, the microstructures to be embedded on the surfaces of the PA gels and PDMS substrates do not have to be MGBs, and depending on the desired cellular studies, any microstructures with well-defined surface shapes may be embedded and the sizes of the embedded microstructures do not have to be the same in a single cell culturing substrate. For examples, micro glass cylinders may be embedded on the surfaces of the PA gels and PDMS to make substrates to study the cellular responses to cylindrical substrates with various diameters; Micro oval bodies may be embedded to study the cellular responses to surfaces with varying curvatures; Square-shaped and rectangular-shaped micro bodies/particles may be embedded to study the cell mechanosensitivities to locally-rigid or -soft substrate regions, etc. And, the material for the embedded micro balls does not have to be glass and again depending on the desired studies, the materials for the embedded micro balls or microstructures with any other surface shapes can be anything (e.g. plastics, hydrogels, ceramics, silicon, or metals, etc.) and the materials for the embedded micro balls or microstructures do not have to be the same in a single cell culturing substrate.

Concave spherical PA gel surfaces

Note that the MGB embedded PA gels only present the C-D convex SSs. Exposed concave spherical PA gel surfaces may be obtained by carefully-removing the embedded MGBs from the MGB embedded PA gels. In our experiments, we have tried this removing process of the embedded MGBs and have observed the exposed concave PA gel surfaces. Although we have not carefully and systematically examined into the exposed concave PA gel surfaces, this removing process presents a method to make non-planar concave PA gel surfaces which may be highly-desirable for some cellular studies since planar PA gels are commonly used as the soft culturing substrates to study cell mechanics and mechanobiology [18,27-34]. After an embedded MGB is removed from a MGB embedded PA gel, ideally, we expect that the shape of the exposed concave SS of the PA gel (due to the removing of this embedded ball) is an exact replica of that of the C-D convex SS of this removed ball. But, due to the strong viscoelastic material behaviors of PA gels (which are hydrogels), the possible significant pulling and pushing forces between an embedded MGB and the PA gel material during the removing process of this ball may induce undesirable significant permanent deformations on the to-be-exposed concave.

SS of the PA gel and then after this ball is removed, the shape of the final exposed concave SS of the PA gel may differ significantly from an exact replica of that of the C-D convex SS of this removed ball. If there are significant permanent deformations on the to-be-exposed concave SS of the PA gel due to the possible significant pulling and pushing forces, a simple qualitative analysis will show that the final concave shape of the exposed SS of the PA gel should be a little flattened compared with an exact replica of the shape of the convex SS of the corresponding removed ball. Also, in our experiments we observed that the PA gels were significantly swollen at 37°C (in the incubator) compared with the same PA gels at room temperature. Then, due to the possible non-uniform swelling and shrinking in the PA gel material induced by various reasons, the shape of

the exposed concave SS of the PA gel (after the removal of an embedded MGB) may vary significantly with temperature changes. These two possible sources of significant shape deviation of the exposed concave SS of a PA gel due to the removal of an embedded MGB, with respect to an exact replica of the shape of the convex SS of this removed ball, will compromise the trustworthiness of the obtained quantitative cellular responses to substrate curvatures if we treat the exposed concave SS of this PA gel as a C-D SS with the radius of the corresponding removed ball.

Concave and convex spherical PDMS surfaces

Again, because the PDMS substrates are much more rigid and their geometrical sizes are much less temperature-sensitive than those of the PA gels, and based on the facts that PDMS is the most commonly used material in soft lithography and the numerous reported PDMS microstructures have been successfully fabricated for cellular studies and other applications by using the casting-onto and peeling-off fabrication process [13,14,21,24,26,35-37], besides developing the above-proposed C-D convex MGB embedded PDMS substrates, C-D concave and convex spherical PDMS surfaces may also be developed for cellular studies and their biomedical applications. The exposed concave spherical PDMS surfaces may be obtained by carefully- removing the embedded MGBs from the MGB embedded PDMS substrates **(Figure 2c)**. The permanent deformations on the to-be-exposed concave SS of a PDMS substrate induced by the possible significant pulling and pushing forces between an embedded MGB and the PDMS material during the removing process of this ball, and the shape variations of the exposed concave SS of a PDMS substrate (after the removal of an embedded ball) due to temperature changes, should not be significant. That is, the exposed concave SS of a PDMS substrate may not have the above-mentioned concern for the exposed concave SS of a PA gel (due to the removal of an embedded ball) on the two possible sources of significant shape deviation with respect to an exact replica of the shape of the convex SS of the corresponding removed ball. Then we may treat the exposed concave SS of a PDMS substrate as a curvature-defined SS with the radius of the corresponding removed ball.

The exposed concave spherical PDMS surfaces may also be obtained by taking advantage of the convex SSs of the exposed parts of the embedded MGBs of the MGB embedded PDMS substrates, through using the casting-onto and peeling-off fabrication process **(Figures 2d and 2e)**. That is, the mixture of the precursor and crosslinker of PDMS at an appropriate ratio is poured onto a MGB embedded PDMS substrate and after curing, the upper newly-solidified PDMS layer is carefully peeled off from the bottom original MGB embedded PDMS substrate. The peeled off upper PDMS layer is a fabricated PDMS substrate having exposed concave SSs. If we take advantage of the concave SSs of this fabricated PDMS substrate by using the casting-onto and peeling-off fabrication process again, a PDMS substrate having convex SSs may be obtained **(Figures 2f and 2g)**. Note that, this newly-obtained PDMS substrate having convex SSs is entirely made of the PDMS material, in contrast with the MGB embedded PDMS substrates where the convex SSs are from the embedded MGBs. Therefore, by using the C-D MGB embedded PDMS substrates, both concave and convex spherical PDMS surfaces may be fabricated. Moreover, by removing some of the embedded MGBs from a MGB embedded PDMS substrate and keeping the rest of the embedded MGBs, we can have both concave and convex SSs presented on a single substrate **(Figures 2h and 2i)**. By using the casting-onto and peeling-off fabrication process to this substrate having both the concave PDMS SSs and convex MGB SSs, we may obtain a PDMS substrate having both concave and convex SSs which are made of the (same) PDMS material **(Figures 2j and 2k)** and again we may treat these obtained concave and convex spherical PDMS surfaces as C-D SSs with the radii of the corresponding original generating MGB. A PDMS substrate having C-D concave and/or convex SSs may also be further fabricated to realize some other desired non-planar substrates with well-defined 3D surface shapes.

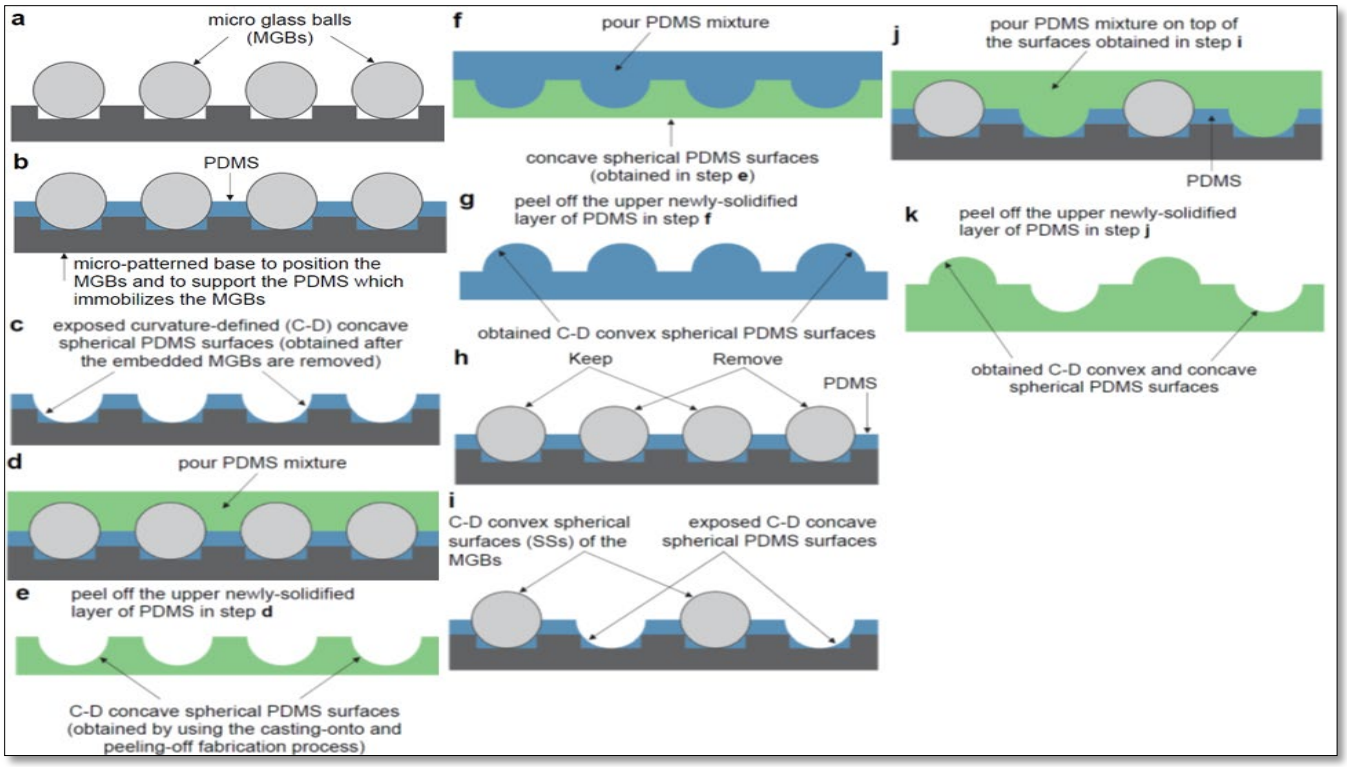

Figure 2. Schematic drawings to illustrate MGB embedded PDMS substrates and the subsequent fabrication processes to obtain curvature-defined (C-D) concave and convex spherical PDMS surfaces.

Substrate curvature modulates cell contractility

It is well-known that, the mean cell spread area and the mean cell contractility (i.e., the mean cellular traction forces) of the hMSCs growing on a PA gel decrease with the decrease of the stiffness of the PA gel [18]. The mean cell spread area and the mean cell contractility of the hMSCs plated on the PDMS micropost arrays also decrease with the decrease of the substrate rigidity [21]. In our experiments, as summarized in Section 2, we found that, overall the mean cell spread area of the hMSCs growing on the MGBs (embedded on the surfaces of the PA gels), decreased with the decrease of the substrate ball diameter. But, unlike the cases of cells growing on the PA gels and PDMS micropost arrays where the cellular traction forces acting on the surfaces of the PA gels and on the tops of the microposts are measured by the displacements of the beads embedded in the PA gels and by the deflections of the microposts, respectively, here the cellular traction forces acting on the surfaces of the MGBs cannot be measured. Nevertheless, according to the theoretical analysis presented [38], cell contractility decreases with the increase of substrate curvature, and then overall the mean contractility of the cells growing on the MGBs should decrease with the decrease of the substrate ball diameter. Therefore, instead of the substrate matrix elasticity and substrate rigidity that the PA gels and PDMS micropost arrays tune respectively to modulate cell contractility, the MGBs here vary the surface curvature to modulate cell contractility while the modulus of elasticity of the material and the rigidity of a substrate glass ball are infinitely high with respect to those of a cell. That is, independent from substrate matrix elasticity and substrate rigidity, substrate curvature presents another substrate mechanical parameter to modulate cell contractility and the decreased cell contractility can be realized on stiff and/or rigid substrates by purely increasing the surface curvature of the stiff and/or rigid substrates.

Arrays of the convex and concave spherical surfaces

The fact that, substrate curvature can modulate cell contractility independently from substrate matrix elasticity and substrate rigidity, may motivate researchers to design and fabricate new curved substrates with well-defined surface shapes and design and conduct new related experiments to study the possible detailed underlying biophysical mechanisms and biomolecular signaling pathways for the observed adhesion, spreading, migration, and division responses of stem cells on curved surfaces and for the observed differentiation responses of stem cells to the mechanical factors including substrate geometries, substrate matrix elasticity, and substrate rigidity. More specifically, by using the well-established and widely-used micro- patterning technologies [39,40], MGBs of the desired diameter may be embedded on the surface of a PDMS substrate in arrays, as illustrated in **Figures 2a and 2b**. Then as done above, a PDMS substrate with the arrays of the C-D concave SSs of

the corresponding desired radius may be obtained by carefully-removing the arrays of the embedded MGBs from the obtained PDMS substrate with the arrays of the embedded MGBs **(Figure 2c)** or by using the casting-onto and peeling-off fabrication process on the entire surface of the obtained PDMS substrate with the arrays of the embedded MGBs **(Figures 2d and 2e)**. A PDMS substrate with the arrays of the C-D convex spherical PDMS surfaces of this same radius may be obtained by using the casting-onto and peeling-off fabrication process again on the entire surface of the obtained PDMS substrate with the arrays of the C-D concave SSs **(Figures 2f and 2g)**. To record the information on which MGB generated which concave SS and which concave SS generated which convex SS, and for the possible future alignment needs between the concave SSs and the corresponding generating MGBs or between the convex SSs and the corresponding generating concave SSs of these obtained PDMS substrates, multiple identification and alignment markers need be made on these PDMS substrates in the fabrication process to precisely memorize the relative positions and orientations of these PDMS substrates when the concave SSs and convex SSs were generated.

C-D convex and concave spherical PA gel surfaces

If the surfaces of the embedded MGBs or the C-D convex or concave spherical PDMS surfaces can be coated with a PA gel of a uniform thickness, a substrate with C-D convex or concave spherical PA gel surfaces may be obtained. Compared with the surfaces of the embedded MGBs and the convex and concave spherical PDMS surfaces, besides the curved nature, convex and concave spherical PA gel surfaces can also mimic the stiffness of the native tissues and measure the cellular traction forces, as the case of planar PA gels which are widely-used for cell culturing. For this purpose, in the following, the PDMS substrate with either the arrays of the embedded MGBs of the desired diameter or the arrays of the C-D convex spherical PDMS surfaces of the desired radius r is called the first PDMS substrate, and the PDMS substrate with the arrays of the C-D concave SSs of the radius r plus the thickness of the to-be-coated PA gel is called the second PDMS substrate **(Figure 3)**. Depending on the thickness of the PA gel to be coated on the surfaces of the embedded MGBs or on the convex spherical PDMS surfaces of the first PDMS substrate (in the following, the surfaces of the embedded MGBs or the convex spherical PDMS surfaces of the first PDMS substrate are stated in short as the convex SSs of the first PDMS substrate), an appropriate amount of the PA solution with florescent beads will be dropped onto the arrays of the embedded MGBs or the arrays of the convex spherical PDMS surfaces of the first PDMS substrate. Later when this first PDMS substrate is used to culture cells, the displacements of the fluorescent beads in the PA gel coated on the convex SSs of this first PDMS substrate will be used to calculate the cellular traction forces, as is done in the case of planar PA gels for cell culturing. Before the polymerization of the PA solution, the first and second PDMS substrates will be oriented and precisely aligned with each other so that each of the convex SSs of the first PDMS substrate will face exactly the same concave SS of the second PDMS substrate which this convex SS faced when this concave or convex SS was originally generated. The fine adjustment of this alignment will ensure that the centerlines of the concave SSs of the second PDMS substrate are precisely aligned with the centerlines of the corresponding convex SSs of the first PDMS substrate. This high-precision alignment between the first and second PDMS substrates may be conducted and may be achieved under an optical microscope with a micro manipulator and with the help of the multiple identification and alignment markers that were specifically made on these PDMS substrates for this purpose. The second PDMS substrate will then be brought to approach to the first PDMS substrate to press the PA solution to uniformly re-distribute the PA solution on the convex SSs of the first PDMS substrate **(Figure 3a1)**. The uniform gap between the C-D convex SSs of the first PDMS substrate and the corresponding C-D concave SSs of the second PDMS substrate will ensure that, after the polymerization of the PA solution the thickness of the formed PA gel on top of the convex SSs of the first PDMS substrate is uniform **(Figure 3a2)**. After the second PDMS substrate is carefully withdrawn from the first PDMS substrate, the convex SSs of the first PDMS substrate are coated with the PA gel of a uniform thickness **(Figure 3a3)**. Then, the first PDMS substrate becomes a substrate with C-D convex spherical PA gel surfaces.

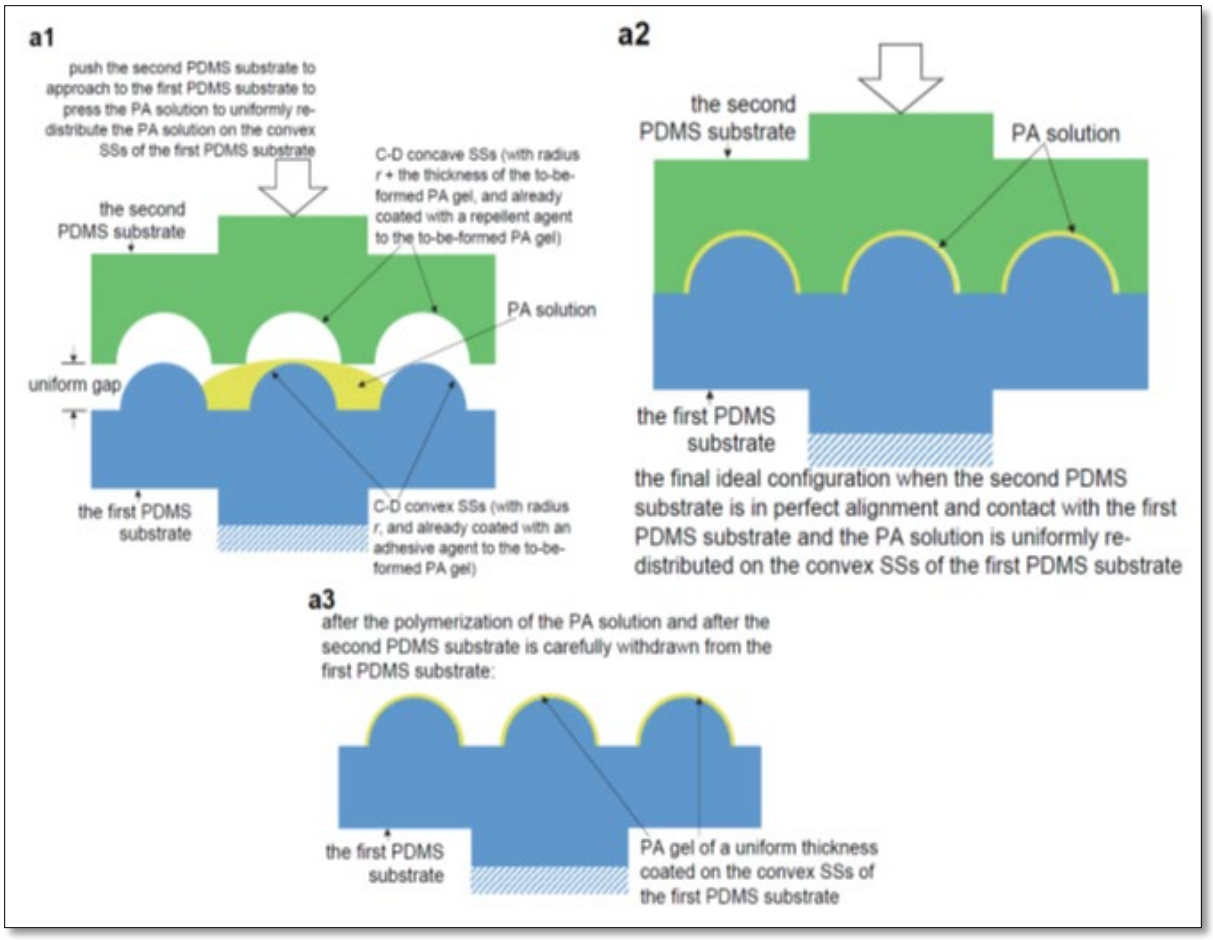

Figure 3a. Schematic drawings to illustrate the fabrication processes to obtain C-D convex (a1, a2 and a3) spherical PA gel surfaces.

Note that, before the dropping of the PA solution, the entire surface of the first PDMS substrate needs to be coated with the appropriate chemical adhesive agent to ensure the strong adherence of the to-be-formed PA gel to the convex SSs of the first PDMS substrate, and the entire surface of the second PDMS substrate needs to be coated with the appropriate chemical repellent agent to ensure the second PDMS substrate can be easily withdrawn or detached from the to-be-exposed convex spherical PA gel surfaces without damaging these to-be- exposed PA gel surfaces. As summarized in the above, we found that, among the used diameters, the minimum diameter of a glass ball on which an hMSC can attach and spread was 500 μm. It is reported that hMSCs increasingly respond to the rigidity of an underlying 'hidden' surface starting at about 10-20 μm PA gel thickness with a characteristic tactile length of less than about 5 μm [29]. Then the thickness of the PA gel to be coated on the convex SSs of the first PDMS substrate for the desired cellular studies may be chosen as small as 20-30 μm. Due to the relatively very small thickness of the coated PA gel with respect to the diameter of the embedded MGBs or the radius of the convex spherical PDMS surfaces and due to the uniform thickness of the PA gel coated on the convex SSs of the first PDMS substrate, unlike the situation of obtaining exposed concave spherical PA gel surfaces by carefully-removing the embedded MGBs from the MGB embedded PA gels, here the possible pulling and pushing forces between the to-be-exposed convex spherical PA gel surfaces of the first PDMS substrate and the concave SSs of the second PDMS substrate during the withdrawing process of the second PDMS substrate may not be significant and then the permanent deformations on the to-be-exposed convex spherical PA gel surfaces induced by these possible pulling and pushing forces may not be significant, and the shape variations of the exposed convex spherical PA gel surfaces due to temperature changes may also not be significant. Therefore, the final convex spherical PA gel surfaces of the first PDMS substrate, formed by coating the PA gel of a uniform thickness onto the surfaces of the embedded MGBs or onto the C-D convex spherical PDMS surfaces of the first PDMS substrate by using the above method (see last paragraph), may be treated as C-D convex SSs.

With respect to an exact C-D convex SS of a desired radius, the shape accuracy of these formed convex SSs of the PA

gel will be highly dependent on the precision of the alignment between the first and second PDMS substrates when the second PDMS substrate presses the PA solution to re-distribute the PA solution on the convex SSs of the first PDMS substrate, and highly dependent on the thickness of the remaining PA solution between the contacting flat parts of the first and second PDMS substrates. A complete squeezing-out of the PA solution between the flat parts of the first and second PDMS substrates is impossible, but a lot of practice may help establish a strategy to minimize the thickness of the remaining PA solution between these contacting flat parts, and to uniformly re-distribute the PA solution on the convex SSs of the first PDMS substrate. The high-precision alignment between the first and second PDMS substrates will ensure that, the gap between the C-D convex SSs of the first PDMS substrate and the corresponding C-D concave SSs of the second PDMS substrate is uniform, and this uniform gap will ensure the thickness of the PA gel formed in the gap and coated on top of the convex SSs of the first PDMS substrate is uniform. The great success of the high-precision alignment between a mask and a silicon wafer in the widely-used traditional micro-patterning technology photolithography [38,39] shows that the high-precision alignment between the first and second PDMS substrates required here will be achievable, and then the high shape accuracy of the above-formed convex spherical PA gel surfaces of the first PDMS substrate may also be achievable.

C-D concave spherical PA gel surfaces may also be obtained by using the same above method if the roles of the above first and second PDMS substrates are exchanged. That is, an appropriate amount of the PA solution with florescent beads will be dropped onto the arrays of the concave SSs of the above second PDMS substrate. The above first PDMS substrate, with the arrays of the embedded MGBs or the arrays of the convex spherical PDMS surfaces, will then be brought to approach to the second PDMS substrate to press the PA solution to uniformly re-distribute the PA solution on the concave SSs of the second PDMS substrate **(Figures 3b1 and b2)**. After the polymerization of the PA solution and after the first PDMS substrate is carefully withdrawn from the second PDMS substrate, the concave SSs of the second PDMS substrate are coated with the PA gel of a uniform thickness **(Figure 3b3)**. Then, the second PDMS substrate is now a substrate with concave spherical PA gel surfaces, and due to the above same reasons, these concave spherical PA gel surfaces may also be treated as C-D concave SSs.

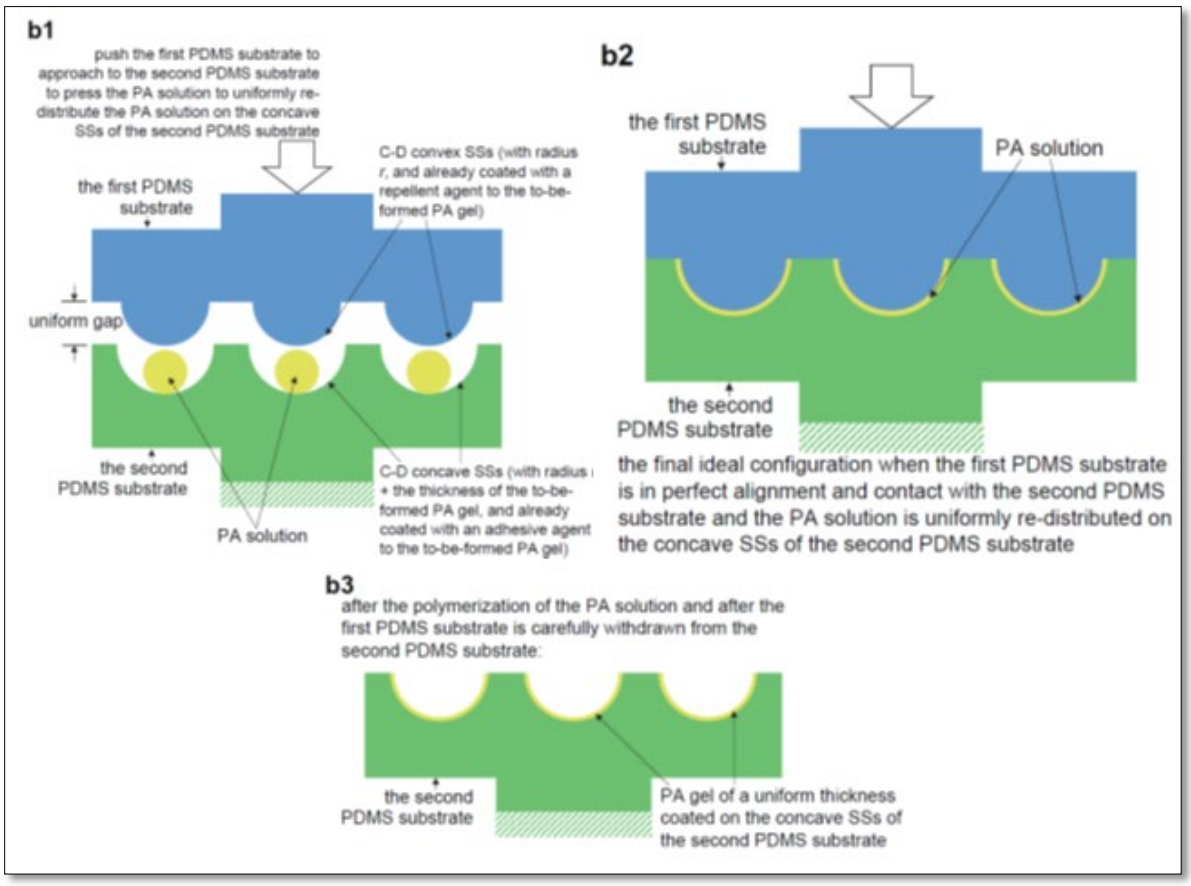

Figure 3b. Schematic drawings to illustrate the fabrication processes to obtain C-D concave (b1, b2 and b3) spherical PA gel surfaces.

Combined effects of substrate curvature and matrix elasticity on cellular traction forces

The above-proposed C-D convex and concave spherical PA gel surfaces, which may be developed by combining the C-D culturing technology here with the existing PA gel technology through the above-proposed fabrication process, can be used to study the combined effects of substrate curvature and matrix elasticity on cellular behaviors and can be especially used to study the effects of substrate curvatures on cellular traction forces. To measure the traction forces of cells growing on curved surfaces [41,42], the confocal laser scanning microscopy will be used to image the cells growing on these convex and concave spherical PA gel surfaces and to image the positions of the fluorescent beads embedded in the PA gel in 3D, an appropriate algorithm will be adopted or developed to track the 3D displacements of the fluorescent beads, and then the 3D cellular traction force field will be obtained by solving an inverse elasticity problem. If the radius of a convex or concave spherical PA gel surface is large enough compared with the geometrical sizes of a cell growing on this SS and if this cell is also growing approximately in the center region of this SS, this SS is virtually flat with respect to the sizes of this cell, and we may then approximately treat this spherical PA gel surface as a flat PA gel surface which is the projection of this spherical PA gel surface onto the horizontal plane. In this case, the 3D cellular traction force field on this spherical PA gel surface may be simplified as the 2D cellular traction force field on the approximated flat PA gel surface which can be obtained by using the existing method for obtaining the 2D cellular traction force field on a flat PA gel surface [43,44]. If the obtained 2D cellular traction force field is regarded as the in-plane components of the real 3D cellular traction force field on this spherical PA gel surface, the errors of this obtained in-plane components of the real 3D cellular traction force field induced by this approximately treating this spherical PA gel surface as the flat PA gel surface should not be significant.

As summarized above, we found that, the attachment of an hMSC is much more sensitive to the large surface curvatures of the small substrate glass balls than that of a fibroblast and the spreading morphology of an hMSC is much more sensitive to the small surface curvatures of the large substrate glass balls than that of a fibroblast. Then it will be interesting to investigate these corresponding mechanosensitivities of an hMSC and a fibroblast cultured on the above-proposed convex spherical PA gel surfaces.

Substrate curvature effects of focal adhesion strength and contractile actomyosin apparatus

The experiments to systematically study the time-lapse curvature-dependent responses of the adhesion, spreading, migration and division behaviors of the stem cells cultured on the above-proposed PDMS substrates with C-D convex and concave SSs (including the MGB embedded PDMS substrates, PDMS substrates with convex spherical PDMS surfaces, PDMS substrates with concave SSs, and PDMS substrates with convex and concave spherical PA gel surfaces) need to be designed and conducted. In these studies, for the three cellular components that determine the cell contractility, namely the focal adhesions, stress fibers and contractile actomyosin apparatus, the dependences of the size, strength, number and distribution of the focal adhesions on surface curvature may be observed or deduced, the dependences of the structure, distribution, pre-stress and tensional and bending mechanics of the stress fibers on surface curvature may be observed or deduced and the dependence of the contractile force generated by the actomyosin apparatus on surface curvature may be deduced [38]. Atomic force microscopy (AFM) indentation and micropipette aspiration may be used to measure the dependences of cell stiffness and cell membrane cortical stiffness on surface curvature, respectively [18,44,45] and these two measurements may further elucidate the roles of surface curvature in modulating cell contractility. The effects of matrix elasticity on the observed surface curvature-dependent cellular behaviors may be identified. The quantitative equivalency in the induced reduction of cell contractility between the increase of substrate curvature and the decrease of substrate matrix elasticity and substrate rigidity, i.e., the quantitative equivalency between the reduced cell contractility of the cells growing on the C-D SSs (for both the convex and concave situations) with smaller radii and the reduced cell contractility of the cells growing on the softer (flat) PA gels and softer (flat) PDMS micropost arrays, in terms of the induced reductions of mean cell spread area and mean in-plane cellular traction force, may be sought. These studies will also enhance our existing understandings on the detailed matrix elasticity-dependent mechanosensing and mechanotransduction processes of the focal adhesions, stress fibers, and contractile actomyosin apparatus of a cell growing on flat PA gels [46-48]. It was reported that, hMSCs actively “escaped” from the concave microstructures [13] and hMSCs on the concave surfaces showed upward stretched cell morphology where a substantial part of the cell body was not attached to the concave surface [15]. The results of the experiments proposed here will determine the minimum radius of a concave SS on which an hMSC can form focal adhesions and the minimum radius of a concave SS to which an hMSC can entirely attach.

Substrates with simple varying surface curvatures

C-D convex and concave SSs have uniform surface shapes, i.e., the through-center normal cross-sections of these SSs are circular and have single invariant curvatures, equal to the inverses of the radii of these SSs, along their circumferences. The following three types of C-D convex **(Figure 4a1-4c1)** and concave **(Figures 4a2-4c2)** substrates may be designed and fabricated to present simple varying surface shapes for cell culturing, i.e., the normal cross-section of the surface of

each of these substrates has a varying curvature or has two or more curvatures. (1). Substrates have revolution surfaces whose through-center normal cross-sections consist of a segment of horizontal straight line (*D*) and two symmetric circular arcs with the desired radius (*r*) tangent to the two ends of this segment of straight line **(Figure 4a)**. The through-center normal cross-section of the surface of each of these substrates has two curvatures with one being the zero curvature of the segment of horizontal straight line and the other being the nonzero curvature of each of the two symmetric circular arcs in this normal cross-section. This type of shape-varying substrates provides smooth surfaces having the shape-variation settings of from a circular flat surface to a curved surface with a defined uniform shape and vice versa. (2) Substrates have cylindrical surfaces whose normal cross-sections (which are perpendicular to the longitudinal directions of these cylindrical surfaces) consist of a segment of horizontal straight line (*w*) and two circular arcs with the desired different radii (*r*1 and *r*2) tangent to the two ends of this segment of straight line **(Figure 4b)**. The normal cross-section of the surface of each of these substrates has three curvatures with one being the zero curvature of the segment of horizontal straight line sandwiched between the other two being the nonzero curvatures of the two circular arcs attached at the two ends of this segment of straight line in this normal cross-section. This type of shape-varying substrates provides smooth surfaces having the shape-variation settings of from a rectangular flat surface to two curved surfaces with defined different uniform shapes at the two longitudinal sides of this rectangular flat surface and vice versa. (3) Substrates have cylindrical surfaces whose normal cross-sections (which are again perpendicular to the longitudinal directions of these cylindrical surfaces) consist of two smoothly-connected (i.e., tangent) circular arcs with the desired different radii (*r*1 and *r*2) **(Figure 4c)**. The normal cross-section of the surface of each of these substrates has two different nonzero curvatures which are the curvatures of the two circular arcs in this normal cross-section. This type of shape-varying substrates provides smooth surfaces having the shape-variation settings of from a curved surface with a defined uniform shape to another curved surface with a defined different uniform shape. The time-lapse curvature-dependent spreading and migration responses of the stem cells cultured on these three types of shape-varying substrates may be investigated. The results of these experiments will reveal the stem cells' abilities to recognize and to respond to surface curvatures and the stem cells' abilities to differentiate and to respond to curvature variations or curvature differences. Since surface curvatures create height differences between different locations on curved surfaces, together with the results of the experiments proposed in the above part for the stem cells cultured on convex and concave SSs, the results of the experiments proposed here will also reveal the stem cells' abilities to differentiate and to respond to the height differences on the surface of a substrate.

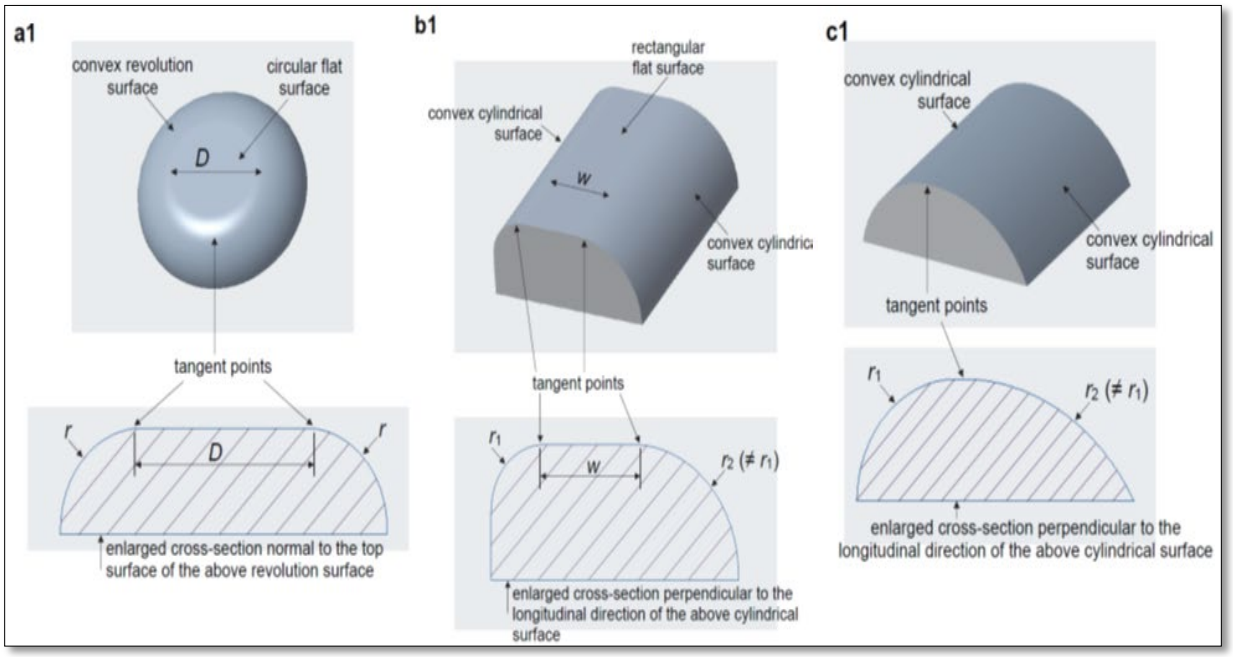

Figure 4(1). Schematic drawings to illustrate the three types of C-D convex (a1, b1, c1) substrates with simple varying surface shapes (i.e., with simple varying surface curvatures) for cell culturing.

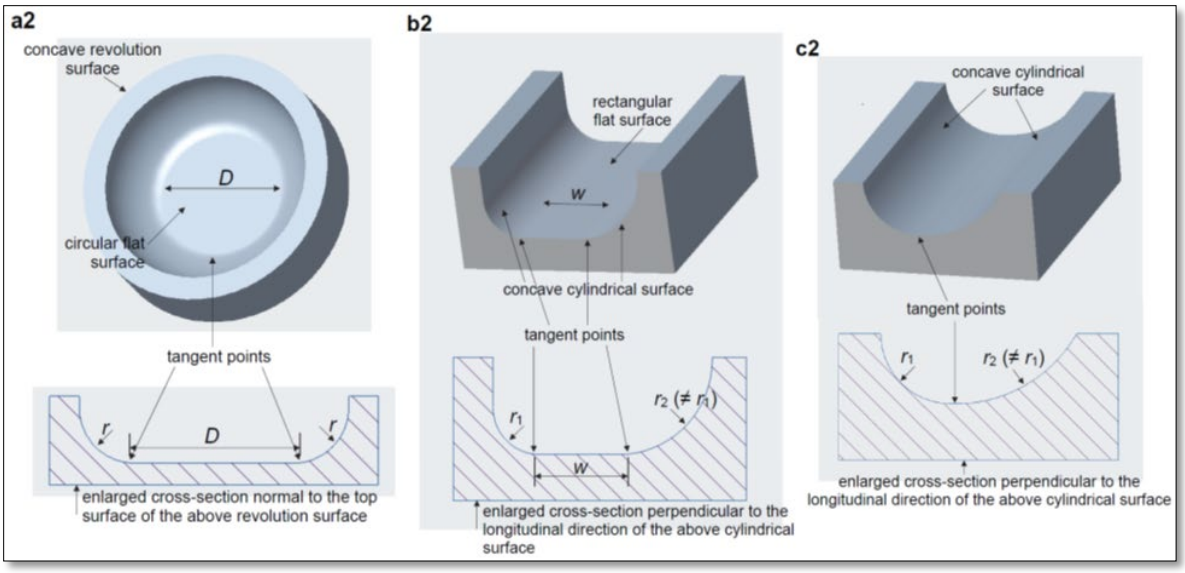

Figure 4(2). Schematic drawings to illustrate the three types of C-D concave (a2, b2, c2) substrates with simple varying surface shapes (i.e., with simple varying surface curvatures) for cell culturing.

Substrate curvature-dependent stem cell mechanics

Based on the results of the above-proposed experiments of stem cells cultured on curved surfaces with normal cross-sections having single invariant curvatures and of stem cells cultured on curved surfaces with normal cross-sections having varying curvatures, the relevant substrate curvature-dependent mechanics and energetics of stem cells at both the continuum and molecular levels may be developed [38,44,47-54] and the following two questions may be specifically answered, how a stem cell senses the curvature and curvature variation of a surface, and how this stem cell makes the decision on which direction to spread and migrate on a curved and curvature-varying surface. The answers to these two questions will be compared with those to the following two similar questions for stem cells cultured on substrates with varying rigidities, how a stem cell senses the rigidity variation of a substrate, and how this stem cell makes the decision on which direction to spread and migrate on a rigidity-varying substrate [28,55]. All these are necessary for understanding the behaviors of stem cells in 3D micromechanical environments and for designing scaffolds to effectively and efficiently control the development of stem cells and the resulting tissues for tissue engineering and regenerative medicine [56-62].

Quantitative equivalency between SSs and PA gels and PDMS micropost arrays in influencing/inducing stem cell differentiation

For the possible biophysical mechanisms and biomolecular signaling pathways of the observed differentiation responses of the stem cells to the mechanical factors, cell contractility plays a critical role in all the observed differentiation responses of the stem cells growing on the PA gels [18-20], PDMS micropost arrays [21] and planar geometrically defined micro-patterns [1-6]. Compared with the case of the PA gels where the different moduli of elasticity of the substrate PA gels are the inducements and the case of the PDMS micropost arrays where the different rigidities of the substrate PDMS micropost arrays are the inducements of the observed different differentiation responses of the hMSCs, here as summarized earlier, substrate curvatures alone can induce differentiation of hMSCs since the MGBs all have infinitely-high moduli of elasticity and infinitely-high surface rigidities with respect to those of the cells. But, since substrate curvature also modulates cell contractility, the observed differentiation response of the hMSCs growing on the MGBs is likely sharing the same or similar fundamental biophysical mechanisms and biomolecular signaling pathways with the observed differentiation responses of the hMSCs growing on the PA gels and PDMS micropost arrays. These same or similar fundamental biophysical mechanisms and biomolecular signaling pathways have to be related to the following observed characteristics of the low cell contractility of the cells growing on the soft substrates (with moduli of elasticity similar to those of fat) with respect to the high cell contractility of the same type of cells growing on the stiff substrates (with moduli of elasticity similar to or much larger than those of bone): low cell tension, low cell spread area, poorly developed focal adhesions and stress fibers, lower levels of lamin-A,C in the nuclear lamina, and transcription factors RAR-γ and YAP/TAZ remain in the cytoplasm, which favor adipogenesis [20]. However, the spreading morphologies of the hMSCs on the MGBs, which are majorly the spindle shapes [17], are very different from those of the hMSCs on the PA gels and PDMS micropost arrays, which can be from the round shapes to the well-spread shapes depending on the

substrate rigidity [18-21]. The sizes, strengths, numbers, and distributions of the focal adhesions and stress fibers of the hMSCs on the MGBs can then be very different from those of the focal adhesions and stress fibers of the hMSCs on the PA gels and PDMS micropost arrays. The detailed mechanosensing mechanism of the bent or misaligned configuration of the contractile actomyosin apparatus of the hMSCs on the MGBs can also be different from the detailed mechanosensing mechanisms of the contractile actomyosin apparatus of the hMSCs on the (flat) PA gels and (flat) PDMS micropost arrays [38]. More fundamentally, cells mechanosense the elasticity of the substrate PA gels and the rigidity of the substrate PDMS micropost arrays only through focal adhesions, and the elasticity of the substrate PA gels and the rigidity of the substrate PDMS micropost arrays modulate the developments of focal adhesions, stress fibers, and contractile actomyosin apparatus at the same time in a coupled fashion, whereas here cells mechanosense the surface curvatures of the MGBs through focal adhesions, stress fibers, and contractile actomyosin apparatus at the same time, i.e., surface curvatures directly and independently modulate the developments of focal adhesions, stress fibers, and contractile actomyosin apparatus at the same time, and the developments of these three cellular components on the curved surfaces also modulate with respect to each other at the same time in a coupled fashion as in the cases of cells on the PA gels and PDMS micropost arrays.

Note that, in making the MGB embedded PA gels, MGB embedded PDMS substrates, PDMS substrates with convex spherical PDMS surfaces, PDMS substrates with concave SSs and PDMS substrates with convex and concave spherical PA gel surfaces, the height of the final convex SSs or the depth of the final concave SSs of a substrate measured from the surrounding flat PA gel or PDMS surface may be controlled. In some experiments, these heights and depths may be decreased to small enough so that a stem cell will spread on both a convex or concave SS and its surrounding flat PA gel or PDMS surface and then the modulation effects of locally-curved substrates or local substrate curvatures on the spreading and on the distributions of the focal adhesions and stress fibers of a stem cell may be studied. This may further elucidate the effects of substrate curvatures on the developments of focal adhesions and stress fibers.

Nevertheless, due to the possibly-same or similar fundamental biophysical mechanisms and biomolecular signaling pathways for the observed differentiation responses of the hMSCs growing on the MGBs and on the PA gels and PDMS micropost arrays, the quantitative equivalency between the decreased cell contractility of the hMSCs on the smaller MGBs or on the C-D convex and concave SSs of the other types (i.e., the above-proposed spherical PDMS surfaces and spherical PA gel surfaces) with smaller radii and the decreased cell contractility of the hMSCs on the softer PA gels and softer PDMS micropost arrays, in terms of the observed matrix elasticity-dependent levels of lamin-A,C in the nuclear lamina and transcription factors RAR-γ and YAP/TAZ in the nucleus [19,20], may also be sought. This quantitative equivalency, between the increase of substrate curvature and the decrease of substrate matrix elasticity and substrate rigidity, in influencing/inducing stem cell differentiation will be correlated to the same quantitative equivalency in terms of the induced reductions of mean cell spread area and mean in-plane cellular traction force discussed above. The threshold diameters or radii of the MGBs or C-D convex and concave SSs of the other types at which hMSCs significantly start to differentiate may be found out, and the relevant quantitative results will largely contribute to the establishments of the possible biophysical mechanisms and biomolecular signaling pathways for the observed differentiation responses of hMSCs to substrate curvatures.

Combined effects of substrate curvature and matrix elasticity on stem cell differentiation

It will also be necessary to investigate the differentiation responses of the hMSCs cultured on the convex and concave spherical PA gel surfaces which will be compared with those of the hMSCs cultured on the MGBs and flat PA gel surfaces, and these comparisons will reveal the combined effects of surface curvature and matrix elasticity on the differentiations of the stem cells. This study will enhance our existing understanding on the specific role of substrate matrix elasticity in inducing the observed stem cell differentiation [20,47]. This study will be useful in identifying the specific roles of cell tension, cell shape, cell spread area, and cell stiffness, the extents of their influences and their combinational effects and the biomolecular signaling pathways of the mechanosensing and mechanotransduction processes for cell tension, cell shape, cell spread area and cell stiffness to play their roles, in inducing the observed differentiation responses of the stem cells to the mechanical factors including substrate geometries, substrate matrix elasticity, and substrate rigidity. This study will also be useful in identifying the direct-involvements and specific roles of focal adhesions, stress fibers and contractile actomyosin apparatus in the biomolecular signaling pathways of the mechanosensing and mechanotransduction processes for the translocations of the relevant transcription factors to the nucleus and for the relevant gene expressions in the nucleus in the observed stem cell differentiations induced by matrix elasticity, which are separate from the established indirect-involvements of these three cellular components in these mechanosensing and mechanotransduction processes through the resulted matrix elasticity-dependent cell contractility (or cell tension, which is believed to be the biophysical quantity that decides the matrix elasticity-dependent stem cell differentiation) [20,47,63].

Deforming the nucleus of a stem cell

It is clear that the deformation of the nucleus induced by the topography of the environment of a stem cell or by the mechanical stresses exerted on a stem cell regulates the gene expressions of the stem cell [64,65]. As summarized earlier, the curvature of the substrate restricts the spreading of a stem cell and this restriction is larger when the curvature of the substrate is larger. Then the curvature of the substrate also naturally indirectly deforms the nucleus inside the stem cell accordingly, and therefore the C-D culturing technology here may also be used as an effective tool to deform the nucleus of a stem cell. The convex and concave C-D surfaces proposed here may be used to and other C-D surfaces may be designed and fabricated to induce some unique and interesting deformations of the nucleus inside a stem cell, and the correlation between the induced-deformation or its resulting shape, size, and tension of the nucleus of a stem cell due to surface curvature and the inducing surface curvature may be sought. The roles of the induced-deformation or its resulting shape, size, and tension of the nucleus of a stem cell due to surface curvature, and the possible corresponding biophysical mechanisms and biomolecular signaling pathways for the involvements of these nuclear parameters in the mechanosensing and mechanotransduction processes for the relevant gene expressions in the nucleus, in inducing the observed differentiation responses of this stem cell, may be studied. This study will add to our existing understandings on the mechanosensing and mechanotransduction processes of the nucleus of a stem cell in gene expression, which clearly constitute the final and decisive step of the entire mechanosensing and mechanotransduction process (which also includes the mechanosensing and mechanotransduction processes of the peripheral cellular components including cell focal adhesions, stress fibers, and contractile actomyosin apparatus) of a stem cell for the observed differentiation responses to the mechanical factors.

CONCLUSION

In summary, it is necessary to study the effects of substrate curvatures on cell behaviors [12]. The micro-convex and concave surfaces generated by a few developed methods for cell culturing are not C-D and the curved surfaces at larger or millimeter scales may not be generated by these methods. We have recently developed a C-D cell culturing technology, a class of MGB embedded PA gels, virtually having no limits on the range of the presented surface curvatures, which provides a unique and effective tool and opens up a systematic paradigm for the studies of cell mechanobiological responses to substrate curvatures and their related applications. The results of our cell culturing experiments on this class of substrates showed that, substrate curvature restricts spreading and induces differentiation of hMSCs and therefore, it is necessary to systematically study the effects of substrate curvatures on stem cell behaviors. Substrate curvature presents another substrate mechanical parameter to modulate cell contractility, which may indicate new experiments to elucidate or reveal the underlying biophysical mechanisms and biomolecular signaling pathways for the observed mechanobiological responses of stem cells to substrate geometries, substrate matrix elasticity, and substrate rigidity. Compared with the geometrically-defined planar or 2D micropatterns where the border lines of the micropatterns are used to restrict the spreading of stem cells and the geometrically-defined 3D pillars for cell culturing where the spaces between adjacent pillars are empty, here the C-D convex SSs of the MGB embedded PA gels are smooth and continuous and have no border lines to restrict cell spreading. Then, the restriction on the spreading of the stem cells generated by the surface curvatures of the MGBs is natural, and the spreading morphologies of the stem cells on the MGBs are naturally formed, which is desirable to mimic the natural cellular environment.

Because of the much larger Young's moduli of PDMS materials compared with those of PA gels, making and using MGB embedded PDMS substrates for cellular studies and their biomedical applications may be the next effort to make this C-D culturing technology much more effective and efficient. By carefully-removing the embedded MGBs, concave spherical PA gel and PDMS surfaces may be obtained. By using the casting-onto and peeling-off fabrication process, both concave and convex spherical PDMS surfaces may be obtained. Treating the obtained concave surfaces of the PA gel as C-D SSs is questionable, but the obtained concave surfaces of the PDMS substrate and the subsequently formed convex PDMS surfaces may be used as C-D SSs with the radius of the corresponding embedded MGBs that were used to generate the concave PDMS surfaces. With the help of the micro-patterning technologies, MGBs may be embedded on the surface of a PDMS substrate in arrays, and then a PDMS substrate with arrays of C-D concave and convex SSs may be obtained. By coating a MGB embedded PDMS substrate or a PDMS substrate with convex or concave spherical PDMS surfaces with a PA gel of a uniform thickness, a substrate with C-D convex or concave spherical PA gel surfaces may be made and then the effects of surface curvatures on cellular traction forces, focal adhesions, stress fibers and contractile actomyosin apparatus may be studied. C-D convex and concave substrates may also be designed and fabricated to present simple varying surface curvatures to study the abilities of stem cells to differentiate and to respond to curvature variations. The relevant mechanics and energetics of stem cells growing on curved surfaces at both the continuum and molecular levels may be developed.

Although the differentiation response of hMSCs growing on MGBs is likely sharing the same or similar fundamental biophysical mechanisms and biomolecular signaling pathways with the differentiation responses of hMSCs growing on PA gels and PDMS micropost arrays, the

detailed mechanosensing mechanism of hMSCs on MGBs can be different from the detailed mechanosensing mechanisms of hMSCs on (flat) PA gels and (flat) PDMS micropost arrays. The quantitative equivalencies between the decreased cell contractility of hMSCs on C-D SSs with smaller radii and that of hMSCs on softer PA gels and softer PDMS micropost arrays may be sought. It will be necessary to study the differentiation responses of hMSCs cultured on convex and concave spherical PA gel surfaces to reveal the combined effects of surface curvature and matrix elasticity on stem cell differentiation. This study will also be useful in identifying the specific roles of cell tension, cell shape, cell spread area and cell stiffness, and their combinational effects, in inducing the observed differentiation responses of stem cells.

Since substrate curvature also naturally indirectly deforms the nucleus inside a stem cell accordingly, the C-D culturing technology here may be used as an effective tool to deform the nucleus of a stem cell. The roles of the induced-deformation or its resulting shape, size and tension of the nucleus of a stem cell, in inducing the observed differentiation responses of this stem cell, may be studied.

CONFLICT OF INTEREST

S.Y. has been granted a United States patent for the invention of the class of cell culture substrates reviewed in this paper. Title of Patent: "Micro and nano glass balls embedded in a gel presenting micrometer and nanometer scale curvature and stiffness patterns for use in cell and tissue culturing and a method for making same", Patent No.: US 8,802,430, Date of Patent: Aug 12, 2014.

S.Y. has been granted a continuation United States patent of the above granted United States patent. Title of Patent: "Micro and nano scale structures disposed in a material so as to present micrometer and nanometer scale curvature and stiffness patterns for use in cell and tissue culturing and in other surface and interface applications", Patent No.: US 9,512,397, Date of Patent: Dec 6, 2016.

S.Y. has been granted a China patent for the invention of the class of cell culture substrates reviewed in this paper. Title of Patent: "Substrates with micrometer and nanometer scale stiffness patterns and curvature patterns," China Utility Mode Patent No.: ZL 2013 2 0124977.5, Date of Patent: Dec 11, 2013.

Based on the obtained cell experimental results reviewed in this paper, S.Y. has filed a United States patent application, entitled "Methods and kits for directing cell attachment and spreading" (Application No.: 15652921, Date of Application: Jul 18, 2017); S.Y. has filed a United States patent application, entitled "Methods and kits for cell sorting" (Application No.:15652928, Date of Application: Jul 18, 2017); S.Y. has filed a United States patent application, entitled "Methods and kits for guided stem cell differentiation" (Application No.: 15652938, Date of Application: Jul. 18, 2017).

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