Video Article

Silk Film Culture System for in vitro Analysis and Biomaterial Design

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Abstract

Silk films are promising protein-based biomaterials that can be fabricated with high fidelity and economically within a research laboratory environment\(^1\). These materials are desirable because they possess highly controllable dimensional and material characteristics, are biocompatible and promote cell adhesion, can be modified through topographic patterning or by chemically altering the surface, and can be used as a depot for biologically active molecules for drug delivery related applications\(^3\-\(^8\)). In addition, silk films are relatively straightforward to custom design, can be designed to dissolve within minutes or degrade over years in vitro or in vivo, and are produce with the added benefit of being transparent in nature and therefore highly suitable for imaging applications\(^9\-\(^13\)). The culture system methodology presented here represents a scalable approach for rapid assessments of cell-silk film surface interactions. Of particular interest is the use of surface patterned silk films to study differences in cell proliferation and responses of cells for alignment\(^12\,\(^14\)). The seeded cultures were cultured on both micro-patterned and flat silk film substrates, and then assessed through time-lapse phase-contrast imaging, scanning electron microscopy, and biochemical assessment of metabolic activity and nucleic acid content. In summary, the silk film in vitro culture system offers a customizable experimental setup suitable to the study of cell-surface interactions on a biomaterial substrate, which can then be optimized and then translated to in vivo models. Observations using the culture system presented here are currently being used to aid in applications ranging from basic cell interactions to medical device design, and thus are relevant to a broad range of biomedical fields.

Video Link

The video component of this article can be found at http://www.jove.com/video/3646/

Protocol

1. Fabrication of Silicone Rubber Molds

1. Produce or purchase a desired topographic surface for casting. For this publication a standard 100 mm etched silicon wafer will be described (Figure 1).
2. Weigh out polydimethylsiloxane (PDMS) potting (component A) and catalyst (component B) solution in a 1:9 ratio (9 g potting and 1 g catalyst) as provided in the purchased kit.
3. Mix solutions thoroughly to initiate curing process.
4. Place silicon wafer surface within a casting dish.
5. Weigh out 4.5 g of PDMS solution onto silicon wafer.
6. Spread PDMS solution as to cover a 100 mm diameter area of the wafer surface.
7. Tilt wafer to spread PDMS solution evenly.
8. Weigh out 4.5 g of PDMS solution onto silicon wafer.
9. Spread PDMS solution as to cover a 100 mm diameter area of the wafer surface.
10. Place cured PDMS/silicon wafer into 70% ethanol bath before removal.
11. Begin removing PDMS from wafer by using razor blade to lift edge (entire circumference) first.
12. Gently pull PDMS off using forceps within a 70% ethanol bath being careful not to tear silicone rubber casting.
13. Punch out individual PDMS molds using 14 mm hole punch. This diameter is designed to fit into a 24-well plate setup.

2. Production of Silk Solution

1. Bring 2 L of distilled water (dH\(_2\)O) to a boil within a glass beaker\(^7\).
2. Cut 5 g of Bombyx mori silkworm cocoons into thirds.
3. Dispose of extensively contaminated cocoons as indicated by excessive insect particulates coating the inner cocoon surface.
4. Measure 4.24 g of sodium carbonate.
5. Add sodium carbonate slowly to boiling dH\(_2\)O volume to prevent boiling over of water, and allow complete dissolution before continuing.
6. Add cocoon pieces to boiling dH$_2$O for 40 min., and use a Teflon coated stir bar to stir cocoons during boiling process.
7. After boiling, carefully drain dH$_2$O into sink and ring out the silk extract by hand to remove excess water.
8. Wash silk extract three times for 20 min. each in 1 L of dH$_2$O in a lab beaker, and use a stir bar to circulate volume within beaker.
9. After washing, ring out the silk extract by hand and place silk fiber extract inside a chemical hood to allow for drying for a 12 hr. period.
10. Next day, weigh the dried silk fibers, which is typically ~3.5 g.
11. Prepare 9.3 M LiBr solution for a 20% w/v solution of silk. Utilize following equations to calculate necessary weight and volumes:
   a. (Silk extract weight from step 10) x (4) = _________mL of total 9.3 M LiBr solution
   b. 

12. Add measured LiBr weight into a glass beaker of the following dH$_2$O volume:
   a. (0.8 x (calculated volume from step 11.a)) = __________ mL of dH$_2$O
13. Pour this solution into an appropriate sized graduated cylinder, and bring solution up to final volume as calculated in part 11.a.
14. Place the silk extract into beaker and pour LiBr solution over the silk fibers making sure the silk fibers are immersed within LiBr solution using a lab spatula.
15. Place the dissolved silk into 60 °C oven for 4 hr:
   Start time: __________
   End time: __________
16. Using an appropriate sized syringe draw up 12 mL of the silk solution. Place an 18G needle on end of the syringe, and then inject the solution into a dialysis case (3,500 MW cutoff, Slide-A-Lyzer, Thermo Scientific). After filling the cassette, draw the remaining air out of the cassette with the emptied syringe.
17. Place filled dialysis cassette within 1 L of dH$_2$O.
18. Change dH$_2$O volume after 1 hr, 4 hr, 8 hr and then every 12 hr 3x for a total of 6 changes:
   a. Begin: __________
   b. 1 hr: __________
   c. 4 hr: __________
   d. 8 hr: __________
   e. 12 hr: __________
   f. 12 hr: __________
   g. 12 hr: __________
   h. End: __________
19. After dialysis, slowly collect the silk solution from cassettes with syringe. Place solution into 10,000 g rated centrifuge tubes.
20. Centrifuge the solution twice at 10,000 g at 4 °C for 20-min each. After each centrifugation place supernatant into a new tube.
21. Store thr silk solution in 4 °C refrigerator.
22. Pipette 2 samples of 0.5 mL silk solution into small weigh dish, and place weigh dishes inside a 60 °C dry oven for 12 hours or until the silk solution dries.
23. Weigh the remaining solid silk film from both samples to measure silk solution percent weight to volume protein concentration:
   a. Weight of combined 2 silk film samples:___________ mg
   b. (Weight from 23.a) x (100) = __________% silk density.

3. Preparation of Silk Films and Culture System Setup

1. Prepare PDMS casting surfaces by cleaning with clear tape to remove dust.
2. Clean PDMS substrates by soaking in 70% EtOH and wash three times with dH$_2$O.
3. Place 14 mm PDMS molds onto holder plate, which is typically a 24 well plate lid.
4. To produce a 50 μm thick film, spread 70 μls of 8% silk solution on PDMS molds using a liquid spreading tool that is typically a 1 mL syringe tip.
5. Allow the silk films to dry uncovered on a clean bench running an air flow pressure of 150 Pa for a period of 90 min or until films are dry.
6. Once films are dry place entire set of films, including PDMS molds, into a water-annealing chamber for >4 hrs to produce a water insoluble film. This is typically an empty desiccator chamber with water in the bottom of the basin pulled at a 25 kPa vacuum.
7. Remove silk films from water-annealing chamber and place onto a clean bench.
8. Prepare a 70% EtOH bath within a 35 mm Petri dish, and place control substrates (i.e. glass or plastic surfaces) and stainless steel rings within wells for at least 10 min to sterilize.
9. Remove silk films from PDMS molds using forceps, dip them into 70% EtOH, and place sample into 24-well plate prefill with 1 mL of 70% EtOH making sure patterned side is facing up to allow for cell adhesion.
10. After placing each silk film sample into a corresponding well place stainless steel ring weights (11.65 mm inner diameter, 1.18 mm thickness) on top.
11. Allow samples with rings to incubate for 10 minutes to ensure sterility.
12. Remove EtOH and wash each sample 3x with 1 mL of PBS. Place the wash at 5 min to allow for complete diffusion.
13. Remove PBS using aspirating glass pipette, while making sure to remove majority of bubbles beneath silk film samples.
14. Prepare cell line for seeding. As an example, trypsinize human corneal-limbal epithelial (HCLE) cell line with 0.25% trypsin and ethylenediaminetetraacetic acid (EDTA) solution for 7-min. Deactivate trypsin using 1:1 volume of Dulbecco's modified Eagle medium (DMEM) passage media with 10% FBS added. Centrifuge trypsinized HCLE cells, add 8 mL of keratinocyte-serum free media (K-SFM) to cell pellet, gently agitate to disperse HCLEs, and generate cell count.
15. Sample 500 μL of HCLE suspension per well typically using a 10,000 cells/cm$^2$ density.
16. Place cultures within incubator at 37 °C and 5% CO$_2$ and run desired experimental protocol.
4. Representative Results

Figure 1. Flow chart illustrating summarized 10-step process of silk film production and culture system preparation.

Figure 2. (A) 21-dye array of patterned line surface topographies produced upon a 90 mm diameter silicon wafer employing standard photolithographic and dry etching techniques. (B) Silk films retain original parallel line patterned features after casting on PDMS molding surfaces. (C) Schematic demonstrating feature size design chosen to promote cellular alignment. (D) Cross-section of silk film illustrating retained parallel lined patterned surface.
Figure 3. Scanning electron micrographs of HCLE cell line adhering to (A) patterned and (B) flat silk films at day 2 in culture. HCLE cultures continue to proliferate to confluence on (C) patterned and (D) flat surfaces by day 8 in culture.

Figure 4. (A, D, G) Patterned and (B, E, H) flat silk films culture HCLE cells comparatively to (C, F, I) glass control substrates at (A-C) day 1, (D-F) day 4, and (G-I) day 8 in culture. (J) CyQuant nucleic acid content and (K) (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolic activity assay data demonstrating HCLE viability on both patterned and flat silk film substrates when compared to glass control surfaces over time (scale bars = 100 μm).

Video 1. Time lapse phase-contrast imaging of HCLE cells migrating over a patterned silk film surface during an 18 hr. time period. Cells were seeded at 10k/cm² density and cultured for 2 hr. before imaging. Click here to view movie.

Video 2. Time lapse phase-contrast imaging of HCLE cells migrating over a flat TCP control surface during an 18 hr. time period. Cells were seeded at 10k/cm² density and cultured for 2 hr. before imaging. Click here to view movie.

Discussion

The use of regenerated silk films as a substrate for cell culture has gained in popularity over the past two decades due to extensive characterization of the material properties of this protein and increased understanding of its biomaterial utility. The culture system described here represents a novel in vitro testing system for assessing cell surface interactions on patterned silk film biomaterial substrates. The system allows for in depth analysis of cellular interactions over time that can be easily adopted for high-throughput data collection. This is largely enabled because silk films possess a number of tunable biomaterial properties that can be modified to directly affect cell function, including: control of surface micro/nano-surface topography; various surface chemistries through covalent modification or adsorption of biologically active...
Silk film substrates are produced in the lab with high fidelity, consistency, and with relatively low cost (Fig. 1). This enables reproducibility in both culture system setup and experimental outcomes. It has been demonstrated that water-anneal processing produces a stable silk film material within culture that has defined degradation rates pending the concentration of proteases in solution. As a result these materials may be used for extended periods of time for long-term cell culture, or remain implanted for months or years depending on the physiological location.

In addition, recent work has shown that both the protein structure and material properties of water-annealed silk films are consistent from batch to batch allowing for reproducible culture results as shown through various mechanical and biophysical testing methods. In addition, the material’s surface has shown great fidelity amongst film batches as indicated by SEM, atomic force microscopy (AFM), and cell culture studies. Material stability and consistency is an important factor to how the cell will sense the culture substrate through the various mechanotransduction pathways, and ultimately produce a desired/undesired cellular response.

Historical standards for culture substrates, such as tissue culture treated plastic or glass, provide adequate substrates for cell attachment. However, these materials are not amenable for further utility in vivo. It can be envisioned that a silk film biomaterial could be customized in vitro and once experimental expectations have been achieved the customized film can be directly translated to an in vivo model. Such paired design between in vitro and in vivo experimentation offers a great advantage for such implantable silk biomaterials over other substrates that are routinely used in vitro.

Disclosures
No conflicts of interest declared.

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