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Title: Generation of Polyacrylamide and Silicone Extracellular Matrix Substrates with Defined Stiffness for Cell Biology Applications

Authors and Affiliations:

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Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**
- 3. Filming location:** Will the filming need to take place in multiple locations? **No**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 22

Number of Shots: 45

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

Videographer's NOTES:

Camera: Sony fs5 – 1920x1080, 29.97 fps, s-log 2
LUT is in folder

Audio (interviews) – Channel 1 – On board shot gun mic (back-up)

Channel 2 – Lav Mic on Authors

Clip0001 – 1.1.1 & 1.2.1 – Take 1 – WS

Clip0002 - 1.1.1 & 1.2.1 – Take 2 – CU

Clip0003 – 1.3.1 – 1.5.1 – Take 1 – CU

Clip0004 - 1.3.1 – 1.5.1 – Take 2 – WS

Clip0005 – Grey Card & White Balance

Clip0006 – Head Shot of FuiBoon Kai

Clip0018 – Head shot of Aisha Almsoud

Clip0019- Head shot of Upama Nyaupane

INTRODUCTION:

~~**REQUIRED:** Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **FuiBoon Kai:** My group studies how ECM stiffness drives pulmonary fibrosis, specifically investigating how lung cells sense stiffness and convert it into pathological signaling.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~**Describe the research gap or limitation in existing methods and how this protocol addresses them.**~~

- 1.2. **FuiBoon Kai:** Traditional ECM fabrication is complex, costly, and poorly reproducible. In contrast, our method is highly cost-effective and reproducible, thus allowing large-scale culture.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **FuiBoon Kai:** This protocol will allow researchers to study the impact of a soft and stiff matrix on cellular signaling and function.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **FuiBoon Kai:** The key challenge is to achieve optimal functionalization chemistry, as over- or under-functionalization can compromise downstream applications.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **FuiBoon Kai:** Tissue stiffening is a hallmark of many chronic diseases. Future studies can use this protocol to explore how tissue stiffness regulates diverse cell behaviors and to better understand how ECM mechanics influence disease progression.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Polyacrylamide Hydrogel Preparation

Demonstrator: Aisha Almsoud

2.1. To begin, mix the polyacrylamide hydrogel components in a 1.7 milliliter microcentrifuge tube, excluding ammonium persulfate and TEMED **[1-TXT]**.

2.1.1. WIDE: Talent mixing the polyacrylamide hydrogel components in a 1.7 milliliter microcentrifuge tube. **TXT: TEMED: Tetramethylethylenediamine**

Videographer's NOTE: Clip0007 – 2.1.0 – Optional Additional Shot – All solution names

***Videographer:** Please obtain CU shot as well for this shot.*

2.2. Degas the polyacrylamide gel mixture, the ammonium persulfate, and the TEMED solutions in a vacuum chamber at 25 millimeters of mercury for 30 minutes **[1]**.

2.2.1. Talent placing the polyacrylamide gel mixture, the ammonium persulfate tube, and the TEMED tube into a vacuum chamber.

2.3. To prepare a hydrophobic top coverslip, apply a water-repellent coating on the coverslip with a cotton swab **[1]**. Ensure the coverslip is free of smudges to maintain consistent gel polymerization **[2]**.

2.3.1. Talent applying a water-repellent coating to a clean coverslip with a cotton swab.

2.3.2. Shot of the smudge-free coverslip.

2.4. Add ammonium persulfate and TEMED to the degassed solution **[1]**. Then, gently pipette the mixture onto the activated coverslip **[2]**.

2.4.1. Talent adding ammonium persulfate and TEMED to the degassed solution.

2.4.2. Talent gently pipetting the mixture onto the activated coversli.

2.5. Place the hydrophobic coverslip on top of the solution to create a uniform thin hydrogel layer **[1]**. Ensure no bubbles are trapped and that the solution does not spill over the

edges [2]. Allow the gel to polymerize for 35 minutes at room temperature [3].

2.5.1. Talent placing the hydrophobic coverslip on top of the solution.

2.5.2. Shot of pointing to the hydrogel layer between the coverslips with no trapped bubbles or spilled solution.

2.5.3. Talent placing the gel aside for polymerization at room temperature.

2.6. Submerge the hydrogel sandwich in PBS to separate the layers [1]. Ensure that the hydrogel remains attached to the activated bottom coverslip and that the hydrophobic top coverslip detaches easily [2].

2.6.1. Talent submerging the hydrogel sandwich in PBS.

2.6.2. Shot of the hydrogel attached to the activated bottom coverslip as the hydrophobic top coverslip detaches.

Videographer's NOTE: Clip0021 – 2.6.2 – Take 1 CU

Clip0022 – 2.6.2 – Take 2 MS

3. Hydrogel Functionalization and Sterilization

3.1. Sterilize the hydrogel by immersing it in 70 percent ethanol [1]. Then, transfer it to tissue culture plasticware with sterile PBS [2].

3.1.1. Talent immersing the hydrogel in 70 percent ethanol.

3.1.2. Talent transferring the hydrogel to tissue culture plasticware with sterile PBS.

3.2. Prepare a master mix functionalization solution composed of water, ethanol, HEPES, and bisacrylamide [1]. Then, dilute the solutions of ultraviolet photoinitiator and tetraacrylate separately in 100% ethanol [2].

3.2.1. Talent preparing a master mix functionalization solution.

3.2.2. Talent preparing the diluted solutions of ultraviolet photoinitiator and tetraacrylate in ethanol.

Videographer's NOTE: Clip0026 – 3.2.2 – Take 1 – Only the ultraviolet photoinitiator

Clip0027 – 3.2.2a – Tetraacrylate in Ethanol

3.3. Weigh the required amount of N-hydroxysuccinimide ester or NHS-acrylate using an analytic balance [1]. Immediately before use, add the NHS acrylate to a mixture of 50 percent ethanol and 50 percent water [2]. Sonicate the solution at 40 kilohertz for 10 seconds at room temperature [3].

3.3.1. Talent weighing the N-hydroxysuccinimide-acrylate on an analytic balance.

Videographer's NOTE: Clip0029 – 3.3.1a – ECU of N-hydroxysuccinimide-acrylate

3.3.2. Talent adding the N-hydroxysuccinimide-acrylate to 50 percent ethanol and 50 percent water.

3.3.3. Talent sonicating the solution.

3.4. Add the ultraviolet photoinitiator, tetra-acrylate, and NHS-acrylate solutions to the prepared master mix [1]. Aspirate the PBS from the gel [2], then add the master mix to the gel, ensuring it is fully covered [3].

3.4.1. Talent adding the ultraviolet photoinitiator, tetraacrylate, and N-hydroxysuccinimide-acrylate solutions to the prepared master mix.

3.4.2. Talent aspirating the PBS from the gel.

3.4.3. Talent adding the master mix to fully cover the gel.

3.5. Expose the hydrogel without the tissue culture dish lid to ultraviolet light for 400 seconds at a wavelength less than 365 nanometers [1].

3.5.1. Talent placing the uncovered hydrogel under the ultraviolet light source.

3.6. Next, remove the gel and place it on ice [1]. Aspirate the functionalization solution [2]. Then, wash the gel 3 times with prechilled sterile 25 millimolar HEPES at pH 6, followed by 3 washes with prechilled sterile 0.9 percent sodium chloride [3].

3.6.1. Talent placing the gel on ice.

3.6.2. Talent aspirating the functionalization solution from the gel.

3.6.3. Talent washing the gel with the prechilled sterile HEPES.

3.7. Incubate the hydrogel with the extracellular matrix ligand of choice in prechilled sterile 100 millimolar HEPES at pH 8 with 100 millimolar sodium chloride overnight at 4

degrees Celsius on an orbital shaker [1].

3.7.1. Talent placing the hydrogel with the extracellular matrix ligand solution on an orbital shaker.

3.8. After 16 hours, carefully aspirate the extracellular matrix ligand solution [1]. Wash the hydrogel 3 times with PBS [2]. Add serum-free base media or PBS to keep the hydrogel hydrated [3], then incubate it overnight in a tissue culture incubator at 37 degrees Celsius to verify sterility before cell seeding [4].

3.8.1. Talent carefully aspirating the extracellular matrix ligand solution from the hydrogel.

3.8.2. Talent washing the hydrogel with PBS.

3.8.3. Talent adding serum-free base media or PBS to the hydrogel.

3.8.4. Talent placing the hydrogel in a tissue culture incubator.

3.9. Seed the cells of interest onto the ECM-functionalized hydrogel [1] and culture them for the desired experimental duration based on the cell type [2].

3.9.1. Talent adding the cell suspension onto the ECM-functionalized hydrogel.

3.9.2. Talent placing the culture dish in a tissue culture incubator.

4. RNA or Protein Lysate Collection

Demonstrator: Upama Nyaupane

NOTE: Show 4.3 here before 4.1

4.1. Using forceps, carefully remove the gel from the culture dish [1]. Submerge the gel in sterile PBS to rinse away the residual culture media and serum [2].

4.1.1. Talent carefully removing the gel from the culture dish with forceps.

4.1.2. Talent submerging the gel in sterile PBS.

Videographer's NOTE:

Clip0048 – 4.1.1, 4.1.2, 4.4.1 – All happens so fast so shot in one go – Take 1

Clip0049 - 4.1.1, 4.1.2, 4.4.1 – All happens so fast so shot in one go – Take 2 – just different focal length

- 4.2. Gently dab the edge of the hydrogel on low-lint wipes to remove excess PBS [1].
 - 4.2.1. Talent gently dabbing the edge of the hydrogel on low-lint wipes.
- 4.3. Add an appropriate volume of RNA extraction buffer to the lid of a tissue culture dish [1].
 - 4.3.1. Talent adding an appropriate volume of RNA extraction buffer to the lid of a tissue culture dish. **Videographer's NOTE: Clip0047 – 4.3.1 – THIS STEP MUST HAPPEN BEFORE 4.1.1**
- 4.4. Place the coverslip gel side down onto the lysis solution [1]. Carefully lift and lower the hydrogel repeatedly to ensure complete lysis across the gel surface [2].
 - 4.4.1. Talent placing the coverslip gel side down onto the lysis solution.
 - 4.4.2. Talent carefully lifting and lowering the hydrogel several times.
- 4.5. Then, gently scrape the gel surface to release the lysate [1].
 - 4.5.1. Talent gently scraping the gel surface to release the lysate.
- 4.6. Store the RNA or protein lysates at minus 80 degrees Celsius until further processing using conventional biochemical methods [1].
 - 4.6.1. Talent placing the RNA or protein lysates into storage at -80 degrees Celsius.

Results

5. Results

5.1. Polyacrylamide gel stiffness generated using the formulations [1] was experimentally tested using a rheometer. The gels with nominal stiffness values of 0.4, 6, and 60 kilopascals [2] had measured stiffness values of approximately 363 pascals [3], 6.41 kilopascals [4], and 36 kilopascals, respectively [5].

5.1.1. LAB MEDIA: Table 1

5.1.2. LAB MEDIA: Figure 4A

5.1.3. LAB MEDIA: Figure 4A. *Video editor: Highlight the bar labelled '0.4 kPa'.*

5.1.4. LAB MEDIA: Figure 4A. *Video editor: Highlight the bar '6 kPa'.*

5.1.5. LAB MEDIA: Figure 4A. *Video editor: Highlight the bar '60 kPa'.*

5.2. The fluorescently labeled fibronectin-coated 0.4 and 6 kilopascal gels exhibited increased fluorescence intensity [1] compared to gels coated with unlabeled fibronectin [2].

5.2.1. LAB MEDIA: Figure 4B. *Video editor: Highlight the 2 bars 'Alexa-594'.*

5.2.2. LAB MEDIA: Figure 4B. *Video editor: Highlight the bar "Unlabelled FN".*

5.3. The cells cultured on 0.4 and 6 kilopascal substrates exhibited distinct morphologies [1], with significantly increased cell spreading observed on the stiffer substrate [2].

5.3.1. LAB MEDIA: Figure 4C.

5.3.2. LAB MEDIA: Figure 4C. *Video editor: Highlight the image labelled '6 kPa'.*

5.4. The quantitative analysis confirmed a stiffness-dependent increase in cell spread area [1].

5.4.1. LAB MEDIA: Figure 4D.

5.5. The phosphorylation of focal adhesion kinase at Tyrosine 397 increased in A549 cells cultured on the 6 kilopascal Polyacrylamide-based ECM substrates [1].

5.5.1. LAB MEDIA: Figure 6B. *Video editor: Highlight the lanes labelled '6.0 kPa'.*

5.6. The ANKRD1 expression was elevated in cells cultured on the 6 kilopascal PA gels [1].

5.6.1. LAB MEDIA: Figure 6C. *Video editor: Highlight the bar labelled '6.0 kPa'.*

1. Polyacrylamide
Pronunciation link: <https://www.merriam-webster.com/dictionary/polyacrylamide>
IPA: /ˌpɑːliəˈkrɪləˌmaɪd/
Phonetic Spelling: pah·lee·uh·KRIL·uh·myd
2. Hydrogel
Pronunciation link: <https://www.merriam-webster.com/dictionary/hydrogel>
IPA: /ˈhaɪdrəˌdʒel/
Phonetic Spelling: HY·druh·jel
3. Microcentrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/microcentrifuge>
IPA: /ˌmaɪkroʊˈsentrɪˌfjuːdʒ/
Phonetic Spelling: MY·kroh·SEN·truh·fyooj
4. Ammonium
Pronunciation link: <https://www.merriam-webster.com/dictionary/ammonium>
IPA: /əˈmʌniəm/
Phonetic Spelling: uh·MOH·nee·uhm
5. Persulfate
Pronunciation link: <https://www.merriam-webster.com/dictionary/persulfate>
IPA: /pərˈsʌlˌfɛt/
Phonetic Spelling: per·SUL·fayt
6. TEMED
Pronunciation link: No confirmed link found
IPA: /ˈtiːˌɛmˌɛd/
Phonetic Spelling: TEE·EM·ed
7. Tetramethylethylenediamine
Pronunciation link: <https://www.merriam-webster.com/dictionary/tetramethylethylenediamine>
IPA: /ˌtɛtrəˌmɛθəlˌɛθəˌliːnˈdaɪəˌmiːn/
Phonetic Spelling: TET·ruh·METH·uhl·ETH·uh·leen·dy·uh·meen
8. Degas
Pronunciation link: <https://www.merriam-webster.com/dictionary/degas>
IPA: /diːˈgæs/
Phonetic Spelling: dee·GAS
9. Hydrophobic
Pronunciation link: <https://www.merriam-webster.com/dictionary/hydrophobic>
IPA: /ˌhaɪdrəˈfoʊbɪk/
Phonetic Spelling: HY·druh·FOH·bik
10. Polymerization
Pronunciation link: <https://www.merriam-webster.com/dictionary/polymerization>

- IPA: /pəˌlɪməˈɹeɪʃən/
Phonetic Spelling: puh·LIM·uh·ruh·ZAY·shuhn
11. Coverslip
Pronunciation link: <https://www.merriam-webster.com/dictionary/coverslip>
IPA: /ˈkʌvərˌslɪp/
Phonetic Spelling: KUV·er·slip
 12. Sterilize
Pronunciation link: <https://www.merriam-webster.com/dictionary/sterilize>
IPA: /ˈsterəˌlaɪz/
Phonetic Spelling: STER·uh·lyz
 13. HEPES
Pronunciation link: <https://www.merriam-webster.com/medical/HEPES>
IPA: /ˈhepez/
Phonetic Spelling: HEP·ez
 14. Bisacrylamide
Pronunciation link: No confirmed link found
IPA: /ˌbaɪsəˈkrɪləˌmaɪd/
Phonetic Spelling: BY·suh·KRIL·uh·myd
 15. Ultraviolet
Pronunciation link: <https://www.merriam-webster.com/dictionary/ultraviolet>
IPA: /ˌʌltrəˈvaɪələt/
Phonetic Spelling: UL·truh·VY·uh·luht
 16. Photoinitiator
Pronunciation link: No confirmed link found
IPA: /ˌfoʊtoʊɪˈnɪʃɪˌeɪtər/
Phonetic Spelling: FOH·toh·ih·NISH·ee·ay·ter
 17. Tetra-acrylate
Pronunciation link: No confirmed link found
IPA: /ˌtetrəˈækrəˌleɪt/
Phonetic Spelling: TET·ruh·AK·ruh·layt
 18. N-hydroxysuccinimide
Pronunciation link: No confirmed link found
IPA: /ɛn haɪˌdrɑːksiˌsʌkˈsɪnɪˌmaɪd/
Phonetic Spelling: EN hy·DROK·see·suk·SIN·ih·myd
 19. NHS-acrylate
Pronunciation link: No confirmed link found
IPA: /ˌɛn ɛɪtʃ ɛs ˈækrəˌleɪt/
Phonetic Spelling: EN·AYCH·ES AK·ruh·layt
 20. Analytic
Pronunciation link: <https://www.merriam-webster.com/dictionary/analytic>
IPA: /ˌænəˈlɪtɪk/
Phonetic Spelling: an·uh·LIT·ik
 21. Sonicate
Pronunciation link: <https://www.merriam-webster.com/dictionary/sonicate>

- IPA: /'sɑ:nəˌkɛrt/
Phonetic Spelling: SON·uh·kayt
22. Aspirate
Pronunciation link: <https://www.merriam-webster.com/dictionary/aspirate>
IPA: /'æspəˌreɪt/
Phonetic Spelling: ASS·puh·rayt
23. Functionalization
Pronunciation link: No confirmed link found
IPA: /ˌfʌŋkʃənələˈzeɪʃən/
Phonetic Spelling: funk·shuh·nuhl·uh·ZAY·shuhn
24. Extracellular
Pronunciation link: <https://www.merriam-webster.com/medical/extracellular>
IPA: /ˌɛkstrəˈseljələr/
Phonetic Spelling: EK·struh·SEL·yuh·ler
25. Ligand
Pronunciation link: <https://www.merriam-webster.com/dictionary/ligand>
IPA: /'lɪɡənd/
Phonetic Spelling: LIG·uhnd
26. Orbital
Pronunciation link: <https://www.merriam-webster.com/dictionary/orbital>
IPA: /'ɔːrbɪtəl/
Phonetic Spelling: OR·bih·tuhl
27. ECM
Pronunciation link: No confirmed link found
IPA: /ˌiːsiːˈɛm/
Phonetic Spelling: EE·SEE·EM
28. Lysate
Pronunciation link: <https://www.merriam-webster.com/dictionary/lysate>
IPA: /'laɪseɪt/
Phonetic Spelling: LY·sayt
29. Rheometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/rheometer>
IPA: /riˈɑːmətər/
Phonetic Spelling: ree·AH·muh·ter
30. Kilopascal
Pronunciation link: <https://www.merriam-webster.com/dictionary/kilopascal>
IPA: /ˌkɪləˈpæskəl/
Phonetic Spelling: KIL·oh·PAS·kuhl
31. Fluorescently
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescent>
IPA: /flɔːˈresəntli/
Phonetic Spelling: flohr·ES·uhnt·lee
32. Fibronectin
Pronunciation link: <https://www.merriam-webster.com/medical/fibronectin>

IPA: /ˌfaɪbroʊˈnektɪn/

Phonetic Spelling: FY·broh·NEK·tin

33. Morphologies

Pronunciation link: <https://www.merriam-webster.com/dictionary/morphology>

IPA: /mɔːrˈfɑːlədʒiz/

Phonetic Spelling: mor·FOL·uh·jeez

34. Phosphorylation

Pronunciation link: <https://www.merriam-webster.com/dictionary/phosphorylation>

IPA: /ˌfɔːsfəːrəˈleɪʃən/

Phonetic Spelling: FOS·for·uh·LAY·shuhn

35. Tyrosine

Pronunciation link: <https://www.merriam-webster.com/dictionary/tyrosine>

IPA: /ˈtaɪrəsiːn/

Phonetic Spelling: TY·ruh·seen

36. ANKRD1

Pronunciation link: No confirmed link found

IPA: /ˌeɪ ɛn keɪ ɑːr diː ˈwʌn/

Phonetic Spelling: AY·EN·KAY·AR·DEE·WUN

37. A549

Pronunciation link: No confirmed link found

IPA: /ˌeɪ faɪv ˈfɔːr naɪn/

Phonetic Spelling: AY·five·FOUR·nine