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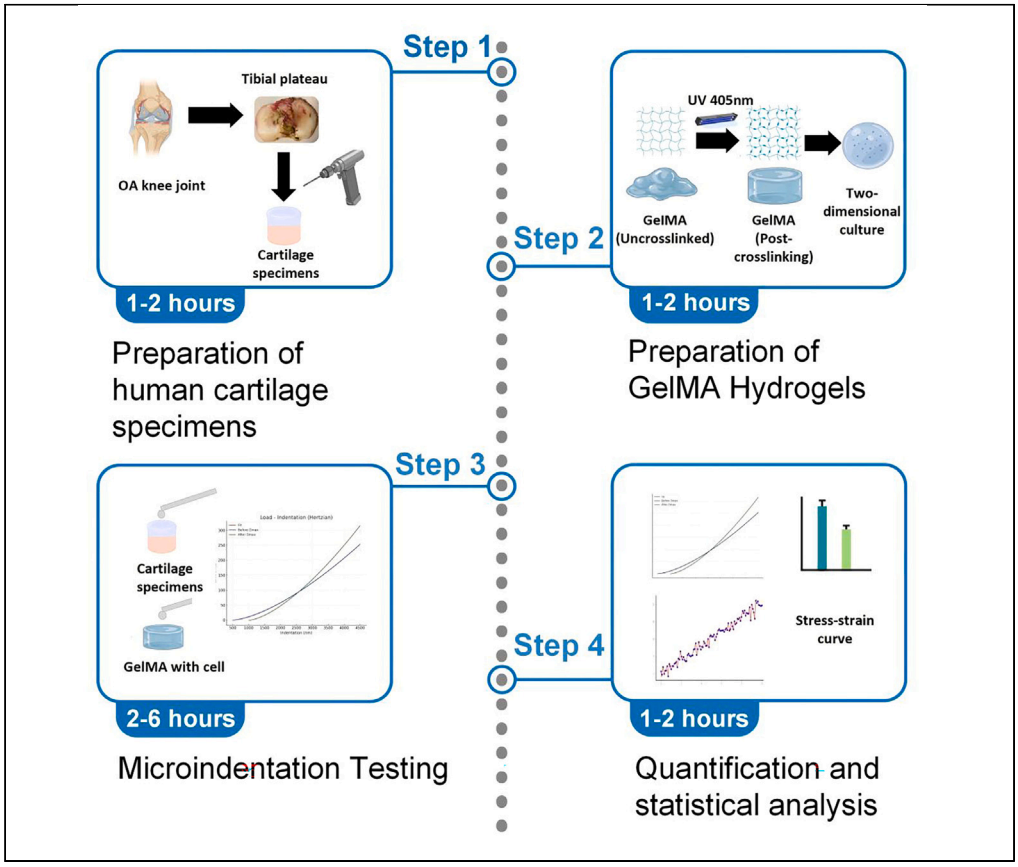
Protocol

Protocol for microindentation analysis of human cartilage and methacrylated gelatin hydrogels with varying stiffness in ATDC5 cell cultures



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Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Steps for culturing ATDC5 cells on hydrogel

Instructions for testing the mechanical properties of GelMA hydrogels

Guidelines for testing the mechanical properties of cartilage samples from clinic

Wang et al., STAR Protocols 6, 103511
March 21, 2025
https://doi.org/10.1016/j.xpro.2024.103511

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Protocol for microindentation analysis of human cartilage and methacrylated gelatin hydrogels with varying stiffness in ATDC5 cell cultures

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https://doi.org/10.1016/j.xpro.2024.103511

SUMMARY

Here, we present a protocol for evaluating mechanical properties of human cartilage specimens and methacrylated gelatin (GelMA) hydrogels with varying Young's moduli for cultures of ATDC5 chondrocytes using microindentation. We describe steps for preparing human cartilage specimens and hydrogels, culturing ATDC5 cells, and assessing the stiffness. This protocol was designed to facilitate reproducibility in biomechanical assays of chondrocytes.

For complete details on the use and execution of this protocol, please refer to Kan et al.[1]

BEFORE YOU BEGIN

1. Prepare microindentation devices and equipment in advance.
2. Prepare hydrogel systems with different concentrations.
3. Advance culture and induction of ATDC5 cells into chondrocytes.

Institutional permissions

This study was approved by the Ethics Committee of the Shanghai Ninth People's Hospital (No. SH9H-2021-T401-2). All the patients were Han Chinese. Sample size requirements were computed to detect a mean difference of 8 in the total points of the Osteoarthritis Research Society International (OARSI) scores, and a sample size of $n = 6$ was determined. All the six patients provided written informed consent.

Preparation of human cartilage specimens

Timing: 1–2 h

4. Sample collection
   a. Obtain fresh or frozen human cartilage tissue from the knee, ensuring that appropriate ethical guidelines are followed and informed consent procedures are conducted during sample collection (Figure 1).

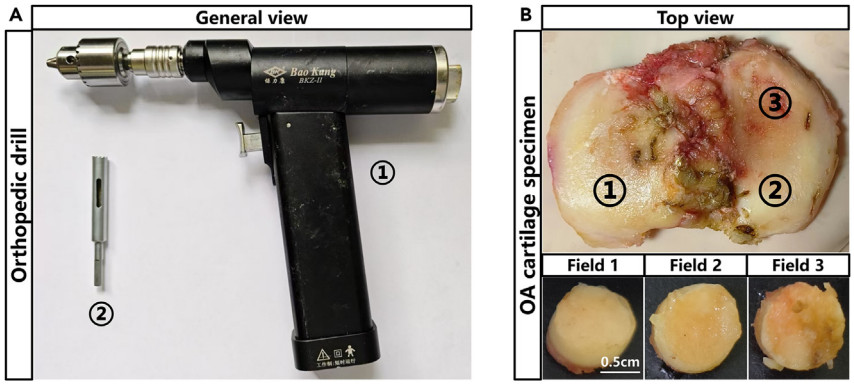

Figure 1. Acquisition and treatment of human cartilage specimen

(A) General view of specimen treatment tools: ① Orthopedic drill; ② Ring drill (d = 1 cm).

(B) Human tibial plateau cartilage specimen and segmented osteochondral explants. Scale bars are 0.5 cm.

b. Keep the samples hydrated by storing them in a phosphate-buffered saline (PBS) solution, ensuring that they do not dry out during transportation or prior to use.

5. Preservation of samples
   a. Store the cartilage samples in PBS or sterile saline to maintain their moisture and mechanical properties if experiments are not planned immediately after collection.
   b. Refrigerate the samples at 4°C to prevent loss of cellular activity or dehydration.

Note: For samples temporarily stored at 4°C, we recommend a storage time of no more than 12 h.

   c. Store samples in liquid nitrogen (−196°C) to effectively maintain their biomechanical properties and viability for long-term storage of cartilage samples.

Culture and chondrogenic differentiation of ATDC5 chondrocytes

Timing: 15–20 days

6. Cell Culture
   a. Obtain the ATDC5 chondrocyte cell line from the Fuheng Center Cell Bank.
   b. Add 25 mL of fetal bovine serum (FBS) and 5 mL of penicillin/streptomycin to 470 mL of DMEM/F-12 basal medium to form the final concentration of complete medium.
   c. Cells are inoculated in 10 cm dishes and ATDC5 cells are cultured in DMEM/F-12 medium supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin.
   d. Maintain the cells in a humidified incubator at 37°C with 5% CO_2 and 21% O_2.
   e. Replace the medium every 2 days. ATDC5 cells are cultured to 100% confluence in the growth medium.

Note: The cell growth time to 100% density depends on the density of the inoculated cells, it is generally considered that the maximum amount of cells that can be cultured in a 10 cm dish is $10*10^6$, if the inoculated density is $10*10^4$, it usually takes 7 days for the cell density to reach 100%. If the number of cells is sufficient for the experiment, there is no need for passaging

7. Chondrogenic Differentiation Induction
   a. Add 1 mL of 1% insulin-transferrin-selenium (ITS) to every 100 mL of complete medium to configure the final medium.

b. Switch the cells to differentiation medium by supplementing the standard culture medium with 1% insulin-transferrin-selenium (ITS).
c. Continue culturing the cells in the differentiation medium under the same incubation conditions (37°C, 5% CO_2, and 21% O_2).
d. Change the differentiation medium every 2 days.
e. Induce differentiation for 14 days. Subsequently, the differentiated ATDC5 chondrocytes can be used in subsequent experiments. ATDC5 chondrocytes that have been induced to differentiate can be detached from the culture dish by trypsin-EDTA and planted on the surface of a GelMA hydrogel for subsequent assays.

Preparation of GelMA hydrogels with varying Young's modulus

Timing: 2 h

8. Preparation of 0.25% (w/v) LAP Photoinitiator Solution:
   a. Add 20 mL of PBS to a brown bottle containing 0.05 g of LAP photoinitiator.
   b. Heat the mixture in a 40°C–50°C water bath for 15 min. The bottle is mixed periodically until the LAP is completely dissolved.
   c. Store the LAP solution at 4°C in a dark environment.

Note: This solution can be stored for up to 12 months.

9. Preparation of GelMA Solution (5–30% w/v recommended):
   a. Weigh the required amount of GelMA (suggested concentrations–5–30% w/v) and place it into a centrifuge tube.[2]
   b. Add the previously prepared LAP photoinitiator solution to the centrifuge tube containing GelMA, ensuring that the GelMA is completely immersed.

Note: Take 10% GelMA hydrogel as an example, take 5 g of GelMA hydrogel powder and add it into a 50 mL centrifuge tube, add 30 mL PBS, and then add 20 mL LAP photoinitiator which has already been prepared, then we can get the final concentration of 10% GelMA hydrogel. Other concentrations can be diluted proportionally.

   c. Heat the mixture in a 60°C–70°C water bath, protected from light, for 20–30 min, mixing periodically to facilitate dissolution. Just shake the container lightly, or use a stirring stick if you have one. If large amounts of GelMA are present, the heating time can be extended accordingly.
   d. After the GelMA is dissolved, centrifuge the solution at 1500 × *g* for 2 min to remove any bubbles.
   e. Filter the GelMA solution immediately using a 0.22 μm sterile syringe filter to sterilize it and to prevent premature gelation at low temperatures.

⚠ CRITICAL: It is important to note that the process needs to be fast enough and avoid light to prevent premature gelation of the liquid hydrogel.

10. Preparation of GelMA Hydrogels with different stiffnesses:
   a. Prepare GelMA solutions at the desired concentrations (10, 15, and 18% w/v) by following the above procedure. Typically, a 0.25% (w/v) LAP photoinitiator solution system is used, diluted proportionally for different hydrogel concentrations.
   b. Transfer 1 mL of the prepared GelMA solution into the bottom of a 6 cm Petri dish and spread it evenly to form a layer of approximately 0.4 mm thick.

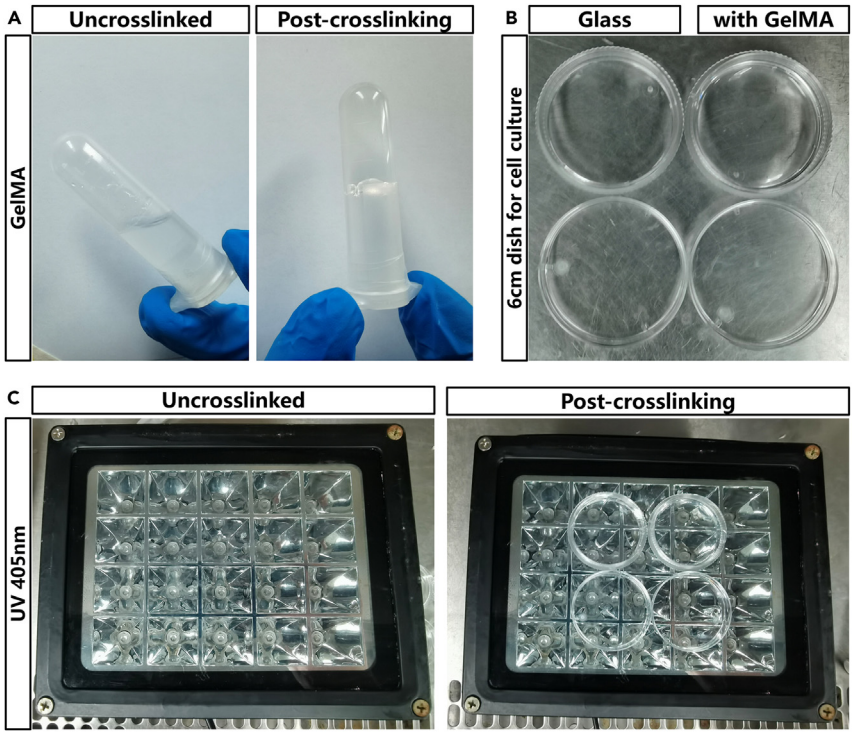

Figure 2. Preparation of GelMA with different stiffness

(A) Before and after GelMA crosslinking in different concentrations.

(B) GelMA is used for cell culture on the bottom of petri dishes.

(C) Representative pictures of uncrosslinked and crosslinked hydrogels.

Note: After adding the hydrogel into the 6 cm Petri dish, the 6 cm Petri dish was slightly shaken in four directions, front and back, left and right, in the horizontal plane, and then left to stand for 1 min to make the hydrogel spread evenly.

11. Photocrosslinking:
    a. Place the petri dishes under a UV floodlight (405 nm wavelength) and expose the GelMA solution to UV light for 1 min to induce photocrosslinking. After crosslinking, the hydrogels with different stiffness values can be used for further experimentation and analysis (Figure 2).

Microindentation testing equipment

Timing: 2 h

12. Verify the calibration of the device, ensuring the indenter is properly installed and the load range is set correctly for the test.

Note: Depending on the predicted stiffness of the hydrogel and the specification of the probe, the load range can be set between 0.025 N and 200 N, and we used a range of 0.025–0.25 N for our tests.

Optional: We used a displacement-controlled PIUMA Nanoindenter (Optics11 B.V., Amsterdam, The Netherlands) for microindenter testing. A probe with 0.45 N/m cantilever stiffness (k) and a 48.5 μm radius (R) spherical tip was used in our study. Depending on the material

to be inspected, different inspection probes can be selected, such as Petri Dish Probes for inspection diameters from 3–25 μm and inspection ranges from 0.025 to 200 N/m.

13. Adjust the microscope for initial observation of the sample surface before testing and for analysis after the microindentation test.
    a. Set up an inverted microscope on an air-suspension anti-vibration table to minimize vibrations.
    b. Adjust the microscope with differential interference contrast (DIC) for enhanced imaging.
    c. Select the appropriate microscope objective and use 100x magnification for high resolution imaging or lower magnification for sample positioning.
    d. Connect the CMOS (Complementary Metal-Oxide-Semiconductor) camera for image capture.
14. Install and configure the control software used to manage the operation of the indenter, including load application and data collection. Configure MATLAB and Micro-Manager software (https://micro-manager.org/) for microscope control and communication with the devices.
15. Place the microhardness tester on an antivibration table to eliminate any external vibrations that could affect the precision of the microindentation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
ATDC5 cells	Cell Bank of the Chinese Academy of Sciences	(RCB Cat# RCB0565, RRID:CVCL_3894)
Software and algorithms		
MATLAB	MATLAB 2023a, Subscription - IISER Pune	R2023a
GraphPad Prism version 8	https://www.graphpad.com/	Prism 8.0.2
Micro-Manager software	https://micro-manager.org/	Micro-Manager 2.0.0
Chemicals, peptides, and recombinant proteins		
Fetal bovine serum	Sigma	F8318
DMEM/F-12 medium	Gibco	11320033
GelMA	Engineering for Life (Suzhou, China)	EFL-GM-30
Lithium phenyl (2,4,6-trimethylbenzoyl) phosphinate (LAP)	Engineering for Life (Suzhou, China)	410896
PBS (1X, premixed powder)	Beyotime	ST447-1L
1% penicillin/streptomycin	Gibco	15140122
1% insulin-transferrin-selenium (ITS)	Gibco	41400045
Other		
Piuma Nanoindenter	Optics11 B.V., Amsterdam, The Netherlands	N/A
XDS-1 inverted microscope	Shanghai Optical Instrument Factory (Shanghai, China)	https://m.sipmv.com/product/microscope/bm/xds-1b/

Materials and equipment

Complete Medium		
Reagent	Final concentration	Amount
DMEM/F-12 medium	94%	470 mL
Fetal bovine serum	5%	25 mL
1% penicillin/streptomycin	1%	5 mL
Total	**100%**	**500 mL**

Note: Complete medium can only be stored at 4°C for 3 months.

Phosphate-buffered saline (PBS)		
Reagent	Final concentration	Amount
PBS (1X, premixed powder)	0.01 mol/L	20 g
Double distilled water	N/A	1 L
Total	**N/A**	**1 L**

Note: We used a premixed powder in our experiments and did not need to add any additional ingredients.

1% insulin-transferrin-selenium (ITS)		
Reagent	Final concentration	Amount
Insulin	1000.0 mg/L	0.1721763 mM
Transferrin	550.0 mg/L	0.006875 mM
Sodium Selenite	0.67 mg/L	0.0038728325 mM
Total	**N/A**	**N/A**

Note: The 1% insulin-transferrin-selenium (ITS) that we use is a commercial product that is already configured for use and only needs to be added proportionally to the culture medium.

0.25% (w/v) LAP photoinitiator solution		
Reagent	Final concentration	Amount
Lithium phenyl (2,4,6-trimethylbenzoyl) phosphinate (LAP)	0.25%	0.05 g
Phosphate-buffered saline (PBS)	N/A	20 mL
Total	**N/A**	**20 mL**

STEP-BY-STEP METHOD DETAILS

Implantation of differentiated cells on the GelMA hydrogel surface

Timing: 2 days

This section focuses on detaching differentiated chondrocytes from the culture dish and planting them on the surface of the GelMA hydrogel at different concentrations to ensure complete growth and keep the cells active for assessing their biomechanical properties.[3]

1. In a sterile environment, detach adherent cells using trypsin-EDTA or an appropriate dissociation method. Add 3 mL of trypsin to each 10 cm petri dish and leave it at 37°C for 5 min.
2. Neutralize trypsin with culture medium containing serum. Centrifuge at 300 × *g* for 5 min to form pellets.
3. Add 10 mL of medium containing serum to neutralize trypsin.

Note: The ratio of trypsin to medium is approximately 3:10.

4. Remove the supernatant and resuspend the cells in fresh culture medium. Adjust the cell concentration to the desired seeding density (e.g., 1×10^5–1×10^6 cells/mL).
5. Gently add the cell suspension directly onto the surface of each hydrogel at different concentrations.

⚠ CRITICAL: Also use complete medium with serum and trypsin to culture the cells, making sure that the medium covers the entire hydrogel, usually adding 10 mL of medium to completely submerge the hydrogel and the cells on the surface.

6. Maintain the cells in a humidified incubator for 24 h at 37°C with 5% CO_2 and 21% O_2.

Preparation of the GelMA hydrogel for testing

Timing: 30 minutes

This section outlines the methodology for culturing the cells on the hydrogel and preparing them for the forthcoming indentation test.

7. Prior to indentation, ensure that ATDC5 cells seeded in the hydrogel reach at least 80% cell density.
8. Immerse the GelMA hydrogels with cells in cell culture media or PBS at 37°C in a CO_2 incubator to maintain cell viability and hydration. Before starting the microindentation test, transfer the hydrogels to PBS or a cell culture medium compatible with the indentation setup.

   Note: Indentation testing can be accomplished with the commonly used 6 cm petri dish, but other petri dishes that are too hard or media that are too viscous may affect the test results. The effect can be eliminated by changing the petri dish when growing the cells or changing the medium to PBS.

9. Inspect the hydrogel surface under a microscope to ensure it is smooth and debris-free.

   Note: Any surface irregularities may interfere with the indentation process, leading to inaccurate force-displacement data.

10. Before implanting cells, reshape or carefully trim the hydrogel surface with a sterile scalpel if it is uneven or contains air bubbles.

    Note: Uneven surfaces can cause the indenter to slip or lead to nonuniform force application during the test.

Microindentation testing on the GelMA hydrogel

Timing: 6 hours

This section outlines the methodology employed in microindentation testing of hydrogels, encompassing the procedures for indentation and withdrawal of the probe, as well as the parameters established for hydrogel analysis.

11. Place the petri dish with GelMA hydrogel onto the stage of the microindentation system. Use sterile forceps to handle the dish to avoid contamination, particularly if the hydrogel contains cells (Figure 3).
12. Focus the microscope's field of view on the region of interest under the indentation probe. For GelMA hydrogels containing cells, focus on areas with uniform cell distribution or specific regions of interest.
13. Capture pre-indentation images to document the hydrogel surface or cell morphology, particularly if cells are present on the hydrogel surface.
14. Using the motorized stage of the microindentation system, guide the indentation probe into close proximity to the hydrogel surface without touching it. Ensure accurate positioning to avoid premature contact with the hydrogel.
15. Fine-tune the probe's position to target specific regions of the hydrogel.
16. Set the desired maximum force or indentation depth based on the experimental goals.

    Note: For hydrogels, typical depths range from 10–15 μm; however, this may vary based on the stiffness of the GelMA. In our experiments, we used 6 cm Petri dishes in which we spread GelMA hydrogel with a thickness of 0.4 mm for testing and tested at a depth of 10 μm.

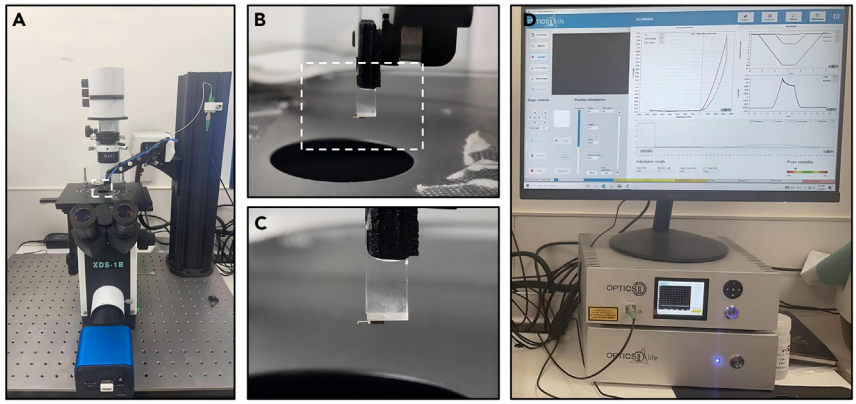

Figure 3. Multi-angle photos of microindentation test system

(A) Microindentation probe and optical microscope.

(B) Microindentation probe and sample table.

(C) Microindentation probe details.

(D) Full view of microindentation test system, including operating interface.

17. Adjust the indentation rate to ensure smooth penetration into the hydrogel material.

    Note: Adjust the speed of approach of the indenter to no more than 10 μm/s to avoid excessive speed leading to test failure.

18. Indentation parameters to be set: Indentation Depth: Maximum depth of 15 μm (adjustable depending on hydrogel stiffness). Loading Rate: 10 μm/s for gradual penetration. Maximum Force: 20 μN.
19. Slowly lower the indentation probe onto the hydrogel surface, recording the force-displacement curve in real time as the probe moves into the gel.

    Note: The indentation depth is recorded as the probe deforms the hydrogel, offering mechanical feedback based on the material properties of GelMA.

20. Stop the indentation once the predefined maximum force or depth is reached and retract the probe slowly from the hydrogel surface to avoid damaging the sample.
21. Perform the indentation at multiple locations on the hydrogel surface, allowing the system to record the force-displacement curve during the loading and unloading phases. Analyze the unloading curve (retraction) to study potential hysteresis or viscoelastic properties of the hydrogel. At the same time, spatial variations in mechanical properties can be assessed, especially in the case of surface-cultured cells.
22. Repeat the procedure across several hydrogels to obtain statistically significant data, ensuring sufficient replicates for reliable mechanical characterization.

Preparation of human cartilage specimens

Timing: 1 h

This section outlines the methodology for preparing samples suitable for testing for cartilage and highlights the key considerations.

23. Use a scalpel or surgical knife to carefully trim the cartilage sample to a diameter of 5 mm and a thickness of 2 mm, maintaining a uniform thickness to ensure consistent results in indentation testing.

24. Post trimming, rinse the sample in PBS to remove any debris or contaminants.
25. Glue the cartilage sample to a rigid substrate, such as a Petri dish or testing platform using a medical-grade adhesive.

Note: The adhesive should be applied carefully to avoid affecting the mechanical properties of the samples. Control the amount of glue used, minimize the amount of glue, and when applying the glue in different locations to avoid large areas of glue. Avoid the area where the glue is applied when selecting the area for testing. Ensure that the cartilage sample remains immersed in a hydration fluid (such as PBS) throughout the mounting and testing processes to maintain the physiological conditions

Microindentation testing on human cartilage specimens

Timing: 6 h

26. Place the petri dish with the mounted cartilage specimen on the microindentation system stage.
27. Use the integrated microscope to center the region of interest under the indenter. Ensure the surface is smooth and debris free.
28. Use the system's "Find Contact" feature to detect the surface of the cartilage. The indenter moves downward slowly, and the starting point for the indentation is recorded by the system when the preset trigger force is reached (typically 0.3 μN).
29. After detecting the contact point, initiate the indentation by moving the indenter tip into the cartilage to the pre-set depth (typically 10–15 μm).
30. Apply force gradually at the defined loading rate (10 μm/s), and record the force-displacement curve in real-time.
31. As the indenter tip displaces the cartilage, the system records the relationship between the applied force and the resulting displacement.

Note: This data will be used to compute tissue stiffness.

32. Once the indenter reaches the target depth, hold it in place for 2–3 s.

Note: This dwell time allows the tissue to adjust to the load and ensures that viscoelastic relaxation does not affect the measurement. For viscoelastic studies, extend the hold time as appropriate.

33. After the dwell time, slowly retract the indenter from the cartilage at the same speed (10 μm/s), while continuing to record the unloading phase of the force-displacement curve.

Note: The unloading data can provide insights into the viscoelastic properties and tissue recovery behavior.

34. Repeat the indentation testing on several cartilage specimens to obtain statistically significant data. The data from multiple tests will improve the reliability of the mechanical characterization.

EXPECTED OUTCOMES

Obtaining the mechanical property data of GelMA hydrogels with Varying Young's Modulus. For each indentation, the system should generate a force-displacement curve. This curve represents the force applied as the indenter moves into the tissue and the resulting displacement. The slope of the force-displacement curve during the loading phase provides key information about the stiffness of the tissue, which can later be analyzed using appropriate mechanical models.[4] For example, the Hertz Contact Model, Hooke's Law Model, Von Mises Yield Criterion. The data should

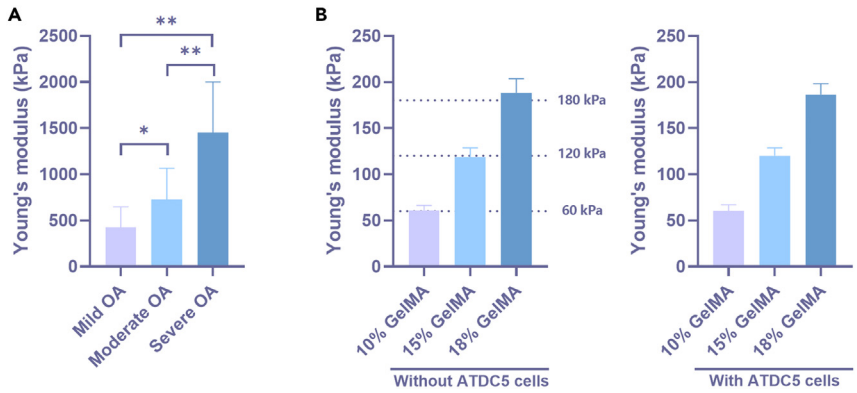

Figure 4. Microindentation test results of human cartilage specimen and GelMA

(A) Microindentation test results of human cartilage specimen. (n = 6).

(B) Microindentation test results of 10%, 15% and 18% GelMA with and without cells (n = 6). Data are shown as means ± SD, *p < 0.05, **p < 0.01, ***p < 0.001.

demonstrate the mechanical properties of GelMA with varying stiffness values in human cartilage samples (Figure 4).

QUANTIFICATION AND STATISTICAL ANALYSIS

1. Collect and analyze force-displacement curves to determine the tissue's mechanical properties. The experimentally observed force-displacement data are compared with the corresponding model to determine the appropriate parameters for the best fit between the experiment and the model. According to the measured indentation depth and contact radius, for example, the indentation depth is 40 μm, the contact radius is 10 μm, enter the parameters in the computer software, use the formula for fitting the model, you can automatically get the results of the stiffness data.
2. Data Modeling: Use models such as the Hertzian contact model to derive the Young's modulus (elastic modulus) based on the indentation data.
3. The Hertzian contact model[5] used in microindentation testing, is based on the assumption of a linear elastic material under small deformation. It is suitable for characterizing materials in the linear elastic regime using a spherical indenter. The model relates the force applied to the indentation depth, allowing computation of the elastic modulus of the material.
4. All statistical analyses were conducted using the GraphPad Prism 9.0 software.

LIMITATIONS

The methods described in this program are capable of measuring only the mechanical properties related to the overall stiffness or viscoelasticity of the GelMA hydrogel (Figure 5). They do not reflect mechanical characteristics related to the physical properties of subtle intracellular structures, organelles, or cell membranes[3] (Figure 6).

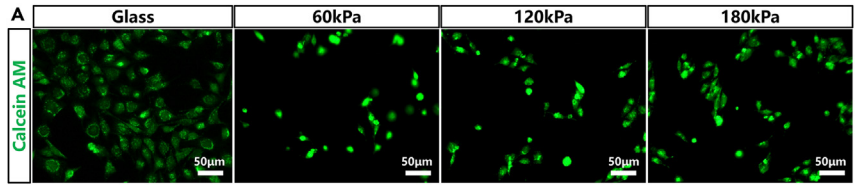

Figure 5. Living cells on hydrogel surfaces with different stiffnesses

(A) Calcein-AM staining of living cells on hydrogel surfaces. Scale bars are 200 μm.

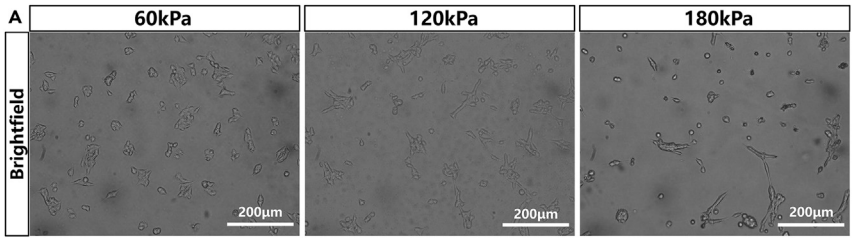

Figure 6. Two-dimensional cultivation of chondrocytes with different stiffness

(A) Bright field images of ATDC5 chondrocytes with different stiffness. Scale bars are 200 μm.

TROUBLESHOOTING

Problem 1

Inconsistent GelMA crosslinking.

Potential solution

One potential solution is to ensure uniform UV exposure during the crosslinking. This can be achieved by optimizing the distance between the UV light source and GelMA sample, using a consistent exposure time, and ensuring that the UV intensity is equally distributed across the surface.

Problem 2

Cell viability reduction post encapsulation.

Potential solution

The cell culture medium can be changed before the experiment to ensure that the cells are in the right environment for growth.[1]

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Zhifeng Yu (zfyu@outlook.com).

Technical contact

For technical information, please contact Mengning Yan (yanmengning@163.com).

Materials availability

This study did not require unique reagents.

Data and code availability

This study did not generate any unique dataset or code.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (12172223, 12272232, 82272997, and 81802679), Cross-Disciplinary Research Fund of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (JYJC202201), Science and Technology Commission of Shanghai Municipality (no. 20ZR1432200 and 21140900102), and Shanghai Key Laboratory of Orthopedic Implants (no. KFKT202203).

AUTHOR CONTRIBUTIONS

H.W. and T.K.: conceptualization, investigation, formal analysis, and writing – original draft. L.S.: investigation and formal analyses. H.L.: investigation and formal analysis. L.W.: methodology and supervision. M.Y. and Z.Y.: conceptualization, supervision, funding acquisition, and writing – review and editing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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