In Vitro Model Integrating Substrate Stiffness and Flow to Study Endothelial Cell Responses

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Abstract

We present an innovative *in vitro* model aimed at investigating the combined effects of tissue rigidity and shear stress on endothelial cell (EC) function, which are crucial for understanding vascular health and the onset of diseases such as atherosclerosis. Traditionally, studies have explored the impacts of shear stress and substrate stiffness on ECs, independently. However, this integrated system combines these factors to provide a more precise simulation of the mechanical environment of the vasculature. The objective is to examine EC mechanotransduction across various tissue stiffness levels and flow conditions using human ECs. We detail the protocol for synthesizing gelatin methacrylate (GelMA) hydrogels with tunable stiffness and seeding them with ECs to achieve confluency. Additionally, we describe the design and assembly of a cost-effective flow chamber, supplemented by computational fluid dynamics simulations, to generate physiological flow conditions characterized by laminar flow and appropriate shear stress levels. The protocol also incorporates fluorescence labeling for confocal microscopy, enabling the assessment of EC responses to both tissue compliance and flow conditions. By subjecting cultured ECs to multiple integrated mechanical stimuli, this model enables comprehensive investigations into how factors such as hypertension and aging may affect EC function and EC-mediated vascular diseases. The insights gained from these investigations will be instrumental in elucidating the mechanisms underlying vascular diseases and in developing effective treatment strategies.

Introduction

Endothelium, lining the inner surface of blood vessels, plays a pivotal role in maintaining vascular health. Endothelial cells (ECs) are central to regulating various cardiovascular functions, including vessel tone control, selective permeability, hemostasis, and mechanotransduction[1,2]. Research has firmly linked EC dysfunction to a primary role in atherosclerosis development. Notably, ECs encounter diverse mechanical forces at the interfaces where they interact with blood flow and underlying vessel tissues[3,4]. Several studies have associated EC dysfunction with abnormal changes in mechanical factors within the vascular environment, such as the fluid shear stress from blood flow and tissue rigidity[5,6,7].

However, prior research has received limited attention in comprehending the combined effects of tissue rigidity and shear stress on EC function. To enhance the ability to translate research outcomes into effective treatments for atherosclerosis and other cardiovascular diseases, it is essential to improve the cellular models used in the field. Significant progress has been made in humanizing cellular models by employing human ECs and subjecting them to either shear stress or substrates with varying stiffness levels[8,9,10]. However, the adoption and refinement of cellular models that integrate dynamic flow environments with EC substrates possessing adjustable stiffness properties has progressed slowly. The challenge lies in devising non-swelling EC substrates to prevent alterations in flow parameters within the flow channel while also facilitating the cultivation of intact and well-adhered EC monolayers. An *in vitro* model capable of overcoming these obstacles could facilitate more effective investigations into how hypertension, aging, and flow conditions collaboratively influence EC mechanotransduction, vascular health, and, ultimately, the development of atherosclerosis. Various methods have been developed to apply shear stress on cells while controlling substrate stiffness, including rotating plates and microfluidic devices. In the rotating plate method, cells are placed between two plates and shear stress is applied through the rotational movement of the plates. This method is less complicated and provides a quick model; however, it suffers from spatial shear stress variation, with zero shear stress at the center and maximum shear stress at the periphery[11].

On the other hand, microfluidic devices represent the new generation of tools with the ability to control substrate rigidity and flow conditions. These systems are suitable for mimicking microvasculatures under laminar flow conditions. However, studying atherosclerosis with such devices is impractical, as atherosclerosis occurs in large vessels with disturbed flow[11]. This paper aims to contribute to the critical research domain of EC studies by presenting a cost-effective system capable of examining the effects of varying stiffness levels in EC substrates under different flow conditions. The system integrates substrates with different stiffnesses to emulate pathological and physiological blood vessels. This protocol outlines the method for creating gelatin-based hydrogels with no swelling and stiffness levels of 5 kPa and 10 kPa, representing physiological and pathological stiffness, respectively. Additionally, the construction of a parallel-plate flow chamber capable of integrating these substrates is detailed. Computational fluid dynamics (CFD) was employed to evaluate shear stress and flow conditions. The preparation of hydrogels for EC culture and the execution of a 6 h flow experiment are described, followed by a discussion on post-experiment immunostaining.

Protocol

1. Synthesis of GelMA

1. Prepare a 0.2 M solution of anhydrous sodium carbonate and a 0.2 M solution of sodium bicarbonate.
2. Mix 46 mL of sodium bicarbonate solution with 15 mL of sodium carbonate solution and add 139 mL of deionized (DI) water. Adjust the pH to 9.5 using 0.1 M NaOH and HCl if necessary.

3. Add 10 g of type A, 300-bloom gelatin from porcine skin to 100 mL of carbonate-bicarbonate buffer at a concentration of 10% w/v.

4. Dissolve the gelatin using a 55 °C water bath while stirring the solution at 700 rpm using a magnetic stirrer. Once fully dissolved, adjust the pH of the gelatin solution to 9.5 using 0.1 M NaOH.

5. To initiate methacrylation, add 938 µL of methacrylic anhydride (MAH) dropwise to the solution. Improve the initial distribution of MAH in the gelatin solution by injecting it at various locations, including different depths and radial distances from the center.

6. Wrap the reaction vessel in aluminum foil to prevent exposure to light. Maintain the temperature at 55 °C and stir the solution at 500 rpm for 1 h to complete the reaction (**Figure 1A**)[12].

   NOTE: The reaction stops at a pH below 7.4.

7. Prepare one 2 L beaker containing 1.8 L of acetone and one 0.6 L beaker containing 0.3 L of acetone.

8. Add the resulting GelMA solution dropwise to the 2 L beaker while stirring at 200 RPM to induce precipitation[13]. To maximize the collection of the precipitated product, place a stainless-steel rod or spatula as a nucleation site for easier transfer.

9. Transfer the product from the larger beaker to the smaller one and allow it to sit for 10 min before proceeding to the drying step. The precipitated GelMA should appear as white fibers.

10. Collect the product on absorbent paper, dry it in a vacuum oven at room temperature (RT), and store it at -20 °C until further use.

    NOTE: Additional details on the chemical characterization of the product via proton nuclear magnetic resonance (^{1}H NMR) can be found in a previously published work[8].

2. Glass salinization

NOTE: Attaching hydrogels to glass slides provides a flat and even surface, facilitating handling and ensuring stability under flow-derived shear stress. Functionalizing the glass with 3-(trimethoxysilyl)propyl methacrylate is necessary to enhance surface properties and enable the covalent attachment of hydrogels during the polymerization process.

1. Precisely cut plain microscope slides (1 mm thickness) into pieces similar to the final hydrogel dimensions using a glass cutter. Wash the cut slides with soap to remove surface contaminants and debris that could obstruct the treatment of the glass surface.

   NOTE: If necessary, sonicate the glass slides for 5 min in ethanol, then air-dry.

2. Prepare a 0.5% solution of 3-(trimethoxysilyl)propyl methacrylate in absolute ethanol. Prepare a 10% glacial acetic acid solution in DI water. Mix the solutions to achieve a final concentration of 3% glacial acetic acid.

   NOTE: The glacial acetic acid solution can be prepared in large quantities and stored.

3. Organize the glass slides in a glass container to ensure the glass surfaces are not obstructed. Pour the resulting solution over the glass slides and keep them for approximately 5 min on a rocker at 80 rpm for the reaction to complete. Ensure both sides of the glass slides are modified by removing any trapped air bubbles.

4. After the reaction is complete, aspirate the solution and wash the glass slides with ethanol 2x. Air dry the slides and store them in the dark at RT.
   NOTE: The glass slides retain their modification for 1 month under these conditions.

3. Hydrogel preparation

1. Fabricate hydrogels using a redox-induced free radical polymerization method.
   1. Assemble two-piece Polytetrafluoroethylene (PTFE) molds with a proper depth and opening windows of 10 mm^2 to shape the final substrate. Account for a 25% shrinkage in the hydrogel's height post-polymerization during the design process. Ensure the molds are flat and are tightly fastened at the bottom and top plates to prevent leakage.
      NOTE: The designed mold dimensions are intentionally 10% larger than the intended hydrogel size. This design serves multiple purposes: it prevents damage to the sides upon mold separation, enhances surface evenness of the hydrogel by pushing impurities or bubbles to the sides, and compensates for the anticipated 25% shrinkage after equilibration due to the syneresis effect, where highly crosslinked hydrogels repel water post-equilibration, causing shrinkage. The shrinkage amount is specified in the protocol[8].
   2. To ensure hydrogels have an even surface and constant height, suspend modified glass slides from the top plate of the mold, allowing a narrow opening for injecting the polymer solution. Use a cover glass to suspend the modified glass slides (**Figure 1B**).
   3. To create the cover glass, cut plain microscopic glass 20% larger than the mold. Attach two spacers to the shorter sides. Calculate the spacer thickness as follows:
      Spacer thickness = 1.33 x final gel thickness + 1 (thickness of the modified glass) - depth of the mold
   4. Apply a thin layer of cell-compatible sealing grease to the longer sides of the cover glass, on the same face where the spacers are attached.
   5. Attach the modified glass slide to the cover glass between the two spacers.
   6. Place the cover glass on the mold so that the spacers sit on the mold and the modified glass extends into the mold. This setup provides a clearance of 1.33 of the final hydrogel's height between the modified glass and the bottom of the mold.
   7. Dissolve GelMA in DI water at 4% and 10% w/v for 5 kPa and 10 kPa hydrogels, respectively, and place it in a 45 °C water bath.
      NOTE: GelMA is a thermosensitive polymer and maintaining the solution temperature at 45 °C prevents solidification before polymerization and reduces solution viscosity, which is crucial for fabricating flat hydrogels.
   8. Add TEMED (24 mM for 5 kPA hydrogel, 6.25 mM for 10 kPa hydrogel) to the polymer solution and mix thoroughly.
      NOTE: TEMED alone does not initiate polymerization, so ensure molds, pipettes, and solutions are prepared before proceeding.

9. Add APS (24 mM for 5 kPA hydrogel, 24.5 mM for 10 kPa hydrogel) to the GelMA solution and mix thoroughly.

   NOTE: Adding APS initiates the polymerization, and hydrogels may form quickly, requiring prompt action to prevent defective hydrogels. Further details on stiffness measurements can be found in a previously published work[8].

10. Carefully pipette the resulting solution to the opening between the suspended glass slide and the mold at RT and allow it to crosslink. Capillary force drives the polymer solution into the mold. Avoid adding bubbles to the gels and stop pipetting when a small amount of solution remains in the pipette tip.

    NOTE: During polymerization, methacrylate groups of GelMA react with methacrylate residues on the modified glass slides, resulting in the chemical attachment of the hydrogel to the glass.

11. After 15 min, the reaction is complete. Detach the substrate from the mold using a sharp object, such as a needle, and slide the substrate away from the cover glass to separate it.

12. Transfer the hydrogels to 1x phosphate-buffered saline (PBS) in a 100 mm Petri dish sealed with plastic film and equilibrate at 37 °C to remove remaining reactants and byproducts.

13. As the hydrogel is slightly larger than the final dimensions, trim any excess gel at the sides to match the dimensions required for the flow chamber window.

14. Use the flow chamber to verify the hydrogel dimensions. Ensure the hydrogel fits properly in the flow chamber without gaps between the hydrogel and the mold or any defects that could affect flow patterns. If there are shape irregularities that may affect flow pattern in the flow chamber, leading to adverse cell behavior, save the hydrogel for static conditions.

15. Transfer four hydrogels to a Petri dish containing 1x PBS for sterilization.

2. Sterilize the hydrogel as described below using 70% ethanol.

   1. Prepare 25%, 50%, and 70% alcohol solutions for gradual hydrogel dehydration.

      NOTE: If gradual dehydration is not carried out, stiffer hydrogels may shrink and break, while wrinkles may form on the surface of softer hydrogels.

   2. Aspirate the 1x PBS solution from the Petri dish containing hydrogels. Add 25% ethanol solution to each Petri dish to submerge the 5 kPa and 10 kPa hydrogels for 15 min.

   3. Aspirate the 25% alcohol solution. Add 50% alcohol solution to each Petri dish to submerge the 5 kPa and 10 kPa hydrogels for 15 min.

   4. Aspirate the 50% alcohol solution. Add 70% alcohol solution to each Petri dish, submerging the 5 kPa and 10 kPa hydrogels for 5 min. Place the Petri dish on a shaker at 100 rpm.

   5. Leave the 5 kPa hydrogels submerged for 40 min and the 10 kPa hydrogels for 20 min.

      NOTE: The 5 kPa hydrogels were observed to be more prone to contamination compared to 10 kPa hydrogels.

6. Transfer the samples to a 6-well plate in a biosafety cabinet and wash the hydrogels 2x-3x using sterile PBS.

4. Coating hydrogels

1. Prepare a 60 µg/mL gelatin solution by dissolving 3 mg of gelatin in 50 mL of sterile PBS (1x with magnesium and calcium salt). Heat the solution in a water bath at 37 °C for 30 min or until all gelatin is fully dissolved.
2. Sterile filter the coating solution using a 0.2 µm syringe filter. Aspirate the 1x PBS solution from each well plate and add gelatin solution to each well, ensuring complete coverage of each hydrogel. To minimize the risk of contamination, add 40 units/mL of penicillin and 10 µg/mL of streptomycin to the coating solution.
3. Incubate each well plate in a 37 °C, 5% CO_2 incubator for 45 min. Wash the hydrogels with 1x PBS solution.
4. Aspirate PBS and add cell culture media.
5. For no longer than two days, keep the hydrogels in cell culture media and incubate them in a 37 °C, 5% CO_2 incubator until cell seeding.
NOTE: Despite fibronectin's excellent cell attachment properties, there is a concern about its use due to ECs depositing endogenous fibronectin in atherosclerotic conditions, which can lead to a proinflammatory response. To avoid chemically stimulated cell responses, we refrain from using fibronectin. Additionally, Orr et al. demonstrated that fibronectin coating, compared to collagen I coating, upregulated atherogenic genes. Specifically, they observed increased expression levels of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and Nuclear factor-κB (NF-κB)[14].

5. Seeding cells on the substrates

1. Prepare the cell suspension following available detachment protocols and count the cells using a hemocytometer[8].
2. Thoroughly aspirate the media from the hydrogels. Add 50,000 cells/cm2 to each sample. Add a proper volume of cell suspension to each sample to prevent cell overflow.
NOTE: Stiffer substrates are more hydrophobic; therefore, minimize the time between steps to improve wettability and cell suspension dispersion on the substrate surface.
3. Incubate cell-seeded hydrogels in a 37 °C, 5% CO_2 incubator for 2 h. Add cell culture media to the samples once the cells are attached to the gels.

6. Flow chamber fabrication

NOTE: The approach for designing the flow chamber is cost-effective and requires minimal expertise for fabrication and utilization.

1. Use poly(methyl methacrylate) for chamber fabrication to visually assess flow quality, hydrogel integrity under shear stress, and potential real-time biomarker studies during flow experiments. The chamber design was created using computer-aided design (CAD) software. The flow chamber has been filed for a patent, and details about this device cannot be disclosed in the present protocol.
2. Computational assessment of flow conditions in the flow chamber

1. Assemble the individual components of the designed flow chamber (.SLDPRT files) to create the final chamber setup.
2. Draw a line along the centerline of the flow tangential to the hydrogel surface to analyze shear stress. Close the inlet and outlet openings to define a closed control volume.
3. Open Flow Simulation from the Add-Ins tab in the CAD software. In the Flow Simulation tab, open the Wizard feature to enter the properties. Specify the unit system and adjust pressure and the stress units to dyne/cm^2.
4. Check **Fluid Flow** and **Gravity** in the Physical Features. Set gravity to -9.8 in Z component; X and Y components should be zero.
5. Choose **Internal** for analysis type in Geometry Handling. Select **Water** as the default fluid.
NOTE: Assume the medium behaves like water.
6. Choose **Laminar and Turbulent** for the flow type. Define the wall as an adiabatic wall and input surface roughness in Wall Condition.
7. Set the temperature to 37.5 °C (310.65 K) in Initial Conditions. Define the computational domain in Flow Simulation projects, ensuring it covers the entire control volume.
8. Select all walls interfacing with the fluid to define the fluid subdomain. In Boundary Condition, designate the inner surface of the inlet lid as Inlet Volume Flow and specify flow rate (Q) and uniform flow. Then, assign the inner surface of the outlet lid sealing as static pressure.
9. Determine the Global Mesh Size based on available computational resources. Hit **Run** to initiate the simulation.
10. Upon completion, review desired results, including shear stress plots and flow simulations.
11. Repeat steps 6.2.8 to 6.2.10 with various flow rates to calculate the applied shear stress at different rates. Use regression analysis to establish the flow rate-shear stress relationship.
12. Assess the system's tolerance for variations in hydrogel dimensions to enhance the simulation's realism.

7. Run uniform laminar flow

1. After culturing cells on the 5 kPa and 10 kPa hydrogels, ensure confluence of the cell monolayer in a 37 °C, 5% CO_2 incubator. A monolayer of cells on each hydrogel should be achieved.
2. Sterilize autoclavable components of the parallel plate flow chamber system (stainless steel screws, spatula, forceps, gasket, and the media reservoir) with a 30 min gravity autoclave cycle. Sterilize other parts (acrylic components and reservoir sealer/dampers) with UV light exposure.
3. Assemble the flow chamber device in a biosafety cabinet. Secure hydrogel samples in the device, ensuring uniformity. Use a filler of equal size to the hydrogels if an insufficient number of samples are available for flow.
4. Pre-wet the inner surface of the chamber and hydrogel surfaces to minimize bubble formation and prevent cell drying during assembly.

5. Add enough media to the inlet reservoir, allowing 20%-30% of the media to flow to the outlet reservoir upon assembly. Seal the inlet reservoir airtight using a damper/ sealer.

   NOTE: Pressure build-up in the inlet reservoir drives flow, and any air leaks alter flow rates. Check for sealer defects if bubbles form in the outer tubing.

6. Set the outlet damper to atmospheric pressure to establish the pressure difference. Place the device and reservoirs in a 37 °C, 5% CO_2 incubator. Connect the device tubing to a peristaltic pump placed outside of the incubator (**Figure 1C**).

7. Turn on the pump to start applying 3.6 dyne/cm^2. Gradually increase the flow rate by 2 mL/min increments (e.g., reach 85 mL/min from 65 mL/min after 10 min) until applying approximately 8 dyne/cm^2.

8. Further increase flow rate by 2.5 mL/min increments until reaching 12 dyne/cm^2 of shear stress.

   NOTE: Allow time for cells to adapt to the dynamic condition, as rapid flow rate increases may detach cells and damage the monolayer.

9. After 6 h, stop the flow, remove the device from the incubator, and disassemble the flow chamber. Subsequently, transfer hydrogel samples to a six-well plate.

10. Wash the samples with ice-cold PBS and either fix the cells for immunostaining or lyse them for protein isolation.

8. Immunostaining setup for confocal microscopy with high magnification

NOTE: To increase study efficiency, a method was developed for immunostaining small portions of hydrogels, enabling the examination of multiple biological targets in a single sample.

1. Prepare biopsy punches or preferred cutting tools with diameters of 3 or 4 mm.

2. Use a pipette tip box as a staining chamber. Humidify the chamber to control staining solution evaporation during extended incubation by placing a wet paper towel at the bottom of the pipette tip box.

3. Reduce antibody consumption by creating small solution pools for individual samples. Cover the tip box rack with transparent film, gently pressing the film against the rack holes with a fingertip to make dimples. Fill the dimples with 70 µL of PBS.

4. Transfer an individual hydrogel to an empty Petri dish and cut it using a biopsy punch or preferred cutting tool. Use a microscope to cut a representative sample area. Cut a full gel cylinder to avoid confocal microscopy issues.

5. Place the cut hydrogel samples into dimples created in step 8.3. Perform staining following standard protocol[15]. After the final staining wash, obtain a silicon rubber sheet that matches the hydrogel thickness.

   NOTE: Preferably, use a sheet with one sticky side for improved sealing. In this study, actin fibers were stained using a fluorescent secondary antibody conjugated to Phalloidin at a dilution of 1:20.

6. Cut the rubber sheet into 15 mm x 15 mm squares. Punch a 6 mm hole using a biopsy punch or preferred cutting tool.

7. Create a sample container by attaching the rubber to a microscopy coverslip (no. 1.5). Add 10 µL of wet mounting media to the dimples while the sample is still present and incubate it in the dark for 10-15 min.
   NOTE: For this study, the mounting media contained 0.9 µg/mL of 4',6-diamidino-2-phenylindole (DAPI) to shorten the staining protocol.
8. Remove the sample from the staining chamber and place it in the container created in step 8.7. Apply 1-2 µL of mounting media on top of the sample.
   NOTE: The quantity of mounting media impacts image quality at high magnifications. Excessive or insufficient mounting media can compromise image clarity.
9. Position another coverslip on top and gently press to ensure the sample contacts the glass. Store the samples at 4 °C in the dark until microscopy imaging.

Representative Results

Figure 1 depicts the experimental setup, outlining the process of GelMA synthesis through a methacrylation reaction. The resulting product was then used to fabricate the hydrogel substrate, onto which ECs were seeded. Subsequently, the cells were introduced into the flow chamber for a 6 h flow experiment at 12 dyne/cm^2.

^{1}H NMR spectroscopy was used to assess the success of the methacrylation reaction **(Figure 2A)**. The presence of a methyl group at 1.9 ppm and a vinylic peak between 5.4-5.6 ppm in GelMA confirmed successful methacrylation. Additionally, the decrease in the lysine peak at 3 ppm in GelMA indicates the consumption of lysine residues, which are replaced with methacrylate residues[12,13,16]. The stiffness of GelMA hydrogels was evaluated using a compression test, which showed that the compression moduli increased with GelMA concentrations **(Figure 2B)**. Hydrogels composed of 4% and 8% (w/v) GelMA were used to mimic physiological (5 kPa) and pathological (10 kPa) matrix stiffnesses, respectively[8].

The flow chamber was engineered to be cost-effective and for easy sterilization, by utilizing acrylic polymer that is UV-resistant. Its transparency facilitates real-time monitoring of hydrogels and flow conditions during experiments. Designed with three distinct layers, the chamber minimizes the risk of hydrogel damage during loading or unloading: the bottom plate provides a sturdy base, the middle layer offers lateral support for the hydrogels, and the top plate, along the gasket, creates the clearance necessary for fluid flow **(Figure 3A)**. Computational simulations were conducted using CFD to assess flow conditions and shear stress within the chamber. The following equation - shear stress = 0.0558 x flow rate - calculated the applied shear stress to the cells based on the flow rate as an input **(Figure 3B)**. Notably, changes in material properties, such as stiffness, did not alter shear stress in the simulations. To count for differences in the hydrogel size in the final experimental setup, the hydrogels were intentionally sized slightly smaller in the computational model. A 0.5 mm gap was created between one side of the hydrogels and the chamber's middle plate walls, perpendicular to the flow direction. This configuration allowed for the analysis of shear stress effects in these gaps. While irregularities in shear stress were observed at gap locations **(Figure 3B)**, their impact was confined to a small area adjacent to gaps, with the remaining hydrogel surface experiencing uniform shear stress **(Figure 3C)**. These insights suggest discarding cells from the edges of hydrogels to minimize the potential impact of turbulent regions. It is worth mentioning that higher shear stress, up to 15 dyne/cm², was experimentally applied to ECs seeded on 5 kPa and 10 kPa hydrogels with no leakage in the device (data not included). However, increasing shear stress further could potentially result in cell detachment and hydrogel failure, emphasizing the need for careful optimization of experimental conditions.

For seeding cells to form a monolayer, it is crucial to use a higher cell density than in traditional cultures. Low seeding density has been shown to hinder the monolayer formation on softer hydrogels[8]. Additionally, pre-coating hydrogels with gelatin before cell seeding enhances initial cell attachment and spreading on softer hydrogels. However, it is important to note that the beneficial effect of this coating is temporary, as it primarily facilitates the initial interaction between the cells and the substrate.

Figure 4 demonstrates how stiffness and shear stress influence the formation of actin fibers. Under shear stress, thicker stress fibers formed, suggesting a stronger attachment to the surface. In softer samples, there were more peripheral actin fibers, which are indicators of physiological conditions. However, in ECs on stiffer substrates, the presence of stronger stress fibers and fewer peripheral fibers could potentially lead to EC dysfunction[17]. This data confirms the effectiveness of the presented system in modulating EC behavior.

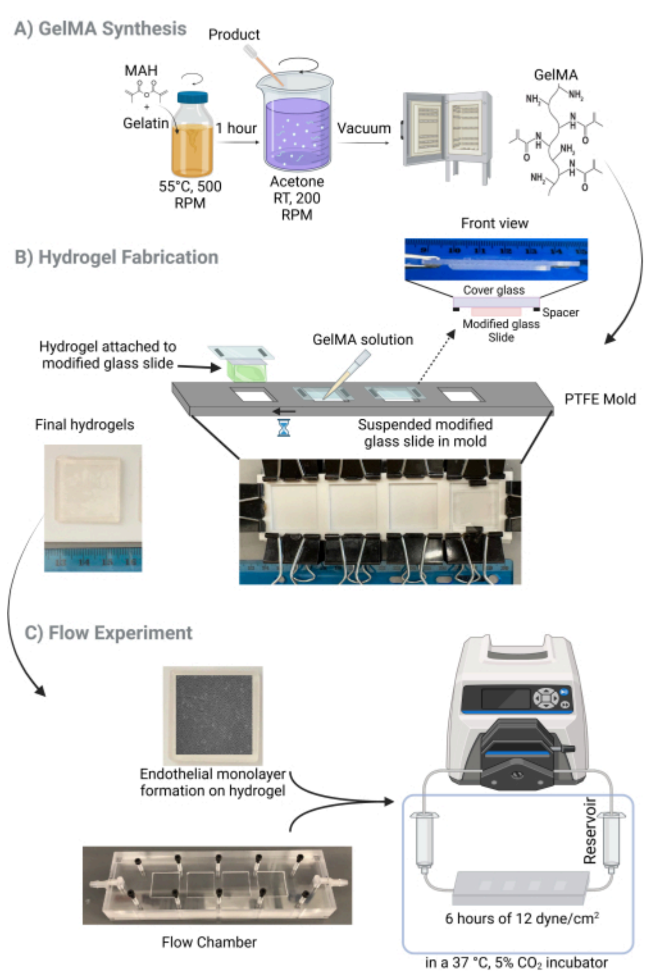

Figure 1: Overview of the current study. (**A**) GelMA synthesis. Gelatin was chemically modified to gelatin methacrylate (GelMA) through a reaction between gelatin and methacrylic anhydride (MAH) at 55 °C. The product was then precipitated in acetone and dried under a vacuum. (**B**) Hydrogel fabrication. The cover glass was prepared by attaching spacers. Then, the modified glass was attached to the cover glass. The cover glass was placed on the mold, with the spacers providing the desired clearance between the modified glass and the bottom of the mold. The GelMA solution containing initiators was added to the opening between the modified glass and the mold, polymerizing to form a hydrogel covalently bound to the modified glass. (**C**) Flow experiment. The resulting hydrogel was used to seed ECs. After forming a monolayer, the cells underwent a 6 h flow experiment at a shear stress of 12 dyne/cm². This figure was created with BioRender.com. Please click here to view a larger version of this figure.

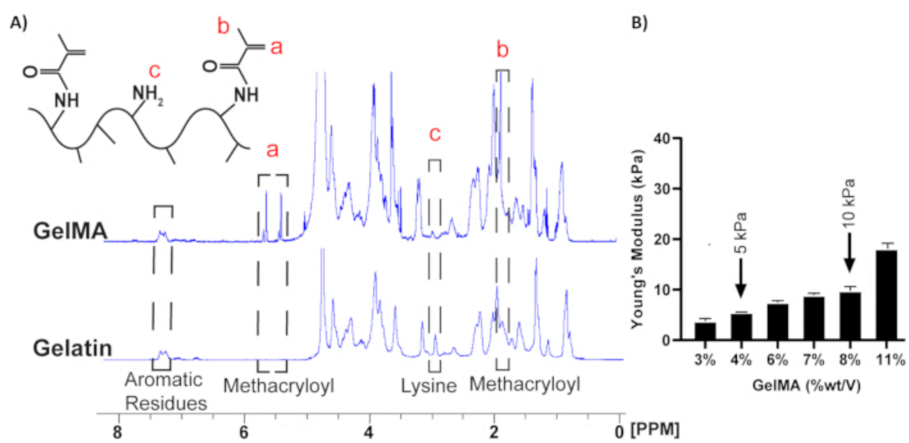

Figure 2: **^{1}H-NMR spectra for gelatin and GelMA pre-polymerization.** (**A**) Refer to dashed-lined boxes for relevant peaks. Methacryloyl peaks (i.e., vinylic and methyl groups) appeared after the chemical modification of gelatin, while the lysine group was used to quantify the degree of substitution following the chemical reaction[18]. (**B**) Young's modulus of the hydrogels was measured by a compression test, and 4% (w/v) GelMA hydrogels were considered as physiological substrates, and 10% (w/v) was considered as pathological substrate (n=4, mean ± SEM). This figure has been modified from[8]. Please click here to view a larger version of this figure.

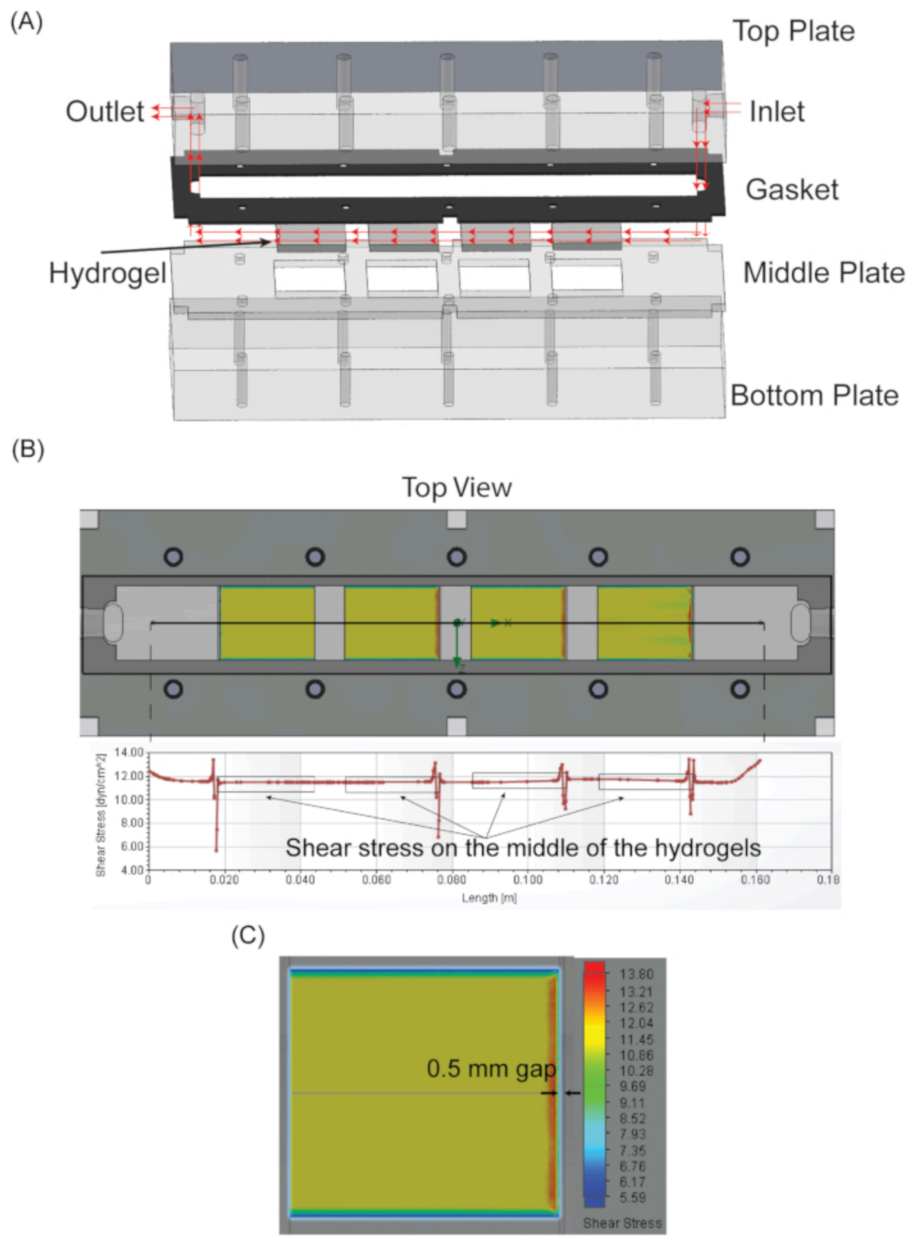

Figure 3: Parallel-plates flow chamber design and computational simulation. (**A**) Three separate plates were used to reduce the possibility of damaging the hydrogels during loading or unloading; where the bottom plate provided a backing surface, the middle surface offered lateral support for the hydrogels, and the top plate and gasket formed the clearance for the fluid to flow. (**B**) The flow chamber underwent computational simulations[11]. When the flow rate is 215 mL/min, the shear stress along the drawn line is approximately 12 dyne/cm^{2}, representing the physiological shear stress. (**C**) The influence of the 0.5 mm gap is confined to a small area adjacent to the gap. The remaining surface of the hydrogel experiences uniform shear stress. Please click here to view a larger version of this figure.

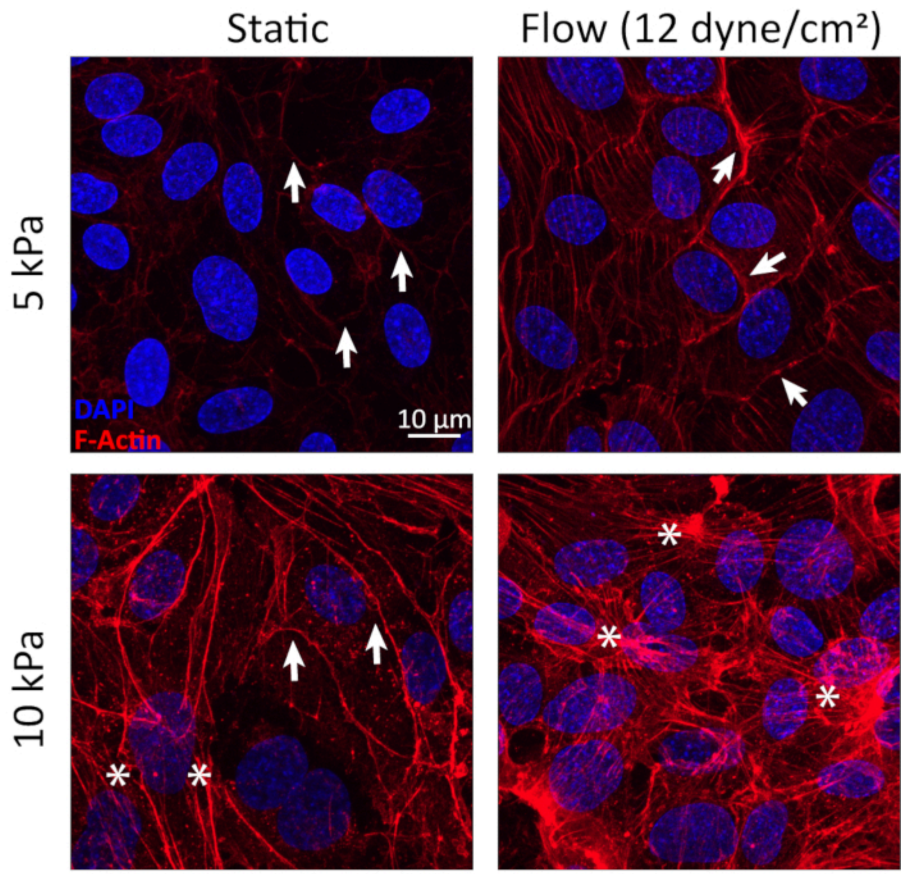

Figure 4: Shear stress and stiff hydrogel increase stress fiber formation. More peripheral actin fibers are formed in ECs on 5 kPa samples under flow. Stronger stress fibers were formed when the cells were exposed to shear stress on 10 kPa hydrogels, showcasing the model's effectiveness on EC's behavior. Arrows indicate peripheral actin, and the asterisks indicate stressed fibers. Scale bar= 10 µm (Blue: DAPI, Red: Actin Fibers). Please click here to view a larger version of this figure.

Discussion

The vascular system is a dynamic environment where various forces significantly influence cellular behavior. Studying biological events in cardiovascular diseases without considering these forces would be inaccurate. Thus, cellular models capable of emulating the vascular mechanical environment are crucial. Researchers have already made significant progress in highlighting the effect of these forces on cellular behavior[11]. However, to understand cell behavior under both pathological and physiological conditions in the human body, it is essential to develop more precise models that more closely resemble the blood vessel's environment. Therefore, we aimed to develop a system that more accurately replicates the blood vessel environment while maintaining ease of access and user-friendliness.

The model can apply controlled flow-derived shear stress to human cells on substrates with varying stiffness levels, producing conditions closer to physiological realities compared to existing models. GelMA was synthesized and utilized in this model to meet the following criteria: 1) tunable mechanical properties, 2) non-swelling behavior, 3) cell compatibility and adhesion, and 4) the capability of embedding vascular cells to model the blood vessels more accurately. The adjustability of mechanical properties was achieved by varying the biopolymer concentration[8] to mimic physiological and pathological conditions. The second criterion was the non-swelling behavior. It is crucial to have a non-swelling substrate to maintain consistent flow chamber dimensions, related flow conditions, and shear stress on the cells. GelMA with a high degree of methacrylation demonstrated non-swelling properties, preserving the hydrogel's shape and surface smoothness throughout the experiment[8]. Importantly, the concentration and stiffness did not affect the swelling behavior, which simplified the model by eliminating the need for separate adjustments for each experimental group. The third criterion was cell adhesion, as proper attachment is necessary to prevent cell detachment and preserve monolayer integrity. GelMA provided cell adhesion, thereby reducing the need for additional steps to conjugate cell-adhesive molecules to the substrate, which is essential for many biopolymers. Furthermore, GelMA's capability for cell encapsulation was considered, although it was not directly tested in this study. The cell encapsulation potential has indications for supporting 3D cell culture and integrating layers of cells, such as vascular smooth muscle cells or pericytes, to enhance the model's accuracy[19]. In addition, the synthesis of GelMA is cost-effective and requires minimal equipment, making it an excellent candidate as a biomaterial for substrate fabrication[20,21,22].

The parallel-plate flow chamber is commonly used to apply shear stress to cells, but it has traditionally only been used with glass coverslips or rigid materials. However, such materials lack physiological relevance[23]. In contrast, microfluidic devices have introduced more geometric complexity and softer substrates by utilizing polymer-based materials. However, these devices often cannot control the flow regime accurately, and their small dimensions limit their capacity to studying only a small number of cells, limiting experimental outcomes[11]. The proposed device combines the benefits of both systems by integrating endothelial cell monolayer seeded hydrogels with a flow chamber applying precisely controlled shear stress.

The device has demonstrated the capability to integrate both flow-derived and solid-derived mechanical forces. When a shear stress of 12 dyne/cm^2 was applied for 6 h, a formation of cytosolic stress fibers was observed, contrasting with the predominance of peripheral actin in the softer substrate group. This is in line with many reports showing fewer stress fibers formed when ECs are cultured on softer surfaces[24,25,26,27]. On the other hand, laminar flow could result in prominent stress fiber formation. It has been shown that cytoskeletal response to flow conditions starts within 1 h of exposure to the flow but requires a remarkably longer time to complete reorganization[28,29,30]. The peripheral actin network is essential for various EC functions, including cell-cell adhesion and barrier functionality[17]. Upregulating this network in a healthy experimental group in comparison to the pathologic group with extensive stress fibers approves the device's successful modeling of healthy and diseased conditions.

One drawback of this device is the potential for damage to the hydrogels, which could disrupt the flow and diminish the success rate of experiments. This issue primarily arises from initial defects in the hydrogels, which, under shear stress, may worsen, leading to the detachment of the sample and partial flow obstruction. Therefore, the sample preparation steps, including polymerization, equilibration, and cutting, should be conducted carefully to prevent any additional damage to the samples. Another challenge in this system is achieving and maintaining the integrity of the monolayer. While coating the hydrogels with gelatin can improve initial cell attachment, our previous work showed that this coating does not affect cell proliferation[8]. Therefore, to enhance monolayer formation, especially considering that cell proliferation is slower on softer hydrogels[31], increasing the seeding density is beneficial. Additionally, the cells may detach due to the shear stress induced by fluid flow. Hence, it is crucial to gradually increase the flow rate, allowing the cells sufficient time to adapt to the new environmental conditions.

In conclusion, the device represents a significant advancement in simulating the vascular environment more accurately due to its ability to simultaneously simulate both fluid-derived and solid-derived mechanical forces. It offers a comprehensive platform for studying EC behavior under various physiological and pathological conditions. This versatility makes it a valuable tool for advancing our understanding of vascular biology and disease progression. This model can contribute to a variety of research studies, including mechanobiology, atherosclerosis, cancer metastasis development, vascular tissue engineering and angiogenesis, and drug delivery and screening.

Disclosures

The authors declare that a provisional patent application (No. 63/634,853) has been filed with the title Flow Chamber with a Mechanically Tunable Substrate, and that no other competing interests exist.

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Materials List for

In Vitro Model Integrating Substrate Stiffness and Flow to Study Endothelial Cell Responses

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Materials

Name	Company	Catalog Number	Comments
(trimethoxysilyl)propyl methacrylate, tetramethylethylenediamine (TEMED)	Invitrogen	15524-010	Hydrogel Fabrication
3-(Trimethoxysilyl)Propyl Methacrylate	Sigma-Aldrich	440159	Glass Salinization
4’,6-diamidino-2-phenylindole (DAPI)-containing mounting media	Vector Laboratories	H-1200	Immunostaining
Acetone	Thermo Fisher Scientifics	A18-4	GelMA Synthesis
Alexa Fluor 555 Phalloidin	Cell Signaling Technology	8953S	Immunostaining
Ammonium Persulfate (APS)	Bio-Rad	1610700	Hydrogel Fabrication
Clear Scratch- and UV-Resistant Cast Acrylic Sheet (45/64")	McMaster-CARR	8560K165	Flow Chamber Fabrication
Confocal Microscope	Carl Zeiss Meditex AG	Zeiss LSM 800	Immunostaining
Covidien Monoject Rigid Pack 60 mL Syringes without Needles	Fisher	22-031-375	Flow Experiment
EC growth kit	American Type Culture Collection (ATCC)	PCS-100-041	Cell Culture
Ethanol 200 Proof	Decon Labs	2701	Glass Salinization
Gelatin Type A (300 bloom) from porcine skin	Sigma-Aldrich	G1890	GelMA Synthesis
Glacial Acetic Acid	Thermo Fisher Scientifics	9526-33	Glass Salinization
High-Purity High-Temperature Silicone Rubber Sheet	McMaster-Carr	87315K74	Flow Chamber Fabrication
Human Umbilical Vein Endothelial Cells (HUVEC)	American Type Culture Collection (ATCC)	PSC-100-010	Cell Culture
M3x30mm Machine Screws Hex Socket Round Head Screw 304 Stainless Steel Fasteners Bolts 20pcs	Uxcell	B07Q5RM2TP	Flow Chamber Fabrication

Masterflex L/S Digital Drive with Easy-Load® 3 Pump Head for Precision Tubing; 115/230 VAC	VWR	#MFLX77921-65	Flow Experiment
Masterflex L/S Precision Pump Tubing, Puri-Flex, L/S 25; 25 ft	VWR	#MFLX96419-25	Flow Experiment
Methacrylic Anhydride (MAH)	Sigma-Aldrich	276685	GelMA Synthesis
Paraformaldehyde	Thermo Fisher Scientifics	043368.9M	Cell Culture
Phosphate-Buffered Saline (PBS)	Gibco	14080-055	General
Sodium Bicarbonate	Fisher Chemical	S233-3	GelMA Synthesis
Sodium Carbonate	Fisher Chemical	S263-500	GelMA Synthesis
SOLIDWORKS educational version			
SOLIDWORKS Student Edition Desktop, 2023	SolidWorks	N/A	Flow Chamber Design
Vascular Basal Medium	American Type Culture Collection (ATCC)	PCS-100-030	Cell Culture