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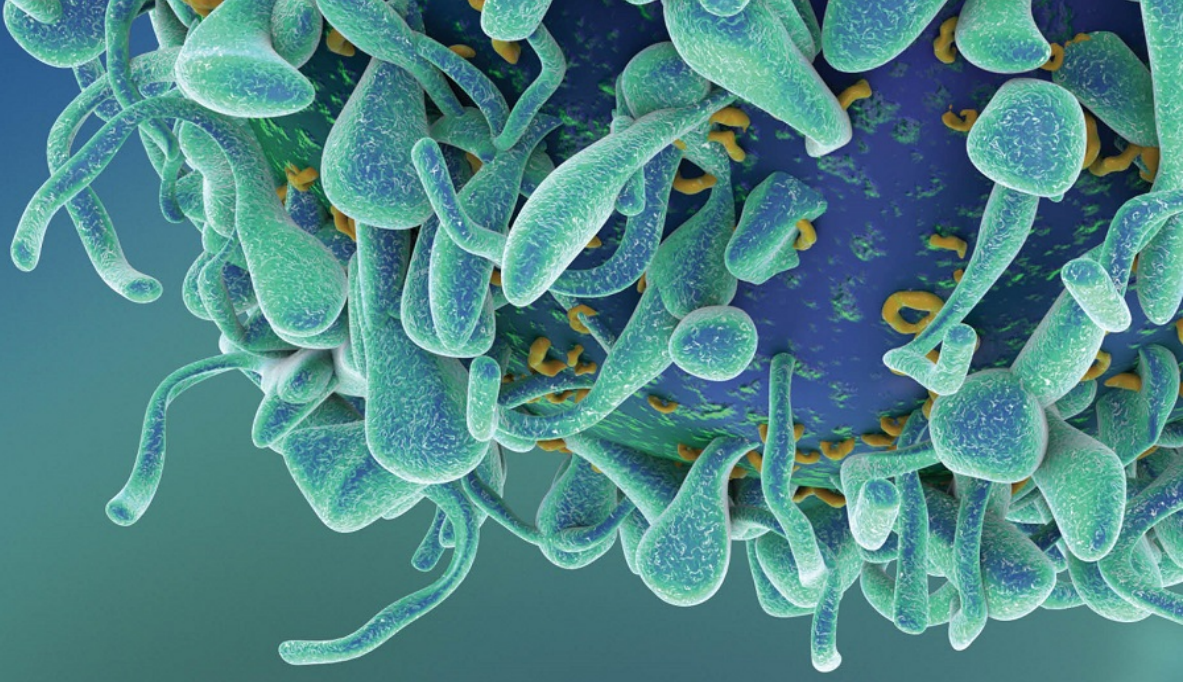

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# Preparation and Mounting of Whole Blood Clot Samples for Mechanical Testing

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Studying and quantifying the mechanics of blood clots is essential to better diagnosis and prognosis of, as well as therapy for, thromboembolic pathologies such as strokes, heart attacks, and pulmonary embolisms. Unfortunately, mechanically testing blood clots is complicated by their softness and fragility, thus making the use of classic mounting techniques, such as clamping, challenging. This is particularly true for mechanical testing under large deformation. Here, we describe protocols for creating in vitro blood clots and securely mounting these samples on mechanical test equipment. To this end, we line 3D-printed molds with a hook-and-loop fabric that, after coagulation, provides a secure interface between the sample and device mount. In summary, our molding and mounting protocols are ideal for performing large-deformation mechanical testing, with samples that can withstand substantial deformation without delaminating from the apparatus. © 2021 Wiley Periodicals LLC.

**Basic Protocol 1:** Cube-shaped blood clot preparation
**Basic Protocol 2:** Sheet-shaped blood clot preparation

Keywords: blood clot • large deformation • simple shear • uniaxial extension • material characterization • mode-I fracture • thrombus

**How to cite this article:**
Sugerman, G. P., Chokshi, A., & Rausch, M. K. (2021). Preparation and mounting of whole blood clot samples for mechanical testing. *Current Protocols*, *1*, e197. doi: 10.1002/cpz1.197

## INTRODUCTION

Blood clots play diametrical roles in our bodies. On the one hand, blood clot formation is vital to occluding severed vessels after injury and thus prevents hemorrhage (Furie & Furie, 2008). On the other hand, blood clots may inadvertently form in vital arteries such as the coronary arteries, cerebral arteries, or pulmonary arteries. Thus, blood clots not only are critical to our survival after injury but also are the source of devastating and deadly pathologies such as strokes, heart attacks, and pulmonary embolisms (Kearon, 2003).

However, mechanical testing of explanted blood clots is challenging, as in vivo samples are heterogeneous and irregularly shaped (Lee, Lee, Humphrey, & Rausch, 2015). Testing in vitro samples is therefore more convenient. However, in vitro samples are soft and susceptible to breakage even with gentle handling. Thus, mounting them on testing



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doi: 10.1002/cpz1.197

equipment remains challenging. This challenge has been overcome by others by directly coagulating blood between mounting surfaces of cone-and-plate rheometers or simple shear test apparatuses (Gersh, Nagaswami, & Weisel, 2009; Kumar, Wang, & Parekh, 2020). However, control of shape and coagulation conditions (e.g., temperature, humidity) is difficult in this configuration. Others have glued samples directly to testing apparatuses (Lee et al., 2015). Although we have not formally investigated the influence of, for example, cyanoacrylate on clot mechanics, we have noted that blood clots in contact with cyanoacrylate undergo changes in color and surface texture indicative of chemical crosslinking (unpub. observ.). Here, we describe a means of securing bovine whole blood clots to mechanical testing apparatuses that has advantages over traditional methods by protecting the sample from chemical crosslinking via cyanoacrylate and by producing regular, homogeneous samples under controlled coagulation conditions (Sugerman et al., 2021; Sugerman, Parekh, & Rausch, 2020).

We present two sample geometries for different testing modalities: the first is a cube-shaped sample designed for simple shear experiments (Basic Protocol 1), and the second is a sheet-shaped sample designed for uniaxial extension and fracture testing (Basic Protocol 2). Both geometries utilize hook-and-loop fabric to securely hold blood clot samples without the need for clamping or gluing.

***BASIC PROTOCOL 1***

## CUBE-SHAPED BLOOD CLOT PREPARATION

The objective of Basic Protocol 1 is to produce a cube-shaped blood clot sample with interfacing hook-and-loop fabric that can be attached to mechanical testing apparatuses without exposing the sample to cyanoacrylate and without directly clamping the fragile material. A schematic of this methodology is provided in Figure 1. This configuration is optimal for bulk tests under simple shear deformation (Sugerman et al., 2021).

### *Materials*

Bovine blood with CPDA-1 anticoagulant (Lampire Biological Laboratories, cat. no. 7200805)
Calcium chloride [$CaCl_2$; 10% (w/v) stock; Fisher Scientific, cat. no. AC349610250]
Cyanoacrylate gel (McMaster Carr, cat. no. 74765A25)

3D printer (e.g., Ultimaker 3 printer)
Appropriate filament (e.g., Ultimaker Tough PLA filament)
Heavy-duty packing tape (Scotch, cat. no. CBGNHW011150)
Hook-and-loop fabric (McMaster Carr, cat. no. 94985K27)

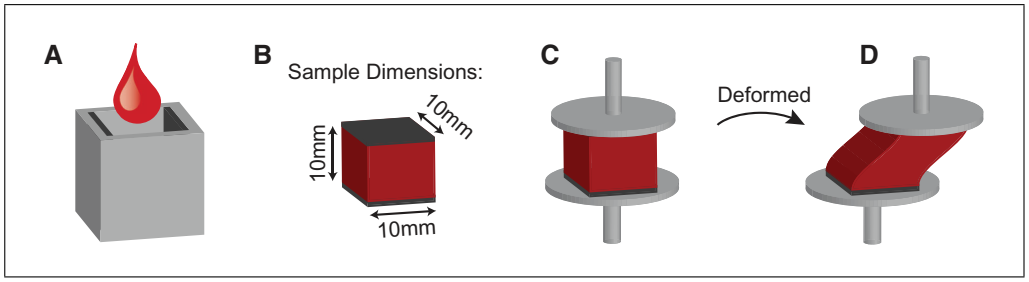


**Figure 1** Sample dimensions and test schematic. (**A and B**) Cube-shaped samples are 10 × 10 × 10 mm, with additional space in the mold for two strips of hook-and-loop fabric. Once coagulated, the blood clot must be carefully removed from its mold and (**C and D**) can subsequently be mounted to a simple shear testing configuration. Figure adapted from Sugerman et al. (2021) and reproduced with permission.

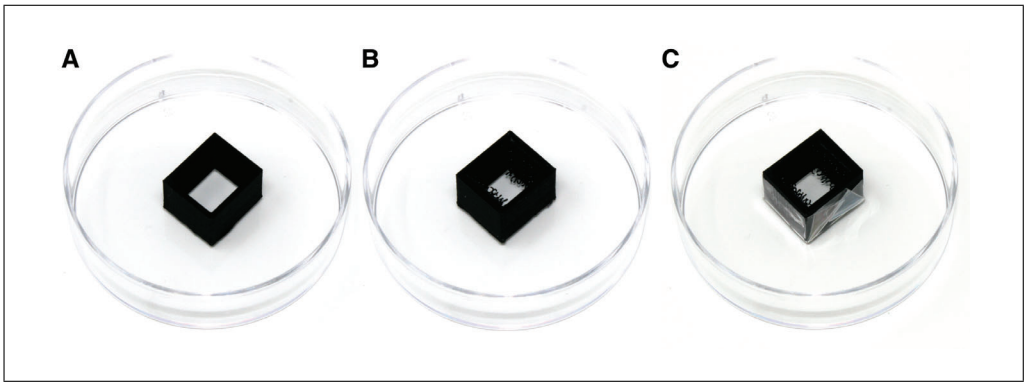


**Figure 2** Preparation of sample molds. (**A**) We used a 3D-printed open rectangular prism as the starting point for our molds. (**B**) Heavy-duty packing tape was used to cover one open side of the mold, with excess tape folding up the sides of the mold to create a reliable seal. (**C**) We fit two squares of hook-and-loop fabric into the mold to later form the interface between the sample and our testing apparatus.

37°C incubator
Thin laboratory spatula (VWR, cat. no. 470149-448)
Mechanical testing apparatus

1. Using a 3D printer and appropriate filament, fabricate a rectangular prism with internal dimensions of 10 × 10 × 12 mm with two open ends, as shown in Figure 2A (see Supporting Information, File 1).

   *We use Ultimaker Tough PLA filament for all molds. We print molds at a resolution of 100 µm and a layer thickness of 0.2 mm.*

2. Set mold on a piece of heavy-duty packing tape to cover one open end of the mold and fold edges up to seal, as shown in Figure 2B.

   *The adhesive side of the tape should face the sample. We do not find that the tape sticks to the clot.*

3. Cut two 10 × 10–mm pieces of hook-and-loop fabric.

   *Note that this is a different fabric than is used in Basic Protocol 2. We cut these shapes by hand, although a cutting machine would improve accuracy.*

4. Fit hook-and-loop fabric squares into opposite ends of the mold, with tines facing inward to later interface with the sample, as shown in Figure 2C.

5. Prepare 1.5 ml bovine blood with CPDA-1 anticoagulant with desired concentration of $CaCl_2$ to reverse the anticoagulant.

   IMPORTANT NOTE: *Work quickly from this point forward to ensure that the sample does not coagulate before placement in the mold.*

   *The blood can be stored for up to 3 days at 4°C.*

   *We use wide-mouth transfer pipets (VWR, cat. no. 414004-032) when working with blood to minimize shear forces on red blood cells.*

   *We have previously demonstrated that there is little effect on stiffness when the $CaCl_2$ concentration is varied between 10, 20, and 40 mM (Sugerman et al., 2021). We use 20 mM $CaCl_2$ as our standard concentration. Record the time at which $CaCl_2$ is introduced if controlling for coagulation time.*

6. Fill mold with blood solution until a dome forms, as shown in Figure 3A and 3B.

   *This requires 1.2 to 1.5 ml blood solution.*

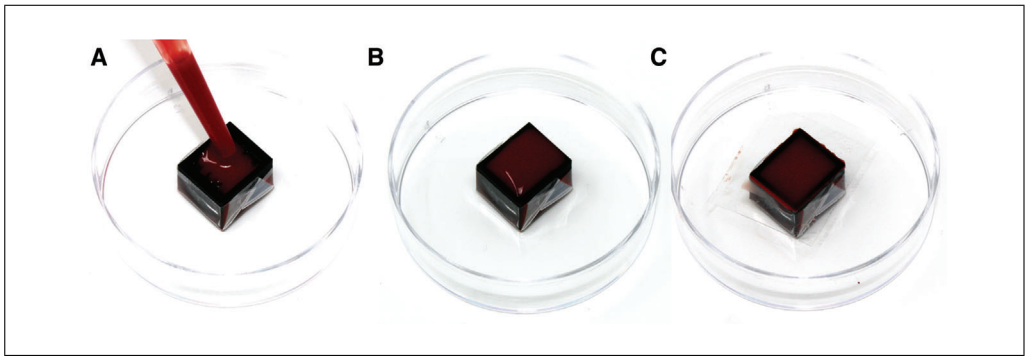


**Figure 3** Casting blood clots into molds. (**A**) We mixed blood with $CaCl_2$ using a wide-mouth pipet and transferred the solution to our molds. (**B**) We intentionally filled molds past the top of the mold to form a dome. (**C**) We placed a piece of packing tape directly on the dome of blood to expel excess fluid and form a flat surface.

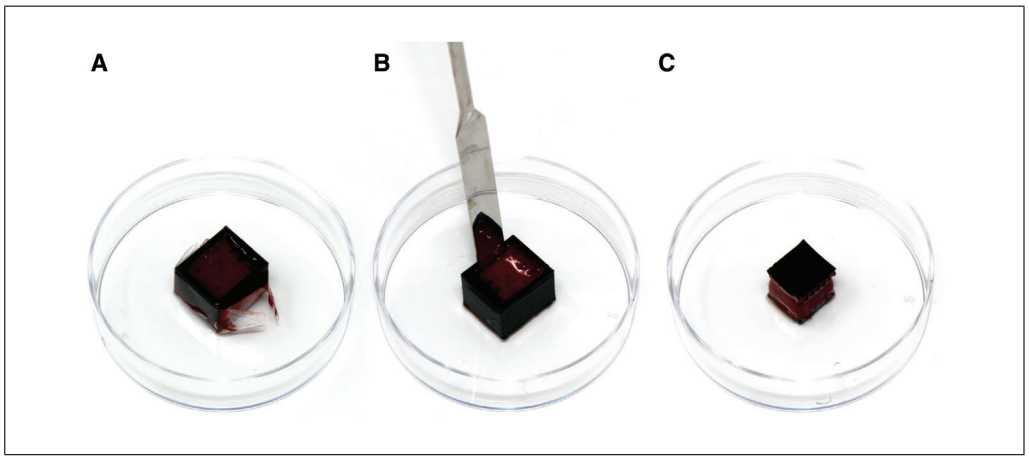


**Figure 4** Removing and mounting blood clot samples. (**A**) We first removed the top layer of packing tape to expose the sample. (**B**) We then used a laboratory spatula to separate the blood clot from the inner surfaces of the mold. (**C**) Completed blood clot samples are sturdy enough for handling and maintain a uniform shape under self-load.

7. Place a small piece of packing tape adhesive side up (i.e., away from the blood) onto dome of blood and press down gently with fingers until tape is flush against the mold, as shown in Figure 3C.

   *Excess blood will be pushed out of the mold.*

   *Placing the tape adhesive side up prevents it from sticking to the tape applied in step 2, which makes removing the sample much easier. We do not find that the tape sticks to the clot itself.*

8. Transfer mold into a 37°C incubator for the desired coagulation time.

   *We have previously demonstrated that there is little effect on stiffness when the coagulation time is varied between 60, 90, and 120 min (Sugerman et al., 2021). We use 60 min as our standard coagulation time.*

9. After coagulation, gently remove tape from the top of the sample to reveal a smooth surface, as shown in Figure 4A.

10. Loosen sides of the sample from the wall of the mold using a thin laboratory spatula, as shown in Figure 4B.

11. Gently remove remaining packing tape.

    *The thin laboratory spatula can also be used to free the tape from the sides of the mold.*

12. Push blood clot sample and interfacing hook-and-loop fabric out of the mold and rest sample on the fabric-covered sides to prevent the sample from sticking, as shown in Figure 4C.

    *The sample will be fragile, so push on the hook-and-loop fabric rather than the center of the sample.*

13. Use a small amount of cyanoacrylate gel to secure hook-and-loop fabric to the mechanical testing apparatus. Allow 1 min for glue to dry.

## SHEET-SHAPED BLOOD CLOT PREPARATION

***BASIC PROTOCOL 2***

The objective of Basic Protocol 2 is to produce a sheet-shaped blood clot sample with interfacing fabric that can be attached to mechanical testing apparatuses without exposing the sample to cyanoacrylate and without directly clamping the fragile material. A schematic of this configuration is provided in Figure 5. This methodology is optimal for uniaxial tensile tests and pure shear tests (Sugerman, Parekh, & Rausch, 2020).

***Additional Materials*** *(also see Basic Protocol 1)*

Distilled water

Dowel pins (McMaster Carr, cat. no. 97395A405)
Hook-and-loop fabric (McMaster Carr, cat. no. 94985K49)
Toothpick, cotton swab with wooden handle, or wooden dowel (VWR, cat. no. 89031-272)

1. Using a 3D printer and appropriate filament, fabricate three-part mold and platen-interfacing pieces (3D print files provided as Files 2 to 4 in Supporting Information and assembly shown in File 5 in Supporting Information).

   *We use Ultimaker Tough PLA filament for all molds. We print molds at a resolution of 100 µm and a layer thickness of 0.2 mm.*

2. Insert dowel pins into holes of the device-facing pieces (Supporting Information, File 2).

3. Cut 37.5 × 2.5–mm rectangles of hook-and-loop fabric.

   *Note that this is a different fabric than is used in Basic Protocol 1. Due to the size of these rectangles, we strongly recommend using a cutting machine to improve accuracy. We use a Cricut Maker (Cricut, cat. no. CXPL301).*

4. Use cyanoacrylate gel to attach hook-and-loop fabric rectangles to the inner surfaces of the two clot-facing pieces (Supporting Information, File 3), as pictured in Figure 6A and 6B.

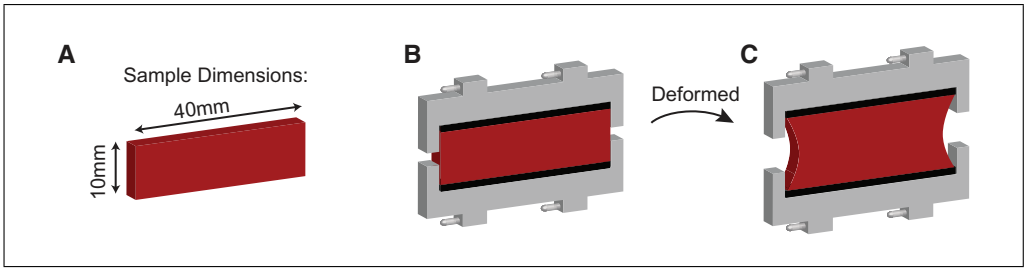


**Figure 5** Sample dimensions and test schematic. (**A**) Sheet-shaped samples are 3 × 10 × 40 mm, with additional space in the mold for two strips of hook-and-loop fabric. (**B and C**) Once coagulated, the blood clot can be extended without removing it from its mold. Figure adapted from Sugerman, Parekh, & Rausch (2020) and reproduced with permission.

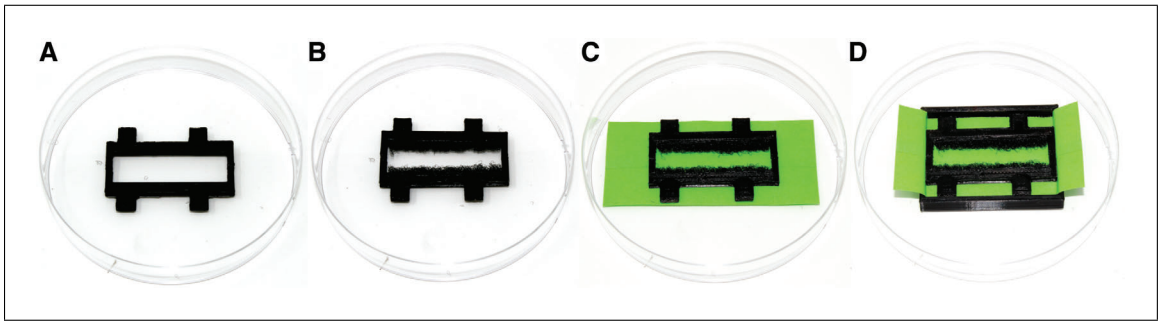


**Figure 6** Assembly of a sheet-shaped blood clot mold. (**A**) Orient two mounting pieces facing each other. (**B**) Use cyanoacrylate to attach thin strips of hook-and-loop fabric to the inner surface of the mounting pieces. (**C**) Seal the back of the mold by applying a piece of packing tape. Ensure that the tape extends past the edges of the mold. (**D**) Place the mold inside a shell to hold the pieces in place. Note: In this figure, we used green tape, as opposed to the transparent packing tape that we usually use, for visualization purposes.

5. Place both clot-facing pieces in their final orientation onto heavy-duty packing tape, adhesive side up, as shown in Figure 6C.
6. Place taped clot-facing pieces into the shell piece (Supporting Information, File 4) to hold them securely in place, as shown in Figure 6D.
7. Fill mold with distilled water to wet the hook-and-loop fabric and then discard water and shake mold to remove excess water.

   *This step is necessary to increase penetration of blood solution into the denser hook-and-loop fabric.*
8. Mix 3 ml bovine blood with CPDA-1 anticoagulant with desired concentration of $CaCl_2$.

   *The blood can be stored for up to 3 days at 4°C.*

   *We use 20 mM $CaCl_2$ and have not specifically tested the influence of changing $CaCl_2$ concentration in this sample configuration. Record the time that the $CaCl_2$ is added as the start of the coagulation time.*
9. Fill mold halfway with blood and use a toothpick, the wooden handle side of a cotton swab, or a wooden dowel to push the blood solution into each corner of the mold, as shown in Figure 7A and 7B.
10. Fill mold completely with blood and remove bubbles that rise to the surface by aspirating with a pipet, as shown in Figure 7C.

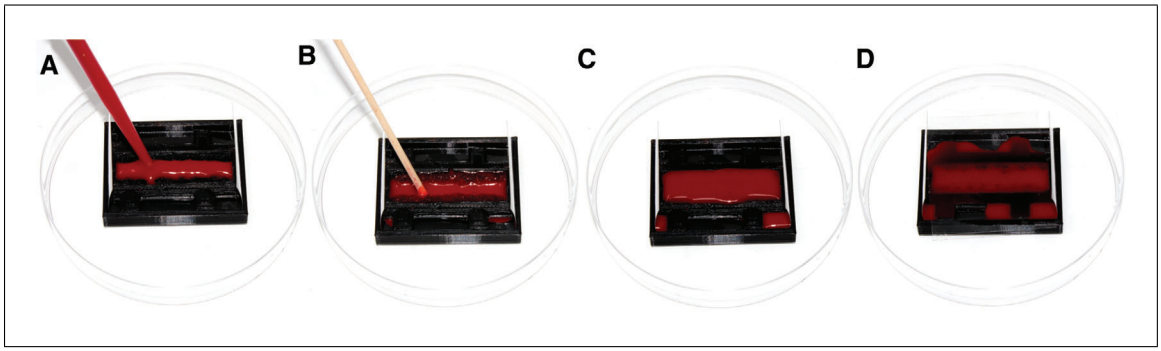


**Figure 7** Preparing a sheet-shaped blood clot sample. (**A**) Transfer blood into the center of the mold with a wide-mouth pipet. (**B**) Use a dowel or toothpick to work the blood into the hook-and-loop fabric. (**C**) Add blood to the mold until a dome forms. (**D**) Flatten the side and seal the mold by placing a piece of packing tape over the entire mold. Excess blood will be pushed out.

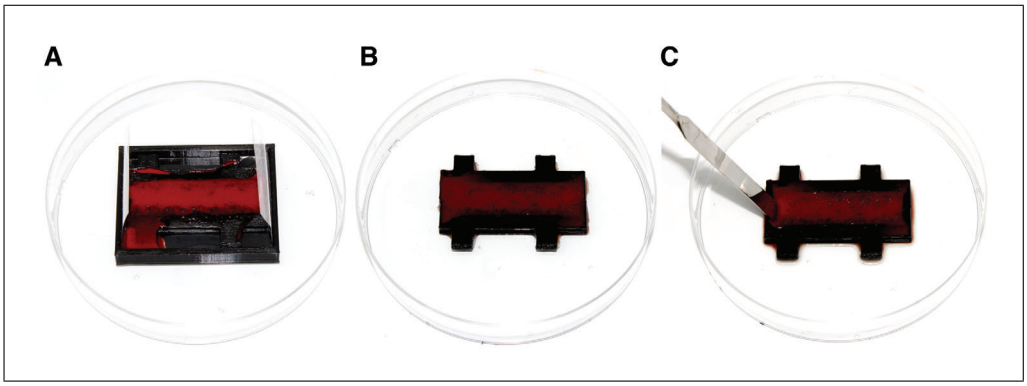


**Figure 8** Preparing a sample for mounting. (**A**) Peel the top layer of packing tape off gently to expose a smooth side. (**B**) Use the overhanging tape to remove the sample from the shell and flip it onto its exposed side and then remove the packing tape from the back of the mold. (**C**) Separate the sides of the sample from the mold using a thin laboratory spatula.

11. Trim and place a piece of packing tape adhesive side down over exposed surface to seal the mold, as shown in Figure 7D.

    *Excess blood will be pushed out.*

12. Place entire mold into a 37°C incubator for the desired coagulation time.

    *We use a 60-min coagulation time and have not specifically tested the influence of varying coagulation time with this sample configuration.*

13. After the coagulation time has elapsed, remove sample from the incubator and use tape to gently lift the sample and clot-facing pieces from the shell, as shown in Figure 8A.

14. Gently remove tape from both sides of the mold to expose the sample, as shown in Figure 8B.

15. Use a thin laboratory spatula to loosen sides of the blood clot from the mold, as shown in Figure 8C.

16. Attach device-facing pieces to platens on the mechanical testing apparatus using cyanoacrylate gel.

17. Mount sample by sliding pins into the pinholes.

## COMMENTARY

### Background Information

Previous investigations of blood clot mechanics have relied on cyanoacrylate or have directly coagulated samples within the mechanical testing apparatus (Gersh et al., 2009, Kumar et al., 2020, Lee et al., 2015). However, using chemical adhesives such as cyanoacrylate necessarily changes the composition of the blood clot and can lead to changes in its mechanics. Although direct coagulation avoids this problem, temperature and moisture control during the direct coagulation process is usually logistically challenging if not impossible. Here, we suggest two alternative protocols (Basic Protocols 1 and 2) that avoid these potential pitfalls.

### Critical Parameters

#### *Hydration and temperature during testing*

The hydration and temperature of the sample can impact the mechanical behavior of whole blood clots by altering stress-relaxation time constants. For example, submerging the sample in a heated 37°C phosphate-buffered saline (PBS) bath during testing decreased the first of two stress-relaxation time constants relative to dry and room-temperature PBS conditions (Sugerman, Parekh, & Rausch 2020). Additionally, sample quality decreases substantially when samples were not fully sealed during incubation time, presumably

due to loss of moisture. For the purposes of our mechanical experiments, we generally exclude such samples.

***Calcium chloride concentration and incubation time***

As mentioned briefly above, we use 20 mM $CaCl_2$ to reverse our anticoagulant and incubate our samples for 60 min. We found no significant effect of varying either the concentration of $CaCl_2$ or coagulation time on sample stiffness. Note that we have observed that coagulation times <60 min paired with $CaCl_2$ concentrations <20 mM led to samples that were not fully coagulated.

***Blood age and species***

Generally, the age of stored blood effects the viability of biological components. For our experiments, we have established that red blood cell concentrations are stable across the first 3 days of storage (Sugerman et al., 2021). Thus, we use blood within this 3-day window to maximize physiological relevance. Anecdotally, we find that blood loses coagulability entirely after ∼4 weeks of storage time. Note that we conducted all of above experiments using bovine blood with CPDA-1 anticoagulant. Using alternative species or anticoagulants may change the effects of storage time and all other conditions.

## Troubleshooting

***Drying***

If samples are not fully enclosed with packing tape, moisture loss will lead to shrinkage and cracking. This can occur when tape is not pressed to seal around the edges of the mold or when molds are printed such that they are not watertight. In the case of molds themselves leaking, we recommend printing at higher resolution. Lower-quality packing tape than the type listed above may also prevent adequate sealing.

***Incomplete filling***

In some sheet-shaped samples, patches of hook-and-loop fabric remain visible after the sample has coagulated. In this case, it is important to include steps 7 and 9 of Basic Protocol 2, which describe pre-wetting the hook-and-loop fabric and pushing blood into the hook-and-loop fabric, respectively. Pre-wetting the hook-and-loop fabric decreases surface tension and allows blood to penetrate the loops more easily. Pushing blood into the corners of the mold using a thin wooden dowel or toothpick helps ensure that the entire mold is filled and no bare hook-and-loop fabric remains.

***Tearing***

Premature tearing is often a result of defects introduced into the sample during unmolding. For this reason, we caution against rushing through this procedure. We suggest using a thin spatula to coax cube-shaped samples (Basic Protocol 1) out of their molds after removing the packing tape. For sheet-shaped samples (Basic Protocol 2), the packing tape used to cover the back of the mold should also be used to lift the sample out of the shell piece. Rather than picking up sheet-shaped samples directly, we slide them to the edge of the bench to avoid disturbing the sample. If tearing persists, it may be worthwhile to explore alternative hook-and-loop fabric options.

## Understanding Results

These protocols enable large-deformation mechanical testing of blood clots. The results of these tests will be dependent on each user's specific test protocol. For our test protocols, we found highly nonlinear behavior, substantial hysteresis, strain-stiffening, and a negative Poynting effect when cubed-shaped samples (Basic Protocol 1) were tested to 50% simple shear strain (Sugerman et al., 2021). When using sheet-shaped samples (Basic Protocol 2) for uniaxial extension, cyclic loading, and stress-relaxation tests, we found that blood clots demonstrate a Mullins-like effect, nonlinear stress-relaxation, permanent set, and strain-rate dependence (Sugerman, Parekh, & Rausch, 2021).

## Time Considerations

3D printing of molds may take up to multiple hours, with the time highly dependent on the printer and print settings used. Using our machine, printing molds for cube-shaped samples (Basic Protocol 1) takes 12 min with 20% infill and 0.2-mm layer thickness. Printing molds and interfacing pieces for sheet-shaped samples (Basic Protocol 2) takes 3 hr and 23 min with 20% infill and 0.2-mm layer thickness.

Preparing solutions and molds takes 3 to 5 min per sample, depending on the cutting method used. When cutting hook-and-loop fabric by hand, this time will be close to 5 min. There is some initial time investment required when using a cutting machine, but after setting the cutting parameters, the time per sample is greatly reduced.

The minimum coagulation time we have systematically tested is 60 min, and we have found negligible influence of extending coagulation time. As such, we recommend using a 60-min coagulation time. This time is measured from the addition of $CaCl_2$ to the start of testing, so molds will need to be removed from the incubator prior to the end of this period. We remove samples from the incubator 7 min before the test begins, though we recommend allowing more time for mounting while initially becoming accustomed to the procedure. When mounting cube-shaped samples (Basic Protocol 1), we allow 60 s for the cyanoacrylate to dry.

Disassembling and cleaning molds takes 1 to 2 min per sample. Molds can be reused until damage is visible.


## Acknowledgments

The authors appreciate funding from the National Science Foundation through CAREER grant #2046148 and grant #2105175.


## Author Contributions

**Gabriella P. Sugerman:** Conceptualization, Data curation, Methodology. **Armaan Chokshi:** Methodology, Writing-original draft. **Manuel K. Rausch:** Conceptualization, Methodology, Writing-original draft.

## Conflict of Interest

None of the authors has conflicts of interest to declare.

## Data Availability Statement

No data resulted from this work.

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