Appendix B

DETAILED EXPERIMENTAL PROTOCOLS

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B.1 Expression of artificial proteins

Overview: This protocol describes the expression of artificial proteins that are encoded by genes on a pQE-80L plasmid harbored within the BL21 strain of *E. coli*. This includes ERE, EPE, EAE, and their variants.

Materials:

LB (Luria-Bertani) liquid medium supplemented with 100 μg mL^{-1} ampicillin

Per liter:

-10 g tryptone/casein hydrolysate (Affymetrix 12855**)**

-5 g yeast extract (Affymetrix 23547**)**

-10 g NaCl

LB/ampicillin agar plate supplemented with 100 μg mL^{-1} ampicillin

-LB liquid medium

-15 g Bacto agar (BD 214010)

2xYT liquid medium supplemented with 100 μg mL^{-1} ampicillin

Per liter:

-16 g tryptone

-10 g yeast extract

-5 g NaCl

Terrific broth supplemented with 100 μg mL^{-1} ampicillin

Per liter:

-12 g tryptone

-24 g yeast extract

-100 mL 10x buffering salts (2.31 g KH_2PO_4, 16.4 g $K2HPO_4 \cdot 3H_2O$) (Note: autoclaved separately from medium)

-8 mL 50% (v/v) glycerol (Note: autoclaved separately for convenience)

IPTG (Biopioneer, C0012-100): 1000x stock at 1 M stock in ddH_2O, sterilized by 0.2 micron filter

Ampicillin (BioPioneer C0029): 1000x stock at 100 mg mL^{-1} in ddH_2O, sterilized by 0.2 micron filter

15 mL culture tube

125 mL Erlenmeyer flask

2.8 L Fernbach baffled shake flask

Lysis buffer: 10 mM Tris, 1 mM EDTA, 100 mM NaCl, 5% (v/v) glycerol, 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate, pH 8

Per liter:

-1.2 g Tris base (Sigma-Aldrich T1503)

-2 mL 0.5 M EDTA (Ethylenediaminetetraacetic acid) (Sigma-Aldrich ED2SS)

-5.8 g NaCl

-5 mL glycerol

-1 mL Triton X-100 (Sigma-Aldrich X-100)

-1 g sodium deoxycholate (Sigma-Aldrich D6750)

(Note: The additives glycerol, Triton X-100, and sodium deoxycholate were included to improve the protein stability, but are not strictly required.)

Equipment:

37 °C incubator: VWR Model 1525 (Spalding 332A)

37 °C shaking incubator(s): VWR Signature benchtop (Spalding 332A) and Thermo Forma orbital (Spalding 312)

Beckman-Coulter Avati J-25 centrifuge with JA-10 rotor (Spalding 332A)

Nalgene 500 mL centrifuge bottles (Thermo Scientific 3141-0500)

Day 1: Streak an LB/ampicillin agar plate with an inoculation loop containing cells from the appropriate glycerol stock in order to obtain single colonies. Incubate the plate overnight (12-18 hr) at 37 °C.

Day 2: Inoculate 3 mL LB/ampillin with a single colony from plate. Incubate the culture at 37 °C, 250 rpm for 8-10 hr.

Prepare an overnight starter culture by inoculating 25 mL 2xYT/ampicillin with 0.25 mL of growing culture in an autoclaved Erlenmeyer flask. Incubate at 37 °C, 250 rpm for 10-12 hr.

Prepare the Terrific broth by adding 12 g tryptone and 24 g yeast extract to 900 mL ddH_2O in the Fernbach baffled shake flask. Autoclave to sterilize.

Day 3: Add 8 mL sterile 50% (v/v) glycerol and 100 mL sterilized 10x buffering salts to the Terrific broth. Supplement with 1 mL 1000x ampicillin stock. Save several mL of the culture to use as a blank for optical density measurements.

Inoculate the Terrific broth with 20 mL (1:50) of the overnight starter culture. Grow the culture at 37 °C, 160 rpm (in Thermo Forma shaker) until the OD_{600} reaches approximately 0.8-1.0. This typically takes 2-2.5 hr.

Induce protein expression by the addition of 1 mL of 1000x IPTG solution (final concentration 1 mM).

After 4-5 hr, harvest the *E. coli* by centrifugation at 6,000 *g*, 4 °C for 8 min.

Weigh the cell pellets to determine the wet cell mass and resuspend in 25 mL of lysis buffer. Freeze at -20 °C.

Expected results: The final OD_{600} after 4-5 hr of expression varies from 4-8. It is typically higher during the expression of EPE and its variants than for ERE. The typical wet cell mass likewise varies from 7 g per L of culture to 15 g per L of culture.

B.2 Inverse thermal cycling purification of artificial proteins with elastin-like polypeptide domains

Overview: This protocol describes the purification of artificial proteins from *E. coli* lysate by inverse temperature cycling above and below the lower crucial solution temperature of the elastin-like domain. Three cycles are typically sufficient to obtain pure protein. This protocol also describes the reduction and desalting of proteins to obtain monomeric artificial proteins with high free thiol content.

Materials:

Phenylmethylsulfonyl fluoride, PMSF (Gold Biotechnology P-470-10)

DNase I (Sigma-Aldrich DN25)

RNase A (Sigma-Aldrich R4875)

$MgCl_2$

β-mercaptoethanol (Sigma-Aldrich M6250)

NaCl

TEN buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8)

Tris(hydroxypropyl)phospine (THP) (Santa Cruz Biotechonolgy, sc-204915)

Zeba™ 7K MWCO spin desalting columns, 10 mL (Thermo Fisher Scientific 89894)

50 mL conical tubes (Corning 430829 or BD Falcon 352098)

water, LCMS Chromasolv® (Sigma-Aldrich/Fluka 39253)

liquid nitrogen (Spalding 312)

Equipment:

QSonica probe sonicator (Spalding 316)

Beckman-Coulter Avati J-25 centrifuge with JA-17 or JA-25.50 rotor (Spalding 332A)

50 mL Nalgene Oakridge centrifuge tubes (Thermo Scientific 3139-0050)

Beckman-Coulter Allegra 25R centrifuge with TS 5.1-500 spinning bucket rotor (Spalding 332A)

Labconco Freezone 4.5 L lyophillzer (Spalding 312)

Day 1: Thaw the resuspended cell pellets in a cold water bath. Add crystalline PMSF to approximately 1 mM.

(Note: PMSF is poorly soluble in aqueous solutions.)

To the thawed cell solution, add 10 μg mL^{-1} DNase I, 10 μg mL^{-1} RNase A, and 5 mM $MgCl_2$. Incubate at 37 °C, 250 rpm for 30 min. (Note: I typically do not weigh PMSF or nucleases. Instead I just add a small spatula tip worth of solid into thawing lysate.)

Sonicate the cell solution. For 25 mL in a 50 mL conical tube, typical sonication conditions are 5 min processing time with 2 sec "on" pulse at 30% power amplitude, and 2 second "off" rest between pulses. For larger volumes (i.e. pooled cells from several liters of culture), increase the processing time.

Add 1% (v/v) β-mercaptoethanol and incubate on ice for 1-2 hr.

(Note: βME is crucial for telechelic artificial proteins with cysteine residues at the termini. Omitting βME or using too little results in chain extension through disulfide formation. For EPE and variants that form physical cross-links, this can cause the pelleted protein fractions in subsequent thermal cycling steps to form physical gels that are difficult to resuspend. Add βME in a fume hood and in subsequent steps only open tubes in the hood.)

Centrifuge at 39,000 *g*, 4 °C for 1 hr. (Note: This is the maximum speed of the JA-17 rotor. The JA-25.50 can spin at higher speeds.) Carefully decant the supernatant into a new centrifuge tube and discard the pellet. The supernatant should be tan and mostly clear.

(Note: When purifying a new artificial protein or when it is necessary to assess the purification by SDS-PAGE, save all fractions. The protein in the pelleted fractions can be extracted with 8 M urea, typically using the same volume as the supernatant that was removed.)

Add crystalline NaCl to a final concertation of 2 M. Incubate at 37 °C, 250 rpm for 1 hr. The solution should become cloudy.

Meanwhile, spin the rotor at 39,000 *g*, 37 °C for 1 hr without any tubes. This allows the centrifuge and rotor to warm up more quickly. The centrifuge does not have a method of heating the rotor, only refrigerating the system. Therefore, it relies of heat or friction generated during a spin to warm above room temperature.

Centrifuge at 39,000 *g*, 37 °C for 1 hr. Discard the supernatant and resuspend the pellet in TEN buffer at a concentration of 100 mg mL^{-1}. Break up the pellet using a spatula. Add 1% (v/v) βME and incubate overnight on the tube rotator in the cold room.

Day 2: Centrifuge at 39,000 *g*, 4 °C for 30 min. Carefully decant the supernatant into a new centrifuge tube and discard the pellet.

Add crystalline NaCl to a final concertation of 2 M. Incubate at 37 °C, 250 rpm for 30 min-1 hr while warming the centrifuge and rotor to 37 °C. The solution should become cloudy again and the proteins may begin to flocculate.

Centrifuge at 39,000 *g*, 37 °C for 1 hr. Discard the supernatant and resuspend the pellet in TEN buffer at a concentration of 100 mg mL^{-1}. If properly reduced, the pelleted fraction will be runny and will resuspend easily. If not properly reduced, the pelleted fraction will be rubbery and difficult to resuspend. Add 1% (v/v) βME and incubate overnight on the tube rotator in the cold room.

Day 3: Centrifuge at 39,000 *g*, 4 °C for 30 min. Carefully decant the supernatant into a new centrifuge tube and discard the pellet.

Add crystalline NaCl to a final concertation of 2 M. Incubate at 37 °C, 250 rpm for 30 min-1 hr while warming the centrifuge and rotor to 37 °C. The solution should become cloudy again and the proteins should begin to flocculate.

Centrifuge at 39,000 *g*, 37 °C for 1 hr. Discard the supernatant and resuspend the pellet in TEN buffer at a concentration of 100 mg mL^{-1}. If this is the final cycle, do not add βME. It is best to round to the nearest multiple of 4 mL at this step.

Once the pelleted fraction is well resuspended (typically 30 min-2 hr), add THP to a final concentration of 5 mM and reduce for 1-3 hr at 4 °C.

To check the reduction, take a 2 μL sample and dilute 1:10 in water. Dilute this 1:10 in SDS loading buffer. As a control, prepare a second sample in the same way but add βME to a final concentration of 5% (v/v). Boil only the control sample containing βME for 2-3 min. Load 5 μL of each solution on a 10-well gel. Run for 45 minutes at 180 V. Stain with InstantBlue to determine whether the protein is monomeric (vs. dimeric or greater) and linear (vs. cyclic, which will have a slightly lower apparent molecular weight). More details are given in B.6.

(Note: Fresh 1x MES/SDS running buffer is critical. Do not reuse buffer. Either a degradation product from the buffer or metals leaching from the electrode cause rapid oxidation of the proteins and can produce false negatives.)

Desalt the protein to remove the TEN buffer and THP according to the Zeba desalting column manufacturers protocol. Remove the storage buffer by centrifugation at 1000 *g*, 4 °C for 2 min. Equilibrate three times with degassed 5 mL LCMS grade water. The first two equilibration steps are done at 1000 *g*, 4 °C for 2 min. The final equilibration should be done at 1000 *g*, 4 °C for 6 min. Apply 4 mL of the protein solution each column and centrifuge 1000 *g*, 4 °C for 4 min, eluting into 50 mL conical tubes.

(Note: LCMS grade water is likely not strictly required, but was used due to potential concerns over the consistency of water from the still. This was likely not an issue.)

Combine the elution from each column into a tared conical tube, freeze in liquid nitrogen, and lyophilize for 3-4 days or until all water is removed.

Expected results: Typical yields vary from 80 mg per L of culture for ERE to >300 mg per L of culture for EPE. The proteins are quite pure, but occasionally have small amounts of lower molecular weight impurities (estimated at <6%). Lower molecular weight impurities are most common in ERE and EAE protein preparations.

B.3 Ellman's assay and non-reducing SDS-PAGE

Overview: This protocol describes the measurement of the free thiol content in purified artificial proteins containing cysteine residues and SDS-PAGE under non-reducing conditions to determine the fraction of protein in the monomeric state.

Materials:

5,5'-dithiobis-(2-nitrobenzoic acid), Ellman's reagent (Sigma D8130)

Reaction buffer:

100 mM sodium phosphate

1 mM EDTA

pH to 8.0

Disposable cuvettes

2x SDS sample loading buffer

β-mercaptoethanol (Sigma-Aldrich M6250)

10- or 15-well Novex NuPAGE Bis-Tris 4-12% SDS PAGE gels (Thermo Fisher NP0322)

20x MES/SDS Running buffer (Boston Bioproducts BP-177)

InstantBlue protein stain (Expedion ISBL1)

Equipment

Cary50 UV/Vis spectrophotometer (Spalding 312)

Heating block set to 95 °C (Spalding 332A)

Gel electrophoresis cell and power source (Spalding 332A)

Typhoon Trio (Spalding 312)

Protocol

Prepare 5 mg mL^{-1} Ellman's reagent in reaction buffer. Reagent must be prepared fresh.

(Note: Weigh Ellman's reagent and protein with a Teflon-coated spatula and forceps, respectively. Metal surfaces could oxidize thiols.)

Pipette 2.5 mL reaction buffer into a cuvette.

Dissolve protein in reaction buffer at a concentration of 5 mg mL^{-1}. (Note: I typically weigh a minimum of 2 mg per sample.) Vortex or sonicate briefly as needed.

Add 50 µL Ellman's reagent solution and 250 µL protein solution to the cuvette containing the reaction buffer. Pipette up and down several times to mix. The solution should turn yellow instantly. At the same time, prepare a blank containing 50 µL of Ellman's reagent solution and 250 µL of reaction buffer without protein. Incubate all samples on benchtop for 15 min. Turn spectrophotometer on.

(Note: The concentration of protein has been optimized in this protocol to detect two thiols per protein. The concentration may require adjustment for proteins with additional thiols.)

Meanwhile, prepare samples for non-reducing SDS-PAGE from the protein solutions prepared above. Add 2 µL protein solution to a microcentrifuge tube containing 10 µL 2x SDS sample loading buffer and 8 µL water. Prepare a second sample in the same way, but also add 1 µL βME as a positive control for reduced protein. Boil only the control sample for 2-5 min on heating block.

Load 5 µL of each sample in a 10-well gel (or 2.5 µL in a 15-well gel). Load 5 µL of the SeeBlue protein marker. Run for 35-45 min at 180V with MES running buffer.

(Note: Fresh 1x MES running buffer is critical. Do not reuse buffer. Either a degradation product from the buffer or metals leaching from the electrode cause rapid oxidation of the proteins and can produce false negatives.)

While gel is running, measure the absorbance of the protein solution with Ellman's reagent. Set the measurement wavelength to 412 nm. From the absorbance values, determine the concentration of free thiols using the extinction coefficient of 2-nitro-5-thiobenzoate, which is 14,150 M^{-1} cm^{-1} under the conditions of this assay. It is assumed that each free thiol reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) to produce one molecule of 2-nitro-5-thiobenzoate.

(Note: A standard curve can be constructed with known concentrations of cysteine reacted with Ellman's reagent. This is useful for determining the linear region of the assay, but somewhat tedious as the standards must be prepared fresh each time.)

When the gel has finished running, rinse with water and stain with InstantBlue protein stain for 15-60 min. Destain with water.

Image the gel on the Typhoon with the red laser, no filter and a PMT of 500 V. If desired, quantify the density of each band using the ImageQuant software.

Expected results: After optimization of the purification protocol, artificial proteins can typically be prepared with 85-95% free thiol content relative to the expected free thiol content. Otherwise the discrepancy between the observed and expected thiol content can arise due to disulfide formation in dimers, trimers, etc. and intramolecular cyclization reactions.

By non-reducing SDS PAGE, the proteins are primarily monomeric. A small amount of dimeric and trimeric protein run are present. A band corresponding to the cyclized monomer occurs at a slightly lower apparent molecular weight than the predominate linear monomer protein band.

B.4 End-linking artificial proteins with 4-arm PEG vinyl sulfone

Overview: This protocol describes the procedure used to form covalent networks by end-linking artificial proteins that contain terminal cysteine residues with 4-arm PEG vinyl sulfone. The same procedure can be applied to end-linking with 4-arm PEG maleimide and 4-arm PEG acrylate, although these reactions occur much faster and slower, respectively, than the reaction with 4-arm PEG vinyl sulfone.

Materials:

Purified artificial protein with high free thiol content (see Protocols 1-3)

4-arm PEG vinyl sulfone (Jenkem USA 4ARM-VS), maleimide (4ARM-MAL), or acrylate (4ARM-ACLT)

Cross-linking buffer:

100 mM sodium phosphate

400 mM triethanolamine (Sigma 90279)

6 M guanidinium chloride (Sigma G4505)

Adjust pH to approximately 7.4 using 6 N HCl.

(Note: Triethanolamine should be omitted or reduced to 4 mM for cross-linking with 4-arm PEG maleimide.)

Sigmacote® siliconizing fluid (Sigma SL2)

Glass slides 75 x 50 mm

Silicone rubber, 1 mm thick (McMaster-Carr 3788T22)

95% ethanol

Binder clips

Protocol

In advance, prepare clean glass slides by treating with 0.5-1 mL SigmaCote® for 1 min. Remove excess liquid, rinse with water, and allow to dry. Clean with 95% ethanol and Kimwipe. Test hydrophobicity by pipetting a droplet of water onto the slide. It should not spread. Cut a rectangle slightly smaller than the slide from the rubber sheet, and then cut a

smaller rectangle out of the middle of this piece to obtain a frame that will serve as a 1 mm spacer between two slides.

(Note: Treated glass slides can be reused for many gels. Clean with 95% ethanol and test hydrophobicity.)

Degas 0.1 M sodium phosphate/6 M guanidinium chloride in a 25 mL side arm flask by pulling under vacuum while sonicating. Repeat with ddH_2O.

(Note: This step is optional and does not appear to be vital. Because dissolving the protein and mixing the gelation reaction requires vortexing, oxygen is likely to be reintroduced into the solution.)

Add triethanolamine to the degassed 0.1 M sodium phosphate/6 M guanidinium chloride to a final concentration of 400 mM. Do the same with the degassed ddH_2O. Adjust the pH of each solution to the desired value, typically 7.2-7.4, with 6 N HCl.

Dissolve protein solution (50 to 250 mg per 1 mL) in cross-linking buffer (0.1 M sodium phosphate/6 M guanidinium chloride/0.4 M triethanolamine). Dissolve the 4-arm PEG vinyl sulfone (50 to 250 mg per 1 mL) in 0.4 M triethanolamine.

(Note: Weigh protein and PEG-4VS with Teflon-coated forceps and spatulas.)

(Note: The 4-arm PEG vinyl sulfone can also be dissolved in cross-linking buffer rather than 0.4 M triethanolamine.)

Sonicate (about 2 min) in ultrasonic bath. Vortex to mix. Centrifuge at 10,000 *g* for 1 min at room temperature or 4 °C. This removes bubbles or foam. Vortex to mix again and repeat centrifugation if necessary.

(Note: After centrifugation, a very viscous, protein-rich phase can form at the bottom of the tube. This may be protein aggregation. It can be resuspended by vortexing.)

Mix the protein solution and PEG-4VS solution at the desired volumetric ratio, vortex, and pipette a droplet onto a treated glass within the rectangular spacer. Place another glass slide on top with the treated side facing the droplet. Carefully clamp the slides together with binder clips, being careful not to introduce bubbles.

(Note: PEG-4MAL cross-linking occurs much too quickly to process in this way. Instead, a droplet of the protein solution can be formed on a treated glass slide. The cross-linker solution should be pipetted directly into this droplet, mixing as best as possible by pipetting up and down in a swirling motion.)

Example: In a typical formulation of hydrogels in this thesis, 160 mg of protein was dissolved in 1.07 mL of degassed cross-linking buffer in a microcentrifuge tube. In a separate tube, 45 mg of PEG-4VS was dissolved in 300 mL degassed 400 mM triethanolamine. The tubes were both sonicated for 2 min, vortexed, centrifuged, and vortexed again. The volumes of the protein solution increased slightly (≈10%) due to the dissolved solids. A similar increase is observed with PEG-4VS. After dissolving completely, 1 mL of the protein solution was transferred to a new tube. To this solution, 231 μL of the PEG-4VS solution was added. This volumetric ratio gives a 1:1 ratio of thiols to vinyl sulfones while maintaining a constant polymer concentration of approximately 15 wt% (actually slightly less when accounting for the volume increases). The gelation mixture was vortexed and pipetted onto a treated glass slide. For swelling experiments, 50 μL droplets were formed. For rheology experiments, 70 μL droplets were formed. For tensile tests, 1 mL droplets were formed. A second glass slide was placed on top, separated by rubber spacers, taking care not to introduce bubbles.

Expected results: Gelation with PEG-4VS at the concentration ranges explored (7.5-25 wt%) occurs within 10-30 minutes; however, longer times are required to completely cross-link the materials. If the slide is treated with SigmaCote and cross-linking reaction proceeds as expected, gels can be easily removed from the slide with a spatula. A small amount of PBS buffer for also helps in removing the gels. Poorly cross-linked gels are sticky and difficult to remove from the slides.

Alternative cross-linking Cross-linking can be performed without guanidinium chloride and without triethanolamine. For cell encapsulation experiments in Chapter 2, protein and PEG-4VS were both dissolved in HEPES-buffered saline (25 mM HEPES, 150 mM NaCl, pH 7.4). The kinetics of the gelation reaction are only slightly slower under these conditions.

B.5. Hydrogel swelling experiments

Overview: Hydrogels swell in the presence of water. According to classical theories, swelling results from a balance of the free energy of mixing polymer chains and solvent and elastic free energy. Electrostatic effects also contribute. The swelling experiments in this thesis are simple measurements of the swollen mass in a specified buffer divided by the dry mass of the cross-linked polymer network. This specific protocol describes the mass swelling measurements of EPE gels swollen in denaturing buffer for 48 hr, followed by swelling in PBS buffer for 48 hr. The first step can be omitted if desired.

Materials:

Hydrogels prepared according to Protocol 4.

Phosphate buffered saline, PBS

1.5 mM KH_2PO_4

4.3 mM Na_2HPO_4

137 mM NaCl

2.7 mM KCl

Guanidinium chloride

Sodium azide (Sigma S2002)

1.7 mL microcentrifuge tubes

Sterile destilled and deionized water, ddH_2O (Spalding 332A)

Liquid nitrogen (Spalding 312)

Equipment

Mettler AE50 balance (Spalding 332A)

Mettler AT201 balance (Arnold lab)

Biorocker (Spalding 332)

Lyophilizer (Spalding 312)

Protocol

Day 1: Remove hydrogels from glass slide cross-linking apparatus and transfer to PBS with 6 M guanidinium chloride, pH 7.4. Typically I have 6 gels per condition and transfer them to 6 mL of buffer in a 35 mm dish. Swell on rocker for desired length of time. Measurements were typically taken at 24 hr and 48 hr. If the swollen mass is unchanged, proceed to the next step. Otherwise wait until equilibrium is reached.

(Note: In poorly cross-linked gels, erosion is often observed and an equilibrium will not be reached.)

Day 2: Measure swollen mass at 24 hr on Mettler AE50 balance. Transfer gels to individual wells of a 6-well plate with 3 mL PBS containing 6 M GndCl.

Day 3: Measure swollen mass at 48 hr.

Dilute buffer with an equal volume of PBS to obtain a final concentration of 3 M GndCl in PBS. Swell for 3 hr, then change to 2 M GndCl in PBS for 3 hr, 1 M GndCl in PBS for 3 hr, and finally PBS buffer overnight.

Day 4: Change PBS buffer to remove final traces of GndCl. Swell in PBS containing 0.02% (w/v) sodium azide to inhibit microbial growth.

(Note: NaN_3 is only required for longer term experiments or if there is concern about contamination. However, I almost always include it out of precaution.)

After 24 hr in PBS, measure the swollen mass.

Day 5: After 48 hr in PBS, measure the swollen mass.

Remove PBS buffer and replace with 5 mL sterile ddH_2O.

(Note: Follow safe disposal of buffer with sodium azide.)

Day 6-7: Replace water to ensure salts are removed. Gels will swell due to charged residues and then begin to shrink (likely due to self-buffering by the charged residues to near the pI).

Day 8: Make a final change of the water in morning. In the evening, transfer to tared microcentrifuge tubes, freeze in liquid nitrogen, and dry on the lyophiziler.

Day 9: Gels are typically completely dried after several hours. Measure the mass of the dry gel and vial, then subtract the vial tare. Masses were measured on the Mettler AT201 balance for higher precision.

Calculate the mass swelling ratio. $Q_m = \frac{swollen\ mass}{dry\ mass}$

Expected results

Hydrogels prepared from EPE and its variants have a typical mass swelling ratio, Q_m, of 11-15 in PBS. ERE hydrogels have a Q_m of 19-21 in PBS. In PBS with 6 M GndCl, the swelling ratios of all gels are typically 29-35.

B.6 Rheological measurements with swollen hydrogels

Overview: This protocol describes sample loading and rheological tests for swollen protein gels as prepared above. The method for loading swollen hydrogels on the rheometer was adapted from T.K.L. Meyvis, S.C. De Smedt, J. Demeester, W.E. Hennink *J. Rheol.* **1999** *43*, 933-950.

Materials:

Protein hydrogels prepared according to Protocol 4.

Biopsy punch, 8 mm diameter (Miltex)

Paraffin oil

Equipment

ARES-RFS strain-controlled rheometer (Kornfield lab)

8 mm parallel plate test geometry

Protocol

Setting the gap height Due to variation in the height of swollen gels, the gap height must be set for each gel. The following protocol was adapted from Meyvis *et al.*

Swell hydrogels in desired buffer. Typically, gels are swollen in decreasing concentrations of guanidinium chloride in PBS, then swollen for 48 hr in PBS with 0.02% (w/v) sodium azide.

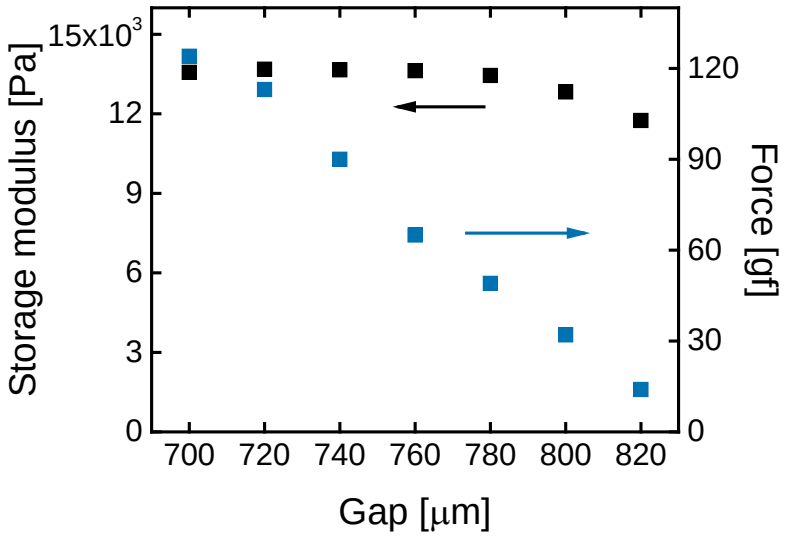
Set ARES-RFS temperature control (25 °C) and turn motor on.

Cut gels to 8 mm with biopsy punch. Place gel on bottom plate and lower test geometry until it contacts the gel, noting the normal force. When the normal force exceeds 10 gf, pipette a thin layer of paraffin oil around the gel to prevent evaporation.

Perform an oscillatory single point measurement at 1% strain amplitude, 5 or 10 rad s^{-1}. Record the storage modulus *G'* and normal force at the beginning of the measurement.

Lower the gap 10 microns, repeating the single point measurement at the new gap height. Continue repeating this process until a *G'* is unchanged (see figure below). This is the gap

height at which data will be collected. Samples were typically compressed 10-30% from the height at which a normal force was first detected.



Strain sweep test Perform a strain sweep experiment from 0.01-20% strain at 10 rad s^{-1}, 25 °C to determine the linear viscoelastic regime. In this regime *G'* or |*G**| is constant. In cross-linked gels, the loss modulus *G"* and the phase angle δ are small.

Non-linear behavior may be a property of the material at higher strains, or may be due to slip between the gel and the plate.

Frequency sweep test Select a strain amplitude in the linear viscoelastic regime. Typical strain amplitudes were 1-5%.

Frequency sweeps were performed from 100 rad s^{-1} to 0.001 rad s^{-1}, starting at high frequency since these measurements are fast. Three decade (100-0.1 rad s^{-1}) tests take 5-10 min. Four decade (100-0.01 rad s^{-1}) tests take 1.5-2.5 hr. Five decade (100-0.001 rad s^{-1}) tests take 12-18 hr. Data were collected at 7 points per decade.

(Note: The loss modulus, *G"*, is difficult to measure accurately for covalent hydrogels. For an elastic solid (steel), the ARES-RFS has an expected phase angle δ of ±0.25 or a tan(δ) = ±0.004. In my experience, the loss moduli become very noisy at tan(δ) < 0.01.)