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COMMUNICATIONS

Published on Web 05/03/2008

Bonding of Macromolecular Hydrogels Using Perturbants

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Received December 21, 2007; Revised Manuscript Received April 16, 2008; E-mail: jtien@bu.edu

This Communication describes the use of low molecular weight solutes to bond patterned macromolecular hydrogels and provides evidence that bonding occurs via reversible perturbation of the gels. The ability to form biologically relevant gels with complex microscale architectures may provide useful scaffolds for studies in cell biology and physiology and for microfluidic devices.[1] One promising route to these structures is layer-by-layer stacking, a widely used technique in the microfabrication of polydimethylsiloxane (PDMS).[2] Passive adhesion between hydrogels, however, is much weaker than that between PDMS structures (most likely due to an intervening thin layer of water). Stacked gels are thus unlikely to resist stresses imparted by mechanical pumping or by cells. Recent work by Stroock and co-workers has shown that alginate gels can be irreversibly bonded by removal and reintroduction of free calcium ion.[3] Here, we describe a general strategy for bonding extracellular matrix gels (such as collagens and fibrin) into mechanically robust structures with a resolution of ~20 μm.

Figure 1 describes the bonding procedure, which started with patterned[4] and flat gels that passively adhered to form a microfluidic network. This adhesion was sufficient to confine a suspension of microspheres to perfused channels. Mechanical stress, however, readily fractured networks along their adhesion planes and allowed microspheres to pass between the gels (Figure 1B).

We reasoned that solutes that can antagonize gelation would serve as effective bonding agents for gels. Given that many macromolecular gels form by self-assembly in water, we tested solutes known to affect hydrogen bonding in water (bond-weakening chaotropes and bond-forming kosmotropes[5]) for their ability to bond gels. Delivery of a candidate solute proceeded by convection through the microfluidic network and subsequent outward diffusion into the gel; after 1 h, a similar procedure with phosphate-buffered saline (PBS) flushed out the solute. Only perfusion and removal of certain solutes resulted in gels that withstood mechanical agitation (Table 1 and Figure 1B) and bursting pressures >80 cm H_2O. For these solutes, bonding occurred up to a maximum concentration, beyond which deformation or disintegration of the gel took place. At the doses indicated in Table 1, these solutes preserved the geometry of microfluidic networks. Cross-sections of treated structures indicated that the sharp features normally obtained by micromolding remained after exposure to solute (Figure 1B, inset).

Our results imply a bonding mechanism that relies on reversible perturbation (de- and repolymerization) of the gel, for several reasons: First, bonding at the indicated solute concentrations invariably led to a loss of opacity in type I collagen and fibrin gels as the solute was introduced, and a return of opacity as the solute was flushed out (Table 1). During exposure of collagen gels to guanidine hydrochloride (GnHCl), second harmonic generation (SHG) output by surface fibrils decreased over time in the forward and backward directions (Movies S1 and S2), with a decrease in the forward/backward signal ratio. The decrease in opacity and SHG output and ratio suggest that bonding solutes decrease the number and thickness of fibrils;[6] the reverse effects occurred during removal of solute. Second, both perfusion and removal of solute were essential to form gels that confined 1 μm diameter microspheres under mechanical stress (Figure 1). Collagen gels that were perfused with GnHCl but not subsequently flushed with PBS led to partial bonding. Third, treatment with bonding solutes led to partial depolymerization of gels. For collagen gels treated with GnHCl, at least 10% of the total protein was solubilized; this effect was kinetically limited, and using a longer treatment with solute (~3 h) decreased the bonding concentration required for bonding by ~10%. Fourth, gels fixed with 1% paraformaldehyde did not bond.

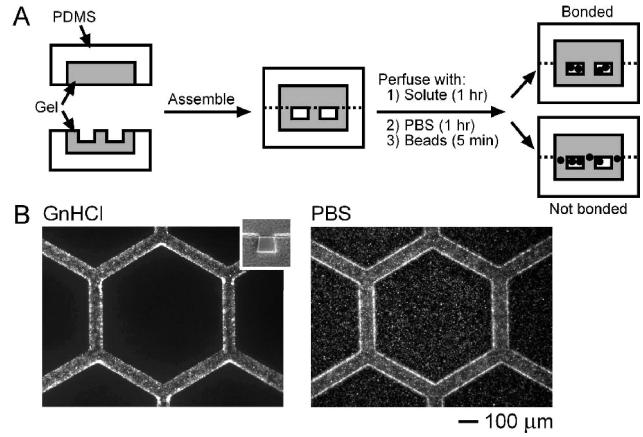

Figure 1. (A) Schematic diagram of perturbant-mediated bonding. (B) Fluorescence images of 80 μm wide hexagonal networks in collagen gels that were treated with 0.42 M GnHCl or PBS, perfused with a suspension of 1 μm diameter fluorescent microspheres and stressed. Inset: phase-contrast image of a cross-section of a GnHCl-bonded gel (same scale).

Table 1. Bonding of Gels by Solutes

solute	bonding concn (M)[a]	decrease in absorbance (%)[b]
Type I collagen (rat)		
GnHCl	0.42	93 ± 2
glycerol	2.1−2.2	93 ± 3
NaSCN	0.82	95 ± 9
(+ 0.2 M NaCl)	0.90	89 ± 5
(+ 0.2 M Na_2SO_4)	1.10−1.20	42 ± 4
NaI	0.82−0.90	98 ± 1
NaBr	2.6−3.0	45 ± 1
NaCl	did not bond	27 ± 2[c]
Na_2SO_4	did not bond	17 ± 2[d]
none	did not bond	0 ± 1
Fibrin (human)		
GnHCl	0.40−0.42	88 ± 4
NaCl	did not bond	18 ± 2[e]
none	did not bond	−2 ± 6

[a] Values are the maximum concentrations in PBS before deformation of gels in three independent experiments. [b] Values are means ± SD (n = 3). [c] Gels were treated with 4.5 M NaCl. [d] Gels were treated with 1.5 M Na_2SO_4. [e] Gels were treated with 0.42 M NaCl.

10.1021/ja711340d CCC: $40.75

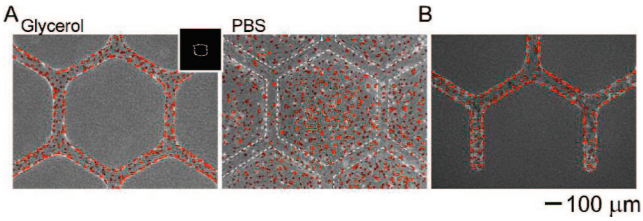

Figure 2. Compatibility of perturbant-treated gels with EC cell culture. (A) Phase-contrast and fluorescence images of a 80 μm wide hexagonal network in collagen gel that was treated with 2.2 M glycerol or PBS (dotted lines indicate channels), seeded with cells, and stained with Hoechst 33342 to visualize nuclei (red). Inset: fluorescence image of a cross-section of a GnHCl-bonded and seeded gel stained with Hoechst 33342 (same scale). (B) Analogous images of a semiopen seeded network.

Nearly all bonding solutes were chaotropes (GnHCl, NaSCN, NaI, NaBr). Addition of the kosmotrope Na_2SO_4 antagonized the bonding effect of chaotropes, while the neutral salt NaCl had no effect (Table 1). The ranking of bonding "strength" for anions followed the Hofmeister series, which correlates with their ability to disrupt macromolecular complexes and fibrils.[7] Although the kosmotrope glycerol did bond collagen gels, this solute acts as a collagen-specific perturbant, by competing for hydrogen bonds between self-assembled helices.[8]

High concentrations of NaCl did not bond gels, ruling out a nonspecific osmotic effect. Treatment of the gels with 0.05% Triton X-100 did not interfere with bonding, implying that bonding is not due to interfacial hydrophobic interactions.

Taken together, our data are consistent with the following bonding mechanism: Solute is rapidly transported throughout the gel (<5 min, by numerical modeling). Depolymerization then slowly occurs to release oligomers, which can slowly diffuse between gels and/or out of the gels. We expect that, as with any linear self-assembling network, a variety of oligomers are released during this phase.[9] Removal of solute allows repolymerization to take place, which interlocks the gels across the interface.

To determine whether bonded gels could serve as effective scaffolds for cell culture, and as an additional test of bonding strength, we cultured cells on the surface of the channels and within the bulk of the gel. We used primary human endothelial cells (ECs) as a model of monolayer forming cells and human fibroblasts as a model of mesenchymal cells. When perfused as a suspension through the microfluidic network in a bonded gel, ECs readily attached and grew to confluence over the span of a few days. These cells remained confined to the surface of the channels, as shown by a nuclear stain (Figure 2A, left and inset). In contrast, cells did not remain confined in untreated gels (Figure 2A, right). When human fibroblasts were *embedded* within collagen gels that were subsequently bonded with glycerol, the levels of cell viability did not differ significantly from those of cells that were not exposed to the solute: For both treated and untreated gels, >90% of embedded fibroblasts remained viable after 2 days. We limited exposure to hyperosmotic stress introduced by the bonding solute to 20−30 min, which may explain why the embedded cells remained largely viable after bonding.[10] Our data suggest that the bonding procedure preserved enough native structure in the gel to support cell adhesion and proliferation, although we cannot rule out the possibility that some epitopes were irreversibly denatured.

The bonding procedure can be extended to semiopen networks, in which the channels do not extend across the full extent of the gel. Here, transport of the solute took place both through the channels and in the bulk of the gel. Because these structures had a higher resistance to water flow than fully open networks did, we perfused them with solute for a longer time (2 h perfusion and 2 h flushing) and at a larger pressure drop. As with the open hexagonal networks, ECs formed a confluent layer in the channels, resulting in semiopen networks in which the cells were confined to channels (Figure 2B).

Our results demonstrate that certain small solutes (chaotropes and other perturbants) can be used to bond macromolecular hydrogels into monolithic structures that preserve the initial geometries of the gels. This technique can be used to form both open and semiopen networks in biologically relevant gels, and the resulting materials support cell culture. Our data are consistent with a bonding mechanism of reversible perturbation, which is similar to the fusion of alginate gels.[3] In theory, other self-assembling macromolecular gels such as Matrigel and agarose may be bonded with similar solutes. Compared with recently described subtractive methods for making microfluidic gels,[11] the additive method described in this work has the potential to form three-dimensional (3D) networks by stacking and bonding layers. The development of methods to register layers in 3D (similar to what has been described for PDMS[2]) will be an important step toward this goal. We believe that the structures described here will provide interesting opportunities to generate spatially complex biological tissues for the study of physiological flows or multicellular biological processes.

Acknowledgment. We thank B. Suki, H. Rhodes, and T. Deans for help with experiments, and E. Evans and V. A. Parsegian for useful discussions. This work was supported by the NIH/NIBIB (EB002228 and EB005792) and the Whitaker Foundation (RG-02-0344). K.K.C. is supported by a Boston University Photonics Fellowship.

Supporting Information Available: Experimental details and time-lapse movies of SHG in treated gels. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA711340D

Supporting Information

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Materials and Methods

Formation of to-be-bonded gels

Each experiment used two separate gels (Fig. 1): one patterned and the other unpatterned (i.e., a flat gel). These gels were formed in shallow PDMS chambers (5 mm x 6 mm x 0.5 mm deep). Patterned and flat PDMS stamps were coated with 1% bovine serum albumin (1 hr) before molding against liquid monomer solutions. For Figures 1 and 2A and Table 1, we used 80-μm-wide, 80-μm-tall hexagonal networks; for Figure 2B, we used semi-open networks that had blind-ended features separating two hexagonal regions. Before use, collagen solution (7 mg/mL; type I from rat tail, BD Biosciences) was neutralized with NaOH, and human fibrinogen (50 mg/mL; Sigma) was activated with 1 U/mL thrombin. Gelation proceeded for 2 hr at 23-25˚C before gentle removal of stamps under phosphate-buffered saline (PBS). All gels were thoroughly washed with PBS for 1 hr before being assembled in pairs to form open or semi-open networks.

Bonding procedure and assay

Flat and patterned gels were carefully brought into passive contact, flat gel on top, and held in place by their surrounding PDMS chambers (Fig. 1A). The assembled gels were exposed to candidate solutes by adding droplets of solutes (200 μL) to one end of the gels and tilting the gels (1 hr at ~12˚ for open networks, 2 hr at ~80˚ for semi-open ones) to induce flow. Droplets were refreshed every 15 min; typical flow rates for the patterns shown in Figure 1 were ~100 μL/20 min. Afterwards, we flushed gels with PBS (1 hr for open networks, 2 hr for semi-open ones).

We deviated from the standard procedure above in three cases: 1) Gels were flushed

with 0.05% Triton X-100 for 15 min before testing whether the gels were bonded (n = 3). 2) Separate gels were fixed with large droplets (~80 μl) of 1% paraformaldehyde for 1 hr and washed with PBS for 1 hr. Gels were then assembled and treated with solute as described above (n = 9). 3) Gels were treated for 1, 1.5, 2, 2.5, or 3 hrs with solute. Gels were tested for bonding immediately (i.e., without flushing out the solute) or after flushing with PBS for 1 hr (n = 3).

To test for bonding, we introduced a 100-200 μL suspension of 1-μm-diameter fluorescent microspheres (Molecular Probes) at one end of the gels and allowed it to flow through for 5 min. Images were captured before and after repeatedly tapping (~20 times) the upper PDMS chamber with fine-tipped tweezers to strain the contacting gels. During tapping, the tips of the tweezers were separated by ~2 mm, and the applied force of each tap was ~0.5 N, causing the gels to cyclically flatten and squeeze outward at both open ends by ~20% (i.e., by ~0.5 mm at either side). The entire tapping procedure required ~10 sec to complete. Gels that still confined beads to the channels after tapping were considered bonded. Gels that displayed deformations before tapping—narrowing of channels or disintegration of the gel—were considered over-bonded. We tested solutes in 5% concentration increments until we observed deformations; the bonding concentration of a solute was taken to be the highest concentration before deformation occurred. The ranges provided in Table 1 were derived from three separate experiments.

We used three different batches of collagen and found that the bonding concentrations varied by ~10% between batches. The data presented in Table 1 are for a single batch of collagen, while all other experiments (paraformaldehyde fixation, time-dependent experiments,

time-lapse imaging) used data from the three different batches. Unless noted otherwise, the concentration of solute used in these experiments was always the bonding concentration for the particular batch of collagen being tested.

Measuring opacity of treated gels

Transmission of wide-spectrum light from a halogen bulb through flat, 1-mm-thick gels was measured on a microscope before and after immersion in solutes. The percent decrease in light absorbance was calculated as $1-\ln(I)/\ln(I^{\circ})$, where I and I° refer to the normalized transmitted light intensities after and before exposure to solutes, respectively.

Imaging second-harmonic generation in collagen fibers

To examine the effect of perturbant on collagen fibers over time, the tops of flat collagen gels were covered with large droplets of GnHCl (~70 μl) and imaged for second-harmonic generation (SHG). Images were captured every minute for one hour, and similarly after replacing the GnHCl solution with a droplet of PBS to induce recovery of fibers. SHG imaging was performed using a custom-built laser scanning microscope with a laser emitting 190 fs pulses at 1030 nm (Amplitude Systèmes t-Pulse) and an Olympus 60×/0.95 NA water-immersion objective. SHG output was collected in forward and backward directions and isolated using narrow-band filters centered at 515 nm (Chroma Technologies). Forward and backward images were normalized using rhodamine-labeled beads.

Determination of bursting pressures

A ~1-mm-diameter hole was drilled into the upper PDMS chamber before assembly and bonding of a pair of collagen gels, thereby exposing part of the gel to atmospheric pressure after bonding. A PDMS lid, threaded with a pair of 100-cm-long pieces of polyethylene tubing, was

placed on the upper PDMS chamber so that the tubing made fluidic contact with both side ends of the bonded gel, as described in refs. 11a and 11b. The other ends of the tubing were placed into a single dish filled with a suspension of fluorescent beads. The dish was gradually raised to increase the hydrostatic pressure within the microfluidic channels in the gel. The bursting pressure of the gels was the pressure at which beads began flowing from the channels into the interior of the pattern (n = 3).

Measuring amount of soluble collagen released from treated gels

Flat collagen gels were treated with a large droplet of GnHCl (~80 μl) for 1, 2, or 3 hrs. The droplet was gently agitated by pipetting before removing the liquid for micro-spectrophotometry at 220 nm with a NanoDrop 1000 Spectrophotometer to determine the concentration of released collagen oligomers.

Culturing cells within collagen gels

Microvascular endothelial cells and fibroblasts from human dermis (Cambrex) were grown as described in ref. 11a. Collagen gels bonded with GnHCl or glycerol were conditioned with media for at least 12 hrs at 37°C before seeding endothelial cells as a suspension through the channels of the gel. In separate experiments, fibroblasts were introduced as a suspension (50000 cells per mL) into liquid collagen that was then molded for 20 min at 23°C and 25 min at 37°C. To bond fibroblast-containing gels, we perfused them for 20 min at 37°C with glycerol in culture media before flushing with glycerol-free media. The gels were cultured at 37°C for 3 hrs or 2 days after bonding, and then labeled by perfusion with propidium iodide (2 μg/mL). Labeled, non-viable cells were counted manually.

Sectioning gels

Cellular and acellular bonded gels were fixed by perfusion with 4% paraformaldehyde for 1.5-2 hrs and then flushed with PBS for 1 hr. Fixed gels were embedded in 5% agarose before sectioning into 100-μm slices using a vibratome. Acellular sections were imaged by phase-contrast microscopy using a Plan-Neo 10×/0.30 NA objective. Cellular sections were stained with Hoechst 33342 to visualize nuclei; five sequential images were obtained at 4.3 μm spacing with a Plan-Apo 10×/0.40 NA objective on an Olympus IX81 inverted confocal microscope, and stacked with ImageJ 1.38 (NIH) to generate a composite.

Modeling diffusion of solutes into gels

The diffusion of solute from the channels (80 μm x 80 μm cross-section) into the interior of the hexagonal pattern was modeled numerically using finite-element software (COMSOL Multiphysics ver. 3.3). A constant concentration at the channel walls and no-flux conditions at the outer walls were chosen as the boundary conditions. An aqueous diffusion coefficient of 1000 $\mu m^2/s$ was used for the model solute.

Movie S1: Time-lapse movie for 1 hr of forward SHG output of collagen gel exposed to GnHCl. The bar refers to 10 μm.

Movie S2: Time-lapse movie for 1 hr of backward SHG output of the same gel shown in Movie S1. The bar refers to 10 μm.