Uniaxial stress–relaxation and stress–strain responses of human amnion

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The mechanical behavior of human amnion is examined under uniaxial tensile loading conditions. Monotonic strain-to-failure and stress–relaxation tests are described for membrane strip samples of amnion obtained by removing the chorion cell layer from specimens of whole chorioamnion. The monotonic behavior of the amnion is characterized by a large stress-free strain (approximately 10%) prior to a quadratic load–displacement response. Substantial stress relaxation behavior (ranging from 20–80%) is observed, described by a two time-constant exponential decay. The effects of the application of a topical antiseptic and of prior straining and relaxation on subsequent monotonic failure properties are examined. The results suggest that while amnion is a remarkably resilient tissue material, its mechanical behavior is typical of nonlinear viscoelastic materials, and depends strongly on its history.

1. Introduction

The chorioamnionic (CA) membrane is the strong and resilient tissue that surrounds a fetus during gestation. The membrane is thus subject to applied stresses and strains during pregnancy; the membrane must support the bulk loads of the fetus and amniotic fluid as well as tolerating local deformation associated with fetal movement. The chorion layer is thick and primarily cellular. The amnion layer is substantially stiffer and stronger than the chorion, with a dense mat of collagen providing resistance to tensile forces [1].

Deformation and failure of the CA membrane occur during labor and delivery; it has been estimated that the CA membrane is strained to 100% (area strain) by delivery [2]. Not all membrane mechanical failure occurs in full-term pregnancies, and there are severe consequences that result from preterm premature membrane rupture [3]. One suspected factor in preterm rupture is membrane prolapse, in which a portion of the membrane experiences excessive local mechanical deformation. A detailed understanding of membrane deformation behavior may help physicians to intervene and prevent local membrane failure after prolapse.

Since membrane rupture occurs *in vivo* by mechanical failure, many studies have been undertaken to study the mechanical behavior of the membrane *in vitro* [4]. Mechanical studies of the membrane under controlled laboratory conditions have examined both the prefailure (deformation) and the failure behavior of control and altered membranes [5–10]. These studies have been performed primarily under uniaxial tensile loading, as such experiments are relatively simple to perform in the laboratory using standard materials testing equipment. The mechanical evaluation of the CA membrane typically has been limited to simple observations based on a single force–deformation or stress–strain curve. For example, although the responses are clearly nonlinear, a tangent is taken and reported as the (pseudolinear) elastic stiffness or modulus. Other frequently reported parameters include failure load or stress, deformation or strain to failure, and work of failure [5–10]. These parameters have been used to compare tissue samples and examine the effects of external influences on membrane behavior.

Limited membrane testing has been performed under biaxial loading conditions such as burst testsing [11–13] or probe puncture [14, 15]. Biaxial testing results in conditions more closely approximating the *in vivo*

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loading compared to uniaxial conditions, but the testing can be more difficult to perform and the results can be difficult to characterize.

There are two prominent characteristics of the mechanical responses of soft biological tissues that separate their behavior from those of standard engineering materials (such as metals or ceramics). First, the responses are time-dependent (viscoelastic), and can undergo rate-dependent stress–strain responses, substantial stress–relaxation at fixed displacement and substantial creep at fixed load. Second, the stress–strain response of soft tissues is typically nonlinear, demonstrating gradual stiffening with increased strain, such that there is no single-valued linear stiffness or modulus describing the entire pre-failure elastic behavior. Although these phenomena (nonlinearity and viscoelasticity) have been demonstrated in CA membranes by previous investigators [5, 11], they have not been explored quantitatively. Ignoring these features of the membrane mechanical response may mask important differences between control and altered membranes.

The aim of the current study was to investigate the baseline uniaxial nonlinear and viscoelastic behavior of the amnion, the strength-bearing portion of the bilayer CA membrane. Quantitative comparisons were made between control membranes and test membranes soaked in Betadine topical antiseptic solution. (This chemical may be applied directly to the membrane in clinical procedures for prolapsed membranes.) In addition, a comparison was made between the stress–strain responses of unstretched (virgin) control samples, as above, and samples for which the stress–strain response was measured following a 200 s static stretching (for viscoelastic stress–relaxation quantification). It was hypothesized that these two treatments, one chemical and one physical, would measurably alter the membrane mechanical behavior.

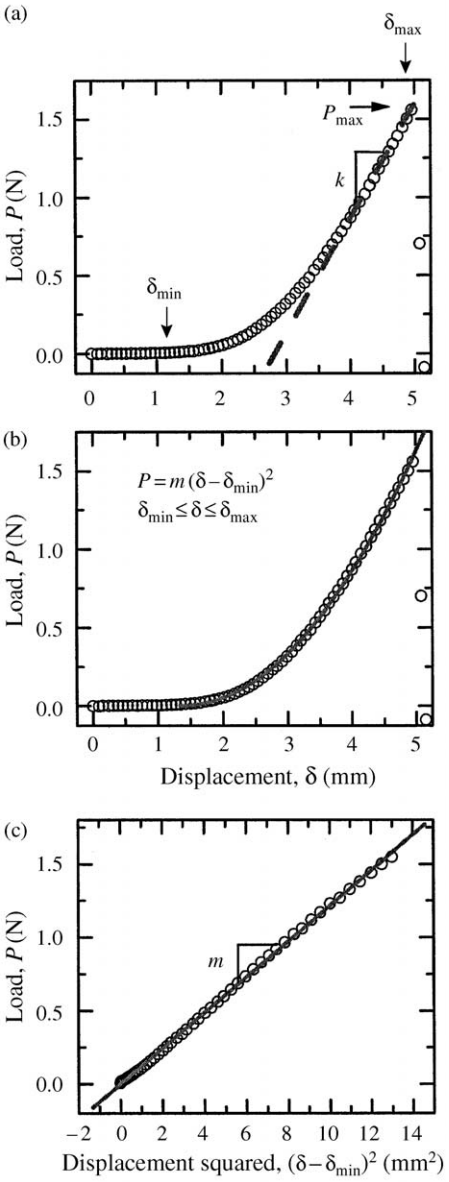

Figure 1 (a) Plot of load, P, versus displacement, δ, for an amnion specimen tested in tension. Arrows indicate the peak force, P_{max}, and the minimum (δ_{min}) and maximum (δ_{max}) displacement levels which were used to calculate the failure strain. A tangent line illustrates the method used to calculate pseudolinear stiffness, k, in the top 20% of the load–displacement response, (b) Plot of load, P, versus displacement, δ, for the same amnion specimen as in part (a). A single coefficient, m, describes the response from δ_{min} to δ_{max} relating the load to the squared displacement (Equation 1). This m coefficient fits the entire curve as opposed to a stiffness k, which only fits a small portion of the curve. (c) Plot of load, P, versus zeroed displacement squared, $(\delta - \delta_{min})^2$, for the same amnion data as shown in parts (a) and (b). The slope of this line gives the parameter m.

2. Materials and methods

Six specimens of human fetal CA membrane were obtained with informed consent at the time of planned cesarean section from patients who were unlabored, full-term, and without clinical evidence of infection. Specimens were stored in sterile saline and tested the day of delivery. Chorion was separated from the amnion using gentle traction and discarded. Each hydrated amnion specimen was cut into approximately 8–16 samples, strips 5–7 mm wide and 25 mm long, for mechanical testing. Amnion samples were tested as-harvested (''control'' group) or soaked 1 h in full-concentrated Betadine (Purdue Frederick Co., Norwalk, CT) (''Betadine-soaked'' group). After 1 h, the Betadine-exposed samples were removed from the solution, rinsed three times in buffered saline, and stored in saline for immediate mechanical testing. Four to eight samples from each single amnion specimen were used for each group (saline or Betadine), with half of the group tested for viscoelastic relaxation and the remaining samples tested for strain-to-failure behavior.

Uniaxial mechanical tests were performed on a horizontal mechanical testing system (MTS MicroBionix, MTS Systems Corp, Eden Prairie, MN) equipped with a 5 N load cell. Custom flat, parallel grips with $\sim$ 11 mm grip-to-grip gage length (L) were coated with sandpaper for soft-tissue gripping and were manually tightened using set-screws. Two different test-types were performed: monotonic strain-to-failure tests and stress–relaxation tests. All tests were performed with the amnion sample submerged in a saline bath.

Strain-to-failure tests were performed at 0.25 mm/s (2.3%/s). Maximum load (P_{max}) and displacement (δ_{max}) were read directly from the load–displacement ($P - \delta$) curve (Fig. 1(a)). Conventional pseudolinear stiffness (k)

was computed from a tangent fit of the final 1 mm of displacement before failure (~60–100% peak load, Fig. 1(a)). The stiffness k was used along with sample gage length L and cross-section area A to calculate the pseudolinear elastic modulus (E) where $E = kL/A$. The amnion width was measured directly with calipers for each sample and thickness was assumed to be 50 μm for all groups [6]. As the load–displacement responses universally showed an initial period of nearly load-free strain, the minimum displacement (δ_{min}) was taken as the point at which the load first clearly deviated from zero (Fig. 1(a)). This minimum displacement was subtracted from the peak displacement to calculate failure displacement, $\delta_f = \delta_{max} - \delta_{min}$.

Inspection of the load–displacement responses (Fig. 1) indicates the likeliness of a quadratic force–displacement or stress–strain constitutive relation. A quadratic constitutive law was proposed,

$$P = m(\delta - \delta_{min})^2 \quad (1)$$

where m is thus defined as a ''quadratic stiffness''. A fit of this relation to the load–displacement data was performed to determine the coefficient m for each sample. In addition, a quadratic modulus D (material property analog to E) was computed from the quadratic stiffness where $D = mL^2/A$. Parameters computed from the strain-to-failure tests $(P_{max}, \delta_f, k, E, m, D)$ were compared using paired t-tests.

Stress–relaxation tests were performed for the control and Betadine-soaked amnion samples at a displacement level of 5.5 mm (approximately 50% engineering strain from neutral closed-grip position). The ramp rate to peak displacement was 1.5 mm/s followed by a 200-s hold. The response was normalized by the peak force (P_0) and fit to the following two time-constant relaxation model using a commercial nonlinear curvefitting algorithm (Microcal Origin, Northampton, MA):

$$\frac{P(t)}{P_0} = 1 - A_1\left[1 - \exp\left(\frac{-t}{\tau_1}\right)\right] - A_2\left[1 - \exp\left(\frac{-t}{\tau_2}\right)\right] \quad (2)$$

where $\tau_1 < \tau_2$. This normalized function has a value of 1 at zero time $(P(0)/P_0 = 1)$ and an equilibrium normalized force value $P_e/P_0 = 1 - A_1 - A_2$. Fitting parameters $(A_1, A_2, \tau_1, \tau_2)$ were averaged for the 2–4 stress–relaxation samples within each group, and these averages were used to make paired comparisons of control and Betadine-soaked samples by individually contrasting each of the four fitting parameters using paired t-tests. For a simple linear viscoelastic material (like many engineering polymers) the magnitude of the peak force would not influence the relaxation response [16]. However, for biological materials and other nonlinear materials, it is common for the relaxation response to be dependent on the peak-force. Therefore, the effect of absolute peak force magnitude (P_0) on time constants (τ_1, τ_2), amplitude coefficients (A_1, A_2), and equilibrium force (P_e/P_0) was examined.

The saline-soaked samples that underwent stress–relaxation testing were strained to failure following the stress–relaxation test. Samples were not removed from the grips between the two different tests. The load–displacement responses for these ''pre-stretched'' samples were compared to the responses of virgin controls (nonrelaxed) from the same amnion using paired t-tests for each parameter after analyzing the load–displacement responses in the same manner as described above.

3. Results

The appropriateness of the quadratic constitutive relation is illustrated for a representative control sample in standard load–displacement space (Fig. 1(b)) as well as in load-squared displacement space (Fig. (1c)). There were no significant differences between strain-to-failure parameters $(P_{max}, \delta_f, k, E, m, D)$ for control or Betadine-soaked samples (Table I).

The stress–relaxation responses for both control and Betadine-soaked amnion exhibited a two-phase relaxation response, with a rapid (<1 s) relaxation followed by a more gradual relaxation. Figure 2 shows representative force–time responses for a control and a Betadine-soaked sample; line fits to the two time-constant relaxation model (Equation 2) are also shown in Fig. 2. There were significant changes in both time constants (τ_1, τ_2) for the relaxation response of Betadine-soaked samples compared to controls (Table I). The short-time amplitude coefficient (A_1) increased (although not statistically significantly), altering the overall relaxation response.

The methodology employed in the relaxation testing yielded variations in the achieved peak force at the onset of each stress–relaxation test, as the peak displacement was controlled and the force–time response was measured. Therefore, both variations in specimen stiffness and variations in gripping gave rise to different peak forces for the same imposed displacement. There was not a significant difference between the average peak force obtained during the relaxation tests for the two groups, although the average peak force was larger for controls (0.80 ± 0.37 N control, 0.54 ± 0.42 N Betadine, $p = 0.2$ for paired t-test). There was a clear dependence of the equilibrium normalized load level (P_e/P_0) on peak load amplitude (P_0) with larger peak loads corresponding to a larger equilibrium level (Fig. 3(a)). There was no dependence of either time-constant on peak load (Fig. 3(b)).

TABLE I Properties obtained from tensile strain-to-failure and stress–relaxation tests for control and Betadine-soaked amnion

	Control	Betadine-soaked
Peak force, P_{max} (N)	1.57 ± 0.62	1.93 ± 0.86
Failure displacement, δ_f (mm)	4.10 ± 0.40	4.23 ± 0.44
Tangent stiffness, k (N/mm)	0.62 ± 0.20	0.74 ± 0.28
Pseudolinear modulus, E (MPa)	23.1 ± 6.3	28.4 ± 7.8
Quadratic coefficient, m (N/mm^2)	0.09 ± 0.03	0.10 ± 0.03
Quadratic ''modulus,'' D (N/mm^2)	38.4 ± 9.8	44.2 ± 8.9
τ_1	0.68 ± 0.09	0.54 ± 0.10*
τ_2	33.1 ± 2.1	26.2 ± 2.3*
A_1	0.29 ± 0.06	0.35 ± 0.05
A_2	0.18 ± 0.03	0.18 ± 0.03

*Statistically different from control by paired t-test at $p < 0.05$.

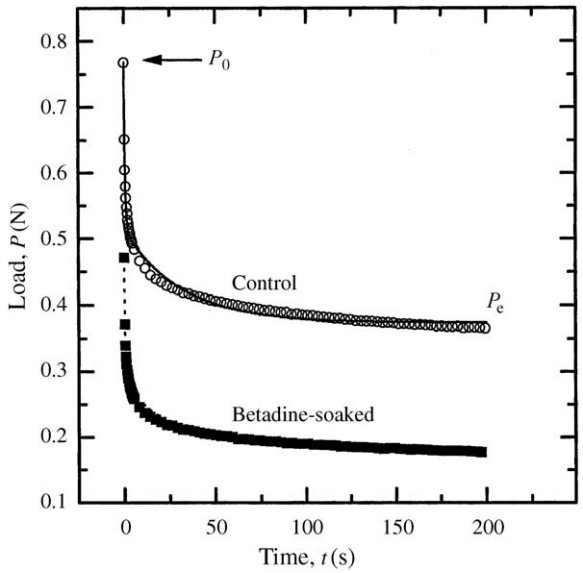

Figure 2 Representative plot of force, P, versus time, t, for single representative control and Betadine-soaked amnion specimens tested in tensile stress–relaxation. A solid line indicates the best fit to the two time-constant model (Equation 2). Values of peak (P_0) and equilibrium (P_e) loads for the control are indicated on the plot.

The strain-to-failure responses for saline control and pre-stretched samples differed, with significant differences in the failure displacement, quadratic stiffness, and quadratic modulus (Table II). It is interesting to note that the average peak *load* did not differ even though there was a substantial decrease in average peak *displacement*.

The pseudolinear tangent stiffness k can be related to the value of m:

$$k = \frac{dP}{d\delta} = 2m(\delta - \delta_{\min}) \tag{3}$$

Substitution for $(\delta - \delta_{\min})$ from the proposed constitutive relation (Equation 1) gives:

$$k = 2m\left(\frac{P}{m}\right)^{1/2} = 2m^{1/2}P^{1/2} \tag{4}$$

This provides an additional check on the validity of the proposed quadratic constitutive law, as a plot of k *vs.* $P_{\max}^{1/2}$ should be a straight line. In this case the measured stiffness values were indeed found to be directly proportional to the square-root of the maximum (failure) force (Fig. 4). The slope of linear fits to the $k - P_{\max}^{1/2}$ data for each group (control 0.78, Betadine-soaked 0.86, pre-stretched 0.93), predict group averages of m to be 0.15, 0.18, and 0.22. These values are slightly larger than the average m-values for the three groups (0.09, 0.10, 0.16, respectively), but rank in the same order by group.

4. Discussion

The uniaxial load–displacement and stress–strain responses for amnion are truly nonlinear. Therefore a single tangent stiffness value, or a single pseudolinear modulus value, is not sufficient to describe the stress–strain response of the tissue. The pseudolinear stiffness depends on the attained peak force; as the tangent of the concave-up curve increases with increasing displacement, the pseudolinear stiffness is greater for greater failure loads. This study found that a quadratic constitutive law adequately described the control amnion response as well as the response following alteration. (The lowest R^2 values for the quadratic fits were approximately 0.98 and in the pre-stretched group. For both control and Betadine-soaked groups, the R^2 values were greater than 0.995 in all cases.) It is interesting to note that although there were *not*

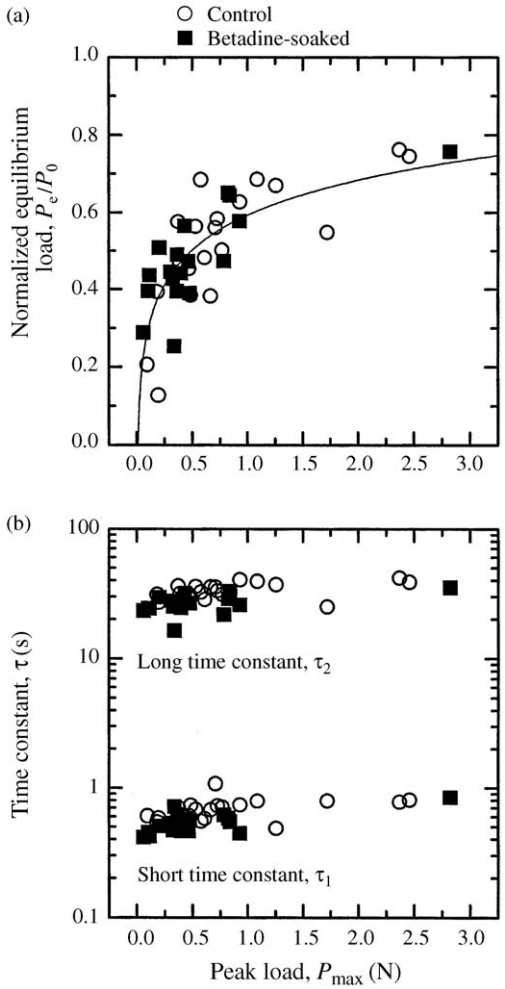

Figure 3 (a) Plot of normalized equilibrium force, P_e/P_0 versus peak force, P_0, for all control and Betadine-soaked amnion specimens tested in uniaxial tensile stress–relaxation. The equilibrium normalized force is $P_e/P_0 = 1 - A_1 - A_2$ where A_1 and A_2 are amplitude coefficients for the relaxation response. The equilibrium relaxation depends strongly on peak force level, with an empirical natural logarithm dependence (solid line) that is independent of group. (b) Plot of time constants, τ_i, versus peak force for the same relaxation tests. There was no apparent effect of peak load on time constants.

TABLE II Properties obtained from tensile strain-to-failure tests for control and pre-stretched amnion

	Control	Pre-stretched
Peak force, $P_{\max}$(N)	1.65 ± 0.66	2.05 ± 0.73
Failure displacement, δ_f(mm)	4.22 ± 0.30	3.67 ± 0.33*
Tangent stiffness, k (N/mm)	0.63 ± 0.22	0.81 ± 0.24
Pseudolinear modulus, E (MPa)	23.5 ± 6.9	29.5 ± 8.1
Quadratic coefficient, m (N/mm^2)	0.09 ± 0.03	0.16 ± 0.04*
Quadratic ''modulus,'' D (N/mm^2)	37.8 ± 10.9	64.0 ± 15.4*

*Statistically different from control by paired t-test at $p < 0.05$.

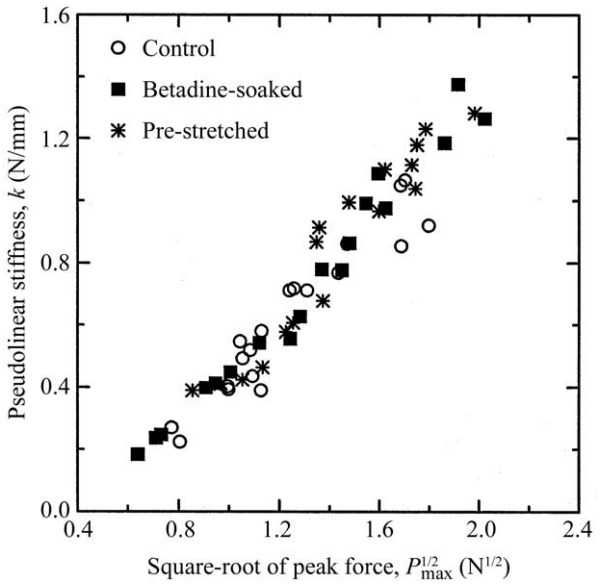

Figure 4 Plot of stiffness, k, versus square root peak force, $P_{max}^{1/2}$, for all amnion specimens tested in tension to failure. Stiffness values are not an independent measure of sample response, but depend linearly on peak force for all groups.

significant differences in the pseudolinear stiffness k (structural linear coefficient), or modulus E (material linear coefficient), there were in fact significant differences both in values of m (structural quadratic coefficient) and D (material quadratic coefficient). This difference highlights the fact that the entire load–displacement response is not necessarily well-represented by analyzing only the ''approximately linear portion'' of the curve, as in other investigation [5, 6]. Notwithstanding the concerns regarding the technique, the approximate pseudolinear modulus values computed in this report agree well with published values in the literature of approximately 29.5 MPa for isolated amnion obtained by C-section [6].

The standard deviations for uniaxial strain-to-failure testing parameters (maximum load, stiffness, failure displacement) are large in this study, consistent with other investigators' results demonstrating large property ranges [5, 6]. In contrast, the standard deviations for the viscoelastic relaxation fitting parameters were small. This is reflected by the coefficient of variation (standard deviation/mean) which is near 10% for the time-constants and is approximately 40% for the maximum force and stiffness. There are several reasons for this difference. The failure load is a property that is flaw-sensitive – it is determined by the weakest link in the component. The tangent stiffness, which was shown to be directly proportional to maximum force in this study, is thus also influenced by flaws. (External experimental factors, such as poor sample gripping or specimen failure at the grips, can also affect the recorded failure properties of a tissue.) In contrast, the time constants and other viscoelastic parameters, such as the normalized equilibrium force, average over the entire sample and are not flaw-dependent. Because the total deformations are smaller, these properties are less likely to be affected by gripping conditions. The viscoelastic testing in this study illustrated differences in the control and Betadine-soaked amnion that were not apparent from the strain-to-failure results.

Previous investigators have explored the phenomenon of biaxial viscoelastic relaxation for chorioamnionic membrane samples at a variety of different pressure levels. The short-time viscoelastic response, which may be associated with physiological loading time frames, was not commented on in previous viscoelastic investigations of the membrane, in which the force–time relation was plotted with a time scale in minutes and no quantitative analysis of the responses was reported [11]. It was observed in the current study that the uniaxial relaxation responses for control and Betadine-soaked membranes were both described with two time constants more than an order of magnitude apart, with a rapid (less than 1 s) initial relaxation response followed by a longer-term (tens of seconds) relaxation response. The time constants for these two relaxation responses were sensitive to the difference between control and altered amnion samples. The relaxation amplitudes corresponding to the two time constants were similar, suggesting that responses in both time frames are of equal importance. In addition, the amplitude terms depended on the magnitude of the attained peak force, highlighting the importance of performing tests over a range of loading conditions.

It has been reported that membranes stretch most rapidly at lower pressures [2]. The results of the current study have shown that there is indeed *more* relaxation in membranes stretched at smaller peak forces compared with larger forces, resulting in a smaller relative equilibrium force level for relaxation tests at small loads. However, there was no dependence on the relaxation rate, as measured by the time constants of relaxation, between tests conducted at different peak force levels.

The strain-to-failure tests here only provided an indirect measure of the fracture properties of amnion; such properties can be elucidated from strength tests, if the dominant flaw size and geometry is known, from direct observation of crack extension, under controlled loading conditions, or from compliance or hysteresis measurements, if both load and displacement are measured and a compliance function is known [17]. In all cases, in order to estimate the fracture resistance or toughness, the nonlinear and viscoelastic tensile properties of amnion need to be included into analysis of measurements to account for the distribution of energy in elastic and (time-dependent) plastic deformation separate from that dissipated in fracture. An empirical method for peforming this accounting has been demonstrated on a similar collagenous membrane [18], indicating substantial toughening on crack extension in a single edge-notched uniaxial loading geometry. Similar techniques should be developed for amnion, but extended to the more realistic biaxial geometry, including retained chorion on the entire CA membrane. Such measurements will provide guidance for the development of chemical treatments, adhesives and patches for intervention or repair of CA and related membranes.

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