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The stress relaxation characteristics of composite matrices etched to produce nanoscale surface features

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Abstract

Many synthetic and xenogenic natural matrices have been explored in tissue regeneration, however, they lack either mechanical strength or cell colonization characteristics found in natural tissue. Moreover natural matrices such as small intestinal submucosa (SIS) lack sample to sample homogeneity, leading to unpredictable clinical outcomes. This work explored a novel fabrication technique by blending together the useful characteristics of synthetic and natural polymers to form a composite structure by using a NaOH etching process that produces nanoscale surface features. The composite scaffold was formed by sandwiching a thin layer of PLGA between porous layers of gelatin–chitosan. The etching process increased the surface roughness of PLGA membrane, allowing easy spreading of the hydrophilic gelatin–chitosan solution on its hydrophobic surface and reducing the scaffold thickness by nearly 50% than otherwise. The viscoelastic properties of the scaffold, an area of mechanical analysis which remains largely unexplored in tissue regeneration was assessed. Stress relaxation experiments of the “ramp and hold” type performed at variable ranges of temperature (25 °C and 37 °C), loading rates (3.125% s⁻¹ and 12.5% s⁻¹) and relaxation times (60 s, 100 s and 200 s) found stress relaxation to be sensitive to temperature and the loading rate but less dependent on the relaxation time. Stress relaxation behavior of the composite matrix was compared with SIS structures at 25 °C (hydrated), 3.125% s⁻¹ loading rate and 100 s relaxation time which showed that the synthetic matrix was found to be strain softening as compared to the strain hardening behavior exhibited by SIS. Popularly used quasi-linear viscoelastic (QLV) model to describe biomechanics of soft tissues was utilized. The QLV model predicted the loading behavior with an average error of 3%. The parameters of the QLV model predicted using nonlinear regression analysis appear to be in concurrence with soft tissues.

Keywords

Stress relaxation; Composites; PLGA; Chitosan; SIS; Scaffolds

1. Introduction

Using biodegradable scaffolds that can support and guide the in-growth of cells have been a promising solution to regenerate tissue parts. Scaffolds generated from natural [1] and synthetic polymers or after removing the cellular components from xenogeneic tissues [2] have been used as scaffolds. Since naturally formed matrices such as small intestinal submucosa (SIS) are constrained by large-scale preparations of reliable, and reproducible products [3], forming synthetic matrices from biodegradable polymers has been an attractive alternative [4].

Synthetic polymers such as poly(glycolic acid), poly(lactic acid), PLGA and naturally derived polymers such as gelatin, chitosan and glycosaminoglycans have been explored in forming scaffolds [5]. However, the dearth of biomaterials that could form scaffolds eliciting controlled cellular responses with essential mechanical properties has necessitated search for novel biomaterials. Blending two polymers is an option to develop scaffolds with wide range of physicochemical properties and cellular interactions [5–9]. Recently, a composite matrix consisting of two porous compartments of chitosan reinforced with a thin membrane of PLGA was reported [10]. The composite was designed to mimic the biological properties, tensile properties and degradation characteristics of SIS. In this configuration, the mechanical and degradation properties can be tailored by selecting appropriate synthetic polymers. Further, biological properties can be tailored independently by altering natural polymer mixture.

Recent advances have shown that the physical properties of the scaffold such as pore size, and void fraction provide cues to guide cell colonization [11], apart from chemical properties such as cell-binding sites necessary for cell attachment. Surface features such as edges, grooves, and roughness also influence cell behavior [12,13]. For example, PLGA membranes etched to produce nanoscale surface features using NaOH are reported to improve cell adherence and proliferation. Furthermore, cells from various origins can react very differently to changes in architectures, such as pore features and topographies [13,14].

Cellular activity is also influenced by *scaffold stiffness* of the substrate [15–17]. Cells show reduced spreading and disassembly of actin even when soluble adhesive ligands are present in weak gels [18,19]. However, it is long recognized that majority of the tissues [20] and extracellular matrix elements in the body behave as viscoelastic materials rather than pure elastic materials [21]. Viscoelastic materials store and dissipate energy within the complex molecular structure, producing hysteresis and allowing creep and stress relaxation to occur. Hence, a full description of the mechanical response of materials requires nonlinear viscoelastic behavior. Interestingly, very few studies have been performed to understand the viscoelastic nature of porous structures used in tissue regeneration [22].

The objective of this study was to assess the stress relaxation properties of the composite scaffolds formed using PLGA membrane with and without the etching process, and after incorporating porous structure of chitosan and gelatin. Stress relaxation behavior of the composite scaffold was also compared to the SIS. A quasi-linear viscoelastic model widely used in biomechanics was adapted. These results show significant differences between composite scaffold and SIS in addition to the etching process.

2. Materials and methods

2.1. Sources for material

Chitosan (200–300 kDa molecular weight, Mw, 85% DO), Gelatin type-A (300 Bloom) are obtained from Sigma Aldrich Chemical (St. Louis, MO). 50:50 Poly(DL-lactide-co-glycolide), ester terminated (nominal) with 90–120 kDa molecular weight was obtained from LACTEL absorbable polymers (Pelham, AL). SLS-1-7 × 10 (10 × 7-cm rectangles) were obtained from Cook® Biotech Inc. (W. Lafayette, IN). Apper Ethyl Alcohol, 200 proof, absolute, anhydrous and chloroform was obtained from Pharmaco.

2.2. Composite layered scaffold fabrication

The PLGA composite scaffolds were prepared in the laboratory using a previously published method with modifications [10]. In brief, a 4% (wt/v) solution of PLGA was prepared in 5 mL of chloroform and allowed to air dry overnight in a chemical hood on a Teflon sheet (United States Plastic, Lima, OH) (6 cm × 8 cm) fixed to a flat aluminum plate. Formed PLGA film

was completely submerged in 1 N NaOH solution for 10 min based on initial analysis. Samples were washed with ample amount of water and holes were punched on the surface in a square pitch 1 cm apart from each other. The PLGA membrane was layered with 3 mL of 0.5% (wt/v) chitosan and 0.5% (wt/v) gelatin mixture dissolved in 0.7% (v/v) acetic acid on both sides and freeze dried to form the porous layer.

Samples were evaluated by JOEL 6360 Scanning Electron Microscope (Jeol USA Inc., Peabody, MA) at an accelerating voltage of 15 kV. Prior to analysis, all samples were subjected to dehydration through a series of increasing concentration of ethanol (0, 50, and 100%) followed by a brief vacuum drying. Samples were attached to aluminum stubs with carbon paint and sputter-coated with gold for 1 min at 40 mA. Images of the scaffold were taken with the scaffold lying normal to the surface of the stub in order to obtain cross-sectional images.

2.3. Cell culture

Human Foreskin Fibroblasts (HFF-1, cell line) were purchased from American Type Culture Collection (Walkersville, MD) and maintained in Dulbecco's modified Eagle medium supplemented with 4 mM L-glutamine, 4.5 g/L sodium bicarbonate, 100 U/mL penicillin-streptomycin, 2.5 g/mL amphotericin, and 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA). The cultures were maintained in 5% CO₂/95% air at 37 °C with medium changes every 48 h. Cells were detached using 0.05% trypsin – 10 μM EDTA was obtained from Invitrogen Corp., (Carlsbad, CA). Viable cell numbers were determined using trypan blue dye exclusion assay. 10,000 cells were seeded onto the control, and 25,000 cells were seeded onto the scaffolds. 0.5 mL of growth medium was added and the cells were incubated for 72 h.

After 72 h, samples were fixed in 3.7% formaldehyde for 30 min at room temperature. Samples were washed thrice with PBS, and permeabilized with –20 °C ethanol overnight at –4 °C. The samples were stained with Alexa Fluor 546 phalloidin (Molecular Probes, Eugene, OR) for 3 h at –4 °C in the dark. Samples were counterstained with DAPI following vendor's protocol (Invitrogen Corp.) and observed under an inverted fluorescence microscope (Nikon TE2000, Melville, NY) and digital micrographs were collected using the attached CCD camera.

2.4. Analysis of surface topography

Surface analysis of dry membrane was done using atomic force microscopy (AFM) using a DI Nanoscope IIIA Multimode Scanning Probe Microscope (Digital Instruments, Veeco Metrology Group, Santa Barbara, CA) at ambient conditions, as described previously [23]. In brief, sample films were attached onto iron AFM substrate disks using double-sided tape. Topographic images were obtained in tapping mode using commercial silicon microcantilever probes (MikroMasch, Portland, OR) with a tip radius of 5–10 nm and spring constant 2–5 N/m. The probe oscillation resonance frequency was ~120 kHz and scan rate was 1 Hz. Images were captured at different locations and the roughness factors were calculated using associated software (Nanoscope, Version 5). The roughness factor R_q is the root mean square average of 'n' number of height deviations (Z_i) taken from the mean data plane

$$R_q = \sqrt{Z_i^2/n} \quad (1)$$

2.5. Thickness characterization

Sample thickness was measured using the previously described procedure [3,10]. In brief, PLGA membranes were cut into small (~2 mm wide) strips, and oriented orthogonal to the plane of view of an inverted microscope so that the cross-section could be viewed. Digital micrographs of the cross-section were recorded using a CCD camera. The cross-sections were

measured using Sigma Scan Pro Software (Systat Software, Point Richmond, CA, USA) which was calibrated using an image of a hemocytometer. For each sample, thicknesses were measured at 40 or more locations and average values were used in all calculations.

2.6. Uniaxial tensile testing

Tensile testing was performed using the previously described procedure [3,7,10]. In brief, 5 cm × 1 cm strips were cut out and strained to break at 10 mm/min cross-head speed using INSTRON 5542 (INSTRON, Canton, MA). Experiments were conducted at 25 °C, hydrated in phosphate buffered saline using a custom made chamber built in-house. Using the associated software Merlin (INSTRON), break stress and strain were determined. The elastic modulus was calculated from the slope of the linear portion of the stress–strain curve.

2.7. Stress relaxation testing

Stress relaxation testing was performed under hydrated condition at 25 °C using the INSTRON 5542 machine on 5 cm × 1 cm strips. The load limits were pre-determined by the linear portion of the stress–strain curves obtained previously under uniaxial tensile testing. Samples were subjected to a constant step tensile strain applied at the rate of 3.125% s⁻¹ for 16 s and the sample was allowed to relax for 60 s, 100 s or 200 s. Four such successive ramp inputs were provided till the sample reached an extension of 200% of its original length.

While performing stress relaxation analyses on SIS, only 15% extension was used per ramp. This was based on limiting the cumulative stress for all four steps to less than the permanent deformation range of 85–90% as determined by the uniaxial tensile testing. For comparison, composite matrices were also strained at 15% extension per ramp.

2.8. Statistical analysis

All experiments were repeated three or more times with triplicate samples for each group. Significant differences between two groups were evaluated using a one way analysis of variance (ANOVA) with 95% confidence interval. When $P < 0.05$, the differences were considered to be statistically significant.

3. Quasi-linear viscoelastic (QLV) model development

The QLV model, first proposed by Fung [24], is commonly used to characterize soft biological tissues [25]. It has the capability of modeling materials with time dependent viscoelastic behavior that undergo large deformations. We need to perform time dependent stress relaxation tests and fit the constants of the function that characterize stress relaxation as well as large deformation. The problem faced by the QLV model earlier was that it was applied to cases where the input ramp strain was subjected instantaneously on the sample. But this problem was solved by Abramowitch and Woo who published a method to fit the QLV model using a ramp time with finite speed [26]. Because linear viscoelasticity did not adequately model the nonlinear tissue behavior, this model is applied to simple strain histories like a single step or a constant rate increase in strain which is then held constant while stress relaxation is observed. The resultant equation for the output stress is then solved in Excel using the Excel Solver tool. The stress values thus generated are then compared with the experimentally generated values.

QLV model description: The QLV theory assumes that the stress relaxation behavior of soft tissues can be expressed as

$$\sigma(t) = G(t) * \sigma^e(\varepsilon) \quad (2)$$

where $\sigma(t)$ is the stress at any time t , $\sigma^e(\epsilon)$ is the instantaneous elastic response (function of current strain and implicitly depends on time), i.e., the maximum stress corresponding to an instantaneous step input of strain ϵ , $G(t)$ is the reduced relaxation function that represents the time dependent stress response of the tissue normalized by the stress at the time of the step input of strain, i.e.,

$$G(t) = \frac{\sigma(t)}{\sigma(0^+)} G(0^+) = 1$$

According to the Boltzmann superposition principle the stress at any time t , $\sigma(t)$ is given by the convolution integral of the strain history and $G(t)$. The complete stress history then at any time is the convolution integral:

$$\sigma(t) = \int_{-\infty}^t G(t - \tau) \frac{\partial \sigma^e(t)}{\partial \epsilon} \frac{\partial \epsilon}{\partial \tau} d\tau \quad (3)$$

where $\partial \sigma^e(t)/\partial \epsilon$ represents the derivative of the instantaneous elastic response, $\partial \epsilon/\partial \tau$ is the strain history.

In the integral the lower limit $t = -\infty$ corresponds to zero-stress state. In practical experiments, the applied strain history begins from time $t = 0$, thus the equation modifies to

$$\sigma(t) = \int_0^t G(t - \tau) \frac{\partial \sigma^e(t)}{\partial \epsilon} \frac{\partial \epsilon}{\partial \tau} d\tau \quad (4)$$

For soft tissues, Fung proposed the following generalized reduced relaxation function equation based upon a continuous spectrum of relaxation

$$G(t) = \frac{\left[1 + \int_0^\infty S(\tau) e^{-t/\tau} d\tau \right]}{\left[1 + \int_0^\infty S(\tau) d\tau \right]} \quad (5)$$

where $S(\tau)$ represents the continuous relaxation spectrum. The general form of the stress response (in terms of the second Piola–Kirchhoff stress) is the hereditary integral equation. $S(\tau)$ has the form

$$S(\tau) = \begin{cases} \frac{C}{\tau} & \text{for } \tau_1 < \tau < \tau_2 \\ 0 & \text{for } \tau < \tau_1, \tau > \tau_2 \end{cases} \quad (6)$$

C is a dimensionless material parameter that reflects the magnitude of viscous effects present and is related to the percentage of relaxation. τ_1 and τ_2 are time constants that regulate the short and long term material responses, relating to the slope of the stress–relaxation curve at early and late time periods, respectively. Eq. (7) can be rewritten as

$$G(t) = \frac{1 + C [E_1(t/\tau_2) - E_2(t/\tau_1)]}{1 + C \ln(\tau_2/\tau_1)} \quad (7)$$

$E_1(t/\tau)$ is the exponential integral function of the form

$$E_1(t/\tau) = \int_{t/\tau}^{\infty} \frac{e^{-z}}{z} dz$$

If τ_1 and τ_2 differ sufficiently such that $\tau_1 \ll t \ll \tau_2$, then Eq. (7) can be written as

$$G(t) = \frac{1 - C\gamma - \ln(t/\tau_2)}{1 + C\ln(\tau_2/\tau_1)} + 0(C) \quad (8)$$

where γ is the Euler constant which has a value of 0.5772. $0(C)$ is small and can be neglected. Thus

$$G(t - \tau) = \frac{1 - C\gamma - \ln((t - \tau)/\tau_2)}{1 + C\ln(\tau_2/\tau_1)} \quad (9)$$

The instantaneous stress response is given by an exponential approximation

$$\sigma^e(\varepsilon) = A(e^{B\varepsilon} - 1) \quad (10)$$

where A is the elastic stress constant with units of stress (MPa), and B is the elastic power constant. Both A and B are determined by curve fitting equation (10). In this study, the engineering stress (MPa)–engineering strain (mm/mm) graph for the loading portion of the experimental data was curve fitted in Sigma Plot software using the user defined equation to determine A and B (Table 1).

The derivative of the nonlinear elastic function with respect to strain is calculated as

$$\frac{\partial \sigma^e(\varepsilon)}{\partial \varepsilon} = \frac{\partial A(e^{B\varepsilon} - 1)}{\partial \varepsilon} = AB e^{B\varepsilon} \quad (11)$$

From $t=0$ to $t=t_0$ (referred as the loading part), there is constant strain rate of loading α and the total strain at any time during the ramp is $\alpha\tau$. Thus, for this time frame the strain time history is

$$\frac{\partial \varepsilon}{\partial \tau} = \alpha \quad (12)$$

While performing experiments, $\alpha = 3.125\% \text{ s}^{-1}$. Over the time period $t = t_0$ to the end of the testing period (referred as relaxation part), the strain level is held constant and thus:

$$\frac{\partial \varepsilon}{\partial \tau} = 0 \quad (13)$$

Upon substituting the equations of the reduced relaxation function and the instantaneous stress response in the convolution integral and solving the integral analytically, separately both for the loading and the relaxation part of the stress relaxation experiment with the limits for the

loading part being 0 to t and that for the relaxation part being 0 to t_0 we obtain the following equations for the output stress:

Loading part:

$$\sigma(0 < t < t_0) = \frac{A}{1 + C \ln\left(\frac{\tau_2}{\tau_1}\right)} \left[(1 - C\gamma)(e^{Bat} - 1) + C \ln t - CB^2\alpha^2 e^{Bat} \ln t - Ce^{Bat}(e^{-Bat} - 1) \ln \tau_2 \right]$$

Relaxation part:

$$\sigma(t \geq t_0) = \frac{A}{1 + C \ln\left(\frac{\tau_2}{\tau_1}\right)} \left[(1 - C\gamma)(e^{Bat_0} - 1) + C \ln t - Ce^{Bat_0} \ln(t - t_0) + CB^2\alpha^2 e^{Bat} (\ln(t - t_0) - \ln t) - C(1 - e^{Bat_0}) \ln \tau_2 \right]$$

The analytically derived equations for the loading and the relaxation parts were used to determine C , τ_1 and τ_2 by performing a nonlinear regression analysis of the respective experimental data using the MS Excel Solver tool and also to determine the stress values at different time. These stress values were designated as the model determined values which were compared with the experimental data for a specific time.

The sum squared deviations (SSD) were mathematically defined for each portion as

$$SSD = \sum \text{Abs}(\sigma_{\text{model data}}^2 - \sigma_{\text{experimental data}}^2)$$

The loading SSD function was denoted as

$$SSD_{\text{loading}} = f(A, B, C, \tau_1, \tau_2, t)$$

The relaxation SSD function was denoted as

$$SSD_{\text{relaxation}} = f(A, B, C, \tau_1, \tau_2, t, t_0)$$

The goal was to minimize the SSD_{loading} and the $SSD_{\text{relaxation}}$ functions by using the MS Excel solver. The value of A , B and t_0 remained constant for both the loading and the relaxation parts and hence the only values that the solver function changed were C , τ_1 and τ_2 . To assess the global minimum, random initial values of C , τ_1 and τ_2 were chosen and the SSD value generated was noted. Several such trials were made wherein random values for the constants are selected from a range for $C = 0-1$, $\tau_1 = 0-50$, and $\tau_2 = 0-10,000$. The C , τ_1 and τ_2 values that resulted in the minimum SSD were obtained.

4. Results

4.1. Effect of etching on roughness

To assess the effect of etching on the surface of the PLGA, membranes were analyzed via SEM. These results showed (Fig. 1A–C) significant difference between unetched and 10 min etched samples. After 15 min etching, clear indentations were visible relative to 10 min sample. However, 5 min of etching did not have significant influence on the surface (data not shown)

roughness and appeared more like unetched sample. Hence, 5 min etching time was not pursued in subsequent analyses.

Surface topographies were assessed by AFM to better understand roughness characteristics. These results showed a marked difference between surface roughness characteristics of the untreated and treated samples (Fig. 1D–F). Air dried PLGA membranes showed an average roughness of 10 nm. After etching for 10 min, the roughness increased to more than 60 nm. After 15 min incubation, the roughness increased to ~70 nm. However, there was no significant difference between 10 min incubation and 15 min incubation.

When formed composite was evaluated under SEM (Fig. 1G), a very good contact between the nonporous PLGA membranes and the porous chitosan–gelatin region was observed. The dry thickness of these composite scaffolds was less than 1 mm and the PLGA membrane contributed only 50 μm . A significant reduction in thickness was achieved relative to composites formed without etching [10]. This reduction is attributed to the roughness created by the etching process which facilitated easy distribution of chitosan–gelatin solution. No separation of layers occurred when the samples were neutralized with ethanol and hydrated in PBS, suggesting that the composite was firmly anchored at the perforations.

4.2. Cell colonization

Cell morphologies were evaluated to understand whether composite matrices support cell colonization. Cytoskeletal organization of HFF-1 was probed via actin staining. These results (Fig. 1H and J) showed that on 3D composite matrices, HFFs showed well spread spindle shape, similar to previous publications [10,27]. HFFs showed peripheral distribution of actin filaments, similar to cells on TCP surface. Counterstaining with DAPI confirmed the presence of nuclei, along with the actin fiber, suggesting that the composite structure promotes cell attachment. The cells were observed in different planes (as seen in out of focus cells in Fig. 1J), suggesting the possibility of colonization in three-dimension. In addition, cellular attachment mimicked the pore morphologies of the scaffold, showing that cell spreading and adhesion appeared to be guided by the porous structure.

4.3. Tensile test analysis

Effect of etching on tensile properties of PLGA membranes has not been understood. Hence, the effect of etching time on the tensile properties was assessed. These results (Fig. 2) showed that the material failed at near about 400–500% of its original extension which is nearly 6–10 times more than many naturally available matrices such as SIS. Previously, it has been shown that the composites formed using PLGA membrane mimic the tensile properties of PLGA. The maximum tensile stress of the material under the tensile loading was in the range of 3.5–4 MPa. With 15 min etching the percentage strain before failure decreased suggesting that the elastic properties of PLGA could be affected. However, 10 min etching did not show significant difference in the percentage strain before failure.

To better understand the etching process, elastic modulus (E) was approximated using a linear relationship in the initial region of the stress–strain curve. These results showed (Table 1) that the 10 min etching increased the elastic modulus, relative to unetched samples, suggesting an increase in hardness. Fifteen minute etching showed no significant difference from the 10 min etched samples.

4.4. Stress relaxation analysis

In order to understand the effect of variable loading time (fast and slow), effect of temperature and also the effect of relaxation time on the stress relaxation behavior, unetched PLGA membrane was tested under

- a) two different strain rates of $3.125\% \text{ s}^{-1}$ and $12.5\% \text{ s}^{-1}$,
- b) three different values of relaxation times were used (60 s, 100 s, and 200 s), and
- c) two different environmental temperatures (25°C and 37°C) in hydrated conditions.

It was observed (Fig. 3A) that the general behavior of unetched PLGA membranes under variable inputs remained the same. Under the similar conditions of temperature and relaxation time (25°C and 100 s) the magnitude of the initial tensile stress changes significantly with changing the rate of loading from $3.125\% \text{ s}^{-1}$ – $12.5\% \text{ s}^{-1}$, suggesting that the rate of loading does have a significant effect on the behavior of PLGA membranes. However, upon changing the relaxation time from 60 s to 100 s, a significant change in the stress accumulation was observed; value of the maximum stress for each cycle reduced with increased relaxation time. However, increasing the relaxation time to 200 s did not change the values considerably as compared to 100 s. Environmental temperature at which the experiments were conducted had a significant effect on the maximum stress reached in each cycle; stresses were lower in magnitude at 37°C than at 25°C . However, the behavior of PLGA membranes still remained the same. Hence all subsequent experiments were conducted at 25°C with a loading rate of $3.125\% \text{ s}^{-1}$ and a relaxation time of 100 s or 60 s.

Next, stress relaxation experiments performed with a fast rate of loading and a relaxation time of 100 s showed (Fig. 3B and C) no significant difference between the unetched, the etched and the composite sample. At a reduced value of relaxation time of 60 s, it was observed that the composite as compared to the unetched sample displayed a much lesser value of the maximum tensile stress and also showed better relaxation behavior with time. This shows that the composite will deform to a much lesser extent in the long run.

4.5. Comparison to SIS

To understand the stress relaxation behavior of the synthetic composite, it was compared to that of small intestinal submucosa (SIS). Stress relaxation experiments were performed on both the composite and the SIS under similar conditions with 15% loading and 100 s relaxation for 4 successive ramps. 15% was chosen as the value of extension for the loading for each ramp because SIS fails at approximately 85–90% of tensile strain. These results (Fig. 3D) show that initially, for the first couple of ramps, the SIS relaxes much faster than the composite; but as the number of ramps increases and in turn the tensile strain increases, the relaxation of the composite is much better. Thus the composite has better relaxation behavior in the long run. Soft tissues are known to strain harden in successive cycles, and SIS also showed similar behavior. As the material was stretched in the first cycle, the stress developed was significantly less than that developed in the fourth cycle. On the contrary, the stress developed in the first cycle for the composite was significantly higher relative to the fourth cycle. This suggests that the composite behaved more like a strain softening material.

4.6. Quasi-linear viscoelastic model

To understand the viscoelastic behavior first, standard linear model was used. The curve fit of the relaxation part of the experiment showed a significant disagreement between experimental results and model results. Hence PLGA membranes did not behave like a standard linear solid. In order to better understand the observed experimental behavior, a QLV model typically utilized in biomechanics was evaluated. The curve fitting results of the QLV model for the unetched, the etched and the composite sample are as shown in Fig. 4. The QLV model is successful in modeling the experimental data and the values of the constant parameters of the model were found by nonlinear regression and curve fitting. These results show (Table 2) that the relaxation times and the equation constants are similar for the etched and the composite but significantly different for the unetched sample. Although the process of etching did not

affect the tensile properties of PLGA, it does affect the viscoelastic stress relaxation behavior. The value of the constant C decreased considerably with the etching process, suggesting that the material is losing its viscous component. However, the process of freeze-drying and scaffold formation did not affect the viscoelastic properties of the PLGA membrane. While determining the model parameters, the τ_2 value of the loading part of the etched sample was not in the range which could be attributed to an error in the optimization routine to detect the global minimum.

5. Discussion

This work explored a novel method of generating composite scaffolds with reduced thicknesses using the technique of etching which produces nanoscale surface features for tissue engineering applications. Composite matrices consisting of two porous compartments of chitosan–gelatin reinforced with a thin membrane of PLGA were formed. Porous compartment provides scaffolding for multilayered cell growth, while the membrane layer provides mechanical strength. In this configuration, the mechanical properties and degradation properties can be controlled by selecting appropriate synthetic polymers. This etching process increased the surface roughness of PLGA, similar to other published reports [28,29], allowing easy spreading of natural polymers on its surface.

Tensile testing analysis showed that 10 min of this etching process did not significantly affect the tensile properties of PLGA while providing sufficient roughness. This condition was used to form composite structures and the formed composites also showed no difference in the tensile behavior of the PLGA scaffold. Human fibroblasts showed spreading characteristics and colonization in three-dimension, similar to our previous publications using chitosan or chitosan–gelatin scaffolds [6,10,27,30]. Previously, we have shown that cells effectively colonize these porous structures and are functional for tissue engineering purposes.

The initial portion of the tensile testing curve displays a nonlinear material behavior. To gauge the behavior of the synthetic matrix representative “ramp and hold” type of tests were carried out at variable set of conditions of temperature (25 °C and 37 °C), slow and fast loading rate (3.125% s⁻¹ and 12.5% s⁻¹) and different relaxation times (60 s, 100 s and 200 s). It was observed that that temperature significantly affects the maximum instantaneous stress values, which are much lower at 37 °C than at 25 °C as expected because the material is now much nearer to its glass transition temperature T_g . The loading rate too has a similar effect on the synthetic PLGA scaffold, the stress values being much lower at 3.125% s⁻¹ than at 12.5% s⁻¹. Both these behaviors are different from that observed for PCL by Duling et al. [22]. The relaxation time, however, does not significantly affect the overall material behavior.

Stress relaxation experiments (ramp and hold) showed wide differences in the stress accumulation of unetched samples to the etched and composite samples, though the overall physical behavior remained similar. Interestingly, the material behavior under stress relaxation for the ramping portion of synthetic matrices (concave downwards) was opposite to that of soft tissues (concave upwards) like ligaments, tendons, etc. [22,31–34]. The stress accumulation for the successive ramps for the synthetic matrix decreased, indicating that the material is softening under the application of constant strain (strain softening) which is found to be in contrast to the natural matrix SIS (strain hardening), which is predominantly made up of collagen type I. Similar curves as that of collagen are obtained with numerous other soft tissues which again are made up of type I collagen [32–35].

The QLV model as proposed by Fung is applied to soft tissues with instantaneous loading, by incorporating the modifications made by Abramowitch and Woo, this can be successfully applied to synthetic membranes (PCL and PLGA) with slow loading as well [22]. The QLV

model was adopted to understand this nonlinear behavior. The model predicts the loading portion of the output curve with an average deviation of 3% or less but gives a relatively poor fit of the relaxation curve. This could be because of the fact that the same A and B constants predicted from the loading portion are used in the relaxation part as well. Better fits have been achieved using a modified Fung's reduced relaxation model with a decaying exponential equation. The values of A (0.5–1 MPa) and B (5–10), the constants of the elastic stress response function were found to be in the similar range in comparison to other soft tissues but with a negative sign, which could be attributed to the use of engineering stress and engineering strain values for curve fitting the exponential equation. Positive A and B values are obtained on using true stress and true strain instead of engineering stress and engineering strain, as seen in some of the previous works [35,36]. The values of the other constants C (~0.01–0.5), τ_1 (0.5–25 s) and τ_2 (1000–7000 s) are in the similar range as that of other soft tissues except for a few discrepancies as in the case of the relaxation behavior of both the etched and the composite samples. This can be because the Excel solver tool does not converge upon a global minimum based upon the range of initial guess provided. Hence there is a need for a more robust and accurate algorithm to detect the correct global minimum.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix

Figures with essential colour discrimination. Parts of Figure 1 of this article may be difficult to interpret in black and white. The full colour image can be found in the on line version, at doi:10.1016/j.biomaterials.2008.10.023.

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Fig. 1.

Effect of etching on surface roughness and composite scaffolds. (A–C) Scanning electron micrographs of the unetched, 10 min etched and the 15 min etched sample. (D–F) Atomic force micrographs (AFM) unetched, 10 min etched and the 15 min etched sample. (G) Scanning electron micrograph of the cross-section of the composite. (H) Fluorescence micrograph of fibroblasts on TCP. (J) Fluorescence micrograph of fibroblasts on the composite scaffolds.

Fig. 2.
Effect of etching on the tensile test properties of PLGA.

Fig. 3.

Stress relaxation behavior of PLGA membranes. (A) Effect of loading rates ($3.125\% \text{ s}^{-1}$ and $12.5\% \text{ s}^{-1}$), relaxation times (60 s, 100 s and 200 s) and different temperature values ($25\text{ }^{\circ}\text{C}$ and $37\text{ }^{\circ}\text{C}$). (B) Effect of etching and scaffold formation at $3.125\% \text{ s}^{-1}$ loading rate, $25\text{ }^{\circ}\text{C}$ and 60 s relaxation time. (C) Effect of etching and scaffold formation at $3.125\% \text{ s}^{-1}$ loading rate, $25\text{ }^{\circ}\text{C}$ and 100 s relaxation time. (D) Comparison of stress relaxation behavior of SIS with the composite scaffold at $3.125\% \text{ s}^{-1}$ loading rate, $25\text{ }^{\circ}\text{C}$ and 100 s relaxation time.

Fig. 4. Comparison of QLV model predictions with experimental data for the first ramp. (A) Unetched PLGA membrane, (B) etched PLGA membrane and (C) the composite scaffold.

Table 1

Young's modulus values for the tensile testing done on the unetched, the 10 min etched and the 15 min etched samples.

Sample type	Young's modulus (MPa)
Unetched	1.228 ± 0.040
10 min treated	2.113 ± 0.090
15 min treated	1.859 ± 0.135

Table 2

Constants obtained in QLV model.

Sample type	A (MPa)	B	C	τ_1 (s)	τ_2 (s)	Sum squared deviation (SSD)
Unetched	-0.994	-5.221				
Loading		0.125		129.344	3005.56	0.011
Relaxation		0.639		23.910	4438.80	0.134
Etched	-0.845	-6.113				
Loading		0.092		26.379	154.173	0.010
Relaxation		-0.064		4993.870	9996.488	0.962
Composite	-0.936	-6.579				
Loading		0.010		24.3699	6314.453	0.002
Relaxation		-0.070		4206.225	6711.053	1.152