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Abstract

Viscoelastic characteristics of acellular dermal matrix (ADM) preparations with various additives were analyzed with creep curves, stress-strain relationships, and the storage modulus with reference to those of ADM preparations crosslinked with glutaraldehyde. Creep curves for all ADM preparations were determined to comply with the Kelvin-Voigt model. The stress-strain plots of all ADM preparations compared were described as linear. The storage modulus of all ADM preparations was maintained at a nearly constant level throughout the range of oscillating frequencies applied. ADM preparations crosslinked with glutaraldehyde showed that both Young's modulus (E) for the spring part and retardation time (τ) in the Kelvin-Voigt model, and hence viscosity (η) for the liquid part, increased with an increasing concentration of glutaraldehyde. Higher Young's modulus and viscosity and a greater extent of the 'solid' response of ADM preparations crosslinked with glutaraldehyde might have been responsible for the longer persistence that was demonstrated after implantation. The increase in ADM concentration and the addition of various additives to ADM preparations, including α-hydroxy acid (citric acid, lactic acid, and glycolic acid) and hyaluronic acid, resulted in similar effects on the viscoelastic characteristics of the ADM preparations, but they were less efficacious compared to those crosslinked with glutaraldehyde. Among them, increasing ADM concentration to higher than 200 mg/mL and addition of glycolic acid at a concentration of greater than 2% improved the viscoelastic characteristics of the resulting ADM preparations such that their level of persistence was closer to that of material crosslinked with glutaraldehyde. On the contrary, the influence on viscoelastic characteristics of adding PVP greatly differed from that of hyaluronic acid and was only apparent when adding concentrations of PVP of greater than 10%. Similarly, viscoelastic characteristics of the ADM preparations examined were also so sensitive to temperature that the persistence of ADM preparations after implantation at body temperature would deteriorate.

中文摘要

利用動態機械分析儀來測試這些可供注射用的無細胞皮膚基質的流變特性。測試的項目包括，潛變曲線，應力應變曲線和儲存模數，我們藉由這些測試的結果來討堅持度的問題。除了測試不同濃度的可供注射用的無細胞皮膚基質的流變特性，還測試 α-hydroxy acids 和其他不同添加物對於流變特性的影響。實驗之後發現，以添加 2 %的甘醇酸其延滯時間最長，所以會呈現最強的剛性和最好的堅持度。因為物質的 τ 值越高，會呈現越固結的特性，相反的，τ 值越低，會呈現越偏流體的特性。而堅持度是影響臨床效果的一個重要指標。另一方面，溫度也會影響流變學的實驗結果。生體相等性試驗藉由與纖維母細胞（3T3）共同培養的體外細胞毒性測試來確定，由觀察細胞的生長狀況和速度來當作細胞毒性的定性判斷。結果發現在實驗期間並沒有發現細胞型態的改變，因此無細胞皮膚基質具有良好的生體相容性，可當作軟組織填充物的發展材料。

Keywords: Acellular dermal matrix, Creep curve, Stress-strain relationship, Storage modulus, α-Hydroxy acid, Hyaluronic acid.

Introduction

Collagen films and sponges developed in recent years for use in various biomedical devices generally require surgery to accomplish specific clinical needs. With the shift toward minimally invasive surgical procedures, the development of injectable implants has become the focus of research interest. Injectability obviates the need for incisions and surgical dissection; in addition, this would allow for more-precise contouring of the implant to suite patients' needs. Recent use of collagen as an injectable material was reviewed by Matton et al. [1]. The clinical use of these injectable materials has been expanded from cosmetic applications [2] and urinary incontinence [3,4] to soft tissue augmentation such as vesicoureteral reflux [5] and laryngeal rehabilitation [6].

The most commonly used injectable materials for soft tissue replacement to date are derived from either cross-linked bovine collagen, autologous skin, or fat tissue. Once injected, bovine collagen persists for an average of only 3-6 months [7,8]. Furthermore, injectable autologous fat has been reported to persist for a shorter period of time than bovine collagen [7]. Mainly, all these injectable implant materials require a mechanical function as indicated by maintaining the size and shape of the implant at the site of injection, designated as persistence of the implant materials. Therefore in addition to persistence, the ideal injectable implants for soft tissue replacement material should be composed of safe, readily available, off-the-shelf, nonmigratory, and easy-to-use materials that match the tissue at the injection site as closely as possible.

Injectable implants based on concentrated viscoelastic collagen formulations first attracted attention due to their improved persistence without sacrificing injectability. However, since these types of implant are free to flow between planes of the host tissue, there is an eventual loss of implant efficiency in augmentation and loss of focal placement control. Slightly higher resistance to flow has been obtained using crosslinking [9,10], which is also known to slow the bioresorption of collagen [11]. Attempts have also been made to cross-link collagen solutions *in situ* after implantation using temperature-activated or photosensitization methods. Laude et al. recently reported a unique collagen formulation that is injectable and compacts after implantation, achieving completely focal applications without crosslinking [12].

Currently, owing to its excellent results in a variety of biomedical applications, the study and performance of acellular dermal matrix, derived from full-thickness skin treated to remove cells and cellular components but retaining the native dermal structure, have attracted attention from many fields [12,13]. Many recent studies have developed several methods for producing acellular dermal matrices (ADMs) from various types of skin. These methods generally include treatment with trypsin, freeze-thawing, and prolonged incubation with enzymes [14,15]. Two commercial products of AlloDerm (derived from human dermis) and XenoDerm (derived from porcine dermis) so processed are available for clinical applications. Micronized or particulate AlloDerm is produced from AlloDerm. It has been reported that processing AlloDerm in liquid nitrogen to obtain the micronized form resulted in less damage to the collagen matrix, and it exhibited longer persistence [13]. This particulate matrix also exhibits rapid repopulation by host cells that should enhance revascularization and remodeling. Previously, a method of processing porcine skin to produce an

acellular dermal matrix (ADM) for biomedical applications was developed and optimized [16]. In this study, the viscoelastic characteristics of ADM so obtained in micronized form were examined, and the influence of various additives on the viscoelastic characteristics was compared with reference to these ADM preparations crosslinked with glutaraldehyde.

Experimental Methods

Acellular Dermal Matrix (ADM)

ADM samples were produced following a method reported previously [16]. In general, fresh porcine skin was obtained from a slaughterhouse. After complete cleaning, cutting of the subdermal fat tissue, and removal of hair, the resulting skin was kept at –20 °C until use. Porcine skin was cut into pieces of 10 x 7 x 0.3 cm^3. The epidermis of the skin was removed by treatment with a 0.25% Trypsin solution at 25 °C for 18 h, and then the dermal part was cut into pieces of 0.5 x 0.5 x 0.3 cm^3. This was followed by treatment with 0.25% Trypsin solution with shaking at 25 °C for 12 h. Porcine skin in both subgroups was then washed with a 0.1% SDS solution at room temperature for 12 h, followed by 560 unit/L of a Dispase solution at 25 °C for 12 h. Sequentially, porcine skin was washed with 0.1% SDS at room temperature for 12 h and then washed with PBS buffer twice (each for 15 min). Samples were freeze-dried and kept in desiccators until used. Gentamicin at 10 μg/mL was added to all solutions used to prevent bacterial growth.

Injectable Implants of ADM Preparations

Before conducting rheological characterizations by dynamic mechanical analysis, the matrices were homogenized with 0.9% sterile saline, then adjusted to the required concentrations. Concentrations of ADM of from 50 to 200 mg/mL in increments of 50 mg/mL were prepared in saline solution. Various additives, including α-hydroxy acids (citric acid, lactic acid, and glycolic acid), hyaluronic acid (0.3%, 0.5%, 1%, and 2% w/w), and polyvinyl pyrrolidone (10%, 20%, and 40% w/w), were added during the process of homogenization at an ADM concentration of 100 mg/mL in saline solution. The added concentrations for citric acid and lactic acid were 2%, 5%, 7%, and 10% w/w, while they were 0.5%, 1%, 2%, 5%, and 10% w/w for glycolic acid. Crosslinked ADM preparations were also prepared by the addition of 0.025%, 0.05%, and 0.1% glutaraldehyde in an ADM solution at a concentration of 100 mg/mL.

Rheological Measurements of ADM Preparations

Rheological characteristics of injectable ADM preparations were measured using a Dynamic Mechanical Analyzer (Perkin Elmer, DMA7e) by monitoring the creep curve, stress-strain relationship, and storage modulus. The cup and plate mode was used in all measurements. A sample volume of 0.5 mL was put into the cup, and then a constant force of 1 mN was applied by compressing the plate for 4 min. The creep compliance plot of J(t) (defined as the strain per unit applied stress: $\gamma(t)/\sigma$) versus time was constructed, and an appropriate model for describing these curves was fitted statistically using mathematical models provided by Sigmaplot (Sigma, USA). Parameters of Young's modulus (E), retardation time (τ), and viscosity (η) were calculated for each sample for comparison. The stress-strain relationship for the same sample was measured by the same cup and plate mode in the DMA instrument. A sample volume of 0.5 mL was added to the cup. A force of from 0 to 4 mN was

applied from the plate by increasing increments of 1 mN/min. When the storage modulus was measured, a 25-mN dynamic force and 30-mN static force were applied to the sample of 0.5 mL in the cup from the plate with increasing oscillating frequencies of from 1 to 2 Hz. The storage modulus was calculated correspondingly, and the plot of the storage modulus versus frequency was constructed.

Results and Discussion

Many polymeric systems exhibit behaviors that combine both elastic (solid) and viscous (liquid) properties, in which the applied stress is proportional to both the resultant strain and the rate of strain [17-19]. Such systems are termed viscoelastic, which has been defined as the 'simultaneous existence of viscous (liquid) and elastic (solid) properties in the materials' [20]. The viscoelastic behaviors of two injectable forms of collagen, nonfibrillar concentrated solutions and reconstituted fibrillar suspensions, have been investigated with shear creep experiments in a parallel-plate apparatus [15]. Extrusion-force measurements on crosslinked collagen suspensions have been used to characterize the rheological properties. The effect of hydrophobic forces on the rheology of concentrated dispersions of collagen fibers in aqueous solution was studied by dynamic rheological measurements over temperatures ranging from 283 to 308 °K [21]. Nonlinear viscoelastic properties of bovine skin collagen gel solutions (diluting 3 mg/mL Vitrogen to 1 or 1.5 mg/mL with DMEM) have been revealed with the use of a servo-controlled linear activator to impose an axial strain on the gel [22]. Mechanical characteristics of engineered collagen fibrils (ECF) have been evaluated from compression and permeability experiments [12]. In this study, the cup and plate mode of dynamic mechanical analysis was applied for the rheological characterizations of ADM preparations.

Since crosslinking of the polymeric nature of collagen with glutaraldehyde was able to slightly increase the resistance to flow between the planes of the host tissue [9,10], improvement in the persistence with crosslinking is evident. Therefore, ADM preparations crosslinked with glutaraldehyde were selected as a reference to compare rheological characteristics for these ADM preparations containing various additives. Figure 1 illustrates the creep curves for ADM preparations crosslinked with various concentrations of glutaraldehyde. The creep compliance plot of $J(t)$ ($\gamma(t)/\sigma$) versus time (t) describes the strain of a viscoelastic body with respect to an applied stress. The mechanical model of a viscoelastic material showing both viscosity of a liquid state and elasticity of a solid state, which can be represented in two different ways by Maxwell and Voigt elements, may be combined into a generalized model to incorporate all possibilities of flow and deformation of non-Newtonian materials. Based on this, the best fitting equation for these curves in Fig. 1 was found to be $y = a^*(1 - e^{-bx})$, which is consistent with the Kelvin-Voigt model represented as $\frac{\gamma(t)}{\sigma} = \frac{1}{E}(1 - e^{-\frac{t}{\tau}})$. Correspondingly, E, Young's (elastic) modulus of spring in the Kelvin-Voigt model, is equal to $1/a$; τ, which is called the retardation time, is equivalent to $1/b$; and η, the viscosity of the dashpot in the Kelvin-Voigt model, is given as $1/ab$ ($= \tau^*E$). Table 1 lists these corresponding parameters, including E, τ, and η, for ADM preparations crosslinked with various glutaraldehyde concentrations. It demonstrates that both E and τ profoundly increase with increasing concentrations of glutaraldehyde, leading η ($= \tau^*E$) to increase with increasing concentrations of

glutaraldehyde.

In the Kelvin-Voigt model, with the spring (solid part) and dashpot (liquid part) attached in parallel, the drag of the viscous fluid in the dashpot (η) simultaneously influences the extension and compression of the spring which characterizes the solid nature of the material (E), while the strain varies in an exponential manner with time. Because of that, a retarded elastic deformation exists in the process, and τ is referred to as the time for retarded elastic recovery. When τ is smaller, a faster decay is observed for a material in a more-liquid-like state, whereas a material in a more-solid-like state will maintain a higher strain value when τ is larger. Therefore, increases in Young's (elastic) modulus (E) for the spring (solid part) and viscosity (η) for the dashpot (liquid part) in the Kelvin-Voigt model, which best describe creep curves of ADM preparations with increasing concentration of glutaraldehyde, reveal that both solid and liquid parts of ADM preparations become more resistant to deformation with an increasing extent of crosslinking by glutaraldehyde. Furthermore, the increase in τ with an increasing concentration of glutaraldehyde demonstrates that the extent of the 'solid' part in ADM preparations increases with an increasing extent of crosslinking with glutaraldehyde. The persistence of collagen crosslinked with glutaraldehyde is possibly attributable to these viscoelastic characteristics.

The stress-strain relationship for ADM preparations crosslinked with glutaraldehyde at various concentrations is shown in Fig. 1B. A linear relationship can be seen for all three concentrations of glutaraldehyde used to crosslink with ADM. The slope of this linear plot reveals the extent of deformation with respect to the unit stress applied. Accordingly, the slope decreases with increasing concentrations of glutaraldehyde, indicating that the resistance to deformation increases with the increasing extent of crosslinking of ADM with glutaraldehyde. This means that the crosslinking of ADM preparations with glutaraldehyde makes them more solid-like, which resists deformation with respect to an applied stress [15]; this function was concentration dependent. The increasing spring effect by linking collagen fibers with glutaraldehyde might be responsible for the increasing extent of the spring part in ADM preparations.

Figure 1C presents the plots of the storage modulus (E') versus the oscillating frequency (Hz) for ADM preparations crosslinked with different concentrations of glutaraldehyde. E' of ADM preparations increases with increasing concentrations of glutaraldehyde. However, the smallest change in E' with respect to the oscillating frequency in the range examined was observed. E' was measured by simultaneously applying static and dynamic forces to the materials with an oscillating frequency which increased from 1 to 2 Hz. E' represents the energy stored per cycle within the sample, i.e., the 'solid' response [23]. Therefore, the higher E' is, as a result of the increased extent of crosslinking with glutaraldehyde, the greater the extent of rigidity or a solid-like status that a material will possess.

Figure 2 illustrates the creep curves (A), stress-strain relationships (B), and storage modulus measurements (C) for ADM preparations containing various concentrations of ADM. Table 1 also lists the corresponding values for E, τ, and η. It similarly demonstrates that both E and τ increase with increasing concentrations of ADM, leading η to increase with increasing concentrations of ADM such as those

crosslinked with glutaraldehyde. Increases in both Young's (elastic) modulus for the spring part and viscosity for the dashpot part with increasing concentration of ADM reveal the resistance of ADM preparations to deformation with increasing concentrations of ADM. The increase of τ from 1.4 min for the ADM preparation at 50 mg/mL to 6.6 min for ADM at 200 mg/mL implies that the liquid-like state at a low concentration of ADM is transformed to a solid-like state at a high concentration of ADM. However, the effects on the increase of these viscoelastic characteristics are more profound when crosslinking with glutaraldehyde than when simply increasing the ADM concentration. Incorporating a higher concentration of ADM in ADM implants would be expected to produce a similar persistence to those crosslinked with glutaraldehyde.

The stress-strain relationship for ADM preparations of various concentrations is shown in Fig. 2B. A linear relationship was demonstrated for all four concentrations of ADM examined. The slope of this linear plot reveals the extent of deformation with respect to the unit stress applied. Accordingly, the slope decreases with increasing concentrations of ADM, indicating that the resistance to deformation increases with increasing concentrations of ADM. This means that increasing the ADM concentration in a preparation makes it more solid-like, and it can thus resist deformation by an applied stress. A linear relationship of strain with respect to the applied stress also reveals that the fewest fiber-fiber interactions leading to entanglement of collagen fibrils exist, or that there is no disruption of the even distribution of collagen fibrils due to there being no tendency of reaching a plateau or suddenly decreasing. Possibly, this is due to the fact that ADM is suspended in saline solution with only a minimal amount of dissolved collagen. Nevertheless, the changing extent of strain with respect to the applied stress is lower (or more resistant) in ADM preparations containing as high as 200 mg/mL than that with crosslinked ADM at a concentration of 100 mg/mL and the highest amount of glutaraldehyde (0.1%). Figure 2C presents the plots of storage modulus (E') versus frequency (Hz) for ADM preparations at four different concentrations. Similarly, E' of ADM preparations increases with an increasing concentration of ADM. The smallest changes in E' were shown with respect to the oscillating frequency in the range examined as well. Since E' increases with increasing ADM concentrations, a corresponding increase in the extent of the rigidity or the solid-like status would be expected. However, ADM preparations only need to be crosslinked with 0.025% glutaraldehyde to have a storage modulus close to that of ADM preparations containing a concentration as high as 200 mg/mL.

It is known that some components of collagen in ADM preparations are acid soluble [collagen isolation]. Rosenblatt et al. also reported that the injectable collagen manufactured by solubilizing type I collagen from cowhide was a pH-sensitive hydrogel [24]. Expectedly, the addition of commonly used α-hydroxy acids in the medium used for the dispersion of ADM would modify its rheological characteristics. Figures 3-5 present plots of the creep curve (A), stress-strain relationship (B), and storage modulus versus frequency (C) for these ADM preparations supplemented with citric acid, lactic acid, and glycolic acid, respectively, in different concentrations at the same ADM concentration of 100 mg/mL. Table 2 also lists the corresponding creep parameters of E, τ, and η for ADM preparations calculated based on the Kelvin-Voigt

model since this model is still considered to be the best model to fit the creep curve for those ADM preparations.

Results show that both E and τ increase with increasing concentration of the three kinds of α-hydroxy acids examined (citric acid, lactic acid, and glycolic acid), except that the change in slope of some creep curves was not sufficiently obvious to allow a better fitting of the curve (those supplemented with 10% citric acid or 5% and 10% glycolic acid). The increase in E means that the spring part of the ADM preparations is strengthened with increasing concentrations of α-hydroxy acid. The increase in τ implies that ADM preparations with added α-hydroxy acid are transformed into a material with an increasing extent of a 'solid' response with increasing concentrations of α-hydroxy acid. The increase in both E and τ with increasing α-hydroxy acid concentrations also results in increased viscosity of the liquid part of ADM preparations. In a comparison of the three α-hydroxy acids examined, only the addition of glycolic acid could modify the viscoelastic characteristics of ADM preparations to an extent similar to those of preparations crosslinked with glutaraldehyde. The clinical use of glycolic acid for the treatment of skin disorders seems to elucidate an interaction between glycolic acid and collagen fibers that is responsible for this effective modification of the viscoelastic characteristics of ADM preparations which contain collagen as the main component [25, 26].

The stress-strain plots in Figs. 3B, 4B, 5B for ADM preparations with added α-hydroxy acids also are closer to linearity as shown by the ADM preparations with different concentrations of ADM. The slopes of the stress-strain plots decrease with increasing concentration of the respective α-hydroxy acids, revealing that the addition of α-hydroxy acids makes ADM preparations more solid-like so that they can resist deformation with respect to the applied stress; the extent was the greatest for glycolic acid. Storage modulus measurements shown in Figs. 3C, 4C, 5C give the same information, i.e., the rigidity or 'solid' response of ADM preparations increases with increasing concentrations of α-hydroxy acids, and this effect is the most profound for glycolic acid at the same concentration as lactic acid and citric acid. In conclusion, the Kelvin-Voigt model is the best fitting model which describes the rheological characteristics of ADM preparations with added α-hydroxy acids. Glycolic acid is the most appropriate additive for increasing the 'solid' response or rigidity of ADM preparations, even improving viscoelastic characteristics to an extent comparable to that of preparations crosslinked with glutaraldehyde.

The effects on the rheological characteristics of ADM preparations at a 100 mg/mL concentration blended with polymeric materials of hyaluronic acid and PVP were compared. Figures 6 and 7 present the respective results of creep curves (A), stress-strain plots (B), and storage modulus measurements (C) for hyaluronic acid and PVP. Table 3 also lists the corresponding creep parameters of E, τ, and η calculated based on the Kelvin-Voigt model, since this model is still considered to be the best model to fit the creep curve for those ADM preparations.

Both E and τ gradually increase with increasing concentrations of hyaluronic acid as evidenced in Table 3. The increase in both E and τ similarly leads to η increasing with higher concentrations of hyaluronic acid. This demonstrates that both the spring and

dashpot parts are strengthened by the addition of hyaluronic acid. However, a smaller τ value (1.94 min for 0.3% hyaluronic acid versus 2.89 min for 0.0% hyaluronic acid) resulted from the addition of hyaluronic acid, implying that the 'solid' response for those ADM preparations supplemented with hyaluronic acid was lower than that of preparations containing no hyaluronic acid. This might be attributed to a different mechanism being responsible for this behavior since hyaluronic acid is well known as an effective plasticizer for collagen in tissue [27, 28]. With increasing concentrations of a plasticizer which increases the molecular insertion of the plasticizer between the collagen fibers, interactions between collagen fibers are hindered and gradually transformed into cross interactions between fibers of collagen and hyaluronic acid. Therefore, increases in both E and η with increasing concentrations of hyaluronic acid can be attributed to the presence of hyaluronic acid as a result of increasing interactions among collagen fibers using hyaluronic acid as an intermediate. The decrease in τ in response to the addition of hyaluronic acid may be attributed to the plasticizing effect of hyaluronic acid on collagen fibers in ADM preparations.

The stress-strain relationship is appropriately described by a linear plot; the storage modulus is nearly constant throughout the range of oscillating frequencies examined; and it becomes larger by increasing the concentration of hyaluronic acid in ADM preparations as shown in Fig. 6B. Similarly, the decrease in the slope of the stress-strain linear plot with increasing concentrations of hyaluronic acid indicates that the resistance to deformation of ADM preparations increases correspondingly. Further, the storage modulus which is used to describe the rigidity or the extent of the 'solid' component increases with increasing concentrations of hyaluronic acid in ADM preparations indicating that the addition of hyaluronic acid strengthens the rigidity or the extent of the 'solid' component of ADM preparations. But modifications of both characteristics with the addition of hyaluronic acid are not as efficacious as that of crosslinking with glutaraldehyde. The persistence of those ADM preparations is improved to an extent comparable to those crosslinked with glutaraldehyde.

On the other hand, the rheological behavior of ADM preparations with the addition of PVP greatly differs from that with hyaluronic acid as shown in Fig. 7 and Table 3. The *E* values for all those ADM preparations with added PVP are comparable to that containing 0% PVP, and the extent of the increase with greater PVP concentrations also occurs to a smaller extent, even when the PVP concentration is increased to as high as 40%. On the contrary, the τ values are minimally changed with increasing PVP concentration, and all are smaller than that without adding PVP. This leads to a slight increase in the η value with increasing concentrations of PVP in ADM preparations, and all are smaller than that without adding PVP as well. The resulting rheological behavior of ADM preparations in the presence of PVP might be attributed to the lubricating effect of the linear polymeric chains of PVP on suspended particles or polymeric fibers [14] and partly to the viscous nature of PVP, which is commonly used as a viscosity-building excipient in the pharmaceutical field. However, the stress-strain relationship shows that the slopes of the linear plots are quite similar to those of ADM preparations with the addition of different concentrations of PVP; however, all are higher than that without adding PVP, which is contrary to the decrease with increasing supplemented concentrations as revealed above. This means that the presence of PVP in ADM preparations can accelerate the deformation of the

resulting preparation, hence improving fluidization of the ADM preparations during injection. Therefore, persistent improvements with the addition of PVP would expectedly be less promising.

The storage modulus (E') at the same frequency for ADM preparations with the addition of 10% PVP was lower than those containing no PVP, whereas that for ADM preparations containing PVP increased with increasing concentrations of PVP of from 10% to 40% for all ranges of frequencies examined. This indicates that the addition of PVP modifies the interactions between collagen fibers, resulting in a decreased storage modulus when PVP is added. The lubricating effect of PVP may also possibly be responsible for this. Furthermore, the rigidity or 'solid' component of ADM preparations in the presence of PVP increases with increasing concentrations of PVP, but not to an extent comparable to that of preparations crosslinked with glutaraldehyde.

The viscoelastic characteristics of telopeptide-poor collagen (TPC) preparations obtained either by further treating ADM or directly from porcine skin with a pH precipitation method reported previously [16] were compared. Figure 8 illustrates the creep curves, stress-strain relationships, and storage modulus measurements for both types of telopeptide-poor collagen mixtures at different concentrations. Table 4 also lists the corresponding creep parameters of E, τ, and η calculated based on the Kelvin-Voigt model since this model is still considered to be the best model to fit the creep curves. Obviously, the tendency for E and τ to increase with increasing TPC concentrations is similar to that for ADM preparations. But the relative changes in both values with respect to the concentration was the greatest for ADM preparations, followed by that for TPC obtained from ADM; the TPC obtained directly from porcine skin was the smallest. The η values of the three preparations and their relative changes with increasing concentrations followed the same order as those for E and τ. The stress-strain relationship as indicated in Fig. 8B and E reveals that resistance to deformation by ADM preparations also followed the same order. Results of storage modulus measurements as revealed in Fig. 8C and F demonstrate that the extent of rigidity or the 'solid' response for TPC preparations obtained from ADM was greater than that of the TPC obtained from porcine skin; but both were less than that for ADM preparations. We concluded that preparations containing a purer form of collagen present different viscoelastic characteristics from those of ADM preparations which contain components of the extracellular matrix in influential amounts in addition to collagen. The particulate form of ADM preparations was also expected to have different rheological characteristics from these two TPC preparations, both of which contain greater amounts of the acid-soluble fraction of collagen. Overall, these two TPC preparations were less efficacious in maintaining the same persistence as that crosslinked with glutaraldehyde.

The effect of temperature on the viscoelastic characteristics of ADM preparations at different concentrations was evaluated, and results are presented in Fig. 9. The corresponding creep parameters of E, τ, and η measured at 25 and 37 °C and calculated based on the Kelvin-Voigt model are listed in Table 4 since this model is still considered to be the best model to fit the creep curves. The increase in E and τ, and hence η, with increasing concentrations of ADM preparations was maintained to be true even if the temperature at which measurements were conducted was increased

to 37 °C. But a significant decrease in E and τ, and hence η, was shown for the same ADM concentration when the temperature of the measurements was increased from 25 to 37 °C. This phenomenon was especially obvious at higher concentrations of ADM preparations. Viscoelastic characteristics of ADM preparations are so sensitive to temperature effects that the persistence of such ADM preparations after implantation at body temperature would seriously deteriorate.

Conclusions

The improved persistence of collagen implants by crosslinking with glutaraldehyde might be attributed to a higher Young's modulus (E) of the spring part, a higher viscosity (η) of the liquid part in the Kelvin-Voigt model, and a greater extent of the 'solid' response as indicated by a larger value for the retardation time (τ). Increasing the concentration of ADM and the addition of various concentrations of additives in ADM preparations may produce a similar influence on these viscoelastic characteristics, but in a less efficacious way than that produced by crosslinking with glutaraldehyde. Increasing the ADM concentration to higher than 200 mg/mL and adding glycolic acid to a concentration greater than 2% would possibly produce the same level of persistence as that crosslinked with glutaraldehyde as evidenced by the resulting viscoelastic characteristics. However, the effect of temperature on the viscoelastic characteristics, especially at higher concentrations of ADM, was so profound that it would be harmful to the persistence of such ADM preparations after implantation at body temperature.

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Table 1. Rheological parameters of ADM gels (100 mg/ml) prepared at various concentrations and crosslinked with glutaraldehyde at various concentrations

	Concentration of glutaraldehyde			
	0%	0.0025%	0.05%	0.1%
a	0.192±0.013	0.043±0.000	0.035±0.005	0.025±0.004
E (Pa)	5.225±0.360	22.736±0.168	28.962±0.269	40.753±0.438
b	0.343±0.077	0.286±0.355	0.225±0.927	0.182±0.283
τ (min)	3.023±0.747	3.485±0.900	4.483±0.713	5.658±0.168
η	16.325±0.310	67.020±1.725	132.523±1.715	213.569±1.423
	Concentration of ADM			
	50 mg/ml	100 mg/ml	150 mg/ml	200 mg/ml
a	0.368±0.009	0.192±0.013	0.132±0.010	0.057±0.004
E (Pa)	2.718±0.006	5.225±0.360	7.565±0.585	17.538±0.171
b	0.707±0.052	0.343±0.076	0.198±0.015	0.150±0.034
τ (min)	1.419±0.108	3.023±0.746	5.051±0.407	6.926±0.803
η	3.860±0.341	16.325±0.309	38.050±0.172	111.969±1.144

Table 2. Rheological parameters of ADM gels (100 mg/ml) prepared with various concentrations of citric acid, lactic acid, and glycolic acid

	Concentration of citric acid				
	0%	2%	5%	7%	10%
a	0.192±0.013	0.179±0.009	0.162±0.008	0.070±0.010	0.851±0.047
E (Pa)	5.225±0.360	5.583±0.296	6.167±0.343	14.075±0.336	1.176±0.065
b	0.343±0.076	0.358±0.044	0.298±0.024	0.228±0.047	0.004±0.000
τ (min)	3.023±0.746	2.813±0.328	3.364±0.289	4.386±0.325	208.8±8.764
η	16.325±0.309	15.482±0.998	20.949±0.620	61.256±0.745	244.639±3.080
	Concentration of lactic acid				
	0%	2%	5%	7%	10%
a	0.192±0.013	0.121±0.147	0.132±0.002	0.072±0.006	0.038±0.022
E (Pa)	5.225±0.360	8.276±0.936	7.577±0.138	13.821±1.125	25.740±1.413
b	0.343±0.076	0.687±0.054	0.290±0.031	0.226±0.050	0.135±0.023
τ (min)	3.023±0.746	1.460±0.117	3.472±0.388	4.594±1.164	7.543±1.263
η	16.325±0.309	12.243±0.573	27.369±2.281	62.045±4.852	191.200±23.587
	Concentration of glycolic acid				
a	0.5%	1%	2%	5%	10%
E (Pa)	0.142±0.0240	0.063±0.003	0.059±0.010	0.469±0.309	0.335±0.280
b	7.152±1.168	15.675±0.817	17.064±2.786	3.038±2.232	9.578±12.787
τ (min)	0.410±0.149	0.336±0.022	0.120±0.0353	0.012±0.011	0.062±0.051
η	2.626±0.797	2.981±0.206	8.819±2.658	265.966±347.937	35.890±40.124

Table 3. Rheological parameters of ADM (100 mg/ml) blended with various concentrations of hyaluronic acid and PVP

	Concentration of hyaluronic acid				
	0%	0.3%	0.5%	1%	2%
a	0.192±0.013	0.090±0.004	0.083±0.001	0.072±0.037	0.072±0.009
E (Pa)	5.225±0.360	11.104±0.571	11.977±0.273	15.885±0.369	17.501±0.256
b	0.343±0.076	0.512±0.047	0.363±0.047	0.413±0.230	0.213±0.039
τ (min)	3.023±0.746	1.962±0.192	2.782±0.393	3.428±0.736	4.813±0.985
η	16.325±0.309	20.884±1.534	34.027±3.417	49.725±3.256	63.914±6.236
	Concentration of PVP				
	0%	10%	20%	40%	
a	0.192±0.013	0.199±0.010	0.189±0.012	0.174±0.005	
E (Pa)	5.225±0.360	5.028±0.258	5.297±0.375	5.733±0.175	
b	0.343±0.076	0.609±0.086	0.591±0.0303	0.576±0.003	
τ (min)	3.023±0.746	1.662±0.222	1.693±0.086	1.737±0.096	
η	16.325±0.309	8.223±0.865	9.199±0.122	9.807±0.370	

Table 4. Rheological parameters of various concentrations of ADM and telopeptide-poor collagen (TPC) preparations obtained either by further treating ADM with pepsin (I) or directly from porcine skin (II)

	Concentration of ADM			
	50 mg/ml	100 mg/ml	150 mg/ml	200 mg/ml
a	0.368±0.009	0.192±0.013	0.132±0.010	0.057±0.004
E (Pa)	2.718±0.06	5.225±0.360	7.565±0.585	17.538±0.171
b	0.707±0.052	0.343±0.076	0.198±0.015	0.150±0.034
τ (min)	1.419±0.108	3.023±0.746	5.051±0.407	6.926±0.803
η	3.860±0.341	16.325±0.309	38.050±0.172	111.969±1.144
	Concentration of TPC (I)			
	50 mg/ml	100 mg/ml	150 mg/ml	200 mg/ml
a	0.528±0.0648	0.219±0.015	0.158±0.003	0.052±0.005
E (Pa)	1.745±0.256	4.458±0.569	6.325±1.077	14.256±1.256
b	0.785±0.0451	0.398 ±0.045	0.255 ±0.098	0.239 ±0.056
τ (min)	1.247±0.158	2.405±0.074	4.056±0.104	4.258±0.147
η	2.258±0.256	11.589±0.589	25.448±0.412	67.589±0.326
	Concentration of TPC (II)			
	50 mg/ml	100 mg/ml	150 mg/ml	200 mg/ml
a	0.843±0.161	0.565±0.037	0.376±0.048	0.213±0.001
E (Pa)	1.212±0.209	1.774±0.114	2.688±0.359	4.673±0.020
b	0.937 ±0.111	0.674 ±0.007	0.457±0.047	0.261±0.004
τ (min)	1.182±0.103	1.449±0.105	2.362±0.664	3.960±0.758
η	1.323±0.504	2.478±0.134	6.225±0.557	15.981±0.116

Table 5. Rheological parameters of ADM gels prepared at various concentrations and measured at 25 and 37 °C

	Concentration of ADM (25 °C)			
	50 mg/ml	100 mg/ml	150 mg/ml	200 mg/ml
a	0.368±0.009	0.192±0.013	0.132±0.010	0.057±0.004
E (Pa)	2.718±0.006	5.225±0.360	7.565±0.585	17.538±0.171
b	0.707±0.052	0.343±0.076	0.198±0.015	0.150±0.034
τ (min)	1.419±0.108	3.023±0.746	5.051±0.407	6.926±0.803
η	3.860±0.341	16.325±0.309	38.050±0.172	111.969±1.144
	Concentration of ADM (37 °C)			
	50 mg/ml	100 mg/ml	150 mg/ml	200 mg/ml
a	0.386±0.014	0.284±0.010	0.256±0.015	0.226±0.027
E (Pa)	2.584±0.009	3.516±0.132	3.909±0.227	4.448±0.507
b	0.800±0.013	0.515±0.043	0.370±0.049	0.386±0.085
τ (min)	1.250±0.021	1.948±0.158	2.637±0.392	2.687±0.659
η	3.233±0.045	6.758±0.231	10.841±0.740	11.278±0.687

Legends

Figure 1. (A) Creep curve, (B) stress-strain relationship, and (C) storage modulus measurements for ADM preparations (100 mg/ml) crosslinked with various concentration of glutaraldehyde (GLA). Key: (●) GLA 0.0%, (■) GLA 0.025%, (▲) GLA 0.05%, (◆) GLA 0.1% (n=3).

Figure 2. (A) Creep curve, (B) stress-strain relationship, and (C) storage modulus measurements for ADM preparations containing various concentration of ADM. Key: (●) 50 mg/ml, (■) 100 mg/ml, (▲) 150 mg/ml, (◆) 200 mg/ml (n=3)

Figure 3. (A) Creep curve, (B) stress-strain relationship, and (C) storage modulus measurements for ADM preparations (100 mg/ml) adding with various concentration of citric acid (CA). Key: (●)CA 0%, (■) CA 2%, (▲) CA 5%, (◆) CA 7%, (▼) CA 10% (n=3)

Figure 4. (A) Creep curve, (B) stress-strain relationship, and (C) storage modulus measurements for ADM preparations (100 mg/ml) adding with various concentration of lactic acid (LA). Key: (●) LA 0%, (■) LA 2%, (▲) LA 5%, (◆) LA 7%, (▼) LA 10% (n=3)

Figure 5. (A) Creep curve, (B) stress-strain relationship, and (C) storage modulus measurements for ADM preparations (100 mg/ml) adding with various concentration of glycolic acid (GA). Key: (●) GA 0%, (■) GA 0.5%, (▲) GA 1%, (◆) GA 2%, (▼) GA 5%, (○) GA 10% (n=3)

Figure 6. (A) Creep curve, (B) stress-strain relationship, and (C) storage modulus measurements for ADM preparations (100 mg/ml) adding with various concentration of hyaluronic acid (HA). Key: (●) HA 0%, (■) HA 0.3%, (▲) HA 0.5%, (◆) HA 1%, (▼) HA 2% (n=3)

Figure 7. (A) Creep curve, (B) stress-strain relationship, and (C) storage modulus measurements for ADM preparations (100 mg/ml) adding with various concentration of PVP. Key: (●) PVP 0%, (■) PVP 10%, (▲) PVP 20%, (◆) PVP 40% (n=3)

Figure 8. (A) Creep curve, (B) stress-strain relationship, (C) storage modulus measurements for TPC preparations obtained by further treating ADM with pepsin and (D) Creep curve, (E) stress-strain relationship, (F) storage modulus measurements for TPC preparations obtained directly from porcine skin. Key: (●) 50 mg/ml, (■) 100 mg/ml, (▲) 150 mg/ml, (◆) 200 mg/ml (n=3)

Figure 9. (A) Creep curve, (B) stress-strain relationship, and (C) storage modulus measurements for ADM preparations containing various concentration of ADM (circle: 50 mg/ml, square: 100 mg/ml, upper triangle: 150 mg/ml, diamond: 200 mg/ml) at different temperature: 25℃ (closed symbols) and 37℃ (open symbols) (n=3).

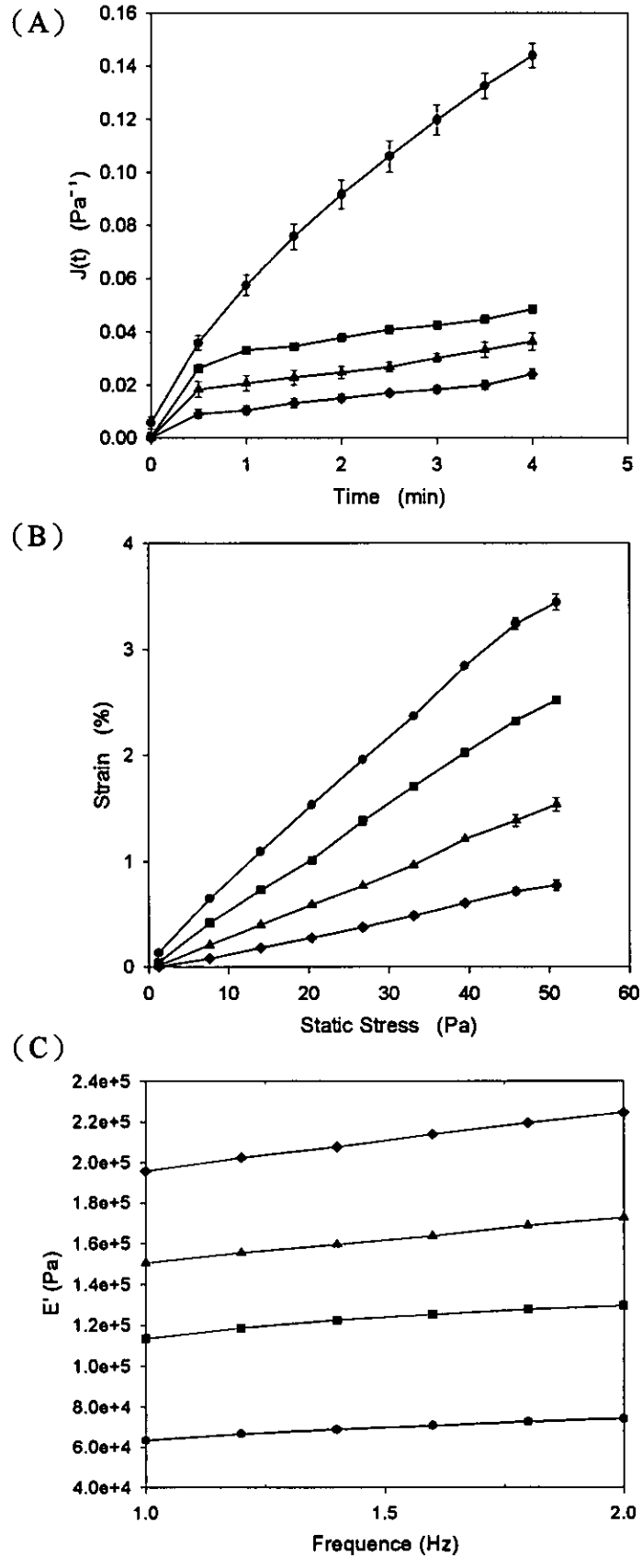


Figure 1

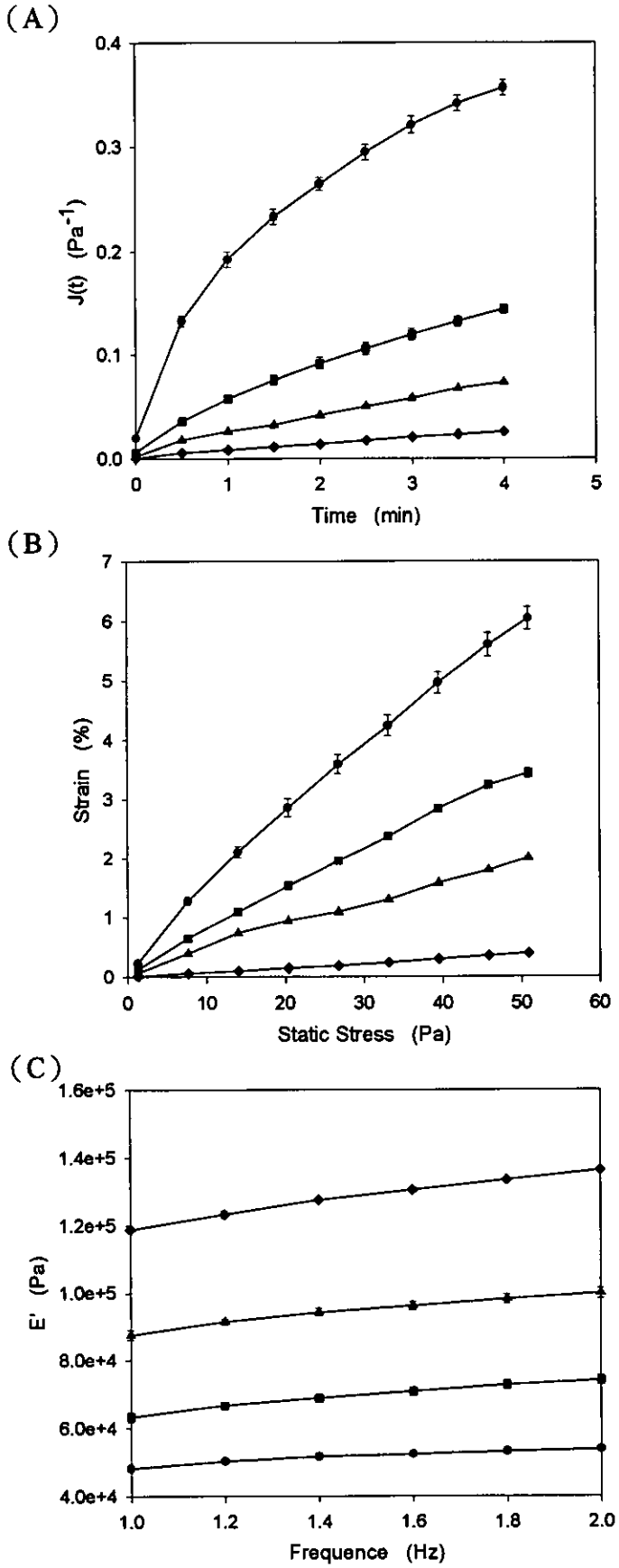
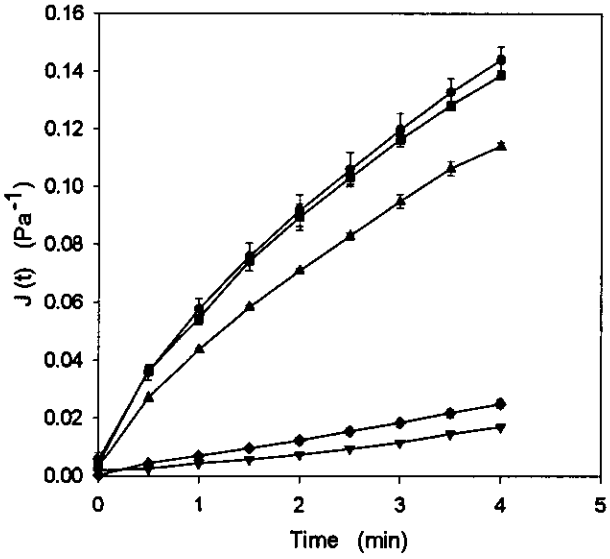
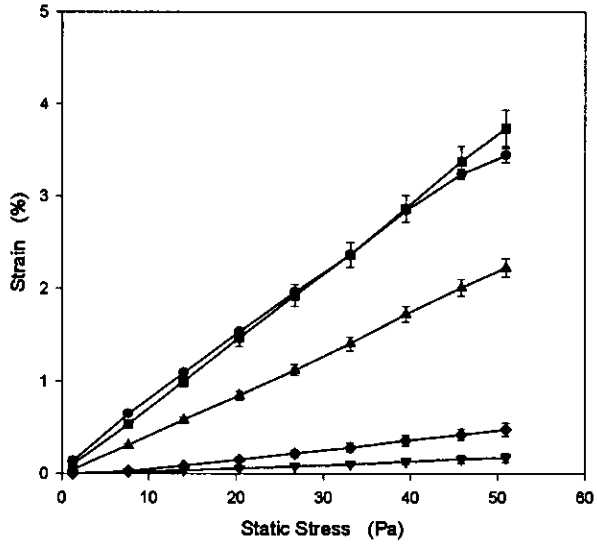

(A)
0.4
0.3
0.2
0.1
0.0
J(t) (Pa-1)
0
1
2
3
4
5
Time (min)
(B)
7
6
5
4
3
2
1
0
Strain (%)
0
10
20
30
40
50
60
Static Stress (Pa)
(C)
1.6e+5
1.4e+5
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
E' (Pa)
1.0
1.2
1.4
1.6
1.8
2.0
Frequence (Hz)

Figure 2

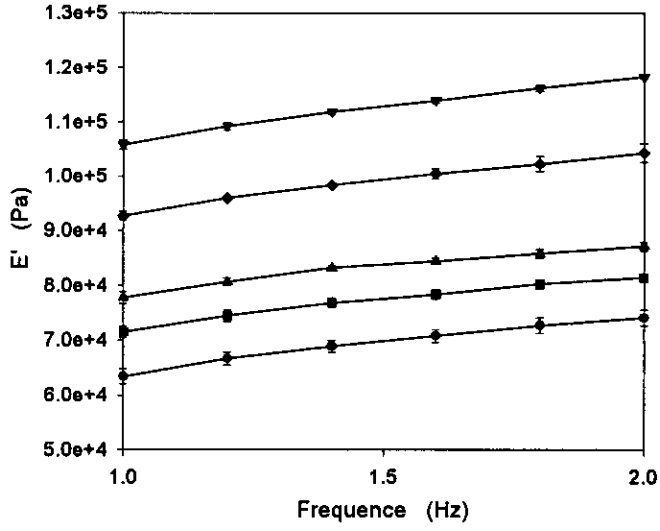
(A)


0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
J (t) (Pa-1)
0
1
2
3
4
5
Time (min)

(B)


5
4
3
2
1
0
Strain (%)
0
10
20
30
40
50
60
Static Stress (Pa)

(C)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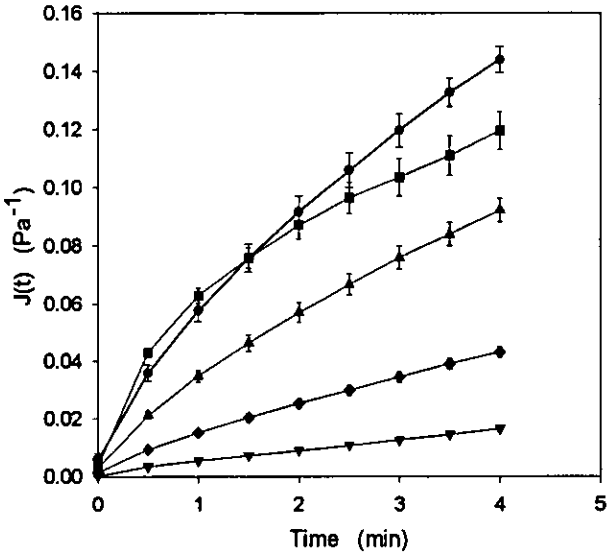
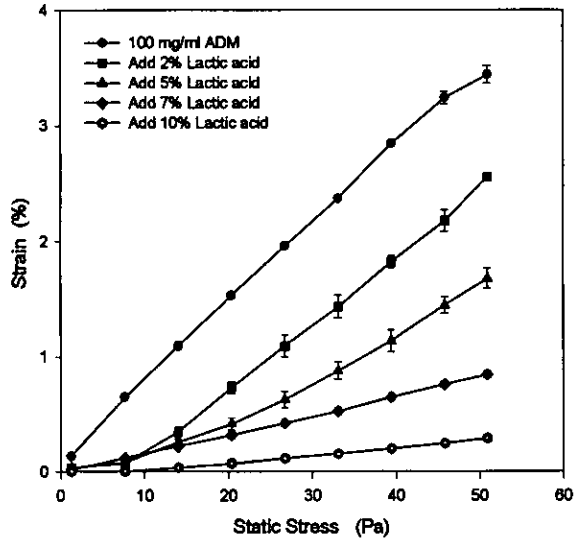
1.1e+5
1.0e+5
9.0e+4
8.0e+4
7.0e+4
6.0e+4
5.0e+4
E' (Pa)
1.0
1.5
2.0
Frequence (Hz)

Figure 3

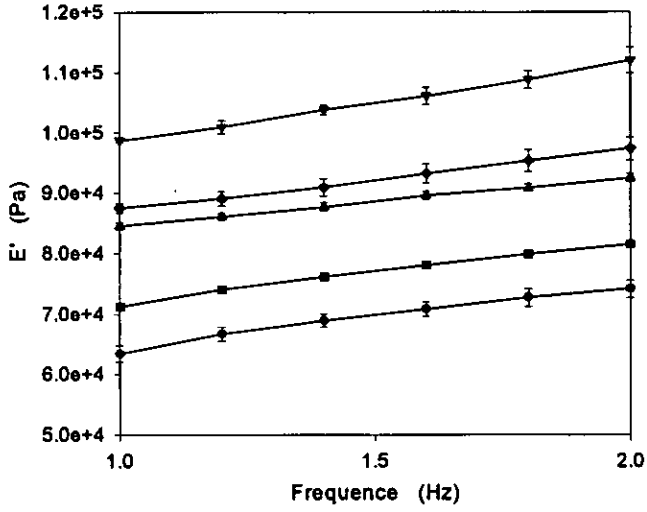
(A)


0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
J(t) (Pa-1)
0
1
2
3
4
5
Time (min)

(B)


100 mg/ml ADM
Add 2% Lactic acid
Add 5% Lactic acid
Add 7% Lactic acid
Add 10% Lactic acid
Strain (%)
0
1
2
3
4
0
10
20
30
40
50
60
Static Stress (Pa)

(C)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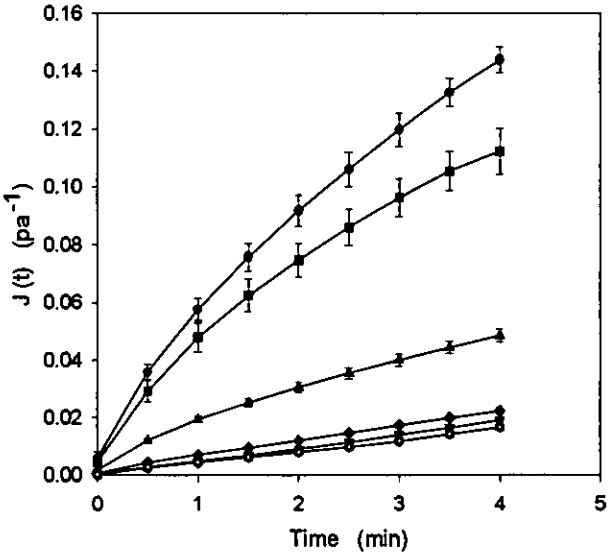
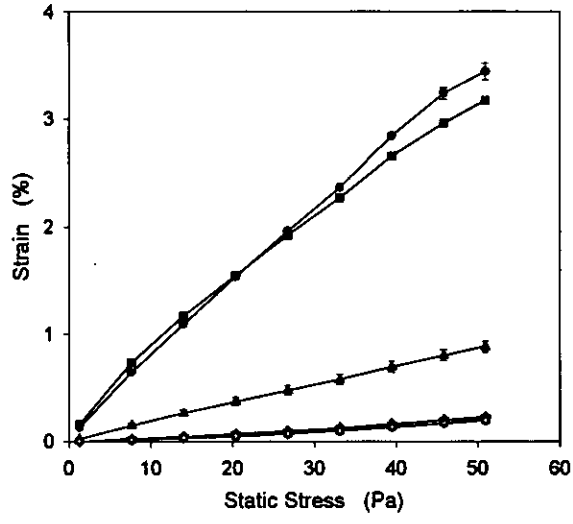
1.1e+5
1.0e+5
9.0e+4
8.0e+4
7.0e+4
6.0e+4
5.0e+4
E' (Pa)
1.0
1.5
2.0
Frequence (Hz)

Figure 4

(A)


0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
J (t) (pa-1)
0
1
2
3
4
5
Time (min)

(B)


4
3
2
1
0
Strain (%)
0
10
20
30
40
50
60
Static Stress (Pa)

(C)

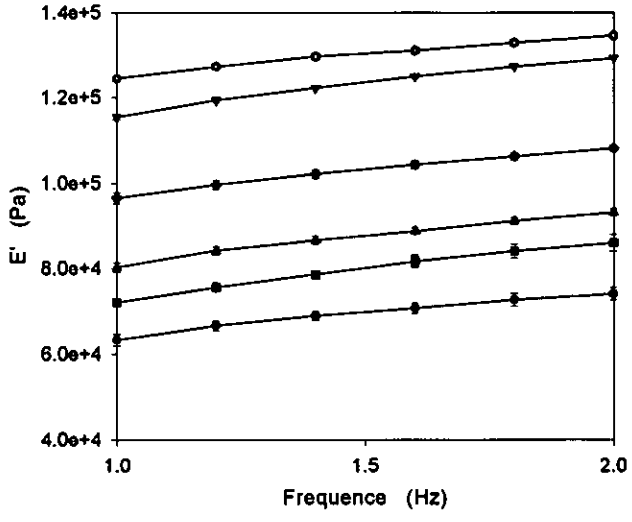
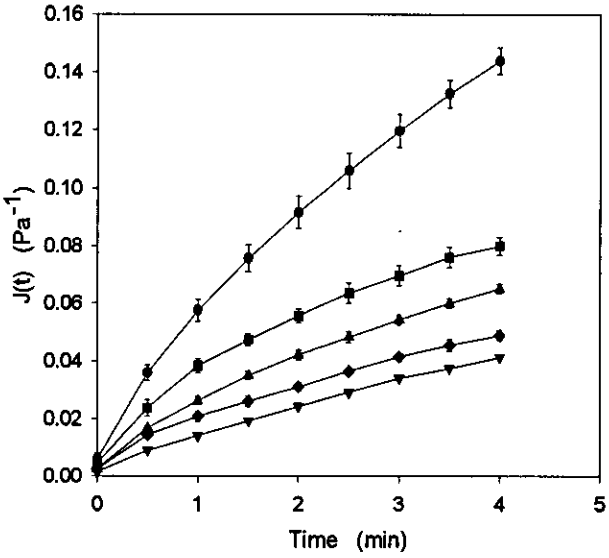
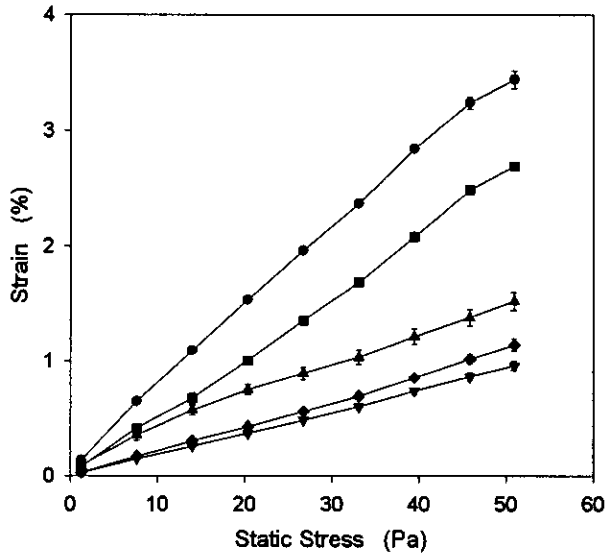

1.4e+5
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
E' (Pa)
1.0
1.5
2.0
Frequence (Hz)

Figure 5

(A)


0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
J(t) (Pa-1)
0
1
2
3
4
5
Time (min)

(B)


4
3
2
1
0
Strain (%)
0
10
20
30
40
50
60
Static Stress (Pa)

(C)

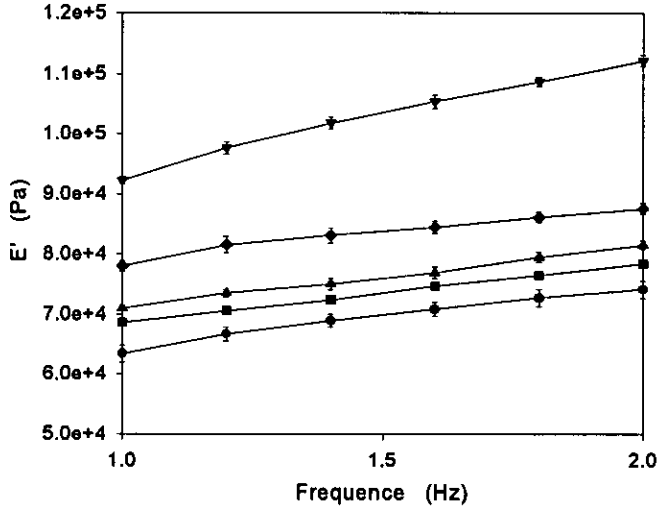

1.2e+5
1.1e+5
1.0e+5
9.0e+4
8.0e+4
7.0e+4
6.0e+4
5.0e+4
E' (Pa)
1.0
1.5
2.0
Frequence (Hz)

Figure 6

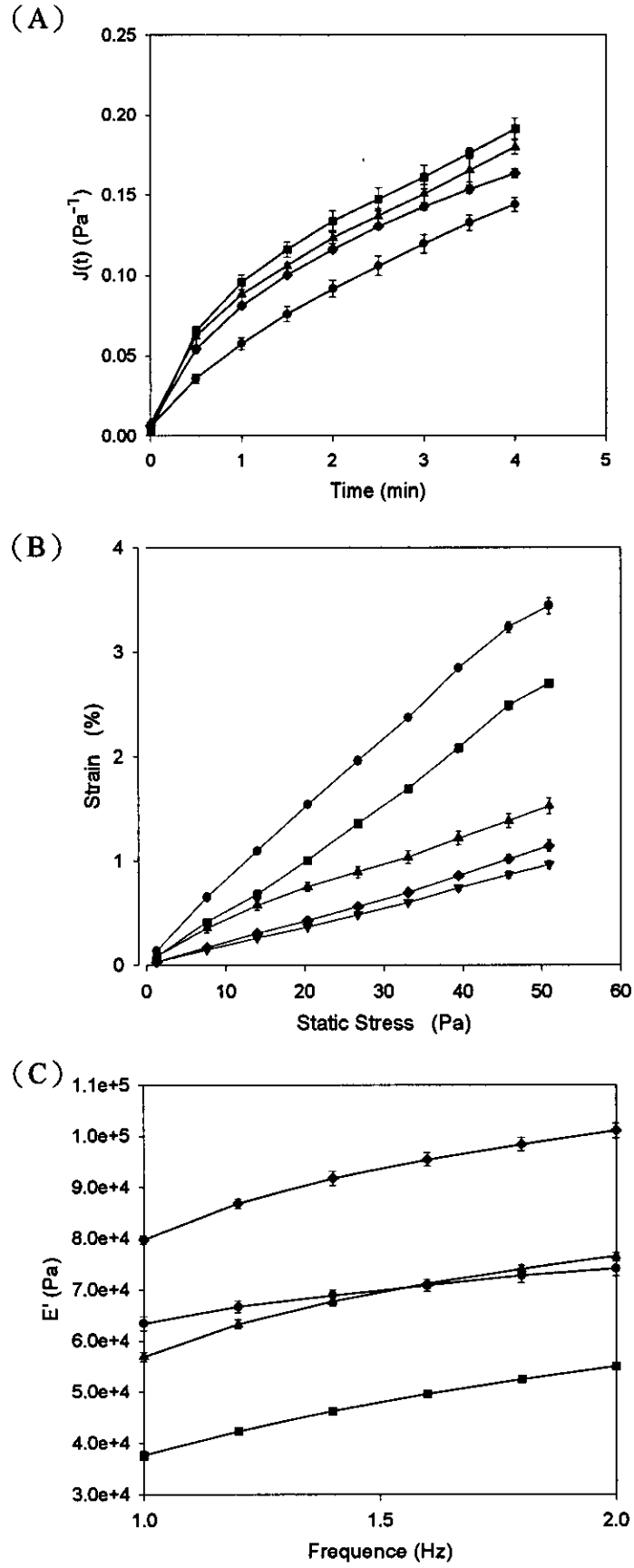

(A)
0.25
0.20
0.15
0.10
0.05
0.00
J(t) (Pa^{-1})
0
1
2
3
4
5
Time (min)
(B)
4
3
2
1
0
Strain (%)
0
10
20
30
40
50
60
Static Stress (Pa)
(C)
1.1e+5
1.0e+5
9.0e+4
8.0e+4
7.0e+4
6.0e+4
5.0e+4
4.0e+4
3.0e+4
E' (Pa)
1.0
1.5
2.0
Frequence (Hz)

Figure 7

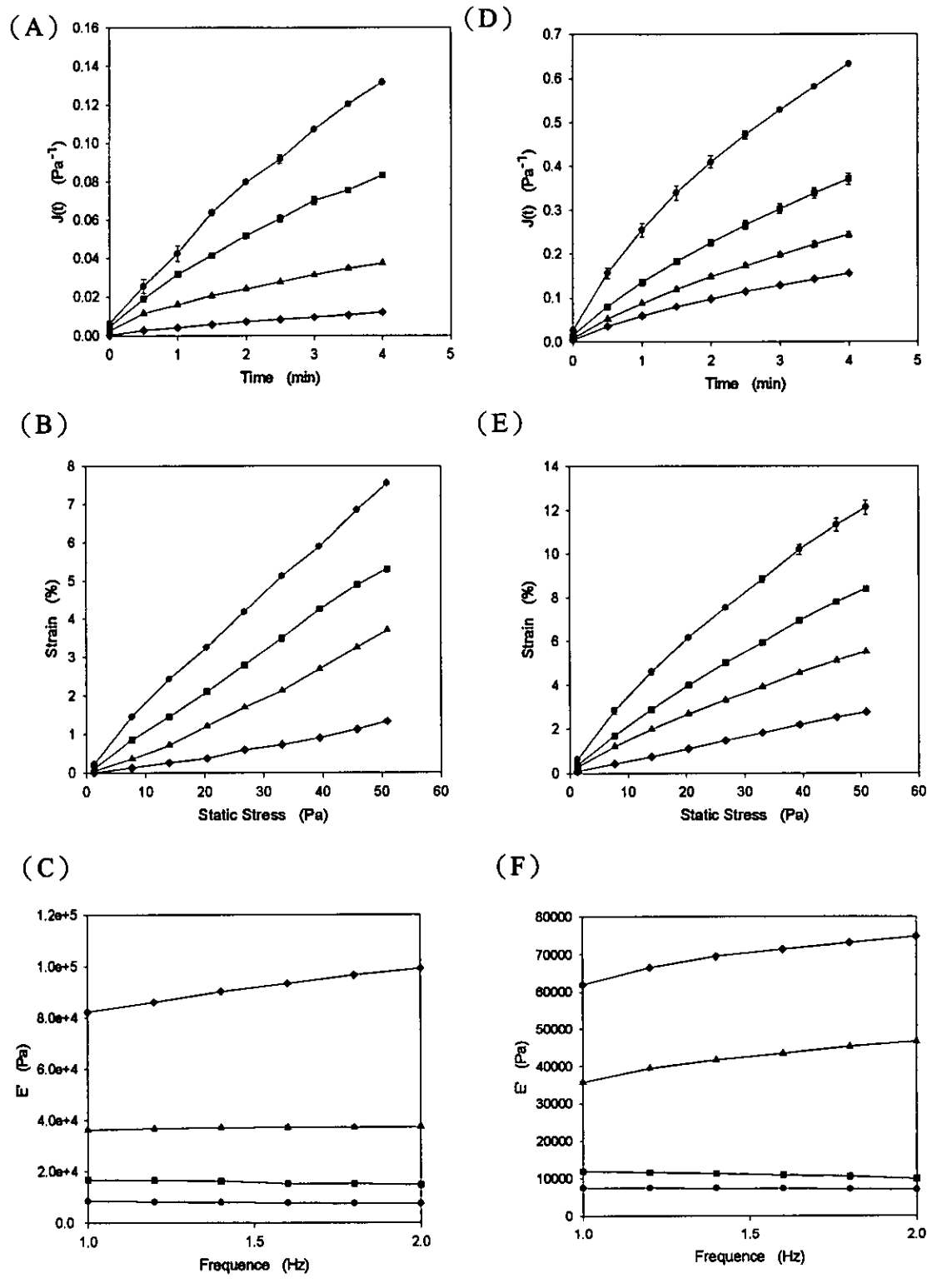
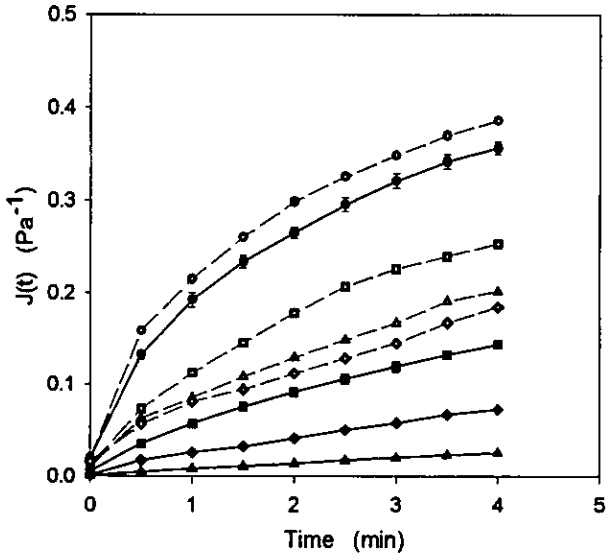
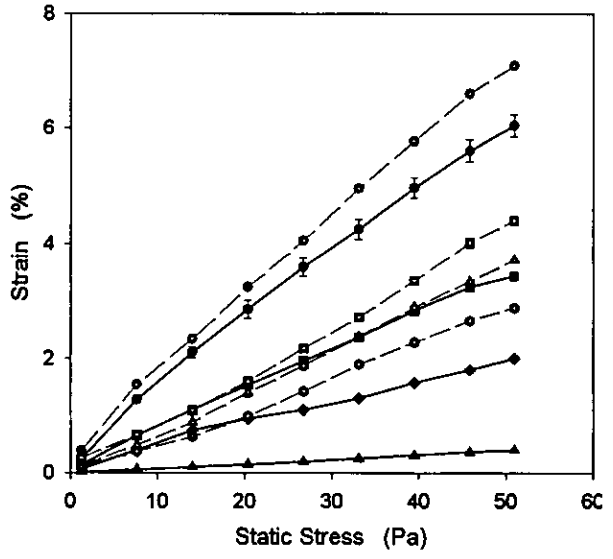

(A)
J(t) (Pa-1)
Time (min)
(D)
J(t) (Pa-1)
Time (min)
(B)
Strain (%)
Static Stress (Pa)
(E)
Strain (%)
Static Stress (Pa)
(C)
E (Pa)
Frequence (Hz)
(F)
E (Pa)
Frequence (Hz)

Figure 8

(A)


0.5
0.4
0.3
0.2
0.1
0.0
J(t) (Pa-1)
0
1
2
3
4
5
Time (min)

(B)


8
6
4
2
0
Strain (%)
0
10
20
30
40
50
60
Static Stress (Pa)

(C)

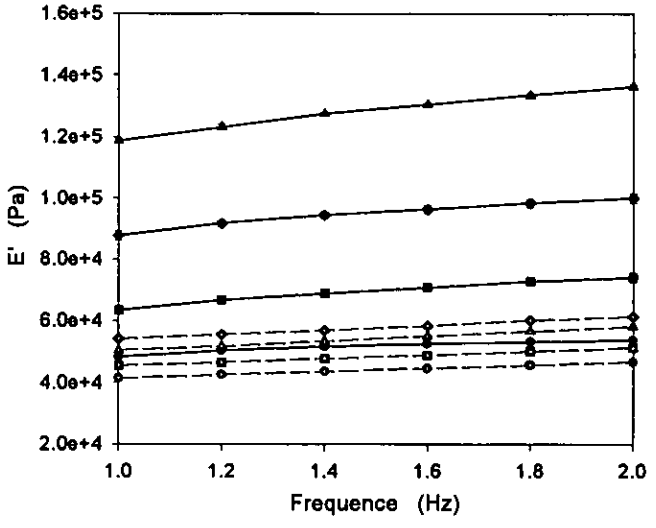

1.6e+5
1.4e+5
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
E' (Pa)
1.0
1.2
1.4
1.6
1.8
2.0
Frequence (Hz)

Figure 9