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**The role of viscoelasticity and the sol-gel transition in the
physiological function of biological fluids**

Hsu, Shan-Hui, Ph.D.

Case Western Reserve University, 1992

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THE ROLE OF VISCOELASTICITY AND THE SOL-GEL TRANSITION IN
THE PHYSIOLOGICAL FUNCTION OF BIOLOGICAL FLUIDS

by
SHAN-HUI HSU

Submitted in partial fulfillment of the requirements
for the Degree of Doctor of Philosophy

Thesis Advisors: Dr. A. M. Jamieson and Dr. J. Blackwell

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May 1992

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*We also certify that written approval has
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Abstract

by

SHAN-HUI HSU

This dissertation comprises three parts. First, rheological studies of gelatin solutions were performed through the sol-gel transition as a function of concentration, pH and ionic strength. At the gel point, power-law frequency dependence of the viscoelastic functions $G'(\omega)$, $G''(\omega)$ and $\eta^*(\omega)$ was observed. The power-law exponents n obtained from these dynamic measurements were confirmed by creep experiments. Under all conditions, the n values fall between 0.64 and 0.72, a range predicted both by a theoretical treatment by Martin et al based on percolation at the Rouse limit, and by a theory by Muthukumar et al for a Cayley tree.

Second, alginate sols and gels were used in a mechanical device (tube model) to simulate the role of viscoelasticity of the lining fluids on the airway reopening process. In contrast to earlier studies of Gaver et al, which used Newtonian fluids, entangled sols or weak gels undergo transitions in the tube, causing either a sudden drop or a plateau in the reopening pressure. Normal upper airway secretions are likely to exhibit a sol-gel transition during the airway opening process, due to their strain-sensitive structure. Simulations of airway reopening in the

bronchial tree were made using viscoelastic functions typical of upper airway secretions. Our results suggest that factors which assist rapid reopening are: a) fluids with small steady shear viscosities, but sufficient to hold the airway walls in apposition; b) fluids with small surface tensions, high elasticities (i.e. low $\tan \delta$), and strong shear-thinning behavior; c) airways with larger radii coated with a thinner mucus layer; and d) a mucus structure which is highly strain-sensitive.

Third, gels made of native collagen were used as a mechanical analog for the connective tissue of skin. Comparative experimental studies demonstrated that specific interactions of dermatan sulfate proteoglycans (DSPG) and hyaluronic acid (HA) with the collagen fibrils, as well as variations in the collagen fibril size distribution and the amount of elastin, can modify the viscoelastic behavior of the model collagen gels. The effects observed in the model system are in good correlation with the current available clinical observations on age-related changes in the mechanical properties of skin, which show progressive decreases in DSPG, HA, and elastin content.

*Dedicated to my parents
in Taiwan*

*and Hsu's clen
in Hsu-chou, China*

The past years of studying in this country turns out to be one of the greatest experiences so far in my life.

I am obliged to my parents for their everlasting confidence in me which helped me overcome some of the misfortunes during the first several years of my Ph. D. study.

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This dissertation comprises three distinct parts: 1) a study of changes in viscoelastic properties accompanying the thermal sol-gel transition of gelatin, 2) an experimental study of the influence of the viscoelasticity of mucous secretions on the mechanics of the upper airway reopening, and 3) an investigation of the viscoelastic behavior of native collagen gels and of the role of the interaction of collagen with other skin macromolecules. These comprise three quite independent problems, but each has the common feature that it concerns role of the gelling process in biological systems. In the first topic, gelatin is used as a convenient vehicle to test molecular theories of the gel point, since the gelling process can be initiated simply by thermal cooling. In the second, viscoelastic fluids, including sols and gels, are prepared from aqueous sodium alginate, a naturally-occurring polysaccharide, by fine tuning the relative concentrations of alginate and calcium chloride, and are used in a mechanical device designed to simulate the effect of the viscoelasticity of the lining fluids on the airway reopening. The results are compared with those from animal models. The third topic focuses on gels made of native collagen, which are used as a mechanical model for skin, and we investigate the influence of various extracellular macromolecular species, found in skin, such as hyaluronic acid, dermatan sulfate proteoglycan, fibronectin and elastin, on gel viscoelasticity as a means to investigate the

influence of intermolecular interactions on the mechanical properties of skin.

The gels utilised in our studies, gelatin, alginate and collagen, all have unique rheological properties, which play an important role in their biological function. By carrying out these three studies, we expected to gain insights as to how the rheological properties of these bio-gels are designed to control the physiological function of the human body.

INTRODUCTION

1. CLASSIFICATION OF GELS

Many naturally-occurring gels exist in the biological system [1]. Their major functions generally are of a mechanical and protective nature. For example, connective tissue matrices are gels comprising mostly collagen and proteoglycans [2-4]. Their primary function is load-bearing [5] and dissipation of mechanical energy. The molecular interaction between collagen, proteoglycans and the other ground substance macromolecules present in the the matrix is believed to play a role in normal physiological functioning [6]. The mucous secretions of lung and the upper airway are known to form viscoelastic gel-like materials [7]. The mucus covers the surface of the airway and one

of its functions is to trap foreign particles in the inspired air which are then removed by the mechanical action of ciliated cells.

The term 'gel' is easier to recognize than to define. Bungenberg de Jong [8] uses the definition: "... (a) system of solid character, in which the colloidal particles somehow constitute a coherent structure, ...". There are different kinds of gels but they all have the common feature of being a continuous network. According to Flory's classification [9], there are four types of gels based on different structure of organisations as follows:

1. Well-ordered lamellar structures. For example, soap gels, phospholipids and clay. There is long range of order because the lamellae are arranged in parallel by either electrostatic, van der Waals forces or dipolar interactions.
2. Covalent polymeric networks; completely disordered. For example, elastin, vulcanized rubbers and condensation polymers formed from reactants with more than two functional groups. Their continuity is provided by a three-dimensional network with chemically covalent links. They are not soluble in chemical solvents.
3. Polymer networks formed through physical aggregation; predominantly disordered, but with regions of local order. Examples are the gelatin and alginate gels used in this research. The mucins (mucus proteins) of tracheobronchial and gastrointestinal mucus may also be included in this category

[10,11]. Chains between the local ordered regions are disordered as in the type 2 gels. The major difference is the physical nature of the bonding in this type of gels. Therefore, they are reversible and can be transformed to the sols by changing the temperature (as in gelatin) or by diluting (as in alginate gels).

4. Particulate, disordered structures. For example, gels formed by aggregation of either fibrillar or globular proteins, such as collagen gel, blood clot and egg white. The forming of the gel is probably non-specific. But the specific interaction causing the gel to form may be a well-controlled biological mechanism.

Most of the bio-gels fall into the type 3 or 4 category of gels. Because of their reversibility and delicate nature, they can be used by the body to achieve a variety of physiological functions.

2. GEL THEORY

The term 'gels' has been defined literally in the previous pages. The essential characteristic of a gel is its solid-like behavior. Many models have been proposed to describe phenomena at the gel point. The widely accepted classical gel theory was developed by Flory, Stockmayer, and Zimm [12-14], represents a mean field theory of gelation. In this analysis, gel is formed by the random crosslinking of macroscopic branched-chain molecules after a certain extent of reaction, predicted by statistics, has been reached. Cyclic bonds, excluded volume

effects, and steric hindrance are neglected. The network formed is a structure called the Bethe lattice or the Cayley tree (fig.0.1.a). Recently, critical exponents and percolation theories [15] have received special attention in this area. Molecules (monomers) occupy the sites of a periodic lattice and bonds are formed randomly between two nearest neighbors of sites (fig.0.1.b). At gel point, some essentially 'infinite' macromolecules formed which are represented by the lines span the whole lattice. The mean field theory can be shown to be a limiting case of the percolation theory and to be equivalent to the percolation model in six dimensions ($d=6$). The percolation model can be extended to predict the dynamics of the gel system near the gel point by combining the fractal geometry concept with theories of the hydrodynamics of polymer solutions, as described below.

According to the scaling theories [16], polymer chains, percolation clusters and aggregates are fractal structures which may have non-integer fractal dimensionalities. Fractal structures are statistically self-similar over an ideally infinite range of length scales. The geometrical structure of fractals is characterised by their fractal dimensionality d_f , which relates the mass to the spatial size. For a molecular cluster, the relationship may be expressed as:

$$M \propto R_g^{d_f} \tag{0.1}$$

(a)

(b)

Figure 0.1. (a) Classical gel theory: structure of the Bethe lattice or the Cayley tree.
(b) Percolation model explained in a two-dimensional square lattice at the gel point. Each bond is shown as a short line connecting two monomers.

where R_g is the radius of gyration and M is the molecular weight. For linear polymers in θ solvents (three dimensional Gaussian chain), described by random walks, $d_f = 2$; for linear polymers in good solvents, described by self-avoiding random walks, $d_f = 5/3$ [17]. Also, the fractal dimension of branched polymers in good solvents, where excluded volume interactions are screened by intervening clusters, is approximated [18] by $d_f \approx (d+2)/2$, where d is the spatial dimension. However, when branched polymers are diluted with a good solvent, intersegmental interactions are unscreened and the excluded volume effect can not be neglected. The fractal dimension d_s of these swollen clusters is given by the so-called lattice-animal model [19], $d_s = 2(d+2)/5$. These percolation results have been utilised in gelation theory and the fractal dimension at the gel point is predicted to be:

$$\begin{array}{ll} d_f = 4, & \text{for Cayley tree (i.e. } d=6) \\ d_f = 2.52, & \text{for percolation cluster (i.e. } d=3) \end{array}$$

Overall, the percolation theory shows that the spatial dimensionality rather than the lattice structure itself determines the critical exponents.

3. VISCOELASTIC BEHAVIOR OF POLYMERS

3.1. STEADY SHEAR AND DYNAMIC SHEAR EXPERIMENTS; CREEP TEST

The viscoelastic properties of polymers have been thoroughly investigated and reviewed by Ferry [20]. A viscoelastic material is one whose behavior is intermediate between a purely viscous liquid (i.e. Newtonian liquid) and a purely elastic solid (i.e. Hookean solid). There are two common ways to characterise such material: steady shear and dynamic shear experiments; and the creep test.

In steady shear experiments, a constant shear strain, σ , is applied at various shear rates, $\dot{\gamma}$, to the polymer samples and generates data in the form of a rate-dependent shear viscosity η , which can be expressed as follows:

$$\sigma = \eta(\dot{\gamma}) \dot{\gamma} \quad (0.2)$$

Most polymers exhibit shear-thinning behavior, also called pseudoplastic, i.e. they show a decrease in the shear viscosity as rate increases. At very low shear rates, it is common to observe a so-called Newtonian plateau where the viscosity is independent of the shear rate (η_0). The onset of the non-Newtonian behavior is determined by the configurational relaxation time of the polymer. Gel-like materials may deform under an applied stress but do not flow unless the network structure is disrupted. Upon removal of the stress, all or part of the network structure may be recovered.

In a dynamic oscillatory measurement, a periodic sinusoidal strain (or stress) is applied to the polymer and the stress (or strain) response is measured. For a purely viscous fluid, the energy is completely dissipated by flow. For a purely elastic material, the energy is stored and once the input is removed, it instantly returns to the original state. So for a elastic solid the output is in phase with the input, whereas for a viscous liquid the output is 90° out of phase with the input. Intuitively, for a viscoelastic material, the output would be lagged behind by a phase angle $\delta(\omega)$ between 0° and 90° . The relation can be expressed as follows:

$$\text{input:} \quad \gamma = \gamma_0 \sin \omega t \quad (0.3)$$

$$\text{output:} \quad \sigma = \sigma_0 \sin (\omega t + \delta) = \gamma_0 (G' \sin \omega t + G'' \cos \omega t) \quad (0.4)$$

$$G'(\omega) = (\sigma_0/\gamma_0) \cos \delta \quad (0.5)$$

$$G''(\omega) = (\sigma_0/\gamma_0) \sin \delta \quad (0.6)$$

$$\tan \delta = G''(\omega)/G'(\omega) \quad (0.7)$$

where γ_0 and σ_0 are the maximum amplitude of strain and stress, respectively, and ω is the frequency of the oscillating strain. G' and G'' which measure, respectively, the in-phase and out-of-phase response are termed the dynamic storage and loss moduli. The ratio $\tan \delta$ is an index, therefore, of the predominance of the liquid-like or solid-like behavior in the viscoelastic material. The complex modulus and complex viscosity are defined as follows:

$$G^*(\omega) = [(G'(\omega))^2 + (G''(\omega))^2]^{1/2} \quad (0.8)$$

$$\eta^*(\omega) = G^*(\omega)/\omega = [(\eta')^2 + (\eta'')^2]^{1/2} \quad (0.9)$$

$$\eta'(\omega) = G''/\omega \quad (0.10)$$

$$\eta''(\omega) = G'/\omega \quad (0.11)$$

The data from sinusoidal experiments can also be expressed in terms of a complex compliance:

$$J^* = 1/G^* \quad (0.12)$$

$$J' = G'/(G'^2 + G''^2) = 1/G'(1 + \tan^2 \delta) \quad (0.13)$$

$$J'' = G''/(G'^2 + G''^2) = 1/G''[1 + (\tan^2 \delta)^{-1}] \quad (0.14)$$

The advantage of a dynamic experiment over steady shear experiment is that it generally does not influence the material structure when used at small strains. Also, it involves the motion of progressively smaller polymer domains when the frequency goes higher. By doing a frequency scanning, information about polymer relaxation over a range of length scales can be obtained. In fact, this information permits calculation of the other functions, such as transient creep compliance and relaxation modulus $J(t)$ and $G(t)$ over wide range of time scale to reflect the variety of molecular motions. But this correlation of information is only feasible when the viscoelastic behavior is linear, i.e. when the strain is sufficiently small. As the strain goes higher, the stress response (or the moduli) may show strain dependence as in steady shear experiment. It is no longer possible to predict the behavior

by linear viscoelasticity theory because the structure has changed irreversibly.

The complex viscosity of dynamic experiment can be related to the steady shear viscosity by the Cox-Merz rule [21]:

$$|\eta^*(\omega)|_{\omega \equiv \dot{\gamma}} = \eta(\dot{\gamma}) \quad (0.15)$$

At higher shear rates, this relation may not be satisfied because the entanglement structure of polymer chains has disengaged at high shear rate but remains in high frequency low magnitude dynamic experiments, especially in linear flexible chains.

Creep test is another popular way to characterise a viscoelastic material. In the creep test, a constant stress is applied to the sample, and the resultant response is measured against time (creep retardation curve). After certain time, the stress is removed. This is called creep recovery. The strain response against time (creep relaxation curve) is recorded. Different strain responses can be associated with molecular structures in the system being tested and rheological models can be applied to describe elastic and viscous behavior [20, 22]. In addition, as mentioned earlier, creep data can be transformed to dynamic data and vice versa [20]. At the extremes, it is to be expected that $\eta'(\omega \rightarrow 0) = \eta_0$ (creep) and $G'(\omega \rightarrow \infty) = G_0$ (creep).

Another useful information which can be extracted from the creep test is the yield stress [23]. Yield stress is defined as the minimal shear stress necessary to apply to a substance for it to flow. Flow curve extrapolation by continuous shear rate experiments are not adapted to the correct estimation of yield stress because the sample is forced to flow [24]. By using a controlled stress rheometer, the sample is subjected to a shear stress that is increased step by step and the resulting deformation is recorded. When the stress is less than the yield stress, the strain versus time remains constant. If the stress is beyond the yield value, the strain will increase with time.

3.2. VISCOELASTICITY OF POLYMER SOLUTIONS

The hydrodynamical properties are strongly influenced by the molecular dimensions of the polymers and their measurement offers a useful tool of molecular characterisation. The study of viscoelasticity gives an insight into the nature of the molecular rearrangement during deformation.

Theories are available to describe the viscoelasticity of dilute linear flexible polymer solutions [20]. They include the Zimm theory for dilute solutions and the Rouse theory at higher concentration. Zimm theory accounts for the intersegmental hydrodynamic interactions. Measurements of the dynamic properties of several polymers at very low concentrations in a

solvent have been shown to correspond well to Zimm theory [25]. At higher concentrations, the intermolecular interactions caused by the overlap of the coils tend to screen the hydrodynamic interaction. There is a transition from Zimm-like behavior to Rouse-like behavior. The Rouse theory neglects the hydrodynamic interaction and the excluded volume effect.

The prediction of the frequency dependence of the dynamic moduli by Zimm and Rouse theories is shown as Figure 0.2. [20]. There are two regions for both theories. At lower frequency regime, known as terminal zone, both theories yield the same:

$$[G'(\omega)]_R \propto \omega^2 \quad (0.16)$$

$$[G''(\omega)]_R \propto \omega \quad (0.17)$$

$$\text{reduced variables } [G']_R = [G']M/RT, [G'']_R = [G'']M/RT \quad (0.18)$$

$$\text{intrinsic variables } [G'] = \lim_{c \rightarrow 0} G'/c, [G''] = \lim_{c \rightarrow 0} G''/c \quad (0.19)$$

At higher frequency, known as the transition zone, the curves become different for two theories. Zimm theory predicts $G''(\omega)/G'(\omega) = \sqrt{3}$ with frequency dependency on $\omega^{2/3}$ in θ solvents and $\omega^{5/9}$ in good solvents; whereas Rouse theory predicts congruent moduli with frequency dependency on $\omega^{1/2}$. Or in another word, in the transition zone, Zimm theory predicts $\eta^* \propto \omega^{-1/3}$ for θ solvents and $\eta^* \propto \omega^{-4/9}$ for good solvents, and Rouse theory predicts $\eta^* \propto \omega^{-1/2}$.

Figure 0.2. Rouse theory and Zimm theory for the prediction of frequency dependence of the dynamic moduli G' and G'' in θ solvents.

Recently, a theory of the viscosity of a solution containing polymeric fractals has been derived by Muthukumar [26]. The intra- as well as interfractal hydrodynamic interactions and the excluded volume effect are included. It predicts the frequency dependence of the complex viscosity in terms of the arbitrary fractal dimension of the molecular cluster. In the region of Rouse limit, where both the hydrodynamic and excluded volume effects are fully screened, the frequency dependence of the viscosity is:

$$\eta^*(\omega) \propto \omega^{-2/(d_f+2)} \quad (0.20)$$

The frequency dependence of the shear viscosity of a solution containing polymeric fractals depends only on d_f . This theory have been shown to reproduce the high concentration behavior of unentangled linear polymers. If it is assumed permissible to apply the same equation to a gel system at gel point, the frequency dependence would be $\eta^* \propto \omega^{-1/3}$ for a Cayley tree model and $\eta^* \propto \omega^{-4/9}$ for a percolation cluster at threshold. This interestingly coincides with the predictions of the Zimm theory in the transition zone of dilute linear polymer solutions in θ solvents and good solvents, respectively.

For dilute solutions, where hydrodynamic interactions and the excluded volume effect are both present, the frequency dependence of the viscosity is [26]:

$$\eta^*(\omega) \propto \omega^{(d_f-d)/d}$$

(0.21)

This gives $\eta^*(\omega) \propto \omega^{-0.16}$ for a percolation cluster with $d_f=2.52$. Therefore, it is concluded that the frequency dependence of the percolation cluster is between $\omega^{-4/9}$ (Rouse limit) and $\omega^{-0.16}$ (Zimm limit).

3.3. EXPERIMENTAL CONSIDERATIONS

The rheological experiments described in the following chapters were performed on either a controlled strain Rheometrics Fluids Spectrometer RFS-8400 or a controlled stress Carri-Med CSL-50 rheometer both utilizing cone and plate geometry shown schematically in Figure 0.3.

For the Rheometrics rheometer, the lower plate is driven by a servo motor which can be programmed for dynamic or steady shear experiments. The magnitude and phase of the torque response on the upper cone are measured by a 10 gm-cm/100 gm-cm alternative transducer supported by a precision air bearing. Sensitivity of the transducer is one thousandth of full scale.

In dynamic mode, the frequency range is 0.01-100 rad/s. Transducer torque, M , is converted into in-phase and 90° out-of-phase components relative to the applied strain:

Figure 0.3. Schematic of cone and plate geometry in a Rheometrics or Carri-Med rheometer.

$$G = G' + i G'' = K (M/\theta) \quad (0.22)$$

where K is a geometric and scaling constant, and θ is the shearing angle in radians. By using equations eqs.(0.7)-(0.14), the other dynamic parameters can be obtained. The geometry constants for the cone and plate fixture are:

$$K = 3 \beta (980.7) / 2 \pi (R/10)^3 \quad (0.23)$$

$$\gamma = \theta / \beta \quad (0.24)$$

$$\tau = 3000 (980.7) M / 2\pi R^3 \quad (0.25)$$

where β is the cone angle in radians, R is the cone radius, γ is the shear strain, and τ is the stress.

In steady shear mode, the shear rate range is 0.01-100 s^{-1} . Transducer torque is converted to steady shear viscosity by the following equations for cone and plate geometry:

$$\eta = MK / \dot{\gamma} \quad (0.26)$$

$$K = 3 (980.7) / 2\pi (R/10)^3 \quad (0.27)$$

$$\dot{\gamma} = \Omega / \beta \quad (0.28)$$

where Ω is the rotational rate in rad/s and $\dot{\gamma}$ is the shear rate in s^{-1} .

For the Carri-Med rheometer, the bottom plate of the cone/plate assembly is equipped with a Peltier system, by which the temperature can be controlled between 5°C and 80°C. The instrument is a drive/displacement measuring combination, comprised of the microprocessor controlled induction motor drive coupled with a low inertia air bearing and a high resolution (sensitivity down to $< 10^{-5}$ rad) digital optical displacement encoder. In addition to controlled stress (or strain rate) steady shear as well as controlled stress (or strain) dynamic oscillation, it also provides the potential for the creep measurement. It has a torque range 0.01 gm-cm to 50 gm-cm. The above equations eqs.(0.22)-(0.28) are still valid except that the stress is controlled and the strain is measured.

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CHAPTER 1. STUDIES OF THE GEL POINT IN THE THERMAL SOL-GEL TRANSITION OF GELATIN

1. BACKGROUND

1.1. RHEOLOGICAL DETECTION OF THE GEL POINT IN A CHEMICALLY CROSSLINKED GEL

There are two approaches to determine the gel point from viscoelastic measurements. In the first, the polymer in its sol state is subjected to steady shear flow. As the molecules in the sol proceed to form junctions with each other, the measured viscosity increases rapidly near the gel point to values beyond the maximum limit of the instrument (still before gel point). Alternatively, beyond the gel point, the polymer in its gel state is subjected either to a specified strain (or an incremental stress) and the equilibrium modulus (or the yield stress) is measured within the minimum sensitivity limit of the instrument. Experiments in the transition region from sol to gel are difficult to interpret. Therefore, these measurements can only give data away from the gel point and the transition point is obtained by extrapolation.

In the second method, dynamic shear experiments are used to obtain the dynamic parameters G' , G'' , η^* and $\tan \delta$. In the initial state of the experiment, the material is sol-like and $G'' > G'$.

As the junctions proceed to form, G' increases steeply until it intersects and exceeds G'' . Both moduli level off as the gel forms a complete network and attains its full strength. This oscillatory shear experiment has the advantage of continuous measurement of the viscoelastic properties from the sol through the transition to the gel state. According to the traditional suggestion [1], the gel point corresponds to when the two moduli intersect (a G' - G'' crossover), or $\tan \delta$ equal to 1. However, this definition poses problems since a G' - G'' crossover occurs in non-gelling solutions of entangled polymers when the frequency $\omega \approx \tau_r^{-1}$, where τ_r is the entanglement relaxation time. Also experiment and theory, discussed below, indicate that the true gel point in gelling systems may occur before or after the G' - G'' crossover at certain frequency.

Recently, two research groups, using different theoretical concepts, derived quite different viscoelastic relationships for the gel point. The fractal dimension concept was utilised in both analyses. Each of these theoretical approaches seeks to derive expressions for the frequency-dependence of G' , G'' and $\eta^*(\omega)$ at the gel point [2-7]. Unfortunately, the prediction for the viscoelastic functions are totally different in each theory.

Winter and coworkers [2] first investigated experimentally the poly(dimethyl siloxane) (PDMS) chemically crosslinking model network system, and they found that the stress relaxation at the gel point follows a power law dependence on time:

$$G(t) = S t^{-n}, 0 < n < 1 \quad (1.1)$$

where the gel strength constant S is determined by the nature of the polymer chains and the crosslink density and n is a network-specific relaxation exponent. Likewise, the complex viscosity follows a power law relation:

$$\eta^*(\omega) = \frac{\pi}{\Gamma(n) \sin(n\pi)} S \omega^{n-1} \quad (1.2)$$

In their chemically crosslinking system, they found that:

$n = 1/2$, for a stoichiometrically balanced network or a
stoichiometrically imbalanced network
with excess crosslinker

$n > 1/2$, for a stoichiometrically imbalanced network
deficient in crosslinker

For a network with exponent $n = 1/2$, congruent moduli are found:

$$G'(\omega) = G''(\omega) = \frac{\pi}{\Gamma(n) \sin(n\pi)} S \omega^{1/2} \quad (1.3)$$

$$\tan \delta = G''/G' = 1 \quad (1.4)$$

This methodology provides a simple approach to determine the gel point. $\tan \delta$ is independent of frequency at gel point, so the gel point is reached when G' and G'' cross each other in a dynamic experiment run at a single frequency. Note that the relations (1.3) and (1.4) are reminiscent of the Rouse theory for a concentrated polymer solution where the overlapped coils result in a shielding of the hydrodynamic interaction. The difference is that Rouse model predicts $G' = G'' \propto \omega^{1/2}$ only over a limited intermediate frequency range, but the experimental congruency in PDMS gels was observed over more than 5 decades of frequency.

By using equation eq.(0.20) in the hydrodynamic fractal theory derived by Muthukumar described previously, the fractal dimension of a gel with exponent n at the gel point (i.e. eq.(1.2)) is derived to be:

$$d_f = 2n / (1-n) \quad (1.5)$$

Therefore, the PDMS cluster at the gel point is calculated to be $d_f = 2$ [8]. This value is closer to the dimension predicted by percolation theory ($d_f = 2.5$, $\eta^*(\omega) \propto \omega^{-4/9}$) than that predicted by the Cayley tree model ($d_f = 4$, $\eta^*(\omega) \propto \omega^{-1/3}$), but is clearly numerically different, within experimental error with both fractal geometries..

However, the detection of the gel point becomes more complicated when the network dynamics has an exponent n which is not equal to $1/2$. According to the Kronig-Kramers relations [9], the dynamic moduli exhibit parallel power-law relationships at the gel point:

$$G'(\omega) = \frac{G''(\omega)}{\tan(n\pi/2)} = \frac{\pi}{2 \Gamma(n) \sin(n\pi/2)} S \omega^n \quad (1.6)$$

At the gel point, $\tan \delta = \tan(n\pi/2)$ and is independent of the frequency. Thus a multifrequency scan experiment must be used to detect the gel point. From studies in nonstoichiometric systems [4], Winter concluded that there is no unique power-law exponent, but a continuous change from $n=0.5$ ($G' = G'' \propto \omega^{1/2}$) to $n=0.66$ ($G''/G' = \sqrt{3}$ and $G', G'' \propto \omega^{2/3}$) when the stoichiometry of the gel (i.e. the ratio of crosslinker to crosslinking sites) decreases below unity. It was concluded [4] that for $r < 1$, there may be an increase in the branch frequency leading to an increase in the fractal dimension. As noted above a Cayley tree has $d_f=4$ and hence would lead to $n = 2/3$.

Recently, Muller et al [5] employed a different chemically crosslinking system, hydroxyl-terminated poly(ethylene oxide) (PEO) with pluriisocyanates as crosslinking agent to confirm the validity of Winter's methodology. Also they extended their investigations to nonstoichiometric systems. Consistent with

Winter's results, they observed $G''/G' = \sqrt{3}$ and $G', G'' \propto \omega^{2/3}$ at the gel point in a system with stoichiometric ratio $r = 0.7$; whereas congruent moduli equivalent to those in stoichiometric systems was found when $r > 1$. They suggested this change is a continuous change from Rouse-like ($G' = G'' \propto \omega^{1/2}$) to Zimm-like ($G''/G' = \sqrt{3}$ and $G', G'' \propto \omega^{2/3}$) behavior, and was due to the lower degree of overlap of the coils near the gel point in systems with $r < 1$. This would lead to a reduction in the screening of the hydrodynamic interactions and hence a change from Rouse-like to Zimm-like behavior.

In summary, the above experimental studies, comprising both Winter's and Muller's crosslinking systems, led to a new definition of the gel point [5] experimentally:

$$\tan \delta = G''/G' = \tan (n\pi/2) \quad (1.7)$$

where n is the common exponent of the power laws at the gel point ($1/2 \leq n \leq 2/3$). Winter [4] attributed the change of n to a change of fractal dimension (i.e. $d_f = 2n/(1-n)$ by Muthukumar [8]). Muller [5] regarded it as a change between Rouse-like ($n=1/2$) and Zimm-like ($n=2/3$) limits. Muller's argument about Zimm-like behavior ($n=2/3$) is explained by Winter as a Cayley tree ($d_f=4$). Moreover, the previous definition of the range of n is based on experimental results and neither of these argument is capable of predicting a specified range of n for a single d_f (e.g. $d_f=2.52$)

changing from Rouse-like to Zimm-like behavior. A better theoretical approach should be based on Muthukumar's theory [10] as described in the previous chapter. For a percolation cluster $d_f=2.52$, the frequency dependence of the viscoelasticity is $\eta^* \propto \omega^{-4/9}$ for the Rouse limit and $\eta^* \propto \omega^{-1/6}$ for the Zimm limit, which gives rise to congruent power-law exponents ranging from $n=5/9$ (Rouse) to $n=5/6$ (Zimm). For a Cayley tree $d_f=4$, the frequency dependence of the viscoelasticity is $\eta^* \propto \omega^{-1/3}$, giving $n=2/3$. $n=1/2$ would be considered as an extra-Rouse experimental special case in this approach.

As mentioned earlier, a quite different set of predictions regarding the viscoelastic properties at the gel point has been proposed based upon a percolation theory model. de Gennes [11] treated the gel network by analogy to resistor-superconductor networks. For zero steady shear viscosity and equilibrium modulus near the gel point, the scaling prediction from percolation theory is $\eta_0 \propto |p-p_c|^{-s}$ for $p < p_c$ and $G \propto |p-p_c|^t$ for $p > p_c$, where p is the extent of reaction near the gelation critical point p_c . Computer simulation [12,13] yields values of t and s . Recently, Martin [6,7] and some other groups [14,15] used further fractal and percolation arguments to describe the dynamics and predict the frequency dependence in the critical region, where $G' \propto G'' \propto \omega^n$. The polymer fractals of mass M_i are treated in the Rouse limit with relaxation time $\tau_i \propto M_i$. In the Zimm limit, cluster relaxation is governed by Stokes-Einstein diffusion. Thus the environment

of any test molecule is composed of smaller fractal clusters relaxing at a faster rate, and larger clusters which are considered immobile on the time scale of the test molecule. Using such a dynamical model, the power-law frequency dependence of G' is predicted to be $n = t/(s+t)$ [6,15]. Application of the de Gennes analogy using computer simulated values of t and s gives $n = 0.72 \pm 0.2$. Martin's theoretical calculation [6] leads to a final result:

$$G' \propto G'' \propto \omega^n, \text{ with } 2d/(d+6) \leq n \leq 1 \quad (1.8)$$

where $n = 2d/(d+6)$ represents the Rouse limit and $n=1$ represents the Zimm limit. For a typical percolation cluster ($d=3$), this gives $2/3 \leq n \leq 1$. For a Cayley tree (mean-field limit, $d=6$), the exponent n must be uniquely 1. Experimentally [7], these authors measured the complex viscosity and complex shear modulus of epoxy resins (diglycidyl ether of bisphenol A, with crosslinker diethanolamine) at 90°C using a parallel plate rheometer, and found $G' \propto G'' \propto \omega^{0.70}$, which is close to the Rouse limit of $n=2/3$. Note, however, that the Rouse limit corresponds to $n=5/9$ in Muthukumar's theory, and $n=1/2$ in Muller's experimental model. Moreover, Martin et al [7] point out that their prediction is not consistent with experimental results on stoichiometric systems or when excess crosslinker is present where $n = 1/2$ is observed. They argue that in gels prepared with the minimum amount of crosslinker necessary to reach the gel point, the crosslinkers barely site percolate and the system is well described by the

percolation model. However, in systems with excess crosslinker there may be a tendency to form rapid difunctional bonds, leading to initial production of long linear chains which subsequently slowly crosslink or vulcanize to form a gel. They suggest that these long interlinking linear chains will contribute interfering Rouse modes which tend to reduce n to 0.5.

Eq.(1.8) gives $n = 2d/(d+6)$ for the Rouse limit. By substituting $d_f \approx (d+2)/2$, this further yields $d_f = (2n+2)/(2-n)$. Comparison between the latter and eq.(1.5) demonstrates that these two theories predict the same $d_f (=2)$ only when $n=0.5$.

It should be recognised that despite utilising analyses based on fractal geometry and an equivalent hydrodynamic description of the polymer in solution, the predicted power law exponents at the gel point, and the associated interpretation are different for the two groups. Neither of the theories predicts $n=1/2$ observed experimentally for chemical gel systems with excess crosslinker. This state of affairs is summarized as follows:

Muthukumar-	(Rouse)	(Zimm)	
Winter [8,10]:	$5/9 \leq n \leq 5/6$		(for a percolation cluster $d_f=2.52$)
	$n = 2/3$		(for a Cayley tree, $d_f=4$)
			(1.9)

(Rouse)

(Zimm)

$$\begin{aligned}
 \text{Martin et al [6]: } 2d/(d+6) \leq n \leq 1 & \quad (\text{for a percolation cluster,} \\
 & \quad d = \text{spatial dimension}) \\
 2/3 \leq n \leq 1 & \quad (d=3, d_f \approx (d+2)/2 = 2.5) \\
 n = 1 & \quad (\text{for a Cayley tree, } d_f=4)
 \end{aligned}
 \tag{1.10}$$

In summary, previous experimental studies of the viscoelastic properties of chemical gels near the sol-gel transition demonstrate power-law frequency dependence in G' , G'' and $\eta^*(\omega)$ in the vicinity of the sol-gel transition. In this critical regime, theoretical analyses also lead to the prediction that at the gel point both the storage G' and loss G'' parts of the modulus are power laws in frequency, $G' \propto G'' \propto \omega^n$. By a Fourier transform it can then be shown that the shear relaxation modulus is $G(t) \propto t^{-n}$, and by a Laplace transform it can be shown that the spectrum of relaxation times scales $H(\tau) d \ln \tau \propto \tau^{-n} d \ln \tau$. Such arguments also lead to the conclusion that, in such materials, the creep compliance $J(t)$ should scale as $J(t) \propto t^n$. Hydrodynamic theories suggest that the power law exponent is related to the fractal dimension d_f of the gel network, thus d_f can be in principle be determined from the frequency dependence of G' , G'' or η^* , or alternatively, via eq.(1.7) from the magnitude of $\tan \delta$ at the gel point. However, because different theories predict different numerical values of n , it is clear that further experimental studies

of the viscoelastic exponent n at the gel point would be of great interest.

1.2. RHEOLOGICAL STUDIES OF A PHYSICAL GEL AT THE GEL POINT

The previous experimental studies by Winter [2-4,8], Muller et al [5], and Martin et al [6,7] pertain to type 2 gels: covalent polymeric networks. Physical gels such as gelatin may behave differently. In one of the very recent work, Lin and Winter et al [16] studied the gel point of the physical gelation process of crystallization-induced gelation in the thermoplastic elastomeric polypropylene and observed the typical scaling behavior expressed in the power law with an exponent n close to 0.13. This low value of n is again not predicted by either theory. In another recent work, Amis [17,18] investigated the dynamic viscoelasticity during thermoreversible gelation of gelatin at concentrations ranging from 0.90 to 5.10 % (w/w) using the Multiple Lumped Resonator technique (MLR) operated at five different frequencies over a range of less than two decades from 272 to 9450 Hz. They found power-law frequency dependence of G' and G'' . The viscoelastic exponents n have the initial value 1.5 and 0.9 for G' and G'' , respectively, prior to gelation and both exponents decrease as the gelation process proceeds. At the gel point, the two exponents are equal, and exhibit a range of values from 0.58 to 0.77 for different experimental conditions. The frequency

independent values of $\tan \delta$ range from 1.3 to 2.5. The average value is close to the result obtained for chemically-crosslinked gels in nonstoichiometric systems. However we note that only five oscillation frequencies covering less than two decades were utilised in their measurement. They conclude that gelation of gelatin can be described by the Rouse-Zimm percolation theory of Martin et al [6], $2/3 \leq n \leq 1$ for a typical percolation cluster. In this chapter, we describe a study of gelatin gelation utilising the Rheometrics fluids rheometer as well as creep experiment by the Carri-Med controlled stress rheometer. Our results extend the range of the previous work to higher gelatin concentrations and hence provide a more extensive test of the theoretical predictions.

1.3. GELATION OF GELATIN

The water-insoluble highly-organized collagen fibers of connective tissue can be degraded or denatured into a water-soluble product named gelatin, with a lower degree of internal order. During the collagen $\rightarrow$ gelatin transition, the ordered triple-helical chains of the fibrous or crystalline collagen are transformed to random coils. This is called the hot form of gelatin. Upon cooling, the random coiled of gelatin partially regains its intramolecular and some chains may locally recover the triple helical structures. This second form of gelatin is called the cold form or the "collagen fold".

The gelation process has been observed and studied for a long time. The development of the collagen fold was characterised by the X-ray diffraction patterns of gelatin and comparison with those of collagen [19]. X-ray diffraction pattern of collagen show three spacings, at 2.85, 4.5 and 11 Å. Gelatin films prepared by drying cold gelatin gels showed only 2.86 Å and 4.4 Å rings with an absence of the longer-range 10-16 Å spacing. This indicates the return of the short-range ordering of the peptide chains. Infrared dichroism can be used as another tool [20]. In both collagen fibers and cold gelatin films, a shift in the N-H stretching frequency from 3300 cm^{-1} to 3300 cm^{-1} is observed due to formation of hydrogen bonds. The dichroism is perpendicular, indicating that hydrogen bonds perpendicular to the peptide backbone chain axis are involved in forming links between the ordered segments during gelation. The initial event appears to be intramolecular reorganization, followed by intermolecular association.

The collagen fold is believed to have a poly-L-proline II helix type of chain conformation [21]. This idea is borne out by the fact that the X-ray diffraction pattern is very similar to that of native collagen [22]. The studies of Grassman [23] and Eagland [24] of a single-strand α -gelatin chain produced distinct models (fig.1.1.) for collagen fold formation. Grassman pointed out that gelatin peptides have regions rich in pyrrolidine nonpolar residues or rich in polar residues. The polar regions can be

(a) by Grassman et al

(b) by Eagland et al

Figure 1.1. Two models for collagen fold formation in a single-strand α -gelatin.

imagined to form an unstable poly-L-proline II intermediate, which serves to nucleate the collagen fold (assumed to be the rate determining step). Dipoles on the same chain attract each other and lead to the formation of compound helices stabilised by hydrogen bonds, electrostatic interactions, and the organized water structure which surrounds the compound helix segment. On the other hand, Eagland proposed that certain single turn helices can occur along the random coil chain to form a nucleated random coil. Subsequently, this coil folds back upon itself twice bring three single-turn helices into juxtaposition and then forms a nucleated triple-helix region (rate determining step). Finally, the remaining portion of the chain loop "zip up" to form extended runs of helix. The precise details of collagen fold formation are not crucial to our gelation studies. What is important is that intermolecular crosslinks will form through aggregation of three peptide chains via collagen fold formation to form a three-dimensional gel network.

Peniche-Covas et al investigated [25] the applicability of the classical theory of gelation (formation of a Cayley tree structure), using a generalised expression for any number of chains involved in a crosslink, formed at random between homodisperse primary chains, to the gelation of aqueous gelatin. The evolution in time of Young's modulus E of gelatin gels was measured in a micro-sphere rheometer. The data were fitted reasonably well to the classical rubber elasticity theory, if a random crosslinking process is

postulated, each crosslink consisting of a rapidly formed helical section comprising three intertwined primary chains.

In spite of extensive studies of gelatin and its gels, the gelation process is still not well understood in terms of the functionality of the interchain connections and the detailed mechanism of gelation.

2. EXPERIMENTAL

2.1. INTRODUCTION

The viscoelastic detection and characterisation of the gel point for gelatin, a typical physical gel, is the focus of this chapter. In the subsequent two chapters, native collagen gels will be used to model the viscoelastic behavior of the connective tissue of skin, and calcium alginate gels will be used to simulate the non-Newtonian mucus in the respiratory airway. Like gelatin, these are physical gels which are typically reversible by readjustment of pH, temperature, ionic strength or concentration of multivalent ions to the original values for the sol state.

As previously mentioned, existing theoretical interpretations of the gel point are controversial. The earlier studies of gelatin gelation by Amis et al [16] indicated confirmation of the general validity of Martin's theoretical

exponent. Power-law viscoelastic exponents were determined for gels containing 0.90 to 5.10 %(w/w) gelatin at frequencies from 272 to 9400 Hz. The deficiencies of their experimental method using Multiple Lumped Resonance are: first, only five frequencies in less than two decades were used, so all exponent values were estimated based on five points regression; second, because the MLR measurement requires about 10 minutes duration, the gelation experiments had to be performed at rather dilute concentrations, and at high temperatures (quenched from even higher temperatures) to ensure sufficiently slow gelation. This is likely to have caused inevitable water evaporation during the measurement. The methodology applied in our current study is complementary to their approach. A Rheometrics Fluids Spectrometer utilising cone and plate geometry (fig.0.3.) was used. This instrument can perform dynamic frequency scans from 0.01 to 100 rad/s. However, in order to confine the measurements to about one minute duration, ten frequencies from 1 to 63.1 rad/s (or 0.16 to 10.04 Hz) were selected. This range represents about two decades of lower frequency compared to the MLR. Due to the sensitivity limit of our transducer, our studies were limited to comparatively high concentrations of gelatin solutions (4 to 12 %(w/v)).

In addition, creep compliance is measured in a Carri-Med controlled stress rheometer. As we mentioned earlier, if $G' \propto G'' \propto \omega^n$, the relaxation modulus can be shown by a Fourier transform

as $G(t) \propto t^{-n}$. As $\tan \delta$ is constant at the gel point, by eqs.(0.13) and (0.14), $J' \propto J'' \propto \omega^{-n}$. In a similar way, the creep compliance can be shown as $J(t) \propto t^n$. Thus the creep compliance measurement can confirm the power-law relation and power-law exponent n in the dynamic measurement.

2.2. MATERIALS AND METHODS

Gelatin (Fisher chemicals) of bloom strength 100 was used. (This gelatin was a gift from the Cleveland Clinic where it was used as a biolized surface coating for the artificial heart.) According to information from the manufacturer, this gelatin was made and purified from cattle connective tissues, including skin and bone. It is an alkali-precursor gelatin with isoionic point in the range pH 4.7-5.1. Its average molecular weight is around 80,000. This gelatin was completely dissolved at 50-60°C in double distilled deionized water to the desired concentrations. The pH of these solutions is 5.5 with only small variations in the concentration range 4-12 % (w/v) utilised in the present research. The pH of gelatin solution was adjusted to a value 3 or 9 by adding small amount of HCl or NaOH. In some experiments, gelatin was dissolved either in phosphate buffered saline (PBS) to pH=7 or in 1 M NaCl or Guanidinium chloride (Gdn·HCl) solution at pH 5.5.

The solutions were maintained at 26-27°C before measurement of viscoelastic properties. For the latter, 670 μ l of solutions were rapidly quenched to the measurement temperature in the Rheometrics Fluids Spectrometer. This instrument has been described in an earlier chapter and a cone and plate geometry with 0.02 radian angle and 50 mm diameter cone was used. The temperature control of the instrument was achieved by a circulating fluid bath of ethylene glycol and water. Because of its most crucial effect on the behavior of the thermally-reversible gelation process, the temperature must be strictly controlled within $\pm 0.05^\circ\text{C}$. The instrument was set in the dynamic frequency sweep mode with 5% strain as input. For each time point, measurement of each set of ten frequencies from 1 to 63.1 rad/s takes about one minute. The frequency dependence of the storage and loss moduli at each time point as well as the time dependence of moduli for each of the ten frequencies were obtained.

From the results, determination of the gel point is performed by plotting $\tan \delta$ as a function of time for each frequencies, and observing the appearance of frequency independent crossover points; or, alternatively, by plotting viscoelastic exponents n' and n'' ($G' \propto \omega^{n'}$, $G'' \propto \omega^{n''}$) obtained from the frequency dependence of G' and G'' at each time point, and observing a crossover where $n'=n''=n$. These results for $\tan \delta$ and n can be used to test the validity of eq.(1.7) proposed by Winter

and Muller. Comparative estimates of the fractal dimension d_f can be determined by the two alternative theoretical models: Winter's treatment using eq.(1.5) and Martin's treatment using eq.(1.8) and $d_f \approx (d+2)/2$.

For the creep measurement, 60 μ l 8% gelatin solutions were quenched from 40°C to the desired temperature in the Carri-Med rheometer. A cone and plate geometry with 1 degree angle and 20 mm diameter cone was used. The temperature is controlled by a Peltier system, located in the lower plate, thus a small temperature gradient exists across the sample. Typical creep curves are shown in Figure 1.2. [26]. In our experimental curves, two portions may be distinguished: initially, creep retardation occurs (0-10 sec), followed by creep relaxation (10-20 sec) on removal of the applied stress. A permanent deformation is defined as $\gamma_{t=20\text{sec}}$, but this may not be a true permanent deformation if the relaxation times are very long, since our observation time (10 sec) is probably not long enough.

The creep retardation curves were fit by power-law time-dependence with exponent n , that is, $\gamma(t) \propto t^n$ or $J(t) = \gamma(t)/\sigma_0 \propto t^n$. Comparisons can be made between these n along gelation and those obtained from the dynamic measurement.

3. RESULTS AND DISCUSSION

Viscoelastic solid

Figure 1.2. Typical shear creep and creep recovery shown schematically for a viscoelastic liquid and a viscoelastic solid.

Table 1.1. shows a list of the dynamic measurement for gels prepared and measured under varying conditions. There were twenty-eight experimental conditions using the methodology described in the previous section. Typical results for one set of experiment are shown in Figure 1.3. In (a), the gel point is determined by observation of a frequency-independent $\tan \delta$ obtained from a multi-frequency plot of $\tan \delta$ vs. gelling time. In (b), determined quite separately from the $\tan \delta$ measurement, the unique power-law exponent (n) at gel point, is obtained from linear regression log-log fits of G' and G'' vs. ω (frequency) plots. In (c), n at gel point is confirmed from the crossover of the power-law viscoelastic exponents for G' and G'' (defined as n' and n'' , respectively) vs. gelling time. The gelation time t_{gel} (i.e. time to transition) at gel point observed from (a) usually matches very well with that from (c).

A list of the results for $\tan \delta$ and power-law exponent n , determined as described in last paragraph, are summarized in Table 1.2. The results (for 12% gelatin at 24.7°C) were demonstrated to be very reproducible when the temperature is strictly controlled. The $\tan \delta$ and n values generated in this way were also used to test the validity of the relation $\delta = n\pi/2$ theoretically predicted as a new definition for the gel point (eq.(1.7)). It was confirmed that the above relation was valid in our experiment with a small experimental variation giving an average relation between δ and n as $\delta = n\pi / (2)(1.038)$. This

Table 1.1. A list of experimental conditions.

conc. w/v %	other component	pH ± 0.3	temp. $^{\circ}\text{C}$	t_{gel} sec
12%		5.5	24.7	960
12%		5.5	24.7	930
12%		5.5	24.7	960
12%		5.5	24.7	930
12%		5.5	24.3	600
12%	PBS	7.0	24.3	5500
12%	1M NaCl	5.5	21.1	3900 ⁺
12%	1M NaCl	5.5	20.4	2640
12%	1M NaCl	5.5	20.4	2940
12%		3	19.0	2880
12%		3	18.3	1170
12%	1M NaCl	3	16.0	3480
12%	1M NaCl	3	15.6	2280
12%	1M NaCl	3	15.2	1200
12%		9	22.3	2040
12%		9	22.3	2100
12%	1M NaCl	9	19.6	2040
12%	1M NaCl	9	19.5	1830
12%	1 M Gdn·HCl	5.5	10.9	2460
12%	1 M Gdn·HCl	5.5	10.5	1860

(continue from the previous page)

conc. w/v %	other component	pH ± 0.3	temp. °C	t _{gel} sec
10%		5.5	23.6	2220
10%		5.5	23.3	1040
10%		5.5	23.3	940 ⁺
10%		5.5	23.0	900
10%	PBS	7.0	23.0	4680
10%	PBS	7.0	22.0	1250
10%	PBS	7.0	21.3	720
8%		5.5	21.7	2430
8%		5.5	21.4	1545
8%		5.5	21.0	1380
8%	PBS	7.0	21.4	3720
8%	PBS	7.0	20.3	1725
4%		5.6	17.5	2700
(sensitivity limit for low conc.)				

⁺ All the solutions were quenched from 26-27°C to the gelling temperatures, except for these two. 12% in 1M NaCl one was first quenched to 24.3°C and 10% one was first quenched to 24.0°C and only small change in viscoelastic properties was observed within 30 minutes under those temperatures. They were then quenched to the final gelling temperatures.

Figure 1.3. A typical set of analytic plots for data from measurement. $\tan \delta$ and n at gel point were determined separately.

- (a) Multi-frequency plot for $\tan \delta$ vs. gelling time.
- (b) Linear regression to get the exponent n at gel point.
- (c) Power-law exponents n' and n'' vs. gelling time.

(b)

(c)

Table 1.2. Results of data analysed by the plots shown in fig.
1.3. Methods are described in text.

conc. w/v%	other salt	pH ± 0.3	temp. °C	t _{gel} sec	tan δ	n	n by eq(1.7) $n=2\cdot\delta/\pi$
12%		5.5	24.7	960	1.76	0.70	0.67
12%		5.5	24.7	930	1.74	0.69	0.67
12%		5.5	24.7	960	1.76	0.70	0.67
12%		5.5	24.7	930	1.78	0.70	0.67
12%		5.5	24.3	600	1.66	0.68	0.65
12%	PBS	7.0	24.3	5500	1.84	0.71	0.68
12%	1M NaCl	5.5	21.1	3900+	1.98	0.72	0.70
12%	1M NaCl	5.5	20.4	2640	1.87	0.70	0.69
12%	1M NaCl	5.5	20.4	2940	1.71	0.70	0.66
12%		3	19.0	2880	1.72	0.69	0.67
12%		3	18.3	1170	1.56	0.69	0.64
12%	1M NaCl	3	16.0	3480	1.61	0.64	0.65
12%	1M NaCl	3	15.6	2280	1.64	0.66	0.65
12%	1M NaCl	3	15.2	1200	1.60	0.66	0.64
12%		9	22.3	2040	1.69	0.70	0.66
12%		9	22.3	2100	1.78	0.71	0.67
12%	1M NaCl	9	19.6	2040	1.70	0.70	0.66
12%	1M NaCl	9	19.5	1830	1.73	0.71	0.67
12%	1M Gdn·HCl	5.5	10.9	2460	1.79	0.72	0.68
12%	1M Gdn·HCl	5.5	10.5	1860	1.71	0.69	0.66

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conc. w/v %	other salt	pH ± 0.3	temp. $^{\circ}\text{C}$	t_{gel} sec	$\tan \delta$	n	n by eq(1.7) $n = 2 \cdot \delta / \pi$
10%		5.5	23.6	2220	1.68	0.68	0.66
10%		5.5	23.3	1040	1.70	0.69	0.66
10%		5.5	23.3	940+	1.69	0.67	0.66
10%		5.5	23.0	900	1.65	0.68	0.65
10%	PBS	7.0	23.0	4680	1.75	0.69	0.67
10%	PBS	7.0	22.0	1250	1.73	0.69	0.67
10%	PBS	7.0	21.3	720	1.61	0.68	0.65
8%		5.5	21.7	2430	1.72	0.69	0.66
8%		5.5	21.4	1545	1.70	0.69	0.66
8%		5.5	21.0	1380	1.56	0.66	0.64
8%	PBS	7.0	21.4	3720	1.77	0.69	0.67
8%	PBS	7.0	20.3	1725	1.71	0.68	0.66
4%		5.6	17.5	2700	1.63	0.67	0.65
					Ave.=	Ave.=	Ave.=
					1.71 $\pm$	0.69 $\pm$	0.66 $\pm$
					0.08	0.02	0.01

small deviation may be caused by the finite times required for measurement. The time taken to scan from low to high frequency has the effect of shifting $\tan \delta$ downward more rapidly than shifting n downward by a factor which is evidently of the order 1.038. Or in other words, $\tan \delta$ is more sensitive to the fact that we have ignored in our analysis that the measuring time produces a spread in the multi-frequency time points from low to higher frequency.

In order to check the self-consistency of the data and the accuracy of the linear regression analysis, another set of $\tan \delta$ and n values (Table 1.3.) was generated by using the linear regression intercepts A and B of G' and G'' vs. ω plots at the gel point as an alternative route:

$$G'(\omega) = A\omega^n \quad (\text{from linear regression}) \quad (1.11.a)$$

$$G''(\omega) = B\omega^n \quad (\text{from linear regression}) \quad (1.11.b)$$

$$\tan \delta = G''/G' = B/A \quad (1.12)$$

$$n = 2 \tan^{-1} (B/A) / \pi \quad (1.13)$$

$$\bar{n} = 1.038 \times \frac{2}{\pi} \times \tan^{-1}(\tan \delta) \quad (1.14)$$

A and B are related to the material strength factor S , by $S = \sqrt{A^2 + B^2} \Gamma(n) \sin(n\pi)/\pi$. By comparing these two sets of results, it was found that $\tan \delta$ values by the alternative method are very close to that determined from the multi-frequency plot as in Figure 1.3.a., and in addition, $\bar{n}$ is very close to n determined from

Table 1.3. Alternative way to determine $\tan \delta$ from linear regression intercepts, and n from equations relating $\tan \delta$ and n .

conc. w/v %	other salt	pH ± 0.3	temp. $^{\circ}\text{C}$	t_{gel} sec	B/A	$\tan \delta$ by eq(1.12)	n by (1.13)	$\bar{n}$ by (1.14)
12%		5.5	24.7	960	4.52/2.53	1.79	0.68	0.70
12%		5.5	24.7	930	5.15/2.97	1.73	0.67	0.69
12%		5.5	24.7	960	5.00/2.83	1.77	0.67	0.70
12%		5.5	24.7	930	4.96/2.80	1.77	0.67	0.70
12%		5.5	24.3	600	5.53/3.36	1.64	0.65	0.68
12%	PBS	7.0	24.3	5500	4.45/2.36	1.89	0.69	0.72
12%	1M NaCl	5.5	21.1	3900+	3.84/1.95	1.97	0.70	0.73
12%	1M NaCl	5.5	20.4	2640	4.65/2.62	1.77	0.67	0.70
12%	1M NaCl	5.5	20.4	2940	5.19/2.96	1.75	0.67	0.69
12%		3	19.0	2880	4.85/2.82	1.72	0.67	0.69
12%		3	18.3	1170	4.62/3.06	1.51	0.63	0.65
12%	1M NaCl	3	16.0	3480	4.85/3.01	1.61	0.65	0.67
12%	1M NaCl	3	15.6	2280	4.52/2.72	1.66	0.66	0.68
12%	1M NaCl	3	15.2	1200	4.55/2.76	1.65	0.65	0.68
12%		9	22.3	2040	4.89/2.99	1.63	0.65	0.68
12%		9	22.3	2100	4.35/2.63	1.66	0.65	0.68
12%	1M NaCl	9	19.6	2040	4.94/2.94	1.68	0.66	0.68
12%	1M NaCl	9	19.5	1830	4.73/2.71	1.74	0.67	0.69
12%	1M Gdn·HCl	5.5	10.9	2460	4.85/2.70	1.80	0.68	0.70
12%	1M Gdn·HCl	5.5	10.5	1860	5.50/3.30	1.67	0.66	0.68
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conc. w/v %	other salt	pH ± 0.3	temp. $^{\circ}\text{C}$	t_{gel} sec	B/A	$\tan \delta$ by eq(1.12)	n by (1.13)	$\bar{n}$ by (1.14)
10%		5.5	23.6	2220	5.17/2.97	1.74	0.67	0.69
10%		5.5	23.3	1040	5.52/3.25	1.70	0.66	0.69
10%		5.5	23.3	940 ⁺	4.71/2.91	1.62	0.65	0.67
10%		5.5	23.0	900	4.06/2.46	1.65	0.65	0.68
10%	PBS	7.0	23.0	4680	4.50/2.44	1.85	0.68	0.71
10%	PBS	7.0	22.0	1250	4.50/2.55	1.76	0.67	0.70
10%	PBS	7.0	21.3	720	4.79/2.91	1.65	0.65	0.68
8%		5.5	21.7	2430	2.78/1.63	1.70	0.66	0.69
8%		5.5	21.4	1545	2.60/1.51	1.73	0.67	0.69
8%		5.5	21.0	1380	3.31/2.07	1.60	0.64	0.67
8%	PBS	7.0	21.4	3720	2.51/1.36	1.85	0.68	0.71
8%	PBS	7.0	20.3	1725	3.10/1.81	1.72	0.66	0.69
4%		5.6	17.5	2700	1.18/0.73	1.62	0.65	0.67
						Ave.=	Ave.=	Ave.=
						1.71 $\pm$	0.66 $\pm$	0.69 $\pm$
						0.08	0.01	0.02

the crossover in the viscoelastic exponent plot as in Figure 1.3.c. It is concluded that the data are very consistent and the linear regression technique based on ten frequencies did not induce large errors, thus experimental power-law exponents n are very reliable.

From Tables 1.2. and 1.3., we deduce that, if the gelling process is too short such that each measurement is a substantial fraction of the gelation time (as e.g., in the case of 12% gelatin at 24.3°C or 10% gelatin in PBS at 21.3°C), $\tan \delta$ tends to shift to a smaller value . The same situation occurs for n . From the experience gained in our experiments, it was determined that gelation times longer than 900 sec are required for accurate measurement of $\tan \delta$ and n (i.e. a single measurement should take less than 7% of the total gelation time). In addition, as previously mentioned, we find that these parameters are exceedingly sensitive to temperature variation.

The data show that the gelatin concentration does not have a significant effect on the power-law exponent. Higher concentrations result only in a higher range of gelling temperatures and a higher gel strength factor. The effect of temperature is mainly on the gelling rate. Its effect on the power-law exponent, if any, is modest.

The effect of pH and increases in ionic strength by mixing 1 M NaCl or PBS on the power-law exponent appears to be negligible too. A change of pH from pH=5.5 (close to the isoionic point) to a lower or higher value and the presence of 1 M NaCl in gelatin caused considerable delay of gelation but has little effect on $\tan \delta$ and n at the gel point. The delay of gelation in PBS buffered (pH=7) gels is probably caused by both the presence of 0.14 M NaCl in PBS buffer and the pH effect. The presence of 1 M Gdn·HCl decreases the gelling rate even more dramatically. These kinetic changes are presumably caused by a decrease in the intermolecular interactions (hydrogen-bonding, dipolar, electrostatic or hydrophobic interactions) which drive collagen fold formation between two segments of gelatin coils.

All of the variables discussed here, including concentration, temperature, pH, ionic strength and the addition of a denaturing agent, apparently do not modify the basic gelation mechanism, in spite of their remarkable influence on the gelation kinetics. Under all conditions we have used, the n values falls between 0.64 and 0.72 (with an average n or $\bar{n} = 0.69 \pm 0.02$), a quite narrow range, and are numerically consistent with the results of Amis et al [17,18]. Based on the percolation model of Martin [6,7,eq.(1.10)], with $d=3$, our experimental n values fall rather close to the Rouse limit ($n=2d/(d+6)=0.667$) and are quite different from the Zimm limit ($n=1$). This indicates that gelatin gels are typical percolation clusters ($d=3$, $d_f=2.52$) at the Rouse limit ($n=0.667$). Our results

are similar to previous experimental results for gelation of epoxy resins ($n=0.70\pm0.05$) [7], polyester ($n=0.69\pm0.02$) [15], polyurethanes ($n=0.69\pm0.02$) [27], and tetraethoxysilane ($n=0.71\pm0.05$) [28,29].

Alternatively, if we apply the analysis of Muthukumar and Winter [8,10,eq.(1.9)], our n values fall between the extremes of the predicted values for a percolation cluster ($5/9 \leq n \leq 5/6$) and are numerically close to prediction for a Cayley tree in the Rouse limit ($n=0.667$). Alternatively, we may consider the explanation, offered by Muller [5] that we are dealing with a nonstoichiometric gel with a deficit of crosslinker so that we have a preponderance of linear chains with Zimm-like behavior at the gel point ($n=0.667$).

In summary, therefore, for the dynamic viscoelastic experiments, we conclude that our data are consistent both with Martin's theoretical treatment based on the percolation model at the Rouse limit, and with Muthukumar's model of a Cayley tree at the Rouse limit, or of a percolation cluster between Rouse and Zimm limits. Under all conditions, the power-law exponent is close to 0.667.

The possibility exists that the finite strains imposed on a gel in a controlled-strain rheometer may disrupt the gel structure and produce anomalous results. It is of interest therefore to carry out

creep experiments in gelling experiments using a controlled-stress rheometer. The creep experiments were performed only on 8% gelatin at 23°C (near room temperature) using 1°/2cm cone at an applied stress $\sigma_0=10$ dynes/cm², in order to minimise the temperature gradient across the sample between cone and plate. A transition from purely Newtonian behavior at gelling time 1 min to mostly elastic behavior at gelling time 74 min was observed, as shown in Figure 1.4. Most of these retardation curves can be fit by a power-law time-dependence with exponent n : $\gamma(t) \propto t^n$. It is found that, as gelation precedes, n decreases from $n=1.118$ at gelling time 1 min to $n=0.563$ at gelling time 43 min. After 43 min, a plateau region (equilibrium compliance) appears in the curves together with an instantaneous recoverable strain, indicative of the presence of a gel network. Observation of an equilibrium compliance makes a power-law fit no longer possible. During this transition process, the permanent deformation (as defined earlier) becomes smaller. When n approaches 0.67 which we anticipate corresponds to the gel point (between $n=0.697$ at gelling time 35 min and $n=0.644$ at gelling time 40 min), the magnitude of the permanent deformation tends to vary irregularly, and so does n . (Note: we did observe curves with a systematically decreasing permanent deformation at 36.75 min and 38.25 min. These data are not shown here since they were obtained using a slow ten-point (1 point per sec) sampling instead of the high-speed sampling technique which gives 280 data points.) The non-systematic variation in the permanent

Figure 1.4. Creep experiment on 8% gelatin at 23.0°C. Series of curves represents a transition from predominantly viscous to elastic response.

deformation probably occurs because the comparatively large strains reached during creep ($\approx 10\%$) disrupt the fragile gel network clusters. Finally, the permanent deformation consistently decreases after 40 min, with a marked instantaneous recoverable strain $\gamma_{t=0\text{sec}}$ and $\gamma_{t=10\text{sec}}$ appearing. From our observations, the gel point falls between 35 min and 40 min with a power-law exponent n in the range 0.66 to 0.72.

A stress level of 10 dynes/cm² is believed to be far above the yield stress of the critical gel formed at the gel point. The yield stress for the critical gel is expected to be very small (<1 dyne/cm²) and cannot be measured by the rheometer due to the requirement of having to overcome the cone inertia. (For example, for a pure gelatin sol, we still observe a small apparent yield stress of around 0.2 dyne/cm².) One ideal method for determining the gel point if the rheometer could distinguish the inertial yield stress and the true material yield stress would be to observe the initial appearance of a yield stress in the creep measurement.

The creep experiments generally confirm what was observed earlier in the dynamic experiments that the gelatin gels exhibit a viscoelastic power-law exponent which is close to the theoretically-predicted limiting value at the percolation threshold 0.667. The variation of the exponent n in dynamic measurement on all systems, 0.69 ± 0.02 ($n=33$ best sets of data), appears to be

non-systematic and defines therefore the range of experimental errors. This behavior is also evident in the creep measurement as a non-systematic variation in n ($0.70 \rightarrow 0.72 \rightarrow 0.67 \rightarrow 0.66 \rightarrow 0.64$) over a narrow range of times (35-40 min) precisely as the sol-gel transition occurs. Both of these variations may be related to the disruption of the early weak gel clusters, which tends to reverse the transition to some extent, resulting in a higher n . Due to this mechanism, there exists a dilemma in controlling the rate of gelation for measurement. As mentioned earlier, if the gelling process is too rapid, n tends to shift to a smaller value. However, if the process is too slow, disruption of percolation clusters may be significant and shift n to a higher value.

Most gels whose gelation mechanism has been found to be consistent with the percolation model are irreversible gels [30]. Gelation of gelatin proceeds by a physical mechanism, and it would be of interest to investigate the transition from the opposite direction, i.e. from the gel to the sol state, by raising temperature. Because the temperature controller for our Rheometrics rheometer cannot change rapidly between two temperatures, such an idea cannot be carried out with this rheometer.

The percolation model assumes that the sol 'monomers' occupy the sites of a periodic lattice, and, between monomers on two nearest-neighbor lattice sites, bonds are formed randomly.

From our previous discussion, it is assumed that gelation of gelatin is brought about by random interchain collagen fold formation between two or three coils where each coil is assumed to occupy a lattice site. Statistically, it seems more likely that two coils are involved in the crosslinking process, as shown in Figure 1.5., where we have shown a possible gelation mechanism based on Grassman's mechanism for helix formation in single-strand gelation (fig.1.1.). A similar mechanism of the Cayley tree model is shown as Figure 1.6.

Figure 1.5. Percolation model for gelation of gelatin via collagen fold formation.

Figure 1.6. Cayley tree model for gelation of gelatin via collagen fold formation.

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CHAPTER 2. VISCOELASTICITY OF AIRWAY SECRETIONS AND THE UPPER AIRWAY REOPENING

1. BACKGROUND

1.1. UPPER AIRWAY REOPENING

Upper airway closure is a common health problem in adults and in infants where it may lead to hypoxemia and respiratory failure. The importance of the mechanical properties of the upper airway in the pathogenesis of sleep apnea, asthma, and other airway diseases has received recent attention [1].

In normal function, the lower airway (bronchi and bronchioles) closes at the end of expiration and reopens at the beginning of inspiration [2]. The time required to reopen the airway is important to ensure sufficient ventilation and gas exchange. Two mechanisms have been proposed for airway closure. The first [3] is film collapse; the airway closes by meniscus formation, and thus a film of the lining fluid obstructs the airway. The second [3,4] is compliant collapse; the walls collapse and are held together by the adhesive properties of the lining fluid. Currently, the evidence [5] is in favor of the latter mechanism, that is, the walls do indeed collapse, probably as a result of negative pressures induced by a film meniscus [6].

In contrast to the situation in the lower airway, the upper airway (i.e. nose, pharynx, larynx and extrathoracic trachea) must be kept as patent as possible. Long-term patency of the upper airway requires a high resistance to collapsing forces and the capability of rapid reopening after episodes of obstruction since infrequent and brief apneas are often present in healthy individuals.

All regions of the upper airway are capable of changing size and shape (i.e. they are compliant). As in all other parts of the respiratory tract, the walls are covered with mucosa and the secretions overlying the mucosal surface which are termed respiratory mucus. The latter is in constant motion via ciliary activity toward the orifice of the esophagus. Airway closure is favored by the negative intraluminal pressure generated by inspiration. Closing pressure is the term used to indicate the negative intraluminal pressures which collapse the airway. The resistance to airway closure is related to tissue elasticity, muscle activity and mucosal factors [7-9]. On the other hand, evidence suggests that airway reopening is not a simple reversal of airway closure [7-11]. Muscle activation still plays a role but the compliance of the wall is apparently not as important as in airway closure. Most significantly, the properties of the airway lining fluids appear to determine the ease of reopening of the airway [8,10-12]. Thus, as observed by Olson and Strohl [11], filling the upper airway with methacholine-induced secretion has the effect

of impeding airway reopening. This leads to the possibility that pathological changes in the viscoelastic behavior of the airway fluids may be involved in the pathogenesis of certain airway diseases.

1.2. RHEOLOGICAL PROPERTIES OF AIRWAY SECRETIONS

As we mentioned above, the viscoelastic properties of the airway lining fluids may affect airway reopening. Not only that, the main function of respiratory mucus is to clear foreign matter from the lung and the airway. A complete understanding of the viscoelastic properties of normal and abnormal respiratory mucus is thus important.

The composition of human respiratory mucus includes about 1% (by weight) salts and other dialysable contents, 1% lipids, 0.5 to 1% free proteins, 0.5 to 1% carbohydrate-rich glycoproteins ('mucin') and 95% or more water [13-15]. A schematic plot for a drop of mucus is shown by Braga [16] as in Figure 2.1. Its rheological properties are mainly attributed to the mucin content [17]. Human respiratory mucins are macromolecules with molecular weight ranging from 2×10^5 to 2×10^7 . Each molecule has a protein core rich in threonine, serine and proline, with many branched oligosaccharide side chains. It consists of 70-80% carbohydrate, 20-30% protein and 1-2% sulfates [17].

Figure 2.1. Schematic representation of a drop of mucus with randomly coiled entangled mucin macromolecules.

The viscoelastic properties of the respiratory mucus have been studied by existing rheological techniques. Most of these observations have shown that airway mucus is a non-Newtonian, viscoelastic gel. However, the reproducibility of such measurement is poor because of the problems involved in collecting sufficient quantities for characterisation and in ensuring homogeneity of the samples [18,19]. For this reason, chemical studies were usually performed on sputum, a form of airway secretion contaminated by saliva, from patients with chronic bronchitis, asthma or cystic fibrosis. Rheological properties of sputum were also studied [20-23] despite that they were regarded unreliable. Early work of uncontaminated airway secretions by Denton [24] determined the apparent viscosity of tracheal mucus and found that certain respiratory disorders are associated with marked change in viscosity. Lutz et al [25] and King et al [26] used magnetically-driven spheres (magnetic microrheometers) to characterise the viscoelasticity of small quantities of mucus from dog tracheas. Lutz et al found $G'(\omega=1 \text{ rad/s}) = 50\text{-}130 \text{ dynes/cm}^2$ with a mean 70 dynes/cm^2 ($n=21$ from 3 dog tracheal pouches). King et al determined $G'=170\text{-}550 \text{ dynes/cm}^2$ with a mean 300 dynes/cm^2 , the mean value of $\eta'=75$ poise at $\omega=1 \text{ rad/s}$, and zero steady shear viscosity $\eta_0 = 3 \times 10^4$ poise ($n=9$ from 7 anesthetized dog tracheas). King et al [18] expanded their rheological studies of tracheal mucus to healthy human subjects, and found $\log G^* = 2.17 \pm 0.36$ and $\tan \delta = 0.28 \pm 0.07$ at $\omega=1 \text{ rad/s}$. Recent work by App and King [27] on

Table 2.1. Viscoelastic data obtained by magnetic microrheometer technique at a frequency of 1 rad/s by King et al.

Year	Ref.	Source	Values at $\omega=1$ rad/s	
1977	[26]	Dog tracheo-bronchial mucus	$G' = 300$ dynes/cm ² (mean) $\eta' = 75$ poise (mean) (precision only mentioned as typically $\pm 25\%$ for G' and $\pm 40\%$ for η')	
1985	[18]	Human tracheo-bronchial mucus	$\log G^* = 2.17 \pm 0.36$ $\tan \delta = 0.28 \pm 0.07$	
1990	[28]	Puppy trachea mucus	lower trachea:	$\log G^* = 2.23 \pm 0.36$ $\tan \delta = 0.30 \pm 0.04$
			sub glottical:	$\log G^* = 1.60 \pm 0.23$ $\tan \delta = 0.61 \pm 0.37$
		Adult Dog trachea mucus	lower trachea:	$\log G^* = 2.49 \pm 0.34$ $\tan \delta = 0.32 \pm 0.05$
			sub glottical:	$\log G^* = 2.05 \pm 0.37$ $\tan \delta = 0.27 \pm 0.05$

+Approximate range of G^* ($\omega=1$) $\approx$ 20-700 dynes/cm²,
 and approximate range of $\tan \delta$ ($\omega=1$) $\approx$ 0.2-1.0.

tracheal mucus of puppies and dogs also showed similar ranges of G^* and $\tan \delta$. Table 2.1. is a list of results for viscoelasticity of tracheal mucus obtained from a series of work by King et al using magnetic microrheometer. As noticed, the ranges of G^* and $\tan \delta$ are relatively wide. Large variations were also observed both between healthy individuals and within the same individual on different days [18]. Edwards et al [28] measured viscosity of canine tracheal pouch mucus by suspending ferromagnetic particles (0.5-1 μm in diameter) in the samples and found the viscosity to be 239 ± 10 poise at 1 sec^{-1} ($n=3$). This value is close to those obtained by King et al.

The dynamic and steady shear viscosities depend on frequency or shear rate. The shear rates that are applied to mucus are uncertain. But the shear rate applied by cilia has been suggested as 1 sec^{-1} by Dulfano [29]. In addition to frequency, the dynamic viscoelastic parameters are strongly affected by the strain applied to the mucus sample during measurement. In the magnetic microrheometer [30], a sample of mucus, into which has been inserted a small steel ball, is placed between the poles of an electromagnet. Then the ball is oscillated at different driving frequencies within the sample. The amplitude of oscillation and the current in the magnetic coil are used to determine the viscoelasticity. The strain is not well specified in these experiments.

There are certain techniques other than dynamic and steady shear experiments to characterise the rheology of mucus. Pucchelle [31] measured elastothixotropy, spinability and adhesiveness (or stickiness). Duvivier [32] used a newly designed rheometer to measure the yield stress of pathological bronchial mucus. The mean yield stress value was 19 ± 12 dynes/cm².

The rheological properties of airway secretions can be physiologically modified. By using a micropipette method to obtain secretions from single submucosal gland ducts *in vivo* in cat trachea, Ueki et al [33] concluded that cholinergic and α -adrenergic stimulation increases fluid secretion from glands. Leikauf et al [34], by using a newly developed capillary microrheometer, further demonstrated that while muscarinic, α -adrenergic or β -adrenergic stimulation all increase the secretory rate, the viscoelastic properties of the secretion induced by muscarinic stimulation were identical to control, whereas α -adrenergic stimulation produced secretion of lower apparent viscosity, and β -adrenergic stimulation produced secretion of higher apparent viscosity and lower elasticity. M. King et al observed that high dose of methacholine chloride [35] increases the secretory rate, $G'(\omega=1 \text{ rad/s})$ and $\eta'(\omega=1 \text{ rad/s})$, all to about twice of the control values, but does not change $\tan \delta$ of canine tracheal mucus; whereas pentobarbital anesthesia [36] causes a 2- to 4- fold increase in G' and η' , and a substantial decrease in the secretory rate ($< \text{one tenth of the control}$) of canine tracheal

mucus. Jeanneret-Grosjean et al [18] also found that atropine significantly alters the mucus viscoelastic properties in human trachea, as evidenced by an increase in $G^*(\omega=1 \text{ rad/s})$ from 248 ± 54 to $764 \pm 182 \text{ dyn/cm}^2$ observed 15 minutes after intravenous injection. Ammann et al [37] found that airway secretion can be modified centrally as well as peripherally by inflammatory mediators, such as substance P, prostaglandin $F_{2\alpha}$, and leukotriene D_4 . All are reported to increase airway secretions. But their effect on the rheological properties of secretion is unknown.

The importance of the rheological properties of respiratory mucus on the mucociliary transport has been well recognised and elaborately studied [27,31,35,36,38-42]. The rheological properties should fall within a given range of values for optimal clearance of secretions. The majority of the literatures on viscoelastic properties of airway secretions are from this field. In our studies, we will investigate the influence of mucus rheology on airway reopening as proposed by the animal experiments [9,11]. The general relation between viscoelasticity and reopening will be first established in an *in vitro* mechanical model, since the viscoelastic data for respiratory mucus available so far (Table 1.1.) have relatively wide range and rather low precision. Then we will characterise the viscoelasticity of airway secretions from the upper airway of animals by using rheometers operated by different principles, and compare with viscoelastic data obtained

by magnetic microrheometer technique. Finally, interpretation can be made based upon these studies.

1.3. THE ROLE OF SURFACTANTS

The essential importance of the pulmonary surfactant for normal lung function has been known for a long time. The detailed mechanisms by which this phospholipid mixture stabilises the alveolar surfaces and prevents alveolar collapse have been described in most physiology textbooks and review papers [43,44]. The equilibrium surface tension of pulmonary surfactant is ~ 25 dynes/cm. Recently, Liu et al [45] observed that the pulmonary surfactant also functions to secure free airflow through a narrow tube and hypothesized that, without pulmonary surfactant, narrow sections of the airway would fill up with a column of liquid which would be a hindrance to free airflow.

There is increased evidence showing that lung is not the only biological locus in the respiratory airway in which surfactant plays an important role [46]. The influence of respiratory surfactants on mucociliary transport has attracted attention [47-49]. They may control rheological properties [50] as well as surface properties [51] of respiratory mucus. It has been shown that surfactants can improve the mucus cough clearance by decreasing mucus adhesion [51,52]. Thus it seems reasonable to assume that surfactants could modify the airway reopening event

by modifying the surface tension and viscosity of the mucus. Indeed, recent work by Miki et al [53] found that lubrication of the pharyngeal mucosa with artificial surfactant facilitates reopening of the obstructed upper airway.

The surface tensions of respiratory tract fluids in fetal guinea pigs have been measured [54]. The mean bronchial fluid surface tensions for fetus weighing 85 to 115 grams was 44.2 ± 7.3 dynes/cm. In the upper and lower trachea, they were 63.8 ± 7.6 and 62.3 ± 9.3 dynes/cm, respectively.

2. EXPERIMENTAL

2.1. INTRODUCTION

Our study is designed to provide mechanistic insight into the role played by the lining fluids on airway function. The hypothesis is that the rheological properties of fluids lining the upper airway will determine the ease with which an airway, once closed, reopens. The approach we used employs a tube model to simulate the airway reopening event *in vitro*.

Gaver et al [55] simulated the reopening of collapsed lower airways in a benchtop airway model designed to mimic bronchial walls held in apposition by the adhesive force of the airway lining fluid. The construct has features similar to those observations

described for inflating a bronchiole [56]. The mathematical analysis of this model contains features of certain classical fluid mechanics problems, such as two-phase displacement in a Hele-Shaw cell [57,58] and the peeling of a flexible strip attached to a substrate by a viscous adhesive [59]. Using fluids of different surface tensions (γ) and viscosities (μ), and utilising thin-walled polyethylene tubing of different radii (R) with different coated film thicknesses (H) and axial wall tensions (T), Gaver et al measured the relationship between the velocity of airway opening (u) and the applied transmural pressure (p), and investigated how the physical characteristics of the lining fluid and of the airway itself affect the opening pressure-velocity relationship. Using dimensionless analysis, they found that T and H only influence the results slightly; all data normalized by surface tension, viscosity and tube radius then can be represented by a nonlinear regression formula. The relative importance of the viscous forces and capillary forces (surface tensions) depends on the capillary number ($Ca = \mu u / \gamma$). When Ca is large ($Ca > 0.5$), viscous forces dominates. When Ca is small (close to zero), the opening pressure is $\approx 8\gamma/R$, and represents a capillary yield pressure. As predicted by their analysis, the opening times are prolonged if lining fluid viscosity or surface tension is elevated.

We considered Gaver's tube model for simulating lower airway reopening would be an appropriate tool to simulate also upper airway reopening. Furthermore, we are interested in

exploring beyond his assumption that the lining fluid is Newtonian. Specifically, we extend the tube model to investigate the behavior of non-Newtonian and viscoelastic fluids, encompassing sols and gels. Thus in addition to the influence of non-Newtonian viscosity and surface tension, we investigate the roles played by the elasticity (G'), and the rheological yield stress of the lining fluids. We also study the effect of surfactants. The results from the *in vitro* tube model will be compared with existing animal experiment results.

The viscoelastic fluids chosen in our study to simulate the non-Newtonian airway secretions are alginates. The advantage of alginates for our purpose is their versatile viscoelastic properties. As shown in Figure 2.2., aqueous solutions of sodium alginate are sol-like (viscoelastic sols); upon addition of divalent cations, they are capable of changing to more gel-like behavior [60]. With increasing concentration of divalent cations, which act as a crosslinking agent, the alginate eventually turns into a true gel. Thus, by fine tuning the relative concentrations of sodium alginate and divalent cation (e.g. in our case, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), a series of fluids of systematically-varying viscoelastic properties encompassing those typical of normal or abnormal mucus can be obtained. The simulation fluids as well as samples of airway secretions isolated from animals have been characterised by rheometry and tensiometry when sufficient quantities are available.

M.W. = 70,000 - 120,000

Sol-like

Crosslinks

Gel-like

Figure 2.2. Upon addition of divalent ions, sodium alginate is capable of changing to more gel-like behavior due to crosslink formation.

Sodium alginates (alginic acid sodium salt) were from Fluka with average molecular weight 70,000-120,000. Ca^{2+} ions in the form of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (M.W.=147) was from Matheson Coleman & Bell. The surfactant utilised is sodium dodecyl (or sodium lauryl) sulfate (SDS; formula $\text{CH}_3(\text{CH}_2)_{10}\text{CH}_2\text{OSO}_3\text{Na}$, M.W.=288.4) and was from Fisher. Thin-walled polyethylene tubing with 0.002 in. wall thickness and 0.923 cm or 1.67 cm radius was supplied by Aabaco Plastics in Cleveland.

Sodium alginate was dissolved in double-distilled water to the desired concentrations. For those sodium alginate solutions containing CaCl_2 or SDS, weighed quantities of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ or SDS were dissolved in water before sodium alginate was added. A small amount of sodium azide was added to prevent bacterial growth. The solutions were stirred in room temperature for 1-2 days to achieve complete dissolution, or for 2-3 days to produce homogeneous gels. The quantities used to express the composition of the alginate fluids in this study are all weight to volume (of water) percentages for either sodium alginate, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ or SDS. Each of the fluids was subjected to the following three tests at the same temperature (22-23.5°C): tube model simulation, rheological analysis and surface tension measurement.

In the tube model experiment, the airway reopening process is modelled by observing meniscus propagation in a collapsed fluid-coated polyethylene tube upon repressurisation at one end. The device utilises a design described by Gaver et al [55], as shown in Figure 2.3. We used initially only a single combination of tube radius ($R = 0.923$ cm), film thickness ($H = 0.85$ mm) and axial wall tension ($T = 1 \times 10^4$ dynes/cm) in order to simplify the analysis process, since their effects on the reopening are relatively small [55]. Later, for some of the fluids tested, we used two values for each parameter to determine their contribution. This includes $H=0.85$ mm and 0.25 mm, $R=0.923$ cm and 1.67 cm, and $T=1 \times 10^4$ and 5×10^4 dynes/cm. The film thickness is controlled by a specially designed stainless steel rolling rod. The wall tension is applied by hanging down a weight W ($T=W/2\pi R$) at the end of the tube. The nonlinear quasi-steady state reopening velocity (u) is measured by a stopwatch recording the time for the meniscus to travel 40 cm distance. The reopening pressure (p) is measured by a well-calibrated Validyne pressure transducer (± 50 cm- H_2O range) connected upstream. For each fluid, more than 30 p - u data points were collected randomly. In viscoelastic sols, we did not observe any hysteresis in the p - u curves; whereas in viscoelastic gels, we did observe a small degree of hysteresis in some of the weaker gels, probably due to a slight destruction of the gel network during the procedure of rolling the rod to control the film thickness. Such disruption of the gel network requires a finite time period to recover the original state. Also, we found that the

Top View

$R = \text{radius of tube} = 0.923 \text{ cm}$

$H = \text{film thickness} = 0.85 \text{ mm}$

Meniscus propagation

Side View

Figure 2.3. Schematic of the tube model simulating the upper airway reopening.

pressure during meniscus procession is relatively constant in viscoelastic sols; whereas in gels, especially stronger gels, the pressure keeps ascending (but usually within 0.3 cm-H₂O) during meniscus procession, representing a back pressure in the unopened part. Under those circumstances, an averaged pressure over the travelling period was used to obtain the p-u curve.

The simulation fluids were characterised by controlled-strain and controlled-stress rheometers described in earlier chapters at the same temperature as when utilised in the tube tests. The dynamic viscoelastic parameters (under 5% strain) and the steady state shear viscosity were measured by the Rheometrics controlled-strain rheometer, using cone and plate geometry with a 0.02 radian angle and 50 mm diameter cone. The viscoelastic functions determined were the complex viscosity (η^*) and the non-Newtonian steady shear viscosity (η). The apparent yield stress of the gels was measured by the Carri-Med controlled-stress rheometer, again in cone and plate geometry, but utilising a 2 degree angle and 4 cm cone. Yield stress is defined as the minimal shear stress necessary to apply to the material to cause viscous flow. The value of the apparent yield stress is defined under certain time frame. In these gels, we measured the apparent yield stress by the controlled stress flow mode, where the stress was incremented in around 100 steps from 0.5 up to 1000 dyn/cm² logarithmically within 4 minutes. The apparent

yield stress is the stress where the angular velocity starts to be $>10^{-4}$ rad/s.

The surface tension (γ) of the fluids was measured with a Fisher Tensiomat-21 surface tensiometer. An apparent surface tension (S) is determined from a meter which records the force needed to lift a metallic ring from the fluid. A correction factor (F) can be calculated from the fluid density and the ring geometry and hence an absolute surface tension, γ , can be obtained ($\gamma=S \times F$).

Dimensionless analysis was applied to reduce dimensional pressure (p) - velocity (u) data to a master curve. This analysis follows that of Gaver et al [55] and is based on choosing the tube radius R as the dominant length scales for both capillary and viscous forces. The dimensionless pressure P is defined as pR/γ . The dimensionless velocity U is defined as the modified capillary numbers η^*u/γ or $\eta u/\gamma$. Since we are interested in studying viscoelastic fluids, we have substituted a non-Newtonian frequency- or shear rate-dependent viscosity in capillary number ($\mu u/\gamma$) for the Newtonian viscosity, μ , used in the analysis of Gaver et al [55]. The distinction is that whereas μ is a constant and does not change with u. The numerical value of η^* or η changes with u. Thus for each u value, there is a corresponding unique value of the capillary number. As a last step, nonlinear regression analysis is used to derive the universal equation showing the relative importance of viscous forces and capillary forces: $P = P_{cap} + P_{vis}$

U^0 , where P_{cap} and P_{vis} are the dimensionless capillary and viscous pressures, respectively.

Finally, the viscoelastic properties of airway secretions from animals have also been characterised by both Rheometers to make connections between the properties of the naturally-occurring lining fluids and the simulation fluids. As mentioned earlier, since wide range of viscoelasticity for normal respiratory mucus was observed by King et al and other groups, a generalized relation between viscoelasticity and reopening in the tube model will be described first in the following results section.

3. RESULTS AND DISCUSSION

3.1. AIRWAY REOPENING AND FLUID BEHAVIOR

3.1.1. Flow Behavior of Non-Newtonian Sols in the Tube Model

Pressure-velocity relation for viscoelastic sols with different viscoelastic and surface properties

Initially, we utilised nine viscoelastic sols in the airway reopening model at specified values of R , H and T . Table 2.2. is a list of the nine sols. A $\tan \delta$ vs. ω plot for each of the fluids is shown in Figure 2.4. Six are predominantly viscous ($\tan \delta > 1$) at all frequencies. Of these, 0.5% and 1% sodium alginate show close to Newtonian behavior. Three systems, specifically 5.5% sodium

alginate, 5% sodium alginate+0.15% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 4.5% sodium alginate+0.15% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ have $\tan \delta < 1$ at higher frequencies and hence show high-frequency elasticity probably because of the existence of an intermolecular entanglement network. (Sometimes, we also observed this phenomenon in 4.5% sodium alginate sols, probably due to variation in concentration or molecular weight of the sodium alginate.) Thus we term such materials entangled sols. In particular, we note that 4.5% sodium alginate+0.15% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ shows behavior typical of a sol very close to a gel point, as $G' \approx G''$ at all frequencies. The G' and G'' vs. ω plots for the three entangled sols are shown in Figure 2.5. As shown in fig.2.5.c, the 4.5% sodium alginate+0.15% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ sol exhibits power-law dependence of G' and G'' on deformation frequency. From the power-law exponent, within experimental error, alginates have a traditional gel point with $n=1/2$ and $\tan \delta = 1$ (or $G'-G''$ crossover) as observed in the chemically-crosslinked gels discussed in the previous chapter. Thus we define a characteristic frequency ω_c for these three sols as the frequency where the $G'-G''$ crossover occurs which corresponds to the transition frequency from sol-like to gel-like behavior caused by transient entanglement network behavior. Theory suggests that $\omega_c \propto 1/\tau$ where τ is the relaxation time of the entanglements.

In all of the nine sols, η^* and η are numerically very similar, i.e. $\eta^*(\omega) |_{\omega \equiv \dot{\gamma}} \approx \eta(\dot{\gamma})$, as shown in Figure 2.6.; that is, these sols obey the Cox-Merz rule (i.e. eq.(0.15)). The surface tensions of the

nine sols were also measured and are shown in Table 2.3. The measurement for highly viscous sols is somewhat difficult since they have long relaxation times.

The dimensional reopening pressure vs. velocity (p vs. u) in the tube model is shown in Figure 2.7. As evident, the data cover about two decades range of pressure and velocity. For the three entangled sols, we observed, at high velocities, a plateau in p and some flow instability. (Thus took the form of pressure oscillations, which were not included in our plots.) This region is followed by a transition or sudden drop of pressure, after which a slow further increase in p and a second plateau are observed. The critical velocities, u_c , defined as the last stable velocity value before the pressure oscillation occurs, appear to be associated with the viscoelastic frequency ω_c , where the transition to high frequency elastic behavior occurs. Dimensionless analysis is performed by transforming p to pR/γ and u to η^*u/γ or $\eta u/\gamma$. We also change the unit of pressure p from cm-H₂O to dynes/cm², a factor of 980. We assume that the surface tension γ is constant in each fluid under all velocities. η^* and η change with shear rate, and thus with meniscus velocity. The shear stress and strain rate ('fields') are location-dependent with largest values at the wall. The shear rate $\dot{\gamma}$ (in fact, average of the strain rate field) and meniscus velocity u are approximated by an engineering point of view :

$$\sqrt{R \times H} \quad (2.1)$$

so η and η^* can be expressed as a function of u approximately:

$$\eta(u) = \eta\left(\dot{\gamma} = \frac{u}{\sqrt{R \times H}}\right) \quad (2.2)$$

$$\eta^*(u) \approx \eta^*\left(\omega = \frac{u}{\sqrt{R \times H}}\right) \quad (2.3)$$

in our case, $R=0.923$ cm and $H=0.85$ mm, so $\eta(u) = \eta(\dot{\gamma}=3.57u)$ and $\eta^*(u) \approx \eta^*(\omega=3.57u)$. $\eta^*(\omega)$ and $\eta(\dot{\gamma})$ can be obtained from interpolation or extrapolation from the plots in fig.2.6. In this way, each u can be transformed into a dimensionless quantity U ($=\eta^*u/\gamma$ or $\eta u/\gamma$). Therefore, dimensionless plots P vs. U for these nine sols can be constructed, as shown in Figure 2.8. The slope of these plots tends to level off at higher U . More importantly, the results indicate three distinct regimes of behavior. Graphical fits using nonlinear regression can be employed to describe the three flow regimes, where we have used the relation as shown in Figure 2.9., $P = P_{\text{cap}} + P_{\text{vis}} U^b$, with P_{cap} , P_{vis} and b as adjustable parameters. We obtain:

$$\text{Regime I.} \quad P = 5.90 + 21.92 U^{0.81} \quad (2.4)$$

$$\text{Regime II.} \quad P = 9.17 + 7.38 U^{0.90} \quad (2.5)$$

$$\text{Regime III.} \quad P = 45.68 + 1.34 U^{1.08} \quad (2.6)$$

Regime II encompasses a wide range of dimensionless velocity, U , and has a numerically similar functional relationship to that observed for Newtonian lining fluids by Gaver et al [55]. These authors obtained $P=8.3+7.7U^{0.82}$, for fluids with low surface tension ($\gamma=24-43$ dyn/cm), with the Newtonian viscosity used to compute U . Thus, even when the lining fluids are viscoelastic, over a wide intermediate range of U values, the flow behavior is still controlled by the non-Newtonian viscous forces and the surface tension, in each case with R as the characteristic length scale. We also deduce that regime II for viscoelastic fluids is still a viscous flow-dominated region.

Regime I was not detected by Gaver et al [55] and occurs at very low U ($0.002 < U < 0.1$) by using fluids of high surface tension and low viscosity (recall that $U \propto \eta/\gamma$). Examples are 0.5% and 1% sodium alginate. (Note: The experiments of Gaver et al [55] utilised U values far above 0.01.) The low velocity anomaly may be a true rheological phenomenon, or it may be an artifact due to instability of flow or poor wettability of the lining fluid. The anomaly can be clearly seen when comparing results using 0.5%, 1% and 2% sodium alginates, as in Figure 2.10. Nonlinear regression of these data by using the yield obtained from 0.5% and 1% still shows better fitting in 2% region than in the lower U regime (fig.2.10.b), thus this regime may not follow a single dimensionless formula relationship. Regime III was also not detected by Gaver et al [55], and is apparently due to the high-

frequency elasticity effect (G') which is not incorporated in the normalization process. The nonlinear 'plateau' portions of the dimensionless plots at the ends of the regime II and III are also believed to be due to the predominantly elastic fluid response at high velocities, and may be viewed, qualitatively, as analogous to catastrophic (brittle) failure of the fluid layer. We also noticed that, in the regime III, the pressure slowly ascended during meniscus procession, which is similar to the behavior observed in gels and is caused by a back pressure arising from the material elasticity. Transition U_c from regime II to regime III is well-defined for all fluids, and clearly it depends on the fluid properties. The dimensional u_c (velocity where transition occurs) for each fluid seems to be associated qualitatively, but not precisely with $\omega_c \approx \dot{\gamma}_c = u_c / \sqrt{R \times H}$. The deviations are probably due to the limited accuracy of the approximate relation between u_c and $\dot{\gamma}_c$, as well as experimental precision. In addition, ω_c , thus u_c , is in fact determined by the strain. The strain dependency of ω_c in 5.5% sodium alginate is shown in Figure 2.11. Increasing strain causes an increase in $\tan \delta$ at all frequencies, so $\tan \delta = 1$ occurs at a higher frequency. The strain dependency appears to be strongly affected by the strength of the entangled structure (magnitude of G'). We found that, for entangled solutions with low G' and η , the transition was not observed within our experimental velocity range, probably because the comparably larger strain in the tube destroys the weak intermolecular entanglements, resulting in a

much larger ω_c and a delay for real entanglement transition to occur. This will be discussed later in more detail.

From the previous experiments and associated nonlinear regression fits, eqs.(2.4)-(2.6), we conclude that, for a given flow regime, P_{cap} is relatively important when U is smaller and P_{vis} gradually dominates when U increases. Fluids which exhibit viscoelastic character show distinct flow regimes. Increasing U increases the relative importance of the fluid elasticity and produces a drop in P_{vis} . A transition from the viscosity-dominated flow regime II to an elasticity-dominated regime III occurs. The nonlinear decrease in slope of the dimensionless plots at the end of regime II and regime III also indicates that the fluid elasticity dominates.

When the surface tension of the fluid is very high, the yield pressure for the tube reopening will also be high since $p=P_{cap}\cdot\gamma/R$ when $u=0$. In addition, according to observations of regime I behavior in fig.2.10., very high surface tension fluids will cause the airway to be relatively difficult to reopen when compared with medium surface tension fluids of the same viscosity. Therefore, if the amount of surfactant in the mucus is not enough, the airway is less likely to overcome the yield pressure for opening.

According to the dimensional and dimensionless plots, lining fluids with high viscosity but low elasticity (high η and high $\tan \delta$) have the highest reopening pressure for a specified u . Therefore, we can draw the conclusion that, for fixed geometry and wall tension, the mucus properties which are conducive to airway reopening (i.e. smaller p , though not necessarily smaller P) are, low surface tension to produce a low yield pressure, plus low viscosity for rapid meniscus propagation velocities, and high-frequency elasticity (gel-like) for lower pressure at a later stage. Low viscosity needs a compromise with high elasticity, since high elasticity (G') contributes to high viscosity ($\eta = \eta^* = (G'^2 + G''^2)^{1/2} / \omega$). We have known that [61] certain amount of mucin content is necessary to form a gel-like mucus. If due to some pathological reason, the mucin content is reduced, only enough to form a sol or entangled sol, or the ionic strength is changed, causing a decrease in the elastic properties or entanglements, the reopening pressure would keep high until a very high reopening velocity, corresponding to a high ω_c , has been achieved. This is hypothesized to be one of the mechanisms of failing to reopen the upper airway.

The effect of geometrical factors R and H, and the effect of T on reopening pressure, transition and dimensionless plot

As mentioned above, when the lining fluid is Newtonian, geometrical factors such as H and R do not alter the dimensionless plot significantly [55]. The tube radius R does not modify the

dimensionless plot because its contribution has been normalized in the analysis. The effect of film thickness H in dimensional as well as in dimensionless plot is dependent on the axial tension T . Decreasing T always shifts p (or P) upward, more so for lower H . Thus the effect of T is also dependent on the magnitude of H . Thus, when T is large, p (or P) are independent of H . When T is small, a decrease in H shifts p (or P) slightly upward. Therefore, the effect of R , H and T on the reopening pressure for Newtonian fluids can be summarized as follows: for certain u (or U , since $\eta = \text{const.}$ here), smaller tubes always give rise to larger dimensional pressure p in a fluid since $P = pR/\gamma = \text{const.}$; whereas smaller film thickness either does not have much influence when T is large, or alternatively gives rise to a larger p when T is small. Likewise, the effect of decreasing p by increasing T is more evident at smaller H . (i.e. R , H and T are inversely proportional to p . The influence of H and T is mutually dependent.) However, the effect of these quantities on non-Newtonian fluids in the tube model is much more complicated, with regard both to reopening pressure and to the transition; it is for this reason that we fixed H and R in the previous analysis to obtain a universal curve.

In a Newtonian fluid, for a certain u , a change in H and R does not alter η , so the relationship between p - u and P - U is easy to compute. This can be demonstrated for 2% sodium alginate which is not too far from a Newtonian fluid. Figure 2.12. shows that the effect of H and T follows the Newtonian rule. Figure 2.13.

shows the influence of R , but this effect can be normalized in a dimensionless plot as evident in Figure 2.14. This figure clearly shows that lowering T and H modifies the dimensionless plot. We believe that the reopening pressure dependence of T is, as suggested by Gaver et al [55], due to the additional force for opening offered by T . When the tube is precisely at the opening point, the wall is not exactly parallel to the tube axis. The tension along the tube wall will have components both normal and parallel to the axis of the tube. The normal component will be essentially zero where the tube is closed. At any given point near the open end, however, this normal component is nonzero, and results in an additional force to pull the wall apart. The influence of H is due to a minor contribution of H in addition to the dominant contribution of R in the characteristic length scale of viscous forces. These two factors are not considered in the dimensionless plot. Thus, the dimensionless plots of Newtonian fluids in the tube model are changed by varying T or H , but not by varying R .

However, for a non-Newtonian fluid, at a certain u , $\eta(u)$ is a function of H and R (eqs.(2.2),(2.3)). This may not have an effect on the construction of the dimensionless plot, but since $\eta(u)$ changes with H and R , the dimensional relationship is different. Since decreasing H or R at a specified U increases the shear rate, $\dot{\gamma} = u/\sqrt{R \times H}$. It follows that $\eta(u)$ decreases since we are in a more shear-thinning region. The effect of increasing p produced by a

decrease in H or R in the Newtonian fluids tends to be neutralized by the shear-thinning effect in the non-Newtonian fluids, especially in the case of decreasing H. Moreover, the velocity where the elasticity starts to play a role (u_c) is related to H and R. For fixed mucus properties (ω_c is constant, $u_c \propto \sqrt{R \times H}$), larger H and R are expected to result in a higher velocity for transition to regime III (u_c). This was confirmed by testing 5.5% sodium alginate in tubes with different H and R. The results are shown in Figure 2.15.-2.17. Figure 2.15. shows that for smaller H, no matter what the tension is, p is slightly lower, which is just opposite to its effect on Newtonian fluids. Figure 2.16. shows that increasing R still reduces the reopening pressure, but not as significantly as in the Newtonian fluids, since less shear-thinning occurs. In addition, we observe that u_c is smaller for smaller H and smaller R. The effect of H on u_c is more dramatic. These results further confirm that the transition from II to III is due to elasticity, since u_c decreased to about half of the original value when H changed from 85 mm to 25 mm, as the mathematical relation $u_c \propto \sqrt{R \times H}$ predicted (i.e. by a factor of 0.54). In figs.2.15. and 2.16., we also notice that the effect of T is not as significant as in the Newtonian fluids. The pressure-velocity relation for different T now depends more on tube radius than on H. For larger tubes, the effect of increasing T to decrease p is more evident (fig.2.16.). The magnitude of R may slightly modify the shear stress and shear rate fields since the fluid is non-Newtonian, and thus the meniscus velocity and average shear rate relationship.

Figure 2.17 is the dimensionless plot for 5.5% sodium alginate. The dimensionless plot was found not to be changed by R , except that U_c (or u_c) is changed. The effect of H on the dimensionless plot is insignificant. When H changes from 0.85 to 0.25 mm, u_c is smaller and p_c is smaller; so are U_c and P_c since $U_c = \eta(\omega_c)u_c/\gamma$ and $P_c = p_c R/\gamma$ with constant $\eta(\omega_c)$, γ and R . Therefore, decreasing H causes almost no change in P for specified U , but produces a decrease in p for specified u ; and also causes a decrease in the II-III transition velocity and pressure. By contrast, decreasing R for specified u slightly decreases P because of the smaller shear-thinning viscosity η and a smaller U . However, since $P = pR/\gamma$, p generally increases with increasing R .

Increasing T results in a smaller p , as for the Newtonian fluids, but less substantially. The dimensionless plot is not significantly changed by T , except in larger tubes with larger wall tension. However, as shown in figs.2.15.-2.17., larger T tends to reduce the oscillation associated with the transition (fewer unstable points before the pressure drop) and results in a more stable flow near transition. Especially in larger tubes, the velocity oscillations which show up at low T have been greatly reduced by increasing T , and no detectable transition to regime III is observed. This is probably due to, either a more even strain rate field distribution within meniscus in the larger tube, or a larger

extent of destruction of the weak entanglements by the larger strain in the larger tube.

In summary, we have established that the dimensionless P-U relationships in regimes II and III, eqs. (2.5)-(2.6), are not modified significantly by changing R, H or T.

Effect of R, H and T in very low U regime

We also discovered, as shown in Figure 2.18., that for lining fluids with high surface tension and low viscosity, such as 0.5% sodium alginate, the H and T dependency of the p-u relation is not the same as that for normal Newtonian fluids. The dependence of H is not observed. The dependence of T is not significant either, especially at small R. However, larger R tends to shift the dimensionless plots to lower P. As evident in Figure 2.19, the dimensionless curves are close to each other except for larger tubes, especially at larger wall tension. Large R and T tends to stabilise the very watery flow, probably by helping hold the walls in apposition. Extrapolation of the larger tube data (under low wall tension $T=1 \times 10^4$ dyn/cm) shows a yield value of 3.09, lower than that for regime I previously deduced by extrapolation of data at smaller tube (i.e. 5.90). This may imply that the regime I is in fact not a distinct rheological regime but an extension of regime II, since the anomaly apparent in fig.2.10. decreases if we use data for 0.5% sodium alginate in the larger tube, as shown in Figure 2.20. This also suggests that the yield value of regime II

may be 3.09 instead of 9.17. If this interpretation is correct, the similar higher yield value of Gaver et al [55] (≈ 8.3) is probably a result from lacking data points in the low U region. In addition, if we combine data from regime I and regime II using the smaller yield value from extrapolation of the large tube data in the low U regime, the combined dimensionless equation for these two regimes will be $P=3.09+13.26U^{0.68}$. The U power dependence in this expression is in excellent agreement with the predicted 0.67 power dependence of pressure on capillary number in Hele-Shaw flow [57,58]. Also, this lower yield ($p=3.09\gamma/R$) is in better agreement with the prediction by Macklem [6] that the critical pressure of meniscus motion in excised canine bronchioli as $2\gamma/R$. Our yield value from experiments in larger tubes under high wall tension $T=5\times 10^4$ dyn/cm is even lower ($P=2.58$, or $p=2.58\gamma/R$).

In fact, if we consider regime I as low U anomaly, the yield value for regime II is highly speculative and can fall anywhere between 3.09 and 9.17, since the nonlinear regression for each of them shows pretty fair correlation coefficient ($R^2=0.972$ for $P=3.09+13.26U^{0.68}$, and $R^2=0.984$ for $P=9.17+7.38U^{0.90}$). For example, if we consider the yield value of regime II to be the same as that obtained previously for regime I (5.90). Then the dimensionless formula is $P=5.90+11.24U^{0.73}$ ($R^2=0.981$). The viscosity-dominated regime has the form between $P=9.17+7.38U^{0.90}$ and $P=3.09+13.26U^{0.68}$. In order for a more universal expression, we chose $P=3.09+13.26U^{0.68}$ as the form for

describing the viscosity-dominated reopening process since it comprises the larger tube data in low U regime. This formula will be used in the later discussion for gels.

The effect of G' and η on transition

Another thing we observed for entangled sols is that, if G' and η are too low, the transition from II to III is substantially delayed, probably due to the destruction of the weaker transient entanglement network structure by the large strain employed in the tube model. This destruction can be noticed as a decrease in G' and η and an increase in $\tan \delta$ in all dynamic viscoelastic measurements when using strain $\gg 5\%$, where $G'-G''$ crossover shifts toward higher frequencies. For this reason, we tried to prepare a sol whose viscoelastic properties show an entanglement transition but which has G' and η about half of the values in the previous three entangled sols, thus the entanglement transition is very much strain-dependent. 3% sodium alginate+0.16% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ is found to be such a system. Figure 2.21.a shows the rheological property for this sol. It is a sol very close to the gel point, as 4.5% sodium alginate+0.15% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. There is a $G'-G''$ crossover in the frequency sweep under 5% strain, but as shown in Figure 2.21.b, in the $H=0.85$ mm tube model (under $T=1 \times 10^4$ dyn/cm), we observe no transition from regime II to regime III in p. Table 2.4. is a comparison list between this entangled sol and the previous three sols. $u_c = k_d \cdot \omega_c \sqrt{R \times H}$, k_d is defined as a delay factor. Thus we believe that the transition u_c is not only

dependent on ω_c (under 5% strain), but also modified by geometric factors H and R, wall tension T, as well as G' and η , and surface tension (see next paragraph). In this case, the transition was substantially delayed because of low G' and η of the entangled structure, which causes no G' - G'' crossover at higher strains until a very high frequency.

The effect of surfactants on transition

The above dimensionless analysis of tube model experiments for different sols incorporated the fluid surface tension and experimentally encompassed fluids with $\gamma = 40\text{-}60$ dyn/cm. The similar observations of Gaver et al [55] included fluids with $\gamma=24\text{-}43$ dyn/cm. Thus any additional effect of surfactant on unentangled sols in the linear portion of regime II is believed to be negligible. We have known from our previous analysis, that the main purpose of a lower surface tension is to give a smaller yield pressure for opening, which could be a significant contribution to the overall pressure. However, we did not investigate entangled model fluids with surface tensions lower than 40 dyn/cm in this previous analysis. Such sols may behave differently in the tube model. Surface tension may be lowered by addition of surfactants to the model fluids. Our interest focused on the effect of surfactant on the entangled sols, especially on the transition from viscosity-dominated regime II to elasticity-dominating regime III. We chose two pure sodium alginate solutions (4.5% and 5.5%) whose surface tension was varied by

adding sodium dodecyl sulfate (SDS). We were unable to add SDS in entangled sols containing CaCl_2 because the presence of SDS, competing with alginate for binding the divalent ions, decreases the viscosity and elasticity of the sols, resulting in no rheological transition.

The properties of these sols are shown in Figure 2.22. and Table 2.5. Figure 2.22. shows that there is no change in viscoelasticity after SDS is added, but there is a significant decrease in surface tension (Table 2.5.). The results of tube model tests (under $T=1 \times 10^4$ dyn/cm) are shown in Figures 2.23. and 2.24. The dimensional plots in 4.5% and 5.5% sodium alginates (figs.2.23.a and 2.23.b) in a $H=0.85$ mm tube model do confirm that the addition of SDS decreases the magnitude of the instability (pressure oscillations) associated with the high frequency elasticity. Thus the transition is smoother, an effect which is similar to that observed at high wall tension. As expected, the dimensionless plot before transition is not modified (fig.2.23.c) by changing the surface tension. However, for 5.5% sodium alginate in a $H=0.25$ mm tube, no difference in the dimensional and dimensionless plots were seen on decreasing surface tension (fig.2.24.). We propose that, surfactants may have an effect on the meniscus geometry (meniscus area), which changes the velocity and shear rate fields. The meniscus velocity and average shear rate relationship is modified, thus u_c is also changed. The effect is particularly evident when film thickness is very large since there

is a larger meniscus area available for the modification. This minor effect does not change the average opening pressure significantly. However, since oscillation can sometimes cause pressure overshooting, the observed effect of a reduction in oscillation may be important. Miki et al [53] observed that lubrication of the pharyngeal mucosa with artificial surfactant facilitates reopening of the upper airway reopening. We suggest that their finding results from a decrease in surface tension of the mucosa lining fluid on adding surfactant, which decreases the dimensional reopening pressure p and, in addition, stabilises the meniscus motion. Alternative possibilities, however, are a decrease in viscosity resulting from dilution by the surfactant, a decrease in mucus work of adhesion (adhesiveness), or a specific molecular interaction between the surfactant and the mucus molecules which modifies mucus viscoelasticity.

The effect of wettability of tube walls

The surface property of tube walls may change flow behavior and opening process. Due to its low surface tension, the surface of polyethylene tube has a more hydrophobic nature than the epithelia of the respiratory tract. We have suspected poor wettability to be one of the causes for the previously described anomaly in regime I occurring when tubes are lined with very watery fluids. To study the effect of surface wettability on reopening, in the current experiment, albumin was coated on the inside wall of the polyethylene tube to improve the wettability.

This procedure was carried out by filling the tube with 1% bovine serum albumin (BSA, from Sigma) in PBS buffer, sitting for 1 hour, and shaking occasionally. Then the tube was rinsed with saline, and dried by circulating a small but steady air flow through it. The control tube was rinsed only with saline and dried by the same way. Contact angles of these two tube walls were measured by a contact angle goniometer (manufactured by rame-hart, Inc.). As seen in Table 2.6., the wettability was improved by coating BSA on tubes, manifested as a decrease in both advancing and receding contact angles.

Two lining fluids have been tested in such tubes ($R=0.923$ cm and $T=1 \times 10^4$ dyn/cm): 5.5% sodium alginate with a thickness $H=0.25$ mm; 0.5% sodium alginate with a thickness $H=0.85$ mm. The results are shown in Figure 2.25. No significant change in opening pressure was observed. Therefore, the contact between surface of tube walls and the fluid does not seem to be important in determining the flow mechanism. The result also suggests that the anomaly in regime I is probably not due to poor surface wettability of those fluids with low η and high γ , but is a result of lacking the adhesive force which holds two walls in apposition. Since the adhesive force provided by those fluids is not sufficient, the film thickness H in the vicinity of the meniscus is modified. The original mechanical as well as mathematical models are no more valid. This explains why a dependence on H was not observed in the case of those watery fluids. In addition, a large

tube radius (i.e. larger gravity force) and a large wall tension each assist in holding the walls in apposition, and thus a decrease in the anomaly occurs under those circumstances, as previously noticed.

Conclusion

We have established the reopening pressure-velocity relationship for viscoelastic fluids in the tube model of Gaver et al [55] which simulates the upper airway reopening event. In contrast to Newtonian fluids, the reopening process exhibits a transition to an elasticity-dominated regime, in addition to the viscous flow regime observed by Gaver et al [55].

From dimensional as well as dimensionless analyses, we conclude that the influence of the system parameters in the airway reopening under the circumstances in which airway fluids exhibit viscoelastic character is as follows. With regard to fluid properties: The fluid elasticity strongly affects the pressure-velocity relationship, helping to reduce the opening pressure. Smaller surface tension gives rise to a smaller yield pressure for reopening, and seems to play an additional role in stabilising the flow. As for other factors: Larger wall tension decreases reopening pressure only slightly and also tends to stabilise the flow. Larger airways are easier to open; whereas smaller airways produce additional shear thinning of the lining fluid. If the surface tension of the lining fluid is sufficiently low to give a low yield pressure p_{cap} , the reopening process may be easier than in

the case of Newtonian fluids. A thinner fluid lining (thinner mucus layer) apparently has beneficial effects contrary to its role in Newtonian fluids. This occurs because a thinner lining contributes to more shear-thinning behavior, resulting in lower reopening pressures at low velocities, and a higher elasticity, providing for lower reopening pressure at high velocity.

3.1.2. Flow Behavior of Gels in the Tube Model

As mentioned in the background section, airway secretions often are weak viscoelastic gels, with $\tan \delta$ smaller than 1 at all frequencies and at small strains. (Note, however that, under physiological conditions when the strains may not be small, they will behave as viscoelastic sols.) For gels, $\tan \delta$ and G' are important parameters to determine their properties, in addition to η^* . G' determines the strength of the gel, and $\tan \delta$ determines the relative level of viscous to elastic response. We have prepared nine viscoelastic gels with different $\tan \delta$ and G' . Table 2.7. is a list of the properties of the nine gels, including η^* , G' and $\tan \delta$ at $\omega=1$ rad/s (under 5% strain), as well as their surface tensions. Figure 2.26. shows the viscoelastic behavior of each gel by dynamic frequency and steady shear rate sweeps. We find that despite being gels, most still obey the Cox-Merz rule (i.e. eq.(0.15)) $\eta^*(\omega) | \omega \equiv \dot{\gamma} \approx \eta(\dot{\gamma})$, as shown in Figure 2.26.c and d, with the exception of 3% Na Alginate+0.2% CaCl_2 and 2% Na Alginate+0.175% CaCl_2 (i.e. gels with both large G' and small $\tan \delta$).

In these systems, there is a small difference between η^* and η at lower ω or $\dot{\gamma}$.

The dimensional reopening pressure vs. velocity relation (p vs. u) in the tube model (at fixed $R=0.923\text{cm}$, $H=0.85\text{mm}$ and $T=1\times 10^4\text{ dyn /cm}$) is shown in Figure 2.27. We observe that the reopening pressures for three stronger gels (3% Na Alginate+0.2% CaCl_2 , 3.5% Na Alginate+0.185% CaCl_2 , and 2% Na Alginate+0.175% CaCl_2) show stronger meniscus oscillations than those for sols at all velocities and that the pressure rises slowly during meniscus propagation at higher velocities. It is believed that these two phenomena are due to the high elasticity. As is clearly evident, two gels (2.5% Na Alginate+0.175% CaCl_2 and 3% Na Alginate+0.18% CaCl_2) exhibit a transition similar to those previously observed for entangled sols. We found that these two gels have G' , η^* and $\tan \delta$ all in the moderate range as compared to the other gels. Also, for the two weakest gels, 1.6% Na Alginate+0.14% CaCl_2 and 1.6% Na Alginate+0.15% CaCl_2 , we noticed that the results are slightly different when comparing measurements performed immediately after coating, and those carried out 5 minutes after coating (fig.2.28.a). Measurements made after 5 min. have slightly lower p values but show more oscillations. There is also a small hysteresis for these two gels (i.e. difference between measurements on increasing pressure and decreasing pressure. The hysteresis effect in 1.6% Na Alginate+0.15% CaCl_2 is shown in fig.2.28.b.). These phenomena

are due to time-dependent destruction and build-up of the gel strength. The effect is minor and is ignored in the following analysis.

Dimensionless analysis was performed by transforming p to pR/γ and u to $\eta * u/\gamma$ or $\eta u/\gamma$, using the procedures established previously for the model sols. The dimensionless results are shown in Figure 2.29. We found that $U=\eta u/\gamma$ is a better quantity for reducing the data for these model gels. Dimensionless curves are close to each other at low U , but start to diverge at higher U ($U \geq 3$). The yield pressure obtained from the low U data are 3.60 and 3.55, extrapolated from the plots vs. $U=\eta * u/\gamma$ and $U=\eta u/\gamma$ respectively. The divergence at higher U appears to be related to differences in elasticity, since we find that if G' is higher, the curve shifts increasingly downward. For a given U , gels with higher G' have smaller P .

For analysis of the gels, the dimensionless equation minus an elasticity term can not accurately describe the behavior. In the sols, where the viscous flow dominates, the following equations were used: $p=p_{cap}+p_{vis}$, and $P=pR/\gamma=P_{cap} + P_{vis}(\eta u/\gamma)^b$, assuming the length scales for capillary and viscous pressure are both equal to R [55]. For the analysis of the gels, the relation may be considered as:

$$P=P_{cap}+P_{vis}+P_{elas}=P_{cap}+P_{vis}+\sigma_0+G'P_{elas} \quad (2.7)$$

$$P = (p_{cap} + \sigma_0)R/\gamma + P_{vis}(\eta' u/\gamma)^b + G'(u)R/\gamma P_{elas} \quad (2.8)$$

$$P - (p_{cap} + \sigma_0)R/\gamma = P_{vis}(\eta' u/\gamma)^b + G'(u)R/\gamma P_{elas} \quad (2.9)$$

where p_{elas} is the contribution to the total pressure due to the shear stress associated with elasticity, σ_0 is the rheological yield stress, η' is the dynamic viscosity, G' is the storage shear modulus, and P_{elas} is the dimensionless elastic pressure. In these gels, γ is larger than 60 dyn/cm, so p_{cap} calculated by utilising the results from viscoelastic sols ($P_{cap} = 3.09 = p_{cap}\gamma/0.923$) is at least 200 dyn/cm². This is much larger than the apparent yield stress of these gels, as shown in Table 2.8. The values of the total yield pressure are different between gels, but all are close to that predicted previously by the dimensionless plot (fig.2.29.). The yield stress does not seem to play a significant part in the total yield pressure in this case. However, for the true mucosal secretions, the surface tension is sufficiently low that the capillary yield pressure is also low. In such a situation, the rheological yield stress can contribute significantly to the total yield pressure.

Eq.(2.9) has too many unknown variables, and consequently it is very difficult to generate a dimensionless relationship using this formula. Therefore, we used the following approximation instead:

$$P - (p_{cap} + \sigma_0)R/\gamma = P_{viscoelas} \cdot (\eta' u/\gamma)^b \quad (2.10)$$

$P_{viscoelas}$ is a factor determined by the relative contribution from viscous and elastic forces. $(p_{cap} + \sigma_0)R/\gamma$ for each gel was obtained from Table 2.8. The nonlinear regression of data by fitting into this formula is shown as Figure 2.30. The curves are somewhat different between each gel (in fig.2.30.a), because for different gels, the relative contribution from elasticity and viscosity is different. When U increases, a sudden jump to the right is frequently observed in these plots, since the relative contribution of elasticity increases with U . For convenience, the gels are divided into three groups: 1) 4 gels, with large G' and η , including 3% Na Alginate+0.2%CaCl₂, 3.5% Na Alginate+0.185%CaCl₂, 2% Na Alginate+0.175%CaCl₂, and 2.5% Na Alginate+0.175%CaCl₂, 2) 3 gels, with moderate G' and η , including 3% Na Alginate+0.18%CaCl₂, 3.27% Na Alginate+0.173%CaCl₂, and 1.6% Na Alginate+0.164%CaCl₂, and 3) 2 gels, with low G' and η , including 1.6% Na Alginate+0.15%CaCl₂ and 1.6% Na Alginate+0.14%CaCl₂. For each group, a non-linear regression formula is obtained (fig.2.30.b). There is slight difference between each group:

$$1) \quad P - P_{yield} = 8.85 U^{0.87} \quad (2.11)$$

$$2) \quad P - P_{yield} = 10.70 U^{0.88} \quad (2.12)$$

$$3) \quad P - P_{yield} = 11.41 U^{0.93} \quad (2.13)$$

If combined data are used (fig.2.30.c), the formula is:

$$P - P_{yield} = 11.01 U^{0.79} \quad (2.14)$$

The formula previously derived for sols was $P=3.09+13.26U^{0.68}$. As mentioned, the yield pressure is rather similar for sols and gels, since $P_{cap} \gg \sigma_0 R/\gamma$. The formulae for gels appear to have a smaller $P_{viscoelas}$ and higher b , probably due to the contribution from a smaller P_{elas} and first order dependency of $G'(u)$.

The effect of H, R and T on the reopening pressure and dimensionless plot

First, we have studied the effect of H on the reopening pressure of gels. The gels utilised in this study are shown in Table 2.9., and their viscoelastic properties are displayed in Figure 2.31. The effect of H on the reopening pressure of these gels is exhibited in Figure 2.32. Smaller H results in a smaller reopening pressure, as observed in viscoelastic sols. It is also evident that, the more elastic the gel (larger G' and smaller $\tan \delta$), the more evident the decrease in reopening pressure on decreasing H , due to a higher level of shear-thinning. However, decreasing H causes meniscus oscillation and the appearance of a transition in gels with moderate G' and $\tan \delta$ (3% Na Alginate+0.18% CaCl_2 and 3.27% Na Alginate+0.173% CaCl_2). The transition is less evident in gels with moderate G' but with a smaller $\tan \delta$ (e.g. 1.6% Na Alginate+0.164% CaCl_2), since, at comparable values of G' , a larger strain is needed to destroy gel structures with smaller $\tan \delta$. The dimensionless plot is not changed significantly by H , except that for those which have a transition, P is lowered after the transition,

as shown in Figure 2.33. This shift toward the right direction on the plots is due to an increase in the relative contribution of elasticity.

We have observed that a change of geometry may result in a modified behavior of gels which depends on their different $\tan \delta$ values. Therefore, a complete study of the effect of H, R and T was conducted in four gels with different $\tan \delta$, as listed in Table 2.10. Their viscoelastic properties are shown in Figure 2.34. Oscillation and transition occurred in the 3% Na Alginate+0.18% CaCl_2 gel at smaller H and R (i.e. $H=25\text{mm}$ and $R=0.923\text{cm}$). The effect of H, R and T on the reopening pressure and dimensionless plot for each of the four gels is shown in Figure 2.35-2.38. Generally speaking, the effect of H, R and T is similar to each other and resembles that of entangled sols in the previous section. Decreasing H and increasing T always reduces the reopening pressure. The effect of H on the dimensionless plot is not as significant as that on the dimensional plot. The dimensionless plot is not significantly changed by T either, except in larger tubes. Comparison of these four gels shows that for gels with larger $\tan \delta$, dimensional reopening pressures for larger R and H are evidently higher; for gels with smaller $\tan \delta$, the dimensionless pressures for larger T are apparently lower. For the gel with moderate $\tan \delta$ and transition (3% Na Alginate+0.18% CaCl_2), the reopening pressures for lower H exhibit plateau regions at higher velocities, which possibly indicates influence of the sol-gel transition of the

fluid. It seems that, as the meniscus velocity reaches levels where the sol-gel transition occurs, the reopening pressure no longer increases with meniscus velocity.

The transition phenomenon in the behavior of gels in the tube model

There is no entanglement transition in gels, but a rheological transition occurs in the tube model similar to that previously observed for entangled sols. We attribute this effect to the occurrence of a sol-gel transition rather than an entanglement transition; however, their essence is similar.

Figure 2.39. shows the strain dependency of $\tan \delta$ of two gels. As mentioned in the earlier chapter, larger strains may disrupt the gel clusters. The critical strain (where $\tan \delta = 1$) is frequency-dependent, i.e. it increases with increasing frequency, as shown in fig.2.39. We also measured the strain dependency of these two gels by Carri-Med controlled stress rheometer. The results are shown in Figure 2.40. The results from two rheometers are somewhat different. The critical strains are rather larger in fig.2.40 (by controlled stress rheometer). We deduce that weaker gels have a true $\tan \delta$ larger than 1 at all velocities in tubes of various geometries, i.e. although defined as 'gels', they are in fact 'sols' when flowing in the tube. For gels with medium $\tan \delta$ (at $\omega = 1$ and 5% strain), the true $\tan \delta$ under the effective strain of the tube may be larger than 1 at low velocities

($\approx$ frequencies); however, $\tan \delta$ gradually approaches 1 as the velocity increases. Eventually, $\tan \delta$ crosses 1 and the fluid becomes a gel. Thus the behavior is analogous to a 'three dimensional' sol-gel transition with strain and velocity (equivalent to shear rate, and frequency) as variables. For strong gels with low $\tan \delta$ ($\omega=1$ at 5% strain), it appears that they behave as true gels in the tube under all circumstances.

The effect of surfactants on gels

We have prepared gels with low surface tensions (~ 45 dyn/cm) by adding surfactants (0.01-0.25% SDS), but have not observed any significant change in tube reopening so far, except that the flow is more stable for gels with surfactants. As suggested in the previous section, their effect on the dimensionless plot of viscoelastic sols is only minor. The decrease in surface tension in gels should reduce p_{cap} as usual, but it should not reduce the apparent yield stress σ_0 .

Conclusion

We conclude that gels and sols behave quite similarly in our tube model experiments. The finite strain in the tube increases the $\tan \delta$ values for alginate gels at all circumstances. $\tan \delta$ may start larger than 1 at low shear rate and approach to values near 1 as the shear rate increases. At certain high velocity, $\tan \delta$ eventually crosses 1 and the sol-gel transition occurs. Thus the alginate gels show a transition phenomenon in the tube model

similar to that observed for entangled sols under similar situations.

A smaller lining thickness H results in a more elastic behavior of lining fluids. In healthy subjects, the layer of mucus lining the respiratory tract epithelia is only $\sim 5\text{-}10\text{ }\mu\text{m}$ thick and rests on a $\sim 6\text{ }\mu\text{m}$ sol phase [62]. The diameter of human trachea is around 1.8 cm [63]. Thus the shear rate of the opening meniscus would be $\sim 2.8\text{-}5$ times that of the shear rate employed in our tube model when $H/2=0.125\text{ mm}$ and $R=0.923\text{ cm}$. In such a case, high frequency elasticity will be more predominant. All weak gels may therefore be near the sol-gel transition within a certain range of velocities. This transition causes a plateau in the reopening pressure. Once the lining fluid passes into the gel state, the rheological yield stress contributes to the yield pressure, especially when the surface tension is comparatively low. G' keeps increasing and contributes more to the reopening pressure. As observed for our alginate gels, the plateau only occurs for gels which exhibit a certain range of viscoelasticity. Therefore, our next goal is to investigate the viscoelasticity of typical airway secretions, and contrast their similarities and differences in order to make contact with the results from the tube model studies of alginate gels.

3.2. CORRELATION WITH ANIMAL EXPERIMENTS

As mentioned in the background section, airway secretions have been studied by various other groups and the resulting data cover a wide range of viscoelastic behavior. Most findings suggest that they are true gels ($\tan \delta < 1$ at all frequencies) in the rheologic ground state, where the method of measurement does not destroy the delicate gel network. During airway opening, the effective strain is probably large enough to disrupt the gel clusters. We do not know the typical strains involved in the airway opening process, thus it is difficult to find out the pertinent viscoelastic properties of secretions under physiological conditions. The focus of our studies is on characterising the viscoelasticity of secretions from different locations of upper airway and the strain dependency of the secretions. Hopefully, this will permit a comparison of the tube model and physiological behavior.

3.2.1. Rheological Properties of Airway Secretions from Animals

We have collected airway secretions from opened airways (mouth, nose, pharynx and trachea) of ventilated, anesthetized dogs and cats. Nine animals (5 dogs and 4 cats) were used. A list of samples is shown in Table 2.11. The amount of all fluids were sufficient to enable repeated measurements in order to get a statistical average, except for three tracheal mucus samples, for which only $\sim 60 \mu\text{l}$ was obtained, enough for one measurement. For nasal secretions ($\sim 5\text{ml}$), surface tensions were also determined. The surface tensions of nasal fluids are 52.3 dyn/cm

and 48.6 dyn/cm for normal and substance P-treated dogs, respectively. These values are lower than values reported for tracheal fluids and higher than those for bronchial fluids obtained from fetal guinea pigs by Enhorning et al [54].

The rheological properties characterised, included yield stress and steady shear viscosity (by the Carri-Med controlled stress rheometer using cone and plate geometry with a 1 degree angle and 2 cm diameter cone), and dynamic viscoelasticity (by either the Rheometrics controlled strain rheometer in cone and plate geometry with a 0.02 radian angle and 5 cm diameter cone or with the Carri-Med rheometer). All measurements are reported at room temperature ($\sim 23.5^{\circ}\text{C}$), since we found no significant temperature-dependence for all secretions from 20 to 40°C .

The apparent yield stress can be measured by two methods: either a creep experiment or a flow curve measurement, both utilizing the controlled stress rheometer. Measurement of the yield stress from a creep experiment has been described in Chapter 1, Section 3.1. (p.12). Determination of the yield stress by controlled stress flow mode has been described in Section 2.2. of this chapter (p.84). Typical creep experiments for measuring the yield stress are shown in Figure 2.41. The shapes of creep curves show poor reproducibility within the same collection. Typical flow curves for each sample are in Figure 2.42. For each collection, the

samples were reloaded to the rheometer and measured several times if the amount was enough. The flow curves were more reproducible within the same collection, except for the oral secretion from dog, which was macroscopically heterogeneous. Thus two flow curves of the oral secretion are shown. For tracheal secretions, only one flow curve was obtained for each sample, due to the scarce amount. We also include the flow curve of human tracheal mucin (HTM, purified from cystic fibrosis (CF) patients [61]) 20 mg/ml in 6 M Gdn·HCl solution for comparison. It seems that upper airway secretions contain less mucin than tracheal secretions, except for the oral secretion, where the airway secretion is contaminated with other mucin in saliva. Complete data for mean apparent yield stresses of airway secretions by these two methodologies are listed in Table 2.12. In general, the values of apparent yield stress from the creep curves are lower than those from the flow curves, due to the greater sensitivity of the former methodology. As shown in the table, there is no statistically significant difference in the apparent yield stress among secretions from different locations. All values of either method fall into a range 2-10 dyn/cm², except for the values of two tracheal samples from flow measurement.

Steady shear viscosities of airway secretions can be calculated from the flow curves, as shown in Figure 2.43. A comparison of average steady shear viscosity between different secretions at $\dot{\gamma}=1 \text{ sec}^{-1}$ is shown in Table 2.13. In spite of similar

yield stresses (Table 2.12.), the viscosities are different from site to site. Pharyngeal and nasal secretions have similar steady shear viscosity, but the nasal secretions show bigger hysteresis loops. Oral and, especially, tracheal secretions are more viscous than nasal or pharyngeal secretions. HTM has similar viscosity as pharyngeal and nasal at low steady shear rate. Since it is less shear thinning than the natural secretions, the viscosity at higher shear rate ($\dot{\gamma} > 10^2 \text{ sec}^{-1}$) for HTM approaches that of tracheal or oral secretions. We believe that the different viscoelastic behavior of airway secretions is due to different mucin contents [17]. However, oral secretions are very likely to contain submaxillary mucin in addition to respiratory mucins, thus become more viscous than pharyngeal or nasal secretions. We also observed 'structure break points' in the curves of two out of four tests of oral secretions and of two tracheal collections (fig. 2.43.). These points are at around 100-300 dyn/cm². They represent sudden loss of structure in the fluid and are suspected to be the structural breakdown of the gel network.

The dynamic viscoelasticity at $\omega = 1 \text{ rad/s}$ is also shown in Table 2.13. As evident, all of the secretions are true gels under small strains. The complex viscosities of tracheal secretions are close to the mean values observed by King et al (Table 2.1., [18,28]). The magnitude of dynamic viscosity generally is numerically similar to that of steady shear viscosity. Typical frequency dependency of dynamic parameters for some secretions

under constant strain and constant stress are shown in Figure 2.44. and Figure 2.45., respectively. It is found from these two figures that once the strain is fixed, $\tan \delta$ decreases slightly as the frequency increases, which is opposite to the previous alginate gels. If the stress is fixed, $\tan \delta$ is in fact determined by the strain under that frequency (fig.2.45.). Strain and stress are crucial factors for the dynamic viscoelasticity, especially at lower frequencies. Toward higher frequencies, their influence is less dramatic. Thus the strain dependency of $\tan \delta$ at different frequencies was investigated and the results are shown in Figure 2.46 and Figure 2.47. It is found that the critical strain where $G' - G''$ crossover occurs depends on the frequency. At fixed frequency, the critical strains for secretions obtained from different locations are different. Table 2.14. is a comparison of the critical strains of different secretions. We observed that tracheal secretions have much higher critical strains than oral, nasal or pharyngeal secretions, in spite of the fact that, under smaller strain (5%), $\tan \delta$ of the tracheal fluids is not significantly different from $\tan \delta$ of the other secretions. This suggests that, during airway opening process, upper airway secretions are more likely to have a sol-gel transition occur than tracheal fluids, due to a more strain-sensitive structure.

3.2.2. Tube Model and the Animal Experiments

Earlier animal experiments [11] observed that filling the upper airway with methacholine-induced secretion has the effect of impeding airway reopening. From the tube model studies, we have concluded that higher viscosity (η) and large fluid layer thickness (H) are not conducive to reopening. Since high doses of methacholine have been found to increase the secretory rate and the viscosity but not $\tan \delta$ of canine tracheal mucus [35], the increase in reopening pressure in the previous animal experiment can be interpreted as due to an increase in viscosity as well as an increase in the fluid film thickness in the tube model. In addition, the behavior of viscoelastic fluids in the tube model suggests that weak gels with medium $\tan \delta$ would have the lowest reopening pressure if there is a sol-gel transition occurring under that specific tube geometry and film thickness. From our animal experiment data, nasal and pharyngeal secretions have similar apparent yield stress as tracheal secretions, but are weaker gels compared to tracheal fluids. This indicates that normal upper airway secretions are more conducive to reopening of the upper airway than tracheal secretions. We can imagine that if we coat the pharyngeal mucosa with the tracheal fluids, the reopening pressure should increase. The comparison can be accomplished by simulating the upper airway reopening pressure utilising the formula obtained in the previous tube model study (section 3.1.) and the rheological data obtained earlier in the current section. The geometrical factors of upper airway are assumed to be $R=1$ cm and $H=20$ μm [62,63,64]. The surface tension is assumed to be

50 dyn/cm, which is chosen because it is close to our measurement on the dog nasal secretions. In this simulation, eq.(2.14), $P - P_{\text{yield}} = 11.01 U^{0.79}$, is used, where P_{yield} is equal to $3.09 + \sigma_0/50$. The values of σ_0 are directly taken from Table 2.12. Steady shear viscosities used in computing U are from fig.2.43 and based on the shear rate approximation, eq.(2.1). By this procedure, a dimensional pressure and velocity relationship (p vs. u) can be generated. The simulation of upper airway reopening time is based on $t = L/u$, where L is the length of the upper airway and is assumed 20 cm [64,65] in this case. Figure 2.48. shows predictions of airway opening velocity and airway opening time as a function of pressure. The pharyngeal p - u curve in fig.2.48.a does not include the 'plateau effect' caused by the possible sol-gel transition for pharyngeal mucus as previously mentioned, since we have no idea at what particular time this will occur. In spite of that, the simulated reopening pressure is much lower for the upper airway coated with pharyngeal mucus ($p = 2.6$ cm-H₂O for $t = 0.01$ sec) than when coated with tracheal mucus ($p = 11.6$ cm-H₂O for $t = 0.01$ sec).

Gaver et al [55] have used their own regression formula for Newtonian fluids to predict the time to open bronchial generations 8-14 in the lower lung assuming a Weibel lung geometry [63,66]. Table 2.15. shows the architecture of the human lung according to Weibel's model, with regularized dichotomy. Generation 8 was chosen according to Martin and Proctor's [67] speculation that

airway closure might occur in 2-mm airways due to their relatively large compliance and small radius. A comparison between Gaver's result and our simulation data for the upper airway is shown in Figure 2.49. Based on their result, in a 'normal lung' (i.e. with $\gamma=25$ dyn/cm and η or $\mu=0.01$ poise), the opening time is very small at a pressure slightly greater than p_{yield} . This gives a 'popping open' phenomenon once the yield pressure is exceeded. The appearance of popping open is not as evident in our simulation as that observed in the normal lower airway by Gaver et al, but is more announced than their prediction on a lung with elevated Newtonian viscosity ($\eta=1$ poise). We regard that popping open is more evident in the fluids with lower steady shear viscosity or higher extent of shear thinning. If sol-gel transition occurs during reopening, it will result in an enhanced popping-open effect, because of a plateau occurs in a plot of p vs. u during the transition.

As seen in fig.2.49., the yield pressure is smaller in our case ($p_{\text{yield}}\sim 0.2$ cm-H₂O) than that predicted for opening of bronchial generations 8-14 by Gaver et al ($p_{\text{yield}}\sim 5.7$ cm-H₂O). This is partially due to the different regression formula. Our P_{yield} ($\equiv p_{\text{yield}}R/\gamma$) is equal to $3.09+\sigma_0/50$, compared to their value of 8.3. More importantly, however, the differences are due to the different morphometry, fluid surface tension (γ) and fluid rheology assumed in each case. The radius of the upper airway is assumed 1 cm in our simulation; while the radii of generations 8-

14 are normally below 1 mm (Table 2.15.). Even in our simulation, when the radius of the upper airway deviates from the assumed value 1 cm, the reopening pressure is dramatically modified. For example, the nasal valve has a cross section only $\sim 30 \text{ mm}^2$ on either side, the smallest in the upper airways [64,65]. Thus the reopening pressure could be higher than that predicted previously by assuming $R=1 \text{ cm}$ (i.e. a cross-section $\sim 314 \text{ mm}^2$), if the rheology of the surface mucus remains similar.

If we assume that lower airway secretions have similar rheology to pharyngeal or tracheal secretions, using our regression formula, we are able to predict opening time for generations 8-14 of the lower airway, assuming a Weibel geometry and $\gamma=25 \text{ dyn/cm}$, the equilibrium surface tension of pulmonary surfactant. This procedure was carried out according to that previously described. We assume that u_n in each generation is independent and that the opening pressures $p_{8-14} = p_8 = p_9 = \dots = p_{14}$, where u_n and p_n represent velocity and pressure in each generation. At given p values, the individual radius R_n is used to calculate U for each generation. Since $U = \eta(u) \times u / 25$, and $\eta(u)$ vs. u curve is known, for each U_n , u_n can be obtained (by trial and error). The overall opening time $t_{8-14} = t_8 + t_9 + \dots + t_{14}$, where $t_n = L_n / u_n$ and L_n represents the individual length. The result of this simulation is shown in Figure 2.50. Again, the yield pressure is smaller in our case ($p_{\text{yield}} \sim 2.2 \text{ cm-H}_2\text{O}$) than that observed by Gaver et al ($p_{\text{yield}} \sim 5.7 \text{ cm-H}_2\text{O}$). We also noticed that popping open is not as

evident in our case, which is due to the much higher steady shear viscosities assumed in our simulation. When the fluid rheology changes from pharyngeal mucus-like to tracheal mucus-like, the opening pressure increases dramatically, especially for rapid opening.

Recent investigations on sleep apnea indicate the locus of obstruction is in the pharynx [65]. In our previous simulation, the normal pharyngeal reopening pressure should be quite low (for $R=1$ cm) and is increased significantly by changing the pharyngeal secretion into a more elastic and viscous gel such as that found in tracheal mucus. The pharyngeal space is susceptible to change in volume through action of the muscles controlling the position of the palate and tongue. The length is ~ 8 cm; the cross section can vary from 4.7 cm^2 to 1.8 cm^2 [64], resulting in an effective radius ranging from about 1.23 cm to 0.75 cm. Such a change causes a modification of the reopening pressure, as shown in Figure 2.51. As predicted from studies of the tube model, the reopening time of the pharynx is prolonged when the pharyngeal size is smaller. The simulated reopening pressures for $R=1.23$ cm and 0.75 cm at $t=0.01$ sec are 0.97 cm-H₂O and 1.8 cm-H₂O, respectively. (c.f. $p=1.25$ cm-H₂O for $t=0.01$ sec, at $R=1$ cm.) Therefore, based on the hypothesis that the pharyngeal lining fluid determines airway patency [65], we conclude that, when the pharynx is obstructed, this can arise due to a change in the rheology of the pharyngeal

secretions and/or incapability of the muscle to provide a larger pharyngeal space.

Table 2.2. A list of the viscoelastic sols used in the study.

1. 5.5% Na Alginate
2. 4.5% Na Alginate
3. 3% Na Alginate
4. 5% Na Alginate + 0.15% CaCl_2
5. 4.5% Na Alginate + 0.15% CaCl_2
6. 3% Na Alginate + 0.15% CaCl_2
7. 2% Na Alginate
8. 1% Na Alginate
9. 0.5% Na Alginate

+ Na Alginate stands for sodium alginate.
 CaCl_2 stands for $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$.

+ All are measured under 5% strain, except for 0.5% Na Alginate (60%) and the first 5 points in 1% Na Alginate (50%) in order to get the data within the sensitivity range of the transducer. Tested by the strain sweep, these points were still in the linear viscoelasticity range.

Figure 2.4. tan δ vs. ω plot for the viscoelastic sols used in the tube model.

5.5% Na Alginate

(a)

5% Na Alginate + 0.15% CaCl_2

(b)

(continue in the next page)

(c)

Figure 2.5. G' and G'' for three entangled sols (5% dynamic frequency sweep): (a) 5.5% Na Alginate (b) 5% Na Alginate + 0.15% CaCl_2 (c) 4.5% Na Alginate + 0.15% CaCl_2 .

Complex Viscosity

Steady Shear Viscosity

Figure 2.6. Viscoelastic properties of nine model fluids: complex and steady shear viscosity. For each fluid,
 $\eta^*(\omega) |_{\omega \equiv \dot{\gamma}} = \eta(\dot{\gamma})$.

Table 2.3. The surface tension of the nine viscoelastic sols.

Fluids	Surface tension γ (dynes/cm)
5.5% Na Alginate	47.9
4.5% Na Alginate	49.6
3% Na Alginate	54.7
5% Na Alginate + 0.15% CaCl_2	51.2
4.5% Na Alginate + 0.15% CaCl_2	51.3
3% Na Alginate + 0.15% CaCl_2	56.7
2% Na Alginate	58.3
1% Na Alginate	63.4
0.5% Na Alginate	68.0
<hr/>	
H_2O	72.2

+ Repeated measurement on the same sols showed that the measured values were always within ± 1 for less viscous fluids and ± 2 for more viscous fluids. The values shown here are averages of results from two preparations and each is an average on three most close values. The surface tension varied more among different preparations, but the difference is no more than 5 dynes/cm.

Raw data plot from Tube Model test

for three entangled sols

Figure 2.7. Reopening pressure vs. reopening velocity (p - u) plot for nine viscoelastic model fluids in the tube model. Three of them show transitions at u_c .

Figure 2.8. Dimensionless plot of fluids in the tube model. Data from different fluids can be reduced to a master curve by the dimensionless analysis.

Regression equations:

(I)	$y = -1.6503 + 0.81009x$	$R^2 = 0.871$
(II)	$y = -2.1232 + 0.89718x$	$R^2 = 0.984$
(III)	$y = -2.8635 + 1.0762x$	$R^2 = 0.943$

Figure 2.9. Non-linear regression of the dimensionless plot shows three regimes of behavior.

Figure 2.10. Low U anomaly. Two low viscosity fluids (0.5% and 1% sodium alginate) do not follow the same P-U relationship as others.

Strain	ω_c (rad/s)
5%	55
10%	70
20%	80
40%	90
50%	beyond 100

Figure 2.11. The strain dependency of the crossover frequency (ω_c) of entangled sol, 5.5% sodium alginate, by dynamic shear experiment.

Pressure-velocity plot
 2% sodium alginate, $R=0.923\text{cm}$
 Effect of H and T

Figure 2.12. The effect of film thickness (H) on the reopening pressure for 2% sodium alginate under different wall tension (T).

Figure 2.13. The effect of tube radius (R) on the reopening pressure for 2% sodium alginate under different wall tension (T).

**Dimensionless plot for 2% sodium alginate
with different T, H and R**

Figure 2.14. Dimensionless relationship for 2% sodium alginate in tubes with different radius, film thickness and wall tensions.

Pressure-velocity plot
 5.5% sodium alginate, $R=0.923\text{cm}$
 effect of H and T

Figure 2.15. The effect of film thickness (H) on the re-opening pressure for 5.5% sodium alginate under different wall tension (T).

Figure 2.16. The effect of tube radius (R) on the reopening pressure for 5.5% sodium alginate under different wall tension (T).

Dimensionless plot
5.5% sodium alginate, effect of H and T

Figure 2.17. Dimensionless relationship for 5.5% sodium alginate in tubes with different radius, film thickness and wall tensions.

Pressure-velocity plot
0.5% sodium alginate, different T, H, R

Figure 2.18. The effect of film thickness (H), tube radius (R) and wall tension (T) on the reopening pressure for 0.5% sodium alginate coated tubes.

Dimensionless plot
0.5% sodium alginate, different H, R, T

Figure 2.19. Dimensionless relationship for 0.5% sodium alginate in tubes with different radius, film thickness and wall tensions.

Dimensionless formula for regime II: $P=3.09+13.26U^{0.68}$

Figure 2.20. A combination of regime I and II by using the data of 0.5% sodium alginate from larger tube (radius=1.67cm). Note that the low U anomaly is reduced.

3% Na Alginate + 0.16% CaCl_2

(a)

(b)

Figure 2.21. 3% sodium alginate + 0.16% CaCl_2 : (a) Rheological measurement shows that there is $G'-G''$ crossover. (b) Tube model test shows no transition.

Table 2.4. The relation between the velocity where transition occurs in the reopening pressure in the tube model and the rheological properties of the fluids (dynamic viscoelasticity under 5% strain).

R: Tube radius = 0.923 cm

H: Film thickness = 0.85 mm

(unless otherwise mentioned)

T: Wall tension = 1×10^4 dyn/cm

$$k_d \text{ is defined by } u_c = k_d \cdot \omega_c \sqrt{R \times H}$$

where ω_c is the G'-G'' crossover frequency under 5% strain (ω_c increases with increasing strain)

Fluids	ω_c (rad/s)	G' ($\omega=\omega_c$) (dyn/cm ²)	η^* ($\omega=\omega_c$) (poise)	u_c (cm/sec)	k_d
5.5% Na Alginate	55	4217	94	25	1.62
5% Na Alginate+0.15%CaCl ₂	35	2915	102	12	1.22
4.5% Na Alginate+0.15%CaCl ₂	16	1615	126	9	2.01
3% Na Alginate+0.16%CaCl ₂	20	76	43	≥50	≥9

5.5% Na Alginate (H=0.25mm)	55	4217	94	8	0.96

$k_d > 1$ indicates that the average strain in the tube model is larger than 5% and causes a delay in the G'-G'' crossover.

Figure 2.22. Rheological properties of two viscoelastic fluids are not influenced by the addition of surfactant SDS.

Table 2.5. Surface tensions of two viscoelastic fluids are both lowered by the addition of SDS.

Fluids	Surface tension γ (dynes/cm)
5.5% Na Alginate	47.9
5.5% Na Alginate + 0.20% SDS	32.1
4.5% Na Alginate	49.6
4.5% Na Alginate + 0.25% SDS	32.3

(a)

(b)

(continue in the next page)

(c)

Figure 2.23. (a), (b) The effect of SDS on the reopening pressure in a tube coated with 0.85mm thickness of 4.5% sodium alginate and 5.5% sodium alginate. (c) The effect on the dimensionless plot.

5.5% Na Alginate with/without SDS
in H=0.25mm tube

Dimensionless plot

Figure 2.24. Transition still occurs in a tube coated with 0.25mm 5.5% sodium alginate with surfactant SDS.

Table 2.6. Contact angles of albumin coated or noncoated polyethylene tubes.

Tube	Advancing angle (°)	Receding angles (°)
Control	83±4	85±2
(n=3)		82±1
		78±4
		71±1
		69±3
Albumin coated	73±3	68±2
(n=3)		60±2
		53±4
		45±3
		38±2

(a)

(b)

Figure 2.25. The effect of surface wettability on the reopening pressure for (a) 5.5% sodium alginate, and (b) 0.5% sodium alginate.

Table 2.7. A list of the properties of the nine viscoelastic gels we used to obtain the dimensionless relation.

Fluids (Viscoelastic gels)	γ , surface tension (dyn/cm)	η^* (poise) $\omega=1$	G' (dyn/cm ²) $\omega=1$	$\tan \delta$ $\omega=1$
3% Na Alginate+0.2%CaCl ₂	73	952.8	892.4	0.37
3.5% Na Alginate+0.185%CaCl ₂	82	679.4	629.5	0.48
2% Na Alginate+0.175%CaCl ₂	81	475.4	461.3	0.25
2.5% Na Alginate+0.175%CaCl ₂	78	311.8	272.7	0.55
3% Na Alginate+0.18%CaCl ₂	65	322.5	264.6	0.70
1.6% Na Alginate+0.164%CaCl ₂	73	255.1	244.5	0.30
3.27% Na Alginate+0.173%CaCl ₂	63	266.9	199.5	0.89
1.6% Na Alginate+0.15%CaCl ₂	67	111.2	99.6	0.50
1.6% Na Alginate+0.14%CaCl ₂	64	73.9	63.2	0.60

+Na Alginate stands for sodium alginate. CaCl₂ stands for CaCl₂·2H₂O. All dynamic parameters shown are obtained under 5% strain as $\omega=1$ rad/s. For dynamic parameter under all frequencies, see Figure 2.26. The surface tension values are only estimates, since it is difficult to measure surface tension accurately in these gels by the ring method utilised by Tensiomat.

Figure 2.26. Viscoelasticity of gels by dynamic measurement (G' , $\tan \delta$ and η^* in (a), (b) and (c), respectively) and steady shear measurement (η , in (d)).

(c)

(d)

Reopening pressure-velocity of gels in the tube model

(a)

Transition occurs in two of the gels

(b)

Figure 2.27. (a) Reopening pressure vs. reopening velocity (p - u) plot for nine model gels in the tube model. (b) Two of them show transition similar to that occurs in sols.

Figure 2.28. The reopening pressure for weaker gels showing (a) structure build-up while waiting after coating the film in the tube, and (b) small hysteresis loop in the upward and downward measurement.

Figure 2.29. Dimensionless plot of model gels in the tube model.
(a) normalized by η^* (b) normalized by η .

Table 2.8. The apparent yield stress of the model gels, as measured by the method described earlier in the text. As shown, the yield stress does not change the total yield pressure significantly.

Fluids (Viscoelastic gels)	Apparent yield stress, σ_0 (dynes/cm ²)	Dimensionless yield pressure (assuming $P_{cap}=3.09$), $P_{yield}=P_{cap} + \sigma_0 R/\gamma$
3% Na Alginate+0.2%CaCl ₂	35±4	3.53
3.5% Na Alginate+0.185%CaCl ₂	30±5	3.43
2% Na Alginate+0.175%CaCl ₂	25±5	3.37
2.5% Na Alginate+0.175%CaCl ₂	25±6	3.39
3% Na Alginate+0.18%CaCl ₂	20±2	3.38
1.6% Na Alginate+0.164%CaCl ₂	30±5	3.47
3.27% Na Alginate+0.173%CaCl ₂	15±3	3.31
1.6% Na Alginate+0.15%CaCl ₂	10±2	3.23
1.6% Na Alginate+0.14%CaCl ₂	10±2	3.23

(a)

Figure 2.30. Regression curves for gels.

(b)

(c)

Table 2.1: List of the properties of the five viscoelastic gels we used to study the effect of H.

Fluids (Viscoelastic gels)	γ , surface tension (dyn/cm)	η^* (poise) $\omega=1$	G' (dyn/cm ²) $\omega=1$	$\tan \delta$ $\omega=1$
1.6% Na Alginate+0.135%CaCl ₂	55	46.5	43.0	0.41
1.6% Na Alginate+0.164%CaCl ₂ ⁺	73	323.9	312.3	0.27
3% Na Alginate+0.18%CaCl ₂ ⁺	67	443.6	384.8	0.57
3.27% Na Alginate+0.17%CaCl ₂	57	63.5	52.7	0.67
3.27% Na Alginate+0.173%CaCl ₂ ⁺	65	269.5	217.2	0.73

+These solutions were re-prepared. Their properties are not necessarily the same as the gels used in the previous nonlinear regression analysis, due to the reason described earlier in text.

++Transitions occurred in 3% Na Alginate+0.18%CaCl₂ ($u=5-23$ cm/s) and 3.27% Na Alginate+0.173%CaCl₂ ($u=8-24$ cm/s) when $H=25$ mm. Some vibration of pressure also happened in 1.6% Na Alginate+0.164%CaCl₂ ($u=7-13$ cm/s) when $H=25$ mm, but not as evident.

Steady shear viscosity

(a)

Complex viscosity

(b)

(c)

(d)

Figure 2.31. Viscoelasticity of gels (for H effect) by steady shear measurement (η , (a)) and dynamic measurement (η^* , $\tan \delta$ and G' in (b), (c) and (d), respectively).

The effect of H on gels

Figure 2.32. The effect of H on the Reopening pressure vs. reopening velocity (p-u) plot for five different gels in the tube model.

Figure 2.33. The effect of H in the dimensionless plot of gels tested in the tube model.

and T on the reopening pressure in the tube model.

Fluids (Viscoelastic gels)	γ , surface tension (dyn/cm)	η^* (poise) $\omega=1$	G' (dyn/cm ²) $\omega=1$	$\tan \delta$ $\omega=1$
3% Na Alginate+0.163%CaCl ₂	56	166.4	121.4	0.94
3% Na Alginate+0.18%CaCl ₂	63	488.6	395.8	0.70
3% Na Alginate+0.19%CaCl ₂	67	650.6	595.1	0.44
2.5% Na Alginate+0.2%CaCl ₂	89	993.1	956.3	0.28

+Transitions occurred only in 3% Na Alginate + 0.18% CaCl₂ when H=25mm and R=0.923cm (u=6-19 cm/s at T=1×10⁴ dyn/cm, and u=19-25 at T=5×10⁴ dyn/cm).

(a)

(b)

Figure 2.34. Viscoelasticity of gels (for studying the effect of H, R and T) by steady shear measurement (η , (a)) and dynamic measurement (η^* , $\tan \delta$ and G' in (b), (c) and (d), respectively).

Figure 2.35. The effect of H, R and T on the (a) reopening pressure and (b) dimensionless plot of 3% Na Alginate+0.163% CaCl_2 gel in the tube model.

Figure 2.36. The effect of H, R and T on the (a) reopening pressure and (b) dimensionless plot of 3% Na Alginate + 0.18% CaCl₂ gel in the tube model.

(a)

(b)

Figure 2.37. The effect of H , R and T on the (a) reopening pressure and (b) dimensionless plot of 3% Na Alginate + 0.19% CaCl₂ gel in the tube model.

(a)

(b)

Figure 2.38. The effect of H , R and T on the (a) reopening pressure and (b) dimensionless plot of 2.5% Na Alginate + 0.2% CaCl_2 gel in the tube model.

Figure 2.39. The strain dependency of $\tan \delta$ for gels, by Rheometrics controlled strain rheometer.

Figure 2.40. The strain dependency of $\tan \delta$ for gels, by Carri-Med controlled stress rheometer.

Table 2.11. A list of collections of upper airway secretions from anesthetized animals for rheological measurements.

Source	abbr.	Treatment	Amount
Dog oral secretion	Or-dog	substance P	3 ml
Dog nasal secretion	Na-dog-1	blank	5 ml
Dog nasal secretion	Na-dog-2	substance P	5 ml
Dog pharyngeal secretion	Ph-dog-1	substance P	2 ml
Dog pharyngeal secretion	Ph-dog-2	substance P	1 ml
Cat pharyngeal secretion	Ph-cat	hypercarbonia	~0.8 ml
Cat tracheal secretion	Tr-cat-1	blank	~60 μ l
Cat tracheal secretion	Tr-cat-2	blank	~60 μ l
Cat tracheal secretion	Tr-cat-3	blank	~60 μ l

rheometer, shown as (a)-(f). (a) is the creep curve using longer retardation and relaxation time (300 sec or 150 sec). (b)-(f) are creep curves using shorter retardation and relaxation time (30 sec). The results of longer and shorter time are close. (c)-(e) shows different runs within the same collection. As noticed from our experience, reproducibility of the creep curves using small stresses within the same collection is not as good as reproducibility of the later part of the flow curves, where the stresses are large.

(a) Ph-dog-1, yield stress= 3 dyne/cm².

(b) Ph-dog-2, yield stress= 5 dyne/cm².

(c)-(e) Ph-cat, yield stress= 3.5, 4 and 5 dyne/cm², respectively.

(f) Tr-cat, yield stress= 3 dyne/cm²

(a)

Ph-dog-2

(b)

Ph-cat

(c)

(d)

(e)

Typical flow curves

Figure 2.42. Typical flow curves from the controlled stress rheometer for each of the collections shown in Table 2.11.

secretions. n represents the number of sample reloading and measurements within the same collection.

Sample (each collection)	yield stress (dyn/cm ²) from creep	yield stress (dyn/cm ²) from flow
Or-dog	3.7±0.2 (n=3)	5.3±1.9 (n=4)
Na-dog-1	3.9±1.7 (n=4)	5.4±1.8 (n=5)
Na-dog-2	4.0±1.6 (n=4)	6.0±2.0 (n=4)
Ph-dog-1	3.5±0.9 (n=4)	6.2±1.0 (n=4)
Ph-dog-2	3.8±1.0 (n=3)	6.2±1.0 (n=6)
Ph-cat	3.9±0.9 (n=6)	7.1±3.1 (n=6)
Tr-cat-1	5	19.4
Tr-cat-2	7	20.1
Tr-cat-3	3	7.6
HTM	5.4±0.9 (n=3)	6.7±1.5 (n=2)

Figure 2.43. Steady shear viscosity curves calculated from the flow curves in fig. 2.42.

stress flow curves and dynamic viscoelasticity at $\omega=1$ rad/s for airway secretions.

Sample	η ($\dot{\gamma}=1$ /s)	η^* ($\omega=1$ rad/s) by controlled strain rheometer at 5% strain	η^* ($\omega=1$ rad/s) by controlled stress rheometer
Or-dog	23.4 $\pm$ 6.4 (n=4)	18.0 tan $\delta=0.43$	106.6 $\pm$ 34.8 (n=3) (stress=5 dyn/cm ² , strain=5.3 $\pm$ 1.7%, tan $\delta=0.47\pm0.18$)
Na-dog-1	7.2 $\pm$ 1.0 (n=5)	7.6 $\pm$ 2.6 (n=2) tan $\delta=0.61\pm.12$	N. A.
Na-dog-2	10.6 $\pm$ 3.3 (n=4)	13.4 $\pm$ 5.8 (n=2) tan $\delta=0.54\pm0.03$	N. A.
Ph-dog-1	11.3 $\pm$ 3.5 (n=4)	13.2 tan $\delta=0.47$	N. A.
Ph-dog-2	8.3 $\pm$ 0.8 (n=4)	9.2 tan $\delta=0.53$	N. A.
Ph-cat	11.9 $\pm$ 3.7 (n=6)	N. A.	N. A.
Tr-cat-1	116.9	N. A.	188.6 (stress=10 dyn/cm ² , strain=5.3%, tan $\delta=0.63$)
Tr-cat-2	124.2	N. A.	178.1 (stress=10 dyn/cm ² , strain=5.6%, tan $\delta=0.49$)
Tr-cat-3	274.4	N. A.	100.9 (stress=5 dyn/cm ² , strain=5.0%, tan $\delta=0.41$)
HTM	8.1 $\pm$ 0.9 (n=2)

(a)

Dynamic modulus of secretions

(b)

(continue in the next page)

Figure 2.44. The frequency sweep of dynamic viscoelasticity of pharyngeal, nasal, oral and tracheal secretions at 5% strain. All data except for the secretion from cat trachea were obtained by the controlled strain rheometer. The tracheal fluid was tested using the controlled stress rheometer by presetting the strain at 5%. Since this instrument is not designed for controlling strain, the variation of data is caused by deviation of the real strain from 5% (in a range 2.5-6.2%) during measurement.

Tr-cat-2

(a)

(b)

(continue in the next page)

(c)

(d)

(continue)

(e)

(f)

(continue)

Figure 2.45. The frequency sweep of dynamic viscoelasticity of tracheal secretion (Tr-cat-2) under different constant stress, in (a)-(d). (e)-(h) are comparisons with the other samples.

Figure 2.46. Strain dependency of $\tan \delta$ under certain frequencies for upper airway secretions from animals, measured by the controlled strain rheometer.

Figure 2.47. Strain dependency of $\tan \delta$ under certain frequencies for airway secretions from animals, measured by the controlled stress rheometer. Two typical measurements were shown for Or-dog (one marked *) since it is heterogeneous.

Table 2.14. Critical strains (γ_c , estimated from figs.2.46.&2.47.) for secretions from different locations of the airway.

Sample	γ_c ($\omega=1$)	γ_c ($\omega=10$ or 50)
By controlled strain rheometer		
Ph-dog-1	13%	23% ($\omega=50$)
Ph-dog-2	15%	
Na-dog-1	16%	29% ($\omega=50$)
Or-dog	35%	
By controlled stress rheometer		
Or-dog	33%	
Or-dog*	48%	
Tr-cat-1	400%	120% ($\omega=10$)
Tr-cat-2	2000%	
Tr-cat-3	2000%

(a)

Figure 2.48. (a) Predictions of airway reopening pressure and reopening velocity relationship; (b)&(c) predictions of airway reopening time. Both using eq. (2.14) and other assumed parameters mentioned in the text. Rheological data for simulation of upper airway coated with tracheal mucus and pharyngeal mucus are from those of Tr-cat-2 and Ph-cat, respectively.

(b)

(c)

Table 2.15. Architecture of the human lung according to Weibel's [63,66] model, with regularized dichotomy.

	Generation	Number	Diam. (mm)	Length (mm)
Trachea	0	1	18	120
Main bronchus	1	2	12.2	47.6
Lobar bronchus	2	4	8.3	19.0
	3	8	5.6	7.6
Segmental bronchus	4	16	4.5	12.7
	5	32	3.5	10.7
Bronchi	6	64	2.8	9.0
	7	128	2.3	7.6
	8	256	1.86	6.4
	9	512	1.54	5.4
	10	1,020	1.30	4.6
Terminal bronchus	11	2,050	1.09	3.9
	12	4,100	0.95	3.3
Bronchioles	13	8,190	0.82	2.7
	14	16,400	0.74	2.3
up to 23 generation

Figure 2.49. A comparison between our simulation data for upper airway reopening time and prediction of airway opening time for generations 8-14 by Gaver et al.

Figure 2.50. A comparison between prediction based on our methodology and that by Gaver et al, of opening time for bronchial generations 8-14.

Figure 2.51. Simulation of pharyngeal reopening pressure and reopening time (assuming $L=8$ cm) for variable pharyngeal space.

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1. BACKGROUND

1.1. EXTRACELLULAR MATRIX OF SKIN

Mammalian connective tissue is a highly complex macromolecular composite. In addition to the cellular components, the matrix contains fibers surrounded by a ground substance, and water. The fibers are mostly composed of the proteins collagen, and elastin. The ground substance contains loosely organised macromolecules including proteoglycans (PG), glycosaminoglycans (GAG) and structural glycoproteins (cell-matrix attachment factors such as fibronectin and laminin) [1]. Typical values for the composition of skin expressed as percentage wet weight are as follows: water 60-72%, collagen 30%, elastin 0.2%, and PG and GAG 0.03-0.35% [2]. Other information showed that skin contains dry weight 75% collagen [3] and 2-5% elastin [4]. The relative amounts of these components vary significantly with age. The collagen network primarily determines the structure and mechanical properties of the skin. But the other non-collagenous components are also believed to play important roles either through their unique viscoelastic property (e.g. elastin) or via their interaction with collagen fibers (e.g. PG).

Collagen

Collagen is a fibrous protein which provides structural integrity of skin as well as other connective tissues due to the high tensile strength of the fibrils. This load-bearing property is due to its unique macromolecular conformation. There are extensive reviews regarding the structure and function of collagen fibrils [5-8].

Collagen is observed as fibrils under electron microscope with diameters in the range 100 to 3000 Å and appears banded with an axial repeating period (called the D period) of 500 to 700 Å, most commonly 640-670 Å [5,8,9]. Wide-angle X-ray diffraction pattern has meridional reflections at 10 Å, 4 Å, and 3 Å and an equatorial spacing of 10 to 15 Å, depending on the degree of hydration of the fiber [9]. The banded structure is assumed to be related to a quarter-staggered array of tropocollagen molecules, as shown in Figure 3.1. The long, thin tropocollagen molecules align side-by-side in such array, to give a fibril of large axial ratio, but limited lateral dimensions. The diameter of the fibrils seems to grow in a multiple of 80 Å ("quantized") [2,8] and is suggested to be related to the tetragonal unit cell by Smith and Fraser et al [10,11]. As indicated by X-ray diffraction data [4,5], each tropocollagen molecule is a coiled coil consisting of three individually helical polypeptide chains twisted together. Each chain is a left-handed, polyproline-II type helix with 3 Å per residue and 3.3 residues per turn. It contains about

Figure 3.1. Quarter-staggered array (Petruska-Hodge packing) of tropocollagen molecules. Dashed lines indicate likely positions for intermolecular crosslinks, involving residue pairs 9-943 and 103-1044. The scale D is ~668 Å or 234 residues.

1000 residues with a length close to 3000 Å. The triple helix is stabilized by the sequence -Gly-Pro-Hyp-, -Gly-Pro-X- and -Gly-X-Pro, where X is some other amino acid.

Fibrillogenesis and fibril morphology will be discussed in Section 1.2. Soluble forms of collagen can be extracted by neutral salt or acid media [12]. The product of extractions carried out at pH 3 to 4 and at low salt concentration has been called acid-soluble collagen or procollagen. The product of those carried out near pH 7 and at high salt concentration has been called neutral salt soluble collagen or tropocollagen. *In vitro* reconstituted collagen fibrils from collagen solutions will also be discussed in Section 1.2.

Elastin

Elastin can also exist as a fibrous structure in connective tissues with diameters ranging from 4-6 µm fibers to 5 nm fine filaments [13]. The X-ray diffraction pattern shows only amorphous rings and no periodicity is apparent in the electron microscope. Two polyfunctional amino acids, desmosine and isodesmosine, serve as the main crosslinks between peptide chains in elastin [9,14]. Others [15] concluded that elastin exists as an amorphous structureless material in elastic fibers in connective tissues.

Elastin serves as an elastic restraint and occurs in association with collagen in tissues to give a variety of mechanical properties [4]. It is prominent in the nuchae ligment of the necks of ruminates and aorta, but is a minor component in most other connective tissues, e.g. skin. Elastin is synthesized with a molecular weight between 70,000 and 76,000 [16], which may be a cleavage product of a higher molecular weight (~100,000-140,000) protein termed proelastin [17]. Because of its insolubility, *in vitro* studies have frequently utilised a chemical degradation product called α -elastin.

α -Elastin, obtained by hot oxalic acid cleavage of purified elastin fibers [18], is a water-soluble protein with a molecular weight of 70,000 approximately. The other fraction of oxalic acid extraction is called β -elastin (M.W.<10,000). The soluble precursor of elastin, tropoelastin, has also been isolated [19]. Upon raising the temperature of an aqueous solution of α -elastin or of tropoelastin to 37°C, there occurs a phase separation. Two phases, i.e. the more dense coacervate phase and a dilute solution phase, are formed. Cox et al [20,21] have demonstrated that coacervation of either α -elastin or tropoelastin results in fiber formation with dimensions resembling the native elastic fiber.

Proteoglycans and Glycosaminoglycans [22]

Proteoglycans (PG) are high molecular weight biopolymers consisting of a protein core to which glycosaminoglycans (GAG)

side chains are covalently attached. GAG are themselves macromolecules containing repeat disaccharides composed of a hexosamine residue and a uronic acid residue, thus all GAG carry a net negative charge which makes them hydrophilic. For example, the repeating disaccharide in hyaluronic acid (HA) is β -D-glucuronic acid + β -D-N-acetyl glucosamine, and that in dermatan sulfate (DS) is β -D-glucuronic acid or β -L-iduronic acid + β -D-N-acetyl galactosamine. HA, found in skin (0.03-0.09% in adult human skin [23]), synovial fluids, and the vitreous humor of the eye, is the only GAG that is not covalently bound to a protein core. HA increases the viscosity of the fluid phase of the matrix which helps to dissipate stress energy. Dermatan sulfate proteoglycan (DSPG) is found in cartilage, skin, lung, aorta, tendon, umbilical cord, and scar tissue. Ground substances may not only have an effect on the collagen network through their interactions with collagen fibers but also may exert an influence during fibrillogenesis [24-27]. Scott et al [24] found that DSPG is located at specific binding sites on the surface of collagen fibrils, most frequently at the d bands and, occasionally, at the e bands. The map of binding sites of proteoglycans along the collagen fibril by Scott et al is shown in Figure 3.2.

Structural glycoproteins (cell-matrix attachment factors)

In connective tissues, these high molecular weight glycoproteins serve as a way to link biosynthetic events within cells to mechanical events in the matrix [1,4]. Fibronectin (FN)

Figure 3.2. Map of binding sites of proteoglycans along the type I collagen fibril (by Scott et al). The a-e banding pattern within the D period of the fibril (lower portion) is shown against the quarter-staggered array (upper portion). DS-PG, dermatan sulfate proteoglycan; KS-PG, keratan sulphate.

and laminin are two glycoproteins that apparently mediate adhesion of cells to fibrous components of the extracellular matrix. Fibronectin is present in an insoluble form at the cell surface of many cell lines in the connective tissue such as fibroblasts, and also in a soluble form in plasma (~300 µg/ml) and other body fluids. The plasma and cellular forms have almost identical amino acid and carbohydrate compositions. They appear to be composed of two polypeptide chains linked through disulfide bonds close to their carboxy terminal ends. These large polypeptide chains have molecular weights close to 220,000, giving the molecule a total molecular weight of ~450,000. The carbohydrate content varies between 5 and 9%, depending on the fibronectin source. A domain structure of fibronectin, implicated in the specificity of binding, has been identified [28,29]. These domains include fibrin-binding sites, heparin-binding sites, *S. aureus* binding site, collagen(gelatin)-binding site and cell-binding site [28,29]. Other GAG, such as HA, also bind to FN [30]. In addition, experiments have indicated a cryptic GAG-binding domain, which enables the binding of DSPG to substratum-bound FN [31]. Ruoslahti et al [32] proposed that the multiple interactions of FN, collagen and GAG, including HA, DS, CS heparin and HS, could play a role in the depositions of these substances as an insoluble extracellular matrix.

Laminin is a component of basement membranes [4,33]. It accelerates the attachment of epithelial cells and endothelial cells

to type IV collagen in basement membranes. It also binds to heparin and heparan sulfate (HS) and mediates their association with collagen. Laminin has a molecular weight of ~900,000. SDS gel electrophoresis indicates two subunits with molecular weights of 220,000 and 440,000 respectively. The carbohydrate content of laminin is 12 to 15%.

The interaction between the fibers and ground substance in the extracellular matrix during fibrillogenesis can determine the morphology and properties of the collagen network, as described in the next section.

1.2. FIBRILLOGENESIS AND FIBRIL FORMATION *IN VITRO*; FIBRIL MORPHOLOGY

During fibrillogenesis (fibril formation *in vivo*), procollagen, the soluble precursor to collagen, produced by the cell, undergoes extracellular modifications to form tropocollagen which self-associates forming collagen fibrils. The exact sequence of events between the synthesis of collagen molecules and the appearance of morphologically-distinct collagen fibrils is still unknown. Experiments [34,35] suggested that stable aggregates of collagen molecules formed intracellularly are transported by exocytosis to the extracellular space where they subsequently undergo further aggregation into fibrils. In embryonic tissues, collagen fibrils grow in an environment rich in hyaluronic acid and proteoglycans

[36], and it is likely that specific interactions between them help decide fibril morphology. There may be correlations between fibril nucleation, fibril radial growth and fusion *in vivo* and the presence of ground substances. *In vivo* work by Parry et al [2,37,38] tended to relate mechanical function of different tissue to growth patterns of collagen fibrils and their ground substance contents, from extensive analyses of fibril diameters and GAG compositions in tissues. It was shown [27,38] that tissues with the smallest diameter collagen fibrils have high concentrations of HA and that tissues with the largest diameter collagen fibrils have high concentrations of DS. The GAG levels appeared to correlate with the functional requirements of the tissue. Recent histological work by Scott [26] has presented ultrastructural evidence confirming that collagen fibrils are aggregates of subfibrils and protofibrils, and that intrafibrillar proteoglycan is originally associated with protofibrils, which coalesce to larger fibrils during development. Thus the function of the large amount of PG and HA in young tissues is seen to keep the collagen protofibrils and subfibrils from coalescing. Note that, most of the knowledge concerning fibril-ground substance interactions is based upon the widespread data of collagen self-assembly *in vitro*.

Collagen fibrils may form *in vitro* in the absence of cells. The neutral-salt soluble collagen molecules, without the aid of the other molecules, can self-assemble into collagen fibrils with a structural periodicity similar to the native fibers by raising the

temperature to 37°C [4,12,39]. Similar assembly occurs in the acid-soluble collagen molecules by raising pH and ionic strength simultaneously [40]. The product of self-assembly is a hydrated collagen lattice called a gel. *In vitro* systems enable studies which develop an understanding as to how collagen assembles in skin and other connective tissues to form structural networks. The kinetics of fibril formation, the morphology and the stability of the fibrils depend on a variety of factors, such as pH, ionic strength, temperature, and phosphate concentration [41-44]. From analysis of turbidity-time curve, the process can be characterized by three phases: a lag (nucleation) phase, a sigmoid growth phase, and a plateau phase [4,45,46]. Early work by Wood and Keech [42], on aggregation of an acid-soluble collagen, followed by turbidimetry and electron microscopy, has demonstrated the effect of a range of experimental conditions (pH, ionic strength, temperature and collagen concentration) on the rate of fibril formation. When the rate was increased by increasing the temperature or lowering the ionic strength, fibril width decreased; but when the rate was increased by reducing the pH, fibril width increased. The influence of pH and ionic strength indicates that the fibril formation process is controlled to a significant extent by electrostatic interactions between the soluble collagen molecules. It was also found that increasing temperature during only the lag phase resulted in a decrease in the fibril width, which suggested that most of the fibrils are formed during the lag period and the ultimate fibril width is decided before any

appreciable precipitation has occurred. Ripamonti et al [47] used x-ray and neutron diffraction techniques to investigate the roles of pH and ionic strength on the structure and stability of collagen fibrils and concluded that electrostatic interactions are essential for the structural stability of the collagen fibrils.

The addition of other extracellular components may change the time schedule of phases and the ultimate sizes of the collagen fibrils formed *in vitro* [48-50], suggesting their possible role in regulating fibrillogenesis and fibril morphology *in vivo*. Most *in vitro* research on morphological and kinetic studies have related the fibril diameter or lag time of fibril formation to the existence of extracellular components. Early work by Wood and Keech [48,49] have suggested that those GAGs which delay fibrillogenesis and which give an extended nucleation phase tend to produce thinner fibrils; those GAGs which increase the rate tend to produce thicker fibrils. Danielsen [51] observed that the mechanical properties of reconstituted collagen fibrils were deteriorated by the addition of dermatan sulfate. Obrink [52-54] found that chondroitin sulphate (CS), DS, HS, haptanin, CSPG and DSPG accelerated the fiber formation, when they were added before the lag phase, due to an effect on the nucleation process. When added after the lag phase, CS and proteoglycans delayed the fiber formation, due to an inhibition of fiber growth from the nuclei. HA accelerated both the nucleation and the growth processes, possibly by excluded volume effects that increased activity of

collagen. Scott [25] deduced that some GAG, especially iduronate-rich GAG (e.g. DS and HS) bind to collagen under physiological conditions via electrostatic bonds between amino groups on collagen and sulfate groups on the GAG, since this interaction is abolished by raising the salt concentration; whereas HA interacts feebly or not at all with collagen at physiological pH and ionic strength. Vogel et al [55] demonstrated that the small DSPG of bovine tendon had a unique ability to inhibit fibrillogenesis. Scott et al [24,56,57] have suggested that PG may have an inhibitory effect on the lateral growth of collagen fibrils in developing rat tail tendon, possibly through interference with the crosslinking between collagen molecules. DSPG (skin) protein core associates with collagen and inhibits fibrillogenesis. Electron microscopic studies have demonstrated binding of the protein core to reconstituted type I collagen fibrils in the gap region [24].

In summary [25], some GAG may interact electrostatically with but are not incorporated into type I collagen fibrils under physiological conditions; strongly interacting GAG accelerate fibrillogenesis. CSPG and DSPG interact strongly with type I collagen, both electrostatically via GAG chains, and by protein-protein interactions; they inhibit fibrillogenesis. Besides, they are incorporated into collagen, if present during fibril growth.

Fibronectin has been reported [58] to inhibit the rate at which collagen fibrils are formed though it does not alter the total

amount of fibril formed. Since fibronectin delays fibrillogenesis, it could regulate the size of fibrils *in vivo*. Brokaw et al [59] suggested that its influence on the fibrillogenesis kinetics may be due to its ability to prevent molecular collisions. The mechanism of fibril formation appeared unchanged. Jilek [60] observed that HA prevented the insolubilisation of native collagen type III by binding to fibronectin. Thus the multiple interactions of FN, GAG, PG and collagen could play a role in the deposition of these substances as an insoluble extracellular matrix.

In addition to collagen, elastin also exists as a fibrous structure in the native tissue. Coacervation of α -elastin can result in fiber formation *in vitro*, with fibrous structures similar to those observed for the native elastin fiber [20]. The filaments, 50 Å across, are generally associated into groups of varying dimensions. The coacervate phase forms on raising the temperature to 37°C, and dissolves again on reducing the temperature. No specific interaction is known to occur between elastin and other extracellular macromolecules.

1.3. AGING OF THE SKIN

Connective tissues are stress-resisting in which the fibrillar element resists pulling forces, and the interfibrillar gel or sol (ground substance) resists compressive forces. An increase in fibril diameter enhances the density of intrafibrillar crosslinking

and gives high tensile strength. On the other hand, a reduction in fibril diameter increases the surface area per mass for interaction between matrix and fibrils and thus inhibits creep. The above-mentioned factors determining the fibril morphology will participate in deciding the mechanical properties. Overall, the mechanical properties of skin and other connective tissue are dependent on the nature of the intramolecular and intermolecular covalent crosslinks, the type and quantity of GAG and PG associated with the collagen fibrils and the content of elastin, water and minerals [2,23,61]. These factors can be functions of age. Systematic age-dependent changes in mechanical properties of human skin are not well-established [62]. Studies in rat skin indicate that all mechanical parameters including elasticity and viscosity decrease during aging [63].

In early development, high tissue concentrations of HA and PG are associated with thin collagen fibrils, and fibril diameter increases during maturation accompanied by a decrease in the concentration of HA and PG [26,36]. Sampaio [64] isolated DSPG-II from human articular cartilage and found the highest content (~4 mg/g wet weight) in 15-25 year-olds and a lowest content (~1mg/g wet weight) in old tissue (>55 years). Since DSPG-II has been shown to interact with collagen network, its reduction may result in a shrinkage of ground substance or a decrease in viscosity and be related to changes in the mechanical properties during aging of the skin.

Additional factors which may influence the mechanical properties of skin with increasing age include the decreasing rate of collagen biosynthesis [65], the decreasing water content, the decreasing elastin content [37,66,67] and a changing stability of the covalent crosslinkages with age [68].

2. EXPERIMENTAL

2.1. INTRODUCTION

From the information surveyed in the background section, it is reasonable to hypothesize that viscoelastic properties of skin are directly controlled by the interaction between macromolecular components of the extracellular matrix, specifically the associated interaction between collagen, elastin, dermatan sulfate proteoglycan, hyaluronic acid, and fibronectin in the dermis. The changes in the relative amount of these species during aging could be the primary reason for the attendant changes in mechanical properties of the skin. So far, no viscoelastic data of a collagen gel network correlated with content of ground substance macromolecules has been given.

The goal of this study is to develop a molecular/morphological model for the extracellular matrix of skin which accounts for the viscoelastic behavior, and if possible, their change

as a function of age. The approach is to investigate the mechanical and morphological properties of model collagen gels *in vitro*. Similar collagen gel systems have been used to study the cell-matrix interactions [69] or used as substrata to study the cell culture behavior [70,71]. The gels consist of a hydrated lattice of collagen fibers, and are formed by quickly raising the ionic strength and the pH of an aqueous acid-soluble collagen solution to physiological levels by adding growth medium and salt solution. The other extracellular components used in this study are skin DSPG-II, HA, fibronectin and elastin. The viscoelastic properties of the collagen network are then be measured by a fluids rheometer. The morphology of the fibrils is examined by transmission electron microscopy (TEM).

2.2. MATERIALS AND METHODS

Preparation of collagen gels [71]

Type I collagen was extracted from rat tail tendons in 0.5 M acetic acid for 2 days at 4°C. Debris was removed by centrifugation at 2000 g for 3 hr. The supernatant was mixed with an equal volume of 10% (w/v) NaCl to precipitate the collagen. The precipitate was centrifuged (2000 g for 3 hr), the pellet dissolved in 0.1 M acetic acid and then dialysed against 0.001 N HCl for 2 days at 4°C. The aqueous solution of collagen was then centrifuged overnight at 2000 g (8°C) and the clear supernatant stored at 4°C. The collagen concentration of the final

stock solution is 1.65 mg/ml and with pH=3. Three-dimensional gels of reconstituted collagen fibers were prepared by mixing 1 ml of 2.75 times-concentrated (or 5 $\times$, 7.5 $\times$ to give different ionic strength I=0.11, 0.18 or 0.25, respectively) Earle's Balanced Salt Solution, 1ml KBM (cell culture medium) and 2.5 ml collagen solution. The mixture (4.5 ml) was pipetted into 60 $\times$ 15 mm petri-dishes precoated with 1% BSA. They were incubated 2 hr at 30-32°C. The final concentration of collagen in the gel is around 0.91 mg/ml. The phosphate concentration (introduced by adding Earle's Balanced Salt Solution and KBM) is approximately 1 mM.

The other matrix components (e.g. GAGs) may be conveniently introduced in the medium [69]. These include dermatan sulfate (DS) 0.25 mg/ml, hyaluronic acid (HA) 0.1-1 mg/ml, DSPG-II 0.13-0.65 mg/ml, fibronectin (FN) 0.0211-0.0422 mg/ml, elastin 0.1 mg/ml and their different combinations. DS and HA were a gift from Dr. M. B. Mathews of the University of Chicago. DS was isolated from hog mucosal tissues. HA was isolated from human umbilical cords. The intrinsic viscosities for DS and HA are 84 and 540 ml/g, respectively. DSPG-II was isolated from fetal calf skin and generously supplied by Dr. H. U. Choi, Montefiore Medical Center, Bronx, NY. The weight average molecular weight is $\sim 61 \times 10^4$ and the hydrodynamic radius is $\sim 32 \times 10^{-7}$ cm in 0.15 M NaCl. The intrinsic viscosity is 339 ml/g assuming an impermeable sphere [72]. FN used in this study was human plasma fibronectin (pFN) [73] provided by Dr. K.

Lewandowska of the Department of Molecular Biology and Microbiology of this university. Elastin from ligamentum nuchae was from Fluka with an average molecular weight ~100,000 and is soluble in water.

Rheological studies of gels

After incubation for two hours, fibril precipitation was essentially complete [74]. The rheological experiments of the gels were performed on a Rheometric Fluids Spectrometer, utilising cone and plate geometry with a 0.02 radian angle and 50 mm diameter cone. The instrument was operated in dynamic mode under 5% strain with the frequency range from 0.1 to 100 rad/s. The dynamic parameters including storage modulus (G'), loss modulus (G''), $\tan \delta$ ($=G''/G'$) and complex viscosity (η^*) were measured. Most of the measurements were conducted at room temperature (25°C). For the gels with elastin, some of the measurements were performed one hour after heating the gel to 37°C.

Data are compared using a collagen gel minus any other matrix component as a control each time, and are interpreted in terms of interactions between matrix components present in the gels.

Transmission electron microscopy (TEM)

Cuprolinic blue, a derivative from quinolinic acid, was used to stain DSPG-II [56,75-77]. Collagen fibrils were positively stained by an electron-dense dye [5,75] such as uranyl acetate or phosphotungstic acid.

Fresh gels, either immediately after rheological studies, or without having been through rheological studies were stained overnight at room temperature or at 37°C in 0.05% Cuprolinic blue (BDH Chemicals) in 25 mM sodium acetate buffer (pH=5.6) containing 0.1 M MgCl_2 and 2.5% (w/v) glutaraldehyde. Unbound dye was removed by rinsing three times for a total 15 min in buffer containing glutaraldehyde and MgCl_2 , followed by further *en bloc* staining for 15 min in 0.5% aqueous sodium tungstate solution. Then the stained gels were washed in 0.5% sodium tungstate in 50% (w/v) ethanol, dehydrated in ethanol, cleared in propylene oxide, and embedded in PolyBed-812 epoxy resin (supplied from Polyscience). Thin sections ~80 nm were cut and mounted on copper grids, unstained or counterstained with 1-2% aqueous uranyl acetate (UA) or 1% phosphotungstic acid (PTA) and examined under a Jeol 100CX transmission electron microscope at an accelerating voltage of 80 kV. For those samples stained at 37°C instead of room temperature, they were allowed to sit at 37°C for an hour before fixing and staining [20,21]. All the chemicals used in the sample preparation except Cuprolinic blue and epoxy resin were from Fisher.

Gels have been found to have reproducible tendency properties if a control collagen gel without any other macromolecules is used for comparison in each experiment. Gels were subjected to both rheological and morphological studies in order to identify the relation between mechanical strength and fibril morphology. The gelling process (fibril precipitation) was essentially complete after 2 hours of incubation at 30-32°C. The $\tan \delta$ values for all the gels are close to 0.2.

Gels formed at different ionic strength were studied first. Figure 3.3. shows the rheological measurements for gels formed at collagen concentration 0.91 mg/ml in pH=7.4 under different ionic strength, $I=0.11$, 0.18 and 0.25. As evident, the gels formed at ionic strength 0.11 have better strength. An increase of ionic strength to 0.18 or 0.25 causes a decrease in the moduli of the gels, $G'(\omega)$ and $G''(\omega)$, and in the complex viscosity η^* . But a further increase of ionic strength from 0.18 to 0.25 does not decrease these parameters much further. The decrease in shear modulus with increasing ionic strength is consistent with similar observations for gelatin gels as described in the first chapter. This phenomenon is probably associated with a retarded precipitation rate and consequent regulation of collagen fibril diameter [42] when increasing the ionic strength. The electron microscopic results of gels formed at different ionic strength are shown in

Figure 3.4. We found that P1A is a darker stain than UA, but they each stains collagen fibrils well. The morphology of gels after rheological studies shows a more dense fibrillar structure than that of gels which have not been subjected to rheological studies. It appears that, loading the gels under the cone and plate geometry, results in extrusion of water and leads to a more compact collagen network. However, in both fresh and sheared gels, similar systematic variation of morphological characteristics with ionic strength was observed. That is, no matter whether the gel was subjected to rheological study or not, it is always found that fewer numbers of fibrils are formed at higher ionic strength (under $I=0.25$), but that the fibers are longer fibrils and have a better banding structure. Thus in all subsequent TEM studies, fresh gels were used for comparison of morphological behavior. In these collagen gels, the fibrils are about 360-450 Å in width and their length varies, up to 2 μ, with bands repeating at 540 Å (the D period). The difference of the D period from the most common value of 640 Å may be due to differences in gel preparation or a true variation in the D period for rat tail tendon collagen, or it may be a result of distortion during TEM specimen preparation or observation [8]. Scott et al [56] reported that D of rat tail-tendon collagen increased from 530 to 630 Å, as the rat matures from 7- to 70-day-old. The diameters of fibrils (360-450 Å) are multiples of 80 Å [2]. But in contrast to the observations of Wood et al [42], the fibril width does not seem to increase with ionic strength. This is probably because it is difficult to

distinguish single fibrils from bundles (aggregates) of fibrils in our photographs. Comparing rheological to morphological results, we found that the formation of a more dense network of shorter fibrils at low ionic strength ($I=0.11$) correlates with larger moduli. However, the degree by which the more dense fibril network enhances the modulus may be partially compromised by an alteration in the strength of single fibrils which are more poorly organised at lower ionic strength, as evidenced by poorer banding contrast. The change of ionic strength from 0.18 to 0.25 did not change the fibril density as significantly as from 0.11 to 0.18. Thus an increase of $I=0.18$ to $I=0.25$ does not give further change in the total viscoelasticity (fig.3.3.).

The viscoelastic behavior of the gels at $I=0.11$ containing additional single macromolecular components has been studied. These include gels with DS (0.25 mg/ml), DSPG-II (0.65 mg/ml), HA (0.1 or 1 mg/ml) and pFN (0.0211 mg/ml). All of them exhibit an increase in G' , G'' and η^* , except for the gels with pFN, which do not have much difference from control gels. Figure 3.5. displays the dynamic viscoelasticity of gels with DS or DSPG-II. The approximate molecular weights of DS and DSPG-II used in this study are 24K and 62K respectively [72]. Thus the molar ratio of DS in the 0.65 mg/ml DSPG-II gel is similar to that in the 0.25 mg/ml DS gel. As observed, the gel with DSPG-II always has higher values than gel with DS. Viscoelastic properties for gels with HA and with pFN are shown in Figures 3.6. and 3.7. The

viscoelastic functions are determined by the sum of contributions from the fibrils network and from the medium. As a rheological model, we assume that the native fibrils are dispersed in an aqueous medium, whose viscosity may increase with the addition of another macromolecular component. To evaluate the influence of the macromolecular additive on the fibrous component, therefore, the dynamic parameters of each gel were normalized to the medium viscosity. This normalization was carried out by dividing the rheological functions by the medium viscosity. The medium viscosities of the gels were either measured or calculated from their intrinsic viscosity, as listed in Table 3.1. The normalized dynamic viscoelasticity of gels with different components are shown in Figures 3.8. and 3.9. It emerges that the addition of DS and HA, to a similar extent, increases each of the dynamic viscoelastic functions of the fibers. However, DSPG-II results in a larger increase than that observed for addition of DS or HA. The small increase in gels containing DS or HA may be due to weak electrostatic interactions between them and collagen fibrils [25]. The larger increase in DSPG-II gels is presumed due to the known specific binding interaction between collagen and DSPG [24], which may change the morphology of the fibrils [56,57]. It remains unclear whether the effect of DSPG is via a change in fibrillogenesis or is due to differences in interfibrillar interactions, since, in our experiment, DSPG was introduced before fibril precipitation. The addition of pFN does not alter the viscoelastic properties of gels at $I=0.11$, in spite of the known presence of a

collagen- or gelatin-binding domain (~40K) on the pFN which has been well characterised by others [28,29].

Figure 3.10. indicates that for gels formed at $I=0.25$, both DSPG-II (0.13 mg/ml) and its combination with pFN (0.0211 mg/ml) increase the dynamic viscoelastic parameters to a comparable level. This again indicates that pFN does not seem to play a role in the mechanical properties. It was suggested earlier [31] that the interaction between pFN and DSPGs occurs through a cryptic GAG-binding domain on pFN, which, however, only allows attachment of DSPG to substratum-bound pFN. This could explain why the combination of pFN and DSPG does not further increase the mechanical properties.

Electron microscopic analyses of collagen gels containing other macromolecular components at $I=0.25$, stained at room temperature, are shown in Figure 3.11. The binding of DSPG-II to the collagen fibrils in gels formed at a DSPG-II concentration 0.13 mg/ml is clearly evident by the presence of regularly-spaced dots along the stained collagen fibrils. The staining bands of collagen gels also exhibit a 540 Å repeat. The diameter of the fibrils is about 360 Å, which is close to that of the control gel. The length of the fibrils varies. The fibrils also show a stronger fibril-fibril association in the DSPG gel. The cuprolinic blue staining clearly demonstrates that DSPG-II is associated with the d band on the collagen fibril and these DSPG-II molecules appear to function as

bridges to align two collagen fibrils parallel to each other, with closely matched banding patterns (fig.3.11.g). PTA is a darker stain for collagen fibrils, resulting in some loss of contrast for the cuprolinic blue stained DSPG-II (fig.3.11.a-d). The visualisation of DSPG-II can be enhanced by using UA stain instead of PTA stain. As observed in fig.3.11.e-g, samples counterstained by UA show better resolution of DSPG-II molecules. When the collagen fibrils were not stained, DSPG-II could be clearly seen as rods perpendicular to the fibril axis at periodic locations along the fibrils (fig.3.11.h). HA in the gel was also stained and is seen as globular particles randomly sticking to the fibrils (fig.3.11.j). The fibril density in the matrix does not seem to be changed by the presence of either DSPG-II, HA (0.5 mg/ml) or pFN (0.0422 mg/ml).

Rheological analyses for gels containing 0.1 mg/ml elastin was carried out at two temperatures. First, the measurements were conducted at 25°C. Then the gel was heated to 37°C for one hour and the viscoelastic analyses performed at 37°C. The temperature was then adjusted so that it returned to 25°C in 10 min. The gel rheology was measured again after being kept at 25°C for 10 min. The results are shown in Figure 3.12. It was observed that all dynamic parameters for gels containing elastin at 25°C were close to the control collagen gel under either 25°C or 37°C. After heating the gel to 37°C for an hour, G' (and G'' , to a lesser degree) increased. This is believed due to the coacervation

of elastin (elastin fiber formation) since the gel is more elastic (higher G'). Unlike collagen fibers, this formation is highly reversible. After reducing the temperature to 25°C, the viscoelasticity of the gel returned to the original values, except that G'' is still a little larger possibly because of some thermal-related change in the collagen network. The rheological property of collagen gel containing HA (0.5 mg/ml), pFN (0.0422 mg/ml), DSPG-II (0.078 mg/ml) and elastin (0.1 mg/ml) was also measured by the same method. The result is similar to the gel with only elastin, and is shown in Figure 3.13. For the above two kinds of gels containing elastin (0.1 mg/ml), $\tan \delta$ decreases from the original value ~ 0.2 at 25°C to ~ 0.15 at 37°C.

The micrographs for collagen gels containing elastin stained at 37°C are shown in Figure 3.14. When collagen gels were stained at 37°C, no significant change in fibril density or morphology was found, except that the banding was a little less distinct than for those stained at room temperature. An example of DSPG-II gel stained at 37°C is shown in fig.3.14.b. It has been suggested that partial degradation of collagen causes alteration in staining [78]. The less distinct banding pattern in collagen gels stained at 37°C (overnight) is possibly the result of a small extent of degradation. The gel containing elastin stained at 37°C shows some electron-lucent spots near the fibrils, which are thought to be elastin coacervate (fig.3.14.a). For the gel with elastin as well as DSPG-II, HA and pFN stained at 37°C, the morphology had a

mottled look (fig.3.14.d and e) caused by DSPG and HA, with less electron lucent spots (fig.3.14.f) than pure elastin gel. We suspect that HA may also stick on elastin and cover those lucent spots. In contrast to HA and DSPG-II, no binding interaction appears to occur between elastin and the collagen fibrils.

From the previous experiments, we found that gel properties were determined by fibril morphology. In our collagen gel system, variation in ionic strength alters the fibril number and size, and influences viscoelasticity to a large degree. Addition of DSPG-II and HA enhances both storage and loss moduli, G' and G'' . The presence of elastin at 37°C increases G' . Elastin is believed to enhance G' by forming elastic coacervate particles (fig.3.14.a). DSPG-II, DS and HA increase the viscoelasticity of collagen gels by interacting with fibrils. The interfibrillar matrix is known to resist compressive forces. Also, it has been presumed that the interactions between the interfibrillar ground substances and the collagen fibrils, together with changes in the collagen fibril size distribution, as well as in the amount of elastin, contribute to changes in the viscoelastic properties of skin in disease and aging. For example, any age-related decrease in the elastin content [66,67] can reduce the elasticity of the skin, which was observed in aged rat skin [63]; age-related decreases in ground substance macromolecules such as proteoglycans [61,64] can not only reduce the ability of skin to inhibit creep, but also cause consequent loss of skin hydration. We note that our observations in the model

collagen gel system are in good correlation with the current available clinical observations [65-67]. The addition of HA and DSPG-II to collagen gels results in an enhanced elasticity due to adhesive binding interactions with collagen fibrils. Addition of elastin results in an enhanced elasticity when the temperature is raised to the coacervation point. This effect is due to the formation of elastic coacervate particles and is not due to any specific interaction with the collagen fibril network.

Thus we conclude, from our studies utilising a collagen gel system, that mechanical coupling between collagen fibrils and ground substance macromolecules can indeed contribute to the overall viscoelastic properties. Also our results support the hypothesis that changes with age in the intermolecular interactions, fibril forming ability (number and size), as well as the elastin content, are major reasons for changes in the viscoelastic properties of the skin during aging.

Figure 3.3. The rheological properties of collagen gel formed at different ionic strength.

(a) $I=0.11$, 4,440 $\times$

(b) $I=0.11$, 49,000 $\times$

Figure 3.4. Electron microscopic photographs for gels formed at different ionic strength. (a)-(f), gels after the rheological studies; (g)-(l), fresh gels. All samples were counterstained by PTA.

(c) $I=0.18$, 4,440 $\times$

(d) $I=0.18$, 49,000 $\times$

(e) $I=0.25$, 4,440 $\times$

(f) $I=0.25$, 49,000 $\times$

(g) $I=0.11$, $4,440\times$

(h) $I=0.11$, $49,000\times$

(i) $I=0.18$, 4,440 $\times$

(j) $I=0.18$, 49,000 $\times$

(k) $I=0.25$, 4,440 $\times$

(l) $I=0.25$, 49,000 $\times$

Figure 3.5. Dynamic viscoelasticity of collagen gels with 0.25 mg/ml of dermatan sulfate (DS) or 0.65 mg/ml of skin DSPG-II.

Figure 3.6. Dynamic viscoelasticity of collagen gels with different amount of hyaluronic acid (HA).

Figure 3.7. Dynamic viscoelasticity of collagen gels with human plasma fibronectin (pFN).

Table 3.1. The medium viscosity of gel matrices.

Gels	Medium viscosity (cp)	Intrinsic viscosity [η] of macromolecule (ml/g)
control collagen gel	1.07	-
gel with DS (0.25 mg/ml)	1.093	84
gel with DSPG-II (0.65 mg/ml)	1.306	339
gel with HA (0.1 mg/ml)	1.128	540
gel with HA (1.0 mg/ml)	1.648	540

+The medium viscosity of control collagen gel (η_0 , control medium) was measured by a Ubbelohde type viscometer (Cannon Instrument Co.). Medium viscosity of all the other gels were calculated based on the approximate relation between viscosity and intrinsic viscosity: $\eta \approx \eta_0 + [\eta]c\eta_0$. The intrinsic viscosity of each macromolecule was from the supplier.

Figure 3.8. Dynamic viscoelasticity of collagen gels with DS (0.25 mg/ml) or DSPG-II (0.65 mg/ml), normalized to their medium viscosities.

Figure 3.9. Dynamic viscoelasticity of collagen gels with 0.1 mg/ml or 1.0 mg/ml HA, normalized to their medium viscosities.

Figure 3.10. Dynamic viscoelasticity of collagen gels with 0.0211 mg/ml pFN or/and 0.13 mg/ml DSPG-II mg/ml.

(a) DSPG-II gel, 4,440 $\times$, PTA counterstained

(b) DSPG-II gel, 9,300 $\times$, PTA counterstained

Figure 3.11. Electron microscopic photographs for gels containing (a)-(h), DSPG-II (0.13 mg/ml); (i)-(j), HA (0.5 mg/ml); (k)-(l), pFN (0.0422 mg/ml). All were at $I=0.25$.

(c) DSPG-II gel, 15,700 $\times$, PTA counterstained

(d) DSPG-II gel, 49,000 $\times$, PTA counterstained

(e) DSPG-II gel, 49,000 $\times$, UA counterstained

(f) DSPG-II gel, 49,000 $\times$, UA counterstained

(g) DSPG-II gel, 49,000 $\times$, UA counterstained

(h) DSPG-II gel, 39,300 $\times$, uncounterstained

(i) HA gel, 4,440 $\times$, UA counterstained

(j) HA gel, 49,000 $\times$, UA counterstained

(k) pFN gel, 4,440 $\times$, UA counterstained

(l) pFN gel, 49,000 $\times$, UA counterstained

Figure 3.12. Dynamic viscoelasticity of the collagen gel with 0.1 mg/ml elastin. Three measurements (at 25°C or 37°C) were performed on the same gel.

Figure 3.13. Dynamic viscoelasticity of the collagen gel with 0.1 mg/ml elastin as well as DSPG-II (0.078 mg/ml), HA (0.5 mg/ml), and pFN (0.0422 mg/ml). The procedures for measurement were the same as those described in fig.3.12.

(a) elastin gel, 49,000 $\times$, PTA counterstained at 37°C

(b) DSPG-II gel, 49,000 $\times$, UA counterstained at 37°C

Figure 3.14. Electron microscopic photographs for (a), gel with 0.1 mg/ml elastin stained at 37°C; (b), gel with 0.13 mg/ml DSPG-II stained at 37°C; (c)-(f), gel with 0.1 mg/ml elastin as well as DSPG-II (0.078 mg/ml), HA (0.5 mg/ml), and pFN (0.0422 mg/ml), stained at 37°C.

8, 20.27.

(c) elastin+DSPG+HA+pFN gel, 4,440 $\times$, UA counterstained

(d) elastin+DSPG+HA+pFN gel, 49,000 $\times$, UA counterstained

(e) elastin+DSPG+HA+pFN gel, 49,000 $\times$, PTA counterstained

(f) elastin+DSPG+HA+pFN gel, 49,000 $\times$, UA counterstained

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Our principal emphasis has been to investigate using model systems the role of the sol-gel transition process and fluid viscoelasticity in the physiological function of biological materials, specifically upper airway secretions and the extracellular matrix of skin.

In the first study, the connection between the viscoelastic behavior of secretions and the airway reopening event was explored. Our conclusions were as follows: (1) In the tube model studies of the viscoelastic sols, three distinct regimes relating reopening pressure to meniscus velocity are observed. Regime II has a similar functional relationship to that observed independently by other workers for Newtonian fluids, but with the non-Newtonian viscosity used to compute the dimensionless velocity. (2) In regime II, rapid opening is mainly due to low viscosity and shear-thinning behavior. A high elasticity and large strain dependency influence the transition from regime II to regime III, and also facilitate opening. (3) The regime I anomaly can be reduced by using a larger tube and/or a larger wall tension, but not by improving the surface wettability, indicating that this phenomenon is a result of insufficient viscous adhesive force to hold the two walls in apposition. (4) Regime III occurs earlier when reducing H and R (i.e. increasing the shear rate), indicating that this regime is a manifestation of high frequency

elasticity due to an entanglement network. (5) Use of gel systems as lining fluids gives similar features to those observed for sol systems in regimes II and III. (6) From airway simulations, we find that the shear-thinning behavior and the sol-gel transition help the airway to reopen quickly. (7) Pharyngeal secretions have lower viscosity and strain-sensitive structures. Together with a wider pharyngeal space, they give a lower reopening pressure in healthy individuals. Thus, under circumstances in which airway fluids exhibit viscoelastic character, our tube model and airway simulations suggest that the following factors are responsible for rapid reopening: a) fluids with small steady shear viscosities, but sufficient to hold the airway walls in apposition; b) fluids with small surface tensions, high elasticities (i.e. low $\tan \delta$), and strong shear-thinning behavior; c) airways with larger radii coated with a thinner mucus layer; and d) mucus with a highly strain-sensitive structure.

In the second study, links between the mechanical properties of skin and the structure and interactions of the macromolecules in the connective tissue matrix were sought by comparison of viscoelastic and electron microscopic studies of model collagen gels. Our results indicate that: (1) Gel properties are determined by fibril morphology (i.e. size, number, and fibril-molecule interactions). (2) Fibril size and number in the gels are altered by ionic strength. (3) DSPG-II, DS and HA increase the viscoelasticity (G' and G'') of collagen gels by specific or

electrostatic binding interactions with the collagen fibrils. (4)
Elastin enhances G' only, by forming elastic coacervate particles in the interfibrillar spaces. These observations in the model collagen gel system are in good correlation with the current available clinical observations on changes in the compositions of macromolecules of the connective tissue of skin on aging. Specifically, progressive decreases in the ratio of DSPG, HA and elastin to collagen are observed in older individuals.

Most biological fluids are viscoelastic in nature, being either a sol or a gel. Our results illustrate the importance of viscoelasticity in physiological function. Moreover, our research indicates that the kinetics and mechanism of sol-gel transition processes in biological fluids can also play an essential role in regulating physiological function.

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