Linear viscoelastic behavior of subcutaneous adipose tissue

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Abstract. Subcutaneous adipose tissue contributes to the overall mechanical behavior of the skin. Until today, however, no thorough constitutive model is available for this layer of tissue. As a start to the development of such a model, the objective of this study was to measure and describe the linear viscoelastic behavior of subcutaneous adipose tissue. Although large strains occur in vivo, this work only focuses on the linear behavior to show the applicability of the described methods to adipose tissue. Shear experiments are performed on porcine samples on a rotational rheometer using parallel plate geometry. In the linear viscoelastic regime, up to 0.1% strain, the storage and loss modulus showed a frequency- and temperature-dependent behavior. The ratio between the two moduli, the phase angle, did not show any dependency on temperature and frequency. The shear modulus was found to be 7.5 kPa at 10 rad/s and 37°C. Time–temperature superposition was applicable through shifting the shear modulus horizontally. A power-law function model was introduced to describe both the frequency dependent behavior at constant temperature and the stress relaxation behavior. In addition, the effect of snap freezing as a preservation method was analyzed. Histological examination demonstrated possible tissue damage after freezing, but the mechanical properties did not change. Since results were reproducible, it is concluded that the methods we used are most probably suited to explore the non-linear behavior of subcutaneous adipose tissue.

Keywords: Mechanical properties, rheometry, hypodermis, time–temperature superposition, power–law model, snap freezing

1. Introduction

The mechanical behavior of subcutaneous adipose tissue, also called hypodermis, is a widely ignored topic in the biomechanics literature. A plethora of papers can be found on properties of skin and skeletal muscle, but only few papers have addressed the properties of the layer in between [6,9,11,16,19]. This seems strange, because adipose tissue plays an important role in the load transfer between different structures in the body during breathing, body movements or exercise, or when exposed to therapeutic...
stretching during physiotherapy and massage. It is already recognized that the subcutaneous fat experiences larger strains than the dermis during suction and that its stiffness is likely to be a few orders less than that of the dermis [4,8]. However, it is still not common practice to take the adjacent adipose layer into account when the combined mechanical behavior of layers like skin, fat and muscle is modeled. Currently it would be difficult to do so, because mechanical parameters of adipose tissue are hardly available, and even when available, they are not consistent. Thus, there is a need to develop a parametric and constitutive model of subcutaneous adipose tissue, which can be implemented in numerical models of the whole skin as well as in multilayer models including skin, fat and muscle. Numerical models including the subcutaneous fat layer are needed in a wide field of applications, e.g. studying skin device contact, needle insertion procedures and the removal of skin adhesives. Rheological experiments are accepted to be a good starting point to develop a constitutive model.

For a meaningful interpretation of the mechanical behavior of the adipose tissue, it is essential to know the tissue composition. The present paper is focused on subcutaneous adipose tissue, which is a type of connective tissue found between the dermis and the aponeurosis and fasciae of the muscles. Subcutaneous adipose tissue is found everywhere throughout the body. However, the fat pads on the palm of the hand and foot are considered to be different, since they contain a much higher ratio of unsaturated versus saturated fatty acids and are therefore morphologically different. Relatively small differences in tissue composition exist at the other body sites. The tissue of interest in the present study mainly consists of white adipose tissue, of which 90–99% is triglyceride, 5–30% water and 2–3% protein. The tissue is a loose association of lipid-filled cells called white adipocytes, held in a framework of collagen fibers. Lipids within the white adipocytes are organized in one droplet. The diameter of the white adipocytes ranges from 30 to 70 µm, depending on the site of deposition [1]. White adipocytes are spherical, thereby allowing for maximal volume within minimal space. Collections of white adipocytes comprise fat lobules, each of which is supplied by an arteriole and surrounded by connective tissue septae. Each adipocyte is in contact with at least one capillary. In healthy adults, only one third of the adipose tissue contains mature adipocytes [1]. The remaining two thirds consists of blood vessels, nerves, fibroblasts, and adipocyte precursor cells.

The subcutaneous adipose tissue of the lower trunk and the gluteal-thigh region is further divided into two distinct layers: the superficial and deep subcutaneous adipose tissue [21,22]. Both morphological and metabolic differences were found between those two layers [3,15,22], but it is not clear if these layers differ in terms of mechanical properties.

To our knowledge, only a few authors studied the mechanical properties of the subcutaneous adipose tissue. Because of an interest in the response of different types of breast tissue regarding the detection of breast cancers, subcutaneous adipose tissue has mostly been studied as part of breast tissue [7,13,18–20,25]. For these studies, mainly indirect and non-invasive measurement methods were used. However, the largest study was recently performed with indentation measurements on about 70 samples of breast fat tissue ex vivo resulting in a Young’s modulus of 3.21 kPa [19]. Linear viscoelastic behavior up to 50% strain during uniaxial tension for abdominal subcutaneous tissue of rats when applying incremental displacement steps of 1 mm followed by a 1 s relaxation period [11]. Patel et al. [16] measured the storage and loss moduli of subcutaneous fat tissue, also from the abdomen, for strains up to 20%. The results show frequency-dependent shear moduli decreasing with increasing strain. As we will see later on, all these measurements are far outside the linear viscoelastic strain range. Recently, the mechanical behavior of subcutaneous adipose tissue of the buttock was measured in relation to pressure ulcers by performing confined compression tests, but no mechanical parameters for modeling could be derived from the results [6,14].
All the above-mentioned studies only give limited descriptions of the mechanical behavior, either because the focus was only on the differences between breast tissue types, or on long term quasi-static behavior [11,14], or because the authors were only interested in a comparison of properties between human fat and a mimicking material [16].

Our ultimate goal is to develop a skin model that includes the mechanical properties of all skin layers separately and can be modeled in a numerical model. Since we expect that the mechanical behavior of adipose tissue contributes considerably to the overall skin behavior, there is a need to develop a thoroughly tested constitutive model describing the mechanical behavior for large strains. The formulation of such a model will be based on rheological experiments in vitro. The first step is to investigate the material bulk properties within the linear viscoelastic strain region. These types of experiments are relatively simple to perform and hence, it is appropriate to design experimental procedures as well as to identify experimental problems. The linear viscoelastic parameters obtained will form the basis for a non-linear viscoelastic model in future work. The concept will be developed for porcine subcutaneous adipose tissue because of the availability and minimal biological variability among specimens. The objective of the current study is to use dynamic mechanical thermal analysis (DMTA) in combination with time–temperature superposition (TTS) to determine the small oscillatory strain behavior of subcutaneous adipose tissue in vitro. DMTA is performed through oscillatory shear experiments up to 100 rad/s at various temperatures. Next we introduce a linear viscoelastic power-law memory function, commonly used for soft-solids, to describe the small strain linear viscoelastic behavior of this biological material.

2. Methods and materials

2.1. Sample preparation

Porcine subcutaneous fat tissue was obtained from a local slaughterhouse. In porcine species, the back fat is divided in an outer, middle and inner layer of subcutaneous tissue because the adipocyte features of these layers differ with respect to size, number and metabolic activity. The porcine middle layer, which is used in the present study, is comparable to the deep subcutaneous layer in the abdominal region of humans [12]. All pigs were Landrace, having a dressed carcass weight of approximately 83 kg and were 14–18 weeks old at necropsy.

Already in the slaughterhouse, the tissue was cut into transverse slices of 1.5–2 mm. Samples were stored at 4°C and used within 48 h. From the slices, circular tissue samples were obtained with an 8 mm diameter cork borer. Next the samples were stored ice-cooled in a PBS solution and tested within the subsequent 4 h. An overview of the number of specimens and the number of samples from each specimen per test is given in Table 1.

Methods of tissue preservation may change the mechanical properties of tissue due to changes in tissue quality [5]. Rapid freezing is thought to be a reliable method of preservation to store tissue. Freezing would be attractive because samples can then be preserved over much longer times. It has already been demonstrated that the fatty acid composition is not affected by freezing [19]. In order to find out whether snap freezing preserves mechanical properties, adipose tissue was also snap-frozen by immersion in 2-methylbutane cooled by liquid nitrogen and stored at −80°C until use for mechanical testing. Thawing of the samples was done slowly within an ice-cooled box. To verify that the storage did not damage the tissue structure, histological sections were examined by light microscopy. For that, the specimens were fixed in 10% phosphate-buffered formalin and processed for conventional paraffin embedding.
The specimens were cut into 5-µm thick sections and stained with hematoxylin and eosin (H&E). Since all lipids were extracted out of the adipocytes by using the conventional paraffin embedding technique, other specimens were embedded in O.C.T. compound (TISSUE-TEC) and frozen for lipid staining. These specimens were cut into 8-µm thick sections at −20°C, stained with oil Red O (Sigma) and counterstained with hematoxylin.

2.2. Rheological methods

To determine the linear viscoelastic properties, oscillatory shear experiments were performed using a Rheometrics rotational rheometer (Advanced Rheometric Expansion System (ARES), Rheometrics Scientific, USA) with a controlled strain mode, and parallel plate geometry in combination with a Peltier environmental control. Measurement methods are based on procedures used for the viscoelastic properties of other soft biological tissues, such as brain [10], muscle [26] and thrombus [24]. Sand-blasted plates were used to prevent slippage. An oscilloscope was used to ascertain that the shape of the torque signal was indeed sinusoidal. Samples were compressed between the plates by lowering the upper plate until an axial force of 0.1 g was reached.

In these experiments a sinusoidal strain $\gamma(t)$ was imposed on the sample, that when in steady state and in the range of linear viscoelastic behavior, resulted in a sinusoidal shear rate, $\gamma(t)$, and shear stress, $\tau(t)$ with a phase shift $\delta$:

$$\gamma(t) = \gamma_0 \sin(\omega t),$$

$$\tau(t) = G_d \gamma_0 \sin(\omega t + \delta).$$

The dynamic shear modulus $G_d(\omega, T)$ and the phase shift $\delta(\omega, T)$ are both a function of the angular frequency $\omega$ and temperature $T$. It is common to separate the dynamic shear modulus into a storage modulus, $G'$, representing the elastic behavior since this describes the stress in phase with the strain, and a loss modulus, $G''$, representing the viscous behavior, $\frac{1}{2} \pi$ out of phase with the strain, i.e., in phase with the strain rate:

$$G_d = \sqrt{G'^2 + G''^2}.$$
The phase shift $\delta$ expresses the ratio between viscous and elastic behavior and is related to Eq. (4):

$$\tan(\delta) = \frac{G''}{G'}.$$  

(4)

The linear viscoelastic regime is defined as the range of strain amplitudes where the material properties are independent of the applied strain.

The time–temperature superposition (TTS) principle is applicable when data can be shifted to and from a reference temperature $T_0$ to form a master curve. The advantage of applying this principle is that the frequency domain can be extended beyond the measurement limits as well as that data can be shifted to other working temperatures. One smooth master curve is obtained by shifting frequency sweep curves obtained at different temperatures horizontally and vertically on the curve obtained at the reference temperature, until all curves overlap. When no smooth master curve can be obtained, the TTS principle is invalid. Normally, the horizontal shift factor $a_T$ is applied to the phase angle $\delta$. Subsequently, the dynamic shear modulus $G_d$, and also $G'$ and $G''$, can be shifted along the horizontal and vertical axis to a reference temperature with the horizontal shift factor $a_T$ and a vertical shift factor $b_T$:

$$\tan(\delta(\omega, T)) = \tan(\delta(a_T\omega, T_0)), \quad (5)$$

$$G_d(\omega, T) = \frac{1}{b_T} G_d(a_T\omega, T_0). \quad (6)$$

2.3. Testing procedure

The linear viscoelastic regime was determined using oscillatory shear experiments with constant frequency and varying strain. Strain sweeps were performed from 0.04% to 10% at frequencies of 1, 10 and 100 rad/s. A constant strain within the determined linear regime of 0.1% was chosen for the subsequent frequency sweep tests.

The frequency sweep was repeated three times to avoid tissue conditioning phenomena, observed during preliminary testing. We did not carry out traditional preconditioning. Instead we performed always three frequency sweeps, increasing the frequency stepwise logarithmically from 1 to 100 rad/s and then performing the data analysis on the third frequency sweep. This protocol was also used to examine the influence of snap freezing and thawing on the mechanical properties of subcutaneous fat tissue. For this purpose, samples from 3 pigs were tested, both fresh and after freezing and thawing. All tests were performed at 20°C.

To investigate whether the TTS principle is applicable to subcutaneous adipose tissue, frequency/temperature sweeps were successively performed at temperatures of 5, 20, 35 and 40°C, at 0.1% strain and frequencies ranging from 1–100 rad/s. Again, two successive frequency sweeps from 1–100 rad/s were performed prior to these frequency/temperature sweep tests. The temperature range is bounded at the low end by the phase transition temperature of water and above by temperatures at which tissue degradation is likely to occur. To check the possible influence of the order of heating or cooling, 3 samples were also subjected to a frequency/temperature sweep with decreasing temperatures. The results were similar to those found for the experiments with heating.

As a control for the applied power–law model, a stress relaxation experiment additional to the frequency sweep tests was done for five samples. In these experiments a step strain of 0.1% was applied during 100 s.
2.4. Statistics

Data obtained on $G'$ and $G''$, were analyzed with the linear mixed model [27] by using the software Splus to determine whether cryopreservation has a significant effect. For this purpose, the log of the frequency sweep data was used. The test was performed with samples from three specimens. The linear mixed model was chosen because it accounts for biological variability among samples and among specimens while analyzing freezing effects.

3. Results

3.1. Small oscillatory strain behavior

Figure 1 shows the results for the strain sweep tests at 10 rad/s for both $G'$ and $G''$. Both moduli and phase shift, which is not shown here, were found to be nearly independent of strain for amplitudes up to 0.1%. Tests at other frequencies revealed similar results and are therefore also not shown.

Preliminary testing showed that tissue conditioning phenomena are avoided by performing two frequency sweeps before the real measurement (Fig. 2). Results for the storage and loss moduli, as functions of the applied frequency, are shown in Fig. 3. The biological variation appeared to be small. Taking all samples from fresh specimens together, the shear modulus $G_d$ is found to be $14.9 \pm 4.8$ kPa at 10 rad/s. The average phase angle is approximately $21.0^\circ$ over all frequencies, indicating more elastic than viscous behavior.

Results of stress relaxation are depicted in Fig. 4. The shear modulus decreases by about a decade over 100 s.

3.2. Model application

The shear stress response for linear viscoelastic behavior is usually described in terms of the Boltzmann integral:

$$\tau = \int_{-\infty}^{t} G(t - t')\dot{\gamma}(t') \, dt',$$

Fig. 1. Average $G'$ and $G''$ demonstrate a linear viscoelastic regime up to 0.1% strain.
where $G(t)$ is the relaxation function and $\dot{\gamma}$ is the shear rate. The results of the frequency sweeps indicate that a power-law relation can adequately describe the storage and loss moduli:

$$G'(\omega) = G'(1)\omega^p$$

with $G'(1)$ and $p$ as constants [17]. The same relation is used for $G''$.

The phase angle can be expressed in terms of the exponent $p$ [17,23]:

$$\tan \delta = \frac{G''}{G'} = \tan \frac{p\pi}{2}. \tag{9}$$
So the small oscillatory strain behavior is captured by an approximation with only two constants $(p, G(1))$. It is known [17] that the relaxation function $G(t)$ in Eq. (7) can be written as:

$$G(t) = G(1)t^{-p}. \quad (10)$$

The constants $G(1)$ is related to $G'(1)$ by:

$$G(1) = \frac{2G'(1)(p!)}{p\pi} \sin \frac{p\pi}{2}, \quad (11)$$

where $p!$ is the factorial function.

The expressions for $G'$ and $G''$ were fitted simultaneously, resulting in one value for $p$ per sample. Next, the exponent $p$ was used to calculate the phase angle corresponding to the frequency sweeps (Fig. 3) and the relaxation modulus for the stress relaxation experiments (Fig. 4). In all cases, the exponent $p$ was in the range from 0.18 to 0.25, the average $p$-value was 0.21.

### 3.3. Time–temperature superposition

Results of the frequency/temperature sweeps show that the phase angle is not dependent on temperature. However, the shear modulus $G_d$ can be shifted along the horizontal frequency axis to obtain a smooth master curve at a reference temperature of 20°C (Fig. 5), in such a way that $G_d(\omega, T) = G_d(a_T\omega, T_0)$. The curves of $G_d$ for different temperatures show well overlapping areas and the frequency domain could be extended to almost 3 decades (Fig. 5). The horizontal shift factors, as a function of the temperatures at which each dataset was acquired, can be captured quite well with an exponential function with a quadratic power:

$$a_T = e^{aT_0^2 + bT_0 + c} \quad (12)$$

with $a = -0.0046 \pm 0.0021$, $b = 2.54 \pm 1.25$ and $c = -351.39 \pm 183.39$ (Fig. 6). Now it can be calculated that $G_d$ at body temperature is approximately 7.5 kPa at 10 rad/s.
3.4. Freezing effects

In histological sections embedded in paraffin severe damage could be observed in 2 out of 12 samples. Either cells were less packed or cell membranes were broken (Fig. 7). However, less or no damage occurred when tissue was embedded in the O.C.T. compound. So it remains unclear, whether the damage was only due to the snap freezing method.

Statistical analysis on the moduli data obtained from the frequency sweeps showed no statistically significant differences for both intercepts of the regression lines (Fig. 8). The slopes of the regression lines of $G'$ were statistically significant. However, the biological variance among all samples is larger than the difference between fresh and snap frozen samples. This can be seen in Fig. 8, where the regression
Fig. 7. (a) Fresh adipose tissue, (b) adipose tissue after snap freezing without damage, (c) tissue damage after snap freezing.

Fig. 8. The biological variation on the slope of the normalized regression lines of \( G' \) is shown. The dotted lines represent the limits of two times the standard deviations on both sides of the belonging regression line.

line of the frozen samples lies within the biological variation of the fresh samples. So from a practical viewpoint, the observed difference of slopes for the two conditions is negligible for \( G' \) (Fig. 8). In the case of the \( G'' \) slopes, there was no statistical difference. Taken this all together means that snap freezing does not show any effects on the mechanical properties compared to fresh tissue.

4. Discussion

The shear moduli we obtained can be shifted to measurement conditions described in the literature when using the time–temperature superposition. From the literature it is known that the linear region for other soft-solids consisting of loosely bounded soft particles is below 1%, which is consistent with our observations. In fact, the linear region is considered to be only up to 0.1% strain. This small strain was the maximum strain that could be still considered as close to linear behavior while the signal-to-noise ratio has become sufficient. Too large strain amplitudes are outside the linear strain regime and reduce the “apparent” modulus, which might explain the difference with Patel’s data [16]. In comparison with Samani et al. [19], who applied a quasi-static loading with a frequency of 0.1 rad/s result-
In a Young’s modulus of 3.2 kPa, our shear modulus is somewhat higher, i.e. $G_d (\omega = 0.1 \text{ rad/s}, T = 20^\circ) = 5.6 \text{ kPa}$. In contrast to Samani et al., our results show an obvious temperature dependency. The reason for this difference is unknown. Furthermore, a specific startup behavior can be observed in our frequency sweeps. These reproducible long term variations in the beginning of a frequency sweep, a change in the slope of $G'$, are not yet understood. Snap freezing may cause tissue damage resulting in less packed cells or broken membranes, but it is more likely that the observed artifacts are caused by the chosen histological technique. Snap freezing did not appear to have an effect on the mechanical behavior. Although the slopes of the regression lines for $G'$ demonstrated significant differences, the observed difference is smaller than the biological variation. Except for the temperature, environmental conditions are difficult to control. Since the snap frozen samples were measured on other days than the fresh samples, the environmental conditions might have influenced the measurement outcomes per specimen. Of course, the unknown environmental conditions can have influenced all other measurements as well. Nevertheless, the experimental results look reproducible and useful for modeling.

In the present study porcine tissue from the slaughterhouse was used. As expected, biological variation appeared to be relatively small for the chosen specimens. The adipocytes of the fattened pigs had a diameter of 70 µm or greater whereas that of human adipocytes varies from 30 to 70 µm. The question rises whether other tissue composites contribute more to the mechanical behavior of the bulk tissue than the adipocytes. Besides blood vessels and the collagen fiber network no other significant composites are present in the adipose tissue. Tissue with visible blood vessels was excluded from testing. Therefore, it might be that the stiff collagen fiber network surrounding the fat lobules plays an important role in the overall mechanical behavior.

To our knowledge, it is the first time that this common rheological model has been applied to biological soft tissue. The power–law model fits the experimental data well. The $p$-values obtained are comparable to those of other soft materials in literature. It should be noticed that the fit on the slope of the stress relaxation behavior can be improved but this optimization is not really necessary to our opinion. More interesting is the fact that we have introduced a model that can be extended to a three-dimensional non-linear model capturing large deformations with the possibility to include the build up and breakdown behavior of initial structures. Nevertheless, experiments in the non-linear strain regime are necessary to prove whether or not this promising model can fit those expectations.

Also, time–temperature superposition is applicable to this type of biological tissue. Mechanical properties measured at any temperature can be shifted to body temperature by applying the time–temperature superposition. However, the applicable temperature range for experiments is physically bound by phase transitions at low temperatures and the solidifying of proteins above 41°C. The measurements already showed a much larger variation at the upper limit of the temperature range, i.e. at 40°C, than at any other temperature. This indicates that it is recommended to avoid this boundary of the temperature range.

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References


