Effect of pH on the viscosity and viscoelastic properties of bile

Nguyen Ngoc Minh*, Hiromichi Obara

Department of Mechanical System Engineering, Tokyo Metropolitan University 1-1 Minami-Osawa, Hachioji, Tokyo 192-0397, Japan

Received: 30 March 2020 / Accepted: 1 June 2020
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Abstract The rheological response of bile (which is produced in the liver) was studied in steady-shear and creep-recovery measurements as a function of pH to promote clinical prediction and prevention of stone formation in the gallbladder. We used bile samples extracted from the gallbladders of pigs. Steady-shear rheological measurements reflected the shear-thinning behavior of bile, and the viscosity of bile increased to high values at high pH values. Therefore, an increase in pH could reduce the emptying process of the gallbladder, which would enhance the risk of gallstone formation risk. Creep-recovery measurements revealed that bile elicited a rapid elastic response and showed strong recovery behavior at high pH. Moreover, the elastic behavior of bile could be predicted by the Burger model.

Keywords creep, recovery, mucus, gallstones, sol-gel transition

1. Introduction

Bile is a greenish-yellow secretion produced in the liver and used to digest fats. Bile comprises three major components: cholesterol, bile salts, and bilirubin. If the gallbladder is not functioning correctly, the components of bile are supersaturated, which leads to the formation of solid crystals (“gallstones”) [1]. The biochemical changes of bile during gallstone formation have been studied. The gallbladder can protect against calcium precipitation and gallstone formation; bile in the gallbladder secretes hydrogen ions because acidifying gallbladder bile increases the solubility of calcium salts [2].

Cholelithiasis is a relatively common disease. Cholelithiasis may occur due to specific regulation of biliary pH and, therefore, of bile saturation with calcium carbonate [3]. Bile in the gallbladder of women is significantly less acidic than bile in the male gallbladder, but both show similar acidification rates. These observations suggest that the amount of mucin gel attached to the mucosa of the gallbladder may be higher in women than that in men, so the diffusion of hydrogen ions through the mucus into the bile will decrease [4]. Moreover, the very high acidic pH can induce polymerization of calcium bilirubinate to form an insoluble polymer, which can reflect the process of pigment-stone formation. Also, the acidification of bile reducing the ability of calcium carbonate to precipitate can be explained by secretion of hydrogen ions, and could prevent gallstone formation [5, 6].

If the pH of gallbladder bile changes, the concentration of calcium ions also changes, so the rheological properties of bile (e.g., viscosity, viscoelasticity) may also change. This action may result in incomplete emptying of bile, which could lead to bile stasis and subsequent gallstone formation [7–10]. Rheological measurements of gallbladder bile also show the importance of pH upon sol–gel transition, which may contribute to the physiological mechanism of gallstone formation. However, detailed studies on the rheological properties of bile to ascertain the importance of pH have not been carried out. Studying the dependence of the frequency of bile release is important because its rheological properties change with the frequency of the applied force.

We investigated the effect of pH on the viscosity and viscoelastic properties of gallbladder bile. We measured shear viscosity and creep, thereby confirming that another factor related to gallstone formation is pH.

2. Materials and methods

2.1 Materials

Gallbladders were extracted from healthy white pigs sacrificed in a conventional slaughterhouse (Tokyo Shibaura Zouki, Tokyo, Japan). Gallbladders were placed in a controlled-temperature chamber. Then, 50–70 mL of bile sample was extracted by cutting the bottom of gallbladder with a scalpel.
as described in our previous study [11]. Each bile sample was stored in a plastic bottle that was insulated to prevent cooling. This bottle was placed almost immediately in a constant-temperature box at 37°C.

2.2 Experimental setup

The pH of extracted bile samples was measured using a compact pH meter (LAQUAtwin pH-33, Horiba Scientific, Kyoto, Japan). This pH meter was calibrated via a two-point method using standard buffer solutions at a pH of 4.01 and 6.86, respectively, at 25°C. A third measurement was made on each bile sample. In general, the pH was measured within 30–60 min of bile samples being extracted from pigs at the slaughterhouse.

A rotational rheometer with cone-plate geometry (HAAKE RS600; Thermo Fisher Scientific, Waltham, MA, USA) was used to study the response of extracted bile samples in terms of steady shear, oscillatory shear, and step stress. The rheometer had a diameter of 60 mm and a cone angle of 1°. Experiments to measure shear oscillation were carried out in the frequency range 1–100 s⁻¹ at 37 ± 0.1°C with 0.01% strain using the same rheometer, and the two surfaces were separated by a 52-μm gap during measurements. And shear viscosity measurements were performed in triple in steady-shear mode in the range 0.01–1000 s⁻¹ at 37 ± 0.1°C.

Creep measurements with an applied shear stress \( \sigma_0 = 10 \text{ mPa} \) were conducted to measure the viscoelastic characteristics of extracted bile samples. The duration of the creep and creep-recovery experiments was 60 s to ensure steady-state conditions. All measurements were carried out in triplicate at 37 ± 0.1°C.

The creep compliance function is defined as \( J(t) = \gamma(t)/\sigma \), where the strain \( \gamma(t) \) varies with time (Fig. 1a) [12]. The instantaneous elastic shear compliance \( J_g \), the delayed elastic compliance \( J_d \), equilibrium shear compliance \( J_e(0) \) and the viscous compliance \( J_v \) were defined. The Burger model consists of the Maxwell model and the Kelvin–Voigt model. The Burger model is very simple, and acceptable results can be obtained to analyze system deformation [13] (Fig. 1b). It was used to investigate creep to characterize the deformation of extracted bile samples at different pH. The relationship between time \( t \) and compliance \( J(t) \) is expressed by the following equation [14–18]:

\[
J(t) = \frac{1}{G_1} + \frac{1}{G_2} \left[ 1 - e^{-t/\eta_2} \right] + \frac{J_v}{\eta_1} \quad \text{when } t < t_c
\]

where \( G_1 \) is the instantaneous elastic modulus of the Maxwell unit and \( \eta_1 \) is the dashpot of the Maxwell unit representing the residual viscosity. \( G_2 \) is the retarded elastic modulus of a Kelvin–Voigt material and \( \eta_2 \) is the dashpot of a Kelvin–Voigt material called the internal viscosity [16] (Fig. 1b). The instantaneous elastic response at \( t = 0 \) and recovery at \( t = t_c \) are governed by the isolated spring \( G_1 \).

The unrecoverable strain, measured as \( t \to \infty \), is governed by the isolated dashpot \( \eta_1 \). By calculating the values \( G_1, G_2, \eta_1, \) and \( \eta_2 \) it is possible to compare the internal structure of different systems. In this way, a mechanical model with behavior in response to deformation can be produced [13].

The compliance at the recovery phase could be described by the following equation:

\[
J(t) = J_v + J_d e^{-Bt/C}
\]

Fig. 1 Typical creep compliance–time curve (a) and Burger model (b)
where the characteristic parameters $B$ and $C$ define the recovery of this system. Equation (2) complies with certain clearly defined limiting conditions. When $t = 0$, which corresponds to the maximum deformation of the dashpots in the Burger model, $J(t)$ is equal to $(J_v + J_d)$. When $t \to \infty$, which corresponds to the irreversible sliding of the Maxwell dashpot, $J(t) = J_\infty$ [13]. The final percentage recovery $J_R$ of the entire system of extracted bile samples was defined by the following equation:

$$J_R(\%) = \frac{J_{\text{MAX}} - J_v}{J_{\text{MAX}}} \times 100$$  

(3)

3. Results and discussion

3.1 Mean pH of bile samples

Thirty extracted bile samples were classified into three groups of gallbladders (Table 1, Fig. 2). In subsequent calculations, bile from gallbladders was grouped together. Table 1 gives the physical properties of the extracted bile for three groups of pH. We also measured and estimated the density of gallbladder bile for each group. Results showed that the density was proportional to pH (Fig. 2).

pH shows the active nature of hydrogen ions on a scale. A high pH means that the hydrogen in a molecule can be removed and ionized in solution readily. Hence, it can react readily with negatively charged ions or bases. Also, the pH alters the components secreted in the gallbladder, and the density changes.

However, acidification of gallbladder bile may prevent nucleation of calcium carbonate by decreasing the carbonate concentration. Hence, a decreasing acidification of gallbladder bile would lead to an increase in the concentration of carbonate in the gallbladder and increase the tendency for nucleation of calcium carbonate. The pH, bicarbonate concentration, and carbonate concentration in bile are related to the Henderson–Hasselbalch equation. Hence, addition of hydrogen ions or elimination of bicarbonate ions would reduce the pH of gallbladder bile and lead to a reduction in the concentration of carbonate. This action would lead to a decline in the ion production of calcium carbonate and reduce the tendency for calcium carbonate to precipitate. Therefore, gallstone formation would be reduced considerably [19]. It was clear that the density was proportional to the pH and that we could predict the density value from the pH; this is an easy method during clinical assessment.

3.2 Influence of pH on the shear viscosity of bile

Figure 3 shows the experimental bile viscosity data together with our curve fitting. The apparent bile viscosity of each pH group measured in steady shear flow was plotted as a function of shear rate (Fig. 3). All extracted bile samples indicated strong shear thinning in the steady shear flow measurements, as reported by our team and other researchers [9, 11]. The apparent viscosity increased with increasing pH at all shear rates. The reasons for the change in viscosity were attributed to altering electrostatic interactions and calcium cations ($Ca^{2+}$). The first, pH influences on electrostatic interactions (ionic interactions) [20]. The increasing pH can disrupt electrostatic interactions. And an increasing ionic strength can reduce electrostatic interactions, also reduce viscosity [20]. The second, an increasing concentration of calcium ions may increase bile viscosity. The effect of the concentration of calcium ions was specific, and calcium ions interacted with specific $Ca^{2+}$-binding sites. Therefore, $Ca^{2+}$ may cross-link and stabilize mucins that increase the viscosity of bile. Moreover, the bicarbonate ions reduce the concentration of free calcium ions and decreases the amount of calcium ions that remains associated with bile mucins [21]. The high concentration of bicarbonate ions (or small pH) enhances the swelling and hydration of mucin by reducing crosslinking of calcium ions in mucin, thereby decreasing its viscosity and probably increasing its transportability [21]. It became clear that the viscosity of bile increased at high pH. Hence, the resistance of bile would increase and, as a result, the risk of gallstone formation would increase.

The rheological model parameters of the apparent viscosity were obtained by determining the best fit of the experimental flow curves. The fluid steady-shear viscosity is generally described well by the Carreau-Yasuda model:
where \( \eta_0 \) is the zero shear rate viscosity, \( \eta_\infty \) is the infinite shear rate viscosity, \( \lambda \) is the relaxation time, \( a \) is the index that controls the transition from the Newtonian plateau to the power-law region, and \( n \) is the power-law index.

The Carreau-Yasuda model can describe the shear thinning behavior of bile. When the shear stress is very small and approaches zero, the bile viscosity will reach a constant value (zero shear viscosity). However, we can see that this model can represent the shear-thinning behavior of the bile and it is only practical when the shear rate (or shear stress) is large enough (in our experiment, the shear rate was greater than 0.01 s\(^{-1}\)), and bile shows non-Newtonian behavior (Fig. 3).

The main advantage of the Carreau-Yasuda model is that it is continuous for all \( \dot{\gamma} \geq 0 \). For bile, \( \lim_{\dot{\gamma} \to 0} \eta(\dot{\gamma}) = \eta_0 \) and \( \lim_{\dot{\gamma} \to \infty} \eta(\dot{\gamma}) = \eta_\infty \); this indicates that the fluid acts as a Newtonian fluid with viscosity \( \eta_\infty \) at high shear rates, and behaves as a Newtonian fluid with viscosity \( \eta_0 \) at low shear rates. The parameters \( a, n, \) and \( \lambda \) control how the fluid behaves in the non-Newtonian regime between these two asymptotic viscosities (\( \eta_0 = 1.69 \) Pa.s, \( \eta_\infty = 0.0056 \) Pa.s, \( \lambda = 2310 \) s, \( n = 0.254 \) and \( a = 2 \)). Therefore, we can predict the bile viscosity according to shear rate by using Carreau-Yasuda model.

We investigated the influence of pH on the shear viscosity at the shear rate of 100 s\(^{-1}\). The relationship between the shear viscosity and pH was calculated by linear regression analysis. To compare mean values among the three groups, analysis of variance followed by the least significant difference was used. Here \( p \) is a probability value calculated with statistical analysis. The \( p < 0.05 \) was considered as statistically significant differences. Figure 4 shows the relationship between the viscosity and pH of extracted bile samples. When the pH increased, the viscosity of bile increased. Shear viscosity could be evaluated by pH parameters using a linear relationship. However, it was necessary to carry out rheological experiments at very low shear rates in steady state flow to characterize the gel-like structure of bile. This experiment would be impractical for aqueous and biological dispersions due to the effects of degradation at long experimental times. Thus, to overcome this effect, low frequencies in shear oscillatory experiments and creep-recovery tests were undertaken on the samples (sections 3.3 and 3.4).

### 3.3 pH-dependent sol–gel transition

Frequency sweeps of extracted bile samples with different pH groups are shown in Fig. 5. These results presented clear evidence of a change in viscoelastic behavior in low-pH groups. The storage modulus, \( G' \), of all pH groups was higher than the loss modulus, \( G'' \), at frequencies >1.0 rad s\(^{-1}\), indicating a gel-like material response. The gel strength and gelation could be confirmed with the phase angle, \( \delta \), given by the relationship \( \tan \delta = G''/G' \) in Fig. 5b. The smaller the value of \( \delta \), the more elastic is the material. In general, \( \delta \leq 45^\circ \) indicates a sample that has gelled, whereas \( \delta \geq 45^\circ \) corresponds to a sample in the sol state [22]. Based on these criteria, all extracted bile samples were also in a viscoelastic state. However, solid like behavior of extracted bile samples at pH 7.0 were stronger than extracted bile samples at pH 6.9 or 6.8.

When pH increased, \( G' \) and \( G'' \) increased. pH could influence the entanglement networks or weak gelation of bile. Ionic strength can contribute significant changes in \( G' \) and \( G'' \). Bile contains mucus, and mucin is the main component of mucus. Therefore, the rheological properties of bile were dependent upon the molecular configuration of mucin, which is related closely to pH and ionic strength. The concentration of ions and pH regulate spontaneous swelling of mucins and, consequently, of mucus, in a manner...
according to Donnan equilibrium properties [23]. Also, the rheological parameters that are related to mucus are not only viscosity and elasticity but also the ratio between viscosity and elasticity (tan δ) and dynamic complex moduli [24]. An increased ionic strength, which reduces electrostatic interactions, also reduces viscosity [20]. The transportability of mucus is inversely related to the elastic modulus of a sample [25]. When the range of tan δ increases, the optimum rheological features depend not only on the overall moduli of mucus but also on the relationship between viscosity and elasticity (tan δ) [24]. A sample that is too rigid (i.e., one with a large elastic modulus) can impede appropriate penetration of hydrogen ions. From this aspect, it became clear that a higher elastic modulus would reduce the diffusion of hydrogen ions through the mucus into bile, and, therefore, precipitation would increase so that the risk of gallstone formation would increase.

### 3.4 Creep and creep recovery of bile as a function of pH

#### 3.4.1 Creep

Creep measurements of extracted bile samples of each pH group are shown in Fig. 6a, where the compliance $J(t)$ of the extracted bile samples is shown as a function of time $t$. Creep and creep-recovery properties were qualitatively similar in the three pH groups, but the maximum values of compliance $J(t)$ indicated differences. In all measurements, the extracted bile samples showed recovery to a certain extent and approached equilibrium compliance values after 60 s. These profiles indicated that the viscous component of the viscoelastic material was dominant at the moment when the stress was removed and the energy dissipated.
irreversibly. These results showed non-zero instantaneous compliance, $J_g$, which is evidence for viscoelastic solid-like behavior (Fig. 6b).

We were able to hypothesize how the microstructure of the extracted bile samples changed. Initially, the polymeric network underwent an elastic deformation within the mechanical limits of the network. Continuous deformations led to dismantling of the network, and the material started to flow [26]. Creep compliance and creep-recovery declined as the pH increased from 6.8 to 7.0, and all samples showed the characteristics of viscoelasticity. The creep compliance $J(t)$ of a viscoelastic material characterizes the softness of the material [27, 28]. A high $J(t)$ value represents a weaker structure, and a low $J(t)$ value denotes a stronger material structure [29, 30]. $J(t)$ values decreased with increasing pH, suggesting the formation and reinforcement of bile mucin hydrogel induced by pH (Fig. 6). The Burger model (Eq (1) and Fig. 1b) was suitable for fitting to the creep data ($R^2 > 0.99$). Hence, the Burger model could describe the viscoelastic characteristic and reflect the internal structure of bile samples.

$G_1$ represents the behavior of strong bonds, such as covalent, ionic and metallic bonds, that are destroyed irreversibly. $G_2$ represents the behavior of weak bonds (e.g., hydrogen bonding) that are destroyed reversibly if stress is applied for a period of time and then removed [31, 32].

Fitting results showed that if the pH increased, then $G_1$ and $G_2$ increased gradually (Table 2). These data suggested that with increasing pH, the mucin structure in bile was enhanced, and the bolstering of intermolecular bonding may have contributed to this effect. When pH increased, the internal hydrogen bonds would become unstable, resulting in unfolding of the macromolecules in bile together with exposure to more reactive bonding sites at the surface and an increase in translational energy [33]. These factors could contribute to enhancement of intermolecular bonding, which would generate strong three-dimensional microstructures with high rigidity and cohesive forces. In particular, the parameter $\eta_2/G_2$ denotes the response of a viscoelastic material to instantaneous application of constant stress [18]. A small $\eta_2/G_2$ shows a fast retarded elastic response. $\eta_2/G_2$ decreased when pH increased from 6.8 to 7.0 (Fig. 7a). Hence, bile obtained a faster elastic response at a high pH than at a low pH. This action could enhance the molecular interaction so as to generate gelation at high pH. This action would reduce the diffusion of hydrogen ions through mucus into bile, which would decrease the solubility of calcium salts, and, as a result, the risk of gallstone formation may increase.

3.4.2 Recovery

Figure 6(a) reveals the influence of pH on the recovery behavior of the extracted bile samples. When the pH increased from 6.8 to 7.0, the overall compliance showed a decrease in the recovery phase of bile. After 60 s of stress application, the bile samples reached the maximum deformation ($J_{MAX}$). When the stress was eliminated, recovery of

| pH  | $G_1$ (Pa) | $\eta_1$ (Pa.s) | $G_2$ (Pa) | $\eta_2$ (Pa.s) | $\eta_2/G_2$ (s) | $R^2$ |
|-----|-----------|----------------|-----------|----------------|-----------------|------|
| 6.8 | 2012      | $1.4 \times 10^{-2}$ | $1.5 \times 10^{-4}$ | $1.9 \times 10^{-2}$ | 126             | 0.998 |
| 6.9 | 2535      | $1.7 \times 10^{-2}$ | $2.0 \times 10^{-4}$ | $2.2 \times 10^{-2}$ | 110             | 0.999 |
| 7.0 | 2562      | $1.8 \times 10^{-2}$ | $5.3 \times 10^{-4}$ | $4.3 \times 10^{-2}$ | 80              | 0.998 |
the sample was observed for an additional 60 s with the compliance value as a function of time.

The experimental values for compliance of extracted bile samples at the recovery phase (in the interval between 60 s and 120 s) were fitted to Eq. (2). The latter was well fitted to the recovery data of extracted bile samples, as indicated by $R^2 > 0.90$. The calculated parameters B and C of recovery stage were altered in a small range of $(3.72–3.85) \times 10^{-8}$ and $(3.795–3.914)$ at all pH values tested, indicating that the intensity of the calculated parameters B and C was independent of pH. In particular, even though $J_g$ remained constant, $J_{MAX}$, $J_v$, and $J_d$ of extracted bile samples decreased with increasing pH, suggesting that an increasing pH reinforced the microstructure of bile. It indicated that gelation of bile maybe happen and that increasing the pH could promote the long chains of mucin to stretch, aggregate, and subsequently hydrophobic interactions [34, 35].

Recovery after creep is an appropriate characteristic of elastic behavior. Figure 6 shows that for bile samples, a positive value for recovery was detected at the pH values tested, which is a clear indicator of the elastic characteristic of the samples. Importantly, in Fig. 7b, the level of recovery for a high pH was higher than that for a low pH, and bile obtained a faster elastic response at high pH. The observation could have been because the mucin concentration in a high-pH sample was high.

4. Conclusions

Rheological properties of bile as a function of pH were investigated. The density and viscosity of bile were proportional to pH. When the pH increased, the elasticity of bile also increased, and bile showed solid-like behavior. Moreover, bile elicited a faster elastic response and stronger recovery behavior at a high pH than at a low pH. The Burger model could describe the creep and recovery behavior of bile.

Acknowledgments

This research was supported by Tokyo Human Resources Fund for City Diplomacy.

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