Evaluation of Gelatin Hardening with Soluble Fraction Determination

Yasuo Tsubai* and Takeshi Minato*

Abstract We selected five typical hardeners for photographic use and investigated their hardening properties as a function of drying temperature. For each gelatin film hardened with them, the amount and the molecular weight distribution of the soluble fraction, the swelling ratio and the alkali-melting characteristics were evaluated. The films hardened with the rapid crosslinkers showed larger amount of soluble fraction, higher swelling and shorter time for alkali melting than those hardened with the slow crosslinkers.

We also discussed the relation between crosslinking reactivity and the content of helices formed during drying. It was observed that higher the retardation of helix formation, faster the crosslinking reaction. The number of crosslinks was calculated by Charlesby’s equation from the soluble fraction content of the films. From the difference of this number between cold- and hot-dried films, we also estimated the contribution of the helix formation to the number of crosslinks. For cold-dried films, we estimated about 5 helices formed per weight average molecule.

Key words: gelatin, hardening, drying condition, soluble fraction, molecular weight distribution, number of crosslinks, peptide coupler, glutaraldehyde, triazine, vinyl sulfone

1. Introduction

Swelling, melting, and resistance to mechanical abrasion are commonly measured to evaluate hardened gelatin film. Although these values are useful to comprehend the character of the layer for practical use, they are not appropriate to describe crosslinks formed with hardeners at molecular level.

On the other hand, soluble fraction, the amount of a layer that is still soluble after crosslinking, is an analytically defined value and its physical and chemical significance has been studied theoretically. Hence, it helps us to understand the character of crosslinks at molecular level.

There are several papers concerning the soluble fraction of hardened gelatin films. Most of the previous studies, however, employed thick films (>20 μm) and longer extraction time (>1 h). In order to understand the properties of real photographic layers, thinner films and extraction within second order are preferable.

The main object of this study is to understand the relation between the reactivity of the hardeners and characteristics of the films. Another object is to obtain the information for the structure of the film containing helices.

Various hardeners are known to be used for photographic layers. In this study, we selected five typical hardeners featuring variety of crosslinking reactivity.

We have already presented the effectiveness to determine the soluble fraction content of hardened films using an optical rotation measurement. In addition to the ease in the operation, this method is applicable to various extraction solutions, not only water but also photographic chemicals such as developer and fixer.

The soluble fraction of the hardened films was determined by optical rotation measurement and its molecular weight distribution was analyzed by gel permeation chromatography (GPC). Melting time and swelling of the films were also measured.

Drying temperature gives significant effects on the characteristics of gelatin films. There are many reports concerning the relation between the drying temperature and the film properties,
Table 1  Hardeners used in this study and their crosslinking reactivity.

<table>
<thead>
<tr>
<th>Hardener</th>
<th>Crosslinking reactivity (Gelling time)</th>
</tr>
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<tbody>
<tr>
<td>WS-CDI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride</td>
<td>50 s</td>
</tr>
<tr>
<td>MCP, 1-(morpholino-carbonyl]pyridinium-4-yl-2-ethansulfonic acid betain</td>
<td>2 min</td>
</tr>
<tr>
<td>GA, glutaraldehyde</td>
<td>6 min</td>
</tr>
<tr>
<td>DCT, 2,4-dichloro-6-hydroxy-1,3,5-triazine sodium salt</td>
<td>not gelled within 3 h</td>
</tr>
<tr>
<td>DVS, 1,3-bis(vinylsulfonyl]-2-propanol</td>
<td>not gelled within 3 h</td>
</tr>
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* Crosslinking reactivity of the hardeners was evaluated from the gelling time for a 5% gelatin solution after addition of 25mmol/100g-gelatin of hardener at 36°C and pH6.0 (for WS-CDI pH4.7).

together with the study of the sol-gel transition phenomena of gelatin solution [10]. In this study, we strictly kept the drying temperature constant during all stages of drying. We altered this temperature at six levels ranging from so-called “cold-dry” to “hot-dry” temperature.

We also estimated the number of chemical crosslinks and the number of helices per weight average molecule to discuss the characteristics of the hardeners.

2. Materials and Experimental Procedure

2.1 Gelatin

Throughout this study, we used a lime-processed de-ionized bone gelatin (Nitta P-3201).

2.2 Hardeners

We selected five hardeners known for practical photographic use as listed in Table 1. WS-CDI and MCP are peptide coupling reagents and regarded as rapid crosslinkers like GA that is well known as a quick dialdehyde hardener. The crosslinking reaction of these three hardeners occurs in a sol state in the course of drying. On the other hand, DCT and DVS are regarded as slow crosslinkers. Their crosslinking reaction chiefly occurs after the film is dried. Crosslinking reactivity is also shown in Table 1.

2.3 Gelatin coating

We coated a 5% gelatin solution containing an anionic surfactant and a corresponding hardener on a non-gelatinous sub-coated PET base with 3g-gelatin/m². The pH of coating solutions were adjusted at 6.0 except for WS-CDI. Since WS-CDI reacts favorably at pH 4.7, this pH was applied for it. In all cases, the hardener was added 20s earlier before the coating.

2.4 Drying procedure

Fig. 1 shows the drying apparatus schematically. The coated films were placed on a thermostatic aluminum block with the aid of water layer for ensuring good thermal contact, and then dried under an air flow with controlled temperature and humidity. The surface temperature of the films was monitored and strictly kept constant through all the stages of drying. The temperature was varied at six levels, 12.5, 15.0, 17.5, 20.0, 25.0 and 30.0°C, ranging from so-called “cold-dry” to “hot-dry” temperature. For the temperatures of 17.5°C and below, we used de-humidified air with the dew point of -4°C. In all cases, air flow was controlled to keep the time needed for complete drying constant at approximately 90s. After being dried, each film was cured at 40°C for 10d. In this report, we call drying at 12.5°C and 15.0°C “cold-dry” and at 25.0°C and 30.0°C “hot-dry”, respectively.

2.5 Soluble fraction of hardened gelatin films

2.5.1 Extraction

Samples were cut into a 5.5cm×9.1cm (50cm²) piece and placed in a shallow tray in a thermostatic bath, and then 10ml of an extraction solution (0.1M-phosphate buffer, adjusted to pH6.0 by NaOH) was poured into the tray. We carried out the extraction for 1,000s at 50°C by shaking the tray gently (twice a
second). We also observed the soluble fraction content as a function of extraction time ranging from 10 s to 10,000 s. There are two reasons for that the pH of extraction is adjusted at 6.0. First one is to minimize the hydrolysis of gelatin during extraction [14]. Second one is to coincide the pH with the eluent of gel permeation chromatography (described below).

Also evaluated was the influence of the extraction temperature and pH to appreciate the extraction behavior fundamentally.

2.5.2 Determination of the soluble fraction

The concentration of gelatin was determined by optical rotation measurement using a polarimeter (JASCO DIP-1000, Na-lamp) with a 10 cm-cell at 30°C. The optical rotation, \( \alpha \), showed good linear relationship to the gelatin concentration ranging from 10 ppm to a few % (Fig. 2). We could then deduce the following equation (1).

\[
C\% = \frac{-\alpha}{1.40}
\]

Since a sample piece (50 cm\(^2\)) contains 15 mg of gelatin, gelatin concentration of the extraction solution is 0.15% when the coated gelatin dissolves completely, corresponding to the optical rotation of \(-0.21\) deg. Therefore, soluble fraction content (%) can be calculated from the observed \( \alpha \) value by use of equation (2).

\[
\text{Soluble fraction content} (\%) = \left(1 - \frac{-\alpha}{0.21}\right) \times 100
\]

It is worthwhile to point out that the optical rotation of dilute gelatin solutions did not substantially depend on pH of the solution ranging from 2 to 12 in phosphate buffers.

There still remains a question whether the original gelatin and its soluble fraction have the same specific rotation. Gross et al. [15] reported 15% lower specific rotatory power at 30°C for the extracted gelatin from non-hardened gelatin gel. We confirmed about 10% lower values for enzymatically degraded gelatin having the molecular weight of \(2 \times 10^4\). Although the soluble fraction thus may have a slightly lower specific rotatory power, we ignored this difference for calculations in this study, because their effects are relatively smaller than that by the reproducibility of the extraction experiment itself.

2.6 Molecular weight distribution

The molecular weight distribution of the original gelatin and the soluble fraction were measured by gel permeation chromatography (GPC) under the following conditions. The extracted gelatin solution is injected to the column with no further treatment.

Column: Asahipak GS-620×2; Eluent: 0.1 M phosphate buffer (pH=6.5); Column temperature: 50°C; Injection volume: 100 µl; Flow rate: 1.0 ml/min; Wavelength of detection: 230 nm (followed by PAGI Method [16])

2.7 Melting time of hardened gelatin films

Melting time measurement is one of the appropriate ways to evaluate hardening of the gelatin films. Especially, alkali-melting method has been widely employed. The sample strip was preliminary immersed in de-ionized water for 10 s at 25°C then soaked in 2M-NaOH at 80°C and the time for melting was measured. For this experiment, we prepared gelatin films containing a carbon black dispersion for easy detection of melting.

2.8 Swelling

A sample sheet was soaked in a 0.1 M-phosphate buffer solution (pH=6.0) at 20°C for 30 s and weighed. The swelling ratio was calculated from the weight gain of the film.
Table 2: Characteristic data of hardened films.

<table>
<thead>
<tr>
<th>Drying Temp. (°C)</th>
<th>Hardener mmol per 100g-gelatin</th>
<th>WS-CIDI</th>
<th>MCP</th>
<th>GA</th>
<th>DCT</th>
<th>DVS</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>12.5</td>
<td>Melting time (s)</td>
<td>Swelling ratio (%)</td>
<td>S.F *</td>
<td>Melting time (s)</td>
<td>Swelling ratio (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>12.5</td>
<td>5</td>
<td>400</td>
<td>20.5</td>
<td>16</td>
<td>265</td>
</tr>
<tr>
<td>15.0</td>
<td>5</td>
<td>400</td>
<td>21.1</td>
<td>16</td>
<td>245</td>
<td>6.6</td>
</tr>
<tr>
<td>17.5</td>
<td>5</td>
<td>400</td>
<td>24.2</td>
<td>14</td>
<td>253</td>
<td>8.5</td>
</tr>
<tr>
<td>20.0</td>
<td>5</td>
<td>353</td>
<td>20.8</td>
<td>13</td>
<td>227</td>
<td>8.5</td>
</tr>
<tr>
<td>25.0</td>
<td>5</td>
<td>313</td>
<td>22.2</td>
<td>12</td>
<td>200</td>
<td>10.9</td>
</tr>
<tr>
<td>30.0</td>
<td>5</td>
<td>320</td>
<td>24.0</td>
<td>11</td>
<td>216</td>
<td>12.8</td>
</tr>
</tbody>
</table>

* Soluble fraction content (%) determined from extraction for 1,000s at 50°C and pH6.0.

3. Results

3.1 Influence of temperature and extraction pH on soluble fraction

In preliminary experiments, we observed the influence of temperature and pH of extraction on soluble fraction. Fig. 3a shows the soluble fractions of the film hardened with GA at pH6.0 for 1,000s extraction as a function of extraction temperature. The numbers in the Figure give the amount of GA added (mmol/100g-gelatin). Below 40°C, the soluble fraction content notably depends on the extraction temperature especially in the case of the films with no hardener and with low amounts of the hardener. These simply correspond to the melting characteristics of the films.

Fig. 3b shows the effect of extraction pH on the observed soluble fraction content. SF% increased in a lower pH region (pH<6) for weakly hardened films, implying that not only the crosslinking but also the ionic interaction was involved in the hardening process. More specifically, the dissociation of the carboxylic group of gelatin decreases at lower pH, and the ionic interaction of gelatin molecule is confined. As a result, the extraction of the molecules is accelerated. The soluble fraction of the tightly hardened films stayed in low level, showing no dependence on extraction pH. On the bases of these results, the temperature and extraction pH are selected at 50°C and 6.0 respectively.

3.2 The effects of hardener reactivity and the influence of drying temperature on the film properties: Soluble fraction, Swelling, and Melting time

Table 2 shows obtained melting time, swelling and the soluble fraction for variously hardened films. The data of hardened with 12.5mmol/100g-gelatin of the hardeners are plotted as a function of drying temperature in Figs. 4a, b, and c.

The films hardened with rapid crosslinkers (WS-CIDI, MCP and GA) gave relatively higher soluble fraction content, greater swelling and shorter melting time than the films hardened with slow crosslinkers (DCT and DVS). The soluble fraction content of the films increased with increasing drying temperature. In contrast, the melting time obviously decreased with increasing drying temperature.
Fig. 5 Soluble fraction content (%) vs. extraction time (Hardener: 12.5mmol/100g-gelatin, dried at 12.5°C).

Fig. 6 Molecular weight distribution of the original gelatin.

Fig. 7 Molecular weight distribution of soluble fractions (Extraction at 50°C, pH=6.0. Numbers beside the curves indicate extraction time (s)).

Fig. 8 Molecular weight distribution of soluble fraction for cold-dried film (12.5°C) and hot-dried film (30°C). Extraction conditions, 50°C, pH6.0 and 1,000s.

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Fig. 5 shows the soluble fraction content as a function of extraction time at 50°C and pH6.0. The observed amount of the soluble fraction of the films hardened with the rapid crosslinkers had a tendency to increase with increasing the extraction time. On the other hand, the films hardened with the slow crosslinkers gave minute soluble fraction, and the contents are almost independent of the extraction time.

3.3 Molecular weight distribution of the soluble fraction

Fig. 6 depicts the GPC elution diagram of the original gelatin. There are several peaks and shoulders at the retention time of 16, 22, 24, 27, and 30min. The peak at 24min is known to correspond to α-component, which has the molecular weight of about 100,000. The shoulders at about 27 and 30min corresponds to the molecular weight of 23,000 and 5,000 respectively from calibration with a series of pullulan as standard molecular weight markers.

Fig. 7 illustrates the GPC elution diagrams of the soluble fractions extracted from the hardened gelatin films for a various extraction times at 50°C and pH6.0. From the films hardened with the rapid crosslinkers, higher molecular weight components of the soluble fraction gradually increased with increasing the extraction time. On the other hand, the film hardened with the slow crosslinkers only gave the soluble fraction with limited molecular weight even for prolonged extraction time.

Fig. 8 shows the GPC elution diagrams of the soluble fractions extracted from cold- and hot-dried films, hardened with MCP, GA and DVS. It is seen that slightly higher molecular weight components were extracted from hot-dried films, independently of the hardener type.

It should be noted that the GPC elution diagrams of the soluble fractions showed peaks at 27 to 30min which correspond to the components with lower molecular weight than the α-chain. Especially, the fraction corresponding to the retention time of 30min, whose estimated molecular weight is 5,000, was easily extracted even from the tightly hardened films. Itoh 6) and Sugiyma 5) also demonstrated the similar GPC diagram in their work on hardening. There are several studies 15-17) regarding non-gelling or weakly gelling low molecular weight components (sol fractions) in gelatin. Takahashi 17) et al. concluded that those were...
4. Discussion

The effect of hardener reactivity and the influence of drying temperature on the characteristics of hardened films were well studied. The data of the content and molecular weight distribution of the soluble fraction, the swelling ratio, and melting time for various hardened films gave us valuable information about hardening.

4.1 Effect of hardener reactivity

The films hardened with the rapid crosslinkers showed higher soluble fraction content, higher swelling ratio, and shorter melting time than those hardened with the slow crosslinkers. It is of considerable interest that the characteristics of the films essentially depend on the hardener reactivity, regardless of the type of chemical reaction mechanism of each hardener. To understand such phenomenon, we should take not only the chemical crosslinks, but also the crosslinks by helix formation into account. Crosslinking reaction of hardeners begins immediately after the addition to a gelatin solution especially for rapid crosslinkers. Coopes \(^{9}\) demonstrated that common organic hardeners, such as formaldehyde and mucochloric acid, caused considerable decline of optical rotation during of drying.

We supplementary investigated the optical rotation change by the hardeners used in this study while cooling down the gelatin gels. The results are depicted in Fig. 9. It is clearly seen that the rapid crosslinkers significantly inhibit change in optical rotation, suggesting strong retardation of helix formation. The rapid crosslinkers are considered to react with gelatin just after the addition and inhibit the helix formation of the gelatin, resulting less helix contained in the film layer. Another supposed mechanism is that the reaction in a dilute sol state promotes intra-molecular crosslinks and hence the number of effective crosslinks decreases.

In contrast, the slow crosslinkers do not retard the helix formation during drying and do react to form crosslinks after the film being dried resulting in high level of hardening.

4.2 Influence of drying temperature

The soluble fraction of the films was increased as the drying temperature increased. Conversely, the melting time of the films decreased with elevating the drying temperature especially for the films hardened with the slow crosslinkers.

In the study of the optical rotation of the gelatin films with regard to the drying temperature, Johnson et al. \(^{11}\) indicated the contribution of both chemical crosslinks and ordered structure (triple stranded helix) to the physical properties of swollen gelatin films. They showed that ordered structure formed in cold-dried film gave distinctive elastic modulus of the film although the amount of reaction with bisacryloylurea was roughly equal for hot-dried and cold-dried films. Sterman et al. \(^{12}\) reported that the chemical crosslinks improved the stability of helices in swollen gelatin films. They concluded that the elastic modulus of the film including chemical crosslinks and ordered structure was much greater than that of the film including chemical crosslinks only. Taking these results into consideration, it is worthwhile to estimate the actual number of crosslinks including the number of helices for the interpretation of the properties of the films and hardening as Johnson et al. did by tensile analysis and amino acid analysis \(^{11}\).

4.3 Estimation of the number of crosslinks and the number of helices

Charlesby \(^{1}\) presented an equation to correlate soluble fraction and the number of crosslinks for polymers with random molecular weight distribution. This equation was applied to hardened gelatin films for estimating number of crosslinks \(^{9}\).
\[ \delta = \frac{2}{S + \sqrt{S}} \]  
(4)

\( S \): amount of soluble fraction  
\( \delta \): number of crosslinks per weight average molecule

Using Eq. (4), we calculated the number of crosslinks for cold- and hot-dried films hardened with 12.5mmol/100g-gelatin of each hardener. According to Johnson et al. 11), we assumed the number of chemical crosslinks for cold- and hot-dried films is equal. Therefore, the difference between the number of crosslinks for cold- and hot-dried films is regarded solely as the difference on the helix content in the chain. The obtained results are summarized in Table 3. The number of helices formed in the cold-dried films hardened with the slow crosslinkers lies from ca 3 to 6, so that we could say that there were roughly 5 helices per weight average molecule.

Since this number was calculated from the soluble fraction determination under the particular conditions (1,000s extraction at 50°C), we should understand it as the number of helices which were effectively immobilized by the hardener under such extraction conditions.

Due to the complex nature of gelatin as a polymeric substance, there is still left to be discussed for applying the equation straightforwardly. We believe that further investigation will refine the procedure and give a more precise picture of gelatin hardening.

5. Conclusions

The properties of the gelatin films hardened with five hardeners were studied through evaluating the amount and the molecular weight distribution of the soluble fraction, the swelling ratio, and the melting time under various drying conditions.

1. Hardener reactivity and drying conditions in the film preparation process strongly affected the film properties.
2. The films hardened with the rapid crosslinkers gave a large amount of soluble fraction, high swelling ratio and short melting time and the films hardened with the slow crosslinkers gave the opposite tendency.
3. The rapid crosslinkers strongly retarded the helix formation.
4. The number of crosslinks of the hardened films was calculated from Charlesby's equation.
5. The number of helices in cold-dried films was estimated from the difference between the number of crosslinks for cold- and hot-dried films.
6. The number of helices formed in the cold-dried films hardened with slow crosslinkers were roughly 5 per weight average molecule.
7. Soluble fraction gives useful information for hardened gelatin films.
8. Optical rotation measurement is a straightforward method to determine the soluble fraction content in hardened gelatin films.

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References