

Elucidation of the time-dependent degradation process in insoluble hyaluronic acid formulations with a controlled degradation rate

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Abstract

Degradation rate of hyaluronic acid to prolong its stability in vivo would be beneficial. We investigated a potential solution for prolonging the stability of hyaluronic acid within the body. We focused on decreasing the swelling ratio to slow the degradation rate of hyaluronic acid by insolubilizing sodium hyaluronate without using potentially harmful substances such as crosslinkers or modifiers. Hyaluronic acid formulations were created with three different swelling ratios and time-dependent morphological changes in hyaluronic acid formulations and were scored based on each swelling ratio. In vivo degradation was modeled in simulated body fluid and the extent of decay of test membranes were monitored over time. Results showed that, by adjusting the swelling ratio, the degradation rate of hyaluronic acid formulation could be controlled. Our research could lead to improvements in many products, not only preventive materials for postoperative adhesions, but also pharmaceutical products such as osteoarthritis treatments and cosmetic medicines.

Keywords

Decomposition rate control, hyaluronan, hyaluronate, conformation

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Introduction

Hyaluronic acid is a glycosaminoglycan¹ that was discovered by Meyer in 1934 as a vitreous component of bovine eyeball.² As a component of the extracellular matrix, hyaluronic acid is distributed in a variety of body tissues, such as cartilage, skin, and joints. This molecule possesses properties of high water retention, contributing to tissue stability, lubricity, and elasticity, and is known to be an in vivo component that exhibits physiological activities such as forming a barrier against pathogen invasion.³ Due to such useful properties, hyaluronic acid has been utilized in a variety of medical products, including for treatment of osteoarthritis of the shoulder joint⁴ and knee joint,^{5,6} material for prevention of postoperative abdominal adhesions,^{7,8} and filler in plastic surgery or cosmetic medicine (hyaluronic acid filler).^{9,10}

To fit various applications, demand for hyaluronic acid with more features as a medical product has been

increasing. In particular, controlling the rates of degradation and absorption represents a significant challenge. This is because the material remaining in the body for the required amount of time and exhibiting its effects continuously would result in more stable management of various diseases.

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How hyaluronic acid formulations are degraded, in addition to the degradation rate itself, is also an important factor. For example, as an important point to consider, there is a difference between whether the shape changes little until just before degradation, or the shape is degraded step by step over time. This difference would become a critical point when investigating a target disease or site. Although studies of hyaluronic acid formulations are ongoing globally, many issues relating to the mechanisms and rate of degradation are unknown.

Adjustment of the properties and the degradation rate of hyaluronic acid is also performed by changing the molecular weight¹¹ and by using crosslinkers.^{12,13} Nevertheless, none of the resulting materials is perfect for use in vivo, necessitating further development. Although the factors that determine the degradation rate of a hyaluronic acid formulation remain unknown, Park et al.,¹⁴ recently reported that a decrease in the swelling ratio may slow the degradation rate of hyaluronic acid formulations. The idea that the swelling rate is an antagonistic factor against in vivo degradation was found in our previous study in which the insoluble hyaluronic acid was inserted subcutaneously into rats as well.¹⁵ The relationship between crosslink density and the swelling ratio of hydrogels is basic hydrogel behavior. However, the crosslinking hyaluronic acid formulations which contain no byproducts have been in developmental stages. When they are developed, it could be very beneficial.

The utility of elucidating the key factors controlling the degradation rate of hyaluronic acid would be incalculable. In addition, there are two important points about the hyaluronic acid formulation created using this unknown factor. The first is how many byproducts this hyaluronic acid formulation would produce during the production process. The second is what kind of degradation process this hyaluronic acid formulation would take.

We developed a technique to control the swelling ratio by applying a method we have been developing to insolubilize sodium hyaluronate without the use of crosslinkers. The greatest advantage of this method is that the generated hyaluronic acid formulation does not contain potentially harmful substances such as crosslinkers or modifiers.

Next, using the above method, we created hyaluronic acid formulations with three different swelling ratios and scored the time-dependent morphological changes in hyaluronic acid formulations for each swelling ratio. This in vivo degradation was modeled in simulated body fluid (SBF). The results showed that, by adjusting the swelling ratio, the degradation rate of hyaluronic acid formulation can be controlled.

Materials and methods

Creation of insoluble hyaluronic acid membrane (test membrane)

In accordance with the method described by Isono,¹⁶ a sodium hyaluronate was treated to create an insoluble test

membrane. One point zero grams of sodium hyaluronate (molecular weight (nominal value): 800,000; Kikkoman Biochemifa Corp., Tokyo, Japan) and 99.0 g of water were mixed and stirred in a beaker to obtain a uniform aqueous solution. The obtained aqueous solution was poured into a stainless steel vat and dried at 30°C to obtain a sodium hyaluronate film. The obtained sodium hyaluronate film was immersed in 100 mL of a treatment liquid (20% by volume acetic anhydride/ethanol solution) and incubated at 50°C for 30 min to apply water-insolubilizing treatment. The film to which the water-insolubilizing treatment was applied was washed with ethanol, 80% by volume ethanol aqueous solution, and water, in this order, to obtain a water-insoluble film. The process for producing a water-insoluble molded body according to the method above makes it possible to simply produce, without using a chemical crosslinking agent, a water-insoluble molded body in which intrinsic characteristics of the hyaluronate are retained. The water-insoluble film obtained was cut into a square, the side of which was 2 cm and was placed in a stainless steel vat, and immersed in a phosphate buffer solution (PBS) (pH of 6.8). The film did not dissolve in the solution and maintained its shape, and could be easily picked up with tweezers. After the membrane was treated, correlations between reaction time, swelling ratio, and infrared spectroscopy (IR) spectra were examined. Based on the results for swelling ratio, three groups were created (Group H, high; Group M, moderate; and Group L, low). Three test membranes were prepared for each group.

Measurement of swelling ratio

A 250 mg section of test membrane was cut out and completely immersed in pure water. The weight of the membrane was measured before and after immersion, and the swelling ratio was determined using the following equation

$$\text{swelling ratio} = \text{wet weight} / \text{dry weight}$$

IR analysis

IR analysis was conducted using Fourier-transform infrared spectroscopy (FT-IR) equipment (PerkinElmer Japan Co., Ltd., Kanagawa, Japan) with the sample placed in the ATR attachment irUniversal ATR sampling Accessory; PerkinElmer). Spectra were scanned four times, every 2 cm⁻¹ from 4000 to 400 cm⁻¹. Peaks were assigned in accordance with a previous report.¹⁷

Creation of SBF

SBF was created following a method described previously.¹⁸ In addition, 100 units of penicillin G sodium (Thermo Fisher Scientific, Tokyo, Japan) and 100 µg of streptomycin sulfate (Thermo Fisher Scientific) were

added to reach the final concentration. Phenol red of a concentration of 10 mg/L was added as a pH indicator.

Observation of test membrane degradation in SBF over time

A 50 mg section of test membrane was cut out and soaked in a Petri dish containing 5 mL of SBF. The test membrane was macroscopically observed once daily and the state of degradation was scored and classified into eight levels (Figure 3). The diameter of the largest bubble and extent of decay (tubularization) were set as criteria. The number of bubbles was not taken into consideration.

SBF was replaced once every 2–3 days. The pH of SBF was maintained at 7.4 and adjusted using 5 mol/L sodium hydroxide solution (for volumetric analysis standardized by the Japanese Pharmacopeia, Wako Pure Chemical 196-05375: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The temperature of the SBF during the degradation study have kept 37°C. Soaking of the test membrane in SBF was started on Day 0, and the test membrane was examined until the score became 0.

Results

Evaluation of insolubilization treatment with IR spectroscopy and swelling rate

Based on a previous report,¹⁷ bands at 1600 cm⁻¹ and 1390 cm⁻¹ belong to C=O and C-O⁻ stretching vibrations of the carboxylate ion (-COO⁻) of hyaluronan, respectively, and the bands at 1740 cm⁻¹ and 1250 cm⁻¹ belong to C=O and C-O-H stretching vibrations of carboxylic acid (-COOH), respectively.

Sodium hyaluronate was treated with the conditions under which the treatment time was set to every 10 min from 0 to 90 min. And then, their IR and swelling rate were investigated. Two absorption peaks (1600 cm⁻¹ and 1410 cm⁻¹ ≈ 1390 cm⁻¹) belonging to the carboxylate ion are observed with the treated for 0 min sodium hyaluronate, but are markedly decreased after insolubilization treatment. Furthermore, two new absorption peaks (1740 cm⁻¹ and 1250 cm⁻¹) belonging to carboxylic acid appeared with insolubilized membrane (Figure 1(a)). After 70 min of treatment with acetic anhydride, the spectral change converged thereafter. Based on these results, the 1740 cm⁻¹ absorption peak that markedly increased was designated as the index for insolubilization treatment.

Investigation of methods to adjust swelling ratio

Sodium hyaluronate were treated for 10, 20, 30, 40, 50, 60, 70, 80, or 90 min to create insoluble test membranes, then IR spectra and swelling ratios of these test membranes were examined. It was plotted on vertical axis for the

transmittance spectra of 1740 cm⁻¹ of Figure A and horizontal axis for the treatment time (Figure 1(b)). Then, these membranes were measured for swelling ratio; a plot was made with the swelling ratio being the vertical axis and horizontal axis being the treatment time. There was a strong correlation between the swelling ratio and the treatment time (Figure 1(c)). The swelling ratio could not be measured because the membrane treated for 0 min was completely absorbed in water, and the membrane treated for 10 min was swelling until it was gel-like.

In order to investigate the relationship between IR spectra and swelling ratio, IR absorption at 1740 cm⁻¹ was plotted on vertical axis and the swelling ratio on horizontal axis. A strong correlation was observed between the two variables ($R = -0.9803$) (Figure 1(d)). These results suggested two things. One is that swelling ratio can be controlled along with the treatment time. Another one is that the 1740 cm⁻¹ absorption peak of IR is a predictable indicator of swelling rate.

Creation of test membranes for investigating degradation process

Based on the results obtained (Figure 1), three types of insoluble hyaluronic acid membrane with different swelling ratios (three for each type) were created: Group H, high swelling ratio (2.58, 2.42, 2.42; mean=2.47; SD=0.08); Group M, moderate swelling ratio (2.02, 2.23, 2.22; mean=2.16; SD=0.10); and Group L, low swelling ratio (2.00, 1.97, 2.05; mean=2.01; SD=0.03). These membranes were used in the investigations of degradation process described below.

Observation of test membrane degradation in SBF over time

Up until Day 1, none of the test membrane groups exhibited changes and all had a score of 7. On Day 2, the score for Group H with the greatest swelling ratio began to decline. No changes in Groups M or L were observed at this point. Score began to decrease on Day 3 or 4 for Group M and Days 5–11 for Group L. Score reached 0 on Day 14 or 15 in Group H, Day 15 for all three membranes in Group M, and Day 16 for all three membranes in Group L. All groups showed a similar trend in changes until complete disappearance after the score was ≤6, when morphological changes to the membrane appeared (Figures 2(a) and 3). In addition, the score and swelling ratio have a strong correlation (Figure 2(b)).

Discussion

The in vivo degradation rate of hyaluronic acid formulation is highly associated with the duration of effect when injected into the body as a pharmaceutical product. Numerous hyaluronic acid formulations for different purposes have thus been developed through various methods and subsequently commercialized. For example, as a method to control the in vivo

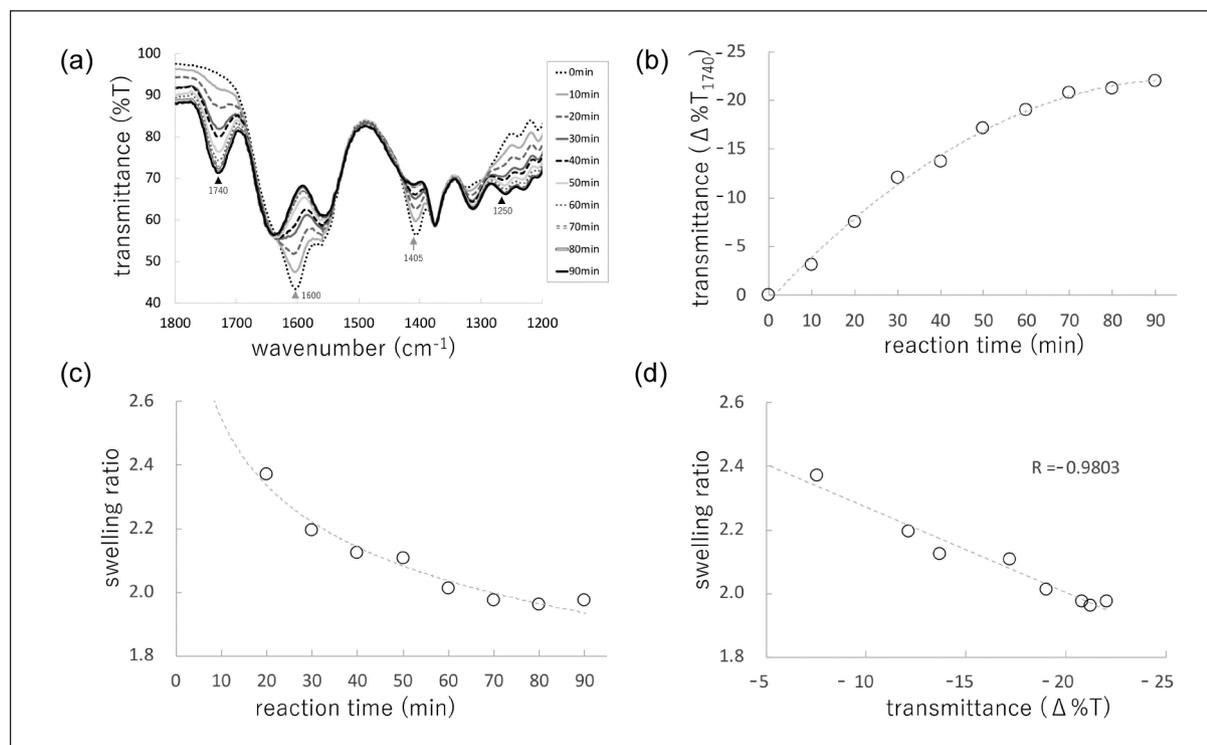


Figure 1. Graph of IR spectra: (a) changes in IR spectra by insolubilization treatment time (0–90 min). Absorption peaks belonging to carboxylate ion (1600 and 1410 cm⁻¹) decreased and the two absorption peaks belonging to carboxylic acid (1740 and 1250 cm⁻¹) increased proportionally with treatment time. After 70 minutes of treatment with acetic anhydride, the spectral change converged thereafter, (b) graph of the treatment time and IR spectra. Transmittance spectra of 1740 cm⁻¹ were plotted on the vertical axis and treatment time on the horizontal axis, (c) graph of the treatment time versus IR spectra. Changes in swelling ratio by insolubilization treatment time (0–90 min). Swelling ratio decreased inversely proportional to treatment time. With approximately 90 min of treatment, the decrease in swelling ratio reached a plateau and (d) Graph of IR absorption at 1740 cm⁻¹ and swelling ratio. A strong correlation was observed between the two variables ($R = -0.9803$).

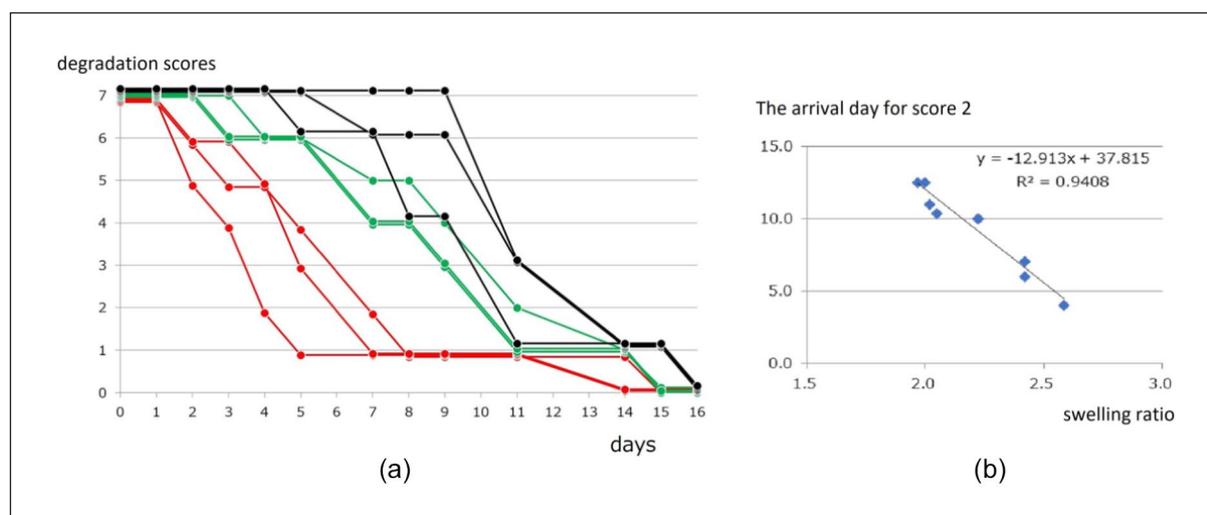


Figure 2. Graph of the swelling ratios and degradation scores: (a) low swelling ratio led to early decomposition. All groups showed a similar trend in changes until complete disappearance after the score reached ≤ 6 , and morphological changes appeared in the membrane (score 7, remain unchanged; score 6, bubble diameter less than 1 mm; score 5, bubble diameter 1–2 mm; score 4, bubble diameter 2–3 mm; score 3, bubble diameter over 3 mm; score 2, partly tubular; score 1, whole tubular; score 0, non solid) and (b) The arrival day for score 2 and swelling ratio. Score and swelling ratio have a strong correlation ($R^2 = 0.9408$).

Score	Criteria
7	 Remain unchanged
6	 Bubble diameter less than 1 mm
5	 Bubble diameter 1 mm-2 mm
4	 Bubble diameter 2 mm-3 mm
3	 Bubble diameter over 3 mm
2	 Partly tubular
1	 Whole tubular
0	Non solid (Unviewable)

Figure 3. Observation of test membrane degradation in SBF over time.

degradation rate of filler, a mixture of high- and low-molecular weight hyaluronic acid formulations is used.¹⁹ In addition, the capability for the material to remain in the body for a long duration (1–2 years) is one reason for its high demand.²⁰

The *in vivo* degradation rate of hyaluronic acid formulation is slower with a high molecular weight than with a low molecular weight.¹¹ For this reason, to prolong the effectiveness as a pharmaceutical product, high-molecular weight hyaluronic acid formulations are commonly used. However, hyaluronic acid formulations created with an excessively high molecular weight have also been reported as locally harmful to the body.²¹

It is possible to control the degradation rate of a hyaluronic acid formulation or its properties using crosslinkers.^{12,13}

However, the use of crosslinkers has many safety issues, as they are toxic to the body.^{22,23} Creating a hyaluronic acid formulation that is both safe and long-lasting in the body has thus been a challenge.

The present study observed the following changes in the test membrane that were proportionate to the treatment time for insolubilized sodium hyaluronate:

1. Decreased swelling ratio
2. Decreased absorption at 1600 cm^{-1} and 1405 cm^{-1} belonging to carboxylate ions, and increased absorption at 1740 cm^{-1} and 1250 cm^{-1} belonging to carboxylic acid on the IR spectra
3. Delayed degradation in SBF

These results demonstrated that the swelling ratio, IR absorption peaks assigned to carboxylic acid/carboxylate ion, and the degradation rate are correlated with each other.

Park et al.,¹⁴ reported the feasibility of slowing the degradation rate of hyaluronic acid formulations by decreasing the swelling ratio. However, what affected the swelling ratio itself was unknown. The present results may suggest that hydrogen bonds are an important factor in the swelling ratio, which determines the degradation rate caused by the decrease in carboxylate peaks and the increase in carboxylic acid group increases. We also verified with nuclear magnetic resonance (NMR) before and after the insolubilization treatment that new covalent bonds and side chains had not formed (data not shown). This strongly suggested that byproducts such as modifiers and crosslinkers had not emerged. That is, the degradation rate of the test membrane can be controlled, and a highly safe formulation that does not contain substances other than hyaluronic acid and sodium hyaluronate can be generated.

We have previously reported a test membrane having a clear effect in preventing postthoracotomy pleural adhesions in dogs.^{24,25} The finding from the present study that the swelling ratio and the degradation rate of hyaluronic acid formulation can be controlled is an extremely beneficial feature of hyaluronic acid formulations. Furthermore, improvements in many products, not only materials to prevent postoperative adhesions, but also pharmaceutical products such as osteoarthritis treatments and cosmetic medicines can be expected to take advantage of these properties.

The limitations of this study were as follows. The test substance was in the form of a membrane and may thus be unsuitable for use as an injectable material under the present circumstances. However, the form of the substance may well be amenable to change when drying during the generation process. That is, during the process of drying the hyaluronic acid formulation, it may be possible to modify the formulation to produce specific shapes, such as small particles, a string, or tubes rather than membranes. Since such modifications would impact on the clinical application, such as the target disease and purpose of use, we plan to conduct further investigations.

Conclusion

In conclusion, the present study revealed one of the controllable factors for the swelling ratio of hyaluronic acid formulations. It is suggested that there have been correlations between IR absorption peak at 1740 cm^{-1} and swelling ratio and between IR absorption peak at 1740 cm^{-1} and degradation speed. In addition, it was shown that the hyaluronic acid formulations are degraded gradually with time. The resulting hyaluronic acid formulation did not contain any by-products. Further studies are needed in order to reveal availability of changing form and reaction

in vivo. However, we believe that the present study suggested a novel approach toward the hyaluronic acid formulations for clinical application.

Authors' note

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