# Crosslinking of hyaluronic acid with water-soluble carbodiimide

**Kenji Tomihata and Yoshito Ikada**
*Research Center for Biomedical Engineering, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606, Japan*

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**Abstract:** Hyaluronic acid (HA) was chemically crosslinked with a water-soluble carbodiimide (WSC) to produce low-water-content films when brought into contact with water. The crosslinking reaction was performed in two different ways; one was by using HA films and the other by casting HA solutions. Both methods produced water-insoluble HA films. The lowest water content of the crosslinked HA films subjected to swelling with water was 60 wt % at 37°C, which was lower than any reported values. Infrared spectra of the crosslinked films suggested that intermolecular formation of ester bonds between the hydroxyl and carboxyl groups belonging to different polysaccharide molecules led to crosslinking. For comparison, pectin which possesses hydroxyl and carboxyl groups in one molecule, similar to HA, was subjected to crosslinking with WSC. The finding on pectin also supported ester formation between different polysaccharide molecules. The crosslinking of HA film with WSC in the presence of L-lysine methyl ester prolonged the *in vivo* degradation of HA film, probably because of amide bond formation as the crosslink. © 1997 John Wiley & Sons, Inc. *J Biomed Mater Res,* **37,** 243–251, 1997.

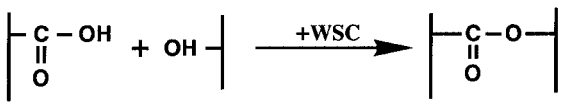
**Key words:** hyaluronic acid; crosslinking; water-soluble carbodiimide; biodegradation; IR spectra

## INTRODUCTION

Hyaluronic acid (HA) is an acidic polysaccharide with extremely high molecular weight. This polysaccharide is an important component of extracellular matrix and connective tissues such as cartilage. Industrially, HA is obtained from animal tissues such as eyeball, cockscomb, umbilical cord, and synovial fluid. Biotechnology also produces HA on a large scale.[1] HA has been used for medical purposes—for instance, as a viscoelastic biomaterial in ophthalmologic surgery[2–10]—since the dilute aqueous solution of HA is highly viscous. This lubricious polysaccharide is also used in orthopedics for the treatment of joint disease through injection of the aqueous solution.[11–16] In addition, HA is applied in cosmetics because of its high water retention capacity[17] and in drug delivery systems because of its biodegradability.[18–21]

As HA is soluble in water at room temperature, some surgical, pharmaceutical, and industrial applications need crosslinking of HA molecules to make them water insoluble. Crosslinking of HA also may be effective in retarding hydrolytic degradation, which takes place rapidly in biological environments. Therefore, many attempts have been made to introduce crosslinks into HA molecules. For instance, Balazs et al.[22] used divinyl sulfone as a crosslinking agent under alkaline conditions, while Malson et al.[4] and Tomas and Bengt[23] used 1,4-butanediol diglycidyl ether and phosphoryl chloride[24] and Francesco and Aurelio[25] used epichlorhydrin. In addition, Yui et al.[21,26] employed ethyleneglycol diglycidylether for the crosslinking of HA.

Although these crosslinking reactions succeeded in making HA water insoluble, the water content of the resultant HA hydrogels was mostly higher than 90 wt %, except for the HA chemically modified through the esterification of its carboxyl groups.[27] To obtain much slower biodegradable hydrogels which are often required for biomedical applications, we have initiated a series of studies on crosslinking of polysaccharides. In this study we describe the results of HA crosslinking by the use of water-soluble carbodiimide (WSC), which does not chemically bind to polysaccharide molecules, in contrast with conventional crosslinking agents.[28] The simplified reaction scheme for crosslinking of HA with WSC is:



(1)

To compare with HA, we also use pectin, which is an acidic polysaccharide containing hydroxyl and car-

Correspondence to: Y. Ikada

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boxyl groups, as the reactive moiety, similar to HA. The chemical structures of HA and pectin are represented in Figure 1, together with that of WSC.

## MATERIALS AND METHODS

### Materials

Hyaluronic acid was produced by a *Streptococcus* mutant of lactic acid bacteria. The sodium salt of HA with an average molecular weight of $1.5 \times 10^6$ was supplied by Denkikagaku Kogyo Co. Ltd. (Tokyo, Japan) as dry powder. Pectin powders were purchased from Nakarai Tesque Co. Ltd. (Kyoto, Japan). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was purchased from Dojindo Laboratories (Kumamoto, Japan) and used as WSC. L-Lysine was purchased from Wako Chemical Co. Ltd. (Osaka, Japan) and L-lysine methyl ester dihydrochloride from Sigma Chemical Co. (St. Louis, MO).

### Preparation of noncrosslinked polysaccharide film

One weight percent of HA or 5 wt % of pectin aqueous solution was prepared from the respective powders using double-distilled water (DDW) and cast into a petri dish. The cast solution was allowed to air dry at 25°C to yield films of 200 $\mu$m thickness.

### Measurement of film swelling in ethanol–$H_2O$ mixtures

Noncrosslinked films were immersed in various ethanol–$H_2O$ mixtures for 20 h at 25°C and then placed between two pieces of dry filter paper to wipe off the excess mixture. The swollen films were weighed, followed by drying in a vacuum oven for 6 h at 60°C under pressure of <0.1 mm Hg. The content of the ethanol–$H_2O$ mixture in the swollen films was calculated by the following equation:

$$\text{Content}(\%) = [(Ws - Wd)/Ws] \times 100 \qquad (1)$$

where $Ws$ is the weight of swollen films and $Wd$ is the weight of dried films.

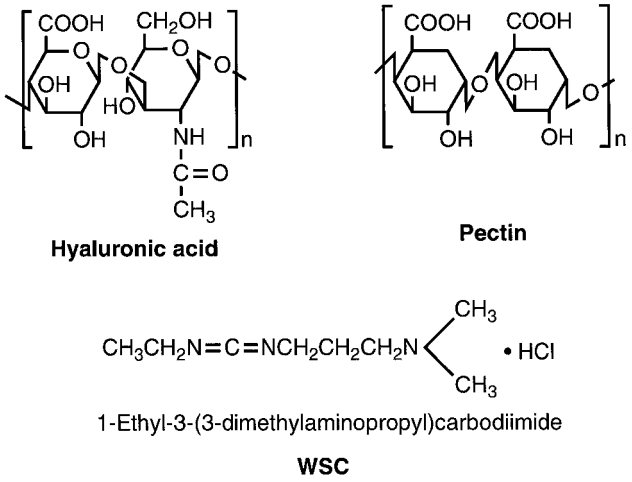



**Figure 1.** Chemical structure of hyaluronic acid, pectin, and the water-soluble carbodiimide (WSC) used in this study.

### Crosslinking by film immersion

Aqueous mixtures containing an organic solvent (ethanol or acetone) of various concentrations were prepared. After addition of WSC to the mixtures, small pieces of noncrosslinked polysaccharide films ($10 \times 10$ mm$^2$) were immersed in the mixtures for 24 h at 25°C for crosslinking and then washed in DDW and dried. In some cases, L-lysine or L-lysine methyl ester was added to the reaction mixtures.

### Crosslinking by solution casting

An aqueous solution of HA or pectin was prepared by dissolving in DDW to a 1 or 5 wt % concentration, respectively. The pH of the solution was adjusted by the addition of 0.1$N$ HCl or 0.1$N$ NaOH. A given weight of WSC was added to each solution and mixed under stirring at room temperature. The polysaccharide solution containing WSC was cast into a petri dish and allowed to air-dry at 25°C for 5 days to yield crosslinked films

### Infrared (IR) spectroscopic measurement

Infrared spectra of polysaccharide thin films were recorded using an adaptor made from silicone rubber with a hole 10 mm in diameter. IR spectra were obtained with Shimadzu Model FTIR-8100 (Shimadzu, Inc., Kyoto, Japan).

### Water content measurement of crosslinked films

Crosslinked polysaccharide films with a known weight were immersed in phosphate-buffered saline (PBS), pH 7.4, at 37°C for 24 hr. After determined intervals of time, they were placed between two pieces of dry paper to wipe off the excess solution, and then weighed. The same films were dried in a vacuum oven for 6 hr at 60°C under a pressure of <0.1 mm Hg and then weighed to determine their water content according to Equation (1).

### Implantation in rats

Crosslinked HA films with a known weight were sterilized with ethylene oxide gas prior to implantation, rinsed with sterilized PBS at pH 7.4, and implanted subcutaneously in the backs of five Wistar rats. After determined intervals of time, the rats were sacrificed and the explanted samples were weighed after drying. The extent of *in vivo* degradation was expressed as a percentage of the weight of films remaining after implantation. For histologic observation, HA films were explanted from the rats together with the surrounding tissue and sectioned with a microtome after fixation with 10% formalin, and stained with haematoxylin–eosin (HE).

## RESULTS

### HA

#### Crosslinking by immersion

Small pieces of dried HA film were used as the starting material for crosslinking with WSC. As they were readily soluble in water at room temperature, a water-miscible nonsolvent of HA was added to the reaction medium containing WSC, to prevent dissolution of HA films into the aqueous medium. The medium penetration into the HA films was assessed from the overall swelling of HA film in the mixed medium. Figure 2 shows the swelling agent content of HA film when immersed in ethanol–$H_2O$ mixtures at 25°C for 24 h. As is seen, dissolution of the HA film in the medium could be avoided when ethanol was added by 65 vol % of the total mixture. No appreciable swelling was observed when HA film was placed in 100% ethanol. In the following experiments, the crosslinking reaction of HA was carried out in aqueous medium containing ethanol of >65 vol %.

The water content of HA film after crosslinking was used as a measure of the extent of crosslinking of HA film throughout this work. Figure 3 shows the water content of the HA films subjected to crosslinking with 50 m*M* WSC in ethanol–$H_2O$ mixtures. Clearly, the film water content markedly depends on the ethanol concentration in the reaction mixture. The lowest water content observed at the ethanol concentration around 80 vol % must correspond to the highest extent of crosslinking of HA. This lowest water content (60 wt %) is lower than the lowest values ever reported for HA, so far as we know. To examine whether the hydroxyl group of ethanol interferes with the crosslinking of HA which also possesses many hydroxyl groups in the molecule, acetone was used as a nonsolvent of HA instead of ethanol. The result of crosslinking HA film in the acetone–water mixtures is also shown in Figure 3. Apparently, acetone gives a result quite similar to that of ethanol for HA crosslinking, indicating that the hydroxyl group of ethanol has no substantial obstructive effect on HA crosslinking under this reaction condition.

The dependence of WSC concentration on the film crosslinking is shown in Figure 4. The reaction was conducted at 25°C for 24 h in the medium containing 80 vol % ethanol and 20 vol % water, which gave the highest crosslinking extent in Figure 3. It is obvious that 10 m*M* WSC is high enough to result in HA crosslinking to the saturated level when assessed from the water content of the crosslinked HA film. To get a deeper insight into the crosslinking mechanism of HA, IR spectra of the HA films crosslinked with 50 m*M* WSC in aqueous media containing different concentra-

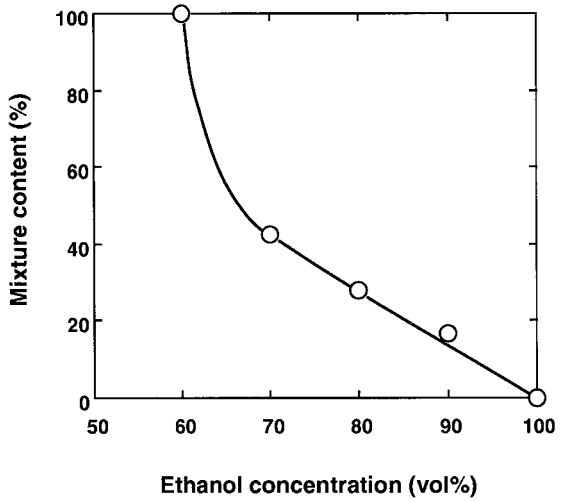



**Figure 2.** The effect of ethanol concentration on the swelling of noncrosslinked HA film in ethanol–$H_2O$ mixtures at 25°C.

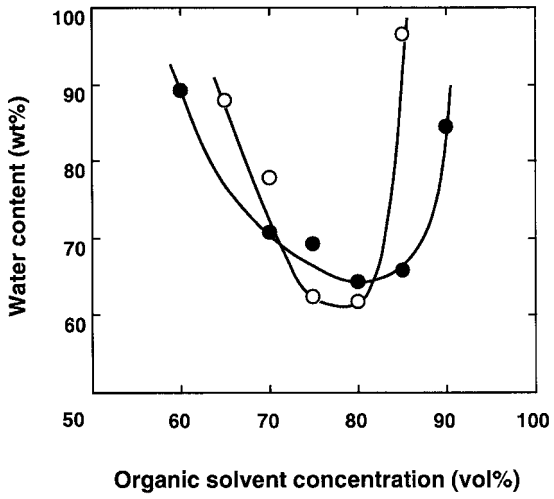



**Figure 3.** The effect of organic solvent concentration on the water content of HA film crosslinked with 50 m*M* WSC in organic solvent–$H_2O$ mixtures at 25°C for 24 h. (○) Ethanol; (●) acetone.

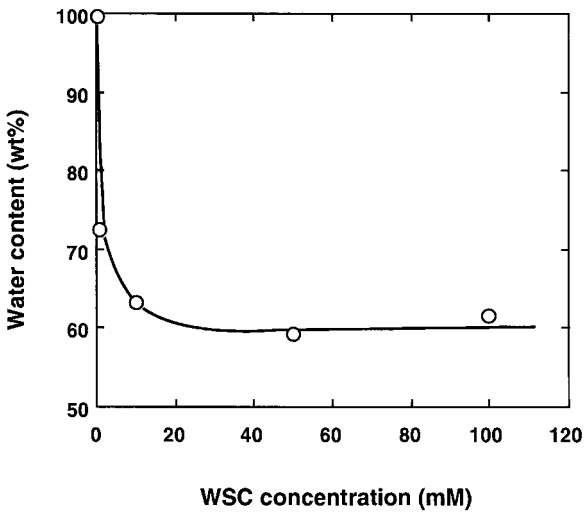



**Figure 4.** The effect of WSC concentration on the water content of HA film crosslinked in an ethanol (80 vol %)–$H_2O$ (20 vol %) mixture at 25° C for 24 h.

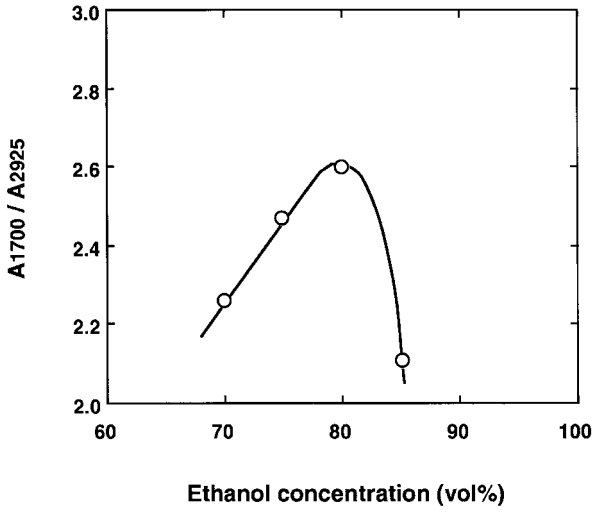



**Figure 6.** The ratio of IR absorbance at 1700 $cm^{-1}$ to that at 2925 $cm^{-1}$ against the ethanol concentration in ethanol–$H_2O$ mixtures used for crosslinking of HA film with 50 m*M* WSC at 25°C for 24 h.

tions of ethanol are shown in Figure 5. As can be seen, the most prominent difference in the spectrum between the noncrosslinked and the crosslinked HA film is noticable at a wavenumber of 1700 $cm^{-1}$, which is assigned to the carbonyl group most likely of ester bond. To quantify the carbonyl formation, we selected the IR spectrum at 2925 $cm^{-1}$ as a control, because the absorption at 2925 $cm^{-1}$ is assigned to the $–CH_2$ group, which remains unchanged during the crosslinking reaction. Figure 6 shows the intensity ratio of the absorbance at 1700 $cm^{-1}$ to that at 2925 $cm^{-1}$, $A_{1700}/A_{2925}$, evaluated from Figure 5. It is interesting to note that maximum $A_{1700}/A_{2925}$ is observed at an ethanol concentration of 80 vol %, which yielded the crosslinked HA film with the lowest water content, as shown in Figure 3. The $A_{1700}/A_{2925}$ dependence of HA film crosslinked in the ethanol (80 vol %)–$H_2O$ (20 vol %) mixture on the WSC concentration is given in Figure 7. Apparently, the semilogarithmic plot gives an almost linear plot. It should be noted that 50 m*M* WSC gives a higher $A_{1700}/A_{2925}$ than 10 m*M* WSC, apparently different from the result shown in Figure 4. To examine the pH effect of the reaction medium on HA crosslinking, a medium was prepared from 20 vol % water of different pHs and 80 vol % ethanol. No significant difference in water content was observed for the resultant HA films when crosslinking was carried out at 25°C for 24 h in the medium containing 20 vol % water of pH 3–11 and 50 m*M* WSC.

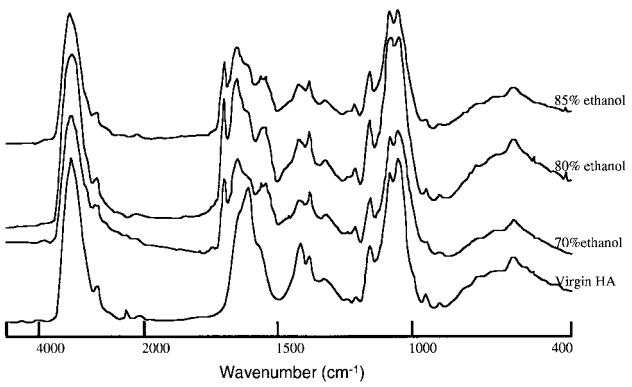



**Figure 5.** IR spectra of HA film crosslinked with WSC in ethanol–$H_2O$ mixtures.

## Crosslinking by casting

In an attempt to introduce crosslinks into HA molecules dissolved in water, dilute HA solutions con-

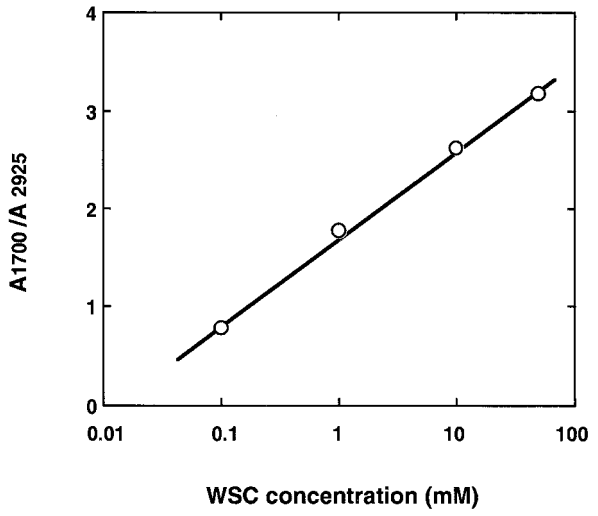



**Figure 7.** The ratio of IR absorbance at 1700 $cm^{-1}$ to that at 2925 $cm^{-1}$ against the concentration of WSC used for crosslinking of HA film in an ethanol (80 vol %)–$H_2O$ (20 vol %) mixture.

taining WSC were allowed to stand at 25°C for a few days, but no gelation was observed on the HA solutions even when the HA and WSC concentratons were raised up to 4 wt % and 200 m*M*, respectively. It was quite difficult to prepare more concentrated HA solutions, owing to the high solution viscosities. However, as demonstrated above, crosslinks could be introduced into HA molecules when noncrosslinked HA films were placed in aqueous media containing WSC. In this case, the polymer concentration in the film swollen, for instance, with an 80 vol % ethanol–20 vol % water mixture was approximately 72 wt %, when evaluated simply from the result shown in Figure 2. It is therefore expected that HA crosslinking will take place if the HA concentration of solutions becomes as high as 70 wt %. Thus, HA aqueous solutions containing WSC were cast onto a glass plate to allow the mixed solvent to spontaneously evaporate at 25°C. After 5 days, the resultant HA films were peeled off from the glass plate and further subjected to drying *in vacuo.* As expected, this casting method yielded crosslinked HA films of low water content. Figure 8 shows IR spectra of the HA film crosslinked by this method. Clearly, new IR absorption appears at 1700 $cm^{-1}$, similar to that of HA crosslinked by placing noncrosslinked HA films in aqueous media containing WSC. The $A_{1700}/A_{2925}$ ratio of HA films was calculated from Figure 8 and is plotted in Figure 9 as a function of the amount of WSC added to the starting HA aqueous solution. The dependence of the water content of the crosslinked HA film on the $A_{1700}/A_{2925}$ ratio was almost the same as that obtained by the film immersion method.

## Crosslinking of HA in the presence of L-lysine and its methyl ester

Chemically reactive groups responsible for crosslinking of HA molecules must be hydroxyl and carboxyl (see Fig. 1). The crosslink bond formed on HA by the use of WSC must be based on these two reactive groups, and hence is probably ester, not ether, because

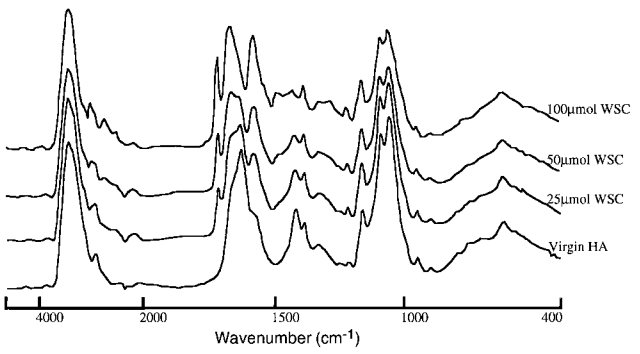



**Figure 8.** IR spectra of HA film crosslinked with different amounts of WSC by the casting method (the HA film weight is 20 mg).

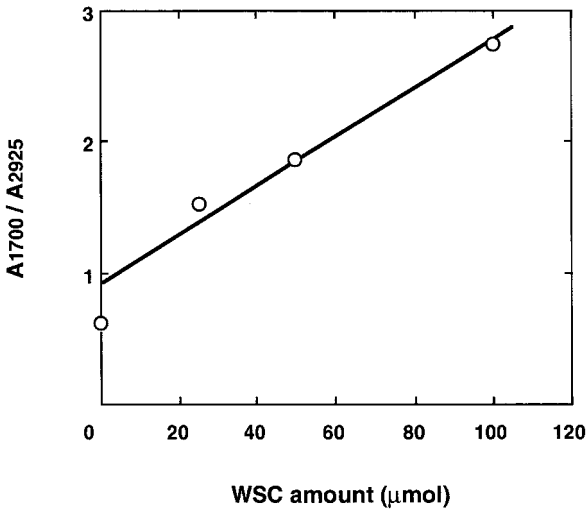



**Figure 9** The ratio of IR absorbance at 1700 $cm^{-1}$ to that at 2925 $cm^{-1}$ against the amount of WSC used for crosslinking of HA by the casting method (the HA film weight is 20 mg).

starch did not undergo crosslinking when WSC was used.[29] Indeed, a slightly crosslinked HA film with an equilibrated water content of 90 wt % was dissolved completely within a few days in a buffered solution of pH 7 at 25°C, while a more strongly crosslinked film with a water content around 60 wt % partly remained 2 weeks after immersion in the buffered solution. The following experiment was performed in an attempt to crosslink HA molecules through an amide bond which is more resistant against hydrolysis than the ester bond. For this purpose, L-lysine methyl ester was added to an 80 vol % ethanol–20 vol % water mixture and the crosslinking of HA film was allowed to proceed in this medium in the presence of 10 m*M* WSC at 25°C for 24 h. The water content of the resultant film is plotted as a function of L-lysine methyl ester in Figure 10, together

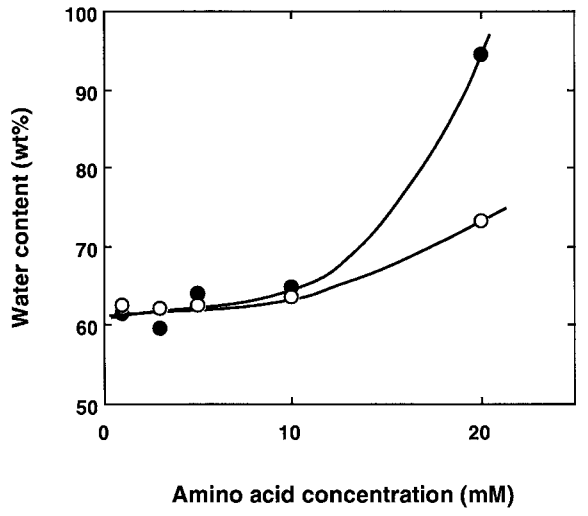



**Figure 10.** The effect of amino acid concentration on the water content of HA film crosslinked with 10 m*M* WSC in an ethanol (80 vol %)–$H_2O$ (20 vol %) mixture at 25°C for 24 h. (○) L-lysine; (●) L-lysine methyl ester.

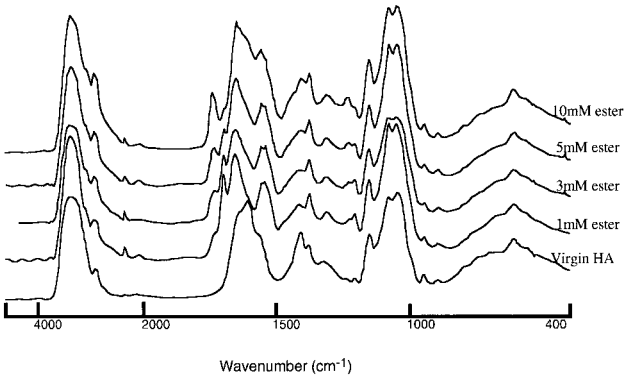



**Figure 11.** IR spectra of HA film crosslinked with 10 m*M* WSC and various concentrations of L-lysine methyl ester in an ethanol (80 vol %)–$H_2O$ (20 vol %) mixture.

with the result of L-lysine. It can be seen that the water content is not lowered by addition of lysine ester, but rather increases with increasing ester concentration. Figure 11 shows IR spectra of the HA film crosslinked in the presence of different concentrations of L-lysine ester. Clearly, the increased concentration of L-lysine ester results in reduction in the absorption at 1700 $cm^{-1}$ and increase in the absorption at 1740 and 1560 $cm^{-1}$. This result suggests that the amide bond is actually formed by an addition of L-lysine methyl ester to the reaction medium.

If HA molecules are crosslinked not only through ester but also through amide, HA degradation is expected to retard hydrolysis, since rapid hydrolysis of amide bond at room temperature and pH 7 requires enzymes, in contrast with that of ester and anhydride bonds, which readily undergo hydrolysis at pH 7 without the help of enzymes. The result of *in vivo* degradation in rats is shown in Figure 12. Evidently, the addition of L-lysine methyl ester to the crosslinking medium suppressed the *in vivo* degradation of crosslinked HA film in the subcutaneous tissue of the rat, providing evidence for the contribution of the amide bond to crosslinking of HA molecules when lysine ester is added to the reaction medium. Addition of L-lysine to the reaction mixture instead of L-lysine methyl ester also yielded a crosslinked HA film with high resistance to degradation, similar to L-lysine methyl ester (data not shown).

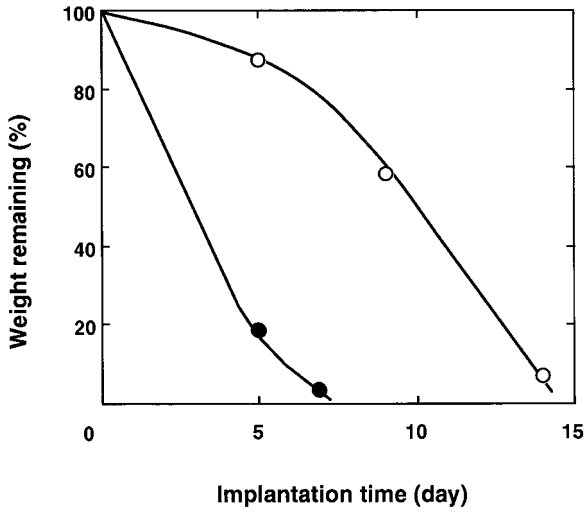



**Figure 12.** *In vivo* degradation of HA film crosslinked in an ethanol (80 vol %)–$H_2O$ (20 vol %) mixture at 25°C for 24 h. (○) 10 m*M* WSC and 3 m*M* L-lysine methyl ester; (●) 10 m*M* WSC without L-lysine methyl ester.

Figure 13 shows a histologic observation of the tissue around the implanted films. Apparently, subcutaneous implantation of the crosslinked HA films elicited no significant inflammatory reaction to the surrounding tissue.

### Pectin

For comparison with HA, pectin was subjected to crosslinking with WSC, because this polysaccharide has both hydroxyl and carboxyl as reactive groups, similar to HA, as shown in Figure 1. Crosslinking of pectin was carried out by the immersion method using dried pectin films as the starting material. Figure 14 shows the water content of the pectin film crosslinked at 25°C for 24 h with 50 m*M* WSC in ethanol–water mixtures of different ethanol concentrations. As can be seen, this film immersion method also produced crosslinked films for pectin. The lowest water content was 65 wt % and the ethanol concentration giving the lowest water content was around 60 vol %. The slight difference in optimal ethanol concentration between HA and pectin crosslinking may be due to the difference in the chemical properties between HA and pectin. IR spectra of crosslinked pectin films were similar to those of HA films. The $A_{1700}/A_{2925}$ ratio for the pectin film crosslinked with 50 m*M* in ethanol–water mixtures at 25°C for 24 h was plotted against the ethanol concentration of the reaction mixture, also in Figure 14. As can be seen, the $A_{1700}/A_{2925}$ dependence of the crosslinked pectin film on the ethanol concentration is again similar to that of HA crosslinking.

## DISCUSSION

As is obvious from the chemical structure given in Figure 1, the reactive groups of HA and pectin responsible for the chemical crosslinking with WSC must be hydroxyl and carboxyl. The need of WSC for the crosslinking of HA and pectin strongly suggests that the intermolecular formation of ester bonds between the hydroxyl and the carboxyl groups in different polysaccharide molecules must have led to crosslinking. IR spectra shown in Figures 5 and 8 support this assump-

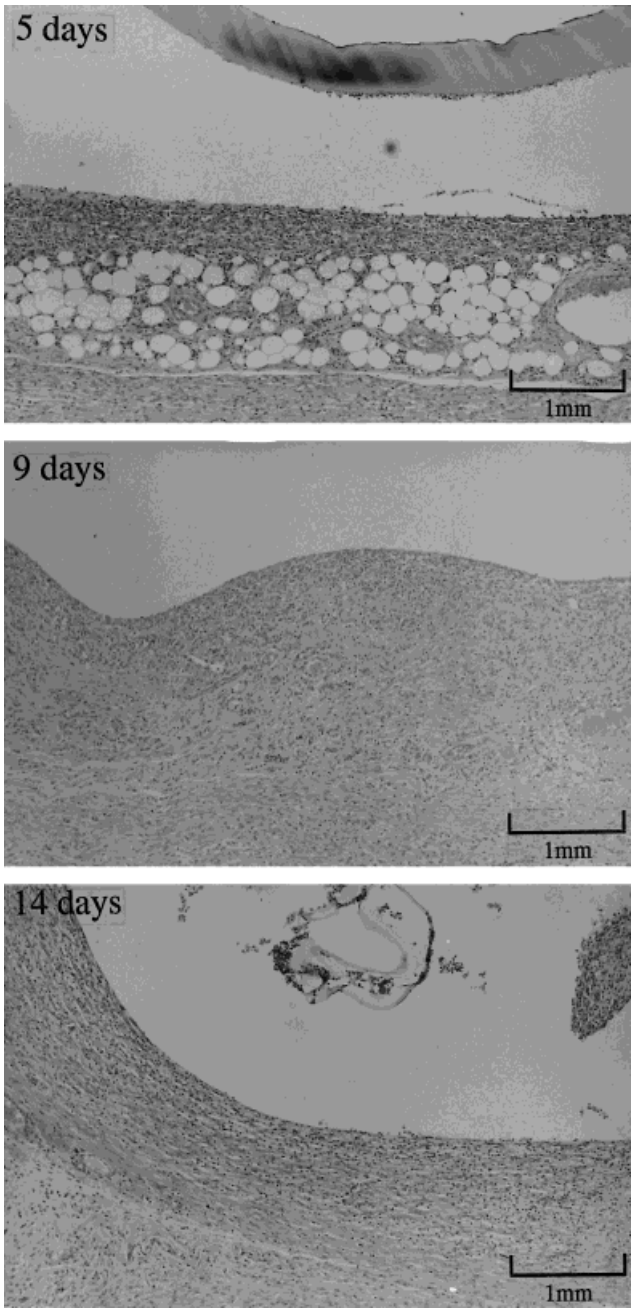



**Figure 13.** Histologic sections of the subcutaneous tissue around the crosslinked HA films implanted in rats for different periods. Crosslinking: 10 m*M* WSC, and 3 m*M* L-lys methyl ester in 80 vol % ethanol–20 vol % water mixture; staining: HE.

tion. As demonstrated in a previous study,[28] WSC seems to mediate acid anhydride formation between two carboxyl groups belonging to the same or different polysaccharide molecules. The resultant acid anhydride may readily react with a hydroxyl group of polysaccharides to yield an ester bond, which functions as a crosslink of polysaccharide molecules. This crosslinking scheme is schematically represented below:

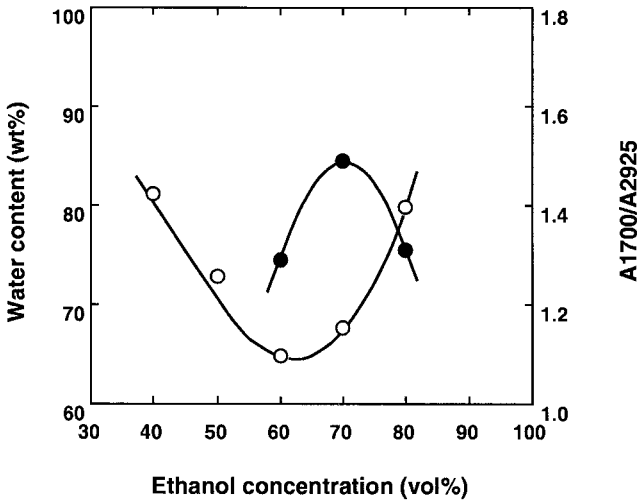
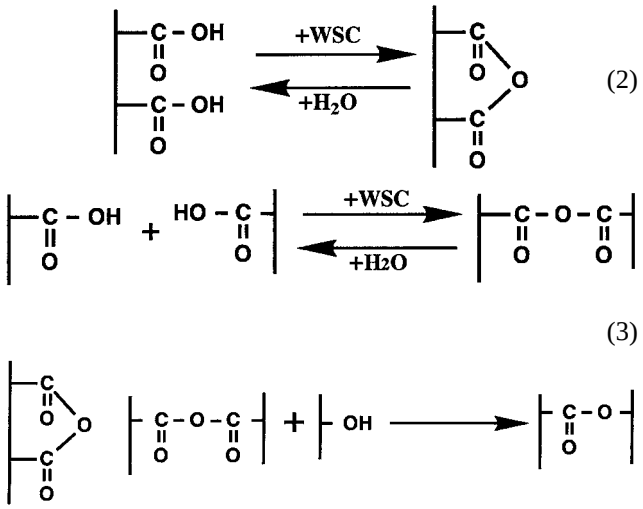



**Figure 14.** The effect of ethanol concentration on the water content and $A_{1700}/A_{2925}$ of pectin film crosslinked with 50 m*M* WSC in ethanol–$H_2O$ mixtures at 25°C for 24 h. (○) Water content; (●) $A_{1700}/A_{2925}$.




It is unclear which reaction is more prevalent: intramolecular reaction (2) or intermolecular reaction (3). Since the acid anhydride formed is very unstable at room temperature in aqueous environments, it will be hydrolyzed back to the original carboxyl groups unless any nucleophilic groups such as hydroxyl quickly encounter the acid anhydride. The IR absorption at 1700 $cm^{-1}$ is not due to the acid anhydride but to the ester bond, as afforded by IR spectra of poly(acrylic acid) (PAA) and poly(L-glutamic acid) (PGA) after their reaction with WSC, as shown in Figure 15. These acidic polymers have only carboxyl groups as a reactive moiety in the molecules. As can be seen, new peaks appear at 1050, 1760, and 1820 $cm^{-1}$ after the reaction of PAA and PGA with WSC, whereas they were not noticed in the IR spectra of HA and pectin when reacted with WSC, suggesting that the new peaks observed for PAA

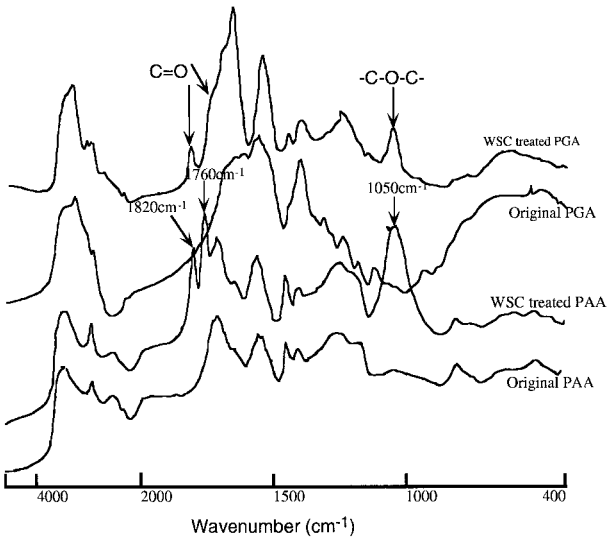


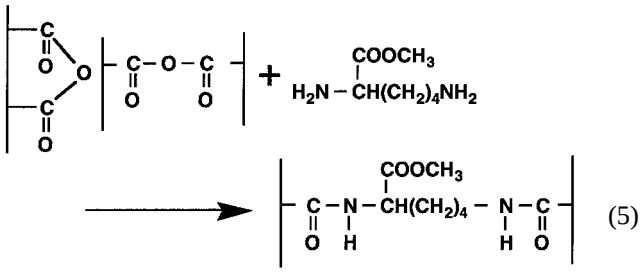

**Figure 15.** IR spectra of poly(acrylic acid) (PAA) and poly(L-glutamic acid) (PGA) treated with 50 m*M* WSC.

and PGA are assigned to acid anhydride. This result supports our assumption that the crosslinking reaction of HA and pectin with WSC proceeds via acid anhydride formation, followed by rapid ester formation between the acid anhydride and the hydroxyl group of other chains of HA or pectin. The anhydride intermediate may be too unstable to remain until the IR measurement, if the surrounding medium contains neither hydroxyls nor amines, but only water.

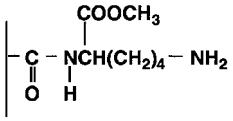
The high instability will also explain why our method by means of film immersion or solution casting is effective for crosslinking of HA and pectin, in contrast with the conventional homogeneous solution method, where the water, not the polymer, is steadily kept at such a high concentration as to prevent acid anhydride formation. The retardation of HA crosslinking reaction at higher ethanol or acetone concentrations seen in Figures 3, 6, and 14 can be explained by the reduced WSC concentration in its reaction medium of high ethanol or acetone concentrations, because WSC has very low solubility in ethanol and acetone.

The aliphatic ester bond formed by reaction (4) is not highly resistant against hydrolysis, although not as unstable as acid anhydride. To enhance the hydrolytic stability of crosslinked polysaccharide films, we attempted to introduce a more hydrolysis-resistant amide bond in the polysaccharide molecules. As shown in Figure 11, IR spectra of the HA film crosslinked in the presence of L-lysine methyl ester showed absorption assigned to amide formation. In addition, *in vivo* degradation of the crosslinked film revealed that the film crosslinked in the medium containing lysine ester underwent slower degradation than that without lysine ester. This finding strongly suggests that an amide bone was formed between the carboxyl group of HA molecule and the amino group of L-lysine methyl ester.

The plausible mechanism of crosslinking of HA in the presence of L-lysine methyl ester is formation of acid anhydride by reaction (2) or (3), followed by reaction (5);




The increased water content of HA film crosslinked in the presence of L-lysine methyl ester of higher concentrations seen in Figure 10 may be caused by the addition of L-lysine methyl ester in excess compared with the counterpart carboxyl group. As 2 mol of anhydride is needed to react with 1 mol of lysine ester, crosslink formation will stop before coming to completion, resulting in formation of side chains with terminal amine as depicted below:




In summary, it may be concluded that HA and pectin can be crosslinked with the use of WSC to yield a product with a low water content such as 60 wt % if the crosslinking reaction is carried out at very high polysaccharide concentrations—in other words, at very low water concentrations. Such a reaction condition can be realized by immersing the polysaccharide films in aqueous swelling media or by casting aqueous solutions of polysaccharide. Addition of L-lysine methyl ester with two amino groups in a molecule to the reaction medium containing WSC can produce polysaccharide films which have higher resistance against hydrolytic degradation than those crosslinked through an ester bond alone.

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