

## Cu(II)-Bridged Crosslink Structure Between Polysaccharide Chains in Gellan Gum Solution Studied by EPR

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It is known that gellan solution forms a gel in the presence of divalent cations, such as Ca(II) and Mg(II) at ca. 35 °C. The gel formation is promoted by chemical bonding between divalent cation and two carboxyl groups belonging to two polysaccharide chains. In this study, we used Cu(II) instead of Ca(II) or Mg(II) and detected EPR spectra to elucidate the gelation mechanism due to divalent cations. Viscoelasticity and CD was observed to confirm the gelation temperature and the helix formation, respectively. We propose the first step of the gelation process in the Cu(II)/gellan system in such a way that the crosslinks via the [-COO>Cu<OOC-] complex are firstly generated between pair of the random coils of gellan and then double helix structures are induced in the vicinity of the crosslinks at much higher temperature (44.7 °C) than the intrinsic coil-helix temperature (27 °C) of gellan solution. The gelation mechanism itself might be similar between due to Cu(II) and due to Ca(II) or Mg(II) except the gelation temperature, because the crosslink via Cu(II) is stabilized in a higher temperature by the strong covalent bonds to carboxyl groups, while the crosslink via Coulombic interaction between alkaline earth ions and carboxyl groups is easily destroyed by a thermal motion of the chains and stabilized only at a lower temperature.

Key words: Cu/gellan, gelation mechanism, EPR, gellan gum, coil-helix transition

### 1. INTRODUCTION

The gelation of polysaccharides in aqueous solution is based on the specific intermolecular interaction between their stiff and regular chains and often is mediated by cations such as alkali or alkaline earth ions. Gellan gum (Fig. 1), a microbial polysaccharide derived from *Pseudomonas elodea*, forms a rigid and stable gel in the presence of divalent cations, such as Ca<sup>2+</sup> or Mg<sup>2+</sup>. It has been suggested that divalent cations contribute to the gelation through making chemical crosslinks between polysaccharide chains via ionic bonds with two COO<sup>-</sup> groups in glucuronic acid unit belonging to two polysaccharide chains.[1] In contrast, monovalent cations such as Na<sup>+</sup> or K<sup>+</sup> was suggested to accelerate the double-helix formation by shielding the ionic repulsion between polysaccharide chains to lead the sol-to-gel transition.[2] These explanations for the gelation mechanism, however, were given based on the experimental results due to viscoelasticity, DSC, CD and NMR, but not on a direct observation of the cation itself. Therefore, it is necessary to elucidate the role of the cations with employing more direct technique for clear understanding of the gelation mechanism of gellan gum aqueous solution.

In this paper we used Cu(II) instead of the divalent alkaline earth cations and directly observed the process of crosslink formation due to Cu(II) by EPR method. EPR parameters such as g-values and hyperfine splitting constants A give information about coordination states of Cu(II) with ligands as well as mobility of the Cu(II) complex. The results by EPR method were compared with those by CD and viscoelasticity measurements to

elucidate the gelation mechanism due to divalent cations, especially a correlation between coil-helix transition, sol-gel transition and crosslink formation.

### 2. EXPERIMENTAL

*Materials:* Gellan gum was supplied by San-Ei Chemical Industries, Osaka, Japan, as the third common sample (NaGG-3) for the research group on gellan gum. A content of metal ions was analyzed as Na 2.59%, K 0.009%, Ca 0.02%, Mg 0.001%, and the loss on drying was 8.7% by atomic absorption and ICP methods.

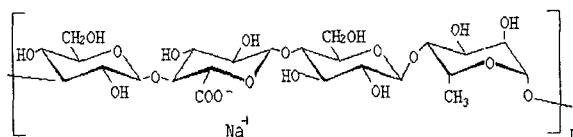


Fig. 1. Repeating unit of gellan gum.

The powdered gellan sample was mixed with distilled water at a concentration 2 (w/w)% and allowed to swell and disperse at 40 °C with stirring overnight. After this process, the resulting homogeneous suspension was heated at 70 °C for 2 h and at 90 °C for 30 min to dissolve completely. Subsequently, the equivalent amount of metal chloride solution with twice the required concentration, which had been kept at 90 °C, was added under vigorous stirring and then the solution was stirred at 90 °C for 30 min, resulting in a homogeneous and transparent solution. Immediately an aliquot of the solution for ESR and CD measurements was prepared in each cell, at 80 °C, respectively. The

final concentration of gellan for all samples was adjusted to 1 (w/w)%.

**Methods:** EPR spectra were measured at varying temperature from 80 to  $-110$  °C with JES-TE300 (JEOL Co.) operating at  $9.15 \pm 0.1$  GHz (X band). A flat cell for aqueous sample (70  $\mu$ l) and a quartz tube (o.d. 5 mm) was used for measurement at temperature above and below 0 °C, respectively. Typical acquisition parameters are as follows; microwave power, 2 mW; field modulation amplitude, 0.5 mT; spectral range, 200 to 400 mT; sweep time, 2 min; time constant, 0.1 s; number of data point, 4096; and number of scan, 10. The sample in the cavity was cooled down in situ from 80 to 5 °C and then heated up to 70 °C. EPR was observed at every 10 °C after keeping the sample at the temperature during 15 min. Accuracy of the temperature is in  $\pm 0.5$  °C. CD measurements were carried out in the range from 250 to 190 nm at a scanning rate of 20 nm/min with a J-720 spectropolarimeter (JASCO Co.) and viscoelasticity measurements were done with RheoStress RS50 spectrometer (HAAKE. Co.) The samples were cooled from 70 to 5 °C at 0.5 °C /min in the latter two measurements.

### 3. RESULT AND DISCUSSION

Figure 2 shows the temperature dependence of the storage modulus ( $G'$ ) and the loss modulus ( $G''$ ) on cooling process of Cu(II)/gellan aqueous system. The  $G'$  and  $G''$  values crossed over at 47.1 °C and then steeply increased with lowering the temperature. Generally, the cross-over temperature is defined as the sol-gel transition temperature ( $T_{sg}$ ), where the crosslinks are cooperatively generated to lead three-dimensional network structure.[3] In CD spectra, a positive peak around 210 nm changes to a negative peak with the structural change from a random coil to a double helix.[4] The molar ellipticity at 210 nm,  $[\theta]_{210}$  plotted to temperature, as shown in Fig. 3, exhibits the change of the fraction of double helix structure. The temperature at which  $[\theta]_{210}$  begins to change drastically was defined as a apparent random coil-helix transition temperature,  $T_{ch}$ . In Fig. 3,  $T_{ch}(\text{Cu}) = 44.7$  °C for the gellan solution with 2 mM Cu(II), and therefore  $T_{sg}(\text{Cu}) > T_{ch}(\text{Cu})$ . As to the gellan solution with 2 mM Ca(II),  $T_{ch}(\text{Ca}) = 34.0$  °C in Fig. 3, which was nearly identical with  $T_{sg}(\text{Ca})$  as reported in our previous paper.[2] This indicates that the conformational transition (i.e., the formation of double helices) and the formation of the network structure are synchronized with each other in the presence of 2 mM  $\text{CaCl}_2$  in 1 % gellan solution, while the formation of the network structure in Cu(II)/gellan system in the same concentration is preceded and then the conformational change occurs. If we compare the trace of  $[\theta]_{210}$  for Cu(II)/gellan system to that for Ca(II)/gellan system in Fig. 3,  $[\theta]_{210}$  for Cu(II)/gellan system shows a characteristic two-step change, namely a gradual decrease from  $T_{ch}(\text{Cu})$ , 44.7 °C and then a steeper decrease below about 27 °C. The latter change of  $[\theta]_{210}$  overlap a trace of  $[\theta]_{210}$  for a coil-helix transition in Ca(II)/gellan system. These findings

suggest that in the Cu(II)/gellan system the helix formation is accelerated together with the formation of effective crosslinks in the first stage of the gelation even in much higher temperature than the intrinsic  $T_{ch}(\text{gellan})$  and then the residual helix formation is promoted with development of crosslinking domain of gellan chains.

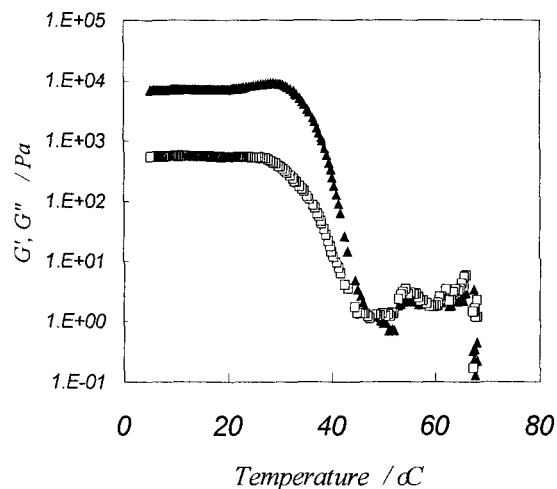


Fig. 2 Temperature dependence of the storage modulus,  $G'$ (▲), and the loss modulus,  $G''$ (□), in cooling (0.5°C/min) of aqueous Cu/gellan solutions.

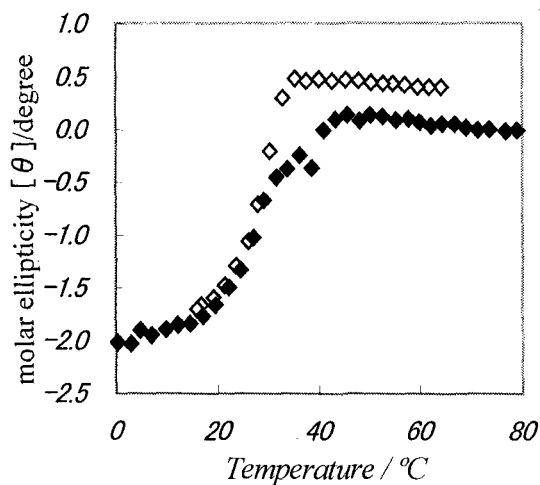


Fig. 3 Plot of the molar ellipticity  $[\theta]$  in Cu/gellan(◆) and Ca/gellan(◇) against temperature in cooling process.

Figure 4 demonstrates the spectral change in EPR with the cooling process. The spectra at high temperatures (80 and 70 °C) indicate the existence of Cu(II) species with high mobility in the system. On the other hand, a typical powder-pattern for Cu(II) in tetrahedral crystal field appeared at  $T_{sg}(\text{Cu})$  and the

intensity of the species increased with the gel-formation in cooling process. The  $g$  values ( $g_{\parallel} = 2.366$ ,  $g_{\perp} = 2.070$ ) and hyperfine splitting constant ( $A_{\parallel} = 13.95$  mT) prove that an unpaired electron in Cu(II) with  $(3d)^9$  electron configuration is mainly located in the  $d_{x^2-y^2}$  orbital in an octahedral crystal field with a tetragonal distortion, i.e., in a square-coplanar coordination. According to the similarity of the EPR parameters to those in Cu(II) coordinated by two carboxylic group, such as glucuronic acid ( $g_{\parallel} = 2.360$ ,  $g_{\perp} = 2.066$ ,  $A_{\parallel} = 13.50$  mT), formate ( $g_{\parallel} = 2.383$ ,  $g_2 = 2.078$ ,  $g_3 = 2.109$ ) [5], and oxalic acid ( $g_{\parallel} = 2.35$ ,  $g_{\perp} = 2.06$ ) [6] at room temperature, it is concluded that Cu(II) in the crosslinks of Cu(II)/gellan system is four oxygen-coordinated in square coplanar by two carboxylic groups. The intensity of these species gradually increases with cooling temperature to 5 °C, indicating that the Cu(II)-carboxylic acid complex is formed between the gellan helices. Even if we reheat the gelled sample, EPR spectrum was never recovered to the original spectrum, for example for the sol state of Cu(II)/gellan system at 70 °C, as shown in Fig. 4. This means that the Cu(II) complexes with two COO<sup>-</sup> groups in crosslinks are quite stable and not thermally reversible. This high thermal stability of the crosslinks of Cu(II)/gellan system is caused to four covalent bonds between Cu(II) and carboxyl groups. On the other hand, the crosslink in Ca(II)/gellan system is formed via specific electric Coulombic interaction between Ca(II) and carboxyl groups and is easily destroyed by a thermal motion of the chains in the high temperature. The difference of the sol-gel transition,  $T_{sg}(\text{Cu}) \gg T_{sg}(\text{Ca})$  is attributed to the difference of the stability of the crosslink structure.

#### 4. CONCLUSION

According to the result by temperature dependence of  $G'$ ,  $G''$  and DSC curves, Miyoshi and Nishinari have reported that Ca(II) seems to be immediately associated with the gellan gum chains at temperatures higher than the conformational transition for gellan solution containing low concentration of  $\text{CaCl}_2$ . [1] In our results by  $G'$ ,  $G''$ , and CD, the situation was not distinguished in Ca(II)/gellans system. As mentioned above, however, EPR spectra of Cu(II) more directly reveal the identical phenomena in Cu(II)/gellan system, namely Cu(II) makes a covalent bonding bridge between a pair of the random coil chain of gellan at temperatures much higher than  $T_{ch}(\text{gellan})$ . Based on our observation, we propose the gelation mechanism in Cu(II)/gellan system in such a way that Cu(II) combines a pair of the random coil chain and then the partial coil-helix transition is immediately induced in the vicinity of the crosslink as the first stage of the gelation at higher temperatures. With decreasing temperature both the population of the helices and the number of the crosslinks via Cu(II) complex formation increase. Finally, Cu(II) causes an association of helices by bridging between helices. This final stage is evidenced by the monotonical increase of concentration of Cu(II) in the complex to 5 °C (data is not shown in this paper). [7] It is not clear from the present experiment

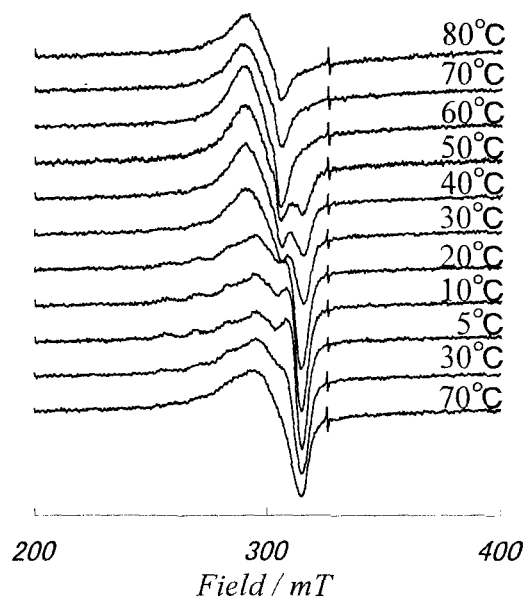


Fig. 4. EPR spectra of 2 mM Cu(II) in 1(w/w) % gellan solution in the cooling and the heating process.

whether the ordered structure is formed in the first stage of gelation or not. The temperature dependence in the EPR spectra of Cu(II) complex with carboxylic groups will give answer for this. The gelation mechanism itself seems to be similar between due to Cu(II) and due to Ca(II) or Mg(II) except the thermal stability of the crosslink and therefore the temperature dependence of the mechanism.

#### 5. ACKNOWLEDGEMENT

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