Visualization of Gellan Network Formation in Different Ionic Environment

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Abstract

Gellan gum is a bacterial polysaccharide with a negatively charged carboxyl ion in its monomer. It forms thermo-reversible gel due to the formation of a biopolymer network in an aqueous medium under suitable conditions, and its properties depend on the type of necessary cations to form a gel. In this study, the Atomic Force Microscopy (AFM) technique has been used to elucidate cation types effect on gellan network formation. AFM images were obtained for gellan gel samples prepared without and with adding salts of monovalent, divalent, and trivalent cations. It was found that gellan gel with monovalent ion did not show any fiber-like network. Instead, it showed a network formed by the association of globular objects. While with divalent ion, the presence of fiber-like objects was seen, and the network was formed due to the crosslinking of these fibers. The mixture of fibrous and globular structures is realized for the sample with added trivalent ion; however, the fibers are thicker than the gellan gel sample with divalent cations.

Keywords: Gellan gum, Polysaccharides, Biopolymer network, Gel, AFM

Introduction

Gellan is an anionic, water-soluble heteropolysaccharide with high molecular weight. It is composed of repetitive units of tetrasaccharide (β- D-glucose, β- D-glucuronic acid, β- D-glucose and α-L-rhamnose), and one carboxyl side group per repeating unit (Jansson, Lindberg and Sanford 1983) (O'Neill, Selvendran and Morris 1983). Gellan forms a physically cross-linked thermo-reversible gel.

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However, at low concentrations or sufficiently high temperatures, the gellan without salts does not form a gel as there is a lack of aggregation because of the carboxyl group (Chandrasekaran, et al. 1988). The carboxyl groups being negatively charged repel each other, which can be changed by introducing a metal cation in the gellan network (Moritaka, Fukuba, et al. 1991) (Moritaka, Nishinari, et al. 1992) (E. Ogawa 1996). The added cations act as the cross-linking agents and hold the helices together, leading to a continuous 3-dimensional gellan network. However, different cations promote aggregation differently. (Moritaka, Nishinari, et al. 1992) showed gellan with added K^+ ion is more effective in promoting gelation than Na^+, and the divalent cation like Ca^{2+} is more effective than any monovalent ion. Moreover, divalent cations can form gel quickly at far lower concentrations than monovalent cations (Moritaka, Fukuba, et al. 1991) (Miyoshi and Nishinari 1999) and have various applications in tissue engineering scaffolds (Xu, et al. 2018), self-healing (Lv, et al. 2019) . Therefore, the type, the valency, and the amount of the cations added to make the solution influence the gel properties of gellan gum. As most of its application depends on its gel-forming ability, the gelation mechanism for gellan is widely studied using differential scanning calorimetry (DSC), Light scattering (Okamoto, Kubota and Kuwahara 1993) ESR (Tsutsumi, et al. 1993), X-ray small-angle scattering (Yuguchi, et al. 1993) osmotic pressure (E. Ogawa 1993), ultrasonic velocities (Tanaka, Sakurai and Nakamura 1993) and viscoelastic measurements (Shimazaki and Ogino 1993) (Nakamura, Harada and Tanaka 1993).The sample used in these studies was a mixture of several different cations such as sodium, magnesium, potassium, or calcium. However, (Kirchmajer, et al. 2014) showed that gelation and mechanical properties of purified gellan are enhanced compared to commercial samples with a trace of different cations. As a result, gellan property can be significantly different for a specific cation than others, and its effect will surely be interesting. Unfortunately, not much research has been done on purified gellan.

To establish the association behavior of the gellan type bio macromolecules several models have been proposed using simulations and experimental observations. Most polysaccharide gelation models have concentrated on the detailed molecular structure of the junction zones within the gel. However, visualizing the bio macromolecules using direct observation by microscopic techniques is still at a very early stage. The cations aggregation of gellan monomer with the carboxyl side group can be directly observed under Atomic Force Microscopy with higher resolution than transmission electron microscopy (Gunning et al. 1996). The AFM image with and without added cation was studied by (Morris, Kirby, and Gunning 1999), and he showed gellan without added cations form helix without side-by-side aggregation. However, when cation is added with the potassium-based gellan aqueous solution, side-by-side aggregation of the

helical filaments and continuous branched network were created. They concluded from this study that the coil-helix transition allows the formation of filamentous structures, which, in the presence of gel-promoting cations, further assemble into branched fibers formed by the association of these filaments.

On the other hand, researchers found observing AFM images of 1.6% (w/w) gellan solution with 0.01 M KCl, CsCl, or 0.001 M $CaCl_2$ salt promotes branched rod-like structures and forms inter helical aggregation (Ikeda et al. 2004). Therefore, Atomic Force Microscopy (AFM) offers a method for investigating such models for gelation and visualizing the long-range distributions of macromolecules within the gel network. Again, most of the study used typically available commercial gellan powder, predominantly in the potassium salt form. The commercial gellan solution can be made free of these cations by dialysis against water and the study of network association of the dialyzed gellan with different types of cation can give an insight into the gellan gelation properties for specific cation.

In the present study, we aim to investigate the network association of dialyzed gellan with and without added three different cation using Atomic Force Microscopy (AFM).

Materials and Method

Materials

Gellan powder was obtained from San-ei Gen (Osaka, Japan) and used in this study without further purification. The metal content present in the sample was analyzed as Na=0.44%, K =4.78%, Ca= 0.34%, and Mg= 0.012% by a LIBERTY Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) system (Varian Inc., Palo Alto, CA, USA). Nanopure water was used to prepare the solution.

Sample preparation

Gellan sample without added salt

The powdered sample was mixed with Nanopure water to form a gellan solution. The mixture was stirred using a magnetic stirrer at 80^{o}C for 1 hour to ensure complete dissolution. The transparency of the solution recognized the complete dissolution. The hot solution was transferred to the cylindrical sample holder. A transparent gel was formed from the uniform solution at ambient temperature. In this way gellan solution of concentration C_p=0.8 and 1.6 wt. % was prepared.

Dialysis of Gellan Sample

The dialysis was carried out using the Wako dialysis membrane, which was cut, soaked in nano-pure water, and filled with gellan solution using a funnel. The dialysis membrane was then put into a beaker with 250 ml nano-pure water and stirred for 3 hours. The dialysis was carried out three times on the sample after a 1-hour interval to remove any existing metal ions from the gellan solution. The metal ion content after dialysis was measured by Shimadzu (AA-6800) Flame Atomic Absorption Spectrometer (FAAS) and the data showed a trace amount of potassium ion still existed at room temperature, 30°C (Table-1). The prepared solution then dialyzed again, but this time heat was applied. The metal ion content after dialysis at 50°C was analyzed yet using FAAS. It showed the existence of the metal contents in the sample are very low, and the effect of those ions can be considered negligible (Table-1).

Gellan Gel with added KCl, $CaCl_2$, and $AlCl_3$ Salt

Gellan with added salt was prepared by dissolving salt in Nanopure water, which was then stirred for 30 minutes at 80°C. The concentration of the stock salt solution made was double than required, and it was then mixed with the same amount of gellan solution. In this way, the salt and gellan solution's concentration was halved than the stock solution and five samples of polymer concentration C_p=0.8wt% without dialysis, C_p=0.8wt% with dialysis and no added salt concentration, C_p=0.8wt% with dialysis and added salt of concentration C_s=0.1M of KCl, $CaCl_2$, and $AlCl_3$ salt was prepared. The polymer concentration was chosen at 0.8wt% because, below this concentration, not all the cations with 0.1M salt concentration could form a gel and this experiment was done to check the effects of the cation in the gel state of gellan.

Atomic Force Microscopy

A small amount of the liquid solution was spread on the glass substrate and stored at room temperature 24 hours or more before visualizing the surface with Flex AFM 5 from nanosurf. This method was followed to prepare C_p= 0.8wt% with and without C_s = 0.1M KCl, 0.1M, and 0.1M gellan sample. After making samples according to the procedure described above, it was ensured that they remain homogenous and fresh enough to take the measurements with fewer errors. The images of the surface of the sample were made in contact mode using Nanosurf Easyscan-2 software. Most of the images obtained in this study contain 256 × 256 points. For each line, 0.7 seconds was given to have decently high-resolution images.

Table-1: Metal ion content in gellan solution after dialysis				
Sample Name	**Na (%)**	**K (%)**	**Ca (%)**	**Mg (%)**
0.8 wt. % Gellan Solution (non-dialyzed)	0.438	4.781	0.336	0.012
Gellan Solution (dialyzed at 30°C)	0.567	3.628	0.258	0.004
Gellan Solution (dialyzed at 50°C)	0.00127	0.0264	0.00696	0.005

Result and Discussion

This experiment explores the effect of monovalent, divalent, and trivalent cations on dialyzed gellan using Atomic Force Microscopy (AFM) images and to analyze the surfaces' morphology and microstructures. A total of 5 samples of the surface of non-dialyzed gellan without added salt, dialyzed gellan without added salt, and dialyzed gel with added monovalent, divalent, and trivalent cations were studied.

For all the samples, a 3μg/ml amount of the solution was deposited on the micro-glass slide and dried at room temperature for 24 hours. Figure 1 shows the micro-glass slide used for the sample deposition visualized by AFM. The average roughness of the glass slides was calculated in the area of 107.8 μm^2 was = 3.70 nm. In contrast, the native mica surface's roughness is 0.13 nm in the area of 670 nm^2 (Senden and Ducker 1992)

In the previous works on gellan gel, the mica surface was used for sample deposition (Gunning, et al. 1996; Ikeda, et al. 2004). Still, in this study, the glass surface was chosen as a substrate as it is rougher than the mica surface, so the solution will adhere to the surface rather than spreading. SEM and X-ray imaging confirms that the gellan molecule with added salt is a long chain and does not exist in a single strand but wraps around and forms a double helix (Yuguchi., Urakawa and Kajiwara 2002). The number of cross-links increases with increasing gellan concentration. But, at a lower concentration, this number is not large enough to build a network at ambient temperature. Hence gellan solution at ambient temperature for concentration less than 1wt% acts like a viscous liquid. Due to the lack of cross-links, all polymer chains clot together in groups and float in solvent forming a colloid solution. AFM image (Figure 2) revealed that C_p= 0.80 wt% for non- dialyzed samples had no continuous coverage of polymers rather than sample had discrete aggregates on the glass substrate.

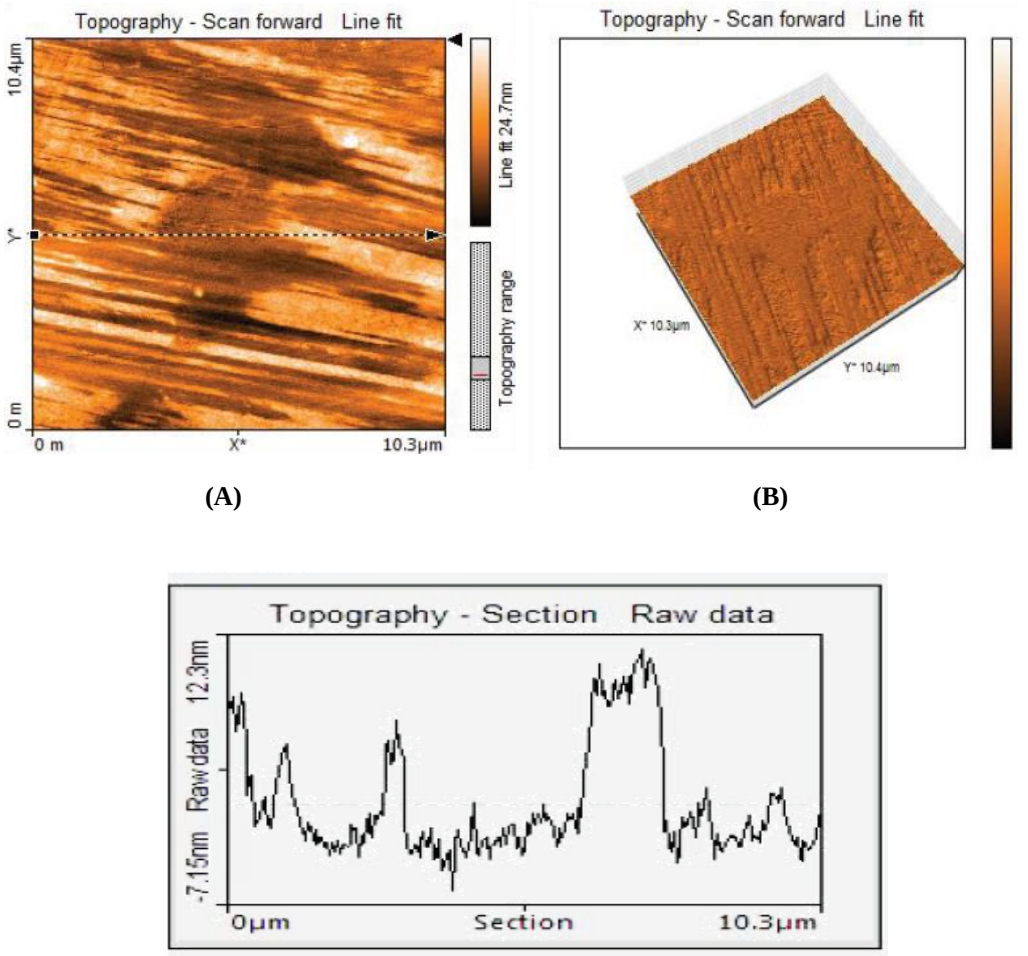

Figure 1: Topographical AFM image of glass substrate; (A) color map, (B) 3D view, and (C) height profile. The scanning size of the image is 10.3µm×10.4µm.

They were randomly oriented in all directions. Without dialysis, a gellan solution can have different types of metallic ions. Therefore, there may be different types of aggregation. In Figure 2, from the image of the non-dialyzed gellan film surface, a significant amount of globules was seen. The average diameter of these globules was 2.28µm. These globules suggest that since the gellan solution was taken at a low concentration, a lack of aggregation of the carboxyl groups caused gellan to not form any continuous network. However, the double-helical chains of gellan aggregated together and formed globules. Figure 3 reveals the surface after dialysis, and it showed there was no uniform network of branched fibers.

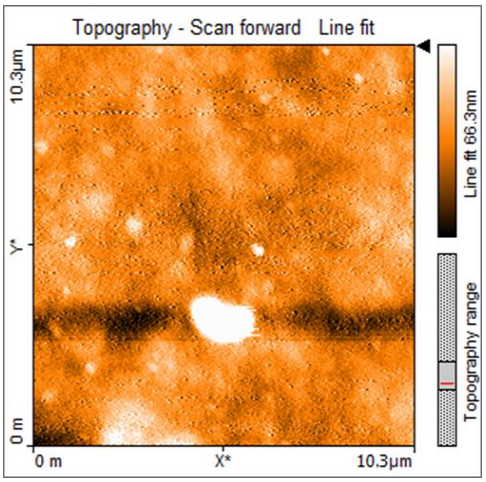

Figure 2: AFM images of 0.80 wt% non-dialyzed gellan gum without any salt. Image size: 10.3µm× 10.4µm.

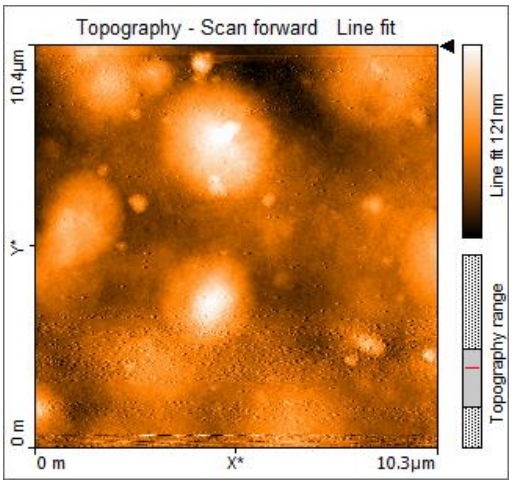

Figure 3: AFM images of 0.80 wt% dialyzed gellan gum without any salt. The scanning size of image size: 10.3µm×10.3µm.

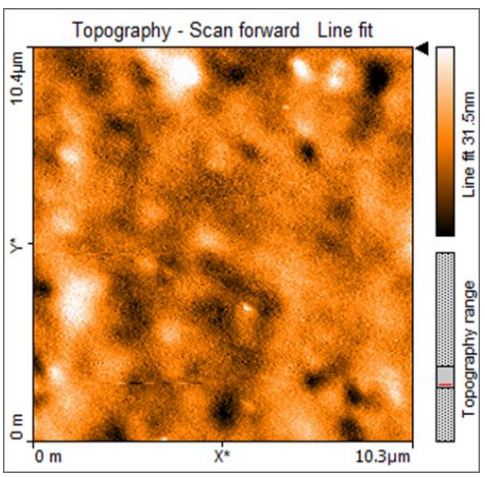

Figure 4: AFM images of 0.80 wt% dialyzed gellan gum with 0.1M KCl. Image size 10.3µm×10.4µm.

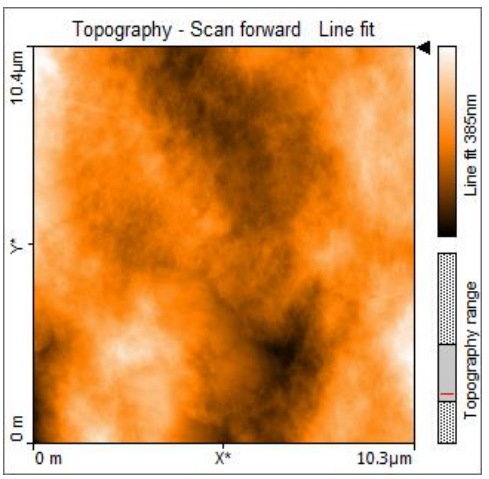

Figure 5(a): AFM images of 0.80 wt% dialyzed gellan gum with 0.1M $CaCl_2$. Image size 10.3µm×10.4µm.

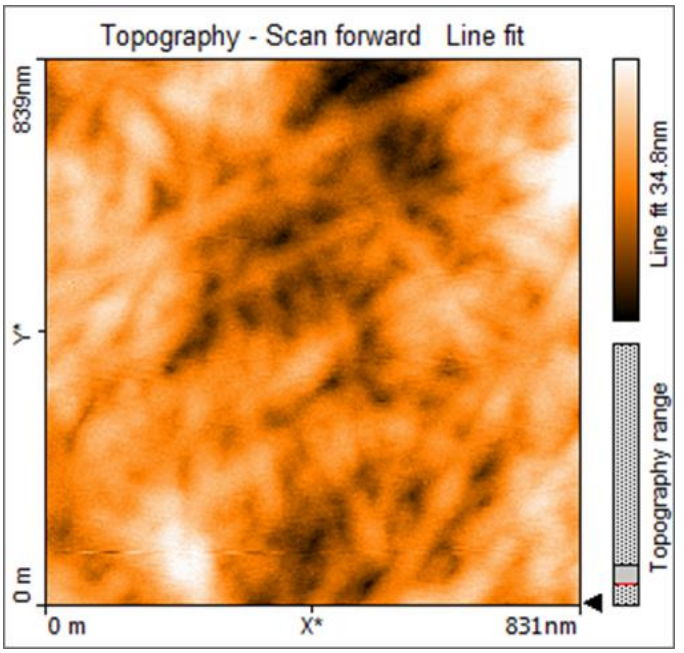

Figure 5(b): AFM images of 0.80 wt% dialyzed gellan gum with 0.1M $CaCl_2$. Image size 831nm×839 nm .

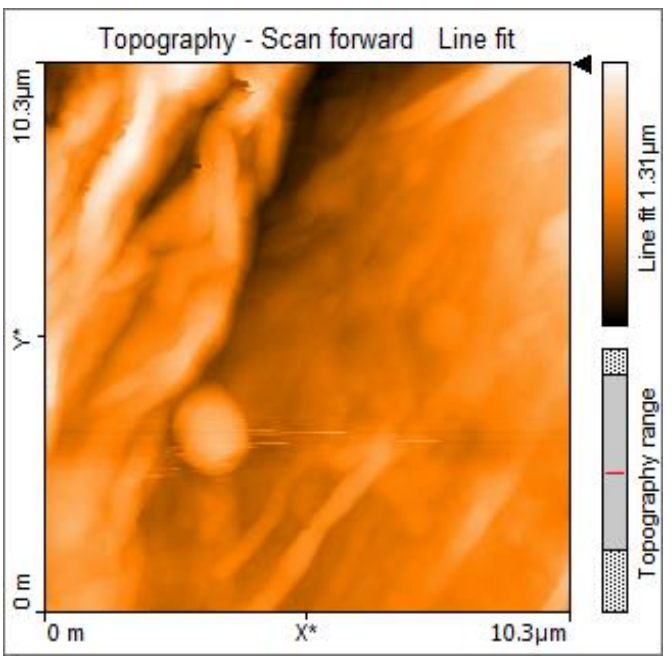

Figure 6: AFM images of 0.80 wt% dialyzed gellan gum with 0.1M $AlCl_3$. Image size 10.3µm×10.4µm.

The double-helical chains of gellan together formed globules, but there was no indication of large aggregates, which suggest the absence of gelation promoting cations (Ikeda, et al. 2004). Adding cations K+, $Ca^{2+,}$ and Al^{3+} to the dialyzed gellan showed a drastic change in the network formation as revealed from the gellan surface. It is known from the previous research that adding metallic ions increases the chances of cross-linking because gellan fibers have active sites, which are the main reason behind cross-linking and branching. When monovalent ions are added to the solution, each ion attaches to the site and shields the repulsion between two carboxyl ions resulting from this aggregation with occasional cross-linking was found (Ikeda, et al. 2004). But Figure 4 shows the chains with ions clot together and form spherical aggregates, and the fiber-like structure was absent for C_p= 0.80 wt% gellan gel with C_s=0.1M KCl salt. It can be attributed to the low concentration of K^+ ions, which were not enough for the huge number of active sites of the gellan solution (Ikeda et al. 2004). In the case of $CaCl_2$, when the sample was evaporated at room temperature, the gellan gum formed a continuous network, as seen in Figure 5 (a) and Figure 5(b). Figure 5(a), the image with scanning size 10.3μm×10.4μm did not show any evident network or fiber but when it was observed in high resolution with scanning size 831nm×839 nm (Figure 5(b)) long fibers and continuous network were visible (Morris, Kirby, and Gunning, 1999). As for trivalent cation, a more effective helix-helix crosslinking was observed with a continuous network formation (Figure 6). For $AlCl_3$, thick and long fibers were seen in high resolution. The strands were thicker and branched fibers than those observed when $CaCl_2$ was used. The network strands were created by side-by-side double helices. They were straight, and there were not any discrete junction jones. In gellan gel with $CaCl_2$ crystals seem to form with gellan fiber in it, and there were no micro particles, but in gellan gel with $AlCl_3$, networks, and micro particles formed together. Again, Al^{3+} is much smaller in size than Ca^{2+}. When Ca^{2+} becomes entangled with fibers, Al^{3+} can move freely between them. Also, irregular aggregates are visible in the sample.

It can be concluded that trivalent metallic ions, like divalent ions, can bond two or more chains together, forming branches and crosslinks. However, $CaCl_2$ makes a more complex network than $AlCl_3$ because of the difference in the ion size. The images are visually similar to that of $CaCl_2$, but the spherical micro particles were large here.

Conclusion

In this experiment, attempts have been made to visualize the association and network formation of gellan polysaccharides by studying the film surface of gellan gel after dialysis using Atomic Force Microscopy (AFM). The gellan concentration was chosen,

C_p=0.80 wt%, with and without added 0.1M of the monovalent, divalent, and trivalent cation. Non-dialyzed gellan without added cations show no formation of any networks because of the low concentration. In the presence of K^+ ion, a visible fiber-like network was not observed but spherical aggregates were observed which suggested that the low amount of fibers with ions aggregated together to form spherical aggregates. Besides, the $CaCl_2$ gellan image showed a continuous network with fiber structure and $AlCl_3$ formed a mixture of fibrous and globular structures. The images are consistent with the fibrous model of gellan gum. Therefore, it can be concluded from the images that two different types of gellan structures were observed; they were fibrous and globular. The fiber-like aggregate was found on the surface of divalent and trivalent gellan films, whereas globular aggregates were found on monovalent gellan film.

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References

Chandrasekaran, R., L.C. Puigjaner, K.L. Joyce, and S. Arnott. 1988. "Cation interactions in gellan: an X-ray study of the potassium salt." *Carbohydrate Research* 181: 23-40.

Gunning, A. P., A. R. Kirby, M. J. Ridout, G. J. Brownsey, and V. J. Morris. 1996. " Investigation of gellan networks and gels by atomic force microscopy." *Macromolecules* 29 (6791–6796).

Ikeda, S., Y. Nitta, T. Temsiripong, R. Pongsawatmanit, and K. Nishinari. 2004. "Atomic force microscopy studies on cation-induced network formation of gellan." *Food Hydrocolloids* 727-735.

Jansson, P. E., B. Lindberg, and P. A. Sanford. 1983. "Structural studies of gellan gum, an extracellular polysaccharide elaborated by Pseudomonas elodea." *Carbohydrate Research* 124: 135–139.

Kirchmajer, D.M., B. Steinhoff, H. Warren, R. Clark, and M. Panhuis. 2014. "Enhanced gelation properties of purified gellan gum." *Carbohydrate Research* 388 (125-129).

Lv, Yukai, Zheng Pan, Cunzheng Song, Yulong Chen*, and Xin Qian*. 2019. "Locust bean gum/gellan gum double-network hydrogels with superior self-healing and pH-driven shape-memory properties." *Soft Matter*.

Miyoshi, E., and K. Nishinari. 1999. "Rheological and thermal properties near the sol–gel transition of gellan gum aqueous solutions ." *Progress in Colloid and Polymer Science* 114 (68–82).

Moritaka, H., H. Fukuba, K. Kumeno, N. Nakahama, and K. Nishinari. 1991. "Effect of monovalent and divalent cations on the rheological properties of gellan gels ." *Food Hydrocolloids* 495-507.

Moritaka, H., K. Nishinari, N. Nakahama, and H. Fukuba. 1992. "Effects of potassium chloride and sodium chloride on the thermal properties of gellan gum gels." *Bioscience, biotechnology, and biochemistry* 595-599.

Morris, V.J., A.R. Kirby, and A.P. Gunning. 1999. "A fibrous model for gellan gels from atomic force microscopy studies." In *Progress in Colloid and Polymer Science*, by K. Nishinari, volume 114. Physical Chemistry and Industrial Application of Gellan Gum.

Nakamura, K., K. Harada, and Y. Tanaka. 1993 . "Viscoelastic properties of aqueous gellan solutions: the effects of concentration on gelation ." *Food Hydrocolloid* 7 (435-447).

O'Neill, M. A., R. R. Selvendran, and V. J. Morris. 1983. " Structure of the acidic extracellular gelling polysaccharide produced by Pseudomonas elodea. Carbohydrate Research, 124, 123–133." *Carbohydrate Research* 123–133.

Ogawa, E. 1996. "Conformational transition of polysaccharide sodium-gellan gum in aqueous solutions." *Macromolecules* 5178-5182.

Ogawa, Etsuyo. 1993. "Osmotic pressure measurements for gellan gum aqueous solutions." *Food Hydrocolloid* 7 (397-405).

Okamoto, T., K. Kubota, and N. Kuwahara. 1993. "Light scattering study of gellan gum." *Food Hydrocolloids* 7 (363- 371).

Senden, T. J., and W. A. Ducker. 1992. "Surface roughness of plasma-treated mica. ." *Langmuir*, 8(2), 733–735.

Shimazaki, T., and K. Ogino. 1993 . "Viscoelastic properties of gellan gum aqueous solutions ." *Food Hydrocolloid* 7 (417-426).

Tanaka, Y., M. Sakurai, and K. Nakamura. 1993. "Ultrasonic velocities in aqueous gellan solutions." *Food Hydrocolloid* 7 (407-415).

Tsutsumi, A., D. Ya, T. Hiraoki, H. Mochiku, R. Yamaguchi, and N. Takahashi. 1993. " ESR studies of Mn(II) binding to gellan and carrageenan gels ." *Food Hydrocolloids* 427-434.

Xu, Zihao, Zhuqing Li, Shan Jiang, and and Kaitlin M. Bratlie. 2018. "Chemically Modified Gellan Gum Hydrogels with Tunable Properties for Use as Tissue Engineering Scaffolds." *ACS Omega* 6998-7007.

Yuguchi, Y., M. Mimura, Kitamura.S., H. Ura kawa, and K. Kajiwara. 1993 . "Structural characteristics of gellan in aqueous solution ." *Food Hydrocolloid* 7, 373-385.

Yuguchi., Y., H. Urakawa, and K. Kajiwara. 2002. "The effect of Potassium salt on the structural characteristics of gellan gum gel." *Food Hydrocolloids* Volume 16, Issue 3; 191-195.