

# Molecular Origin for Strong Agarose Gels: Multi-Stranded Hydrogen Bonding

Masakuni Tako<sup>1,\*</sup>, Takeshi Teruya<sup>1,2</sup>, Yukihiro Tamaki<sup>1,3</sup>, Keiko Uechi<sup>1</sup>, Teruko Konishi<sup>1</sup>

<sup>1</sup>Department of Subtropical Bioscience and Biotechnology, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan

<sup>2</sup>TaKaRa Bio Inc., 7-4-38 Nojihigashi, Kusatsu, Shiga 525-0058, Japan

<sup>3</sup>Molecular Bioscience Research Center, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan

\*Corresponding author: [tako@agr.u-ryukyu.ac.jp](mailto:tako@agr.u-ryukyu.ac.jp)

Received May 05, 2021; Revised May 12, 2021; Accepted May 14, 2021

**Abstract** Agarose gels are currently used in separation, purification, and characterization of DNA, RNA, proteins, and polysaccharides in gel electrophoresis, gel filtration, affinity chromatography, and ion chromatography techniques. Specifically, it is used in PCR (Polymerase Chain Reaction) test. Although, double stranded intermolecular hydrogen bonding between OH-2 and 3,6-ring oxygen atoms of 1,4-linked anhydro- $\alpha$ -L-galactopyranose residues on different molecules take place, triple- or multi-stranded secondary association occur with increasing concentration. The multi-stranded gelation mechanism of agarose molecules is the first to report. The associated agarose molecules play a dominant role in the centre of tetrahedral cavities that are occupied by ice-like hydrogen bonded water molecules which are caused thermodynamically by cage and hydrophobic effects. Many investigations the gelling properties of the polysaccharides have been undertaken to elucidate the structure-function relationship, but no other researchers have established the mechanisms at the molecular level including water molecules. There are structural and theoretical consistencies in our investigation. This paper provides important information not only academia, but also to industrial fields, such as bio-physicochemical analysis, food, cosmetics, agriculture, pharmaceuticals, drug delivery, drug storage, tissue engineering, and biotechnology.

**Keywords:** agarose, multi-stranded hydrogen bonding, gelation mechanism, principles, biotechnology

**Cite This Article:** Masakuni Tako, Takeshi Teruya, Yukihiro Tamaki, Keiko Uechi, and Teruko Konishi, "Molecular Origin for Strong Agarose Gels: Multi-Stranded Hydrogen Bonding." *Journal of Polymer and Biopolymer Physics Chemistry*, vol. 9, no. 1 (2021): 13-19. doi: 10.12691/jpbpc-9-1-2.

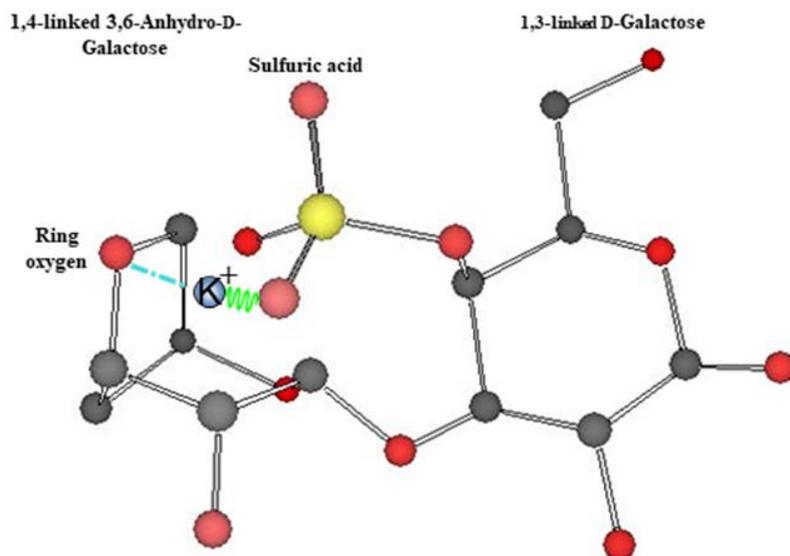
## 1. Introduction

Agarose isolated from family of red seaweeds has many useful characteristics, such as hydrophilicity, biodegradability, biocompatibility, and strong thermoreversible gelation ability. It is currently used in drug delivery [1,2] and tissue engineering [3]. In the course of rheological study of industrial useful polysaccharides, gelation mechanisms of  $\kappa$ -carrageena [4,5],  $\iota$ -carrageenan [6], agarose [7], gellan gum [8,9], amylose [10,11], curdlan [12], alginate [13,14], and deacetylated rhamnan gum [15] where hemiacetal oxygen atom, OH-, CH<sub>3</sub>-, COOH-, or sulfate groups of sugar residues with hydrogen bonding, van der Waals forces of attraction, ionic bonding, or electrostatic forces of attraction involving have been reported at molecular level. The co-gelation mechanism of xanthan [16,17,18] and plant galactomannan [19-24], and glucomannan [24,25] have also been reported. The tri-saccharide side chains of the former contribute to the interaction with the main chains (mannan) of the latter that is for the D-mannose-specific. Furthermore, the gelatinization and retrogradation mechanisms of rice [26,27], potato [28]

and wheat [29] starches have been discussed. The short side-chains of amylopectin contribute to the interaction with the amylose molecules. The molecular origin for thermal stability of schizophyllan [30] and rice amylopectin [31,32,33] have also been reported. We discuss herein the structure-function relationship of agarose in comparison with those of carrageenans and propose multi-stranded hydrogen bond contributing its strong gelling characteristics.

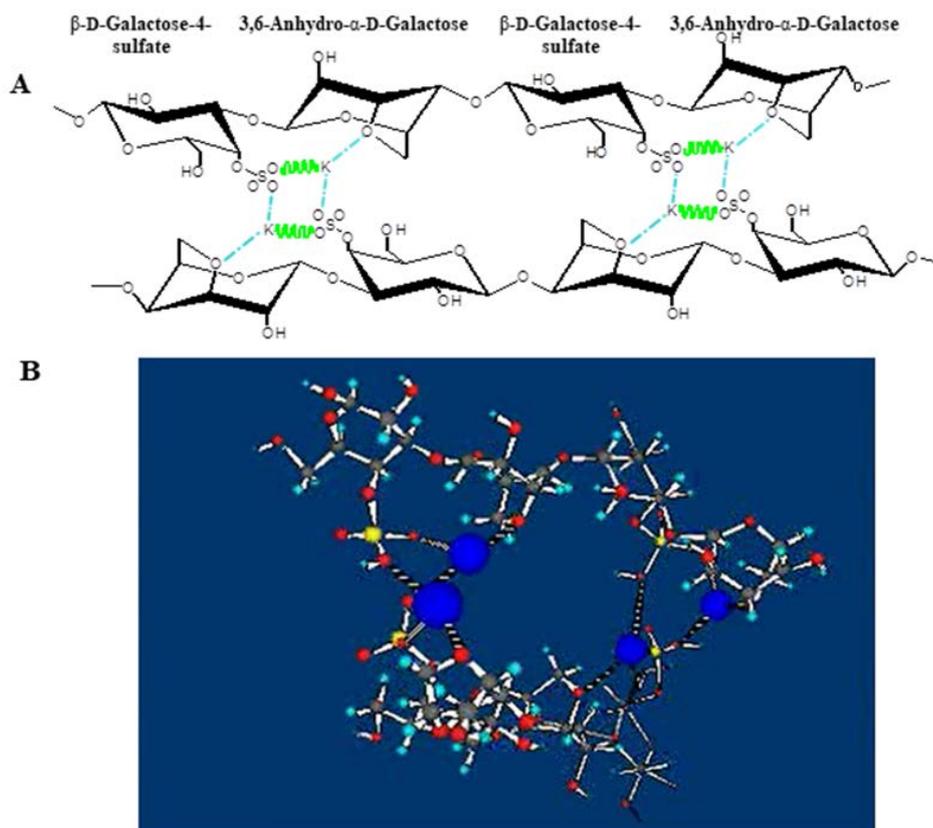
## 2. Potassium Induced Gelation Mechanism of $\kappa$ -Carrageenan

$\kappa$ -Carrageenan is a cation-selective binding polymer [4,5,34], such as 18-crown-6-ether, which gels in the presence of the large-site cation, K<sup>+</sup>, but does not do so in the presence of the small cation Na<sup>+</sup>. An intramolecular K<sup>+</sup>-bridge with electrostatic forces of attraction has been found between the 3,6-ring oxygen atom of 4-linked anhydro- $\alpha$ -D-galactopyranose and the sulfate oxygen atom at C4 of 3-linked  $\beta$ -D-galactopyranose as shown in Figure 1 [4]. This was the first report at the molecular level.



Atoms: Carbon, grey; Oxygen, red, Sulfur, yellow. Small cation,  $\text{Na}^+$  (van der Waals radius, 0.227 nm) is not able to associate, but large cation,  $\text{K}^+$  (0.275 nm) to build up. Chemical bonds: ionic bonding; electrostatic forces of attraction.

Figure 1. Intramolecular  $\text{K}^+$ -bridge on  $\kappa$ -carrageenan molecule



(A) Intra- and intermolecular associations, Chemical bonds, ionic bonding and electrostatic forces of attraction. (B) 3D-Model for gelling  $\kappa$ -carrageenan molecules. Atoms, Carbon, grey; Oxygen, red; Hydrogen, blue; Sulfur, yellow.

Figure 2. Potassium induced gelation mechanism of  $\kappa$ -carrageenan

The intermolecular association between  $\text{K}^+$  and oxygen atoms of sulfate groups on different molecules was thus discovered (Figure 2A) [5]. As shown in Figure 2B, anti-parallel double helical conformation take place at gelling state where potassium cations are involved in

inside of the helix. The cloudy gel melted above the transition temperature ( $25^\circ\text{C}$ ). Specifically, the polysaccharide showed synergistic strong gel with plant galactomannan [5,35]. The polymer is widely used in food industry as gelling, thickening and binding agent.

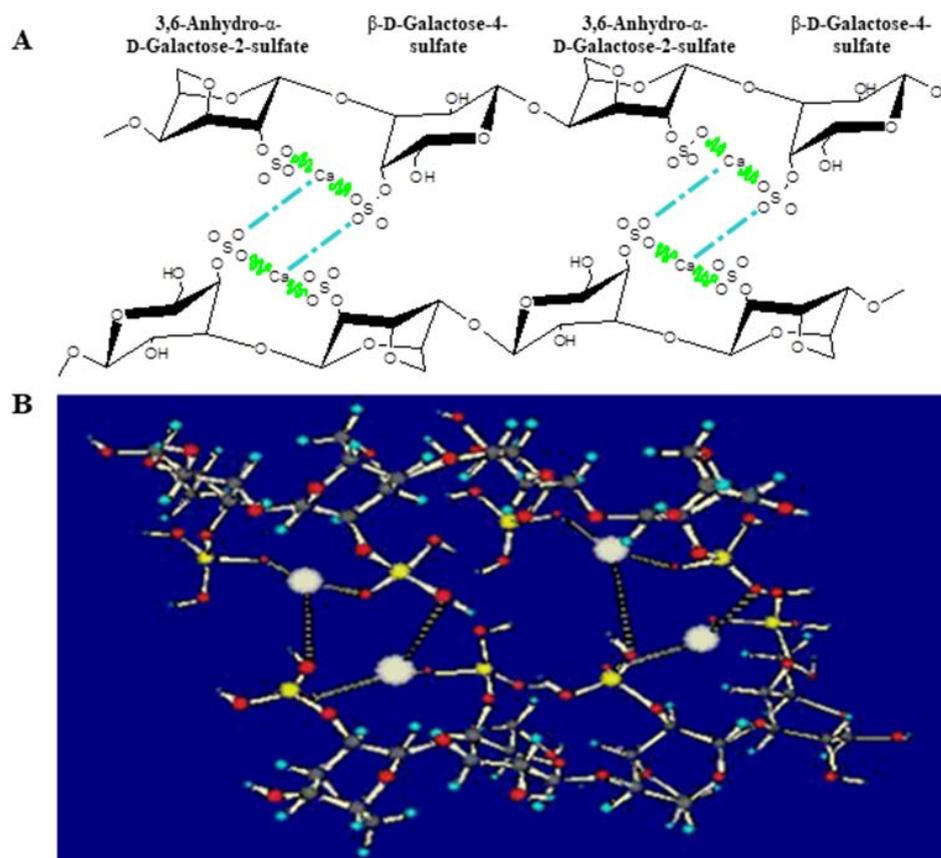
### 3. Calcium Induced Gelation Mechanism of $\iota$ -Carrageenan

$\iota$ -Carrageenan [6,36,37] is more highly sulfated galactan (30%) and is used in food and cosmetic. We concluded that this polymer involves an intramolecular association through  $\text{Ca}^{2+}$  cation between sulfate oxygens substituted at C-4 of  $\beta$ -D-galactopyranose and C-2 of anhydro- $\alpha$ -D-galactopyranose with ionic bonding and intermolecular  $\text{Ca}^{2+}$ -bridges on different molecules with electrostatic forces of attraction (Figure 3A) [6]. 3D-Model for gelling  $\iota$ -carrageenan is presented in Figure 3B. The model corresponds to anti-parallel double stranded helix. The calcium atoms are involved in inside of the helix.

The intra- and inter-molecular calcium bridges of  $\iota$ -carrageenan differs essentially from that of the  $\kappa$ -carrageenan, because the former consists of double ionic forces and electrostatic forces, whereas the latter consists of single ionic force and triple electrostatic forces. These different mechanisms provide an explanation for the conformational transition of  $\iota$ - and  $\kappa$ -carrageenan in aqueous solution. Since ionic forces are stronger than electrostatic forces of attraction, the molecular chain become rigid even at intermediate temperature (45 °C). The polysaccharide also showed a cloudy gel.

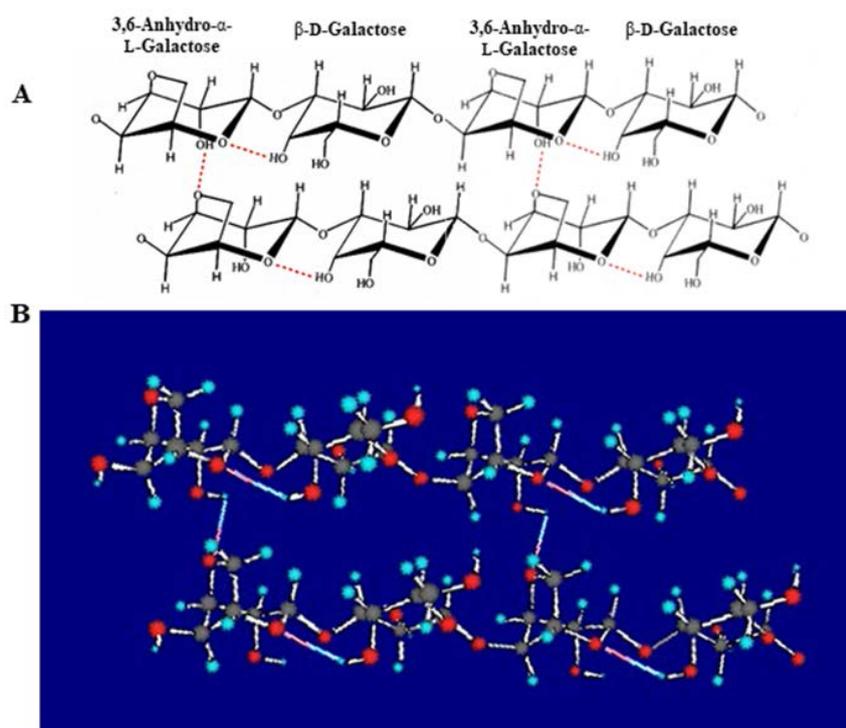
### 4. Hydrogen Bonding in Agarose Molecules

Agarose is the major components of agar [7,38,39,40,41], the structure of which is similar to that of the former polymers except for the sulfate content and L-configuration. The polymer exhibits reversible sol-gel transition upon heating and cooling, and gels at a minimum concentration of 0.08% or 0.13% (W/V) at low (4°C) or room temperature [7]. A very large elastic modulus ( $G'$ ) occurred in 0.08% agarose solution with the addition of  $\text{MgCl}_2$  (13.3 mM), which was 4-fold that after the addition of  $\text{NaCl}$  (16.9 mM),  $\text{KCl}$  (13.3mM), or  $\text{CaCl}_2$  (9.0 mM) caused by the salting-out effect. This result indicates that  $\text{Mg}^{2+}$  cations more easily form tetrahedral hydrogen-bonded water molecules ( $\text{H}_2\text{O}$ ) like ice than  $\text{Na}^+$ ,  $\text{K}^+$ , or  $\text{Ca}^{2+}$ . The gels melted down at high temperatures (>60°C). Furthermore, the small elastic modulus was observed with addition of urea (4 M). On the basis of the results, gelation mechanism for agarose molecules has been reported [7]. Intramolecular hydrogen bonding takes place between the hemiacetal oxygen atom of 1,4-linked 3,6-anhydro- $\alpha$ -L-galactopyranose and OH-4 of the adjacent 1,3-linked D-galactopyranose residue, which involved even at high temperatures (>60°C).



(A) Intra- and intermolecular associations, Chemical bonds,  ionic bonding and  electrostatic forces of attraction. (B) 3D-Model for gelling  $\iota$ -carrageenan molecules. Atoms, Carbon, grey; Oxygen, red; Hydrogen, blue; Sulfur, yellow; Calcium, white.

**Figure 3.** Calcium induced gelation mechanism of  $\iota$ -carrageenan



(A) Intra- and intermolecular associations, The red dotted lines represent hydrogen bonding. The models correspond to double stranded conformation.  
 (B) 3D-Model for gelling agarose molecules, Atoms: Carbon, grey; Oxygen, red; Hydrogen, blue.

**Figure 4.** Intra- and intermolecular hydrogen bonding of agarose at low concentration

Intermolecular hydrogen bonding also takes place between the 3,6-ring oxygen atom and OH-2 which are oriented in the axial configuration of anhydro-L-galactopyranose residues on different molecules, as shown in Figure 4A and 4B [7]. The optically transparent gel was observed in agarose molecules due to the formation of intermolecular hydrogen bonds associated in parallel, similar to a lattice, along the polymer molecules where no absorption of visible light occurred, as shown in Figure 4A and 4B. Such structures are observed in daily life on the cutting end of agarose (agar) gels where involving alternate phases of the associated polymer strands and water molecules. The transparent gel was also obtained in  $\kappa$ -carrageenan-like agarose which isolated from *Gracilaria coronopifolia* [40], but broke down 20 min after preparation caused by substitution with sulfate group at C-4 of  $\beta$ -D-galactopyranose residue. The intra- and intermolecular hydrogen bond have been supported by  $H^1$ - and  $C^{13}$ -NMR analysis [42]. Consequently, the gelling mechanism of  $\kappa$ -carrageenan,  $\iota$ -carrageenan and agarose molecules are quite different.

## 5. Principles of Agarose Gels

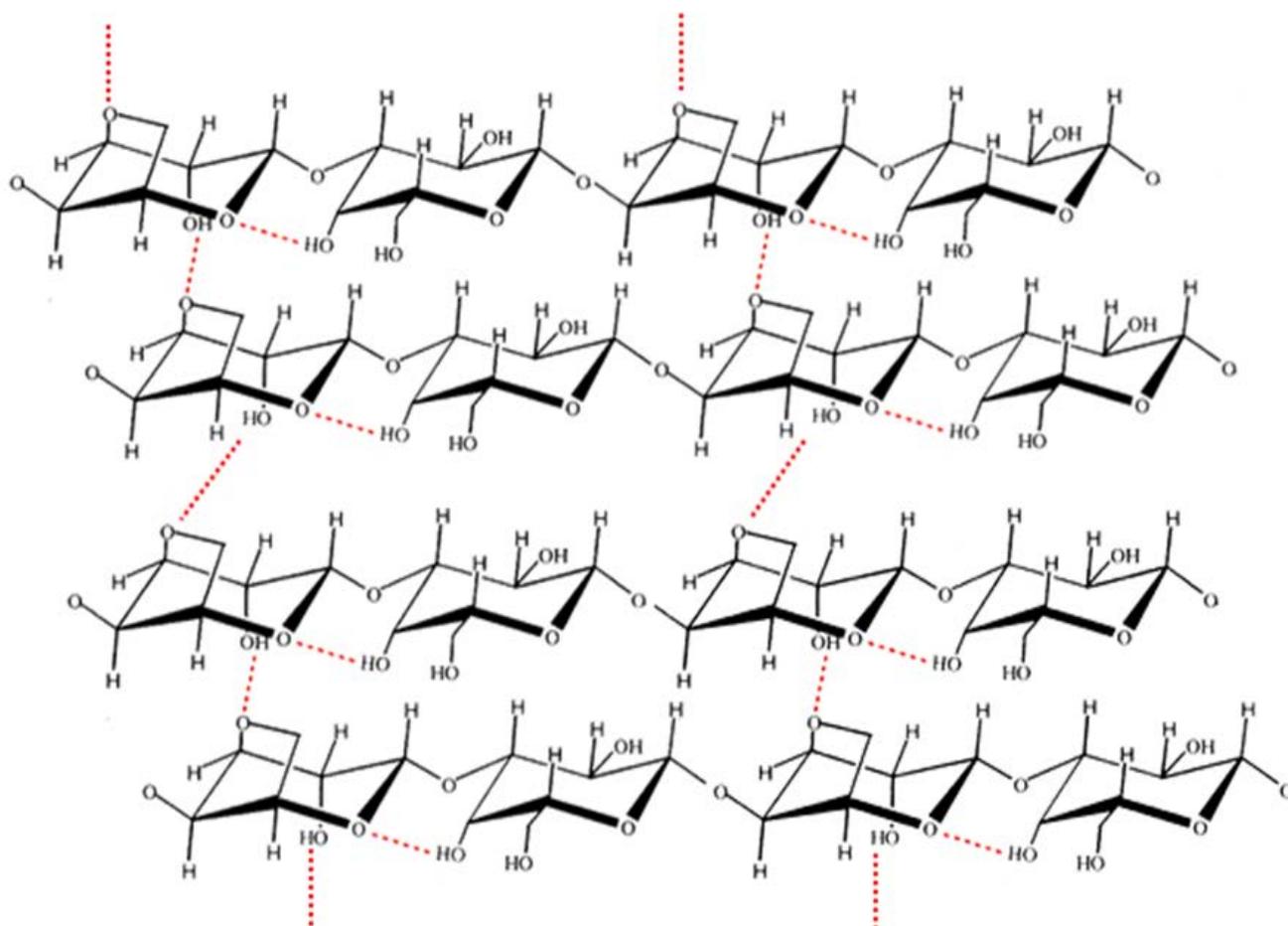
On the basis of this and previous analyses [7,43,44,45], the agarose molecules play a dominant role in the centre of tetrahedral cavities that are occupied by water molecules. This arrangement is similar to a tetrahedral ice-like structure and should lead to a cooperative effect. This effect extends the regions to ice-like hydrogen bonding with water molecules and then forms a cluster. The cage and hydrophobic effects thermodynamically contribute to

gel-formation processes where HO- and hemiacetal oxygens participate in the hydrogen bonding that occurs between the polymer and water, and between water molecules caused by a decrease in entropy.

## 6. Multi-stranded Hydrogen Bonding in Agarose Molecules

The  $H_2O$  molecule can participate in four hydrogen bonds two of them involving the two hydrogens and the lone pairs of electrons of the oxygen with two neighboring hydrogens. This tetrahedral directed hydrogen bonding is involved in the gelling waters in agarose solution. At a minimum concentration of 0.08- 0.13% (W/V) in water (99.92-99.87%), the intra- and intermolecular hydrogen bonded agarose molecules changed into an ice-like structure with the formation of hydrogen bonding between the polymer and water, and between water molecules, which subsequently resulting in gelation even at room temperature ( $>0.13\%$ ). Such dramatic changes from liquid into gels have generally been understood at the molecular level.

Consequently, as the active groups, 3,6-ring oxygen atoms and OH-2 of anhydro- $\alpha$ -L-galactopyranose residues face the other side (see Fig.4 and 5), the gelling agarose molecules adopt double-, triple-, and/or multi-stranded (Figure 5) conformation in parallel depending on the polymer concentration. It is well understood that the strong gels are attributed to the multi-stranded hydrogen bonding. Although the multi-stranded gelation mechanism has been suggested in previous study [7], the scheme is the first to report.



Red dotted lines represent hydrogen bonding.

Figure 5. Multi-stranded hydrogen bond in agarose molecules at high concentrations

The multi-stranded gelation mechanism explains that agarose gels are currently used in the separation, purification, and characterization of biomacromolecules (DNA, RNA, proteins and polysaccharides) in gel electrophoresis, gel filtration, affinity chromatography, and ion chromatography techniques.

As the OH-2 and OH-6 of the 3-linked  $\beta$ -D-galactopyranose residues are free from secondary associations, these groups can be substituted with active or functional agents (ingredients) or group to make derivatives. Furthermore, the cavities of polymer strands are occupied by tetrahedral-directed hydrogen bonds between water molecules so that drug or functional agents (ingredients) are available to easily become entrapped into the strands.

## 7. Conclusions

The followings summarize the principles of agarose gelling processes:

- 1) The intramolecular hydrogen bonding between OH-4 of 1,3-linked  $\beta$ -D-galactopyranose and the adjacent hemiacetal oxygen atom of 1,4-linked 3,6-anhydro- $\alpha$ -L-galactopyranose preferentially takes place to rigidify molecules. The bonding is stable even at high temperatures ( $>60^{\circ}\text{C}$ ).

- 2) The intermolecular hydrogen bonding between OH-2 and the 3,6-ring oxygen atoms of the  $\alpha$ -L-galactopyranose residues is built on different molecules in parallel contributing in the transparent gel. The number of molecular chains associated increases in concentration from double- to multi-stranded.
- 3) Tetrahedral directed hydrogen bonding as that observed in ice is thermodynamically built up between OH- and the hemiacetal oxygen atoms of sugars and water, and between water molecules themselves by cage and hydrophobic effects that correspond to a cluster.
- 4) Consequently, the agarose gels are the total of the cluster.

There are structural and theoretical consistencies in our investigations. This paper provides important information not only academia, but also to industrial fields, such as bio-physicochemical analysis, food, cosmetics, agriculture, pharmaceuticals, drug delivery, tissue engineering, and biotechnology.

## Acknowledgements

Tako, wishes to thank the late Professor Danji Nomura his supervisor in the Doctoral Course in Food Science and

Technology of Kyushu University for encouraging discussions about Nobel Laureate Hans von Euler-Chelpin (Chemistry, 1929). Emil Fischer, van't Hoff, Max Planck, Hermann Nernst and Svante Arrhenius were influential in this work as professors of von Euler at Berlin, Göttingen and Stockholm Universities.

Professor Nomura was also encouraging Tako talking about Dr. Masuzo Shikata Professor of Kyoto University and Dr. Umetaro Suzuki Professor of The University of Tokyo and Institute of Physical and Chemical Research.

The study of the structure-function relationship of polysaccharides first began when listening to the First Movement of Piano Sonata No. 14 Op. 27-2 "Moonlight" composed by Ludwig van Beethoven.

## Statement of Competing Interests

The authors declare no competing interests.

## References

- [1] Witzler, M., Ottensmeyer, P. F., Gericke, M., Heinze, T., Tobiasch, E. and Schulze, M. Non-cytotoxic agarose/hydroxyapatite composite scaffolds for drug release. *Int. J. Mol. Sci.*, 2019, 20 (14), 3565-3583.
- [2] Stefani, R.M., Lee, A. J., Tan, A.R., Halder, S.S., Hu, Y., Guo, X.E., Stoker, A.M., Ateshian, G. A., Marra, K. G. and Hung, J.C.T. Sustained low-dose dexamethasone delivery via a PLGA microsphere-embedded agarose implant for enhanced osteochondral repair. *Acta Biomaterialia*, 2020, 102 (1), 326-340.
- [3] Zarrintaj, P., Manouchehri, S., Ahmadi, Z., Saeb, M.R., Urabanska, A.M., Kaplan, D. L. and Mozafari, M. Agarose based biomaterials for tissue engineering. *Carbohydr. Polym.* 2018, 187 (1), 66-84.
- [4] Tako, M. and Nakamura, S. Indicative evidence for a conformational transition in  $\kappa$ -carrageenan from studies of viscosity-shear rate dependence. *Carbohydr. Res.* 1986, 155 (1), 200-205.
- [5] Tako, M.; and Nakamura, S. Synergistic interaction between  $\kappa$ -carrageenan and locust bean gum in aqueous media. *Agric. Biol. Chem.*, 1986, 50 (11), 2817-2822.
- [6] Tako, M., Nakamura, S. and Kohda, Y. Indicative evidence for a conformational transition in  $\iota$ -carrageenan. *Carbohydr. Res.*, 1987, 161 (2), 247-255.
- [7] Tako, M. and Nakamura, S. Gelation mechanism of agarose. *Carbohydr. Res.* 1988, 180 (2), 277-284.
- [8] Tako, M., Sakae, A. and Nakamura, S. Rheological properties of gellan gum in aqueous media. *Agric. Biol. Chem.* 1989, 53 (3), 771-776.
- [9] Tako, M., Teruya, T., Tamaki, Y. and Konishi, T. Molecular origin for rheological characteristics of native gellan gum. *Colloid Polym. Sci.* 2009, 287 (9), 1445-1454.
- [10] Tako M. and Hizukuri, S. Evidence for conformational transition in amylose, *J. Carbohydr. Chem.* 1995, 14 (4-5), 613-622.
- [11] Tamaki, Y., Konishi, T. and Tako, M. Gelation and retrogradation mechanism of wheat amylose. *Materials*, 2011, 4 (10), 1763-1775.
- [12] Tako, M. and Hanashiro, I. Evidence for a conformational transition in curdlan. *Polym. Gels Networks*, 1997, 5 (2), 241-250.
- [13] Tako, M. and Kohda, Y. Calcium induced association characteristics of alginate. *J. Appl. Glycosci.*, 1997, 44 (2), 153-159.
- [14] Teruya, T., Tamaki, Y., Konishi, T. and Tako, M. Rheological characteristics of alginate isolated from commercially cultured *Nemacystus decipiens* (Itomozuku). *J. Appl. Glycosci.*, 2010, 57 (1), 7-12.
- [15] Tako, M., Tohma, S., Taira, T. and Ishihara, M. Gelation mechanism of deacetylated rhamnan gum. *Carbohydr. Polym.* 2003, 54 (3), 279-285.
- [16] Tako, M., Nagahama, T. and Nomura, D. Non-Newtonian flow and dynamic viscoelasticity of xanthan gum., *Nippon Noeigakagaku Kaishi.*, 1977, 51 (8), 513-518.(in Japanese).
- [17] Tako, M. and Nakamura, S. Rheological properties of deacetylated xanthan gum in aqueous media, *Agric. Biol. Chem.*, 1984, 48 (12), 2987-2993.
- [18] Tako, M. Molecular origin for rheological characteristics of xanthan gum. *ACS Symp. Ser.*, 1992, 489, 268-281.
- [19] Tako, M., Asato, A. and Nakamura, S. Rheological aspect of the intermolecular interaction between xanthan gum and locust bean gum in aqueous media, *Agric. Biol. Chem.*, 1984, 48 (12), 2995-3000.
- [20] Tako, M. and Nakamura, S. Synergistic interaction between xanthan and guar gum. *Carbohydr. Res.* 1985, 138 (1), 207-213.
- [21] Tako, M. and Nakamura, S. Synergistic interaction between xanthan and D-galacto-D-mannan. *FEBS Lett.* 1986, 204 (1), 33-36. (Special issue at 17<sup>th</sup> FEBS Meeting, August 24-26, 1986, West Berlin, West Germany).
- [22] Tako, M. Synergistic interaction between xanthan and tara-bean gum. *Carbohydr. Polym.*, 1991, 15 (3), 227-239.
- [23] Tako, M. Binding sites for D-mannose-specific interaction between xanthan and galactomannan, and glucomannan. *Colloid Sur. B Biointerface*, 1993, 1 (2), 125-131.
- [24] Tako, M., Teruya, T., Tamaki, Y. and Ohkawa, K. Co-gelation mechanism of xanthan and galactomannan. *Colloid Polym. Sci.*, 2010, 288 (10-11), 1161-1166.
- [25] Tako, M. Synergistic interaction between xanthan and konjac glucomannan in aqueous media. *Biosci. Biotechnol. Biochem.*, 1992, 56 (8), 1188-1192.
- [26] Tako, M. and Hizukuri, S. Gelatinization mechanism of rice starch. *J. Carbohydr. Chem.*, 1999, 18 (5), 573-584.
- [27] Tako, M. and Hizukuri, S. Retrogradation mechanism of rice starch. *Cereal Chem.*, 2000, 77 (4), 473-477.
- [28] Tako, M. and Hizukuri, S. Gelatinization mechanism of potato starch. *Carbohydr. Polym.*, 2002, 48 (4), 397-401.
- [29] Tako, M., Tamaki, Y., Konishi, T., Shibanuma, K., Hanashiro, I. and Takeda, Y. Gelatinization and retrogradation characteristics of wheat (Rosella) starch, *Food. Res. Int.*, 2008, 41 (8), 797-802.
- [30] Tako, M. Molecular origin for thermal stability of schizophyllan. *Polym. Gels Network.*, 1996, 4 (4), 303-313.
- [31] Tako, M. and Hizukuri, S. Molecular origin for thermal stability of rice amylopectin. *J. Carbohydr. Chem.*, 1997, 16 (4-5), 655-666.
- [32] Tako, M. Molecular origin for thermal stability of waxy-rice (Kogane) starch. *Starch*. 1996, 48 (11-12), 414-417.
- [33] Tako, M. and Hizukuri, S. Molecular origin for thermal stability of Koshihikari rice amylopectin. *Food Res. Int.*, 2000, 33 (1), 35-40.
- [34] Rees, D. A. Shapely polysaccharides. *Biochem. J.* 1972, 126, 257-273.
- [35] Tako, M., Qi, Z. Q. and Toyama, S. Synergistic interaction between  $\kappa$ -carrageenan isolated from *Hypnea charoides* and galactomannan on its gelation, *Food Res. Int.*, 1998, 31 (8), 543-548.
- [36] Arnott, S., Scott, W. E., Rees, D. A. and McNab, C. G. A.  $\iota$ -Carrageenan: Molecular structure and packing of polysaccharide double helices in oriented fibres of divalent cation salts. *J. Mol. Biol.*, 1974, 90 (2), 253-267.
- [37] Morris, E. R., Rees, D. A. and Robinson, G. Cation-specific aggregation of carrageenan helices: Domain model of polymer gel structure. *J. Mol. Biol.*, 1980, 138 (2), 349-362.
- [38] Arnott, S., Fulmer, A., Scott, W. E., Dea, I. C. M., Moorhouse, R.; and Rees, D.A. The agarose double helix and its function in agarose gel structure. *J. Mol. Biol.*, 1974, 90 (2), 269-272.
- [39] Mohammed, Z. H., Hember, M. W. N., Richardson, R. K. and Morris, E. R. Kinetic and equilibrium processes in the formation and melting of agarose gels. *Carbohydr. Polym.*, 1998, 36 (1), 15-26.
- [40] Tako, M., Higa, M., Medoruma, K. and Nakasone, Y. A highly methylated agar from red seaweed, *Gracilaria arcuata*, *Botanica Marina*, 1999, 42 (5), 513-517.
- [41] Tako, M. and Konishi, T. Discovery of  $\kappa$ -carrageenan-like agarose from red seaweed, *Gracilaria coronopifolia*. *Int. Res. J. Pure Appl. Chem.*, 2018, 17 (2), 1-11.
- [42] Gamini, A., Toffanin, R., Murano, E. and Rizzo, R. Hydrogen bonding and conformation of agarose in methyl sulfoxide and aqueous solutions investigated by <sup>1</sup>H- and <sup>13</sup>C NMR spectroscopy. *Carbohydr. Res.* 1997, 304 (3-4), 293-302.

- [43] Tako, M. Structural principles of polysaccharide gels, *J. Appl. Glycosci.* 2000, 47 (1), 49-53.
- [44] Tako, M., Tamaki, Y., Teruya, T. and Takeda, Y. The principles of starch gelatinization and retrogradation, *Food Nutr. Sci.* 2014, 5 (3), 280-291.
- [45] Tako, M. The principle of polysaccharide gels. *Adv. Biosci. Biotechnol.* 2015, 6 (1), 22-35.



© The Author(s) 2021. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).