Aging effect on network structure in agarose gel studied by NMR

Master's Thesis

AGING EFFECT ON NETWORK STRUCTURE IN AGAROSE GEL STUDIED BY NMR

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修士学位論文内容要旨
Abstract

専　攻 Major	Food Science and Technology	氏　名 Name	Descallar Faith Bernadette Arocha
論文題目 Title	Aging effect on network structure in agarose gel studied by NMR アガロースゲルの網目構造に対するエージング効果のNMRによる研究		

Food gels are commonly used as food additives as thickener and stabilizers and are stored at different storing conditions as based on the convention of how it is processed in food hence it is a common practice that food hydrocolloids are stored and aged. However there is a poor knowledge on how aging effect would affect the structural changes in gels. It is therefore of considerable interest to investigate the aging behavior of food gels and agarose is known as the model of food hydrocolloids. Agarose is a linear and sulfate-free polysaccharide extracted from red seaweeds. It is widely used in food and separation technologies due to its gelling properties. At high temperature, agarose chains appear to be in random coil conformation, and upon reaching gelling temperature, coils reorder to form helices and subsequently aggregate to form a gel network. Thick bundles of aggregates form in the network of this polysaccharide thereby forming rigid and turbid gels. Agarose gels also show considerable degree of hysteresis between its melting and setting temperatures. In this present work, aging effect in agarose gels was studied using diffusion measurements in NMR.

Nuclear Magnetic Resonance Spectroscopy is a powerful tool to elucidate network dynamics and changes of gels. In this research, the pulsed-field-gradient stimulated echo (PFGSTE) ^{1}H NMR method is used to determine the network structure and aging behavior of agarose solutions in different storage conditions. The decay of the echo signal intensity with increased gradient strength reflects the displacement of a molecule due to self-diffusion which is used as the molecule's diffusion coefficient relating to the viscosity and the local interspatial environment of the medium. A dendrimer which is a highly branched spherical molecule containing COONa terminal group is used as a probe molecule in the agarose gel.

1.2 wt% and 2.4 wt% of Agarose type A2929 were used as samples added with 0.1wt% of dendrimer with generations 1.5 and 6.5, respectively. The samples were stored

at refrigeration temperature from 0-150 days and 0-90 days. NMR diffusion experiment shows there is a slight increase in the diffusion coefficient of the generation 1.5 of the dendrimer in the early storage. This possibly suggests that probe molecule diffuses easily in the medium with increasing storage time. However, changes in the dendrimer peaks with increasing storage time were observed and were assumed as highly influenced due to decomposition of the probe polymer. GPC measurements revealed multi-distribution of molecular weight of the dendrimer which supports that dendrimer has decomposed over storage. For generation 6.5 dendrimer, there was no significant difference in the diffusion coefficient in the probe. This could possibly be due to the size of the probe which is quite smaller as compared to the interspaces in the network which in turn show no significant changes in the diffusion coefficient and small degree of degradation. An increase in the diffusion time shows a decrease in the diffusion coefficients which suggest that dendrimer were not in free diffusion but were diffusing in restricted spaces. It is believed that further aggregation of agarose happened in the network as aging proceeds however, it is not clearly observed in this study. To clearly verify the aging behavior of agarose, a stable probe polymer is required.

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1 Introduction

1.1 Rationale of the Study

Gel is a state of matter that consists of the polymer network and huge amount of solvent (Flory, 1953). Gel materials are formed from colloidal polysaccharides, proteins and synthesized polymers (Tako, 2015). Because of the structure, these have been applied in food, bio-medical, pharmaceutical, cosmetic, paint, and chemical industries. Different gelling functionalities have paid attention for the use of polysaccharides to numerous applications. Among these polysaccharides, agarose has a long history as a gel material which is used in a wide range of aids. Agarose are widely used in biomedical and separation technology; and in food industry as a gel, thickener and stabilizer.

Different researches have been devoted to studying the mechanical properties and gelation mechanism of the agarose gels. However, much less attention is devoted to the aging of this polymer and the changes in the mechanical properties that may result from various causes such as spontaneous structural rearrangements (Draper et. al, 2015), phase

separation and spinodal decomposition (Morita et. al, 2013), variation of external parameter such as temperature (Mao et. al, 2017) and drying and/or water-loss (Divoux et. al, 2015). Understanding the aging mechanism of agarose gels would give cognition how to manipulate this polysaccharide to its better usage.

1.2 Agarose: Origin and Properties

Agar is the generic name for a family of structurally related polysaccharides from red algae (Rhododphyceae) and is built up of alternating D- and L- galactopyranose units (Araki, 1956). It is composed of two polysaccharides, agarose and agaropectin (Araki, 1956; Arnott et. al, 1974; Tako et. al, 1988; Arndt et. al, 1994). Agarose is the sulfate-free and neutral (non-ionic) gelling fraction of agar. It is consisting of repeating units (agarobiose) of alternating 1, 3-linked β-D-galactopyranose and 1, 4-linked 3, 6 anhydro-α-L-galactose units (Figure 1) (Araki, 1956). Agaropectin has the same backbone as agarose but contains anionic groups such as sulfate, pyruvate, and glycuronate (Rinaudo, 2008).

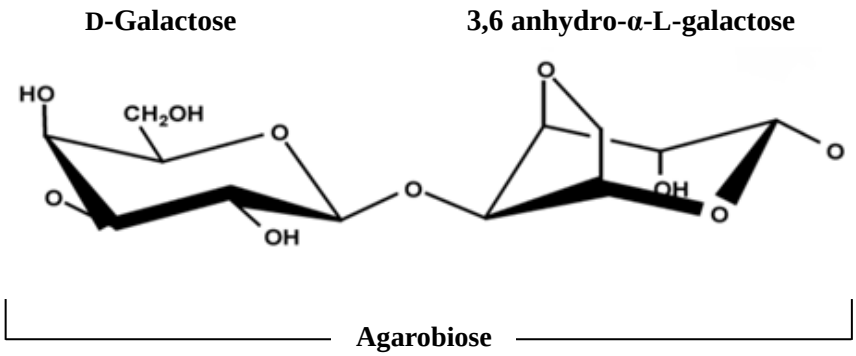

Figure 1. The agarose molecule consisting of repeating units (agarobiose) of alternating 1, 3-linked β-D-galactopyranose and 1, 4-linked 3, 6 anhydro-α-L-galactose units (adapted from Araki, 1956).

Agarose forms thermoreversible gels when dissolve in water. Gels are typically rigid and prone to the phenomenon of “weeping” or spontaneous loss of water on standing known as syneresis (Arnott et. al, 1974). Agarose chains assume a random and stiff coil confirmation at high temperature. The coils reorder to form helices that subsequently aggregate into a three-dimensional network of thick bundles to form a gel upon cooling below the gelation temperature (Figure 2) (Dai et. al, 2013). Gels are very turbid and show considerable degree of hysteresis, which is observed between the melting temperature corresponding to the gel-sol transition observed on heating and gelling temperature corresponding to the sol-gel phase transition on cooling. The hysteresis is a feature of the chemical structure (nature and quantity of the substituents) (Rinaudo, 2008).

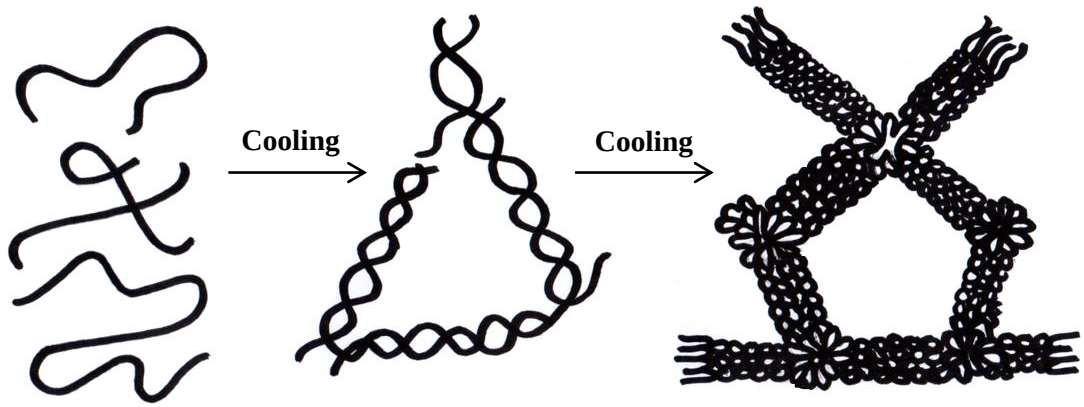

Figure 2. Coil-to-helix gelation mechanism of agarose (adapted from Arnott, 1974; Laas, 1975).

Agarose adopts a single (Foord et. al, 1989) or double (Arnott et. al, 1974) helical conformation. Gelation occurs from aggregation of double helices at a temperature which depends on the methoxyl and sulfate contents that modulate gelation and the three-dimensional network of agarose fibers held together by hydrogen bonding (Dea et. al, 1972; Djabourov et. al, 2013). From the structure of agarose, an intramolecular hydrogen

bonding between OH-4 which is oriented at axial configuration of the β-D-galactopyranose and the adjacent hemiacetal oxygen atom of the 3,6 anhydro-α-L-galactopyranosyl residues which is a cage-like sugar. An intermolecular hydrogen bonding also happens in between the ring O-3,6-atom and the OH-2 which is oriented at axial configuration of 3,6 anhydro-α-L-galactopyranosyl residues on different molecules (Figure 3) (Tako et. al, 1988). The mode of intra- and intermolecular hydrogen bonding of agarose molecules has been supported by ^{1}H and ^{13}C NMR Spectroscopy (Gamini et. al, 1997).

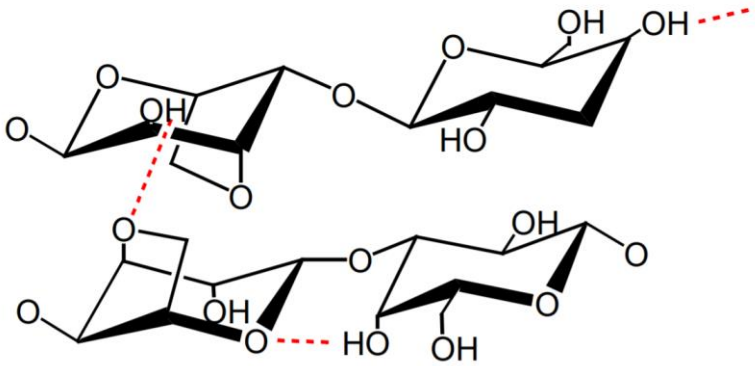

Figure 3. Gelation mechanism of agarose: intra- and intermolecular hydrogen bonding (---) (adapted from Tako, 2015).

Agarose play a dominant role in the center of tetrahedral cavities (cages) that are occupied by water molecules. The said arrangement is similar to a tetrahedral ice-like structure leading to a cooperative effect and the formation corresponds to a total cluster (Figure 4). The intra- and intermolecular hydrogen bonding of agarose molecules result in gelation up to a high temperature (60˚C), and their periphery is surrounded by hydrophobic carbon and hydrogen atoms, which are required to play a role in the hydrophobic effect. This hydrophobic effect leads to hydrogen bonding that easily occurs within water

molecules because of decrease in entropy. Therefore, hydrogen bonding also takes place easily with water molecules on the outside of the polymer helices (Tako, 2015).

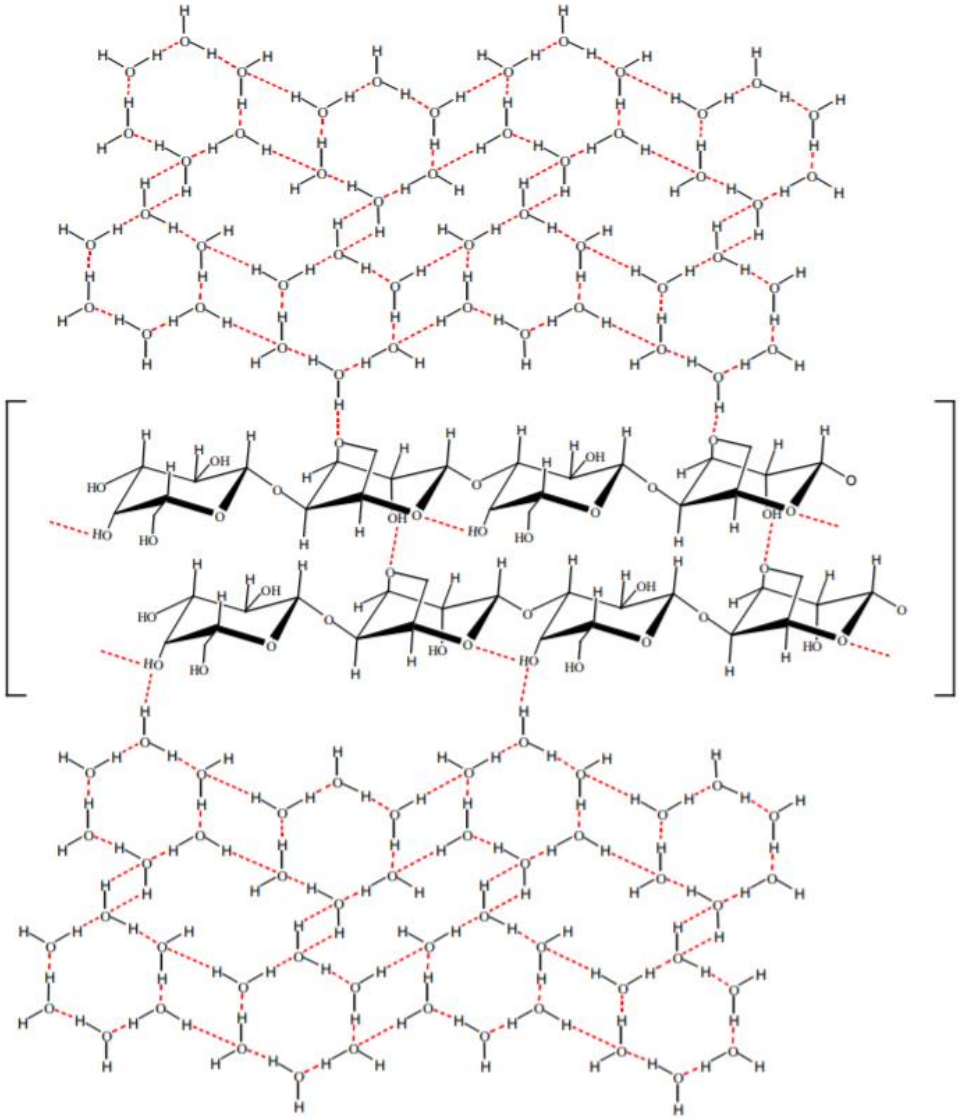

Figure 4. Gelling mechanism of agarose in water molecule where the red dotted lines (---) represent hydrogen bonding (adapted from Tako, 2015).

1.3 Purpose and Significance of the study

The mechanical properties and gelling behaviors of agar and agarose have been extensively studied for the past years using different techniques: optical measurements

studied using X-ray technique (Arnott et. al, 1974; Foord et. al, 1989; Matsuo et. al, 2002), optical rotation (Dea et. al, 1972), light scattering (Fujii et. al, 2000; Bulone et. al, 2004), and microscopy (Waki et. al 1982; Pernodet et. al, 1997; Morita et. al 2013); rheological measurements (Watase et. al, 1983; Tako et. al, 1988; Normand et. al, 2003; Fernandez et. al, 2008; Almrhag et. al, 2013); electrophoresis (Gosnell et. al; 1993; Pluen et. al, 1999) and NMR measurements (Derbyshire et. al, 1973; Albanese et. al, 1987; Gamini et. al, 1997; Chavez et. al, 2006; Dai et. al, 2013; Brenner et. al, 2016). However, much less studies on the aging behavior and structural changes of agarose. A research has reported studies on topological evolution of agarose (Xiong et. al, 2005) but purely focused on the gelation stages in short time frames. Others also reported on the aging effects in the mechanical property of agar (Mao et. al, 2017) and agarose in the presence of co-solutes (Deszczynski et. al, 2003) but only focused on the bulk aspect and studied in a short period of time, i.e. couple of hours. Matsukawa et.al, 2009 reported a change in the mesh size of agar chains and assumed a progressive aggregation of the network however; it didn't confirm how the structure changes in a longer storage time.

As food gels are continually introduced to industrial food products, it is important to know the physical property not just in macroscopic aspect but most importantly in microscopic view. The behaviors of food gels are directly related up to the minute property of it hence it would affect the release of flavors and textural properties (Brenner, 2014). Food gels are commonly used as food additives i.e. as thickener and stabilizers, and are stored at different storing conditions as based on the convention of how it is processed in food; hence it is a common practice that food hydrocolloids are stored and aged not just for hours but up to a couple of weeks and months. However, there is a poor knowledge on how aging effect would affect the structural changes in gels. It is therefore of considerable

interest to investigate the aging behavior of food gels as it would affect the proof of the palate of the consumers, and agarose is known as the model of food hydrocolloids. Moreover, agarose is widely used in separation technology i.e. gel electrophoresis, where agarose is used as a medium for separation of DNA, RNA and other biopolymers hence this study would also give interest to biomedical industry.

Self-diffusion is the most fundamental form of molecular transport (Dai et. al, 2013). It is related to hydrodynamic properties such as molecular size and shape and intermolecular interactions. Diffusion of probe polymers depends upon the structure and mobility of matrix as well as the diffusing molecules and interactions between the diffusing molecules and the matrix (Walderhaug et. al, 2010). Therefore, diffusion of probe polymers reflects the surrounding local viscosity which relays information about the local environment of interspaces in the gel network. When no direct intermolecular interaction occurs between the diffusant probe polymer and network chains, diffusion coefficient of the probe polymer decreases because of hydrodynamic interaction via solvent movement (Matsukawa et. al, 2009).

In this present work, aging effect in agarose gels was studied using diffusion measurements in NMR. NMR techniques provide information about the mobility of molecules in gel. Pulsed-Field-Gradient Nuclear Magnetic Resonance (PFG-NMR) technique was used. To shed further light on the aging process of agarose, a dendrimer, which is a highly branched spherical molecule with a narrow molecular weight distribution with COONa terminal groups, was used as probe polymer in the polysaccharide gel system. The diffusion coefficient *D* of the dendrimer was related to the microscopic environment and aging mechanism of the agarose gel.

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2 Nuclear Magnetic Resonance

2.1 Introduction to NMR

Nuclear Magnetic Resonance (NMR) Spectroscopy is a powerful tool to study the physical, chemical, and biological properties of matter. It is used in different applications in several areas of science and routinely used to study the chemical structure and properties of polymers (Hornak, 2011). Time domain NMR spectroscopic techniques are used to probe molecular dynamics in solutions while solid state NMR spectroscopy is used to determine molecular structures of solids. Others also developed NMR methods of measuring diffusion coefficient.

The versatility of NMR makes it pervasive in the sciences and application of NMR is very essential in the pursuit of knowledge. In food hydrocolloid systems, NMR techniques play an important role in characterizing the structures and dynamics of food polymers.

2.2 Diffusion Measurement by NMR

NMR technique is suitable to study polymer's dynamic properties like self-diffusion, flow and relaxation. In the very early years of NMR, pioneering works of Hahn, 1950 pointed out that echo amplitude would be influenced by molecular diffusion (Brownian motion) because of the fluctuations of the local magnetic field.

2.2.1 Concept of Diffusion

Self-diffusion is the random translational motion of molecules driven by internal kinetic energy (Price, 1997). Translational diffusion is the basic mechanism by which molecules are distributed in space and is considered to play a central role in any chemical reaction since the reacting species have to collide before the reaction can occur. The classical description of diffusion is via Fick's laws. Fick's first and second law of diffusion worked out for systems in which an initial concentration gradient is established (Callaghan, 1991). The Fick's First Law postulates that the flux of material across a given plane is proportional to the concentration gradient across the plane,

$$J = -D\frac{\partial C(x,t)}{\partial x} \qquad [1]$$

where J is the conditional probability flux, D is the diffusion constant for the material that is diffusing in the specific solvent, and $\frac{\partial C(x,t)}{\partial x}$ is the concentration gradient. The diffusion constant of the material is expressed in the units of length^2/time and the negative sign indicates that the impurities are flowing in the direction of lower concentration. Fick's First Law does not consider the fact that the gradient and local concentration of the impurities in

a material decreases with an increase in time which is an important factor to diffusion processes.

Fick's Second Law states that the change in the concentration over time is equal to the change in local diffusion flux, or

$$\frac{\partial C(x,t)}{\partial t} = -\frac{\partial J}{\partial x} \qquad [2]$$

or, from Fick's First Law,

$$\frac{\partial C(x,t)}{\partial t} = -D\frac{\partial^2 C(x,t)}{\partial x^2} \qquad [3]$$

supposing that diffusion coefficient is independent of position.

In an isotropic system, without thermal or concentration gradients, the average molecule displacement in all three directions is zero but the mean square displacement is non-zero and is given by

$$\left\langle r^2 \right\rangle = 6Dt \qquad [4]$$

Following the Stoke's law, the force needed to move a small sphere of radius R through a continuous medium of viscosity η with a velocity V is

$$F = 6\pi\eta RV \qquad [5]$$

The Stoke's-Einstein equation describes the way that diffusion increases in proportion to temperature and is inversely proportional to the friction force experienced by a molecule where f is given by the Stoke's formula for friction, $f = 6\pi\eta R$.

$$D = \frac{k_B T}{f} \qquad [6]$$

Combining equations [5] and [6] leads to the well-known relation

$$D = \frac{k_B T}{6\pi\eta R} \qquad [7]$$

In NMR measurements, PFG-NMR is the widely known method in measuring self-diffusion in polymers. Self-diffusion data provide detailed information about molecular organization and phase structure. Self-diffusion rates are quite sensitive to structural changes as to binding and association phenomena. Experimental self-diffusion values are directly related to molecular displacement.

2.2.2 Pulsed-Field-Gradient NMR (PFG-NMR)

The Pulsed-Field-Gradient Nuclear Magnetic Resonance (PFG-NMR) method was first theoretically and experimentally demonstrated by Stejskal and Tanner, 1965 and is still in its original form as one of the main NMR methods for obtaining information, such as diffusion coefficient.

When two field gradients for dephase and rephase are applied, the NMR signal decays due to the displacement of nuclei during the interval between the two field gradients. This allows calculation of the diffusion coefficient for Fickian diffusion in free space.

The Larmor precession frequency depends on the magnetic field experienced by the nucleus, and is dependent on nuclei position under the magnetic field gradient. The spatially dependent Larmor frequency $\omega(\mathbf{r})$ at the position **r** under a spatially linear field gradient **g** is expressed as follows

$$\varpi(\mathbf{r}) = \gamma(H_0 + \mathbf{gr}) = \varpi_0 + \gamma\mathbf{gr} \qquad [8]$$

where γ is the gyromagnetic ratio, a constant for each nucleus (for protons $\gamma = 267.522 \times 10^6$ rad s^{-1} T^{-1}), H_0 is the externally applied magnetic field and **gr**=0 at the position of **r**=0. It follows that the phase angle ϕ is a function of the spatial position of the nucleus. If the field gradient duration is δ, then ϕ is given as

$$\phi(\mathbf{r}) = \gamma \mathbf{g} \mathbf{r} \delta \qquad [9]$$

The distance in the direction of **g** where ϕ (r) = 2π is

$$q^{-1} = \frac{2\pi}{\gamma g \delta} \qquad [10]$$

q^{-1} is the characteristic length scale of the field gradient.

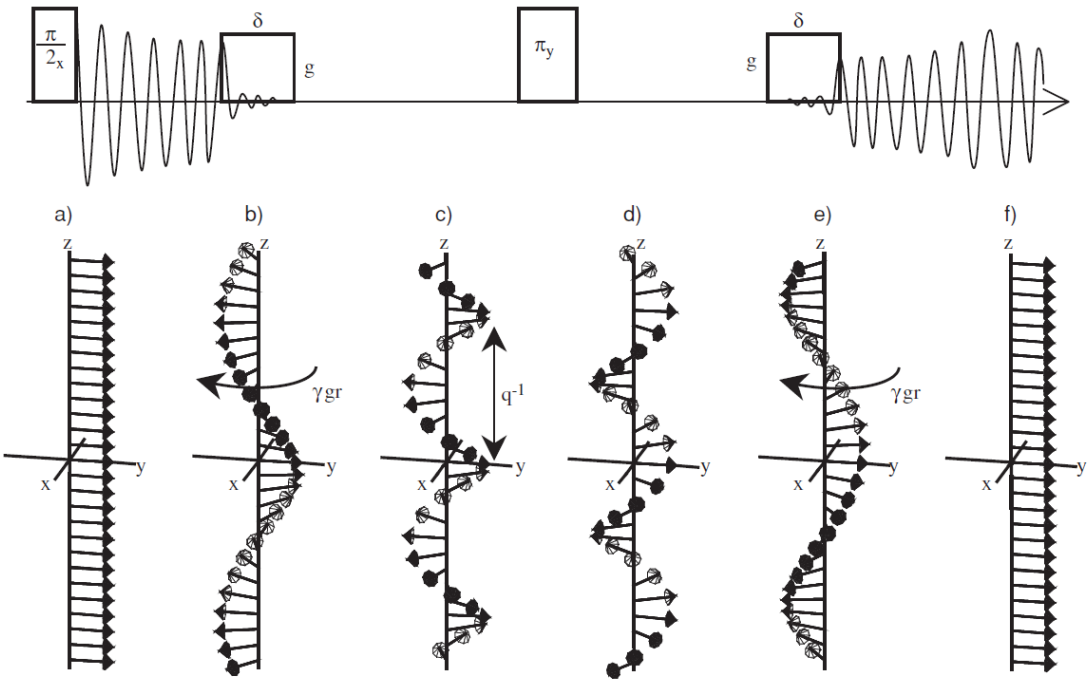

Figure 5. A typical pulse sequence with two PFG of rectangular shape and the dephasing and rephasing behavior of the magnetization. (a) The magnetizations are aligned along the *y*-axis by an rf *π*/2 pulse. (b) Under the first PFG, the magnetizations precess at the angular velocity of γ**gr** corresponding to the *z* coordinate. (c) At the end of the first PFG, the magnetizations are spirally twisted at a pitch of *q*−1. (d) An RF *π* pulse along the *y*-axis rotates the individual magnetizations along the *y*-axis through 180 degree. (e) Under the second PFG, the individual magnetizations precess at the same angular velocity with that under the first PFG. (f) At the end of the second PFG, the magnetizations are aligned along the *y*-axis. (Adapted from Matsukawa, 2006)

For diffusion coefficient measurements, a second field gradient is applied in order to rephase the dephase magnetization. Figure 5 shows a typical pulse sequence with two rectangular-shaped pulsed field gradients (PFG) along the z axis (Stejskal & Tanner 1965), and the dephasing and rephasing behavior of the magnetization when individual spins do not change positions in the interval Δ between the two PFGs. (a) The magnetizations are aligned along the y axis by an RF $\pi/2$ pulse. (b) Under the first PFG, the magnetizations precess at an angular velocity of $\gamma\mathbf{gr}$ corresponding to the individual positions in the rotating frame. (c) At the end of the first PFG, the magnetizations are spirally twisted at a pitch of q^{-1}. (d) The application of an RF π pulse along the y axis rotates the individual magnetizations 180° about the direction of y axis, which yields a mirror-symmetrical arrangement of the magnetizations with respect to the y-z plane. (e) Under the second PFG, the individual magnetizations precess at the same angular velocity with that under the first PFG. (f) At the end of the second PFG, the magnetizations are aligned along the y-axis.

Pulsed-field-gradient is turned off before the echo is formed and the acquisition of the second half of the echo is performed without any pulsed-field-gradient present. If the nucleuses do not move in space during the time interval Δ, the spins will refocus at the time 2τ and loose intensity only according to unavoidable spin-spin (T_2) relaxation effects. However, if the nucleuses move to another point in space because of self-diffusion during Δ, a loss of echo intensity will be produced because the spins will not refocus completely. This loss can be calculated theoretically by taking into account that nucleus will be displaced a root-mean-square displacement of Δz in the z-direction during a time interval Δ due to self-diffusion according to $\langle \Delta z^2 \rangle = 2D\Delta$. D is the self-diffusion coefficient of nucleus and its phase angle is

$$\phi(\Delta z) = 2\pi \frac{\Delta z}{q^{-1}} = \gamma g \delta \Delta z \qquad [11]$$

The echo signal intensity $I(2\tau,g\delta)$ at 2τ is proportional to the vector sum of magnetizations in the sample, therefore, it is expressed as follows

$$\mathrm{I}(2\tau, g\delta) = \mathrm{I}(2\tau,0)\iint \cos\left(\phi(\Delta z)\right)\rho(\mathbf{r})p(\mathbf{r},\Delta z)d\mathbf{r}d\Delta z \qquad [12]$$

where $\rho(\mathbf{r})$ is the density of the nucleus and is constant for homogeneous sample, $p(\mathbf{r},\Delta z)$ is the probability of the displacement during Δ for the nucleus at $\mathbf{r}$ and $I(2\tau,0)$ is the total signal intensity without PFG and expressed as follows

$$\mathrm{I}(2\tau,0) = \mathrm{I}(0,0)\exp\left(\frac{-2\tau}{\mathrm{T}_2}\right) \qquad [13]$$

where $I(0,0)$ is the initial signal intensity just after the RF $\pi/2$ pulse. For free diffusion in an isotropic medium, $p(\mathbf{r},\Delta z)$ becomes a Gaussian distribution as follows

$$p(r,\Delta z) = (4\pi D\Delta)^{-1/2}\exp\left(-\frac{\Delta z^2}{4D\Delta}\right) \qquad [14]$$

where D is the diffusion coefficient. Taking the diffusion during δ into account, $I(2\tau,g\delta)$ is rewritten as follows

$$\mathrm{I}(2\tau, g\delta) = \mathrm{I}(0,0)\exp\left[-\frac{2\tau}{\mathrm{T}_2} - (\gamma g\delta)^2 D\left(\Delta - \frac{\delta}{3}\right)\right] \qquad [15]$$

In order to decrease the effect of residual field gradient, the pulse sequence of Pulsed-Field-Gradient Stimulated Spin-Echo (PFGSTE) is frequently used for D measurements (Karger et al. 1988; Callaghan 1991; Price 1997 and 2009). The diffusion coefficient values are determined from the decay of echo signal intensities, expressed as

$$I(2\tau_2 + \tau_1 g\delta) = I(2\tau_2 + \tau_1,0)\exp[-\gamma^2\delta^2 g^2(\Delta - \frac{\delta}{3})D] \qquad [16]$$

where $I(2\tau_2+\tau_1,g\delta)$ and $I(2\tau_2+\tau_1,0)$ are echo signal intensities at $t = 2\tau_2+\tau_1$ with and without the field gradient pulse, respectively. It should be noted that $I(2\tau_2+\tau_1, 0)$ has decayed from

the initial intensity, $I(0, 0)$, (i.e., the signal intensity immediately after the first π/2 RF pulse) by T_1 and T_2 relaxations

$$I(2\tau_2 + \tau_1, 0) = I(0,0)\exp[\frac{-2\tau_2}{T_2} - \frac{\tau_1}{T_1}] \qquad [17]$$

From equation [17] it follows that a decrease in the relaxation times leads to a decrease in $I(2\tau_2+\tau_1,0)$

For the diffusion in restricted spaces, the D value obtained by applying equation [16] is an apparent diffusion constant

$$D_{app}(\Delta) = \frac{\langle \Delta z^2 \rangle(\Delta)}{2\Delta} \qquad [18]$$

where $\langle \Delta z^2 \rangle(\Delta)$ is the mean square of Δz^2 during Δ. $\langle \Delta z^2 \rangle(\Delta)$ is proportional to Δ for the free diffusion, however, it becomes smaller than the proportional value due to the spatial restriction which gives the information of the space size of the restriction (Price, 2009).

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3 Materials and Methods

3.1 Agarose Sample Preparation

3.1.1 Materials

Agarose used in this study was a purified, low EEO-type (≤ 0.08) agarose (A2929, Sigma Aldrich) with a sulfate content of ≤ 0.20%. The 20% and 5% methanol solutions of poly(amidoamine) PAMAM dendrimer with generations of 1.5 and 6.5, respectively, were purchased from Sigma-Aldrich. The dendrimer has a COONa terminal group (PAMAM-COONa). The methanol solutions of dendrimers were repeatedly diluted with D_2O and were air-blown with dry air so as to evaporate the methanol from the solution.

3.1.2 Sample preparation

The agarose gels used were from stored pouched samples made from previous study of Dai et.al, 2013. The agarose gel was prepared by dispersing agarose A2929 powder in D_2O stirred at room temperature overnight. It was then dissolved completely by stirring at 95°C for 5.5 h. 1.2wt% and 2.4wt% agarose solutions added with PAMAM dendrimer with generations of 1.5 and 6.5, respectively, were used for the diffusion measurements.

Appropriate amounts of agarose and dendrimer solutions were mixed and stirred for 30mins at 80°C to prepare 1.2wt% agarose with 0.1 wt% 1.5-generation dendrimer and 2.4wt% agarose with 0.1 wt% 6.5-generation dendrimer. The resulting solutions were immediately transferred into 8mm NMR tubes and were quenched at room temperature and stored at refrigeration temperature of 5°C for 0-150days. 1.2wt% agarose added with 0.1wt% generation 1.5 dendrimer showed syneresis after 150days of storage. Agarose content in the solution was measured by drying the sample at 105°C for about 12h and residues were then weighed.

3.2 PFG-STE NMR Measurements

Self-diffusion coefficient (D) measurements were carried out on a Bruker Avance II 400WB spectrometer operating at 400.13 MHz for protons using the pulsed-field-gradient stimulated spin-echo (PFGSTE) pulse sequence (Figure 6). The gradient field strength g varied from 5-400 G/cm. The gradient pulse length δ and the interval Δ between the two gradient pulses were 1ms and 10ms, respectively. The repetition time was 5s and the temperature was set at 25°C.

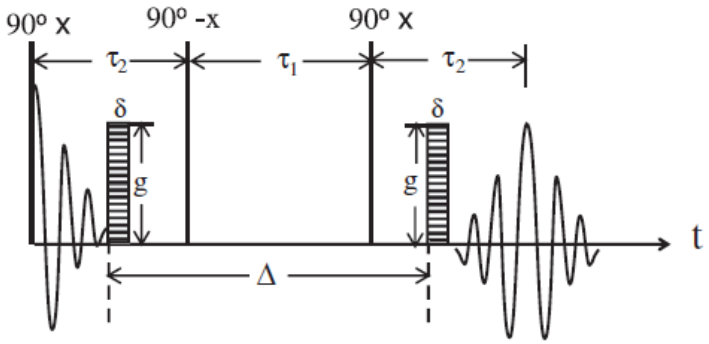

Figure 6. PFG-STE NMR pulse sequence. (Adapted from Dai, 2012)

The self-diffusion coefficients were calculated from the decrease in peak intensity with increasing gradient strength (Stejskal et. al, 1965; Johnson Jr., 1999). The attenuation of the peak intensity in the spin-echo spectra is expressed as

$$I(g) = I(0)\exp[-\gamma^2\delta^2 g^2 D(\Delta - \frac{\delta}{3})] \qquad [19]$$

Where *I(g)* and *I(0)* are the echo intensities at $t = 2\ \tau_2+\tau_1$ with and without field gradient, respectively, and γ is the gyromagnetic ratio of ^{1}H.

3.3 GPC Measurements

Molecular weight distributions were measured using gel permeation chromatography (GPC) machine equipped with refractive index detector (HLC-8120 Tosoh Co. Ltd, Japan GPC). The column system consisted of TSK-gel G6000WXL Columns (7.8 x 300 x 1.3 mm) and TSK-gel G4000PWXL Columns (7.8 x 300 x 1.0 mm) in series.

Aged agarose gels with dendrimers were cut into small pieces and were diluted with 0.1M $NaNO_3$. Samples were centrifuged for 10 mins at 3000rpm at 5°C to obtain supernatants containing dendrimers. The samples were filtered using 0.45μm filter just before the measurements. Elution was performed using a 0.1M $NaNO_3$ solution as the mobile phase at a flow rate of 1.0mL/min. The temperature of the column system was maintained at 80°C. GPC calibrations were performed using a Pullulan (Shodex, Japan) standard sample.

4 Results and Discussion

4.1 Agarose Samples

The gels prepared at 1.2wt% agarose added with 0.1wt% generation 1.5 dendrimer and 2.4wt% agarose added with 0.1wt% generation 6.5 dendrimer are shown in Figure 7. Agarose gels show turbidity when set at room temperature.

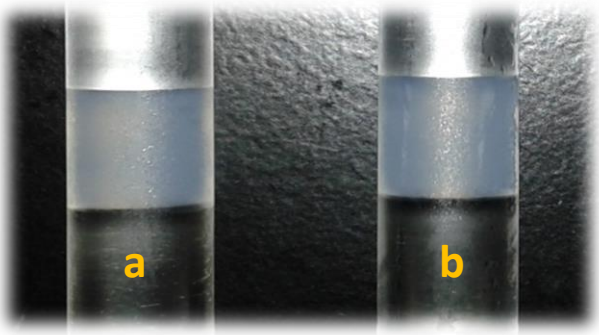

Figure 7. Agarose gel samples: (a) 1.2 wt% agarose added with 0.1wt% 1.5Generation dendrimer; (b) 2.4 wt% agarose with 0.1wt% 6.5Generation dendrimer.

4.2 PFG-STE NMR Measurements

Figures 8 and 9 show the stacked NMR spectra of 1.2wt% agarose added with 0.1wt% generation 1.5 dendrimer and 2.4wt% agarose added with 0.1wt% generation 6.5 dendrimer, respectively.

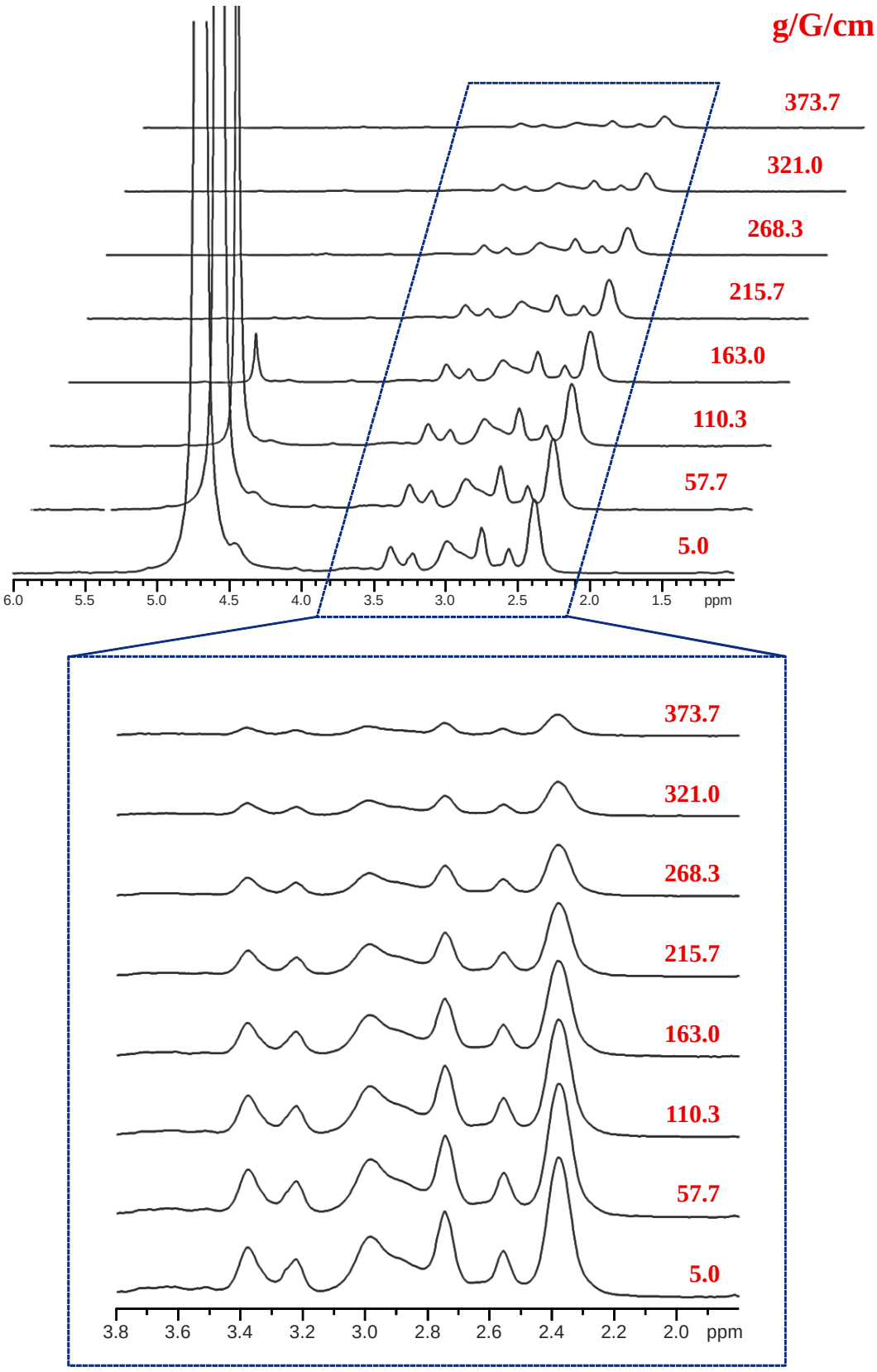

Figure 8. Stacked NMR spectra of 1.2 wt% agarose added with 0.1wt% 1.5Generation dendrimer at 25°C with increasing gradient strength g.

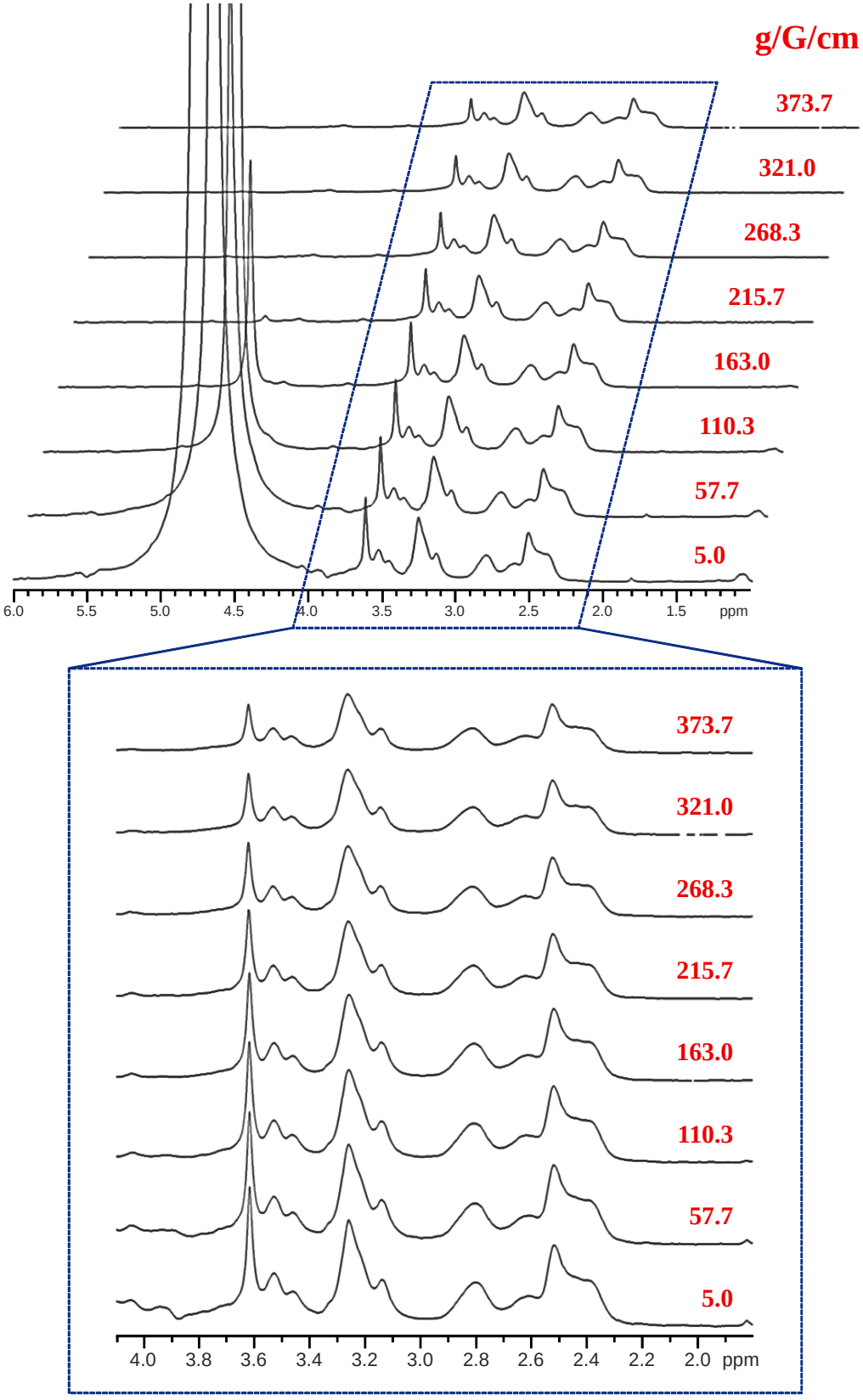

Figure 9. Stacked NMR spectra of 2.4 wt% agarose added with 0.1wt% 6.5Generation dendrimer at 25°C with increasing gradient strength g.

From Figures 8 and 9, the peaks due to HDO at 4.7ppm markedly decrease due to the large diffusivity of water. The peaks at 2-3.5ppm from 1.2wt% agarose added with 0.1wt% generation 1.5 dendrimer and 2-3.8ppm from 2.4wt% agarose added with 0.1wt% generation 6.5 dendrimer were assigned to the ethylene protons of the dendrimer. At the measuring temperature of 25°C, agarose has formed a gel. The agarose chains involved in aggregation have strongly restricted segmental mobility because of the rigid structure (Arnott, 1974), resulting in a short ^{1}H T_2 values which in turn the corresponding agarose peaks disappear by their fast decay during echo times (Dai, 2013).

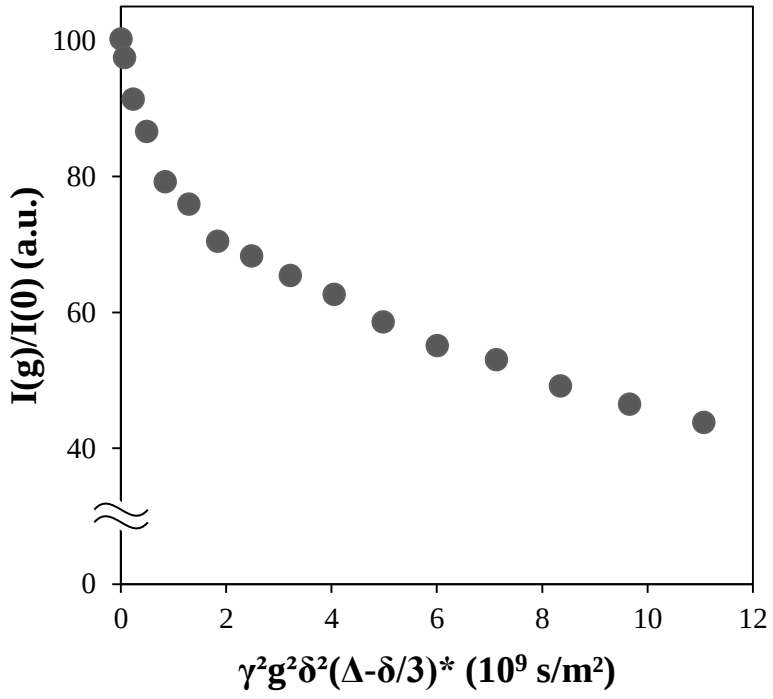

Figure 10. Diffusional spin-echo attenuation of dendrimer peaks plotted as a function of $\gamma^2 g^2 \delta^2 (\Delta - \delta/3)$. Both *I(0)* and *D* were determined using equation [19].

The echo attenuation of the peaks at 2-3.5ppm (1.5 generation) and 2-3.8ppm (6.5 generation) from the dendrimer were plotted as a function of $\gamma^2 g^2 \delta^2 (\Delta - \delta/3)$. An example of the plot is shown in Figure 10, which was obtained for various values of g at fixed δ and Δ. $I(0)$ and D values of dendrimers were determined by fitting the data points to equation [19]. The experimental data obtained for 1.5generation and 6.5generation dendrimers showed 2-component diffusion. The slower component of D was taken as the diffusion coefficient of the dendrimer. The faster component of D was attributed to the possible degraded small parts of the dendrimer which gives a higher diffusivity. This degradation will be discussed later.

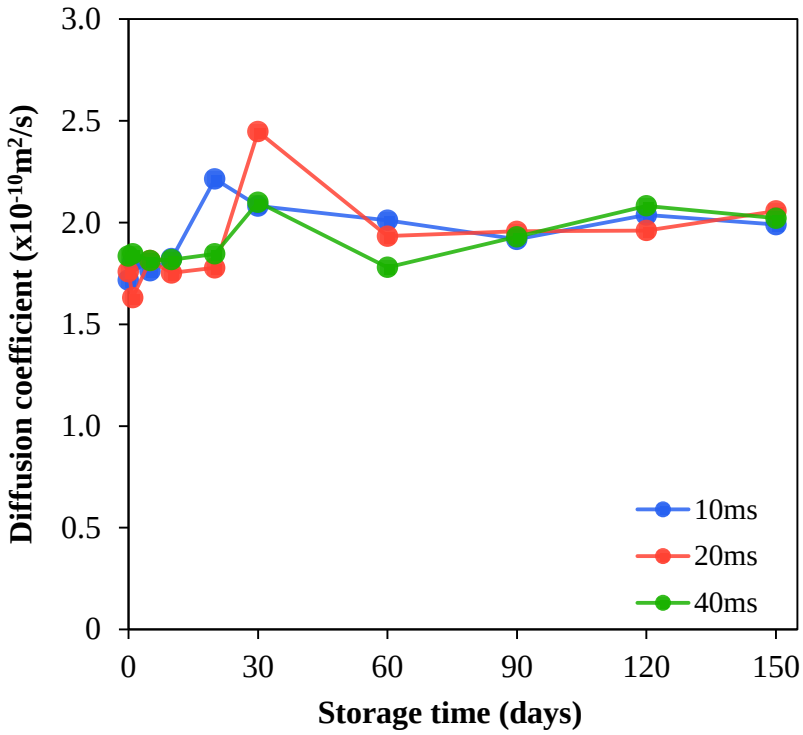

Figure 11. Storage time dependency of D of 1.5generation dendrimer in 1.2wt% agarose with increasing storage time.

The slower components of diffusion coefficient D were plotted as a function of storage time, as shown in Figures 11 and 13. Figure 11 shows the storage time dependency of D of 1.5 generation dendrimer in 1.2wt% agarose. D showed a slight increase to 30days of storage and showed no significant increase in the succeeding days. This possibly suggests that 1.5 generation dendrimer diffuses quite easily in the gel network. However, it is assumed that the increase in D is highly influenced by the decomposition of the dendrimer inside the agarose gel.

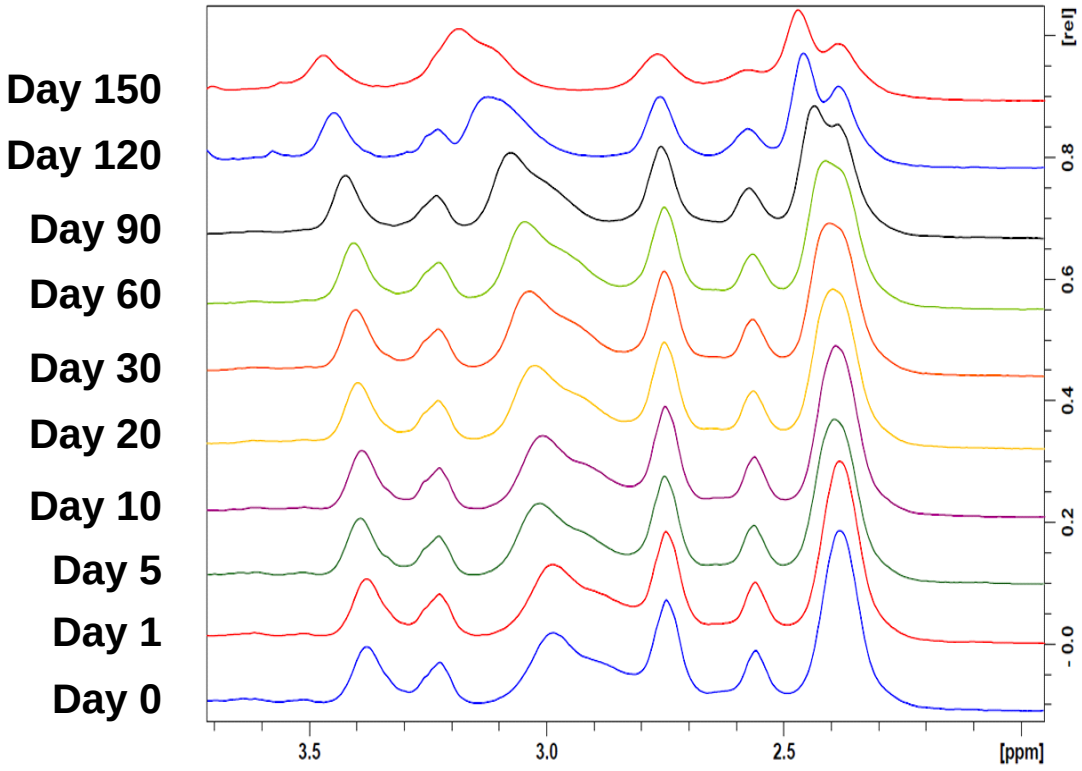

Figure 12. Changes in the dendrimer peak of 1.5generation dendrimer in 1.2wt% agarose with increasing storage time.

Figure 12 shows changes in the dendrimer peak during storage. The peak at 2.4 ppm showed a broadening at days 30 and 90. An appearance of a new peak was detected at days 90-150. The appearance of new peaks suggests degradation of dendrimer in the

agarose gel. The increase in D of 1.5 generation dendrimer in the gel is possibly due to the degradation of the said probe polymer.

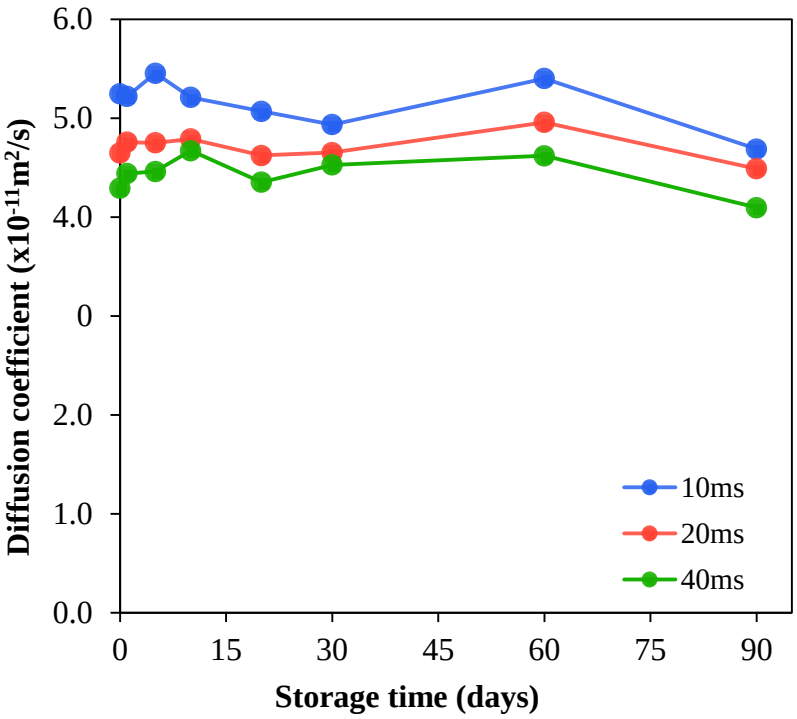

Figure 13. Storage time dependency of D of 6.5generation dendrimer in 2.4wt% agarose with increasing storage time.

Figure 13 shows the storage time dependency of D of 6.5 generation dendrimer in 2.4wt% agarose. D decreases with increasing diffusion time which suggests that dendrimer are not in free diffusion but diffusing in restricted spaced inside the agarose gel network. There is no significant difference in the D of 6.5 dendrimer as aging proceeds. This could possibly be due to the size of the probe which is quite smaller as compared to the interspaces in the network hence no significant changes in the diffusion coefficient. Figure 14 shows no notable changes in the 6.5dendrimer peaks with increasing storage time.

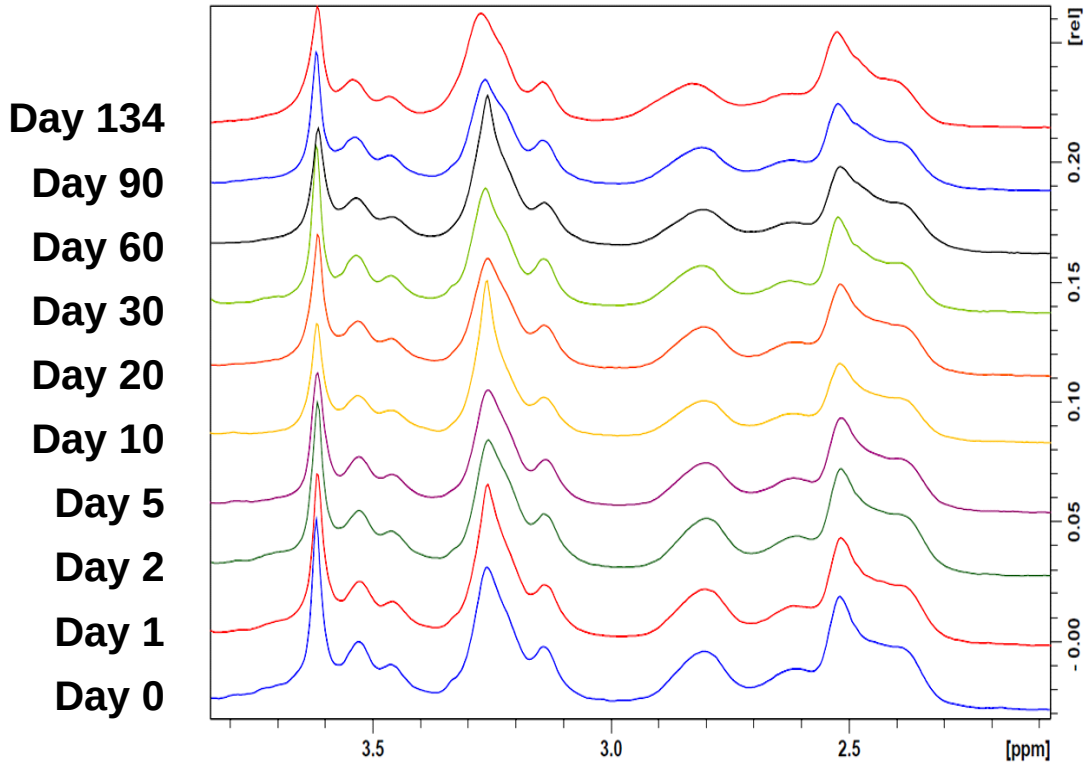

Figure 14. Changes in the dendrimer peak of 6.5generation dendrimer in 2.4wt% agarose with increasing storage time.

4.3 GPC Measurements

Molecular weight distribution of 1.5 generation from stored agarose gels (200days) and pure 6.5 generation dendrimers are shown in Figure 15. Molecular weight of 1.5 Dendrimer shows a multi-distribution which supports degradation of the probe polymer as stored for couple of months inside the agarose gel. 1.5 generation dendrimer shows severe degradation. This infers the changes in the NMR spectra of 1.5 generation dendrimer. On the other hand, pure 6.5 generation show small traces of smaller molecular weights. From the results, 6.5 generation didn't show significant changes in the D could also possibly be due to the degradation of the probe polymer beforehand. Dendrimer is a highly branched

molecule. The smaller molecular weights came from the detached branches of dendrimer from the central core. The same with the observed 2-component diffusion, the faster component may come from small-sized detached branches of polymer while the slower component may come from the intact inner core. The original molecular weights of 1.5generation and 6.5 generation dendrimer are 2934.56 Da and 106,196.91 Da, respectively.

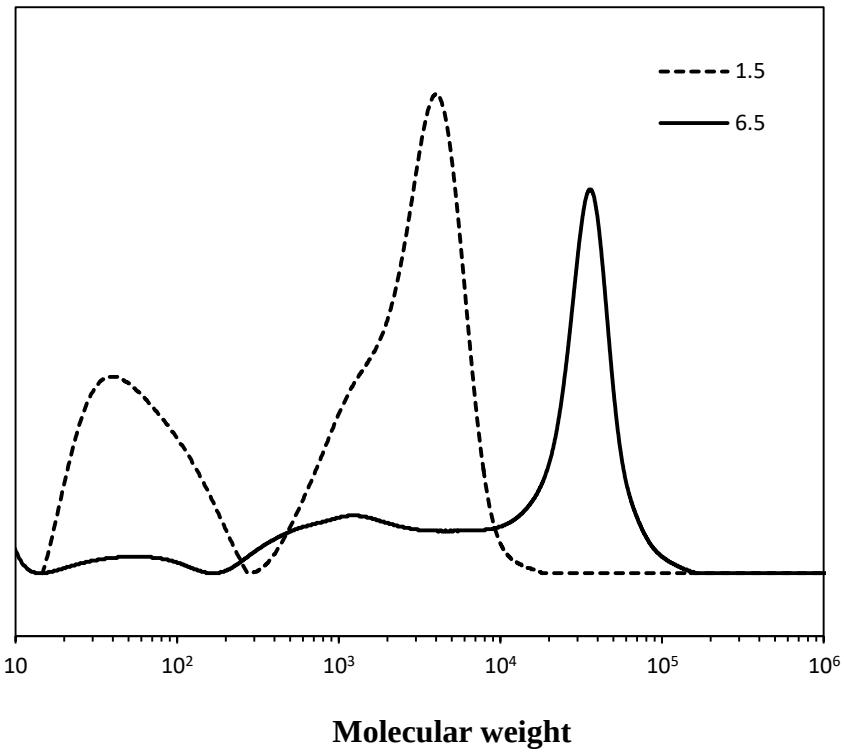

Figure 15. Changes in the dendrimer peak of 6.5generation dendrimer in 2.4wt% agarose with increasing storage time.

5 Conclusion

On the basis of the experimental results, it has been demonstrated that NMR is a powerful tool for the investigation of molecular mobility in the gels. Diffusion coefficients of the dendrimer gave information on the behavior of the probe polymer in the agarose gel and the corresponding limitations. The slight increase of diffusion coefficient of 1.5 generation dendrimer is attributed to the degradation of the dendrimer in the 1.2wt% agarose gel. The said degradation is confirmed by the changes in the NMR spectra observed with an appearance of a new peak in the dendrimer region. No significant change in the diffusion coefficient with 6.5generation dendrimer which is assumed that the size of dendrimer is not comparable enough to the size of the interspaces of the aggregates and small degree of degradation of the sample. Dendrimer is a highly spherical branched polymer which is highly soluble in methanol solution. It is inferred that dendrimer is not stable and easily degraded in D_2O solutions. Multi-distribution of molecular weights of dendrimer from aged agarose gels also confirmed the instability and degradation of dendrimer. The smaller molecular weights come from the detached small parts of dendrimer. Furthermore, from the observed 2-component diffusion of dendrimer, the faster component may come from small-sized detached branches of the probe polymer while the slower component may come from the intact inner core of dendrimer. It is believed that further aggregation of agarose happened in the network as aging proceeds however, it is not clearly observed in this study. To clearly verify the aging behavior of agarose, a stable probe polymer is required.

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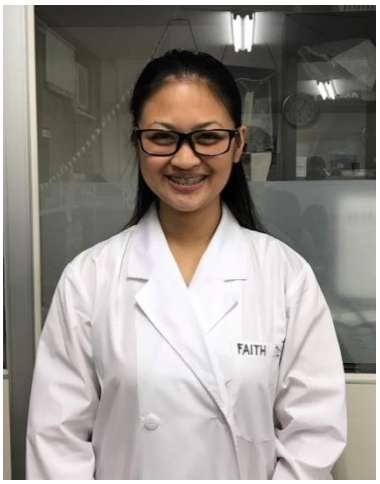

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