Determination of agarose gel pore size: Absorbance measurements vis a vis other techniques

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Abstract. The absorbance measurements in the wavelength range 700 nm to 800 nm were used to probe the agarose gel topology evolution and extract the pore size of the trapped solvent. By following the changes in absorbance and pore size, the gelation process could be clearly divided into three stages - induction stage, gelation stage and pseudo-equilibrium stage. The gelation mechanism is explained as a nucleation and growth process. Following the kinetics of gelation using dynamic light scattering is complicated by multiple scattering (for high concentrations) and large fluctuations in intensity and relaxation time. Comparatively, scanning the absorption spectrum is fast and the method is suitable for a wide range of concentrations and setting temperatures. Pore size determination using absorbance is a fast and non-invasive method when compared to the DNA electrophoresis measurements, which extend over several hours and use probe diffusion.

1. Introduction

Agarose is a marine polysaccharide, often used as the model biopolymer in gelation. It forms thermo reversible gels consisting of thick bundles of agarose chains linked by hydrogen bonds, with large pores holding water [1]. As a hydrogel, agarose finds numerous applications in gel electrophoresis, tissue culture, pharmacy and food technology. The mechanical properties of the gel are decided to a large extent by the network mesh-size (pore size), which in turn depends on the agarose type and concentration, setting temperature etc. Several methods can be employed in the characterization of pore size of agarose gels. With the techniques of atomic force microscopy (AFM) and scanning electron microscopy (SEM) there is experimental difficulty associated with the possible destruction of the gel network, especially during freeze-etching of SEM. While small angle x-ray scattering is a powerful tool for characterization of materials, determination of morphology requires interpretation of the resulting complex Fourier transform of electron density with model parameters. The other possible techniques are dynamic light scattering (DLS), gel electrophoresis and the wavelength exponent (WLE) method as outlined by Aymard et al [2].

Agarose gelation is also important for the many conceptual aspects, such as phase transition and scaling associated with the sol-gel transition and the kinetics of the network topology evolution. We

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investigated the agarose gelation mechanism using absorbance and rheological measurements [3]. Absorbance spectrum was used to calculate the turbidity $\tau(\lambda)$ at various wavelengths, $\lambda$. From $\tau(\lambda)$, WLE and pore size were calculated. The different stages of gelation were identified by monitoring the absorbance and pore size. In this paper we discuss the merits of using absorbance measurements to follow sol-gel transition and pore size determination vis a vis other techniques.

2. Experimental details

The agarose used in this experiment was SeaKem LE Agarose (high-melt, gelling temperature for 1.5%: 34.5-37.5 °C) and Bio-Rad Certified low-melt agarose. Agarose solutions were prepared by dispersing agarose powder in deionized water at room temperature at various concentrations and heating in oven for thirty minutes at 110 °C for high melt (HM) agarose and 90 °C for low melt (LM) agarose. The solutions were subsequently stirred and sonicated for one hour and held in the oven for 30 min. The hot solutions were loaded quickly into cuvettes and cooled down to varying final temperatures. Absorption spectra were measured for a wavelength range 700 to 800 nm using the Cary 50 UV-Vis spectrophotometer. Turbidity is calculated as $\tau(\lambda) = 2.3 A(\lambda)/L$, where $A$ is the absorbance and $L$ is the optical path length. ($L = 1$ cm). The wavelength exponent, i.e., $\text{WLE} = \frac{d \log \tau(\lambda)}{d \log \lambda}$ is obtained from the linear regression of a double logarithmic plot of turbidity against wavelength. A plot of WLE in the range of -4.117 to -2.117 corresponding to correlation length (= pore size, $\xi$) values of 1 nm to 100 μm is given by Aymard et al [2]. For $\xi$ values of 1 to 30 nm and 2 to 100 μm, WLE shows marginal variation with $\xi$ thereby introducing large error in the estimation of pore size. In the intermediate range, the measurement of WLE provides a fast and non-invasive estimation of the pore size of the agarose gel.

3. Results and discussion

HM agarose sol (0.5% w/v) was quenched to 21 °C at a cooling rate indicated in figure 1(a). From the absorbance recorded at 750 nm and the WLE and the pore size $\xi$, calculated from the turbidity spectrum, three stages, indicated as I, II and III in figure 1, could be identified. Stage I corresponds to the sol state where the absorbance is very low and no meaningful data can be extracted for WLE and $\xi$. In stage II, absorbance, WLE and $\xi$ increase rapidly before leveling off gradually in Stage III.

![Figure 1](image-url)  
**Figure 1.** Gelation kinetics of 0.5% w/v HM agarose solution

The three stages are identified respectively as induction, gelation and pseudo-equilibrium stages. The gelation mechanism is explained as a nucleation and growth process as follows. Upon quenching, liquid-liquid phase separation occurs with the formation of nuclei composed of polymer-rich phases with a characteristic induction time. During this induction stage, the sample remains in the sol state and absorbance remains very low. As gelation progresses, the nuclei grow and form a network of
polymer-rich phases. At this point, absorbance, WLE and $\xi$, start increasing rapidly. The polymer-rich phases tend to coagulate so as to minimize the interface between the polymer-rich and polymer-poor phases, thereby reducing the interfacial free energy. The rigidity of the agarose chains and the aggregation of the chains within the polymer-rich phase prevent large scale coagulation. Local merging of the polymer-rich phases leads to continuously increasing pore size. In the later part of gelation stage most of the agarose chains are immobilized in the fibrous junctions and local coagulation becomes more difficult. However, aggregation of surrounding agarose chains from polymer-poor phase to the fibrous network leads to gradually increasing absorbance in the pseudo-equilibrium stage, while the pore size remains almost constant.

The existence of an induction time and its compliance with the 3D nucleation model are necessary to support the nucleation and growth mechanism of the gelation process. According to 3D nucleation models, the nucleation rate $J$ (the number of critical nuclei generated per unit time-volume) can be expressed as [4]

$$J = B \exp \left[ -\frac{16\pi^2 \Omega^2}{3(kT)^2(\Delta \mu / kT)^2} f \right]$$  \hfill (1)

where $k$ is Boltzmann’s constant, $\Omega$ is the volume of growth units, $\gamma$ is the interfacial tension between crystal and fluid, $B$ is a constant for a given system, $f$ is the factor describing the structural correlation between foreign bodies and the nucleating phase, $\Delta H_{\text{diss}}$ is the molar dissolution enthalpy of the nucleating phase, $T_{\text{eq}}$ is the melting point for the 0.5% w/v HM agarose gel (~334.2 K according to our measurements) and $T$ is the final setting temperature. If agarose gelation is initiated through the nucleation and growth mechanism, the induction time, $t_i \sim 1/J$ and according to equations (1) and (2),

$$\ln(t_i) \propto \frac{1}{T_{\text{eq}}^2 T^{-1}(T_{\text{eq}} - T)^{-2}}$$

Figure 2 is the plot of variation of WLE with time as gelation sets in when the cooling rate shown in figure 1(a) is used for HM and LM agarose of same concentrations. The melting point, $T_{\text{eq}}$, of LM agarose is about 10 °C lower than the HM agarose. Thus when quenched to the same temperature, $T$, the induction time for LM agarose must be larger than for HM agarose of same concentration. This can be observed for both 0.5% and 1% concentrations.

The pore size determined by turbidity measurements after equilibrating the samples for 24 h at the room temperature was verified by using electrophoresis [3, 5]. Bio-Rad EZ load 1 kB molecular ruler was used to determine the electrophoretic mobility, $\mu$, for electric field strength, $E$, in the range 0.7 to 5.0 V/cm. By extrapolation, $\mu_{E \to 0}$ can be obtained for different DNA chains of base pair length, $M$.\[85\]
The pore size can be calculated using the expression $\xi = (6MbP\mu_{E\rightarrow 0}/\mu_0)^{1/2}$, where $b = 0.34$ nm is the inter-base pair distance along the DNA helix, $P = 50$ nm is the persistent length of the DNA modeled as a Gaussian chain and $\mu_0 = 4.3 \times 10^{-4}$ cm$^2$V$^{-1}$s$^{-1}$ is the intrinsic mobility of DNA in TBE buffer solution. This model is valid only for DNA molecules of chain length much larger than the pore size. This restriction along with the requirement that $E\rightarrow 0$ results in very long durations of electrophoresis (typically several hours). In comparison, pore size determination using absorbance measurements is a fast and non-invasive method.

![Figure 3. Variation of pore size of HM and LM agarose with concentration for various setting temperatures.](image)

The absorbance measurements are useful in pore size determination of agarose gel over a large concentration and temperature range. This is shown in figure 3. The pore size increases with the setting temperature due to melting of the weak junctions. With increase in concentration, $C$, the pore size decreases due to increased rate of nucleation and closer packing of the chains. Our results suggest $\xi \sim C^\nu$, with $\nu$ being $\approx 1.6$. For chemically cross-linked networks and gels, $\nu \approx 0.7$. However, physical gels such as agarose can exhibit significantly different exponents [6]. Dynamic light scattering (DLS) is generally used to study the dynamics and relaxation processes in sol-gel systems [6]. The normalized intensity autocorrelation function of the scattered light is measured and used to calculate the dynamic structure factor. From the evolution of the dynamic structure factor with the sol-gel transition, the various relaxation modes can be analyzed which characterize the viscoelastic properties of the sol-gel system. The pore size can be calculated from the diffusion coefficient of the fast mode. Though a powerful technique, DLS measurements are complicated by large fluctuations in intensity and relaxation time as gelation progresses and multiple scattering if the concentration is large. Comparatively, absorbance measurements are fast, less complicated and more suitable for concentrated samples.

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