Polymer 50 (2009) 5585–5588

Contents lists available at ScienceDirect

Polymer

journal homepage: www.elsevier.com/locate/polymer

Hierarchical structures in agar hydrogels

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ARTICLE INFO

Article history:
Received 2 April 2009
Accepted 12 September 2009
Available online 17 September 2009

Keywords:
Agar
Hydrogel
Small angle neutron scattering

ABSTRACT

Small angle neutron scattering experiments were performed on agar solutions and gels to explore their differential microscopic structures. In solution state, the wave vector, q, dependence of static structure factor, $I(q)$, could be described by $I(q) = I_g\exp(-q^2R_g^2/3)+I_Rq^{-\alpha}$. Statistical analysis gave: $R_g = 18$ nm and $\alpha = 0.85 \pm 0.07$ indicating the existence of rod-like rigid structures of length, $L = \sqrt{12}\,R_g \approx 63$ nm. In gels, $I(q) = I_G\exp(-q^2\Xi^2)+I_Fq^{-\beta}+(I_P/q)\exp(-q^2R_c^2/2)$ which had discernible Gaussian, power-law and Kratky–Porod regimes in the low, intermediate and high-q regions. Regression analysis yielded a characteristic length, $\Xi = 3.3 - 4$ nm for gels with agar concentration, $c = 0.1 - 0.3\%$ (w/v). The exponent $\beta = 1.2 \pm 0.2$ and the cross-sectional radius of cylindrical fibres, $R_c = 1.5 \pm 0.3$ nm remained invariant of agar concentration. This assigned a value 5 nm to the persistence length of the fibres in the solution phase that reduced to 3 nm in the gel phase indicating differential hydration of the fibres.

1. Introduction

Agar is a polycationic biopolymer which provides the most popular media for cell culture. The molecular structure of this polysaccharide is rather well known [1]. Agar comprises mainly of alternating β-(1-4)-D and α-(1-4)-L linked galactose residues in a way that most of α-(1-4) residues are modified by the presence of a 3,6 anhydro bridge [2]. Other modifications commonly observed are mainly substitutes of sulphate, pyruvate, urinate or methoxyl groups. The gelation temperature of agar is primarily decided by the methoxy content of the sample. Agar sols form thermo-reversible physical gels exhibiting large hysteresis between melting ($T_m \approx 85\,^\circ$C) and gelling ($T_g \approx 40\,^\circ$C) temperatures with the constituent unit being anti-symmetric double helices [3,4]. The internal structure of agar gel matrix is quite complex. Even in dilute solutions agar is known to form fibre bundles through extensive intermolecular hydrogen bonding [5]. Presence of microgels was reported in sol phase; these fibres and microgel domains aggregate in the gel phase to generate large network structures. Fluorescence correlation spectroscopy (FCS) and small angle neutron scattering experiments performed on agarose gels revealed the following facts: there is pore size distribution in the gel phase (size range 1 nm–900 nm) and there is presence of supra-molecular structures comprising of cylindrical fiber bundles [6,7]. The heterogeneous internal structure seen in electron micrographs implied the existence of microvoids randomly distributed in an ensemble of fibrous regions.

This immediately raises a pertinent question: Are the agar gels structurally different from other physical gels, particularly those comprising of biopolymers? Not many reports, of SANS experiments performed on these gels, are available in the literature. In this work, it is intended to compare the SANS data obtained from agar solution and gel. The objective is to seek for characteristic neutron scattering profiles of these phase states and establish internal microstructure of the gel matrix. It must be realized that polysaccharides have low neutron scattering cross-section and the scattering profile is often associated with a large incoherent background even in deuterated solvents. This problem can be addressed by careful background subtraction, a procedure that eventually allows extraction of meaningful structural information.

2. Materials and methods

The details of the materials used and the preparation of agar hydrogels is described elsewhere [8]. For SANS studies samples were prepared in D_2O. SANS is a diffraction technique, which involves scattering of a monochromatic beam of neutrons from the sample and measuring the scattered neutron intensity as a function of the scattering angle. These experiments were performed on the spectrometer at the G.T laboratory, Dhruva reactor (Bhaba Atomic Research Centre, Trombay, India). Further details of the SANS spectrometer at Dhruva are discussed in ref. [9]. The wavelength of the neutrons used covered the scattering vector (q) range, $q = 17 \times 10^{-3} \le q \le 3.2 \times 10^{-1}$ (Å)$^{-1}$ where $q = (4\pi/\lambda)\sin\theta/2$, λ is the wavelength (=5.2 Å) of neutron and θ (~0.5–15°) is the scattering angle. Thus, the instrument is well suited for the study of wide

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doi:10.1016/j.polymer.2009.09.033

range of systems having characteristic dimension between ≈10 and 200 Å. The gel samples were made in rectangular quartz cells of thickness 2 mm and scattered intensity, $I(q)$, was measured as function scattering vector, q. The measured intensity was corrected for the background and the empty cell contribution, and the data were normalized to get the structure factors. Furthermore, the two dimensional isotropic scattering data were azimuthally averaged. This was converted to an arbitrary unit scale using the incoherent scattering data of pure water. Details of the data normalization and background subtraction which is critical in data analysis are discussed in ref. [9,10]. Though, the counting statistics was low as is expected from polysaccharides in deuterated solutions, but it was noticeably larger than the incoherent scattering of H-atoms in agar. Special care was paid for background subtraction and this allowed unambiguous separation of the structure factor data into various q-regimes.

3. Results and discussions

The scattering profile obtained from a 0.05% (w/v) dilute agar solution (gelation concentration = 0.3% w/v) is shown in Fig. 1. Normally, polyelectrolytes are associated with small neutron scattering lengths, thus these are poor neutron scatterers. This poses a major hindrance that limits the structural information derivable from a typical SANS data. This is clearly seen from data presented in Fig. 1. However, it is possible to extract structural information from such data and this has been attempted here. A strong incoherent background in the $q > 0.1$ $(Å)^{-1}$ region, in fact, limits the overall experimental window available. The $I(q)$ versus q data was spliced into three regions: first region ($q < 0.04$ $(Å)^{-1}$) was least-square fitted to a Guinier function given by $I(q) \sim \exp(-q^2R_g^2/3)$ where R_g (=18 nm) is the radius of gyration of the scattering moiety, the following region ($0.04 < q < 0.1$ $(Å)^{-1}$) was fitted to a power-law function, $I(q) \sim q^{-\alpha}$ with $\alpha = 0.85 \pm 0.07$, and beyond this the incoherent regime prevailed.

Agar solutions are known to contain cylindrical fibres that are bundles of agar double helices and these stiff structures can be modeled as rods of length L and cross-sectional radius R_c. Assuming that $L \gg R_c$, R_g can be related to L as $R_g^2 \approx L^2/12$ which immediately gives $L \approx 63$ nm. The q_{cutoff} that separates the Guinier from the power-law regime was located at $q \approx 0.04$ Å that corresponds to a length scale [11] $L_p \approx 6/(\pi q_{cutoff}) \approx 5$ nm. This defines the persistence length of the fibres. For $q > q_{cutoff}$ it is expected that rod-like behaviour must manifest indicating $I(q) \sim q^{-1}$ dependence. The intermediate-q region did show a power-law dependence with $\alpha = 0.85 \pm 0.07$ which is close to the theoretical limit 1.

Thus, the scattering profile for the agar solution can be described by the generalized functional form

$$I(q) = I_g \exp\left(-q^2R_g^2/3\right) + I_R q^{-\alpha} \quad (1)$$

Small angle X-ray scattering data [12] assigns a length ≈ 100 nm to these fibre bundles (rods) and diameters ranging from 3 to 12 nm. Agar extracted from different sources have varying amount of β-D-galactose substituted by sulfate O-methyl groups, and the anhydride substituted by sulfate, O-methyl or pyruvate groups. Such substitution has significant bearing on the physical properties of the gel. Thus, our value of $L = 63$ nm is acceptable and comparable to the literature data. The data on hand allows us to estimate the characteristic ratio (C_∞) of the fibres from the relation [13]

$$C_\infty = \left(\frac{2L_p}{l_{chem}}\right) \quad (2)$$

where l_{chem} is the square root of the mean of the square of four chemical bonds comprising the main chain repeat unit [13]. Agar chain comprises of alternating β-D-galactopyranose and α-L-galactopyranose units joined by C–O–C bonds. This assigns an approximate value of 0.286 nm to l_{chem} (each C–O bond is ≈ 0.143 nm long) which yields $C_\infty \approx 34$ which reflects a large degree of local persistence (normal value is 5–12, see ref. [13]).

The generalized scattering function for gels normally assumes one of the following functions:

$$I(q) = I_G \exp.\left(-q^2\Xi^2\right) + \frac{I_L}{\left(1+q^2\xi^2\right)} \quad : \text{(Gaussian and Lorentzian)} \quad (3)$$

$$I(q) = \frac{I_{ex}}{\left(1+q^2\zeta^2\right)^2} + \frac{I_L}{\left(1+q^2\xi^2\right)} \quad : \text{(Debye—Bueche and Lorentzian)} \quad (4)$$

$$I(q) = I_F q^{-d_f} + I_V q^{-d_v} : \text{(Both power-law)} \quad (5)$$

The amplitudes of various components are given by I_G, I_L, I_{ex}, I_F and I_V defined at $q \to 0$. The second term (Lorentzian also called Ornstein–Zernike (OZ) function) in Eqs. (3) and (4) is normally referred to as gel mode since it accounts for gel osmotic moduli and mesh size (correlation length) through the parameters I_L and ξ respectively. In many SANS experimental data a strong long wavelength (small-q) scattering component is observed that can not be accounted for by the gel mode and must be treated separately. This excess scattering component is treated either as a Gaussian function (first term in Eq. (3)) or a Debye–Bueche (DB) function (first term in Eq. (4)). The characteristic size associated with the Gaussian function is Ξ and the same with the DB function is ζ. This size is normally attributed to the size of heterogeneities present inside the gel matrix. The heterogeneities observed in gels are the sum of at least two main affects: static concentration fluctuations generated by different degrees of local swelling of the polymer network, and clusters of aggregates (or bundles) embedded in the network.

In some gels, the scattering moieties are fractal structures that exhibit self-similarity over a wide range of length scales accessible

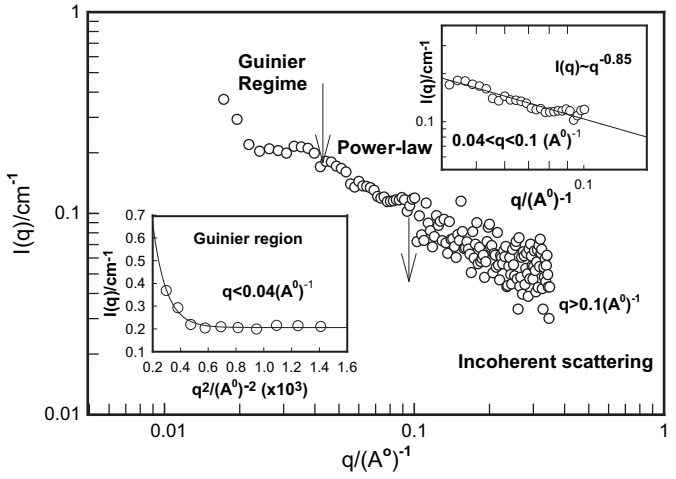

Fig. 1. Logarithmic plot of $I(q)$ versus q obtained from SANS experiments performed on agar solution samples at room temperature (25 °C). The low-q ($q < 0.04$ Å) and intermediate-q regimes (0.04 Å $< q <$ 0.1 Å) are fitted to Guinier and power-law functions respectively as shown in the insets. The noisy data pertaining to $q > 0.1$ Å could not be fitted to any functional form.

to SANS through change in q-vector. In our case, the fractal structure will be accessible from SANS data if self-similarity prevails in some spatial range below ≈20 nm. Near the borders of the fractal region the scattering function needs to be treated differently. The scattering function is described by Eq. (5) if the gel comprises of such structures and voids with the fractal dimensions of the components given by d_f and d_V respectively.

Chemical gels [14] faithfully yield SANS data that follow the q dependence given by Eqs. (3) and (4). Some physical gels like gelatin do follow the same pattern [15,16]. It has been observed that networks formed by statistical cross-linking of long chains (large persistence length) generate a novel gel matrix that is devoid of long range spatial correlations [17]. Such gels do, however, exhibit self-similarity over a well defined length scale. The behaviour of such gels can not be accommodated by the standard theory applicable to gels. The random cross-linking present in such networks creates a inhomogeneous gel matrix where crosslink density is strongly dependent on the spatial coordinate. This contributes to considerable loss in spatial order and such gels are called statistical gels [18,19].

Normally, the neutron scattering profile of a semi-dilute solution of a polymer is identical to that of the gel except in the low-q region where gel samples scatter selectively. Existence of junctions in gels increases their elasticity (raise shear modulus). This contributes to the reduction of dynamical fluctuations which generates a defect-free homogeneous gel [17]. However, the SANS data obtained from agar hydrogels clearly reveal anomalous behaviour. A set of SANS data is shown in Fig. 2 that was obtained from gels having agar concentration 0.1, 0.2 and 0.3% (w/v). This data could not fit to any of the functional forms described through Eqs. (3)–(5).

Beaucage et al. [11] have shown that if the SANS data shows local chain persistence, the scattering from the system can be globally fit with Gaussian scaling. In this picture, the chain structure is decomposed into two distinct levels: Gaussian and persistence regime. We have customized this concept to adapt it to our data in order to facilitate the analysis.

Excellent data fitting was observed when the following global fitting function was used.

$$I(q) = I_G \exp\left(-q^2\Xi^2\right) + I_F q^{-\beta} + \frac{I_P}{q}\exp\left(-q^2R_c^2/2\right) \quad (6)$$

The low-q region was fitted to a Gaussian function given by first term of Eq. (6) which is shown in the inset of Fig. 2. The data fitting yielded, $\Xi = 3.3$, 3.5 and 4.1 nm for 0.1. 0.2 and 0.3 (w/v) % gels respectively. The intermediate and high-q regions were fitted to functional forms given by second (power-law) and third (Kratky–Porod) terms of Eq. (6). Figs. 3 and 4 depict the typical fitting of the data in the intermediate and high-q regions which gave $\beta = 1.2 \pm 0.2$ and $R_c = 1.5 \pm 0.3$ nm. However, neither of these showed any concentration dependence.

According to the scaling theory, the correlation length exhibits a concentration dependence given by [20]

$$\xi = R_g\left(c/c^*\right)^{\frac{\upsilon}{1-3\upsilon}} \quad (7)$$

where c^* (=0.3% w/v) is the overlap concentration and the υ is the excluded volume exponent. The present studies reveal that there is no correlation length-like parameter in agar gels, However, if one replaces Ξ for ξ in Eq. (7) and substitutes the values obtained for Ξ corresponding to various gel concentrations one gets the excluded volume parameter, $\upsilon \approx 0.76$. This is much higher than the value expected for gels made in good ($\upsilon \approx 0.6$) and theta solvents ($\upsilon \approx 0.5$). In statistical gels this exponent assumes a value [17,18] ≈0.77 which matches with our result quite well.

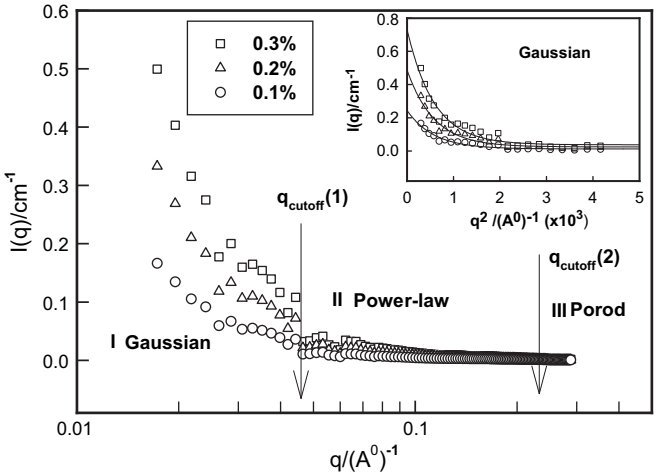

Fig. 2. Semi logarithmic plot of $I(q)$ versus q obtained from SANS experiments performed on agar gel samples at room temperature (25 °C). The low-q ($q < 0.065$ Å), intermediate-q regimes (0.065 Å $< q <$ 0.22 Å), and high-q regions ($q > 0.22$ Å) are discernible as far as statistical analysis was concerned. The low-q data was fitted to Gussian function as shown in the inset.

The data shown in Fig. 2 clearly shows two q-cutoffs that separates Gaussian from power-law, and power-law from Kratky–Porod regime. These are identified as $q_{cutoff}(1) = 0.065$ Å^{-1} and $q_{cutoff}(2) = 0.22$ Å^{-1}. Correspondingly, the persistence length of the fibres in the gel phase was estimated [11] to be $L_p(\text{gel}) = 6/(\pi\, q_{cutoff}(1)) \approx 3$ nm. Thus, it appears that the fibre stiffness reduces as the sol undergoes gelation probably due to strong fibre-solvent interactions. This is supported by the fact that $\beta = 1.2$ which is ≈40% higher than the similar parameter, α, for solutions, that had a value 0.85. This exponent assumes various values depending on the specificity of geometrical conformation of the scattering object found in the continuous medium. For example, $\alpha = 1$ for rigid rods, 1.7 for flexible chains in good solvent and 2 for Gaussian chains. Thus, an increase in the value of this exponent, as is seen in the present case, does signify gain in flexibility. The persistence length of fibre bundles in the gel phase is associated with a characteristic parameter, $C_\infty = 20$ indicating enhanced flexibility. These were found to have a cross-sectional radius, $R_c = 1.5 \pm 0.3$ nm as deduced from Kratky–Porod plot (Fig. 4). X-ray scattering studies

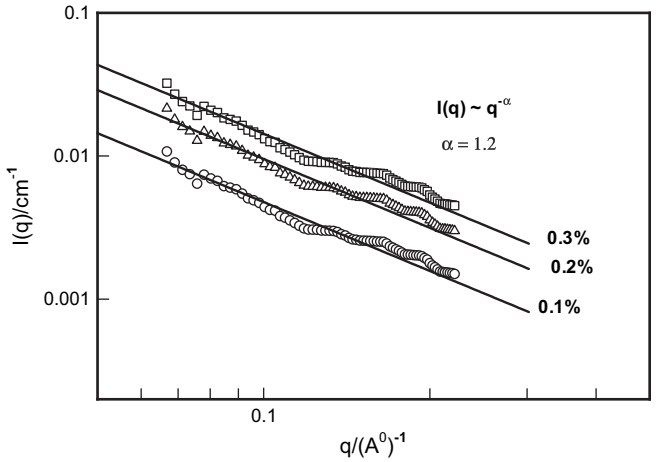

Fig. 3. Logarithmic plot of $I(q)$ versus q obtained from SANS experiments performed on agar gel samples at room temperature (25 °C) in the intermediate-q regimes (0.065 Å $< q <$ 0.22 Å). The data is fitted to the power-law expression, $I(q) \sim q^{-\beta}$. The exponent $\beta = 1.2 \pm 0.2$ which was found to be independent of agar concentration in the gel.

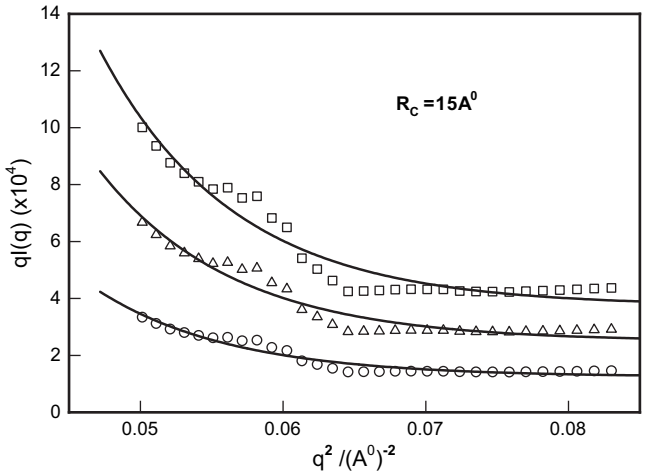

Fig. 4. Plot of $qI(q)$ versus q^2 obtained from SANS experiments performed on agar gel samples at room temperature (25 °C) in high-q regime ($q > 0.022$ Å). The data is fitted to $qI(q) = I_P \exp(-q^2R_c^2/3)$ which gave $R_c = 1.5 \pm 0.3$ nm which was invariant of agar concentration in the gel.

[12] have shown that the fibre bundles comprise of hexagonal rod structures with cross-sectional radius in the range 1.5–6.0 nm. Significant polydispersity was noticed in the cross-sectional diameter indicating various physical assembly of the double helix units that constitute the bundles.

In one of the reports [6,7], the SANS data from agar gel samples could be fitted to both Eqs. (3) and (5). The analysis yielded, the size of crosslinked domains, $\Xi = 6$ nm and pore size = 70 nm. In the power-law analysis, the results obtained were $\gamma = 1.71 - 1.81$ (low-q) and $d_f = 2.21$ (intermediate-q). Our results gave $\Xi = 3.3 - 4$ nm which is much less than the reported data. We could not find a q-region that could be fitted to a Lorentzian function which is normally called the gel mode. This raises several questions. Why are the scattering profiles different for gels made of agar molecules extracted from different sources? A continuous and sharp increase in the low-q region implies prevailing cross-linking in the gel matrix particularly in statistical gels. In our data, this was not identifiable. Agar is a typical low functionality gel where the fibre bundles are randomly distributed inside the gel phase. Each of these bundles is formed by the lateral aggregation of six double helices that forms the basic co-operative unite [12]. Higher order arrangements are possible that contribute to polydispersity in cross-sectional size.

4. Conclusions

Biopolymers are poor neutron scatterers and because of this not many reports of SANS experiments are reported in the literature. However, it has been shown that even when the scattering statistics is not appreciably large, it is possible to extract various structural information from such data. SANS data revealed that agar solution comprises of fibre bundles of typical length, $L = 63$ nm and these were associated with a persistence length, $L_p = 5$ nm. The scattering data in the intermediate-q region could be described by a power-law with exponent, $\alpha = 0.85$. The persistence length of fibre bundles was found to be 3 nm implying change in the specificity of fibre-solvent interactions. This is supported by the fact that the power-law exponent increased to a value 1.2 in the gel phase. The cross-sectional radius of these bundles was found to be 1.5 nm that indicated lateral assembly of multiple double helix units. The scattering profile of agar solution was observed to be significantly different from that of its gel phase. No correlation length (mesh size) could be attributed to these gels and the gel matrix was found to be associated with a hierarchy of structures each contributing to the scattering process uniquely. The scattering profile for the gel was observed to be anomalous, but close to that predicted for statistical gels. Though it has been argued that statistical gels have a random coil conformation, the absence of a discernible correlation length does attribute statistical gel-like features to Agar hydrogels which clearly necessitates further investigations.

Acknowledgement

This work was supported by a IUC-DAE-CSR research grant of Government of India. SB acknowledges receipt of a research fellowship from the University Grants Commission, Government of India. We are thankful to Dr. V. K. Aswal for assistance with SANS measurements.

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