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Studies on Probe Diffusion and Accessibility in Amylose Gels

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ABSTRACT: The concentration dependence of the porosity of aqueous amylose gels was determined using two complementary experimental approaches. In the first, the volume of the gel accessible to probe species was investigated and an average pore size calculated. The probe species included globular proteins, flexible coil polysaccharides, and latex spheres. In the second, the effect of the gel network on retarding the diffusion of a globular protein (bovine serum albumin) was examined. The retardation in diffusion was considered to be due to hydrodynamic screening. The observed concentration dependence of screening length was compared to that predicted by recent theoretical approaches.

Introduction

The diffusion of solutes through porous media, including polymeric matrices, has many practical applications including the controlled release of pharmaceuticals and agrochemicals.[1-3] Usually the solute is a low molecular weight species. The rate of release may be manipulated by controlling the extent and rate of swelling of the matrix and hence the pore size. It is also of interest to study the diffusion of macromolecular species in similar matrices. In some applications, the controlled release of enzymes may be an objective; in others, enzymes may be used to degrade the polymer matrix which contains the active ingredient. In either case, in order to manipulate the rate of release, it is necessary to understand the relationship between the pore size of the matrix and the diffusion rate and accessibility of the macromolecule.[2,4]

The study of the diffusion of probe species in semidilute macromolecular solutions and gels is a topic of continuing research.[5-9] An early theoretical approach modeled the polymer solution or gel as a random collection of fixed rigid rods. A diffusive step was not allowed if it would have involved collision with obstacle.[10] More recently, hydrodynamic screening has been thought to be a more appropriate description of the physical process responsible for the retardation of diffusion.[11-13] In these approaches, semidilute solutions were considered as a three-dimensional fishnet characterized by an average mesh size, ξ, which corresponds to the distance between two entanglement points. The hydrodynamic behavior of a probe molecule depends strongly on the ratio of the probe hydrodynamic radius, R_H, to the mesh size, ξ. When $R_H \ll \xi$, the matrix appears as a continuum, and the Stokes-Einstein relationship

$$D = k_B T / 6\pi\eta R_H \quad (1)$$

may be used to describe the diffusion process, where D is the diffusion coefficient of the probe, k_B and T have their usual meanings, and η is the macroscopic viscosity of the polymer solution. When R_H and ξ are of comparable scale, the hydrodynamic behavior of the probe molecule diverges from eq 1 and shows a strong dependence upon polymer concentration. There has been recent interest, both theoretical[14,15] and experimental,[5,6,8,9] on the influence of polymer concentration on mesh size and on the diffusion of probe particles in polymer networks. There have been fewer studies on probe diffusion in gel matrices, although the diffusion of dextrans in agarose gels has recently been examined by quasi elastic light scattering (QELS).[16] Polymer gels are usually pictured as infinite three-dimensional networks, within which the polymer molecules mobility is rather small. In this case, when $R_H \gg \xi$, the probe particle is totally excluded from the gel. Usually the mesh size varies over a quite wide range, and the gel may be more or less accessible to probe species of a varying size range.

The study of the effect of networks on diffusion and accessibility of macromolecular probes is important to understanding factors that might limit the enzymic degradation of porous biopolymeric materials and is thus relevant to biotransformations, nutrition, and some controlled release applications. In this study the porosity of a gel network of the starch polymer amylose was examined.

Amylose is an essentially linear polysaccharide composed of α-(1-4)-linked D-glucose units. Concentrated aqueous solutions of amylose form turbid gels on cooling to room temperature.[17,18] The turbidity of the gels

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indicates the presence of aggregated material on the order of 1 μm in size. Dilatometry has been used to follow this aggregation process, and it was found that the volume change occurred at the same rate as the development of gel stiffness.[18] The amylose gel is poorly crystalline, the crystallinity developing over a much longer (~2 days) time span than the development of modulus (<2 h). As a result, it was proposed that the primary event in the gelation of amylose is a phase separation which produces an interconnected polymer-rich phase.[19] Subsequently, a limited crystallization of the amylose occurred in this polymer-rich phase. An alternative view[20] has been expressed in a recent study where it has been proposed that the connectivity of amylose gels is due to double-helix formation. Recent electron microscopic examination of vitrified gel slices, prepared by very rapid freezing, revealed the presence of a fibrillar network, the individual strands of which had a radius of 5–10 nm and therefore consisted of assemblies of chains.[21] In the present study, the effect of the microstructure of an amylose gel network on the diffusion of a macromolecular probe (bovine serum albumin, BSA) and the accessibility of the gel to probe species of varying size were examined. Retardation in the diffusion and accessibility were investigated as a function of gelling polymer concentration.

Experimental Section

Materials. D-Glucose, maltose, stachyose, and crystalline preparations of the proteins lysozyme, chicken ovalbumin, and catalase were obtained from Sigma Chemical Co. (Poole, U.K.). The proteins' purity was checked by chromatography on a Pharmacia Superose 12TM column, 30 × 1 cm (Uppsala, Sweden). In each case a single peak, accounting for >95% of the protein applied on the column, was obtained in elution with trizma buffer (pH 8) at a flow rate of 1.5 mL/min. L-[1-^{14}C]Glucose was obtained from Amersham International plc (Amersham, U.K.), and dextran fractions were obtained from Sigma and pullulans from Polymer Laboratories Ltd. (Loughborough, U.K.). Latex microspheres of radius 0.55 nm were obtained from Polysciences Inc. (Warrington, PA, U.S.A.). Amylose was prepared by aqueous leaching of smooth-seeded pea followed by precipitation of the amylose as its 1-butanol complex (Analar grade 1-butanol, BDH, Poole, U.K.). Amylose gels were prepared as described elsewhere[17] and stored for 3 days at 1 °C.

Lysozyme, ovalbumin, and BSA were dissolved in 0.223 M acetate buffer, pH 4.6, and catalase was dissolved in 0.2 M phosphate buffer, pH 7.6. The proteins used in this study have been extensively characterized and their sizes determined from X-ray scattering experiments.[22]

The pullulan and dextran fractions were chromatographed on a TSK-GEL GMPWXL column (30 × 0.4 cm^2) (Toyo-Soda Ltd., Tokyo, Japan) at a flow rate of 1 mL/min. From the known polydispersity of the pullulan fractions (M_w/M_n ranging from 1.14 to 1.07) the polydispersity of the dextran fractions was calculated. The intrinsic viscosities of these polysacchardie fractions were determined from measurements of the specific viscosity of dilute aqueous solutions using Ubbelohde suspended level viscometers. The hydrodynamic radius of the polysaccharide fraction was then calculated using manufacturers' data on molecular weight and polydispersity. The values obtained are summarized in Table I.

Accessibility Experiments. An aqueous amylose gel (~1 cm^3) was immersed in 1 cm^3 of a 0.1% w/w aqueous solution of the macromolecular probe at 25 °C containing 0.02% sodium azide as a preservative. The macromolecule was allowed to diffuse into the gel for 1 week after which time an equilibrium existed between the concentration of probe molecule in the gel and in the external solution. At the end of this time, the concentration of macromolecule in the external solution was determined using a Lowry–Hartree method[23] for the proteins and a phenol–sulfuric method[24] for the polysaccharides. When a colorimetric iodine binding method[25] was used, the solubilization of amylose from the gel was not detected. From a knowledge of the initial and final concentrations of macromolecule in the external solution and an accurate value of the gel volume it was possible to calculate the volume of the gel that was accessible to the macromolecules. The volume of solvent trapped within the gel network was calculated from measurements of the gel volume and partial specific volume of the amylose. After the experiment it was possible to elute quantitatively the macromolecule from the gel, indicating the absence of macromolecular adsorption on the gel network.

Table I
Molecular Weight (MW, g·mol^{-1}) and Hydrodynamic Radii (R_H, Å) of the Probe Molecules Used in the Accessibility Experiment

protein	lysozyme	ovalbumin	BSA	catalase
MW	14 000	45 000	65 000	250 000
R_H	20.6	27.6	36.1	52.2
pullulan				
MW	5800	186 000	380 000	853 000
R_H	16	90	129	193
dextran				
MW	65 600	472 000	2 000 000	
R_H	60	150	270	

Diffusion Experiment. Concentrated aqueous solutions of amylose were cast as gel cylinders (5 × 0.8 cm^2) in open-ended syringes. The gels were preequilibrated with 0.223 M acetate buffer, pH 4.6, for 48 h at 25 °C. After equilibration a thin slice was cut from the gel cylinder to expose a fresh surface, which was placed in contact with 15 mL of the 0.5% w/w BSA solution in acetate buffer. BSA was allowed to diffuse into the gel for 72–90 h exactly, after which time, the first 1.5 cm of the gel was cut into thin slices ~0.15-cm thick and accurately weighed. The abscissa of each slice was calculated from its weight and the gel cylinder dimension. Acetate buffer (1.2 mL) was then added to each slice to allow reverse diffusion. Protein concentration was measured 24 h later by a Lowry–Hartree test.[23] Protein was not detected in slices cut from the upper part of the gel; therefore, the diffusion process can be represented as a diffusion into a semiinfinite medium. This method was chosen as the best suited for amylose gels, as their opacity prevented the use of optical methods. In a similar way, the diffusion of radioactively labeled L-glucose into the gel cylinder was investigated using liquid scintillation counting to determine the concentration of glucose.

The translational diffusion coefficient, D_T, of BSA in 0.223 M acetate buffer, pH 4.6, was measured by QELS as described elsewhere.[26] In the concentration range 0.3–1.0% w/w the measured D_T at 25 °C was 6.4 ± 0.13 × 10^{-11} m^2 s^{-1}. No significant concentration dependence of D_T in this concentration range was observed.

Results and Discussion

Preliminary Considerations. The physical principles governing the accessibility of a gel to probe species are similar to those for the process of size-exclusion chromatography (SEC). Theoretical treatments of SEC have often started from the assumption that the elution volume of the solute is determined by its equilibrium partitioning between interstices of macroscopic dimensions and the microporous chromatographic medium. If energetic interactions between the probe and the support are small, then partitioning is expected to arise solely as a result of size differences. For macromolecular probes various dimensions can be used to described their size. From a theoretical viewpoint the mean external length of the probe species is thought to be the most appropriate quantity to use.[27]

For spherical particles this is equivalent to the hydrodynamic radius, R_H. With increasing asymmetry R_H is no longer the appropriate dimension,[27] and large discrepancies have been observed between the true Stokes radius measured by hydrodynamic methods and the apparent value derived from SEC experiments.[28] For markedly

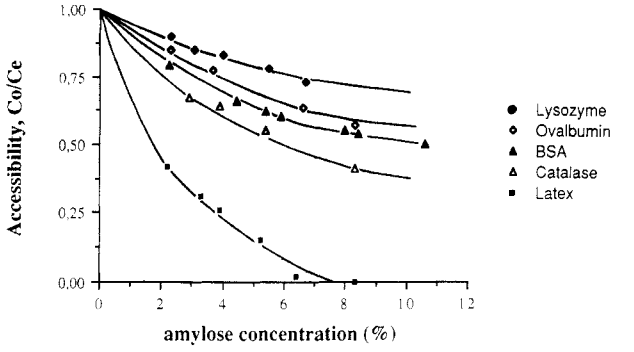

Figure 1. Plot of accessibility expressed as C_o/C_e, as a function of amylose concentration for the globular proteins and latex beads.

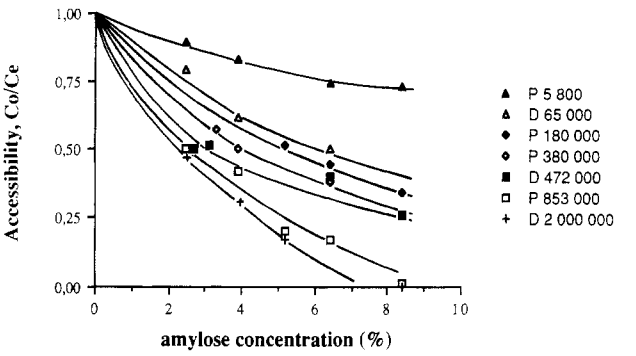

Figure 2. Plot of accessibility expressed as C_o/C_e, as a function of amylose concentration for the flexible polysaccharides: dextran (D) and pullulan (P).

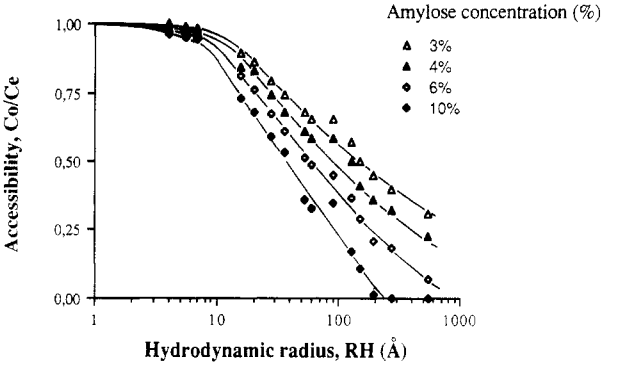

Figure 3. Plot of accessibility versus hydrodynamic radius, R_H, of probe species as a function of amylose gel concentration.

asymmetric particles this discrepancy can be large. For example, the native fibrinogen has a true Stokes radius of 108 Å while that estimated from SEC experiments is 71 Å.[28] For the globular proteins used in the present study which do not deviate substantially from spherical symmetry the hydrodynamic radius should give a good estimate of the size parameter determining its accessibility to different regions of the gel. The characteristics of the proteins are summarized in Table I.

For the flexible polysaccharides the hydrodynamic radius was again chosen as the size parameter (Table I). Flexible polymers having the same hydrodynamic volume usually have the same elution volume in SEC. Although R_H may not always control size-exclusion effects,[29] many experimental studies have found it represents these effects well enough.[30]

A further consideration is the assumption that there are no energetic interactions between the probe and the porous medium. It is known that such interactions can markedly affect partitioning.[31,32] To minimize charge interactions between the protein and the neutral amylose network, experiments were conducted, when possible, at a pH close to the isoelectric point of the protein. For the ionic strength used, charge interactions between probe and network should be small. The polysaccharides chosen for the study are, at high concentrations, incompatible with amylose in aqueous solution,[33] this arises as a result of a weak repulsive interaction. In the present study, dilute solutions of the probe polysaccharide were used to minimize this interaction.

Accessibility Studies. When a polymer gel is initially immersed in a solution containing a macromolecular probe, a concentration gradient exists. With time, an equilibrium will be reached between the external probe concentration and the probe concentration in accessible regions of the gel. The accessibility can therefore be described by the ratio C_e/C_o where C_o would represent the probe concentration if the volume of the solvent trapped within the gel network was totally accessible and C_e the measured probe concentration in the gel. The accessibility ranges from 1 (totally accessible structure) to 0 (totally inaccessible structure). Plots of accessibility versus the amylose concentration are shown Figure 1 for the globular proteins and the latex beads and Figure 2 for the polysaccharide probes. The gel accessibility shows a sharp decrease, when the polymer concentration and/or probe size are increased.

These results are summarized in Figure 3, which presents plots of accessibility versus hydrodynamic radius of probe species, R_H, for amylose gels in the concentration range 3–10%. For probes such as the carbohydrates glucose (R_H = 4 Å), maltose (R_H = 5.5 Å), and stachyose (R_H = 7 Å), where R_H < 10 Å, the volume of solvent trapped within the network is completely accessible to the probe. With increasing hydrodynamic radius, accessibility is reduced, but only for the more concentrated amylose gels (above 7% w/v) is the largest probe (a latex sphere, R_H = 550 Å) completely excluded from the gel. Accessibility is a reasonably continuous function of hydrodynamic radius for each of the gels, giving an indication that the assumptions involving size of probe species, and the lack of energetic interactions between probe and gel are reasonable. The small deviations that are present for the polysaccharide fractions may be due to their polydispersity. These experiments on the dependence of accessibility on particle radius of the probe give therefore a useful measure of pore size. It is of interest to examine the dependence of this pore size on concentration. The value chosen to represent an average pore size was the value of hydrodynamic radius for which 50% of the volume of solvent trapped within the gel was accessible to the probe species. The concentration dependence of this average pore size, R_P, is shown in Figure 4 as a double logarithmic plot with a dependence of R_P on concentration of $C^{-0.82}$ (r = 0.99).

Diffusion Studies. For the experimental conditions chosen, the diffusion process can be described as a diffusion into a semiinfinite medium, with the boundary between the gel and the protein solution being at an effective constant concentration C_o and the initial concentration being zero throughout the medium. In this case, the appropriate solution of Fick's law is[34]

$$C_x = C_o \operatorname{erfc}[x/2(Dt)^{1/2}] \quad (2)$$

where C_x is the concentration at a distance x from the gel surface, D the diffusion coefficient, and t the time. The values of the error function, erfc, were found from tables. The validity of the expression in eq 2 for the

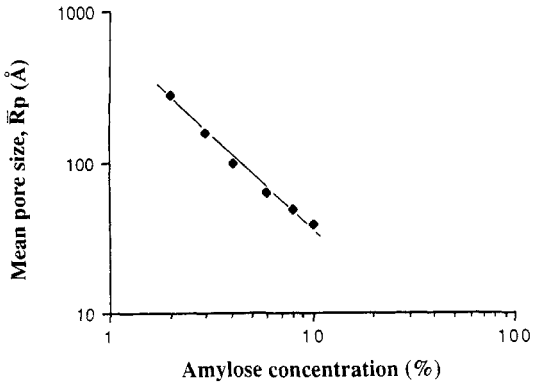

Figure 4. Double logarithmic plot of average pore size, R_P, versus concentration of amylose in the gel.

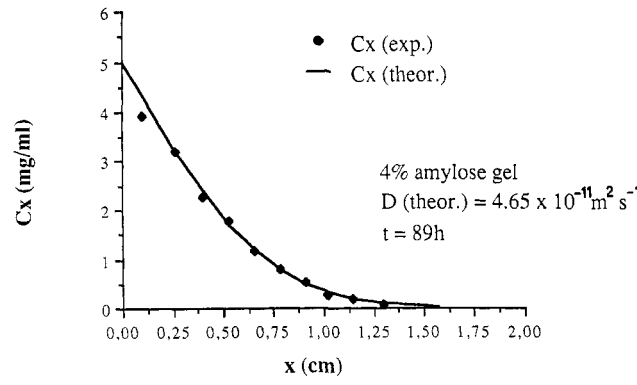

Figure 5. Comparison of the theoretical values of C_x, calculated from the equation $C_x/C_0 = \text{erfc}\,(x/2(Dt)^{1/2})$ with the experimental C_x, expressed as a function of x.

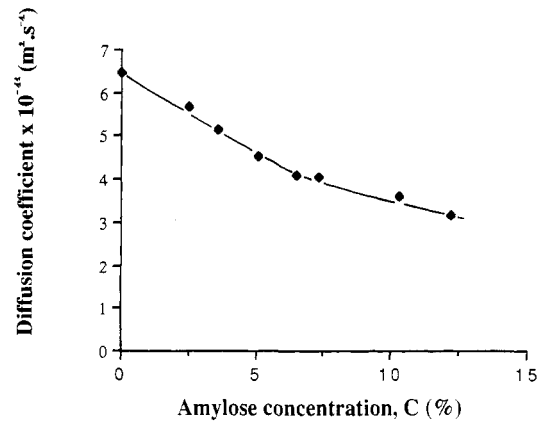

Figure 6. Plot of diffusion coefficient of BSA as a function of concentration of amylose in the gel, allowance for the extent of accessibility being made.

boundary conditions used in the experiment was checked by measuring the diffusion of L-glucose into the gel. The dependence of concentration on distance x was well described by eq 2, and the measured diffusion coefficient of 6.15×10^{-10} m^2 s^{-1} at 25 °C was in good agreement with the published values.[35]

For molecules of a larger size a retardation effect of the amylose network on the diffusion process is expected. To gain information on the extent of retardation and its dependence on concentration of amylose, BSA was chosen as the probe species. The reasons for this are 2-fold. First, its diffusion behavior has been extensively characterized both by classical techniques[36,37] and more recently by QELS.[38] Second, BSA, as a globular protein, is similar in size to many amyloyltic enzymes.[39] Its diffusion behavior therefore should be comparable, and this study should give useful insights into diffusion processes involved in the α-amylolysis of solid forms of starch.

When classical techniques (e.g., diaphragm cell) were used, the measured mutual diffusion coefficient, D_M, of BSA at an extrapolated zero concentration was found to be 5.0×10^{-11} m^2 s^{-1} at 1 °C in 0.5 M KCl, pH 5.14. When the Stokes–Einstein relationship is used, corrected values for D_M of BSA in water at 20 and 25 °C are 5.9×10^{-11} and 6.6×10^{-11} m^2 s^{-1}.[36] Similar values have been obtained in other studies.[37] More recently, QELS has provided a rapid method for measuring the translational diffusion coefficient, D_T.[38] While there is still debate on the equivalence of D_M and D_T particularly at high concentrations, values of D_T obtained after extrapolation to zero concentration are in very good agreement with values of D_M obtained by classical techniques.[38] In the present study, the corrected value for D_T in water at 25 °C was 6.4×10^{-11} m^2 s^{-1}. Preparations of BSA can contain dimers or higher aggregates, usually in the range 4–10% w/w. The purity of the BSA used in the present experiments was >95% as assessed by SEC. The values of D_T obtained for this BSA preparation, using QELS, are in excellent agreement with accepted values of D_M obtained by classical techniques. Aggregates, if present, do not therefore have a strong effect on the observed diffusive behavior. As the QELS experiment is potentially more sensitive to the presence of aggregated material than the layer analysis experiment, errors introduced into the latter experiment through the presence of BSA aggregates are expected to be rather small.

The solvent trapped in an amylose gel is not wholly accessible to the BSA, as clearly shown previously (Figure 1). The accessibility decreases from 80 to 50% when the polymer concentration is increased from 2 to 10% w/v. When the diffusion coefficient is calculated from eq 2, allowance for the inaccessible part of the gel (polymer + associated solvent) was made through a simple correction of C_x. Figure 5 shows experimental and predicted behavior for the dependence of BSA concentration (C_x) on distance (x) for a 4% w/w amylose gel after 89 h at 25 °C. A very good fitting (standard deviation 0.15) of the predicted curve to the experimental data points was observed over the range of amylose gel concentrations studied. Results obtained on gel columns of the same amylose concentration were within ±5% of each other. A number of points can be made. C_x is a simple function of x, indicating that there are no discontinuities in structure or sharp changes in pore size as a function of x. Deviations from predicted behavior were not observed under the experimental conditions of the study. Surface effects, such as adsorption of BSA on the gel network and continuous changes in pore size with x, are not significant. Figure 6 shows a plot of diffusion coefficient, D, of BSA as a function of gelling polymer concentration. The diffusion coefficient of BSA changes from 5.53×10^{-11} m^2 s^{-1} to 3.70×10^{-11} m^2 s^{-1} on increasing amylose concentration from 2 to 10% w/w.

This retardation effect expressed as D/D_0, where D_0 is the expected diffusion coefficient in pure solvent at the same temperature, is predicted to depend on a screening constant, κ

$$D/D_0 = \exp(-\kappa R) \qquad (3)$$

where R is the particle radius.[11] The concentration dependence of the screening length is of interest. Figure 7 shows a plot of ln (−ln (D/D_0)) against ln C; the plot is linear and indicates a dependence of screening length on concentration of $C^{0.73}$ (r = 0.98).

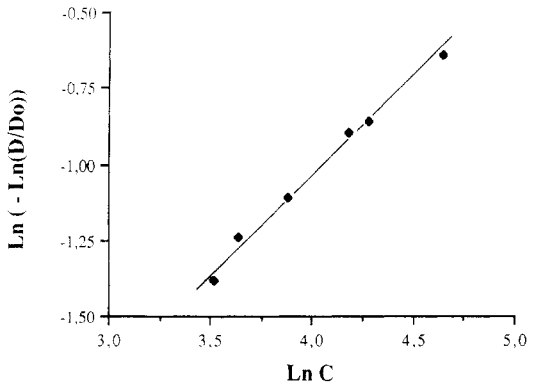

Figure 7. Plot of retardation of diffusion of BSA, expressed as ln [−ln (D/D_o)] as a function of ln C, the amylose concentration.

General Discussion. A large number of recent studies have examined the diffusion of probe species in semidilute polymer solutions.[5-9] There have also been recent theoretical developments that indicates that hydrodynamic screening is the main physical process responsible for retardation of diffusion.[11-13] The screening constant, κ, is a measure of the resistance to the fluid flow due to the polymer network. Experimental studies have often used latex particles as the probe species with flexible or rodlike polymers in solution.[5,6,8,9] Equations of the same form as eq 4 have usually given a good representation of the experimental observed behavior,[7] i.e.

$$D/D_0 = \exp(-AC^{\nu}) \quad (4)$$

with A and ν as adjustable parameters. The exponent ν is generally found to vary between 0.5 and 1.0, although values outside of this range have been reported.[6,40] The predicted value of ν for a polymer solution consisting of rodlike molecules is 0.5[11] whereas for solutions containing flexible coils, no single value has been predicted. The value of the exponent may lie in the range 0.5–1.0 depending on factors such as the quality of the solvent[11] (e.g., 1.0 for a coil in a Θ solvent, 0.75 in a good solvent) or assumptions made in theoretical treatments.[15] Reciprocal pore size, R_P^{-1}, is expected to have a similar dependence concentration.

Experimental studies may test these predictions; in some cases, experimental agreement with predicted behavior has been observed.[8] More usually the experimentally determined value of the exponent ν is at variance with predicted values.[6] There may be a number of reasons for this. First, most experimental studies have been conducted on semidilute polymer solutions, the network is temporary, and the diffusion of the probe particle may perturb the network. Second, electrostatic interactions between probe species and network can affect the observed concentration dependent behavior. It is also important to note that the most commonly used experimental technique for monitoring diffusion has been quasi elastic light scattering (QELS).[8,16,40] A probe such as a latex sphere may diffuse within a "cage" formed by the polymer molecules or diffuse from cage to cage. The QELS experiment will contain information on both processes, yet in this study only the latter is probed.

In the present study, R_P^{-1} and κ show a $C^{0.82}$ and a $C^{0.73}$ dependence, respectively. The amylose gel can be considered as a randomly branched microfibrillar network, for this rodlike network a $C^{0.5}$ dependence of κ and R_P^{-1} is expected. The reasons for the differences between predicted and observed behavior are not clear. For a neutral polymer network and a neutral or weakly charged probe species interactions between probe and network are expected to be weak. The value of the exponent obtained is within the range expected for a network formed from flexible network strands. This does not accord with proposed models of the structure of amylose gels.

Despite the lack of agreement between predicted and experimental behavior, the simple scaling dependence of pore size and screening length gives a valuable indication on how the rate of enzymic degradation of these materials might be affected by concentration of network polymer.

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Registry No. D-Glucose, 50-99-7; maltose, 69-79-4; stachyose, 470-55-3; amylase, 9005-82-7; lysozyme, 9001-63-2; catalase, 9001-05-2; pullulan, 9057-02-7; dextran, 9004-54-0.