

Diffusion of Macromolecules in Agarose Gels: Comparison of Linear and Globular Configurations

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ABSTRACT The diffusion coefficients (D) of different types of macromolecules (proteins, dextrans, polymer beads, and DNA) were measured by fluorescence recovery after photobleaching (FRAP) both in solution and in 2% agarose gels to compare transport properties of these macromolecules. Diffusion measurements were conducted with concentrations low enough to avoid macromolecular interactions. For gel measurements, diffusion data were fitted according to different theories: polymer chains and spherical macromolecules were analyzed separately. As chain length increases, diffusion coefficients of DNA show a clear shift from a Rouse-like behavior ($D_G \propto N_0^{-0.5}$) to a reptational behavior ($D_G \propto N_0^{-2.0}$). The pore size, a , of a 2% agarose gel cast in a 0.1 M PBS solution was estimated. Diffusion coefficients of the proteins and the polymer beads were analyzed with the Ogston model and the effective medium model permitting the estimation of an agarose gel fiber radius and hydraulic permeability of the gels. Not only did flexible macromolecules exhibit greater mobility in the gel than did comparable-size rigid spherical particles, they also proved to be a more useful probe of available space between fibers.

INTRODUCTION

The diffusion of macromolecules through porous medium is important in many fields of the biological sciences. The phenomenon is relevant to biotechnological separation methods such as electrophoresis and size exclusion chromatography. Moreover, it has important implications for understanding how macromolecular drugs and naturally occurring macromolecules are transported in living tissue. The development of therapeutic proteins and genes and of liposome and polymer-based drug carriers has made it important to understand how well such agents diffuse through tissue. For example, the failure of gene vectors to penetrate the extracellular matrix (ECM) surrounding cells may contribute to the overall failure of delivery that has plagued gene therapy trials to date (Jain, 1994, 1998; Verma and Somia, 1997).

A quantitative description of macromolecule penetration in relation to the ECM structure would be a valuable tool for evaluating and developing viable macromolecule-based therapies. At present there is no validated model that reliably predicts how penetration depends on the macromolecule and tissue properties. Even in artificial model systems, there is uncertainty about the appropriate model for permeability of a porous medium to macromolecules (Johnson et

al., 1996; Saltzman et al., 1994; Tong and Anderson, 1996; Williams et al., 1998).

The physical resistance of a medium such as the ECM to macromolecule motion is related to the available volume between fibers. We hypothesize that a “characteristic matrix pore size” can be defined to provide a measure of this resistance, even in a complex matrix composed of fibers of different sizes. The goal of this study was to ascertain whether such a characteristic pore size could consistently describe the diffusion of a wide range of macromolecules in a structurally well-characterized artificial fiber-matrix (agarose). To this end the diffusion of macromolecules in agarose gels was observed by the fluorescence recovery after photobleaching (FRAP) technique (Berk et al., 1993), a technique widely used to determine in vitro or in vivo diffusion of fluorescently labeled macromolecules (Berk et al., 1997; Johnson et al., 1996; Pernodet et al., 1997; Pluen et al., 1998). Although proteins and dextrans are commonly used in biological transport studies (Berk et al., 1997; Chary and Jain, 1989; Johnson et al., 1996; Tong and Anderson, 1996), and DNA has been extensively studied to understand mechanisms of electrophoresis (Tinland et al., 1998; Pluen et al., 1998; Smith et al., 1996a; Tinland et al., 1996), to our knowledge this study is the first to probe all these macromolecules and span a large range of molecular size in a single set of experiments.

Theoretical models for diffusion in a porous matrix

The structure model proposed by Ogston et al. (1958, 1973) describes the diffusion of macromolecules through an array of straight cylindrical fibers of radius, r_f , and fiber volume fraction, ϕ . The solution is derived from stochastic arguments by considering the probability that a molecule hydrodynamically equivalent to a sphere of radius, R_H , would

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encounter spaces large enough to permit its movement. A similar approach was used by Giddings et al. (1968) to evaluate the partition coefficient, K , of spherical molecules in cylindrical pores, but here the fiber radius is related to the pore radius, r_p . However, agreement between these models and experimental data is unsatisfactory (Doi and Edwards, 1986; Johnson et al., 1996; Moussaoui et al., 1992; Tong and Anderson, 1996; Williams et al., 1998).

In the Ogston model, hydrodynamic interactions between the mobile solute and the fibers are not taken into account. Phillips et al. (1989, 1990) proposed that diffusivity could be estimated from Brinkman's equation by treating the fiber array as an effective medium characterized by its Darcy permeability, κ . The square root of the Darcy permeability is a hydrodynamic screening length of the order of the fiber spacing. Kosar and Phillips (1995) argued that the essential parameter is the Darcy permeability and that macromolecular diffusivities in gels or other fibrous media can be predicted from a single macroscopic measurement. Following this approach, Brady (1994) proposed that the hydrodynamic (permeability) and steric (fiber radius) effects influencing macromolecular diffusivities can be separated in two multiplicative factors.

Although the above approaches may be applicable for small spherical macromolecules, the diffusion of polymer chains must be addressed using the polymer physics theory (de Gennes, 1979; Doi and Edwards, 1986). The gel is considered as a network of overlapping or cross-linked chains in which "pore size" or "mesh size" is a defining parameter of the system. The diffusion of a polymer in a fixed network is defined as the movement of a chain in a tube; thus the chains forming the gel are treated as fixed obstacles (Doi and Edwards, 1986; de Gennes, 1979). The gel fibers are assumed to be sufficiently thin to eliminate static contributions so that only the dynamic contributions need to be considered.

The diffusion of polymer chains based on statistical conformations describes the chain as a function of its monomer unit number, N_0 , and the obstacle density, c_{obs} . Computational studies (Yamakov and Milchev, 1997) have clearly shown that an increase in obstacle density shifts the chain diffusion dependence from a Rouse-like behavior in which the chain migrates in an ellipsoid conformation to a reptational behavior in which the chain flexibility allows its elongation. The characteristic pore size is reflected by the chain length at which the crossover between these two behaviors occurs. Separate measures of the effective pore dimension can be extracted from the value of diffusion coefficient measured for each chain length within the reptation domain.

Applying the theoretical models

Diffusion measurements were performed in free solution and in 2% agarose gels. Measurements in buffer solution at suitably low macromolecule concentrations served the pur-

pose of providing the free diffusion coefficient in 0.1 M PBS solvent and the corresponding hydrodynamic radius.

The analysis of data was performed as follows: the diffusion coefficients of the polymer chains (DNA) were analyzed with the Zimm-Rouse model (Doi and Edwards, 1986) and the reptation model (de Gennes, 1979), yielding an evaluation of the effective pore size of the 2% agarose gels. Although dextrans are polymer chains, these molecules are not large enough to exhibit behavior in the two regimes previously mentioned. Thus, they were analyzed with the globular macromolecules. Protein and polymer bead diffusion coefficients were treated with the Ogston model (1958, 1973), Renkin model (1954), Giddings model (1968) and the effective medium model (Phillips et al., 1989, 1990) based on Brinkman's model (1947) to estimate the agarose fiber radius, pore radius, and the hydraulic permeability of these gels. Finally, a general picture concerning the transport of macromolecules in random porous media is proposed.

MATERIALS AND METHODS

Macromolecules

Three fluorescently labeled proteins (bovine serum albumin (BSA), ovalbumin, and lactalbumin) purchased from Molecular Probes (Eugene, OR) and three neutral fluorescently labeled dextrans (4000 MW, 71,200 MW, and $2 \cdot 10^6$ MW), all labeled with fluorescein isothiocyanate, were purchased from Sigma (St Louis, MO.). Three different sizes of green fluorescent polymer microspheres (diameter: 26 nm, 57 nm, 103 nm) were purchased from Duke Scientific Corp. (Palo Alto, CA). For DNA, λ DNA (48502 bp), λ HindIII digest DNA (eight fragments: 23,130 bp, 9416 bp, 6557 bp, 4361 bp, 2322 bp, 2027 bp, 564 bp, 125 bp), and plasmid Φ X174 (5486 bp) were purchased from New England Biolabs (Beverly, MA), and T2 (164,000 bp) bacteriophage DNA was purchased from Sigma (St. Louis, MO). The native plasmid Φ X174 (5486 bp) was linearized using the restriction enzyme *Pst*I.

Labeling of DNA molecules

YOYO-1 (Oxazole Yellow homodimer, Molecular Probes, Eugene, OR), an intercalating fluorescent probe, was stocked in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) at a stock concentration of 0.01 mM. To label DNA, YOYO-1 was added to the DNA solution at a ratio 1:50 basepairs: this ratio is high enough to provide a good signal yet low enough not to modify the structure of dsDNA (Carlsson et al., 1996; Gurrieri et al., 1997). Fluorescein-12-dUTP (Boehringer Mannheim, Mannheim, Germany) was used following the protocol given by Boehringer Mannheim (reference catalog 1373 242). The degree of labeling is in that case between 1:25 and 1:50. The excess of free Fluorescein-12-dUTP was eliminated by electrophoresis in dense polyacrylamide gels (10%T 5%C).

Gel

Agarose gels were prepared by adding the desired volume of 0.1 M PBS buffer (phosphate-buffered saline, Sigma) to the measured amount of agarose powder (type VII: low gelling temperature, Sigma). The resulting slurry was heated to 90°C until complete dissolution of agarose, and then slowly cooled. Before gelation, the viscous agarose solution was cast, then slowly cooled at room temperature. Once the gel was formed, it was immersed in 0.1 M PBS solution and allowed to equilibrate. To calculate

the fiber radius (Ogston et al., 1973) or the permeability (Phillips et al., 1989), the volume fraction of fibers for agarose gels must be known. The volume fraction of agarose fibers, ϕ , was obtained with the following formula:

$$\phi = c_{\text{agarose}} / (\rho_{\text{agarose}} \omega_{\text{agarose}}) \quad (1)$$

where c_{agarose} is the concentration of agarose in the gel (w/v), ρ_{agarose} is the dry agarose density (1.64 g/ml; Laurent, 1967); and ω_{agarose} is the mass fraction of agarose in a fiber, calculated to be 0.625 (Johnson et al., 1995). Equation 1 takes into account the internally bound water in each fiber that is not accessible to macromolecules. From Eq. 1, the volume fraction of fibers of a 2% agarose gel is $\phi = 0.0195$.

Diffusion measurements

Solutions of fluorescently labeled macromolecules were prepared at various dilutions in 0.1 M PBS, then drawn into glass capillary microslides (Vitro-Dynamics Inc., Rockaway, NJ). Diffusion measurements were performed using an image-based FRAP with a spatial Fourier analysis (SFA) technique developed in our laboratory and fully described previously (Berk et al., 1993).

For measurements in agarose gels, we used three different techniques to introduce the fluorescent macromolecules into the matrix. Proteins, dextrans, and small beads were allowed to diffuse into the porous medium by placing the gel in a dilute solution of tracer in 0.1 M PBS. The characteristic diffusion time for the largest-size polymer bead (diameter 103 nm) was too great; therefore, these beads were mixed into the agarose solution before gelation. To verify that this procedure did not modify the measured diffusion coefficients, the same procedure was repeated with the 26- and 57-nm diameter polymer beads and produced no significant change in the diffusion coefficients. The different DNA molecules were introduced into the agarose gel by electrophoresis ($E \approx 1$ V/cm). A 30-min waiting period was observed after electrophoresis and before diffusion measurements were performed. The measurements were conducted at room temperature ($T = 298$ K).

THEORETICAL BACKGROUND

In free solution and in the absence of any interactions with other macromolecules, the diffusion process is controlled by the size of the macromolecule as described by the Stokes-Einstein relation:

$$D_0 = k_B T / (6\pi\eta R_H) \quad (2)$$

where k_B is Boltzmann's constant, T the temperature in Kelvin, η the solvent viscosity, and R_H the hydrodynamic radius.

In gels, diffusion phenomena are explained by different models essentially linked to the nature of the diffusing macromolecule (rigid or flexible). Although R_H (or r_s , the molecular spherical radius) is the defining parameter for proteins or polymer beads, for linear polymer chains like DNA the usual parameter is the monomer number or base-pair number, N_0 .

Flexible chains

In flexible chains, the important macromolecular dimension is the gyration radius R_g , which is directly related to the

monomer number as follows:

$$R_g^2 = N_0 b_0 p / 3 \cdot [1 - 3 \cdot p / (N_0 b_0) + 6 \cdot (p / (N_0 b_0))^2 - 6 \cdot ((N_0 b_0) / p)^3 \cdot \{1 - \exp((N_0 b_0) / p)\}] \quad (3)$$

where p is the persistence length of the chain ($p \approx 50$ nm in our case (Maret and Weill, 1983; Smith et al., 1996b) and b_0 is the interbase spacing ($b_0 \approx 0.34$ nm (Olmsted et al., 1991)). The ratio R_H / R_g of the two different radii represents the chain swelling coefficient and reflects the chain-excluded volume and the solvent quality (Doi and Edwards, 1986).

In the polymer theory, the main parameter of gels is the mean gel pore size, a , defined such that the volume between fibers can be treated as a sphere of radius a . The diffusion of polymer chains (de Gennes, 1979; Doi and Edwards, 1986) is separated into two regimes of behavior based on the chain length relative to pore size.

The first behavioral regime for a Gaussian chain is observed when $R_g < a/2$; the diffusion is described by the Zimm model (Doi and Edwards, 1986) and the macromolecule migrates in an ellipsoidal conformation (Kuhn, 1934):

$$D_G = 0.196 \cdot k_B T / \eta R_H \approx N_0^{-1/2} \quad (4)$$

When $R_g > a/2$, the reptation theory, first proposed by de Gennes (1979), describes the movement of an unattached chain by Brownian motion in a many-chain or gel system. The lateral movements of the chain are limited by obstacles formed by the gel. The resulting loss of entropy induces the chain to migrate inside a tube (with the length of the tube $L_t = Na$, where N is the number of occupied pores). De Gennes (1979) defines a disengagement time of the tube, τ_D , and the corresponding diffusion coefficient of the chain, D_G .

$$D_G = k_B T a^2 / (3 N_K^2 \zeta_K b^2) \quad (5)$$

where a is the pore size, N_K the number of Kuhn segments, ζ_K the friction coefficient of a Kuhn segment, and b the Kuhn length ($b = 2p$).

The definition of diffusion and electrophoretic mobility used for DNA transport mechanisms during electrophoresis allows us to rewrite Eq. 5 as follows (see Appendix):

$$D_G = a^2 k_B T \mu_0^{\text{sol}} / (6 N_0^2 b_0 p q_{\text{eff}}) \quad (6)$$

where μ_0^{sol} is the DNA electrophoretic mobility and q_{eff} is the DNA effective electric charge per basepair.

Equation 6 gives a definition of the diffusion coefficient in gel, D_G , in which the only unknown parameter is the pore size, a . This formula provides a useful tool to determine agarose gel pore.

Rigid particles

For proteins and rigid polymer beads, the stochastic model proposed by Ogston et al., (1973) was the only model available for many years. It addresses the diffusion of spher-

ical particles in an array of fibers. The diffusion coefficient in gel is described as a function of the diffusion coefficient in solution D_0 , the fiber radius, r_f , and the volume fraction of fibers, ϕ :

$$D_G/D_0 \equiv \exp(-\phi^{1/2} R_H/r_f) \quad (7)$$

Estimates of the gel pore size based on this model can be determined by calculating the equivalent permeability, κ , which will be discussed below with the following equation:

$$\kappa/r_f^2 = -3/(20\phi) \cdot (\ln\phi + 0.931) \quad (8)$$

Equation 8 was determined by Jackson and James (1986) after reviewing experimental measurements of these two parameters in light of the different models (Ogston and effective medium models). Although Eq. 8 is intended to link fiber radius and permeability, its validity, especially at low gel concentrations, is not certain (Johnson and Deen, 1996). The determination of a pore size will be done following calculations described later.

A simpler and rough way to determine the gel pore size is the sieving model used in electrophoresis, as derived by Slater and Guo (1996) from the Ogston model. The gel pore size, a , is closely related to the largest molecule able to diffuse inside the matrix:

$$a \approx 2(R_H + r_f) \quad (9)$$

Other models exist to estimate gel physical characteristics. In a recent paper, Williams et al. (1998) gave the name of pore theory to two models: Giddings et al. (1968) gave an evaluation of the partition coefficient, K , which is roughly equal to the ratio D_G/D_0 for spherical macromolecules in cylindrical pores:

$$K = (1 - \phi) \cdot (1 - R_H/r_p)^2 \quad (10)$$

where r_p is the gel pore radius; this equation is only valid for $R_H/r_p \leq 0.6$.

Renkin (1954) described the restricted diffusion of spherical molecules within cylindrical pores

$$D_G/D_0 = 1 - 2.1444(R_H/r_p) + 2.08877(R_H/r_p)^3 - 0.94813(R_H/r_p)^5 - 1.372(R_H/r_p)^6 + 3.87(R_H/r_p)^8 - 4.19(R_H/r_p)^9 \quad (11)$$

One factor not considered in Eq. 7 is the hydrodynamic interaction between matrix fibers and diffusing macromolecules. Phillips et al. (1989) suggested the use of the "effective medium" equation of Brinkman (1947) to account for hydrodynamic interactions:

$$D_G/D_0 \equiv 1/\{1 + (R_H^2/\kappa)^{1/2} + (R_H^2/\kappa)/3\} \quad (12)$$

where κ is the Darcy permeability to water flow, which is a pure property of the material.

It is tempting to use the Darcy permeability to calculate hydrodynamic screening distance (κ) as an estimate of gel pore size. However, a more rigorous estimate of pore size is

obtained by applying a hydrodynamic model that relates permeability to the structural properties of the fiber matrix (Levick, 1987). The Carman Kozeny equation (Carman, 1937) provides the following relation between permeability, κ , and the mean hydraulic radius, r_H :

$$\kappa = \epsilon \cdot r_H^2/k \quad (13)$$

where ϵ is the porosity of the gel ($\epsilon = 1 - \phi$) and k is the Kozeny factor, which depends on channel shape and tortuosity. The Kozeny factor increases with the porosity and can be evaluated using the following theoretical formulas (Happel and Brenner, 1965; Levick, 1987) in which k is separated in its parallel (k_{\parallel}) and normal (k_{+}) components with respect to the flow coordinates.

For cylinders parallel to the flow:

$$k_{\parallel} = 2\epsilon^3/\{(1 - \epsilon) \cdot [\ln(1/(1 - \epsilon)) - 3 + 4 \cdot (1 - \epsilon) - (1 - \epsilon)^2]\} \quad (14)$$

For cylinders at right angles to the flow:

$$k_{+} = 2\epsilon^3/\{(1 - \epsilon) \cdot [\ln(1/(1 - \epsilon)) - ((1 - (1 - \epsilon)^2)/(1 + (1 - \epsilon)^2))]\} \quad (15)$$

Finally, for the cylinders oriented randomly in the 3D space, the Kozeny factor is:

$$k = (2k_{+} + k_{\parallel})/3 \quad (16)$$

RESULTS AND DISCUSSION

Diffusion in solution

Diffusion coefficients in solution, D_0 , were measured for each macromolecule at concentrations dilute enough to avoid interactions between macromolecules (de Gennes, 1979; Dwyer and Bloomfield, 1993). DNA concentrations (e.g., $c^{\phi X 174} = 0.04$ g/l or $c^{\lambda} = 0.01$ g/l) were determined using the study of Tinland et al. (1998) on diffusion coefficients as a function of DNA concentration, whereas for dextrans and proteins concentrations were based on data of Muramatsu and Minton (1988) and were lower than 0.2 g/l. Table 1 provides the extracted hydrodynamic radii of the proteins, polymer beads, and dextrans calculated with Eq. 2, assuming a temperature $T = 298$ K and the solvent (0.1 M PBS) viscosity equivalent to that of water ($\eta_{0.1 \text{ M PBS}} = 0.87$ cP).

The hydrodynamic radii obtained for proteins are larger than typical values reported previously using other methods (Anderson et al., 1978; Bor Fuh et al., 1993; Gaigalas et al., 1992; Gibbs et al., 1991; Lebrun and Junter, 1993; Tong and Anderson, 1996), whereas dextran radii seemed to be in good agreement. Therefore, the introduction of a correction factor (1.1) could easily compensate for the discrepancy (Fig. 1). The underestimation of diffusion coefficients is most likely a 3D effect related to the geometry of the photobleaching laser beam. The FRAP analysis is based on

TABLE 1 Diffusion coefficients, D_0 , in 0.1 M PBS at $T = 25^\circ\text{C}$ of different macromolecules (proteins, dextrans, and polymer beads) and their corresponding hydrodynamic radius, R_H , based on a viscosity value $\eta = 0.8705$ cP

Sample	Diffusion Coefficient D_s ($\text{cm}^2 \text{s}^{-1}$)	Calculated Radius (nm)	Nominal Radius (nm)	Reference
Lactalbumin	$(1.14 \pm 0.1) \cdot 10^{-6}$	2.20 ± 0.05	1.90	(Saltzman, 1994)
Ovalbumin	$(7.8 \pm 0.8) \cdot 10^{-7}$	3.20 ± 0.40	2.12 2.80	(Johnson et al., 1996) (Saltzman, 1994)
BSA	$(6.4 \pm 0.4) \cdot 10^{-7}$	3.93 ± 0.20	3.00 3.0 3.59	(Johnson et al., 1996) (Saltzman, 1994) (Johnson et al., 1996)
Dextran 4400	$(1.35 \pm 0.10) \cdot 10^{-6}$	1.86 ± 0.15	1.5	(Granath and Kvist, 1967)
Dextran 71,200	$(4.4 \pm 0.2) \cdot 10^{-7}$	5.71 ± 0.35	5.7	(Lebrun and Junter, 1993)
Dextran 2,000,000	$(9.6 \pm 1.0) \cdot 10^{-8}$	26.0 ± 3.0	27.2	(Lebrun and Junter, 1993)
Polystyrene bead (26 nm)	$(1.8 \pm 0.2) \cdot 10^{-7}$	14.3 ± 2.1	13.0	(Duke Scientific Corp.)
Polystyrene bead (57 nm)	$(9.5 \pm 0.4) \cdot 10^{-8}$	26.5 ± 2.0	28.5	(Duke Scientific Corp.)
Polystyrene bead (103 nm)	$(3.8 \pm 0.9) \cdot 10^{-8}$	61.0 ± 8.0	51.5	(Duke Scientific Corp.)

Hydrodynamic radii are compared to literature data extracted from diffusion data with their respective temperatures and viscosities.

an assumption that the photobleaching beam is cylindrical, and hence the fluorescence recovery is two-dimensional. Because the beam actually diverges above and below the plane of focus in the sample, axial gradients are created that act to prolong the recovery. This problem affects other implementations of FRAP for diffusion measurement in thick samples and is usually addressed by the use of a correction factor determined from the recovery of a standard tracer such as BSA (Moussaoui et al., 1991; Saltzman et al., 1994). We chose to use the uncorrected diffusion coeffi-

cients and hydrodynamic radii in this study rather than introduce a correction factor.

Diffusion of proteins and polymer beads

Fig. 2 shows the diffusion coefficients measured in 2% agarose gel for all nonflexible particles. The values are plotted as a function of the hydrodynamic radius determined from diffusion measurements in buffer solution alone. Fig. 2 clearly shows that above a critical hydrodynamic radius, $R_H \approx 30$ nm, a drastic decrease of the diffusion coefficients is observed while increasing the nominal polymer bead diameter from 57 nm to 103 nm. (The greater error bars

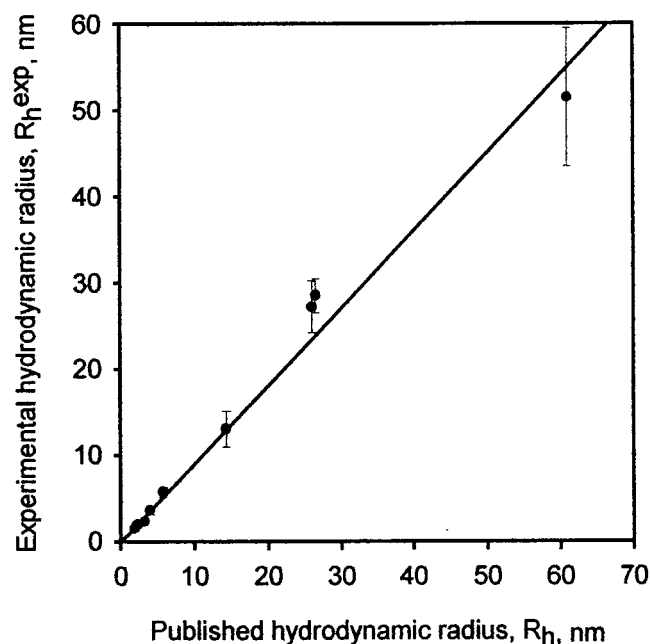


FIGURE 1 Experimental hydrodynamic radius, R_H^{exp} , extracted from the diffusion coefficients measured in solution for all ellipsoidal macromolecules in 0.1 M PBS at $T = 25^\circ\text{C}$ as a function of the hydrodynamic radius published in the literature, R_H^{lit} . The solid line represents the experimental dependence. Experimental hydrodynamic radii are 10% larger than literature data.

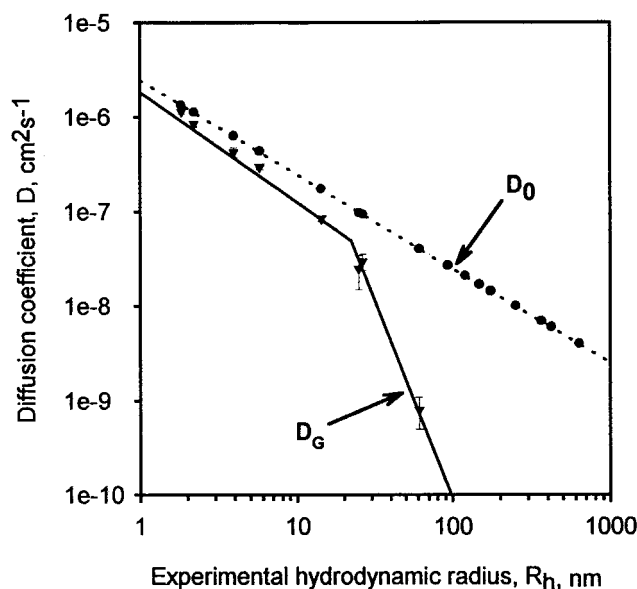


FIGURE 2 Diffusion coefficients of all nonflexible macromolecules studied as a function of their hydrodynamic radius, R_H , obtained in 0.1 M PBS (●) and in 2% agarose gels (▼) and $T = 25^\circ\text{C}$. A sharp decrease of the diffusion coefficients is observed above $R_H = 30$ nm, suggesting higher interactions between the matrix and the macromolecules.

associated with these large beads suggest that the motion of these large particles is perhaps more sensitive to heterogeneity of the gels). The substantial drop in mobility observed for the 103-nm bead suggests particle trapping, and indeed we could detect no penetration or motion for the next-larger bead size (254 nm diameter). This observation suggests a high entropic cost of motion in the gel, even if the average pore size is larger than the polymer beads.

Diffusion of DNA molecules

The diffusion coefficients of DNA molecules in the “dilute regime” are plotted as a function of the number of basepairs, N_0 , in Fig. 3. The slope of the diffusion coefficients in solution, D_0 , as a function of the number of basepairs, N_0 , gives a scaling exponent of -0.50 , which is in good agreement with Zimm’s predictions (Doi and Edwards, 1986) of -0.50 for a θ solvent and -0.60 for a good solvent, and also in agreement with other experimental observations (Tinland et al., 1998; Smith et al., 1996a).

The diffusion coefficients in gels, D_G , (Fig. 3) obtained for the different DNA reflect the two theoretical behavioral regimes predicted for a chain in a porous medium (de Gennes, 1979; Doi and Edwards, 1986) and confirmed by computer simulations (Yamakov and Milchev, 1997). The diffusion coefficients in gels for the small DNA (up to 6000 bp) give a scaling exponent of -0.52 , in good agreement with the predictions of Zimm (Doi and Edwards, 1986). Longer DNA chains fall within a second regime, characterized by a steeper dependence of diffusion coefficient on chain length. According to the polymer theory and previous

computer simulations, the second regime should correspond to the reptational regime (de Gennes, 1979) for which the theoretical scaling exponent is -2.0 (see Eq. 6). As shown in Fig. 3, three of the DNA samples (9416 bp, 23,132 bp, and 48,502 bp) seem to fall in the reptation regime. The actual fit of the data for these three chain lengths gives an experimental scaling exponent of -1.55 , which is close to the published measurements given by Bansil et al. (1990), Tinland et al., (1998), and Pluen et al. (1998) for different types of polyelectrolyte chains within the reptation regime. Based on the hypothesis that the behavior of these three DNA chains is in the reptation regime, we proceeded to apply the corresponding polymer dynamics equations to each DNA to estimate the effective pore size of 2% agarose gel in 0.1 M PBS.

Estimation of gel parameters

Our two sets of results for spherical or globular molecules and for DNA permit the use of the different existing models to estimate various structural or functional features of the agarose gel, such as pore size or fiber radius, and the permeability. The results obtained with the DNA macromolecules will be treated using the reptation model, which includes the gel pore size parameter (de Gennes, 1979), and the Zimm-Rouse model to a lesser degree (Yamakov and Milchev, 1997). Results obtained with the spherical or globular molecules will be treated with the Ogston model (1973), Renkin model (1954), Giddings model (1968), and the Brinkman/effective medium model (Brinkman, 1947; Phillips et al., 1989, 1990) to provide estimates of the fiber radius, pore radius, permeability, and finally the gel pore size.

The gel pore size

For chain lengths of at least 2000 bp, which can be considered as Gaussian ($L = N_0 b_0 \gg p$), we have:

$$\langle h^2 \rangle \equiv Na^2 \equiv N_K(2p)^2 \equiv 2N_0 b_0 p \quad (17)$$

As the chain size grows, a transition is observed between the Zimm regime (scaling exponent -0.52), where the chains migrate in globular conformation in the fiber array, and the reptation regime, where the gyration radius exceeds the pore size and obliges the chain to occupy many pores. Thus, we have the opportunity to evaluate the mean pore size of a 2% agarose gel using different approaches summarized in Table 2.

The effective gel pore size seen by a diffusing DNA chain can be extracted for every DNA chain in the reptation regime. Table 2 gives the effective gel pore size, a , calculated with the published data of $q_{\text{eff}} = e/7$ for a DNA basepair (Smith and Bendich, 1990) and the free solution electrophoretic mobility, $\mu_0^{\text{sol}} = (3.6 \pm 0.4) \cdot 10^{-4} \text{V}^{-1} \text{cm}^2 \text{s}^{-1}$ (Stellwagen et al., 1997; Tinland et al., 1996).

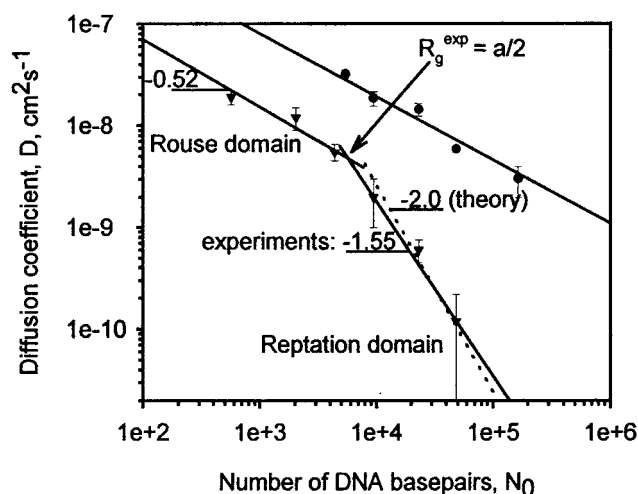


FIGURE 3 DNA diffusion coefficients, D_G , in 2% agarose gels (\blacktriangledown) and the DNA diffusion coefficients, D_0 , in solution (\bullet) as a function of their basepair number, N_0 , in 0.1 M PBS at $T = 25^\circ\text{C}$. The slope indicated for the diffusion coefficients in gels corresponds to the scaling exponent -0.52 , which is in good agreement with Zimm’s predictions. The scaling exponent -2.0 is the theoretical scaling exponent given by the theory (Eq. 8) whereas the scaling exponent -1.55 is the result of the experimental fit. The value $R_g = a/2$ corresponds to theoretical change of regime between the Rouse regime and the reptation regime for the DNA in the gels.

TABLE 2 Pore size extracted from the diffusion coefficient of the DNA with the different methods considering an effective electric charge ($= e/7$) per basepairs in 0.1 M PBS at $T = 25^\circ\text{C}$

Method	Intersection		Reptation	
Basepair number, N_0	6500 \pm 500	9416	23,130	48,502
Pore size, a (nm)	470 \pm 18*	528 \pm 80 [#]	701 \pm 85 [#]	667 \pm 150 [#]

*Pore size was calculated with Eq. 8 for the intersection between the Zimm and reptation regimes.

[#]Pore size was extracted from Eq. 6 for the reptation regime.

However, the experimental intersection of the two regimes is obtained around $N_0 = 6500 \pm 500$ bp. Assuming this intersection corresponds to an ideal chain filling the mean gel pore, we should be able to estimate the mean pore size with Eq. 17. Considering a chain length of $N_0 = 6500 \pm 500$ bp filling $N = 1$ pore, we obtain a mean value $a = 470 \pm 18$ nm (see Table 2).

These calculations of the agarose gel pore size were checked by estimating the friction coefficient of a DNA Kuhn length and then inserted in Eq. 5. A complete description of this approach is given in the Appendix. In short, following Tanford's calculations (1961), we evaluated the friction coefficient of a cylinder that has a length equal to a Kuhn length and the diameter of a DNA chain. Varying both diameter and Kuhn length in reasonable proportions, the friction coefficient, ζ_K , ($\zeta_K = (2.69 \pm 0.36) \cdot 10^{-10}$ Pa \cdot m) was inserted in Eq. 5 and the effective agarose pore size was evaluated for each different DNA in the reptation regime (Table 3). The pore sizes in 2% agarose gel given in Tables 2 and 3 are consistently higher than those determined from the crossover between the Zimm-Rouse regime and the reptation regime. This difference can be understood considering a polydisperse gel system in which the intersection of the two behavioral regimes leads to a mean pore size, whereas large DNA molecules in reptational behavior use the available larger pore to diffuse.

Compared to a study using a similar approach (Tinland et al., 1996), our data (Table 2) indicate larger pores. We attribute the larger pore size detected in our gels to the greater ionic strength used during preparation. An influence of the ionic strength on the agarose gel pore size was observed by Waki et al. (1982) by electron microscopy and by Pernodet et al. (1997) and by Maaloum et al., 1998 by

TABLE 3 Estimation of the 2% agarose gel pore sizes from the experimental diffusion coefficients, D_G , of each DNA in the reptation regime (see Fig. 3) using Eq. 5

Basepairs number, N_0	9416	23,130	48,502
Kuhn length, N_K	32 \pm 6	79 \pm 15	164 \pm 32
Pore size, a (nm)	635 \pm 128	854 \pm 171	800 \pm 160

To solve Eq. 5, we considered the following parameters: the Kuhn friction coefficient $\zeta_K = (2.69 \pm 0.36) 10^{-10}$ Pa \cdot m (see Table A1), the Kuhn length $b = 2p = 100 \pm 25$ nm, the chain diameter $d = 2.4 \pm 0.6$ nm, and the temperature $T = 25^\circ\text{C}$.

atomic force microscopy (AFM). Apparently the ionic strength affects fiber formation; a change in the ionic strength after casting did not have a noticeable effect on the agarose structure. Our data also indicate an increase of the effective pore size as a function of DNA chain length, as previously observed by Maaloum et al. (1998). This is easily understood by considering a Gaussian distribution of the gel pore sizes as observed by different methods: AFM (Pernodet et al., 1997), NMR (Chui et al., 1995) and electron microscopy (Waki et al., 1982). As chain length increases, the population of pores "tested" by the entropy of the chain (Zimm, 1991) is restricted more to the larger pores.

Fiber radius, permeability, and pore radius

In the determination of the fiber radius, pore radius, and permeability, relying on the results obtained with the proteins, dextrans, and the polymer beads, the essential data analysis was conducted with the Ogston and Phillips models, although Renkin and Giddings/partition models were also used. Even if the Ogston model may not be self-consistent in agarose (Moussaoui et al., 1992; Johnson et al., 1995), this model is typically used as a reference for the estimation of the fiber radius, r_f . Phillips et al. (1989, 1990) proposed a new model in which the main parameter is no longer the fiber radius but the permeability, κ , based on the Brinkman's equation (1947).

In the Phillips model (1989, 1990), the permeability is used to express the ratio of the friction coefficient of a sphere in a gel over the friction coefficient of the same sphere in a solution (Eq. 12). Their model is valid as long as $R_H^2/\kappa \leq k/\epsilon$ according to Eq. 13. The porosity of our 2% agarose gel is easily determined knowing that $\phi = 0.0195$ and $\epsilon = 0.9805$. With Eqs. 14–16 a theoretical value of the Kozeny's factor is determined: $k = 28.4$ for our 2% agarose gel. Therefore, FRAP-determined ratios, D/D_0 , were fitted using Eq. 12. Taking into account error bars and uncertainties in the k -value, the condition $R_H^2/\kappa \leq k/\epsilon$ was fulfilled for all macromolecules. In Table 4, the permeability value obtained with these macromolecules ($\kappa \approx 367 \pm 99$ nm²) is in agreement with the data of Johnson et al. (1995) obtained by permeability measurements ($\kappa = 616$ nm²) or FRAP-diffusion ($\kappa \approx 86$ nm²).

To compare the different models, a gel pore size, a , was determined. Using the k -value and porosity value previously determined ($k = 28.4$ and $\epsilon = 0.985$), the mean hydraulic radius, r_H , was extracted from Eq. 13: $r_H = 103 \pm 13$ nm. We can therefore estimate the hydraulic diameter (or the interfiber spacing) which is close to the pore size for the flexible chains: $a \approx 2r_H = 206 \pm 26$ nm. The estimation of the diameter using the Brinkman method gives a value lower than for the flexible chains ($\langle a \rangle = 490$ nm). This is consistent with the work of Johnson et al. (1995) that the ratio D/D_0 underestimated the κ -value: for a 2% agarose gel, permeability measurements extrapolated at zero flow value gave $\kappa = 616$ nm². From Eq. 13 and as previously

TABLE 4 Fiber radius and permeability values obtained using Eqs. 7, 8, and 12 calculated with Brinkman and Ogston models

Structural Parameter	Ogston Model	Brinkman Model	Renkin Model	Partition	Sieving
Fiber Radius (nm)	3.3 ± 0.4	$4.0 \pm 0.5^{\#}$	—	—	—
Permeability (nm ²)	$249 \pm 57^*$	367 ± 99	—	—	—
Pore Radius (nm)	85 ± 9	103 ± 13	73 ± 7	62 ± 6	—
Pore Size, a (nm)	170 ± 18	206 ± 26	146 ± 14	124 ± 12	$126 \pm 10^{\S}$

Pore radius and sizes for Renkin model and partition model were calculated with Eqs. 10 and 11.

*Fiber radius calculated with the Carman Kozeny relation (Eq. 17) for Ogston and Brinkman models.

[#]Permeability calculated with the Carman Kozeny relation (Eq. 17) for Ogston and Brinkman models.

[§]Pore size value from the sieving model was estimated with Eq. 9.

described, a gel pore size, a , is obtained ($a \approx 2r_H = 274$ nm), which is closer to the average value determined for the flexible chains mentioned above.

However, the Ogston model (1973) was used to determine the fiber radius and then an equivalent gel pore size, a . The value obtained for the fiber radius (Table 4 and Eq. 7) is in agreement with small-angle x-ray scattering experiments performed by Djabourov et al. (1989). Ogston's model (1973) gives $r_f = 3.3 \pm 0.4$ nm for our data, whereas Djabourov published a range of values between 1.5 and 4.5 nm (with an average fiber radius $\bar{r}_f = 1.9$ nm). The equation by Jackson and James (1986) (Eq. 8) was used and its validity checked. Table 4 presents permeability and fiber radius values obtained from the Ogston and Brinkman models. As previously pointed out by Johnson et al. (1995), Eq. 8 does not seem to be verified for agarose gels, although it seems to be valid for cross-linked gels (polyacrylamide) (Williams et al., 1998). The gel pore size from the Ogston model, a , was calculated as previously described in the previous paragraph: $a = 170 \pm 18$ nm.

Considering other models to analyze our data, a first attempt was made with the sieving model (Slater and Guo, 1996). With its basic concept it should give a rough estimation of the pore size: as long as the molecule migrates, pores are large enough. Using Eq. 9 (Slater and Guo, 1996), a pore size, a , is calculated to be ~ 126 nm (Table 4). This estimation is in good agreement with data previously published by Griess et al. (1989, 1993). Using the sieving model during gel electrophoresis, they determined the following relation: $r_p = 111 c_{\text{agarose}}^{-0.74}$ which, for a 2% agarose gel, leads to a pore size $a \approx 140$ nm (Griess et al., 1989). More recently, these results were correlated with a study of agarose gels by electron microscopy and led to a new relation, $r_p = 111 c_{\text{agarose}}^{-0.74}$ and, for a 2% agarose gel, a pore size $a \approx 150$ nm (Griess et al., 1993).

An exact determination of the gel pore size using the sieving properties assumes that we are able to reach an asymptotic diffusion regime (which is not the case in our study) in which macromolecules are trapped in gel. This behavior is difficult to achieve: the energy cost is too high for macromolecules diffusing without a "helping force." Therefore, the pore size determined using this method with the present set of data will be below the "real" gel pore size.

In Table 4 are also presented pore radius, r_p , and gel pore sizes, a , obtained with the Renkin model (1954) and Gid-

dings model (1968). Although a poor correlation between data and model is observed in Fig. 4, pore radius calculated from Eqs. 10 and 11 and gel pore size are consistent with previous results. Gel pore sizes are closer to the sieving value than the Brinkman/Ogston values: $a = 146 \pm 14$ nm (Renkin), and $a = 124 \pm 12$ nm (Giddings).

Fig. 4 clearly shows the fitting differences among the different models. Our results tend to demonstrate that to evaluate a "gel pore size" the Brinkman model seems to provide a better description of the interactions of diffusion within the gel than do the Renkin and Giddings models, although it is difficult to directly compare with a partition model like Gidding's. Surprisingly, the Ogston model gives interesting fitting results, although not as good as the ones obtained from the effective medium theory. They support results of Moussaoui et al. (1992) and Johnson et al. (1995) concerning its inconsistency for agarose gels. But, in contrast to Johnson et al. (1995), the use of a steric term in Brinkman model to analyze our data did not improve the determination of the permeability. For a similar range of ratio R_H/r_p , our conclusion concerning the models is similar to Williams et al. (1998) although the nature of the gel was different.

CONCLUSION

The diffusion of different types of macromolecules in agarose gels was observed. The Zimm-Rouse and reptation regimes were observed for the DNA chains while different models were used to explain the diffusion of spherical-ellipsoidal macromolecules (Ogston and effective medium). This experimental study allowed us to determine different characteristics of the agarose gel structure with many different models (reptation, Ogston/effective medium, partition, and Renkin). The mean agarose gel pore size determined using DNA diffusion coefficients is in good agreement with published data, including measurements by different microscopic techniques. The other pore size estimates obtained using the Brinkman or Ogston or other models tend to give lower values, but during data analysis, a better correlation of the pore size values was obtained with the effective medium theory. Allowing a good matrix parameter estimation and a relative simplicity of the technique of permeability measurements, the Brinkman model appears

to be a useful experimental tool. However, this study also points out the fact that for every model used, one or more parameters were lacking: effective DNA electric charge and the free mobility for the reptation model, a true determination of the fiber radius or the largest bead able to move in the matrix for the Ogston model or the exact value for the Kozeny's factor. Thus, no one model completely independent of adjustable parameters seems to be able to give an exact match. As fluorescent molecules and models give rather different gel structures, light scattering seems to be an interesting tool to elucidate agarose gel structure or formation (Manno et al., 1999).

Advantages of flexible macromolecules in comparison to the rigid ones were shown in this experimental study; despite the same hydrodynamic radius in solution, the diffusion coefficients are greater for flexible macromolecules while rigid and/or spherical macromolecules quickly become and remain trapped in pores. This result strongly suggests that to ensure the transport of large amounts of information in gene therapy, semi-flexible macromolecules like DNA should be used. Whereas diffusion coefficients of polymer beads or proteins failed to explain the structure of agarose gels, they may be relevant to describe the effectiveness of drug delivery.

APPENDIX

This section describes the procedure we followed 1) to transform Eq. 5 into Eq. 6, and 2) to evaluate the friction coefficient of a DNA Kuhn segment.

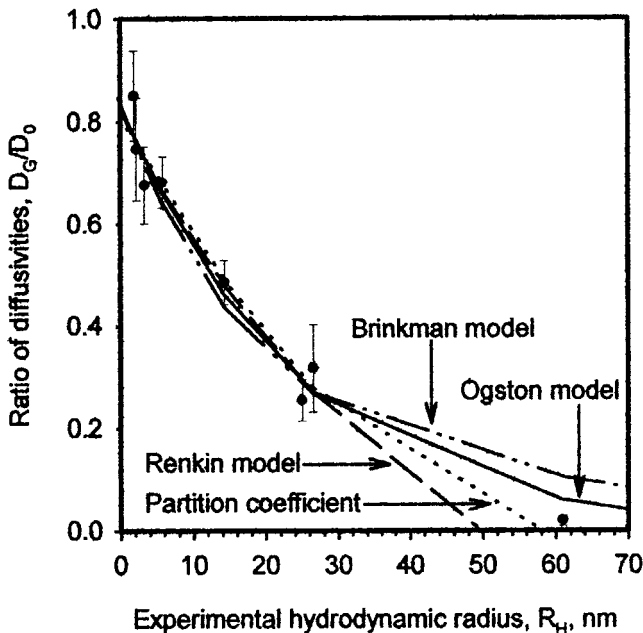


FIGURE 4 Ratio of diffusivities in solution (0.1 M PBS) and in 2% agarose gels as a function of the hydrodynamic radius of different macromolecules (proteins, dextrans, beads). We assumed $\epsilon = 0.985$ for all models. The different models used are effective medium theory/Brinkman model (Eq. 12), Ogston model (Eq. 7), Renkin model (Eq. 11), and partition model (Eq. 10). Boundary limits mentioned in the theoretical part of this paper were taken into account in this figure.

Estimation of D_G using the biased reptation model

In the theory of flexible polymers, the diffusion coefficient of a chain in a gel is defined as:

$$D_G = k_B T a^2 / (3 N_K^2 \zeta_K^2) \quad (5)$$

All parameters in this equation are known except for the friction coefficient of a Kuhn length, ζ_K , and the pore size, a . Taking into account the definitions of the diffusion and the electrophoretic mobility used in DNA transport mechanisms during electrophoresis, the diffusion coefficient in a gel can be reformulated to have only one unknown parameter, the pore size. Basic definitions of diffusion and mobility are:

$$D = k_B T / \zeta_t \quad (A1)$$

and

$$\mu = Q_{\text{total}} / \zeta_t \quad (A2)$$

where Q_{total} is the total effective charge of the DNA chain ($= N_0 q_{\text{eff}}$), q_{eff} is the effective electric charge for one DNA basepair in our experimental conditions, and ζ_t is the total friction coefficient of the DNA chain. Equations A1 and A2 can be rewritten as follows:

$$D_G / \mu_{E=0} = k_B T / N_0 q_{\text{eff}} \quad (A3)$$

where $\mu_{E=0}$ is the electrophoretic mobility in the gel without any electric field.

The electrophoretic mobility is unknown but can be estimated by extrapolating the biased reptation model (BRM) (Slater and Noolandi, 1986). The BRM, used in electrophoresis, describes a chain as a series of N blobs of mean square end-to-end distance a^2 (a is the mean gel pore size). The motion of the chain is a succession of jumps along the tube axis.

From the BRM, we have:

$$\mu_{E=0} / \mu_0^{\text{sol}} = \langle h^2 \rangle / (N a^2) \quad (A4)$$

where h is the end-to-end vector of the chain, $\mu_{E=0}$ is the electrophoretic mobility at zero electric field in the gel, and μ_0^{sol} is the DNA electrophoretic mobility in solution (Olivera et al., 1964; Ross and Scruggs, 1964; Stellwagen et al., 1997; Tinland et al., 1996). The latter has an average value of $\mu_0^{\text{sol}} = 3.6 \pm 0.4 \cdot 10^{-4} \text{ V}^{-1} \text{ cm}^2 \text{ s}^{-1}$.

In the absence of any electric field and in the reptation domain, we have:

$$\mu_{E=0} / \mu_0^{\text{sol}} = 1/3N \quad (A5)$$

where N is the number of occupied pores.

The chain will still remain Gaussian, therefore we have:

$$\langle h^2 \rangle = N a^2 = N_K (2p)^2 = 2 N_0 b_0 p \quad (A6)$$

Inserting Eqs. A4–A6 in Eq. A3, we obtain Eq. 6, which is only a function of the pore size for a defined DNA chain:

$$D_G = a^2 k_B T \mu_0^{\text{sol}} / (6 N_0^2 b_0 p q_{\text{eff}}) \quad (6)$$

Estimation of the friction coefficient, ζ

As in the first section of this Appendix, to obtain the gel pore size, a , from Eq. 5, the friction coefficient, ζ , was replaced as previously described. In this section, an evaluation of the friction coefficient will be done. The DNA chain is the sum of N Kuhn segment (Kuhn length is twice the persistence length) and each Kuhn segment has a friction coefficient, ζ . According to Tanford (1961), the friction coefficient of a prolate ellipsoid (semi-axes M , m , m) can be estimated by the following equation:

TABLE A1 Estimation of the friction coefficient according Eq. A8 for different Kuhn lengths and chain diameters

Kuhn Length [= 2 <i>p</i> (nm)]	Diameter (nm)		
	2.0	2.4	3.3
80	2.18×10^{-10} Pa · m	2.28×10^{-10} Pa · m	2.41×10^{-10} Pa · m
90	2.38×10^{-10} Pa · m	2.49×10^{-10} Pa · m	2.63×10^{-10} Pa · m
100	2.58×10^{-10} Pa · m	2.69×10^{-10} Pa · m	2.84×10^{-10} Pa · m
125	3.07×10^{-10} Pa · m	3.20×10^{-10} Pa · m	3.37×10^{-10} Pa · m
150	3.55×10^{-10} Pa · m	3.69×10^{-10} Pa · m	3.87×10^{-10} Pa · m

Assumed values in our experimental conditions based on experimental data in literature (Maret and Weill, 1983; Olmsted et al., 1991) are given in bold.

$$\frac{\zeta}{\zeta_0} = \frac{\zeta}{6\pi\eta R_0} = \frac{(1 - m^2/M^2)^{1/2}}{(m/M)^{2/3} \ln((1 + (1 - m^2/M^2)^{1/2})/(m/M))} \quad (\text{A7})$$

where ζ and ζ_0 are, respectively, the friction coefficients of the ellipsoid and the volume equivalent sphere; η is the solvent viscosity; and R_0 is the radius of a sphere whose radius is equal to the prolate ellipsoid volume, i.e., $4/3\pi R_0^3 = 4/3\pi Mm^2$. Equation A7 will be used, considering that our prolate ellipsoid will closely approximate a long cylindrical rod of length b and diameter d . The equivalence of length and volume may be stated as $b = 2M$ and $4\pi Mm^2/3 = \pi bd^2/4$. Previous conditions can be summarized as follows:

$$M/m = \sqrt{2/3} \cdot (b/d) \quad \text{and} \quad R_0 = \sqrt[3]{Mm^2} \quad (\text{A8})$$

Inserting M/m and R_0 in Eq. A7 using results of Eq. A8, the friction coefficient of the cylinder is:

$$\zeta = \frac{6\pi\eta R_0 \sqrt{1 - 3/2 \cdot d^2/b^2}}{\sqrt[3]{3/2(d/b)^2} \cdot \ln((1 - \sqrt{1 - 3/2 \cdot d^2/b^2})/(\sqrt[3]{3/2d/b}))} \quad (\text{A9})$$

Regarding our experimental conditions and published data (Carlsson et al., 1996; Maret and Weill, 1983; Smith et al., 1994), basic parameters for the evaluation of the friction coefficient ζ will be $b = 100$ nm ($b = 2$ persistence lengths, p) and $d = 2.4$ nm (diameter of a DNA chain). As both DNA diameter and length may vary with experimental conditions (ionic strength, intercalating dye ratio), Table A1 gives an evaluation of the friction coefficient, ζ , for different b and d values.

GLOSSARY

a	Mean pore size (pore diameter)
b	Kuhn length equivalent to two persistence length
b_0	Interbases spacing
D, D_0, D_G	Diffusion coefficient, diffusion coefficient in solution, diffusion coefficient in gels
e	Coulomb charge
h	End-to-end chain vector
$k, k_{ }, k_{\perp}$	Kozeny factor, Kozeny factor for cylinders parallel to the flow, Kozeny factor for cylinders normal to the flow
k_B	Boltzmann constant
L, L_t	Contour length of the polymer chain, polymer tube length reptating in the gel
N, N_0, N_K	Number of occupied pores by a chain in the gel, number of basepairs, number of Kuhn length of the chain
p	Polymer persistence length
q_{eff}, Q	Effective charge per DNA basepair, total electric charge of the DNA chain
r_D, r_p, r_H	Gel fiber radius, gel pore radius, and hydraulic radius
R_g, R_H	Polymer gyration radius, polymer hydrodynamic radius
T	Temperature in Kelvin
ϵ	Gel porosity
ϕ	Volume fraction of fibers

κ	Darcy permeability
μ_0	Electrophoretic mobility
η	Solvent viscosity
ρ	Dry agarose density
τ_D	Disengagement time of the tube created by a reptating polymer
ω	Mass fraction of agarose in a fiber
$\zeta_K, \zeta_0, \zeta_t$	Friction coefficient of a Kuhn segment (prolate ellipsoid), of the equivalent sphere and of the total chain.

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