# Nuclear Magnetic Relaxation Study of Hindered Rotational Diffusion in Gels

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Polymeric gels are widely used in biotechnology, primarily for purification purposes in separation processes, but also as matrices for the physical entrapment of cells and macromolecules, or for the covalent attachment of enzymes and ligands (Mosbach, 1976). The restricted diffusion of molecules through these gels is an important concern in the design and operation of chromatographic separations, bioreactors, electrophoretic media, etc. Yet the fundamental aspects of the interactions between the gel fibers and the diffusing substances are still poorly understood. In this research note, nuclear magnetic relaxation is shown to be a useful probe of the tumbling motions of molecules embedded in a gel. The technique is applied to study the rotational diffusion of cyclic guanosine monophosphate (cGMP) in polyacrylamide and agarose matrices.

### *Definition of rotational diffusion*

Thermal agitation in solution causes rotational Brownian motion. This phenomenon is described in a manner similar to the more familiar translational motion. If $\Omega$ is the set of angles which determines a molecule's orientation, the probability density function $P(\Omega, t)$ obeys a diffusion equation analogous to Fick's second law. For spherically isotropic bodies, rotary diffusion is characterized by a scalar $D_r$, the rotational diffusion coefficient, and the diffusion equation reduces to (Favro, 1960)

$$\frac{\partial P}{\partial t} = -D_r \mathbf{L}^2 P$$

where $L$ is the quantummechanical angular momentum operator. $D_r^{-1}$ is a characteristic decay time for any anisotropy in the distribution of molecular orientations. Stated another way, $D_r$ measures the effective tumbling rate due to Brownian impacts.

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### *Common retardation mechanisms in gels*

Several mechanisms are thought to contribute to the overall reduction in the mobility of Brownian particles (such as macromolecules) trapped in a random array of fibers (such as a gel), and theories have been developed which highlight only some of these factors. Ogston's theory (Ogston, 1958; Rodbard and Chrambach, 1970) for rigid molecules accounts only for the physical exclusion effect. For translational diffusion, this amounts to neglect of all the influences of the matrix, save tortuosity. This obstruction theory alone, however, cannot explain the low values of the diffusion coefficient observed for many moderately sized solutes (Muhr and Blanshard, 1982). Similarly, for large molecules of very high aspect ratio, such as chains and rods, steric hindrance by the gel causes the "tubular motion" characteristic of reptation theories (de Gennes, 1971; Doi and Edwards, 1986). An electric birefringence study of short DNA segments in polyacrylamide gels (Wijmenga and Maxwell, 1986) confirms some scaling laws derived from these theories, but additional retardation mechanisms have to be invoked to explain the observed dependence of $D_r$ on the gel concentration and on the electric field. In a model study focusing on the increase in the hydrodynamic drag upon confinement, the diffusion coefficent of a sphere trapped in a long, cylindrical tube was calculated (Brenner and Gaydos, 1977). It was found that the diffusivity along the pore axis drops dramatically even when the sphere dimensions are much smaller than the tube radius. Although the cylindrical pore model is certainly not adequate for gels, increased hydrodynamic friction undoubtedly plays a dominant role in the slowing of molecular diffusion in gels. The intricate geometry of the medium makes an exact solution of this problem very complex, however, and current theories rely on scaling arguments (Cuckier, 1984).

A number of other factors can affect the diffusive motion of probe molecules through gels: the molecular conformation may be altered by its confinement in the gel, the solvent microviscosity may be changed in narrow pores, or the gel matrix may be

distorted by the presence of the diffusing species. In addition, it has been reported that the mobility of the fibers themselves is an important aspect of diffusion through polyacrylamide gels (Sellen, 1985). Most of the interactions mentioned affect rotational as well as translational diffusion. However, for molecules that are small compared to the average fiber separation, the diffusing molecule need not "disengage" from its constraints before it can reorient. Consequently, frictional effects are expected to dominate the retardation. The results of this study indicate that this increase in the hydrodynamic drag experienced by the diffusing species upon entrapment can cause a significant diminution of its tumbling rate.

### *Rotational diffusion measurements by nuclear magnetic relaxation*

Although laser light scattering and fluorescence depolarization are often used to observe the dynamics of macromolecules in solution, optically opaque materials cannot be accessed by these methods. Electron spin resonance (ESR) and nuclear magnetic resonance (NMR) on the other hand, operate in the microwave and radiofrequency range of the electromagnetic spectrum, and therefore do not suffer this limitation. ESR, however, is restricted to the use of free radicals. In addition, since water strongly absorbs microwave radiation, aqueous solutions can only be investigated in very thin capillaries, in which wall effects may become important for the gel structure and for the molecular diffusion. Hence, NMR is the technique of choice.

Molecular motions influence the rate of nuclear magnetic relaxation by causing fluctuations in the magnetic field experienced by the nuclei. As the molecule tumbles, the change in the environment of the observed spin modulates the local magnetic field strength. These oscillations can cause transitions between spin states, leading to relaxation. For a typical "inversion recovery" experiment (Martin et al., 1980) the magnetization reaches equilibrium exponentially, with a time constant $T_1$. If the effective rotational diffusion coefficient $D_r$ is much larger than the Larmor frequency of the observed nucleus, the spin-lattice relaxation time $T_1$ is directly proportional to $D_r$ (Abragam, 1961). This condition known as "extreme narrowing" in the NMR literature, is always satisfied by small solutes in solvents of low viscosity.

## Materials and Methods

All samples were prepared using 50 mM solutions of cyclic guanosine monophosphate (cGMP) in 3:1 $H_2O/D_2O$. The solutions were buffered at pH = 7.5 using 50 mM Tris(hydroxymethyl)aminomethane hydrochloride, and 1 mM ethylene-diamine-tetraacetic acid (EDTA) was added to scavenge trace amounts of metal ions. Either NN'methylene bisacrylamide (Bis) or NN'bisacryloylcystamine (BAC) was added as a cross-linker in polyacrylamide at a concentration of 4% (relative to the total acrylamide content). The samples were degassed under vacuum, and polymerization occurred at room temperature upon addition of 1 $\mu$L/mL of a freshly made 40% ammonium-persulfate solution and 0.3 $\mu$L/mL of N,N,N',N' tetramethylethylenediamine (TEMED) under nitrogen. Agarose gels were prepared by dissolving the appropriate amount in previously degassed and buffered cGMP solutions at 55°C, followed by quenching in a waterbath at 20°C under nitrogen. Agarose was purchased from Sigma (Type XI).

All gels were formed directly in 10 mm outer diameter NMR sample tubes. The longitudinal relaxation time $T_1$ of the single phosphorus atom was measured by the inversion recovery technique (Martin et al., 1980) at a Larmor frequency of 81 MHz. The relaxation behavior was monitored using 18 relaxation delays, evenly spaced on a logarithmic scale between 50 ms and approximately $7T_1$. Broadband proton decoupling was applied during the evolution and the acquisition times. The signal-to-noise ratio exceeded eight in all cases because of signal averaging. A non-linear least squares algorithm was used to fit the data (Leipert and Marquardt, 1976). The recovery was exponential at all gel concentrations. Although the uncertainty in the experimental parameters limits the accuracy of $T_1$ to within 5–8%, the quality of the fits and the reproducibility of multiple experiments suggest an interval of confidence of 3–4%.

## Results and Discussion

Experiments were performed, monitoring $^{31}P$ relaxation of cGMP trapped in two types of gels. The results for several concentrations of polyacrylamide are shown in Figure 1. As expected, the relaxation time decreases with increasing gel concentration, reflecting reduced mobility in the denser gels. If the diffusion of cGMP is approximated by that of an equivalent sphere with a single coefficient $D_r$, and the condition of "extreme narrowing" is assumed to prevail at all gel concentrations, i.e., if $D_r \gg 8 \times 10^7$/s, then $T_1$ is a direct measure of $D_r$ over the entire range of gel densities. Hence, a more than threefold decrease in the rotational diffusivity of cGMP was observed when the gel concentration was brought from 0 to 28% by weight. [A breakdown of the assumption of extreme narrowing would imply an even larger drop in the rotational diffusion coefficient. This is highly unlikely in view of the small size of cGMP and the low (micro)viscosity of the solvent used.] The validity of the diffusion equation itself should be questioned in a fibrous environment. The presence of an impermeable matrix creates a potential field in which the probe molecule undergoes Brownian

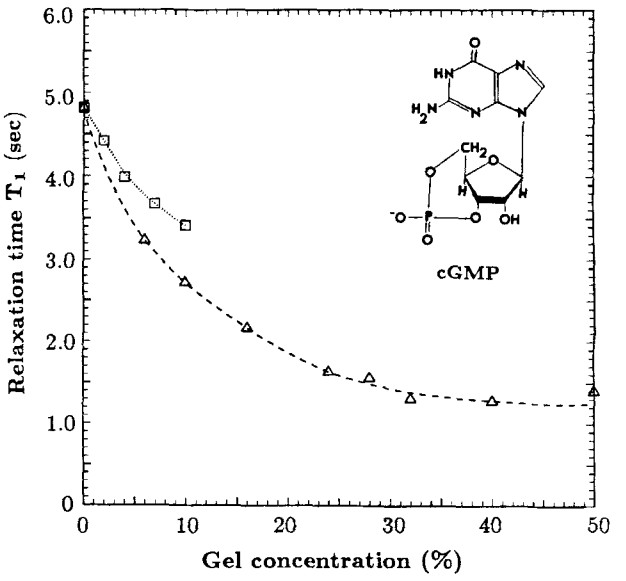


**Figure 1. Spin-lattice relaxation time $T_1$ of cGMP in polyacrylamide (△) and agarose (□) gels as a function of the gel concentration in w/v.**

motion. The average (long-time) rotational displacement, taken over all possible network configurations, is expected to be diffusive, but more complex short-time behavior cannot be excluded. One should therefore be cautious in interpreting $D_r$, and merely regard it as a measure of the molecule's mobility, or as the inverse of a characteristic decay time for the orientational correlation function of cGMP.

The gel composition at 40% and 50% polyacrylamide was not homogeneous (the gel was not translucent). These results should therefore be considered with caution, since they reflect a different gel structure. Nonetheless, the data obtained seem consistent with the general trend. Note that the initial drop in $T_1$ is steeper than the decrease observed at higher fiber densities. This indicates that even fairly porous gels hinder the tumbling of relatively small molecules. This behavior also reflects the fact that the effective pore size does not decrease linearly with increasing gel concentration.

A series of similar experiments was performed using agarose in concentrations up to 10%. The results reproduced in Figure 1 show less dramatic changes in the relaxation time than for polyacrylamide. This agrees with previous observations that the pores in agarose are larger than in polyacrylamide (Serwer, 1983). Agarose chains aggregate in "suprafibers" consisting of 7–30 double helices (Arnott et al., 1974; Waki et al., 1982). This bundling leaves relatively large voids in the structure, up to 120 nm wide in very dilute gels (Ackers and Steere, 1962). The magnitude of the retardation of a molecule as small as cGMP in such a spacious network is therefore quite remarkable.

There is much less consensus on the porosity of polyacrylamide gels. From molecular sieve chromatography, the estimated average pore radius is of the order of 1–2 nm for the crosslinker concentrations used in this work (Fawcett and Morris, 1966; Chrambach and Rodbard, 1971). At the other extreme, micron-sized cellular structures have been reported using electron microscopy (Rüchel and Brager, 1975; Rüchel et al., 1978). However, these estimates seem to be artifacts of the freezing technique employed (Gressel and Robards, 1975); the most carefully prepared electron micrographs of polyacrylamide gels indicate pore sizes in the range of 6–70 nm (peaked about 10 nm) (Hsu and Cohen, 1984). This contrasts greatly with the value of 160 nm reported recently (Nishio et al., 1987), but is in good agreement with model calculations based on macromolecular scaling theory (Wijmenga and Maxwell, 1986). An equal decrease in the rotary diffusion of cGMP was observed for both 5% polyacrylamide and 10% agarose gels. Excluding the unlikely possibility of retardation by specific chemical interactions between the probe molecule and the fibers, the relevant physical characteristics of these two gels should be similar, and their pore dimensions in particular should be of the same order of magnitude. From data on agarose (Ackers and Steere, 1962), the effective pore size of a 5% polyacrylamide gel should therefore be about 17 nm. Of course, this estimate is rather crude, since many other factors which codetermine the retardation effectiveness of the gel have not been considered, such as the rigidity of the network and the thickness of the fibers.

Since $T_1$ is proportional to $D_r$ which is itself, according to the Stokes-Einstein formula, inversely related to the drag coefficient of the diffusing particle, the contribution of the fibers to the effective friction factor of the trapped molecule can be evaluated from $(T_1)^{-1} - (T_1^{sol})^{-1}$. Here, $T_1^{sol}$ denotes the measured relaxation time in the absence of gel. A double logarithmic plot

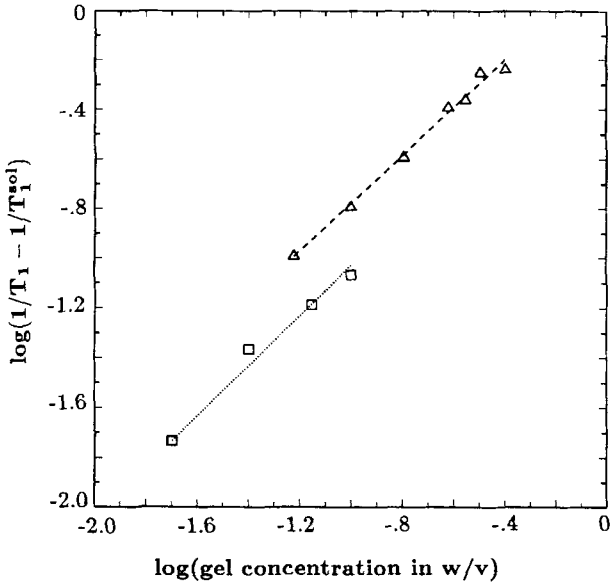


**Figure 2. Double logarithmic plot of the difference in relaxation rates inside a gel ($1/T_1$) and in solution ($1/T_1^{sol}$) vs. the polyacrylamide (△) and agarose (□) concentrations.**

The data for both gels lie on lines with a slope very close to unity.

of this difference in relaxation rates versus gel concentration, yields a slope very near unity for both polyacrylamide and agarose, Figure 2. This similarity suggests that the same mechanisms account for the slowing of the molecule in both gels. It is interesting to note that an analogous linear relation between the rotational relaxation time and the gel concentration was observed for short DNA fragments in polyacrylamide (Wijmenga and Maxwell, 1986). This may point to a common phenomenon, most likely the increasing hydrodynamic interactions with the static fibrous matrix as the gel gets denser. More experimental data, and a rigorous treatment of the fluid mechanics involved, however, will be needed to support this conclusion. The flexibility of nuclear magnetic relaxation experiments is a great advantage in that respect. The spectrum of motional frequencies to which the technique is sensitive can be enlarged by observing different nuclei (any spin other than hydrogen will do), by varying the magnetic field, or by a clever selection of the experiments: the transverse relaxation time, $T_2$, and the spin-lattice relaxation in the rotating frame, for example, reveal the low frequency components of the motions.

## NOTATION

$D_r$ = effective rotational diffusion coefficient, $s^{-1}$
$\mathbf{L}$ = quantummechanical angular momentum operator
$P(\Omega, t)$ = probability density function for the instanteneous molecular orientation, $s^{-1}$
$t$ = time, s
$T_1$ = longitudinal (spin-lattice) relaxation time, s
$T_2$ = transverse (spin-spin) relaxation time, s
$\Omega$ = set of coordinates describing molecular orientation

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