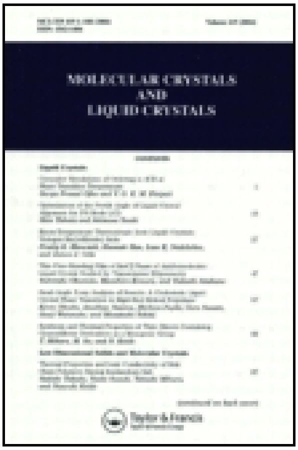

Molecular Crystals and Liquid Crystals Science and Technology. Section A. Molecular Crystals and Liquid Crystals
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl19

Single Molecules Solvated in Pores of Polyacrylamide Gels

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Version of record first published: 04 Oct 2006.

To cite this article: Robert M. Dickson , D. J. Norris , Yih-Ling Tzeng , R. Sakowicz , L. S. B. Goldstein & W. E. Moerner (1996): Single Molecules Solvated in Pores of Polyacrylamide Gels, Molecular Crystals and Liquid Crystals Science and Technology. Section A. Molecular Crystals and Liquid Crystals, 291:1, 31-39

To link to this article: http://dx.doi.org/10.1080/10587259608042727

Mol. Cryst. Liq. Cryst., 1996, Vol. 291, pp. 31–39
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SINGLE MOLECULES SOLVATED IN PORES OF POLYACRYLAMIDE GELS

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Abstract Individual fluorescent molecules and individual singly-labeled proteins have been observed in the water-filled pores of poly(acrylamide) gels with far-field microscopy. The molecular range of motion is dramatically reduced by the gel framework, thus allowing single molecules to be studied in an aqueous environment for long periods of time. For the small fluorophores, the gel restricts Brownian motion by approximately two orders of magnitude in each direction, thus greatly enhancing the molecule's detectability. In contrast to dry polymeric hosts, the gel is composed primarily of water and the majority of molecules remain in solution, thus making these gels an ideal medium in which to utilize single molecule detection methods for the study of biological systems *in vitro*.

INTRODUCTION

Since the initial studies of single molecules in condensed media at low temperatures,[1,2] much information about the individual chromophores themselves and their immediate surroundings has been obtained. Employing high spectral selectivity, low-temperature studies continue to yield fine details of the molecular interactions,[3] while room-temperature studies have recently provided analogous information on coarser environmental differences.[4,5] Far-field and near-field scanning (NSOM) microscopy have been employed at room temperature with fascinating results[6,7,8,9], but the perturbing effects of the metal-coated near-field tips have limited the utility of NSOM in some cases. Conversely, far-field microscopy allows subwavelength resolution (diffraction-limited to about 200 nm) and very high collection efficiencies without perturbing the system of interest.[5,10,11]

Immobilizing the single fluorescent molecule of interest has been one key to successful observations of individual chromophores. For example, authors have reported images of single molecules immobilized in polymeric hosts[10] or non-specifically stuck on surfaces.[5] By examining the spectral emission characteristics of such molecules, a wealth of novel behavior has been observed, but dry surfaces and polymers are rather limited in their application to many chemical and biological problems. Recently, the pioneering work by Funatsu, et al. has opened surface-bound biophysics to the realm of single-molecule study.[12] Other studies with biological significance have been performed on single fluorophores bound in two-dimensional lipid bilayers,[13,14] and on two chromophores on a single poly(peptide) capable of energy transfer and thus distance measurement.[15] Individual DNA molecules have also been detected in solutions[16] and during capillary electrophoresis[17] as the molecule diffused through the focal volume of the optical system. This technique has also been applied to the observation of multiple individually labeled oligonucleotides in a relatively concentrated ethylene glycol solution.[18] These useful studies demonstrate that solution-based single-molecule detection can be performed; however, extracting detailed information about the molecule and its interactions with its surroundings requires that the molecule be localized long enough for the interaction of interest to occur multiple times.

In the hope of opening more areas of biophysics to single-molecule studies, we have employed as a host matrix the easily manipulated poly(acrylamide) (PAA) gels, which polymerize into cross-linked matrices. Depending on the acrylamide and crosslinker concentrations, the pores enhouse water-filled cavities ranging in diameter from two to several hundred nm.[19-21] As our initial studies of single molecules in gels showed, polyacrylamide provides a matrix which restricts the motion of water-soluble molecules, while allowing the molecules to retain solution-based behavior.[22] These properties of PAA gels have allowed us to image singly-labeled molecules in an aqueous environment amenable to prolonged biochemical or physical study.[22]

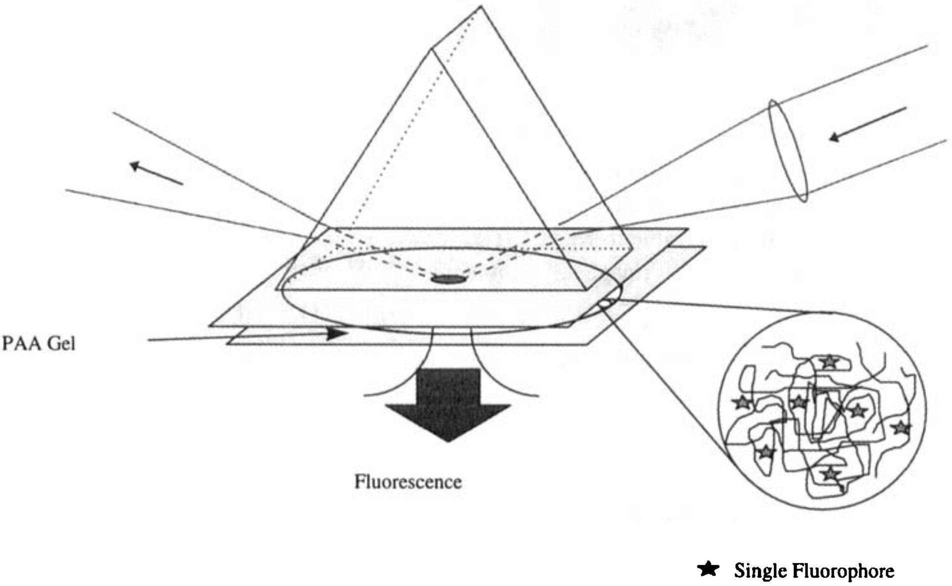

FIGURE 1 Total internal reflection excitation at 532 nm of a schematic fluorophore-doped PAA gel.

EXPERIMENTAL PROCEDURES

In order to study single fluorophores in the pores of PAA gels, samples were prepared containing 10^{-10} M nile red in aqueous acrylamide/methylenebisacrylamide solution

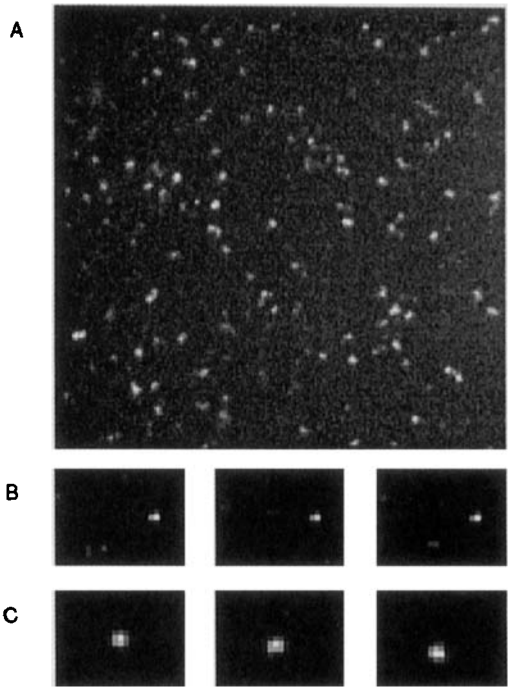

FIGURE 2 (A) Fluorescence image of single nile red molecules in a 18 % PAA gel over a spatial field of 43 μm by 41 μm with ~200 nm resolution with a 1 s exposure time. (B) Three successive expanded images of a molecule fixed in spatial position from frame to frame. (C) Three successive expanded images of a molecule moving in position from frame to frame.

according to standard sodium dodecyl sulfate gel preparation methods.[23,24] Each sample consisted of a 2 μL aliquot which polymerized rapidly between glass coverslips and remained optically transparent. Even high concentration PAA gels consist of a porous network that allow small molecules to diffuse through. The nile red molecules observed in this study were observed in 18 wt. % acrylamide gels with 5 wt. % crosslinker yielding average pore diameters of ~2 nm.[19]

The fluorophore-doped gels were excited with the evanescent wave generated by total internal reflection (TIR) of a 532 nm cw laser at the coverslip-gel boundary (Fig. 1). The evanescent field intensity, I, falls off exponentially with distance z from the interface, as[25]

$$I = I_0 \exp\left[-\frac{4\pi\, nz}{\lambda}\left(\left(\frac{\sin\theta}{\sin\theta_0}\right)^2 - 1\right)^{1/2}\right] \qquad (1)$$

where I_0 is the field intensity at the coverslip-gel interface, λ is the vacuum wavelength, n is the refractive index of the less-optically-dense medium, and θ and θ_0 are the experimental angle of incidence and the critical angle giving TIR, respectively. In our experiments, the exponential decay length was 125 nm; hence only a thin layer of material close to the interface was excited. The chief advantage of TIR is that the volume capable of producing background is greatly reduced.[12] The emitted fluorescence was filtered and observed with a liquid N_2-cooled CCD as shown in Fig. 2A.

RESULTS AND ANALYSIS

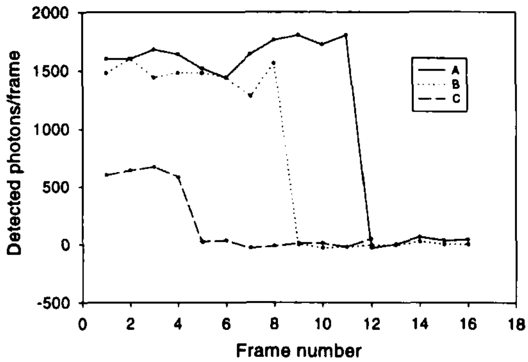

FIGURE 3 Digital bleaching of three molecules trapped in the polymer matrix, excited with 5kW/cm^2 at the gel/coverslip interface. Exposure time 100 ms/frame.

For many images similar to those shown in Fig. 2A, most of the bright spots exhibited transverse (x-y) and axial (z) motion from frame to frame (as in Fig. 2C). A few single molecules were immobilized in the gel matrix in extremely small pores (Fig. 2B). Confirming that these stationary bright spots were single molecules, the observed signals remained essentially constant from frame to frame, then suddenly and permanently fell to the background level ("digital" bleaching), resulting from an irreversible photochemical reaction (Fig. 3). In separate measurements with fixed molecules, the expected strong polarization anisotropy of the absorption was also confirmed.

In contrast to the spatially-fixed molecules, most of the molecules observed in these gels diffused in and out of the field of view, staying for multiple frames, then disappearing, simultaneously defocusing and losing brightness. If a molecule emitted the same number of photons in each frame, drifting out of the depth of focus would merely spread these photons over more CCD pixels, leaving the total (spatially integrated) signal unchanged. In our experiments, the integrated signal from a given bright spot changed significantly from frame to frame while staying in focus. This is consistent with the evanescent field intensity being reduced by a factor of 8 for motion of the molecule by one focal depth (approximately 250 nm). By using the total number of detected photons from a molecule during an exposure as a measure of the exciting intensity, the exponentially decaying evanescent field arising from our TIR excitation scheme enabled three-dimensional (3-D) imaging of single fluorophores in solution.[22] It is important to note that the strong distance dependence of the evanescent field which allows the 3-D imaging of these molecules simultaneously biases our measurements in the z-direction. We can only analyze molecules which linger in the focal volume for several successive frames, meaning that the axial (z) motion must remain very small (on the order of 200 nm, such that the evanescent field retains sufficient intensity to excite the molecules); therefore, only the transverse motion is analyzed in detail.

Brownian motion would require that a single nile red molecule move nearly 30 μm in a 1 second interval, t, according to the well known expression $\langle x^2 \rangle = (kT/3\pi\eta a)\,t$, where k is Boltzmann's constant, T is the absolute temperature, η is the solvent viscosity, and a is the molecular radius. Average squared displacements ($<x^2>$ and $<y^2>$) were obtained by following 16 different single molecules (Table 1). The transverse mean square displacements (Table 1) clearly indicate that the gel matrix hinders the Brownian motion of single molecules. The discrepancy between observed motion and theoretical Brownian motion persists for all the molecules studied. For 1 sec

TABLE 1 Mean square displacements of single molecules in successive exposures at two different data acquisition rates.

1 Second Exposures at 0.4 Hz*			0.1 Second Exposures at 1.5 Hz*		
molecule	$<x^2>$ (μm^2)	$<y^2>$ (μm^2)	molecule	$<x^2>$ (μm^2)	$<y^2>$ (μm^2)
A	0.0893	0.214	I	0.333	0.125
B	0.0536	0.259	J	0.0714	0.286
C	0.0417	0.0729	K	0.125	0.125
D	0.135	0.0521	L	0.286	0.179
E	0.0625	0.0750	M	0.200	0.100
F	0.0938	0.281	N	0.125	0.125
G	0.0781	0.219	O	0.179	0.143
H	0.292	0.125	P	0.107	0.0714
Avg.	0.105 ± 0.075	0.162 ± 0.085	Avg.	0.178 ± 0.085	0.144 ± 0.061

*Typically 6 frames were used for each mean-square measurement exposures, the distance traveled due to diffusion was reduced by a factor of approximately 100 (or, equivalently, the diffusion constant, $D = (kT/6\pi\eta a) = \langle x^2\rangle/2t$, was reduced by a factor of 10^4) in each dimension by the presence of the porous gel matrix. We also found that the average mean-square excursions for longer exposures (1 sec) and those for shorter exposures (0.1 sec) with a four times higher frame rate were essentially equal. This failure of the mean-square displacement to scale with time is consistent with the strong departure from Brownian behavior we have observed, thus making these PAA gels suitable for long-time observation of single molecules in solution. Whether or not the motion of the molecules is Brownian ***between*** wall encounters, however, poses a difficult experimental problem. It requires short time information which would be very helpful in understanding the molecular motion in PAA gels. The distances traversed by the observed molecules, however, would occur due to simple Brownian motion in only 150 μs, a much faster time scale than present single molecule imaging methods are capable of measuring.[11,14]

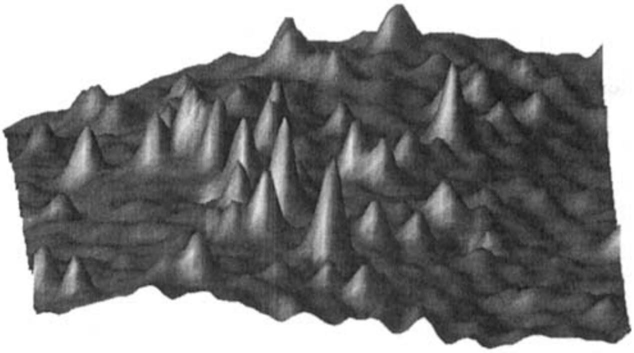

FIGURE 4 Image of individual Cy-3 labeled BSA proteins in a 10% PAA gel. Exposure time 1s, 20 μm x 25 μm region, excitation intensity ~ 3 kW/cm^2.

In order to further probe the utility of these polyacrylamide gels for the study of singly-labeled proteins in aqueous environments, singly-labeled bovine serum albumin (BSA) protein molecules were isolated in 10% polyacrylamide gels which should have pores large enough to freely accommodate the labeled BSA. An image of the single proteins is given in Figure 4. Confirmation that these are single proteins is given by the fact that they exhibit digital bleaching similar to that shown in Fig. 3. Assuming a 5 nm diameter sphere for the three-dimensional structure of this 62 kD protein, one would expect it to move nearly 15 μm in 1 second in free solution. Unlike the individual chromophores, however, the labeled proteins are seen to be completely stationary. The proteins in the gels are individual molecules, sufficiently large to be locked in place by the gel matrix. The fluorophore label, however, does show large (30-50%) fluorescence intensity fluctuations. This is presumably due to the chromophore attached to the protein being able to change its orientation relative to the polarization of the exciting laser field. Based on the fact that individual nile red chromphores locked in the gel matrix (Fig. 3) yield relatively stable intensities, and that the labeled protein nearly completely fills the gel pore, the tethered chromophore may be able to slowly change its conformation, being hindered by the gel matrix and the nearby protein conformation, thus giving rise to the observed intensity fluctuations.

Our experiments indicate that poly(acrylamide) gels offer great promise as hosts for single molecule studies of biological systems.[22] Ease of preparation and controllable pore size make this host material a particularly flexible matrix which should facilitate the study of singly-labeled proteins, electrophoresis,[26] and individual reactions in solutions. For small fluorophores like nile red, gels as hosts hinder the distance traveled due to Brownian motion by approximately two orders of magnitude in each direction for observations on the order of 1 second. This means that the signal from any one solvated molecule is concentrated in 1/10,000th of the area of a similar molecule in free solution, thus providing a large increase in detectability solely from the use of the poly(acrylamide) matrix.

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27. We thank B. H. Zimm for helpful discussions. D. J. N. was supported in part by a National Science Foundation Postdoctoral Fellowship, and R. S. was supported in part by a Damon Runyon Postdoctoral Fellowship. L. S. B. G. is an Investigator at The Howard Hughes Medical Institute.