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Feature article

Single Macromolecule Diffusion in Confined Environments

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We consider the behaviour of single molecules on surfaces and, more generally, in confined environments. These are loosely split into three sections: single molecules in biology, the physics of single molecules on surfaces, and controlled (directed) diffusion. With recent advances in single molecule detection techniques, the importance and mechanisms of single molecule processes such as localised enzyme production and intracellular diffusion across membranes has been highlighted, emphasising the extra information that cannot be obtained with techniques, which present average behaviour. Progress has also been made in producing artificial systems that can control the rate and direction of diffusion, and because these are still in their infancy (especially in comparison to complex biological systems), we discuss the new physics revealed by these phenomena.

Introduction: single molecule diffusion

The motion of the very small has been studied for a long time,[1,2] beginning with the initial microscopic observations of pollen on water in 1827 and continuing in the present day with a vast array of molecular diffusion studies. Initially such studies concentrated on the average

motion of an ensemble of molecules as techniques such as fluorescence recovery after photobleaching[3] (FRAP) were not sensitive enough to observe an individual particle. Despite this, averaging techniques have been, and continue to be, very successful in probing protein dynamics[4-6] and protein-protein interactions,[7,8] as well as determining average diffusion coefficients of molecules within a small region.[9,10]

Recent advances in experimental techniques have allowed the motion and interactions of single molecules to be studied with improved accuracy. Some of these techniques, such as atomic force microscopy,[11,12] total internal reflection fluorescence (TIRF) imaging,[13,14] and super-resolution imaging,[15-17] have allowed direct images to be produced which provide insight into the orientation,[18] clustering, or changes to a molecule within a system. Time-stop imaging techniques have also been used to determine the kinetic properties of a system.[19] However these are somewhat limited by the equipment in terms of exposure time, acquisition rate, and size of detection region. In general, imaging is useful as it provides visual confirmation of the region under study. We show in **Figure 1** data exemplifying why single molecule imaging reveals more information than ensemble averaging techniques; here molecular motion is smeared out of the signal when the data are averaged (box 4 in Figure 1), but time-stop imaging reveals a complex molecular trajectory (box 3 in Figure 1).

Techniques designed to examine the diffusive properties of molecules within a sample, such as neutron spin echo,[20-22] dynamic light scattering,[23,24] fluorescence correlation spectroscopy (FCS),[25-28] and single mode optical fibre detectors,[29] generally do not involve imaging in real space, but require spectroscopic determination of signal intensity and the timescales of fluctuations that occur within the sample. However, one can also use fluorescence spectroscopy for single molecule imaging and tracking.[30-34]

Previously single molecule techniques have allowed direct observation of molecules in their environment or to indirectly determine diffusive characteristics. Recently Leslie and colleagues[35] have developed a modification to wide-field microscopy, termed convex lens-

induced confinement (CLIC), that restricts molecules to a nanoscale wedge-shaped gap (formed between the lens and a coverslip) which both increases imaging quality by significantly rejecting background fluorescence whilst simultaneously increasing the time a diffusing molecule can be measured, by restricting motion to within the focal plane. Whilst such a development is still in its infancy, this simple concept is a promising step forward in producing simultaneous imaging and diffusion measurements of a high quality. However due to the nanoscale dimensions of the wedge required for this technique it will not be viable for measurements in samples of comparable or larger size. Furthermore the low surface to (observation) volume ratio means the technique is highly susceptible to artefacts caused by the surface chemistry of the lens and the coverslip.

The importance of single molecule motion

In the absence of any external influences, a molecule in fluid will undergo Brownian motion, taking a random path with a speed that depends on temperature. This simplest process defines the diffusion coefficient of a spherical particle of radius R through a medium of viscosity η, through the Stokes-Einstein relation,

$$D = \frac{k_B T}{6\pi\eta R}, \qquad (1)$$

where T is the (absolute) temperature of the system at equilibrium. There have been numerous modifications to this theory to account for variations in molecular shape, as well as interactions with the surrounding environment, but in essence the processes described using this equation are stochastic.

Biological systems have become adept at harnessing random thermal motion, for example in the molecular apparatus associated with cellular chemotaxis and haptotaxis, or in the motion of a bacteriophage as it approaches a potential target.[36] Nevertheless, biological systems need to sense and respond to their environment in order to function. Molecular motors, such as the kinesins, are an excellent example of how evolutionary mechanisms have produced a

means of restricting the directionality of a diffusing protein in order to perform a specific task. Some studies suggest that such molecular motors can achieve near-perfect efficiency[37] although this is a widely disputed with other studies suggesting less than perfect efficiency of order 25-50%.[38-39]

It is not the efficiency of these motors that is of interest in this article, but in the imbalance of forward and backward step probabilities; why does a kinesin protein, when attached to a microtubule, preferentially walk forwards? Sindelar and Downing[40] have recently produced maps of kinesin motion at atomic resolutions (0.8-0.9 nm) using cryoelectron microscopy, and found that a unique arrangement on the nucleotide-sensing and switch II regions at the filament binding face of a kinesin provide the preference for forward stepping. This asymmetry is entropic in nature and can reach $6k_BT$ under ambient conditions[41] although this depends upon applied load and environmental conditions.[14,42]

There are some systems in which regions of decreased or inhibited molecular diffusion provide the means for certain processes to occur. Rafting, in which signalling molecules cluster to improve the efficiency of signal transduction, is important in, for example, cell signalling and growth,[43] immune responses,[44] and cell adhesion.[45] In some cases a protein receptor is trapped in a phospholipid bilayer to form a raft. IP_3 (triphosphoinositol) binds to this receptor and activates downstream components in the calcium signalling pathway.[46] This pathway is used to modulate a wide range of important biological processes such as muscle contraction. Video rate imaging of the membrane-anchored diffusion of CD59 rafts induced by IgG-gold complexes show an unexpected effect that would be very difficult to detect with ensemble based diffusion techniques. The rafts exhibited both the expected Brownian diffusion and periods of temporary immobilisation, the regions of which are highlighted in the video-rate trajectory recorded in **Figure 2**. This immobilisation, termed STALL (***S****timulation-induced* ***T****emporary* ***A****rrest of* ***L****atera****L*** *diffusion*), has been linked with the recruitment of other enzymes or proteins onto the raft that are essential for other processes to occur.[48]

PLCγ2 is an enzyme that produces IP_3, and is found to attach to the CD59 raft only during the STALL periods. The exponential decay time of these STALLs (0.57 s) is sufficiently long enough for the production of 20-50 IP_3 molecules so whilst the number of STALL instances is low, sufficient IP_3 is produced at each event to facilitate the intracellular signal.

The ability of cell membranes to selectively control diffusion between extracellular and cytosolic surfaces is important for producing artificial membranes, although current systems are yet to accurately mimic their biological counterparts. Saffman and Delbrück[49] proposed a model for translational diffusion of a cylindrical molecule (such as a protein) in a two dimensional continuum fluid, in which the diffusion varies logarithmically to the ratio of the height and radius of cylinder in contrast to radial Stokes-Einstein diffusion that gives an inverse relation between radius and diffusion coefficient. An important consequence of the Saffman-Delbrück model, in contrast to the Stokes-Einstein relation, is that increasing the number of monomers by a factor of 100 (by forming a complex or using a much larger polymer) only results in a 40% decrease in diffusion coefficient (for a 0.5 nm monomer size). In other words, diffusion is poor at separating out pre- and post-binding molecules as translational diffusion is insensitive to the size of the diffusant, which is not what is observed when membrane molecules form complexes or oligomers; their diffusion rates drop significantly.[50] A study of the unsaturated phospholipid L-α-dioleoylphosphatidylethanolamine (DOPE) in normal rat kidney (NRK) epithelial cells[51] using traditional video-rate observations (with time resolution of 33 ms) and single-particle tracking (25 μs - 33 ms resolution) found that the single diffusion coefficient measured at the longer time scales is in fact an average of three diffusion processes. Such an effect offers support to the theories that membranes partition to form compartments, in which a molecule can (a) diffuse within the compartment away from boundaries, (b) diffuse near boundaries, hopping compartments, and (c) long time-scale hops between compartments. Kusumi and colleagues found that the average dwell time of DOPE in NRK compartments was 11 ms,

well below the resolution of traditional methods. Other studies have shown similar dwelling events in membranes as the mechanism for a variety of processes.[52-54] In short, the diffusion of single molecules must be studied over a wide range of time scales as traditional averaging methods or low-resolution techniques do not highlight individual processes.

Macromolecular diffusion in synthetic systems

In the biological sciences molecular motors, rafting, and other single molecule systems are intricately suited to perform a specific task. The aim of much current research is to produce an artificial system that provides a similar level of intricate response and control, but biological systems have had millions of years to develop, refine, and evolve into the structures we observe today; developing such sophisticated systems is far beyond the current level of technology, scientific understanding, and timescales available. Nevertheless merely by considering the simplest form of diffusion theory, namely the Stokes-Einstein relation given in Equation 1, it is clear that by manipulating basic environmental conditions one can influence molecular diffusion.

Progress has been made in understanding and utilising the response of polymer hydrogels to their environment with the aim of developing "smart" drug-delivery systems; hydrogels that degrade in the presence of the site-specific β-mannanase enzyme can target the colon,[55] whereas complex gel matrices are being developed to control the rate of release of therapeutic agents like growth hormone,[56] paracetamol,[57] and nicotine.[58] Similar delivery systems include molecular aggregates such as micelles, whose behaviour in living cells has also been studied.[59] These developments are promising, but are in essence ensemble systems (i.e., the total molecular diffusion is important, rather than the behaviour of an individual diffusing molecule) and therefore will not be considered further in this article.

Zhao and Granick[60] have recently investigated the change in diffusion of poly(ethylene glycol) (PEG) adsorbed onto self-assembled monolayers (SAMs) of methyl-terminated *n*-octadecyltriethoxysilane using cross-correlation FCS and found that a sharp peak in diffusion

coefficient occurs when the surface concentration is slightly in excess of the predicted overlap concentration (**Figure 3**). Following the peak there is a sharp decrease of nearly one order of magnitude which suggests that the interaction of adjacent PEG molecules drastically reduces the diffusion of the molecules; the diffusion peak is a matter of current interest with studies suggesting reptation is not the mechanism of increased motion,[61] and others proposing a combination of compaction and fractal contours providing a more directed path.[62-63] Nevertheless the region of increased diffusion around the point where polymer overlap is believed to occur indicates that there is an optimal concentration for maximising surface molecular diffusion.

The understanding of how single molecules behave on surfaces is of great interest and importance, but future work on controlling polymer behaviour will require us to be able to create surfaces with specific properties. One such functional surface was prepared with the aim of controlling the direction of the diffusion of adsorbed molecules.[25] SAMs of dodecanethiol (DDT) and mercaptopropanoic acid (MPA) were used as model hydrophobic and hydrophilic surfaces respectively, and gradients between the two were created using ultraviolet light from an optical fibre to degrade the thiol and allow its replacement with a complementary thiol. This methodology can be controlled to create a gradient with a size determined by the experimental parameters, i.e. the distance between the fibre and surface, and exposure time.[64] In this case, a ~6 μm gradient between the two surfaces was fabricated, and directed diffusion of PEG between the two surfaces was measured.

As one would expect, away from the gradient, the diffusion coefficients are isotropic with the diffusion coefficient for the PEG on MPA being greater than that on DDT (D_{MPA} = 4.7±0.4 $\mu m^2 s^{-1}$ versus D_{DDT} = 0.45±0.05 $\mu m^2 s^{-1}$ due to different molecular conformations; the PEG on the hydrophilic surface has fewer contact points than with the hydrophobic surface.

However once a molecule moves into the gradient region a significant anisotropy arises between the diffusion along and orthogonal to the gradient (**Figure 4**). Orthogonal diffusion

remains similar to that of the homogeneous region (pure surfaces) and yet the diffusion along the gradient increases dramatically, with coefficients measured at (for one typical example) 23 and 1.5 $\mu m^2 s^{-1}$ (along and orthogonal to the gradient respectively) for region II and 56 and 0.7 $\mu m^2 s^{-1}$ for region III. This increase in diffusion coefficient along the gradient can be explained by the gradient in adsorption energy providing a force that can be equated to the Stokes drag, $6\pi\eta vR$ (v is the speed of the molecule), which is in addition to any thermal (Brownian) motion.

Another treatment of the issues that face the directed motion of small particles on surface energy gradients has been provided, for the case of nanoparticles on micron-sized gradient surfaces created using a photomask.[65] These experiments showed that the hydrophobic dye-loaded polystyrene beads adsorbed preferentially onto the more hydrophobic regions, and when situated at the boundary between hydrophobic and hydrophilic regions, there was preferential motion from the hydrophilic region to the hydrophobic region, due to the increase in the hydrophobic interaction along the gradient. Here the gradient was treated as a capillary interaction, but the Stokes drag, considered crucial for the directed PEG diffusion, was shown to be not significant in this case, being replaced by a surface (frictional) interaction, retarding the motion of the beads. Because the nanoparticles are fluorescent, their motion can be followed in real space, as shown in **Figure 5**.

Of course, directed diffusion must be only a starting point for more programmed means. The scanning near-field optical lithography used to create the gradient surfaces for PEG diffusion[64] could be used to create more interesting structures, but much more complex surfaces can be made using DNA.[66] An example of directed single molecule diffusion was demonstrated with streptavadin on a surface created by this technique of DNA origami.[67] Here, the streptavadin consists of four (single-stranded) DNA arms, three of which can react with strands of DNA on the origami surface. Binding events are specific to one of the "legs" and the surface strand, and cleave the surface DNA strand, which means that the molecule is

not tethered to the surface. The other legs can interact with other surface DNA strands located elsewhere, but within reach of the streptavadin, which results in directed motion. These binding/cleaving events change the surface, so the streptavadin can only move in a forward direction. The location of strands to bind to on the origami surface define the direction of motion, and allow the molecule to change direction. The motion is curtailed only when the molecule reaches the end of the path.

These previous examples of controlled single molecule diffusion compared modified yet static surfaces, with dramatic results. It is therefore not surprising that more recent work has also involved the effect of switchable surfaces on the diffusion of an adsorbed molecule. The response of surface-grafted polymers (polymer brushes) to environmental switches such as pH, salt, or temperature, has been well studied. Wang and Zhu[68] prepared brushes of poly(*N*-isopropylacrylamide) (PNIPAm) and observed the surface diffusion of Rhodamine 6G (R6G) and Rhodamine 123 as a function of grafting density and thickness. The diffusion on PNIPAm layers is measured to be slower than that on a hydrophobic monolayer despite the stronger interfacial (hydrophobic) interaction of the latter. The interest in the difference is that such an effect is in contrast to what would be expected from previously published work studying the diffusion of prodan (6-propionyl-2-dimethylaminonaphthalene, a dye) on different SAMs and a silanated-PEG surfaces.[69] Given that the PNIPAm experiments were performed with water-immersed brushes, whereas those of the prodan diffusion measurements were performed in a nitrogen environment, the main question should perhaps have been why the diffusion coefficients for rhodamine on PNIPAm (in water) are similar to those measured for prodan on PEG (in nitrogen). Nevertheless, these are different diffusing species, and perhaps one can over-analyse such results.

In **Figure 6** we show data from the rhodamine diffusion study described above.[68] Here the authors varied the thickness of the brush at constant grafting density and found that a maximum in R6G diffusion occurs in the region h = 1.7-7.1 nm. This effect is believed to be

due to the conformation of the brush, which reaches an optimal density of end-segments within this region, shown schematically in Figure 6 whereby the region of greatest diffusion (labelled II) has a relatively smooth surface made up of the ends of PNIPAM chains. Given that polymer brushes can be produced with a wide variety of chain properties and overall responsivity, this avenue of research is very appealing for those interested in controlling the diffusion of single molecules.

Conclusions and outlook

In this article the importance of single molecule diffusion in many systems is highlighted, noting that the more traditional averaging ensemble type techniques overlook important features of the diffusion, although one should not forget that ensemble averaging does not allow the scientist to confuse single events with a more general behaviour. Improvements in video-rate imaging and fluorescence techniques have confirmed that incredibly complex systems can be produced and controlled with only a small number of molecules undergoing a minor change in diffusion. However biological systems, as complex as these tend to be, have had millennia of evolutionary pressure to refine and as such it is no surprise that recent artificial attempts are comparatively crude. Nevertheless even the past few years have seen great strides in producing artificial systems that have some degree of control over the diffusion of individual molecules.

Future developments in this field will take place in three routes. The DNA nanotechnology route will continue to thrive. Progress in controlling motility in DNA-inspired surfaces will not take long to reach the third dimension. There is nothing in principle difficult about creating three-dimensional DNA nanostructures; this was reported as long ago as 1996.[70] Where this will all lead us is an entirely different question, because this work is a step into the unknown. At the moment, much of DNA nanotechnology demonstrates proof of principle and the limits of what man can actually achieve. How it will be useful in medicine, or indeed, in other disciplines is to be debated. Nevertheless, given that Drexlerian nano-assemblers are

now understood to be highly unlikely in the form originally envisaged,[71] DNA offers perhaps the best hope for programmable nanomachines, and an important part of that work is in moving molecular cargo from A to B. In fact, some progress has been made to that end,[72] although this has not been discussed here because the high degree of user involvement required in the relevant molecular processes is orthogonal to our desire to discuss (autonomous) molecular behaviour.

The theme of directed single molecular diffusion is not restricted to DNA. Indeed the simple experiments of PEG diffusion on surface gradients[25] reveal a rich amount of fundamental physics that is perhaps hidden by the experiments on DNA surfaces. These experiments, as well as those of the fluorescent nanoparticles on hydrophobic SAMs[65] present beautiful test cases for understanding how molecules interact with surfaces, because the molecules are given a *choice*. The molecules will move to the location that minimises their overall (free) energy, and this will allow a better understanding of molecular behaviour than comparative experiments between different surfaces. The experiments on PEG reveal the important role of molecular conformation that is unique to single polymer experiments, and how molecules adsorb on surfaces can be linked to their motility.

Finally, single molecular motion is expected to be increasingly important in biology. As we have seen in this article, understanding single macromolecular behaviour has been mostly driven by the biological rather than the physical sciences community; for example techniques such as FCS have only recently been adopted by physical scientists, despite being relatively mature technologies. Periods of Brownian motion separated by specific biological functions, such as the STALL processes undertaken by single molecules that are described in this feature article are a particularly important area where continued research will take place. Single molecule tracking is thus likely to play an increasingly dominant role over complementary methods, such as FCS, although there will always be a place for different techniques.

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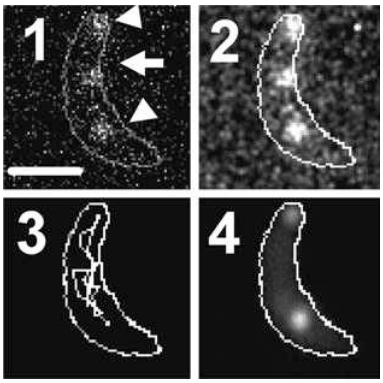

Figure 1. Three fluorescent proteins within a *Caulobacter* cell (outlined with a white line) are here shown detected with a single fluorescence image (boxes 1 and 2 are raw and smoothed images respectively). The diffusion trajectory of the central protein is shown in box 3, but the average of 450 sequential images show that only the two stationary proteins are seen in an averaged image (box 4), demonstrating a significant advantage of single molecule detection over ensemble techniques. Reproduced with permission.[19] Copyright 2006, National Academy of Sciences of the USA.

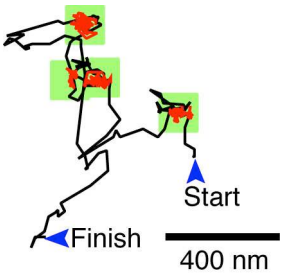

Figure 2. Video-rate imaging of CD59 rafts shows that periods of confined diffusion, termed STALLs (marked by the squares), occur in addition to the expected standard Brownian motion. STALL events are doubly efficient as the enzymatic process that inhibits the diffusion of the raft also induces the production of IP_3 molecules.[47] Data kindly provided by Dr Kenichi Suzuki.

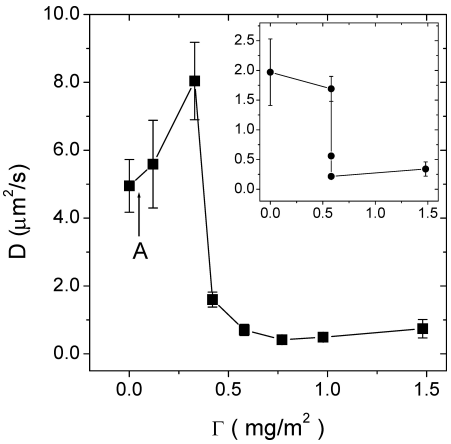

Figure 3. Surface diffusion of poly(ethylene glycol) (PEG) on self-assembled monolayers of methyl-terminated *n*-octadecyltriethoxysilane as a function of surface concentration for M_w = 10 000 g mol^{-1} (main figure) and M_w = 20 000 g mol^{-1} (inset), with error bars representing the standard deviation of 20 or more measurements at different surface regions. The point marked A indicates the estimated concentration at which the "pancake-like" adsorbed molecules begin to overlap one another. Reproduced with permission.[60] Copyright 2007, American Chemical Society.

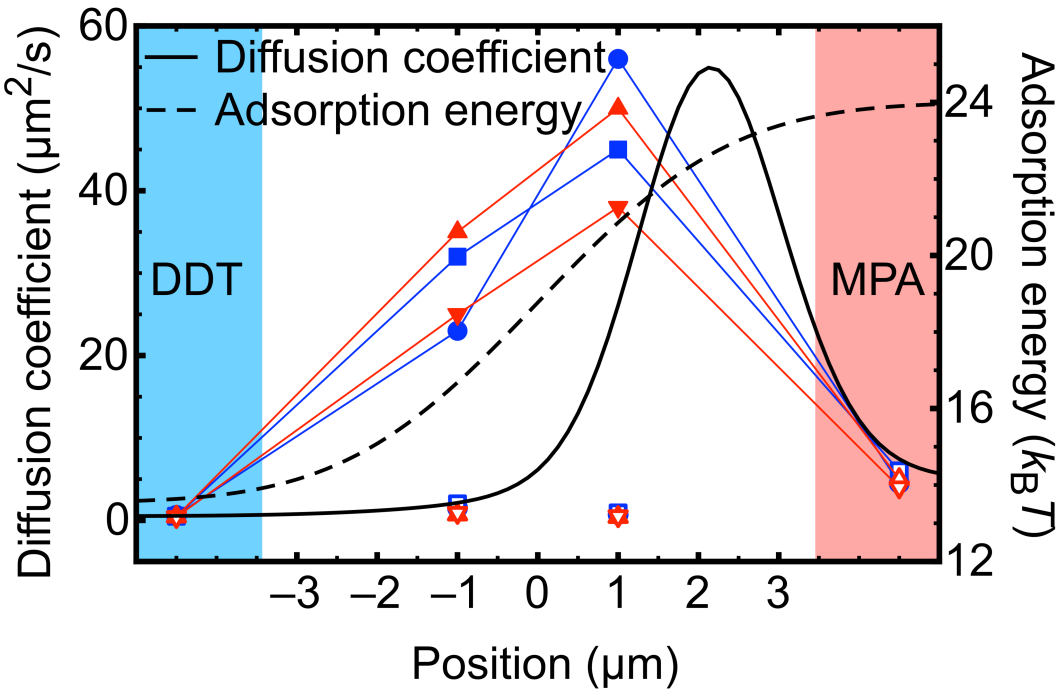

Figure 4. A surface that changes from hydrophobic (DDT) to hydrophilic (MPA) over micrometre length-scales drives the diffusion of adsorbed molecules along the gradient of adsorption energy (dashed line). Closed symbols indicate diffusion along the gradient whereas open symbols represent diffusion of molecules along an orthogonal path. Roman numerals at the top indicate the position on the surface at which the measurements were taken. The blue symbols are for experiments where the gradient was between a DDT channel in an MPA matrix, whereas the red symbols are for an MPA channel in a DDT matrix. Reproduced with permission.[25] Copyright 2009, American Chemical Society.

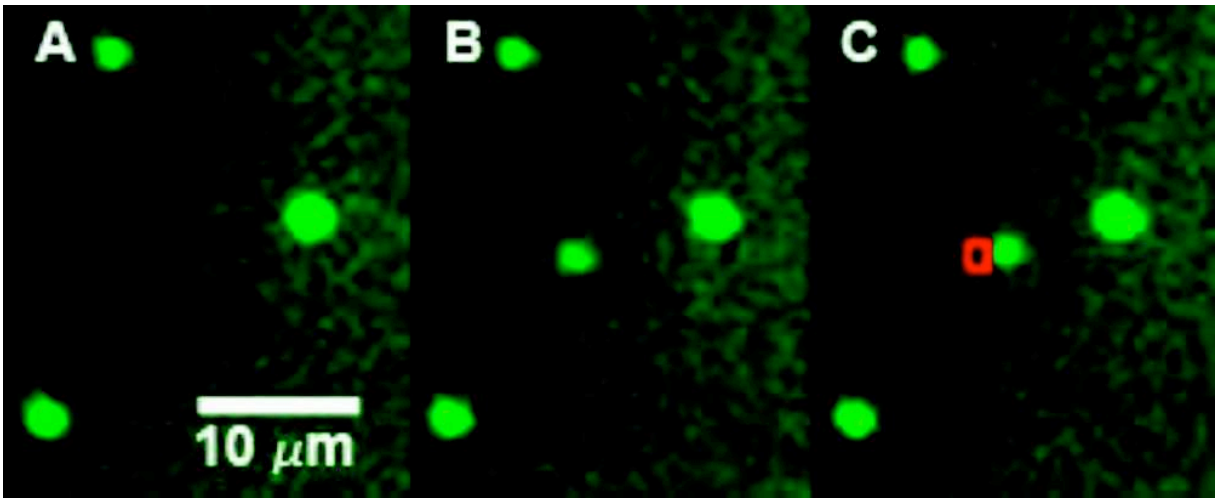

Figure 5. The directed diffusion of 20 nm hydrophobic nanobeads is visualised here by total internal reflection fluorescence microscopy. On either side of the gradient, there is negligible motion, but particles at the boundary will move to the more hydrophobic side, as can be seen by the comparison between images A and C, where the red square shows the original position of the particle; the other three particles in the image, not close to the gradient, do not move. The nanoparticles are not several microns across as the scale bar might suggest; the imaging resolution of the optical technique is limited, and makes the particles seem bigger. Reproduced with permission.[65] Copyright 2010, American Chemical Society.

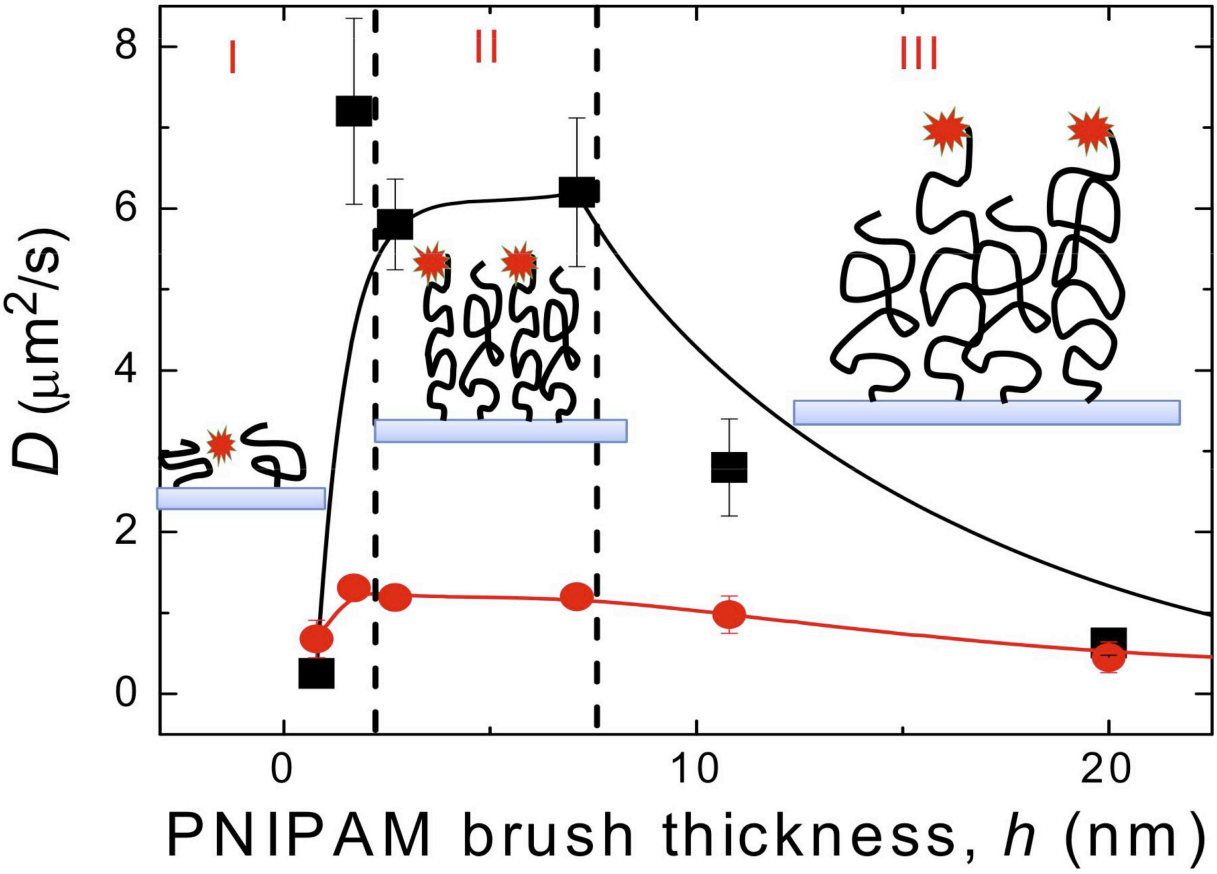

Figure 6. Controlling the thickness of a PNIPAm brush produces a non-monotonic change in the diffusion of an adsorbed rhodamine molecule. A maximum diffusion arises from the conformation at which the end-segment density is optimal. Black markers indicated measurements at 25ºC and red at 45ºC. Reproduced with permission.[68] Copyright 2010, Royal Society of Chemistry.

Matthew Mears was born in Peterborough, UK, in 1984. He studied Physics with Astronomy at the University of Sheffield, obtaining an MPhys. After this he commenced his PhD in 2006 supervised by Professor Mark Geoghegan and his thesis, entitled "Diffusion of Macromolecules in Confined Environments" was submitted in 2011. His primary interests include confined diffusion, polymer adsorption, and glass transitions in thin films.

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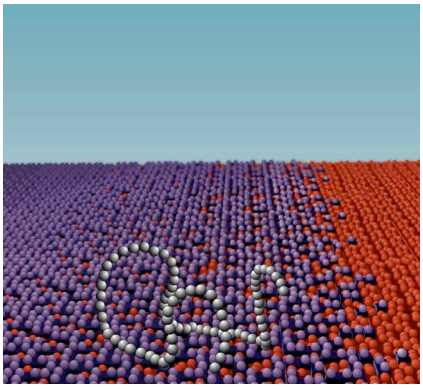
Single macromolecular diffusion on surfaces and, more generally, in confined geometries reveals new physical insights into molecular behaviour. Biomacromolecules have been well studied, but experimental improvements mean that the study of synthetic analogues is now feasible. Recent experimental developments are reviewed, with a view to highlighting areas in which future progress is likely.

M. Mears, D. S. Tarmey, and M. Geoghegan* ((same order as byline))

Title Single Macromolecule Diffusion in Confined Environments



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