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Powering Motion with Enzymes

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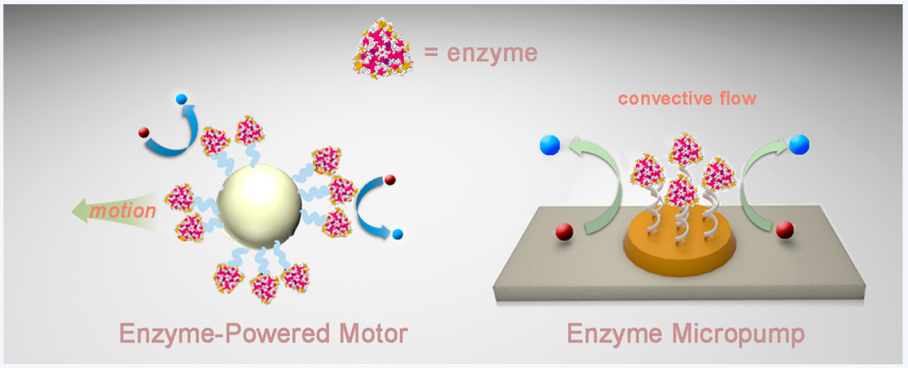

CONSPECTUS: Enzymes are ubiquitous in living systems. Apart from traditional motor proteins, the function of enzymes was assumed to be confined to the promotion of biochemical reactions. Recent work shows that free swimming enzymes, when catalyzing reactions, generate enough mechanical force to cause their own movement, typically observed as substrate-concentration-dependent enhanced diffusion. Preliminary indication is that the impulsive force generated per turnover is comparable to the force produced by motor proteins and is within the range to activate biological adhesion molecules responsible for mechanosensation by cells, making force generation by enzymatic catalysis a novel mechanobiology-relevant event. Furthermore, when exposed to a gradient in substrate concentration, enzymes move up the gradient: an example of chemotaxis at the molecular level. The driving force for molecular chemotaxis appears to be the lowering of chemical potential due to thermodynamically favorable enzyme−substrate interactions and we suggest that chemotaxis promotes enzymatic catalysis by directing the motion of the catalyst and substrates toward each other.

Enzymes that are part of a reaction cascade have been shown to assemble through sequential chemotaxis; each enzyme follows its own specific substrate gradient, which in turn is produced by the preceding enzymatic reaction. Thus, sequential chemotaxis in catalytic cascades allows time-dependent, self-assembly of specific catalyst particles. This is an example of how information can arise from chemical gradients, and it is tempting to suggest that similar mechanisms underlie the organization of living systems. On a practical level, chemotaxis can be used to separate out active catalysts from their less active or inactive counterparts in the presence of their respective substrates and should, therefore, find wide applicability. When attached to bigger particles, enzyme ensembles act as "engines", imparting motility to the particles and moving them directionally in a substrate gradient. The impulsive force generated by enzyme catalysis can also be transmitted to the surrounding fluid and molecular and colloidal tracers, resulting in convective fluid pumping and enhanced tracer diffusion. Enzyme-powered pumps that transport fluid directionally can be fabricated by anchoring enzymes onto a solid support and supplying the substrate. Thus, enzyme pumps constitute a novel platform that combines sensing and microfluidic pumping into a single self-powered microdevice. Taken in its entirety, force generation by active enzymes has potential applications ranging from nanomachinery, nanoscale assembly, cargo transport, drug delivery, micro- and nanofluidics, and chemical/biochemical sensing. We also hypothesize that, *in vivo*, enzymes may be responsible for the stochastic motion of the cytoplasm, the organization of metabolons and signaling complexes, and the convective transport of fluid in cells. A detailed understanding of how enzymes convert chemical energy to directional mechanical force can lead us to the basic principles of fabrication, development, and monitoring of biological and biomimetic molecular machines.

INTRODUCTION

Through the conversion of chemical energy into mechanical force, biological motors in living systems are able to perform precise tasks both spatially and temporally.[1−3] Processive

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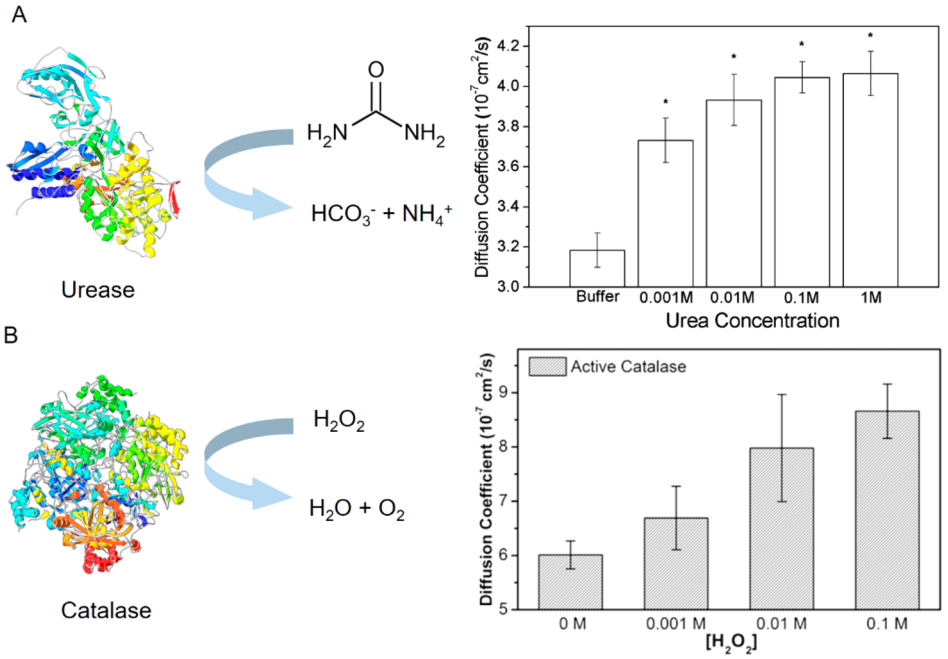

Figure 1. Diffusion of urease (A) and catalase (B) increases in a substrate concentration dependent manner when (A) urea and (B) hydrogen peroxide are converted to their respective products. Reproduced with permission from ref 6 and 7. Copyright 2010 and 2013 American Chemical Society.

motors like kinase and dynein transport cargo along microtubules, and nonprocessive motors like myosin II contract actin filaments.[4,5] However, force generation through chemical catalysis is not restricted to traditional motor proteins; a wide variety of free swimming enzymes have been shown to transduce chemical energy into mechanical force. Moreover, "back of the envelope" calculations suggest that the impulsive force generated per substrate turnover (~10 pN) for these enzymes is comparable to that observed for motor proteins, raising the intriguing possibility that motor proteins evolved from free swimming enzymes and nature put them on "tracks" to allow directed motion in the face of Brownian randomization.

In this Account, we summarize the evidence for catalysis-induced force generation by free swimming enzymes, discuss possible applications arising from this phenomenon, and speculate on the role of enzymatic force in biology.

■ ENHANCED ENZYME DIFFUSION

We and others have shown that the diffusion of active free swimming enzymes increases during substrate turnover. The increase is related to the reaction rate and follows the classic Michaelis–Menten behavior (Figure 1.).[6−10] The enhancement in diffusion disappears when the enzyme is inactivated or when the substrate is consumed. Typically, the enzymes are fluorescently labeled, and their diffusion coefficients are measured using fluorescence correlation spectroscopy (FCS).

Using Langevin/Brownian dynamics simulations, we determined that 12 pN and 9 pN of impulsive force per turnover were sufficient to cause the enhancement in diffusion of urease and catalase, respectively.[6,11] The calculations are based on the known turnover rate and a 10 ns impulse time per reaction, approximating the time it would take for the ions to diffuse one Debye length from the enzyme, thereby dissipating the force. The force from these enzymes is comparable to the force produced by motor proteins and are within the range to activate integrins, biological adhesion molecules responsible for mechanosensation by cells, making force generation by catalysis a potentially novel mechanobiology-relevant event.[1,11−13]

Possible mechanisms behind catalysis-induced enhanced diffusion of enzymes remain an open question, although several hypotheses, described below, have been put forward to account for the experimental observations, including phoresis, local and global thermal effects, and reaction-induced conformational changes in enzymes.

Phoresis

For an enzyme like urease, which converts neutral urea to ionic products (ammonium and bicarbonate ions), a local electric field can form due to the difference in diffusivities of the cation and anion. The self-generated electric field can propel the negatively charged urease. However, catalase, which disproportionates hydrogen peroxide into water and oxygen, also shows enhanced diffusion during catalysis, suggesting that the electrophoretic force cannot be the universal reason for enhanced enzymatic diffusion. Golestanian has suggested that for reactions involving neutral molecules, the diffusion of enzymes may also increase due to asymmetric distribution of reactant and product molecules, which can lead to chemical gradients near the enzyme.[14] This results in diffusiophoretic motion of the enzyme, the direction of which depends on the gradient and the strength of the interaction of the enzyme with the reactant and product molecules. However, the radii of most enzymes are in the range of a few nanometers, meaning that the rotational diffusion times of enzymes are on the order of 10^{-8} s. Thus, the rapidly rotating active site is unlikely to generate a substrate/product gradient in a specific direction. Even if a concentration gradient is formed, it is not expected that a small enzyme molecule will sense the gradient before Brownian randomization rotates it.

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Local and Global Thermal Effects

Bustamante et al.[10] observed reaction-induced enhanced diffusion of exothermic enzymes with reaction enthalpies ranging from −45 to −100 kJ/mol (catalase, urease, and alkaline phosphatase), but not for the thermoneutral triose phosphate isomerase (ΔH = −3 kJ/mol).[15] Based on the above observations, they proposed that the rapidly released thermal energy from the exothermic reaction formed asymmetric pressure waves resulting in enhanced enzyme motility (chemoacoustic effect). However, in this theory they neglect the rapid heat transfer to the surrounding solvent, and Golestanian[16] has argued that this effect should lead to an increase in the bath temperature and a decrease in the viscosity of the solvent, leading to an increase in enzyme diffusivity. When measured experimentally, however, only a 0.2 K increase in temperature was measured for a reaction involving 1 μM urease and 1 M urea in buffer,[17] suggesting that thermal effects do not play an important role in the observed enhanced enzyme diffusivity. In support of this, we also observed a significant reaction-rate-dependent increase in the diffusion of fructose-bisphosphate aldolase,[18] which catalyzes an *endothermic* aldol splitting reaction (ΔH = 30−60 kJ/mol).[19,20]

Conformational Changes

Hydrodynamic force, resulting from conformation changes during catalysis, is another potential reason for enhanced enzyme diffusion. Kapral, Mikhailov, and others[21−23] have proposed that enzyme motion is caused by fluid flows that result from enzymes reversibly deforming during substrate binding and product release at the active site during the catalytic cycle.

In the above scenario, it is assumed that catalysis is essential for the observed enhancement in enzyme diffusion. However, Minteer and we[18,24] have observed that reversible binding and unbinding of substrate or competitive inhibitor (a species that competes with substrate binding at the active site and induces enzyme shape change without undergoing a catalytic reaction) can also lead to diffusion enhancement. This suggests that conformational changes alone are the reason for diffusion enhancement, and actual catalytic turnover may not be necessary. Based on these observations, a new model was recently proposed that uses binding-induced conformational changes to explain the motion.[18] In the presence of substrate, the enzyme stochastically switches between two equilibrium states, either free or bound to the substrate. The relative enhancement of diffusivity can be written as

$$\Delta D/D_0 = \mathcal{A}S/(S + K)$$

where $\mathcal{A}$ is a dimensionless coefficient that depends on the internal degrees of freedom of the enzyme that are affected by binding and unbinding, S is the concentration of binding molecule, and K is the binding equilibrium constant. In this model, the diffusion coefficient of the enzyme is related to conformational fluctuations in a substrate concentration-dependent manner and is independent of the overall catalytic turnover rate.

■ ENZYME CHEMOTAXIS

Given that an enzyme through substrate binding and unbinding can generate sufficient mechanical energy to cause its own movement, the question that arises is whether it can move directionally in the presence of its substrate concentration gradient, paralleling cellular chemotaxis. Yu et al. reported that a DNA template associated with RNA polymerase in solution displayed biased movement upon encountering a gradient of its substrate NTP.[25] We have employed microfluidic devices to examine the directional motility of free swimming enzymes in imposed substrate gradients. A two-inlet one-outlet microfluidic channel was initially used, and fluorescently labeled enzyme was flowed through one inlet and substrate solution through another. A distinct chemotactic shift of the enzyme toward the substrate was observed.[7] To further underscore the nonequilibrium behavior, a different microfluidic channel setup with three inlets and one outlet was designed. A mixture of hexokinase (which phosphorylates D-glucose using ATP), D-glucose, and Mg^{2+} was flowed in through all three channels while ATP was added to the fluid in the middle channel. In response to the presence of ATP, hexokinase was found to move into the central channel from the side channels resulting in enzyme focusing (Figure 2).[26] The phenomenon was not observed when D-glucose was replaced with the nonsubstrate, L-glucose.

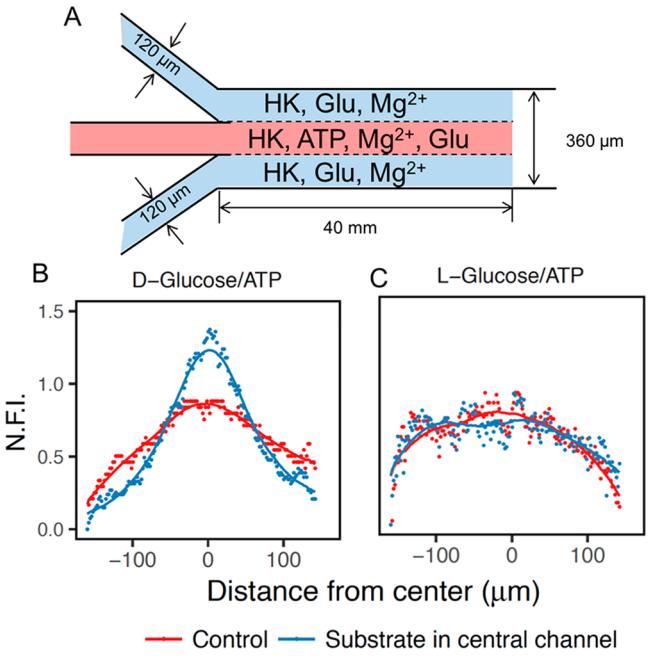

Figure 2. (A) Microfluidic device is a three-inlet one-outlet channel. (B) Hexokinase, D-glucose, and cofactor Mg^{2+} were passed through all three channels. When ATP was added to the middle channel, molecules of hexokinase focus toward the central channel. *Y*-axis represents the normalized fluorescence intensity across the channel. (C) The focusing phenomenon was not observed when D-glucose was replaced with L-glucose. Reproduced with permission from ref 26. Copyright 2018 Nature Publishing Group.

Practical applications for the chemotactic behavior of enzymes have been reported. One particular study used chemotaxis to separate a mixture of active and inactive enzymes.[27] A two-inlet five-outlet microfluidic channel was used for the experiment. Fluid containing a mixture of fluorescently labeled active and inactive enzymes was passed through one inlet while a solution containing the enzyme substrates was passed through the other inlet. Active enzymes migrated toward the channel containing substrate more than inactive enzymes, which allowed for simple separation of the inactive and active enzymes. A paper-based device for

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chemotactic separation of enzymes has also been reported, allowing for a more facile separation.[28]

Additionally, sequential chemotaxis of reaction-linked enzymes may lead to the formation of metabolons in cells. Enzymes that participate in reaction cascades have been shown to assemble into metabolons in the presence of the first enzyme's substrate.[23] We applied microfluidic and fluorescence spectroscopy techniques to study the coordinated movement of the first four enzymes of the glycolysis cascade: hexokinase, phosphoglucose isomerase, phosphofructokinase, and aldolase.[26] We found that each enzyme independently follows its own specific substrate gradient, which is produced by the preceding enzymatic reaction. The extent of enzyme migration is proportional to the time the enzyme is exposed to the substrate gradient. Significantly, the chemotactic migration of enzymes is fairly rapid even under conditions that mimic cytosolic crowding. The observed rate was very similar to the reported rate of enzyme diffusion in living cells.[29] Thus, chemotaxis may be a basis for the organization of metabolic networks in the cytosol of the cell.

Interestingly, while studying the chemotaxis of enzymes participating in cascades, we also observed that enzymes "flock" in response to the presence of substrate, an example of collective behavior at the molecular scale.[26] We found that when hexokinase and aldolase were exposed to the substrates for hexokinase, as well as the other two enzymes in the glycolysis cascade, aggregates of the two enzymes formed over time and the trajectories of the hexokinase and aldolase aggregates were found to be highly correlated.

A theory for chemotaxis was first proposed by Schurr et al.[30] It explains the thermodynamic tendency of probe macromolecules to climb up the concentration gradient of binding ligands in solution. Later, Guha et al. used a slightly modified version of this expression to explain the chemotaxis of small dye molecules toward high concentrations of interacting polymers in solution.[31] In another theoretical work followed by experimental verification, Sitt et al. predicted that when there is a gradient of binding sites on a surface, the probe molecule will move toward the region with a higher density of binding sites.[32,33] Essentially, binding was identified as the thermodynamic origin of molecular chemotaxis in those systems.

Using a modification of Schurr's theory, we proposed a model to describe the chemotactic behavior of active enzymes, based on reversible binding of the substrate.[26] According to this model, chemotactic drift arises from a thermodynamic driving force that lowers the chemical potential of the system due to favorable ligand binding. The substrate gradient-induced enzyme chemotaxis by cross-diffusion is in the opposite direction of enzyme Fickian diffusion, transferring enzymes toward regions of *higher* substrate concentration. The diffusive flow for the concentration c_e of enzyme, e, in the presence of its substrate, s, can be written as

$$J_e = -D\nabla c_e - D_{XD}\nabla c_s$$

where D is the Brownian diffusion coefficient of enzyme, D_{XD} is the "cross-diffusion" coefficient, and ∇c_e and ∇c_s are gradients in enzyme and substrate concentrations, respectively. The cross diffusion coefficient, D_{XD}, is a function of the local substrate concentration, c_s, the diffusion coefficient, D, and the equilibrium constant, K, for substrate binding to the enzyme:

$$D_{XD} = -Dc_e\frac{K}{1 + Kc_s}$$

Combining these two equations gives

$$J_e = -D\left(\nabla c_e - c_e\frac{K}{1 + Kc_s}\nabla c_s\right)$$

This equation highlights the factors that drive cross diffusion flux. The first term inside the parentheses is the traditional diffusive flux toward lower concentrations of enzyme. The second term is the chemotactic flux of the enzyme, which has the opposite sign, showing that this flux is toward higher concentrations of substrate. In addition to the substrate gradient, this term's magnitude is determined by the diffusion coefficient D, the enzyme concentration c_e, and a factor proportional to the fraction of binding sites occupied by substrate at a given time.

Contrary to previous findings, Jee et al. have recently reported that urease and acetylcholinesterase move away from their respective substrates, termed as antichemotaxis.[34] On the theoretical side, the possibility of enzyme antichemotaxis has been hypothesized.[35,36] Agudo et al. suggest that enzyme chemotaxis is the net result of two competing phenomena, phoretic motion due to "nonspecific" interactions resulting in chemotaxis[37] and enhanced diffusion due to enzyme−substrate binding, resulting in antichemotaxis. However, experimentally it has been observed that when there is no binding, there is no chemotaxis, although "nonspecific" interactions may still exist (e.g., hexokinase chemotaxes toward D-glucose but not L-glucose).[26]

In contrast, our proposed model involving binding-induced cross-diffusion is general and has no adjustable parameters.[26,38] Moreover, changes in enzyme diffusion affect the magnitude but *not* the direction of the cross-diffusion flux and predict the occurrence of chemotaxis for favorable binding interaction between any two molecular species.[38]

■ ENERGY TRANSFER TO THE SURROUNDINGS

There have been several reports that both living and synthetic micrometer-sized active particles (e.g., bacteria,[39,40] algae,[41,42] and bimetallic rods[43,44]) transfer their momentum to the surroundings, resulting in enhanced diffusion of suspended tracers. Interestingly, these motors all seem to transfer energy to the surroundings in a similar manner with tracer diffusion increasing with increasing number density and speed of the active particles, despite the differences in their propulsive mechanisms. We wanted to probe this further by examining the phenomenon at the molecular scale in the ultralow Reynolds number regime. Urease and aldolase were used in the study, and it was determined that, in their presence, passive tracers with sizes ranging from nanometers to micrometers showed enhanced diffusion in an activity dependent manner.[45] Furthermore, when the catalytic reaction rate was held constant, tracer diffusion enhancement was found to be inversely correlated to the size of the tracer particles. Based on our observations, we hypothesize that enzymes play a crucial role in the dynamic environment of the cytoplasm, as the impulsive force generated by free enzymes in the cytoplasm during catalysis might be a source of cytoplasmic stochastic motion[46] and glass−liquid transitions in bacterial physiology.[47] It is important to note that these phenomena persist, even if the traditional motor proteins are not active. Proteins must be

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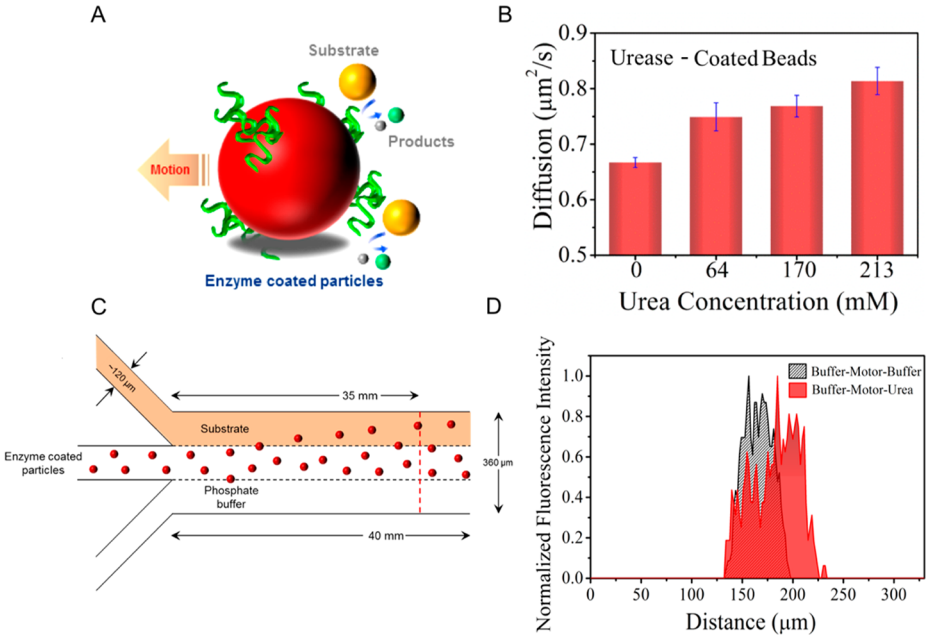

Figure 3. (A) Motion of a polystyrene bead (red sphere) conjugated with enzymes (green spirals) is induced when substrate is converted to product. (B) Diffusion of urease coated 0.8 μm polymer beads when exposed to various concentrations of urea, demonstrating that diffusion of the beads increases in a substrate concentration manner. (C) Microfluidic channel employed to study directional migration of enzyme-coated beads induced by enzyme catalysis. (D) Urease conjugated to 2 μm polystyrene beads chemotax up the substrate gradient in a three-inlet one-outlet channel.[17] Reproduced with permission from ref 17. Copyright 2015 American Chemical Society.

able to function in different organelles, making translocation within the matrix of the cytoplasm a crucial requirement.[48] The transfer of momentum from active free swimming enzymes also suggests that membrane-bound enzymes may exert forces on cell membranes, amplifying mechanically induced signaling mechanisms.[49]

APPLICATIONS OF ENZYME-GENERATED FORCE

Enzyme-Powered Motors

Because of their ability to generate force during catalysis, enzymes can act as "engines" for inactive particles and propel them.[50,51] For example, Dey et al. attached enzymes to micrometer-sized polystyrene beads via a biotin−streptavidin linkage and then measured their diffusion when exposed to different substrate concentrations using optical microscopy, dynamic light scattering (DLS), and microfluidic experiments (Figure 3.).[17] They determined that the enzyme-powered particles also experienced enhanced diffusion in the presence of substrate and chemotaxed directionally in a substrate gradient. This was significant, as it demonstrated that enzyme ensembles are powerful enough to move particles that are orders of magnitude larger than the enzymes themselves. Furthermore, by employing stochastically optical reconstruction microscopy (STORM) to quantify the amount of enzyme conjugated on the surface of a micromotor, Patiño et al. demonstrated that apart from the asymmetric distribution, the propulsion of the particle is also determined by the number of attached enzyme molecules.[52]

Another study by Ma et al.[53] demonstrated that enzymes can be conjugated to more complex particles with different functionalities. The authors bound urease to the silica side of a hollow Janus nanoparticle and incorporated an "on/off" switch. When inhibitors, such as Ag^{+} or Hg^{2+}, were added to the system, the velocity of the particle was significantly reduced or stopped. The motion could be recovered, however, after the addition of a thiol (DTT) that scavenges the inhibitor. This is illustrated in Figure 4.[53]

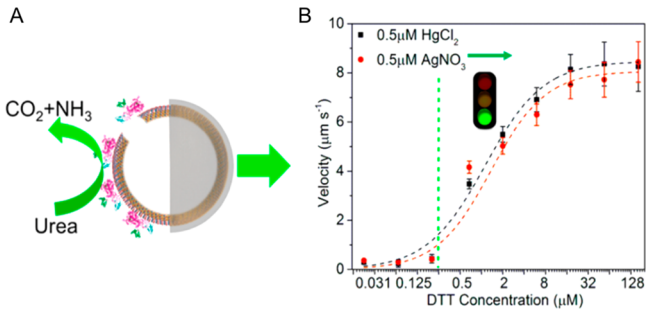

Figure 4. (A) Urease functionalized Janus hollow microsphere that is self-propelled by the decomposition of urea to its products. (B) At low concentrations of DTT and in the presence of 0.5 μM inhibitor, there is little to no motion of the urease powered microspheres. However, motion is recovered with increasing concentrations of the DTT.[53] Reproduced with permission from ref 53. Copyright 2016 American Chemical Society.

In a recent *in vivo* study, Hortelão et al. studied urease-powered doxorubicin (an anticancer drug) loaded core−shell silica spheres, termed nanobots, inside cancer cells.[54] Here, the urease catalyzed decomposition of urea serves multiple purposes, the first being the nanobot's ability to propel itself and the second being the faster release rates of doxorubicin from the bot (due to the urease-driven fluid flow through the porous particles).

Enzyme Pumps

When immobilized onto a surface, enzymes are able to transfer the reaction-generated force to the surrounding fluid and induce movement of the fluid and particles that are suspended

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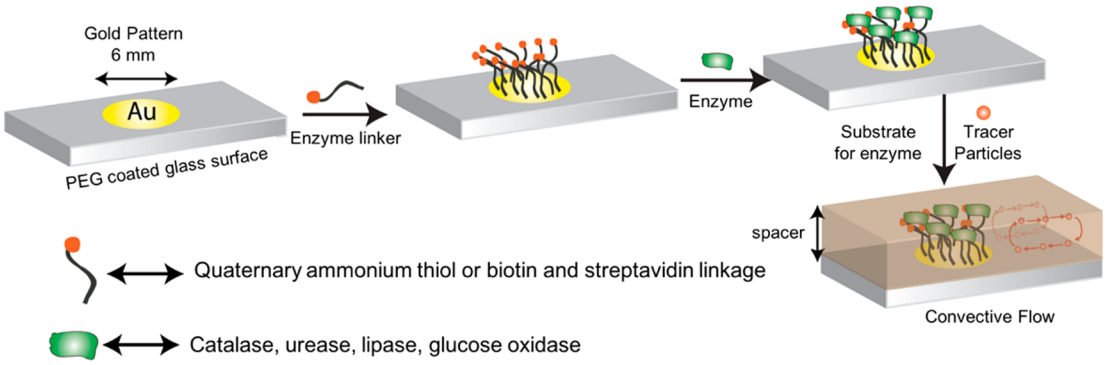

Figure 5. Experimental setup for the enzyme powered micropumps. Glass slides were coated with PEG, and gold was patterned on them. The gold patch was functionalized with a linker, and enzyme was attached to the linker via electrostatic attractions or through a biotin−streptavidin linkage. When the enzyme's substrate is added, convective flow is observed in a sealed hybridization chamber.[55−58] Adapted with permission from ref 55. Copyright 2014 Nature Publishing Group.

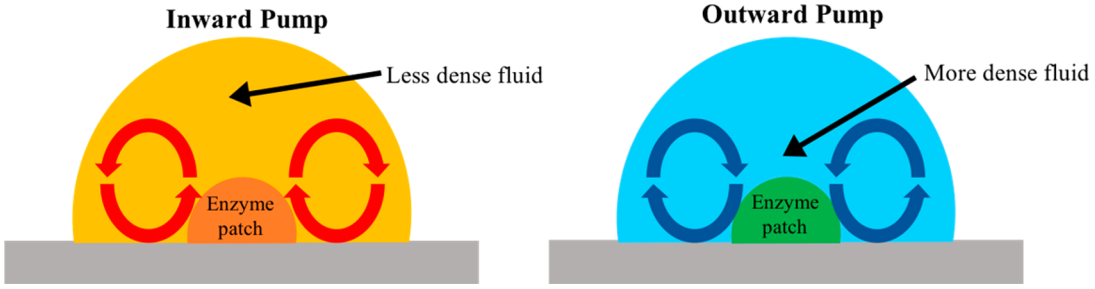

Figure 6. Demonstration of the solutal buoyancy mechanism. In the inward pump, the products are less dense than the reactants, so the local fluid rises and then due to convective flows, falls back down and moves toward the patch on the glass slide. In the outward pump, the enzyme products are denser than the reactants, causing the fluid to locally become denser and slide down and away from the patch.[56,57]

in the fluid.[55] Enzymes were attached to a gold patch via a thiol monolayer attachment[54] or biotin−streptavidin linkage (Figure 5).[56−58]

When the substrate solution was introduced into the closed system, pumping was induced and was found to be dependent on the substrate concentration. Additionally, flow direction could be changed depending on the enzyme that was used in the system. In all cases studied, when the pumps were inverted, the fluid flow directionality was reversed, confirming that the fluid flow is density driven and eliminating other possible mechanisms such as diffusiophoresis or osmophoresis. Since the enzymes employed catalyzed exothermic reactions, the role of thermal effects was investigated for phosphatase-based micropumps using a combination of theory and experiments. It was concluded that the heat produced from the enzymatic reaction was not sufficient to account for the experimental pumping speeds.[56] Rather, the predominant factor that governs the direction and speed of fluid pumping was the density differences between reactant and product molecules. When the products were denser than the reactants, the fluid settled and slid down and away from the micropump. Conversely, when the products were less dense than the reactants, the fluid would rise up and away from the pump (Figure 6).[56,57]

The enzyme pumps constitute a novel platform that combines sensing and microfluidic pumping into a self-powered microdevice. Several potential applications have been demonstrated. This includes release of insulin from a gel with anchored glucose oxidase at a rate that depends on the concentration of glucose in the ambient solution.[55] The dependence of the flow speed on reaction rate also allows the detection of substances that inhibit the enzymatic reaction. Using this principle, sensors for toxic substances, like mercury, cadmium, and cyanide, were designed using urease or catalase-powered pumps, with limits of detection well below the concentrations permitted by the Environmental Protection Agency (EPA).[58] Finally, enzyme pumps have been harnessed for the directional delivery of microparticles in micro-chambers.[59]

CONCLUSION

As Astumian comments, almost any enzyme can, in principle, function as a molecular machine,[60] and a detailed understanding of how enzymes convert chemical energy to mechanical force can lead us to the basic principles of fabrication, development, and monitoring of biological and biomimetic molecular machines.

As a search strategy, directional chemotactic transport of molecules is distinctly superior to simple diffusion in locating specific targets and is relevant to numerous binding events occurring in biological systems. In general, enzymatic reactions require favorable binding interactions between the catalyst and the substrate, and chemotaxis may play a hitherto unsuspected role in promoting these reactions. The sequential chemotaxis in catalyst cascades allows time-dependent self-assembly of specific catalyst particles participating in the cascade. This is an example of how information can arise from chemical gradients and it is tempting to suggest that similar mechanisms underlie the organization of living systems.

How do enzymatic forces affect cellular life?

It is clear that enzymes are able to (1) generate motive force during catalysis, (2) display directional movement in the

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presence of a substrate gradient, and (3) transport the surrounding fluid and entrained particles. Active free-swimming enzymes may be responsible for the stochastic motion of the cytoplasm, the organization of metabolons and signaling complexes, and the convective transport of fluid in cells.[11,46−49]

Technological Applications of Enzymatic Motors

There have been many examples of self-powered micro- and nanoparticles inspired by the well-organized molecular machines in living cells.[61−63] However, many of them require the use of an external field or use fuels that are toxic or require harsh conditions. The biocompatibility of enzymes and their substrates make them ideal for applications in drug delivery,[54] environmental sensing,[64] and biological assays.[65] There are multiple examples of enzymes being used as an engine for complex particles, such as enzyme functionalized polymersomes,[66] stomatocytes,[67] carbon nanotubes,[68] gold nanorods,[69,70] macroscale carbon fibers,[71] mesoporous nano- or microparticles,[72] and more.[63,73−76] Despite the success of these studies, there are still issues that need to be addressed, including the accurate control of motion directionality and the ability to move against fluid flow.

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Notes

The authors declare no competing financial interest.

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