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ABSTRACT

In this thesis, I explore and discuss the possibility of designing a nanomotor which converts the energy of an asymmetric enzymatic reaction to mechanical motion. This work is an important step in development of nanomotors which can be incorporated into biomedical research. The nanomotor described here has a spherical geometry with one side functionalized with an enzyme (urease or carbonic anhydrase) and the other side covered with a thin film of gold. Carbodiimide chemistry was used for enzyme coupling. Protein loading was determined by total nitrogen content. Also, the activity of the enzymes was measured before and after the coupling procedure. The activity of the enzymes significantly decreased upon immobilization. Then, the diffusion of enzyme-functionalized particles was studied in the presence and absence of substrate using light scattering microscopy. Based on the experimental results, the activity of the enzymes significantly decreased upon immobilization, and enhanced diffusion of enzyme-functionalized particles was not observed regardless of the presence of substrate. According to the literature, large particle size and low catalytic activity of immobilized enzymes could retard particle motion. I have proposed two strategies for improving motor design: decreasing particle size and improving catalytic activity of immobilized enzymes by introducing a linker between the enzyme and the particle.
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**Introduction**

**Locomotion on the microscale**

Design of molecular machines capable of propelling themselves by converting chemical energy into mechanical movement has been a popular topic in the past several years. The existing biomotors such as kinesin, dinein, ATPase, and motile bacteria have been a great inspiration for developing man-made nanomachines. The majority of biomotors utilize the energy of ATP hydrolysis to power their motion. For example, kinesin uses ATP hydrolysis for translational movement along a microtubule. The energy of such biochemical reactions has been an attractive possibility for powering the motion of synthetic nanomotors. These reactions offer a large variety of propulsion mechanisms, among them diffusiophoresis, interfacial tension gradients, and self-electrophoresis.

Motion of small-scale objects is limited by viscous forces (low Reynolds numbers) and the ubiquity of Brownian diffusion. Previously, our group reported the autonomous movement of platinum-gold (PtAu) rods in hydrogen peroxide solution. This system was able to overcome viscous forces via self-electrophoresis, which was powered by catalytic redox decomposition of hydrogen peroxide on both ends of the rod (Figure 1). The directionality to the motion was introduced either by using PtAu
nanorods with nickel segments in a magnetic field or by creating a non-uniform peroxide gradient. \(^4, 6\)

![Diagram of catalytic platinum-gold nanorod motor](image)

**Figure 1:** Self-electrophoresis of catalytic platinum-gold nanorod motor in the presence of hydrogen peroxide fuel.\(^6\)

Combining the ideas of the catalytic nanorod motor from our group with the self-diffusiophoretic spherical motor from Golestanian et al. (Figure 2) we decided to explore new catalyst-fuel systems which could expand application of nanomotors. \(^7\)

![Diagram of self-diffusiophoretic swimmer](image)

**Figure 2:** Self-diffusiophoretic swimmer.\(^7\)

Mano and Heller reported the first biomotor which used glucose as a fuel for locomotion. \(^8\) Although it is attractive for biomedical applications due to its fuel, glucose,
the major disadvantage of such a motor is its inability to function outside of the water-oxygen interface. All existing chemically driven catalytic motors, which can move fully immersed in liquid, are limited to using hydrogen peroxide and/or hydrazine derivatives for fuel\(^2\) and are affected by their local environment (ionic strength of aqueous solutions and viscosity). In order to incorporate synthetic nanomotors into biomedical research (i.e. drug delivery and bioimaging), several factors should be considered. Among them are the high viscosity and ionic strength of body fluids, fuel availability, target specificity, and cytotoxicity.\(^9,10,11\) In order to address the aforementioned concerns, we decided to design a new motor which will combine advantages of existing inorganic and biological motors.

Enzymes could be good candidates for powering particle propulsion because they are natural catalysts with a wide variety of substrates. The energy released during the enzymatic reaction could drive the self-propulsion of enzyme-functionalized particles via the dynamic and asymmetric distribution of the products in the particle vicinity.\(^7\) In addition to high catalytic turnover and a wide selection of substrates, enzymes are essential components of all living cells and, therefore, enzyme-functionalized particles could be biocompatible.\(^11\) Thus, enzyme-based nanomotors could become good alternatives to the existing inorganic ones.

Since catalytic turnover is an essential part of particle movement, the choice of the catalyst is very important for our experiment. The major criteria for choosing the enzymes were high \(k_{cat}/K_m\) value, single-site turnover of reactants to products, type of enzymatic reaction (i.e. asymmetric, ionic products), stability during and after immobilization, and the commercial availability of the enzymes and their respective substrates and inhibitors. The ratio of \(k_{cat}\) to \(K_m\) is important for comparing enzymes
because it displays affinity ($K_m$) and catalytic ability ($k_{cat}$) of an enzyme. If $k_{cat}/K_m$ is in the range of $10^8$ to $10^9$ M$^{-1}$s$^{-1}$, the enzyme is called catalytically perfect or diffusion controlled.\textsuperscript{12} For our study we chose one catalytically perfect enzyme, bovine carbonic anhydrase (BCA), and another near catalytically perfect, urease, in order to explore the influence of enzyme catalytic activity on motility. Other important characteristics of chosen enzymes are shown below.

**Urease from Canavalia ensiformis (jack bean)**

Urease catalyzes hydrolytic decomposition of urea by the following reaction:

$$\text{CO(NH}_2\text{)}_2 + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{NH}_3$$  \textbf{Equation 1}

This enzyme is widely distributed among many bacteria, algae, fungi, and number of higher plants.

All ureases are metalloenzymes which contain two nickel ions per subunit. The native state of jack bean urease is homohexamer (Figure 3), but it exists as monomers, trimers, and hexamers of identical 91 kDa subunits.\textsuperscript{13}
Urease is known to be a fairly robust enzyme. Thus, it retains catalytic activity in the form of monomer, trimer, and the native hexamer state.\textsuperscript{14} The catalytic properties of urease have been thoroughly investigated as well as the mechanism of catalysis (Figure 4).
Figure 4: Schematic representation of the urea hydrolysis mechanism proposed to occur in the urease from *Klebsiella aerogenes* active site.\textsuperscript{15}

Some important catalytic parameters for free urease are its $K_m$ of 3.28 mM and its $k_{cat}$ of $2.2 \times 10^4$ s$^{-1}$ (at 38 °C and pH = 7).\textsuperscript{16} Major inhibitors are 2-mercaptoethanol, acetohydroxamate, phosphoramidate, fluoride ion, 1,4-benzoquinone,\textsuperscript{17} $N$-ethylmaleimide,\textsuperscript{18} and pyrocatechol.\textsuperscript{19}
Carbonic anhydrase from Bos taurus

CA is a widely distributed zinc metalloenzyme whose physiological role is to catalyze the reversible hydration of CO₂.

\[
\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+
\]  

**Equation 2**

Mammalian CA is a monomeric protein with molecular weight of 30 kDa and one zinc cation per molecule (Figure 5).²⁰

Figure 5: Crystal structure of bovine carbonic anhydrase II.²⁰

Among seven known isozymes, CA II is the fastest one, based on its kinetic parameters such as its \(K_m = 15 \text{ mM (pH range 5.9–10.2)}\) and its \(k_{cat} = 8.3 \times 10^5 \text{ s}^{-1} \) (pH range 6.85–10.2, at 25 °C).²¹ Sulfonamides and sulfamates are two major classes of carbonic anhydrase inhibitors.²² The mechanism of carbon dioxide hydration is presented in Figure 6.
Figure 6: Mechanism of carbonic anhydrase. The zinc-bound hydroxide mechanism for the hydration of carbon dioxide catalyzed by carbonic anhydrase.²³

**Biomotor design**

In this research we designed the biomotor (Figure 7) and studied its motion in the presence and absence of fuel (substrate). As shown in Figure 7 our biomotor consists of a polystyrene sphere fully or partially covered with urease or carbonic anhydrase. We chose a spherical shape over a rod-like one in order to avoid velocity reduction as a result of the aspect ratio and also to find out the importance of asymmetry for powered motion.²⁴
Figure 7: Design of a biomotor.

We proposed that the catalytic power of immobilized enzymes would help our biomotors to overcome viscous forces and that introduced asymmetry (Janus particle) would bring directionality to their movement. In order to characterize motion of enzyme-functionalized particles, we measured their diffusion coefficients in buffer with substrate, in buffer, in deionized (DI) water, and with substrate in buffer containing inhibitor. We expected to observe enhanced diffusion in the substrate solution whereas water, buffer, and substrate in the presence of inhibitor would serve as three negative controls in our experiments. N-ethylmaleimide was chosen for urease inhibition because it covalently modifies cysteine residues on the flexible flap covering the active site of the enzyme and causes irreversible loss of enzymatic activity. An acetazolamide was employed as a reversible inhibitor of BCA due to its noncompetitive kinetics which alters the enzymatic reaction rate ($V_{max}$) but not the affinity to the substrate.
Experimental

Materials and methods

Carbonic anhydrase II (EC 4.2.1.1) from bovine erythrocytes, urea, acetazolamide, HEPES, and N-(3-dimethyl-aminopropyl)-N′-ethylcarbodiimide (EDC) were purchased from Sigma-Aldrich. Urease (EC 3.5.1.5) from Canavalia ensiformis (jack bean), N-ethylmaleimide, and N-hydroxysuccinimide (NHS) were purchased from Fluka. Purity of the purchased enzymes was confirmed in the lab by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Uniform carboxyl modified polystyrene microspheres, 0.51 µm diameter, were obtained from Bangs Laboratories, Inc. and 0.5 µm diameter spheres were obtained from Polysciences, Inc. The size of the purchased microspheres was verified by field-emission scanning electron microscopy (FESEM).

All chemicals were of reagent grade and used without further purification. Nanopure DI water was used for all experiments and preparations of solutions. All measurements were made at room temperature (25 °C) unless mentioned otherwise. The UV-Visible measurements were performed using an Agilent 8453A diode-array UV-visible spectrophotometer with ChemStation UV-vis software.
Crystal structures of enzymes were visualized using the protein data bank (pdb) files, 2UBP (urease) and 1V9E (BCA II), in the UCSF Chimera molecular modeling program.\textsuperscript{26}

**Preparation of gold-polystyrene Janus particles**

A monolayer of particles was prepared according to procedure by Goldenberg et al.\textsuperscript{27} The glass slides were placed on the bottom of a Petri dish. A suspension of microspheres in ethanol (5% w/w) was introduced to a water-hexan-es interface in the dish. After the monolayer was formed the glass slide was lifted up through the monolayer with tweezers. The microsphere-covered glass slides were left at room temperature to dry overnight. Following the drying, a thin film of gold (99.99\% – Kurt J.Lesker) was evaporated onto the above-mentioned glass slides. The spheres were partially coated, and the thickness of the gold film was 12 nm. The obtained Janus particles were removed from the glass slide by gentle sonication. The particles were washed in DI water several times before further use for immobilization. FESEM was used to confirm the synthesis of Janus particles.

**Immobilization of enzymes on the carboxyl modified microspheres**

Urease and carbonic anhydrase were immobilized on the carboxyl modified microspheres by covalent zero-length crosslinking. The coupling procedure was slightly modified by addition of NHS in the equal amount to EDC.\textsuperscript{28} The schematic
representation of the coupling is illustrated in Figure 8. Urease- and BCA-functionalized particles were stored at 4 °C in 10 mM HEPES and 50 mM HEPES respectively.

Figure 8: Scheme of zero-length EDC/NHS coupling of an enzyme to the carboxyl-modified polystyrene microspheres. • is the polystyrene microsphere.$^{29}$

The amount of immobilized protein was determined by measuring the total nitrogen content of the particle suspension after digestion with perchloric acid.$^{30}$ This method directly measures the amount of the protein bound to the spheres. The sample (5–10 µL particle suspension) was digested by the perchloric acid at 210 °C. Following the digestion, the sample was cooled to the room temperature and was allowed to react with phenol reagent (1.7 g of phenol, 0.1 g sodium nitroprusside, 80 mL DI water) and alkaline hypochlorite (2.8 mL of sodium hypochlorite, 97.2 mL 2.5 M NaOH). The absorbance of the final solution was measured at 578 nm.
Determination of enzymatic activity

Urease activity was assayed using the indophenol blue method. Five to eight microliters of urease-functionalized microspheres were added to 1 mL of 100 mM urea solution, and the mixture was incubated for 30 minutes at room temperature with constant mixing. A twenty microliter aliquot was withdrawn from the reaction mixture and combined with 1 mL solution of each reagent A (1 g phenol, 5 mg sodium nitroprusside per 100 mL of solution) and reagent B (0.5 g of sodium hydroxide, 0.84 mL of sodium hypochlorite per 100 mL of solution). The resulting mixture was further incubated at room temperature for 30 minutes. The absorbance of the final solution was measured at 625 nm, and the urease activity was calculated from the production rate of NH₃ over a period of 30 minutes:

$$U = \frac{N_t}{t \cdot m_{enz}}$$

where $U$ is a unit of enzyme activity; $N_t$ is number of micromoles of NH₃ produced in 30 min; $t$ is the time of enzymatic reaction (30 min); $m_{enz}$ is the mass of immobilized enzyme in mg.

The activity of CA was measured using a kinetic assay of CO₂ hydration. The end point of this assay was determined by the color change of the pH-sensitive dye bromthymol blue, and the assay was carried out at room temperature. A saturated solution of CO₂ in deionized water was prepared by bubbling CO₂ through the water for 30 minutes with cooling from an ice bath. The saturated CO₂ solution was left at room temperature for at least 20 minutes before use in the assay. The concentration of CO₂ in solution was determined by adding an excess of standardized 0.04 M Ba(OH)₂ and back
titrating the resulting solution against standardized 0.01 M HCl. Ten microliters of CA-functionalized particles (or water in case of the blank) were added to 0.5 mL of bromothymol blue buffered solution (0.08 M in 50 mM HEPES, pH 7.8). The reaction was initiated by addition of 0.5 mL of saturated CO₂ solution to the above mixture. The time for the color change from blue (pH~7.8) to yellow (pH~5.8) was monitored. The activity of CA was calculated using following formula

$$U = \frac{(t_{\text{blank}} - t_{\text{enz}})}{t_{\text{enz}} \cdot m_{\text{enz}}}$$

Equation 4

where $U$ is a unit of enzyme activity; $t_{\text{blank}}$ is the time of the uncatalyzed reaction and $t_{\text{enz}}$ is the time of an enzyme-catalyzed reaction; $m_{\text{enz}}$ is the mass of immobilized enzyme in mg.

**Light scattering microscopy: Nanosight LM 10**³²

Diffusion coefficients of particles were measured using a Nanosight LM 10 nanoparticle tracking instrument with Nanoparticle Tracking Analysis (NTA) 2.0 software.³³ The Nanosight LM 10 (Figure 9) was used to collect videos of populations of moving nanoparticles in a liquid when illuminated by laser light.
Flare spot where the laser beam emerges

Particles seen most clearly here

Particle visualization zone

Microscope with LM 10 unit

Figure 9: Nanosight LM 10.
The particles which pass through the laser beam path are seen by the instrument as a small points of light which undergo Brownian motion (Figure 10A).

Figure 10: NTA video analysis: (A) CCD camera capture; (B) NTA analysis screen.

The videos are further analyzed by NTA software (Figure 10B) which identifies and tracks each particle independently on the frame-by-frame basis. The software also calculates and subtracts a bulk flow in the system. The mean square displacement is calculated for each particle for as long as it is visible (i.e. if the particle blinks the
software will consider the same particle as two separate ones). All analysis data are exported to Excel files which could be used for further calculations.

**Diffusion coefficient measurements**

At least 5 videos of 30 seconds in length were recorded for each solution of interest in order to obtain statistically significant populations of particles. Because the normality of the data was confirmed by the Shapiro-Wilk test (0.05 confidence level), all experimental results were further treated as a normal distribution. In order to eliminate multiple contributions of the same particle to the analysis, the particles were discriminated based on the path length (i.e. mean length plus two standard deviations). The diffusion data were combined from each of the aforementioned videos and represented the results of *one experiment* for one solution of interest. Diffusion of functionalized particles was measured in the following solutions: DI water, buffer, buffer with substrate, and substrate in the buffer containing inhibitor (Table 1). Prior to the measurements, the enzyme-functionalized particles were pre-incubated with inhibitor for 30 minutes.

Table 1: Experimental conditions for measuring diffusion coefficients.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate*</th>
<th>Buffer</th>
<th>Inhibitor†</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCA</td>
<td>28.28 (41.33) mM CO₂</td>
<td>25 mM HEPES (pH=7.8)</td>
<td>0.5 mM acetazolamide</td>
</tr>
<tr>
<td>Urease</td>
<td>100 mM urea</td>
<td>10 mM HEPES</td>
<td>5 mM N-ethylmaleimide</td>
</tr>
</tbody>
</table>

*Substrate concentration for BCA functionalized Janus particles is shown in the parenthesis
†Substrate concentration for urease functionalized particles was 100 mM urea; for BCA functionalized uniform particles it was 29.40 mM CO₂ and 41.33 mM for Janus ones
The diffusion coefficients were measured in triplicate for each solution of interest (i.e. 15 total 30-second movies for each sample: substrate in buffer, buffer, water, and substrate in buffer containing inhibitor) in order to assess reproducibility.

**Results and Discussion**

**Preparation of Janus particles**

The synthesis of Janus particles was confirmed by FESEM. Figure 11 compares FESEM images of Janus and uniform particles, which were later used for enzyme immobilization and diffusion studies. The “cap-wearing” particles in Figure 11B are the prepared gold-polystyrene Janus particles. Due to the thin film of gold on the Janus particles, the overall spherical symmetry was preserved, while the coupled enzyme was asymmetrically distributed because the immobilization procedure (EDC coupling) requires a carboxyl modified surface.

![FESEM images of Janus particles](image)

**Figure 11:** FESEM images of (A) uniform polystyrene carboxylated particles; (B) Janus gold-polystyrene carboxylated particles.
According to the FESEM results the average size of the purchased particles was 400 nm in diameter.

**Immobilization and characterization of enzymes**

Carbodiimide chemistry is widely used for immobilization of proteins and enzymes on polymer supports. In this procedure, proteins usually couple through the N-terminal amine or primary amine of a lysine side chain. Therefore, coupling should be efficient if there are lysine residues on the protein surface. The molecular visualization software confirmed that both enzymes have available lysine residues on their surface, and none of the enzymes have lysine in the catalytic site. Thus, we were able to use carbodiimide chemistry for enzyme immobilization.

The major disadvantage of using EDC alone is its fast hydrolysis in aqueous solutions. This problem was solved by addition of NHS which extends the half-life of the active intermediate (Figure 8). Another important factor for consideration was the selection of a buffer solution in order to avoid side reactions, which can have a significant impact on the yield of the protein immobilization. The nonspecific binding of enzymes to the gold surface was minimized by performing coupling for only 2 h followed by extensive washing, centrifugation and sonication. In addition, we ran the control experiment with a gold-covered glass slide to demonstrate a lack of the protein binding to the gold surface under our coupling conditions.

Following immobilization, the amount of the protein coupled to the microspheres was determined by total nitrogen content. The total nitrogen method was chosen because
it directly measures the amount of the protein bound to the spheres. Also protein absorbance at 280 nm (absorbance of amino acids with aromatic rings) was not used for quantification because the crosslinking reagents (i.e. NHS and EDC) and products of their hydrolysis strongly absorb at 260–280 nm.\textsuperscript{36} The amount of enzyme bound to the microspheres is shown in Table 2.

Table 2: Quantification of a protein load.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Uniform particles, mg protein/mL particle suspension</th>
<th>Janus particles, mg protein/mL particle suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCA</td>
<td>0.6 ± 0.1</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>Urease</td>
<td>0.36 ± 0.01</td>
<td>0.05 ± 0.01</td>
</tr>
</tbody>
</table>

In addition to the enzyme load quantification, the catalytic activity of the enzymes after immobilization was determined. Since the catalytic power is essential for further experiments, the activities of enzyme-functionalized particles and free enzymes were determined, and the results are presented in the Table 3.

Table 3: Enzymatic activity.*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Free enzyme, units/mg</th>
<th>Uniform particles, units/mg</th>
<th>Janus particles, units/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCA</td>
<td>12000 ± 3000</td>
<td>23 ± 8</td>
<td>31 ± 8</td>
</tr>
<tr>
<td>Urease</td>
<td>130 ± 8</td>
<td>0.13 ± 0.08</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

* Units of activity were defined in the experimental section. Final substrate concentration was 25 mM carbon dioxide for BCA and 100 mM urea for urease

The data in Table 3 show that the catalytic activity was reduced dramatically after immobilization, possibly due to distortions in enzyme structure since the employed coupling procedure lacks directionality and specificity in orientation of the catalytic site.
Diffusion studies

In order to fully evaluate the experimental data, theoretical diffusion coefficients were calculated using the following equations:

\[ D_{\text{translational}} = \frac{k_B T}{6 \pi \eta r} \quad \text{Equation 5} \]
\[ D_{\text{rotational}} = \frac{k_B T}{8 \pi \eta r^3} \quad \text{Equation 6} \]

where \( r \) is the hydrodynamic radius of a particle; \( D \) is a diffusion coefficient; \( T \) is temperature; \( \eta \) is the viscosity of solution, and \( k_B \) is the Boltzmann constant. Equations 5 and 6 assume that a particle has a spherical shape. For a 400 nm (diameter) particle \( D_{\text{translational}} \) equals 1.23 \( \mu \text{m}^2\text{s}^{-1} \) and \( D_{\text{rotational}} \) is 23 \text{s}^{-1}.

Averages of diffusion coefficients from experimental measurements are illustrated in Figures 12 and 13.
Figure 12: Diffusion of enzyme-functionalized uniform particles.

Figure 13: Diffusion of enzyme-functionalized Janus particles.
Figures 12 and 13 illustrate the average diffusion coefficients of BCA- and urease-functionalized particles in different solutions. Originally, we proposed that enhanced diffusion of the enzyme-functionalized particles should be observed only in the solution of substrate, whereas buffer, water, and inhibitor samples should serve as negative controls. Our hypothesis was based on the fact that energy of the chemical reaction could power particle motion, and was supported by previous results in our group using nanorods and by Golestenian and colleagues’ use of platinum-polystyrene (PS-Pt) Janus spheres.\(^5,24\) Despite slight differences between the average diffusion coefficients (Figures 12 and 13), all diffusion data lie within the error range, and, therefore, enhanced diffusion was not observed. The difference between the experimental and theoretical diffusion coefficients can be explained by the size distribution of the particles. According to the experimental data, for Janus and uniform particles, neither the presence of immobilized catalyst, nor particles asymmetry was sufficient to cause powered motion of the particles.

Although we were not able to observe enhanced diffusion for the 400 nm particles, preliminary data obtained in our group showed enhanced diffusion of the urease molecule in the buffer containing urea. The single enzyme diffusion was studied using fluorescence correlation spectroscopy (FCS). A working mechanism of the observed enhanced diffusion seen in the single enzyme case is the possible dependence of the diffusion coefficient on the size of the double layer surrounding the enzyme. According to Schumacher G.A. et al., a change in diffusion can be observed only if the electrical double layer is comparable to the radius of the particle it surrounds (i.e. \(\kappa a=1\), where \(\kappa^{-1}\) is double layer thickness and \(a\) is the sphere radius).\(^37\) Since the size of the double layer
is on order of several nanometers, the change in its thickness will have a greater impact on the single enzyme than on enzyme-functionalized particles. According to the same theory, at high zeta potentials, the size of counterion primarily determines the degree of change in the diffusion coefficient.

Therefore, a significant change can be observed in the case of neutral substrate molecules and charged products. For example, the hydrolysis of urea by urease produces a variety of charged species among them are ammonium and carbonate. Production of these ions decreases the double layer thickness and thus changes the rate of particle diffusion. So the more active enzyme, the more ions it can produce, and the more its double layer contracts. Thus, one would expect that diffusion of carbonic anhydrase in the presence of carbon dioxide should increase due to the high turnover number of the enzyme and the ionic products (bicarbonate ion and proton).

Another approach to power the motion of the enzyme-functionalized particles is to enhance the catalytic properties of immobilized enzymes. According to Golestanian et al., propulsion velocity of the particle with immobilized catalyst directly depends on the rate of the chemical reaction it catalyses. Golestanian and collaborators demonstrated that PS-Pt Janus particles exhibit enhanced diffusion in hydrogen peroxide solutions. In the case of PS-Pt particles, the rate of the platinum-catalyzed peroxide decomposition followed Michaelis-Menten kinetics and, thus, made the catalysis similar to the kinetics of an enzymatic reaction. Moreover, their observed velocity was directly proportional to the effective surface reaction rate which implies that the higher reaction turnover creates faster particle movement.
In addition to the catalytically driven motion, the local change in the osmotic pressure gradient due to the asymmetric chemical reaction \(2H_2O_2 \rightarrow 2H_2O + O_2\) contributed to the motion of Golestanian’s particles.\(^{24}\) If Golestanian’s mechanism is applicable to our biomotor, the movement of the enzyme-functionalized particles should be possible in the presence of low salt concentrations (buffers) as long as the enzyme catalyzed reaction is fast and asymmetric (Figure 14).

![Figure 14: Enzyme functionalized Janus particle moves upon catalyzing asymmetric decomposition of substrate into products.](Image)

For both urease and BCA, the reactions are asymmetric (Equations 1 and 2), and the catalytic activity is high for the free enzymes. However, since we observed loss of the enzyme activity after immobilization, one could expect that particle diffusion will be affected. Therefore, if the catalytic properties of immobilized enzymes can be preserved or improved, the enhanced diffusion of the enzyme-functionalized particles should be observed.
Based on the discussed mechanisms the best approach to power the motion of the enzyme-functionalized spheres is to use half enzyme coated microspheres several nanometers in size and with enhanced catalytic properties of immobilized enzymes.

Future directions

Further investigations of the double layer mechanism

Several experiments could be performed in order to study Schumacher’s theory as applied to enhanced diffusion of enzymes. For example, if the enzyme produces neutral species (i.e. catalytic decomposition of hydrogen peroxide by catalase), these species should not have an effect on the double layer and, therefore, no effect on the diffusion either. Another way to test this theory is to study an enzyme such as triose phosphate isomerase which catalyses interconversion of ketone (dihydroxyacetone phosphate) into aldehyde (D-glyceraldehyde 3-phosphate).\(^4^0\) The case of triose phosphate isomerase is interesting because the substrate and the product are both ions and the only species in the reaction.

Improvement of catalytic properties

The possible improvement in the catalytic activity of immobilized enzymes can be done through modification or change in the coupling procedure.\(^4^1\) For example, having a linker between the enzyme and particle will introduce better accessibility of catalytic site for the substrate. Another way to preserve the catalytic properties of
immobilized enzymes is by expressing an enzyme with polyhistidine or avidin tag.\textsuperscript{42} This approach can conserve the native conformation of the enzyme, prevent catalytic residues involvement in immobilization, and facilitate favorable orientation of enzyme catalytic site. In addition, some recombinant enzymes demonstrate the enhancement in the catalytic properties compared to the wild-type ones.\textsuperscript{43} Thus, the catalytic properties of immobilized enzymes can be improved by modifying the coupling procedure or by using recombinant techniques.

**Possible applications**

**Separation of biocatalysts**

The catalytic efficiency often limits the effectiveness of the industrial bioreactors. Therefore, it would be beneficial to use highly active immobilized biocatalysts for large-scale bioprocessing.\textsuperscript{44} If catalytically active particles do indeed diffuse faster than non-catalytic ones, it should be possible to separate beads based on their catalytic activity. The scheme of separation follows (Figure 15):
Figure 15. Separation of catalysts based on their catalytic activity.

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If the desired enhancement in the diffusion of the enzyme functionalized particles is achieved, particle movement should be studied under conditions that resemble physiological ones, especially high salt concentrations. After successful in vitro studies, particle toxicity and biocompatibility should be investigated in vivo. Our biomotor has two favorable factors of biocompatibility: small size and enzyme covered surface. Thus, if the enzyme-functionalized particles can demonstrate directional catalytically powered motion inside of the human body without provoking an immune response, these particles can be used for drug delivery, bioimaging, and, perhaps, nanosurgery.\textsuperscript{1,45}
Conclusions

In this work we have investigated enhanced diffusion of enzyme-functionalized particles in substrate solutions. For our study we used two types of particles: fully enzyme covered polystyrene carboxylated microspheres and half enzyme-coated gold-polystyrene carboxylated Janus particles. EDC/NHS coupling was used for enzyme immobilization. Protein load on the particles was determined by total nitrogen content, and the activity of immobilized and free enzyme was measured in the appropriate substrate solutions. The activity of immobilized enzymes was much lower than for free enzymes. We found that the average diffusion coefficients of the enzyme-functionalized particles are similar regardless of the substrate presence and lie within the experimental error limits. Based on the preliminary data from the single enzyme studies and enzyme assays we think that we did not observe enhanced diffusion because of the large particle size and the low catalytic activity of immobilized enzymes. Therefore we propose to decrease the size of the particles and preserve the enzyme activity in order to observe enhanced diffusion.
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