Dynamic Loading and Unloading of Proteins in Polymeric Stomatocytes: Formation of an Enzyme-Loaded Supramolecular Nanomotor

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Supporting Information

ABSTRACT: Self-powered artificial nanomotors are currently attracting increased interest as mimics of biological motors but also as potential components of nanomachinery, robotics, and sensing devices. We have recently described the controlled shape transformation of polymersomes into bowl-shaped stomatocytes and the assembly of platinum-driven nanomotors. However, the platinum encapsulation inside the structures was low; only 50% of the structures contained the catalyst and required both high fuel concentrations for the propelling of the nanomotors and harsh conditions for the shape transformation. Application of the nanomotors in a biological setting requires the nanomotors to be efficiently propelled by a naturally available energy source and at biological relevant concentrations. Here we report a strategy for enzyme entrapment and nanomotor assembly via controlled and reversible folding of polymersomes into stomatocytes under mild conditions, allowing the encapsulation of the proteins inside the stomach with almost 100% efficiency and retention of activity. The resulting enzyme-driven nanomotors are capable of propelling these structures at low fuel concentrations (hydrogen peroxide or glucose) via a one-enzyme or two-enzyme system. The confinement of the enzymes inside the stomach does not hinder their activity and in fact facilitates the transfer of the substrates, while protecting them from the deactivating influences of the media. This is particularly important for future applications of nanomotors in biological settings especially for systems where fast autonomous movement occurs at physiological concentrations of fuel.

KEYWORDS: supramolecular chemistry, stomatocytes, nanomotor, biofuel, autonomous movement

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Biological motors are fascinating structures involved in almost every biological process such as cell division, muscle contraction, and relaxation. Their intricate movement and architecture have been a source of inspiration for scientists from a wide range of disciplines, who have tried to mimic biological motor function using both top-down and bottom-up strategies. Various motor designs have been reported in the literature, such as nanorods/wire motors, tubular motors, Janus motors, polymer-based motors, and polymeric self-assembled nanomotors. These systems have opened the door to various biomedical and environmental-related applications; however for such motors to be truly successfully applied in a biological context, a nanosized motor has to be constructed that can propel itself in biological fluids and at biologically relevant fuel concentrations, employing a fuel-selective catalytic system. Until now most of the biohybrid designs have focused on the replacement of bare metal surface motors with soft matter alternatives such as carbon-based microfibers, polymeric materials, enzyme molecules, and micropumps. They showed the ability to propel themselves in different media, however at quite high fuel concentrations and low efficiency. We have recently reported a supramolecular approach to construct catalytic nanomotors via the shape transformation of polymersomes under osmotic shock, followed by metal catalyst entrapment in the inner compartment of the bowl-shaped structures (stomatocytes). This design merges the properties of polymeric vesicles to enclose different types of drugs for in vivo intracellular delivery with the advantages of a locomotive self-propelling nanomotor system. However, for its use in biological setting, efficient movement by a naturally available energy source and at biological relevant concentrations is required. Biocatalysts such as enzymes are biological molecules capable of converting with remarkable efficiency and...
their degradation in the presence of proteases, usually prevalent in biological systems. Furthermore, the PEGylated locomotive capsule is made of a soft self-assembled material and provides stealth behavior by preventing protein adsorption onto their surface, which is expected to occur in hard micrometer-size metal nanomotors, leading to reduced efficiency. The self-assembled nanomotors should also create a soft interface with the living systems, particularly important for biological applications. Finally this method of entrapment is applicable to other enzymes and is reversible, which broadens significantly the scope of our nanomotor design for future biological applications.

RESULTS AND DISCUSSION

Solvent Addition Method for Stomatocyte Formation under Mild Conditions. Entrapment of enzymes and other proteins inside the bowl-shaped polymersomes (stomatocytes) requires a fast shape transformation of the spherical polymersomes into stomatocytes with minimal solvent exposure to the proteins to prevent their denaturation. We therefore set out to develop a method that would meet these criteria. We started out with the standard preparation of rigid spherical polymersomes from PEG-PS amphiphilic block copolymers.

The addition of water to a solution of polymer in THF/dioxane (4:1 v/v) promotes the self-assembly into polymersomes. Dialysis against Milli-Q for at least 24 h results in solvent removal and polymersomes with glassy membranes. In order to fold the membrane inward, the bilayer was made flexible by the addition of small volumes of THF/dioxane (4:1 v/v) via a syringe pump. A shape transformation into stomatocytes with a large opening occurred quickly after the addition of 150 μL to a 500 μL polymersome solution (10 mg mL⁻¹).

Addition of a second aliquot of the organic solvent (vial 2) induced a decrease in the size of the opening until the structures were almost completely closed after 90 min. When more organic solvent was added, the flexibility and permeability...
of the bilayer membrane increased, to the extent that complete
138 equilibration of the osmotic pressure over the membrane
139 occurred, followed by the recovery of the spherical polymer-
140 some morphology (Figure 2 and Supplementary Figure 2).36,37
141 This method enables a fast shape transformation of polymer-
142 somes into stomatocytes with different openings in a controlled
143 and reversible manner with a minimum amount of solvent
144 necessary for the transformation. Stomatocytes with different
145 size openings were preserved during the transformation cycle
146 by quenching the structures at different time points (30, 60, 90,
147 or 120 min) in a 2 mL aliquot of Milli-Q water. Most
148 importantly, once rigid, these intermediate large-opening
149 stomatocytes could be reshaped into the smaller opening
150 structures by repeating the transformation cycle, this time
151 requiring even less time and organic solvent for the
152 transformation to occur (Figure 2). For example, the 103 ±
153 9 nm opening glassy stomatocyte batch (500 μL colloidal
154 solution, 10 mg mL⁻¹ concentration) obtained after 60 min of
155 organic solvent addition required only 150 μL of organic
156 solvent to undergo the shape transformation into the closed
157 structure, in only 30 min. This methodology presents obvious
158 advantages in reducing both the solvent exposure time and
159 amount of organic solvent required, which are mandatory to
160 prevent denaturation from taking place during enzyme
161 encapsulation (vide infra).

Supramolecular Assembly of Enzyme-Driven Nanomotors. The solvent addition method thus allows for the
162 closing of wide-opening stomatocytes to almost completely
163 closed structures in the time frame of only 30 min using a
164 procedure in which 150 μL of organic solvent is added to 500
165 μL of colloidal solution (Figure 2 and Supplementary Figure 3) followed by fast removal of organic solvent via spin filtration
166 and dialysis. This method therefore provides the appropriate
167 conditions for enzyme encapsulation and the assembly of a
168 biohybrid nanomotor. The catalytic activity of enzymes
169 entrapped in this way during the transition to the closed
170

Figure 2. Stomatocyte formation via the solvent addition method. (a, b) TEM and (c) cryo-TEM of glassy polymersomes at the beginning of the shape transformation cycle; (d, e) TEM and (f) cryo-TEM of widely opened glassy stomatocytes obtained after the addition of 150 μL of solvent (THF/dioxane, 80:20 v/v) over a period of 30 min and quenching of the structure in 2 mL of water; (g, h) TEM and (i) cryo-TEM of opened glassy stomatocyte obtained after the overall addition of 300 μL of solvent (THF/dioxane, 80:20 v/v) over a period of 60 min and quenching of the structure in 2 mL of water; (j, k) TEM and (l) cryo-TEM of almost closed glassy stomatocytes obtained after the addition of 450 μL of solvent (THF/dioxane, 80:20 v/v) during 90 min and quenching of the structure in 2 mL of water. Alternately, the same structure could be obtained from the opened glassy structures after the addition of only 150 μL of solvent over a period of 30 min and dialysis against salt solution. Note that the opened glassy stomatocytes were first brought back to the original concentration before restarting the transformation cycle. (m) Schematic representation of the shape transformation of polymersomes into stomatocytes and back into polymersomes via both the shaping and reshaping protocol using the solvent addition method. All scale bars correspond to 200 nm.

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Figure 3. Characterization techniques to prove enzyme encapsulation. (a) Asymmetric field flow fractionation (AFFF) of enzyme-filled stomatocytes purified by spin filtration and dialysis and their comparative elution to the controls: polymersomes, polymersomes obtained via reshaping of filled stomatocytes, empty stomatocytes, and pure enzymes. Note that GOx and catalase are very close in size and therefore very difficult to separate by FFF; however, tuning the FFF method to low molecular weight compounds allowed for a slight differentiation between the two enzymes (inset). (b) AFFF coupled with static (multiangle) light scattering and dynamic light scattering techniques to determine the ratio between the radius of gyration ($R_g$) and hydrodynamic radius ($R_h$) (Supporting Information, Figures 5 and 7), which gives information about the mass distribution within the structure. Note the clear distinction between the fitting of the $R_g$-$R_h$ ratios of filled and nonfilled stomatocytes. (c) Transmission electron microscopy (TEM) coupled with energy dispersive X-ray spectroscopy (EDX) showing the mapping of iron (Fe) of the heme group present in catalase and sulfur (S) specific to the cysteines and methionines in both catalase and GOx enzymes, and their localization inside the stomatocyte cavity.

Figure 3a shows the experimental setup for the asymmetric field flow fractionation (AFFF) of enzyme-filled stomatocytes. The figure includes a diagram of the AFFF method, with different states of the enzyme-filled stomatocytes, such as Release of Catalase, GOx-Cat filled Stoma, Cat filled Stoma, Empty Stomatocytes, and Empty Stoma + GOx + Catalase. The figure also includes a time line showing the release of catalase, GOx-Cat filled stomata, and cat filled stomata over a period of 30 minutes.

Figure 3b shows the results of the AFFF coupled with multiangle light scattering (MALS) and dynamic light scattering (DLS) techniques. The figure includes a graph showing the relationship between time (min) and fluorescence (RFU). The graph shows the fluorescence (RFU) over a period of 26 minutes, with two distinct peaks indicating the separation of the enzyme-filled stomatocytes from the empty stomatocytes.

Figure 3c shows the results of the transmission electron microscopy (TEM) coupled with energy dispersive X-ray spectroscopy (EDX). The figure includes an image of the enzyme localization inside the stomatocyte cavity, showing the mapping of iron (Fe) of the heme group present in catalase and sulfur (S) specific to the cysteines and methionines in both catalase and GOx enzymes.

The enzyme activity was assessed using standard colorimetric enzymatic assays (Supporting Information, Table 3). Several analysis techniques were used to demonstrate the successful entrapment of the enzymes as well as to determine the structure, stability, and size distribution of the supramolecular assembly.

Asymmetric field flow fractionation (AFFF) coupled to multiangle light scattering (MALS) and dynamic light scattering (QUELS) is a powerful technique that uses both separation and light scattering to analyze samples. It allows for the separation and analysis of a large distribution of particle sizes, i.e., from nano- to microscale, without relying on a stationary phase. In this case, the separation/fractionation occurs in a flow and depends on the diffusion coefficient of the particles with the smallest ones eluting first. Enzyme-filled stomatocytes and several controls such as empty stomatocytes, mixtures of the stomatocytes and the enzymes, polymersomes obtained by the reverse engineering of the stomatocytes, and the pure enzymes were eluted with AFFF (Supporting Information, Figures 4 and 6). Optimization of the cross-flow program by using an exponential gradient allowed for both small enzymes (average size 11 nm) and stomatocytes (average size 500 nm) to be efficiently separated within the same run inside the AFFF channel. Aliquots of catalase, GOx, and their mixture with empty stomatocytes were
Control experiments with both pure enzymes and stomatocytes showed the same elution volumes as the mixture of stomatocytes and free enzymes. This result is a good indication that under the applied encapsulation conditions both enzymes do not adsorb to the outer surface of the stomatocytes. This result was also confirmed by zeta potential measurements on the empty and enzyme-filled stomatocytes, which allowed for the measurement of the net charge on the surface of the structures before and after entrapment. Both measurements gave almost the same result, i.e., a negative value of the zeta potential of –26 ± 2 mV. Stomatocytes in which catalase and a combination of GOx and catalase were entrapped during the shape transformation and further purified showed only one peak corresponding to the filled stomatocytes and the complete disappearance of the enzyme peaks at low retention times. When compared to the empty stomatocytes, both catalase- and GOx–catalase (3:1)-filled stomatocytes showed a slight delay in the elution time, indicating that the encapsulation led to the formation of larger stomatocytes possibly due to a templating effect induced by the clustered enzyme molecules inside the stomatocytes. This hypothesis was further confirmed by the in-flow hydrodynamic radii measurements of the closed-neck empty and catalase-filled stomatocytes, showing in all cases a larger size for the enzyme-filled stomatocytes compared to the empty ones. As mentioned above, the solvent addition method is reversible and can regenerate the polymersome morphology from the stomatocytes. This property was exploited in order to demonstrate the presence of the enzymes inside the stomatocytes by releasing their content during the shape transformation back into polymersomes. As expected, the AFFF data showed the presence of two peaks, one corresponding to the catalase and the other corresponding to the polymersomes. The coupling of the AFFF system to MALS and dynamic light scattering (DLS) allowed for further characterization of the physiochemical characteristics of the particles. While DLS determines the hydrodynamic radius ($R_h$) of the separate peaks and implicitly of the filled and nonfilled stomatocytes, the MALS system enables the calculation of both the molecular weight of the assemblies and the radius of gyration of the particles ($R_g$), which gives information on the distribution of mass within the particles.$^{41}$

The ratio $R_g/R_h$ of spherical objects provides information with respect to their composition. While for an ideal empty sphere $R_g$ equals $R_h$ due to the lack of mass inside the structures, filled objects have been shown to have a smaller radius of gyration due to the concentration of mass inside the structures, leading to $R_g/R_h$ values of 0.775.$^{42}$ Cryo-TEM measurements confirmed the overall spherical geometry of our polymersomes and stomatocytes and the applicability of these studies to our system. The measurements on catalase-filled and GOx–catalase-filled stomatocytes clearly showed consistently smaller $R_g$ and $R_g/R_h$ values over the entire peak compared to the controls, indicating the presence of the enzymes inside the structures (Figure 3b). Enzyme entrapment inside the stomatocytes was also evidenced from experiments in which the energy dispersive X-ray technique in combination with TEM was used. This technique proved the presence of both enzymes in the cavities of the stomatocytes by position mapping of specific atoms in which the enzymes are rich, such as iron for catalase and sulfur for both GOx and catalase, as is shown in Figure 3c. Furthermore, a population element mapping by TEM-EDX showed that there were hardly any empty stomatocytes present, demonstrating that the enzyme-filled stomatocyte formation process is near quantitative (Supporting Information, Figure 10). The TEM and cryo-TEM show the presence of almost completely closed stomatocytes, while the enzymes are tightly packed inside the structures. We think this is due to the confining effect of the stomatocytes, which allows for the enzymes to assemble in tightly packed clusters. The formation of these clusters could also be responsible for the high encapsulation efficiency via a possible templating effect mechanism.

**Enzyme-Driven Supramolecular Nanomotors: Movement Analysis.** To test the autonomous movement of the enzyme-filled nanomotors, we analyzed their behavior in the presence of hydrogen peroxide and glucose at different concentrations. We used nanoparticle-tracking analysis (NTA), a technique complementary to DLS that uses laser light scattering in combination with a charge-coupled detector (CCD) and a microscope, to provide individual particle-by-particle analysis of colloidal particles instead of an assemble size distribution as shown by DLS. The Stokes–Einstein equation is then used to determine the size of the structures by correlating the tracking coordinates from the Brownian movement to the particle size as shown in our previous study on platinum-driven nanomotors.$^{11}$ In this equation the hydrodynamic diameter of the supramolecular nanomotor $d$ is inversely related to the time-dependent particle diffusion coefficient $D(t)$, which however is valid only when there is no fuel present in the system and the particles move under Brownian motion ($D(t) = TK_B/3\eta d$, with $K_B$ the Boltzmann constant, $\eta$ the viscosity, and $T$ the temperature). Since the technique provides additional visualization of the particles, it is also suitable, as we showed previously, for tracking the non-Brownian motion observed when fuel is added to the self-assembled structures. First, the fast directional autonomous movement of the nanomotors in the presence of the fuel makes their sizes “appear” smaller compared to the same structures before adding the fuel, due to the inverse relation between diffusion and (apparent) size.$^{337}$

To test the expected directional movement in our enzyme-driven nanomotors and make sure that the fuel addition was not responsible for the change in the size, we investigated the effect of the addition of hydrogen peroxide and glucose to empty stomatocytes. As expected, no change in their Brownian motion and trajectories was observed (Supplementary Video 1, Supplementary Figure 11). However, addition of hydrogen peroxide of different concentrations to the catalase-filled stomatocytes solution resulted in a clear shift in their “apparent” sizes to smaller values compared to the same structures in the absence of fuel (Supplementary Figure 13). Additionally a clear change of their trajectories from a non-directional Brownian motion to a propulsion directional movement was observed (Supplementary Figure 12). When the fuel was fully consumed, the structures recovered their original size as measured by NTA, demonstrating that the effect was due to the propulsive movement of the nanomotors.$^{347}$

Furthermore, the addition of hydrogen peroxide to a mixture of 90% empty stomatocytes and 10% catalase nanomotors ($v$) showed simultaneously the autonomous directional movement of the nanomotors and the expected Brownian motion of the empty stomatocytes (Supplementary Video 2; note the
In this case the size and trajectories of the empty stomatocytes (90% control structures) are not affected by the fuel addition.

This experiment further confirms that the movement of the assembled nanomotors is autonomous and is not caused or affected by any drift or flow within the chamber, which is only expected at much higher fuel concentrations than used in our system, due to the fast accumulation of gases within the chamber.

As shown in our previous report on platinum-driven nanomotors, the ability of the NTA technique to measure the trajectories and $x,y$ coordinates of the single-particle nanomotors allowed for a closer analysis of their movements by studying their paths and their average mean square displacements (MSD).\textsuperscript{11,43} We used the self-diffusiophoretic model proposed by Golestanian and co-workers to determine the speed of the nanomotors.\textsuperscript{45} The model indicates that the directional movement of micrometer-size Janus sphere motors is the result of both rotational and translational diffusion. The model has two limiting forms, a parabolic component for short periods of observation and a linear component for long periods. The fitting of the experimental MSD data of our enzyme-driven nanomotors allowed only for the observation of the parabolic component. This was due to the limitations of the nanosight system in the movement analysis of nanomotor-scale objects that prevented the tracking of the nanomotors for long periods of time and at high capture rates. Both the trajectories and the average MSDs of 105 nanomotors at three hydrogen peroxide concentrations (11, 50, and 111 mM) were measured, and the propulsive and directional movement of the nanomotors was determined from the fitting of the parabolic fit of the MSD dependency on time according to the equation $\langle r^2 \rangle = 4D \Delta t + (vt)^2$ (Figure 4a and supplementary Figure 14) with $D$ being the diffusion coefficient and $v$, the speed of the nanomotors.

The movement of the nanomotors without fuel (controls) showed only a linear $\langle r^2 \rangle = 4D \Delta t$ dependency, typical for a Brownian motion. The average speeds of the nanomotors at these concentrations were found to be 15, 26, and 60 $\mu$m/s (Figure 4a). The biohybrid catalase-driven nanomotor therefore runs at remarkably high speeds of 176 body lengths/s in 100 mM hydrogen peroxide concentrations, which is 3 times higher than the speed of our previously reported platinum-driven nanomotors.\textsuperscript{11} This high efficiency is most probably due to the combination of high catalytic activity of the catalase molecule and the excellent encapsulation efficiency of the enzyme compared to the stomatocytes filled with the catalytically active platinum nanoparticles. We also think this is due to the special design of our nanomotor system, which confines the enzymes in a small compartment with a very small opening while the gases are expelled through a nanometer pore resembling the nozzle of a rocket. This design is much different from the traditional Janus particle, where the substrates are released from a larger surface.

We subsequently tested the stomatocytes containing the two-enzyme cascade system based on glucose oxidase and catalase with glucose as a fuel (Figure 4b). The ratio between catalase and GOx was selected to be 1:3 (w/w), taking into account the known difference in activities of the two enzymes.

The GOx–catalase nanomotor was observed to become more active in time and increased its speed several seconds after the addition of the glucose. We attribute this behavior to the slower GOx enzyme, which requires oxygen to start the catalytic process.

Figure 4. Movement analysis of the one- and two-enzyme-driven supramolecular nanomotors. (a) Velocity of catalase-filled stomatocytes at different fuel concentrations; the velocity was extracted from the fitting of the average MSD of the catalase-filled stomatocytes at different concentrations (11–111 mM H$_2$O$_2$), calculated from the tracking coordinates of on average 105 particles. (b) Velocity of GOx–catalase two-enzyme-driven nanomotors at different fuel concentrations; the velocity was extracted from the fitting of the average MSD at different concentrations (5 and 10 mM glucose), calculated from the tracking coordinates of on average 100 particles. (c) Schematic representation of the size-dependent inhibition and protecting effect of the stomatocytes in GOx–catalase two-enzyme-driven nanomotors. The small inhibitor sodium azide is able to diffuse inside of the nanomotors, deactivating the enzyme, while large proteases are not able to get in. (d) MSD of GOx–catalase two-enzyme-driven nanomotors in the presence of catalase or trypsin added externally to the mixture. The velocity was extracted from the fitting of the average MSD of the curves. No change in the speed of the nanomotors is detected.

For this reason we used aerated Milli-Q water to perform the rest of the experiments. The movements of particles at two glucose concentrations are shown in Figure 4b and supplementary Figure 15. As can be seen, the two-enzyme nanomotor is able to propel itself at very low concentrations of glucose, even down to 5 mM. This is a much lower value when compared to a previously reported example where carbon nanotubes to which the same catalytic enzyme combination was attached were used. In that case a much higher concentration of glucose, 100 mM, was required.\textsuperscript{30} We think this is because the reaction in the stomatocytes is concerted in the nanocavity, and thus faster transfer of substrates between different enzymes occurs. This is not the case if the enzymes are chemically attached to the surface of the motors, as transfer of byproduct substrates relies on slow diffusion in solution. Furthermore, the design of the stomatocyte nanomotor facilitates the protection of the enzymes by preventing any inhibition of their activity or denaturation in the presence of other proteins such as proteases, often prevalent in biological systems (Figure 4c,d and supplementary Figure 16). To our knowledge this is the first example of an enzyme-driven supramolecular nanomotor that is able to propel itself at low concentrations of biological proteins.

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In a series of experiments we investigated whether the motion of the supramolecular nanomotors could be manipulated by controlling the activity of the entrapped enzymes. Sodium azide is a known small inhibitor of catalase. It binds to the heme iron center in the active site of the enzyme. As expected, the addition of sodium azide irreversibly inhibited the decomposition of hydrogen peroxide and consequently the production of the propelling oxygen gas necessary for the functioning of the stomatocyte motor. After the addition of the inhibitor, both the trajectories of the nanomotors and their sizes indicated the recovery of the Brownian motion characteristics of the nanomotors in the absence of fuel. The inhibition of the catalase inside the stomatocytes was possible due to the small size of the sodium azide, which was able to diffuse inside the stomach. In the case where the inhibitor was a proteolytic enzyme, for instance, trypsin, its larger size should prevent it from diffusing inside the nanomotors to inhibit the activity of the enzyme (Figure 4c,d). To test the protecting effect provided by the stomatocyte, we exposed the GOx–Cat nanomotors to 434 μM trypsin, a proteolytic enzyme, and analyzed the movement of the nanomotors after protein addition (Figure 4d). Both enzymes (GOx and Cat) were able to work in a cascade inside the stomatocytes to produce the propelling gas; therefore the presence of the proteolytic enzyme did not have any remarkable effect on the function of nanomotors (Figure 4c,d). A small decrease in the speed of the nanomotors was observed, most probably due to the decrease in the concentration upon protein addition. Besides their nanometer size, the encapsulation of the enzyme inside the stomatocytes is of great importance, as it provides protection against deactivating elements present in biological environments, such as proteases. The nanomotor design therefore offers a clear advantage compared to other enzyme nanomotors, especially when applying these nanomotors in biologically related applications due to their high efficiency and activity at very low concentrations of naturally occurring fuels.

In summary, we have developed a strategy to incorporate sensitive proteins or enzymes with very high encapsulation efficiencies inside the cavity of polymeric stomatocytes via a process of shape transformation of polymersomes under mild conditions, while fully retaining their activity. Using this procedure we have constructed self-assembled nanometer-scale enzyme-driven motors capable of propelling themselves with ultrahigh speeds using biologically relevant fuels and concentrations. The encapsulation of the two supplementary enzymes GOx and catalase allows the nanomotors to propel themselves using glucose as an alternative fuel for hydrogen peroxide at biologically relevant concentrations, i.e., only 5 mM. This efficiency is probably attributed to the compartmentalization and confining of the enzymes in such a nanovector. This strategy for enzyme entrapment is highly efficient and can also be conveniently applied to the entrapment of other enzymes. The morphology of these nanomotors provides protection of the enzymes within their cavities from proteolytic enzymes that are available in biological species, thus providing a broader scope to the nanomotor design for biological applications, e.g., in living cells. Besides its application for nanomotor assembly, this strategy of encapsulation, release, and protection of proteins within a nanovesicle containing a large pore (stomatocyte) could be useful to other fields such as drug/protein delivery or nanoreactor applications. The unique features of the nanomotors and further control in movement and directionality could be further useful for other applications such as biosensing, protein and DNA isolation and detection, or immunoassays. Nanomotors could rapidly in situ recognize, isolate, and enrich target biomolecules, such as DNA, proteins, and cells, in untreated biological samples. In addition, nanomotors can be a promising tool for treatments in nanotechnology. Future research is focused on demonstrating the functioning of nanomotors in biological media and their collective movement in a gradient of fuel. This will lead to intelligent, self-propelled, and self-guided drug carriers that can follow the chemical clues given by tumor cells. High chemotactic efficiencies are however required, and attractants besides hydrogen peroxide are needed for future applications.

Our nanomotor assembly and the strategy of encapsulation provide high flexibility in the cargo-load and holds therefore considerable potential for future research in the biomedical field.

**METHODS**

All chemicals and enzymes were used as received unless otherwise stated. For the block copolymer synthesis, styrene was distilled before use to remove the inhibitor. Anisole and N,N,N′,N″,N″-pentamethyldiethylenetriamine were purchased from Sigma-Aldrich. Ultrapure Milli-Q water, obtained with the help of a Labconco Water Pro PS purification system (18.2 MΩ), was used for the procedures of polymersome self-assembly and the dialyses experiments. Dialysis Membranes MWCO 12–14 000 g mol⁻¹ Spectra/Por were used where required. Ultrafree-MC centrifugal filters (0.22 μm) were purchased from Millipore. Sodium nitrate was purchased from Merck. Catalase (E.C. 1.11.16) from bovine liver, lyophilized powder 2000–5000 U mg⁻¹, was purchased from Sigma-Aldrich. Glucose oxidase (E.C. 1.1.3.4) from Aspergillus niger type II lyophilized powder (228.25 U mg⁻¹) was obtained from Sigma-Aldrich. Peroxidase from horseradish (E.C. 1.11.1.7) type I, 50–150 U mg⁻¹ solid, and Amplifi Red were purchased from Sigma-Aldrich.

**Synthesis of Poly(ethylene glycol)₄₄-b-poly(styrene)₄₄.** This compound was synthesized using atom transfer radical polymerization as previously reported in the literature. The length and the polydispersity of the polystyrene block were determined by ¹H NMR and GPC. The synthetic details are described in the Supporting Information.

**Preparation of Glassy Wide-Opened-Neck Stomatocytes via the Solvent Addition Method.** Block copolymer poly(ethylene glycol)₄₄-b-poly(styrene)₄₄ (20 mg) was dissolved in 2 mL of THF-dioxane (80:20 v/v), and 3 mL of Milli-Q was added to the solution at a rate of 1 mL h⁻¹. The generated polymersomes were dialyzed against Milli-Q for at least 24 h. The volume of the formed colloidal solution was adjusted to 2 mL, and 500 μL of this solution was transferred to a 5 mL vial, which was sealed with a septum. Subsequently, 300 μL of a 56 THF/dioxane (80:20 v/v) mixture was added at a rate of 300 μL h⁻¹, while there was a 0.6 mm needle inserted through the septum throughout the whole experiment. After the addition the resulting solution was quenched with 2 mL of water and the volume reduced to 500 μL by spin filtration.

**Encapsulation of Enzymes inside the Stomatocyte Cavity.** Catalase (6 mg) or a mixture of catalase (2 mg) and GOX (6 mg) was dissolved in 500 μL of the colloidal open-neck stomatocyte solution. Subsequently, 150 μL of THF/dioxane (80:20 v/v) was added to the mixture at a rate of 300 μL h⁻¹ while there was a 0.6 mm needle inserted through the septum throughout the whole experiment. The structures were purified from the free enzymes via dialysis and spin filtration using an aqueous 5 mM NaNO₃ solution.

**Autonomous Movement of the Stomatocyte Nanomotors.** The concentrations of the enzyme-containing stomatocytes were...
Release of Enzymes from Stomatocyte Cavity. Encapsulated enzymes were released from the stomatocyte cavities by the direct addition of 150 μL or higher amounts of THF/dioxane (80:20 v/v). Subsequently, the samples were dialyzed against a 5 mM NaNO₃ solution to remove the organic solvent. The presence of free enzymes was checked by injecting 20 μL of the colloidal solution into the AFFF, which eventually resulted in two peaks, one corresponding to the enzymes and the other to the formed formersomes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.5b07689.

Additional information regarding polymersome shape transformation with detailed cryo-TEM images, EDX mapping images, nanomotor tracking analysis (PDF)

Nanoparticle tracking video (MPG)
Nanoparticle tracking video (MPG)
Nanoparticle tracking video (AVI)
Nanoparticle tracking video (AVI)

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Notes

The authors declare no competing financial interest.

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