Scientists over the world are inspired by biological nanomotors and try to mimic these complex structures. In recent years multiple nanomotors have been created for various fields, such as biomedical applications or environmental remediation, which require a different design both in terms of size and shape, as well as material properties. So far, only relatively simple designs for synthetic nanomotors have been reported. Herein, we report an approach to create biodegradable polymeric nanomotors with a multivalent design. PEG-PDLLA (poly(ethylene glycol)-b-poly(D,L-lactide)) stomatocytes with azide handles were created that were selectively reduced on the outside surface by TCEP (tris(2-carboxyethyl)phosphine) functionalized beads. Thereby, two different functional handles were created, both on the inner and outer surface of the stomatocytes, providing spatial control for catalyst positioning. Enzymes were coupled on the inside of the stomatocyte to induce motion in the presence of fuel, while fluorophores and other molecules can be attached on the outside.
Self-propelled nanomotors are small machines that are powered by catalytic reactions in fluids. The design and demands of these motors depend strongly on their tasks, for example, for biomedical applications, the motors should be made from soft biocompatible and/or biodegradable materials and should be adaptive like biological motors in nature. An adaptive system requires a multivalent design that combines mobility together with other functionalities, like sensing, recognition, and/or other active components, to ensure that the motor can respond to changes in the environment. So far, the design of existing nanomotors is relatively simple, limiting their application possibilities. Implementing multiple functionalities in one system is still challenging and laborious, yet should be easy and straightforward, especially when going for more complex settings, i.e. mimicking life-like materials. A crucial factor in the design of nanomotors is positional control of the catalyst, which is difficult to achieve at the nanoscale.

For the design of biodegradable nanomotors it is crucial to consider its materials. To date, bulk of research has focused on creating artificial motors comprising metals (metal oxides) and/or non-degradable components and are therefore not relevant for biomedical applications. Furthermore, to mimic natural motors, a controlled bottom up approach is necessary to create soft supramolecular motors. Our group has developed the first supramolecular nanomotors based on polymersomes. Polymersomes are artificial bilayer vesicles made from amphiphilic block copolymers. The block copolymers self-assemble in aqueous solutions into spherical vesicles. Introducing osmotic pressure can induce shape transformation of the polymersomes into various morphologies. The shape transformation depends on the reaction conditions, such as the polymer composition, polymer length and organic solvent(s). By controlling these conditions, different polymersome sizes and shapes can be made, including stomatocytes; bowl-shaped vesicles with an opening on one side.

Encapsulating a catalyst inside the stomatocyte is still challenging and laborious, yet should be easy and straightforward, especially when going for more complex settings, i.e. mimicking life-like materials. A crucial factor in the design of nanomotors is positional control of the catalyst, which is difficult to achieve at the nanoscale.

Results and Discussion

Formation of PEG-PDLLA stomatocytes with azide handles. Three different PEG-PDLLA polymers were synthesized using ring opening polymerization (ROP), including an azide functionalized polymer; mPEG32-PDLLA60, mPEG44-PDLLA90 and N3-Peg67-PDLLA75. The product compositions were calculated from their respective NMR and GPC spectra (Supplementary Table 1 and Supplementary Fig. 1). The three polymers were mixed in the following weight ratio; 10:9:1 (for mPEG32-PDLLA60, mPEG44-PDLLA90 and N3-Peg67-PDLLA75, respectively) and dissolved in organic solvent (1:4 tetrahydrofuran (THF) to dioxane). An equivalent of water was added slowly at 1 mL/h, inducing self-assembly into spherical polymersomes. After dialysis against 10 mM NaCl, stomatocyte morphologies with open necks were obtained, as shown by cryo-transmission electron microscopy (cryo-TEM) (Fig. 2). The opening of the stomatocytes was measured from the cryo-TEM images to be around 30 nm (Supplementary Figs. 2 and 3).
treated with the TCEP beads and was expected to contain around half of the azide groups. The third group was reduced with free TCEP, which would theoretically lead to complete reduction of the azide groups, as the TCEP is able to enter the stomatocyte cavity. The final sample consisted of only PEG-PDLLA polymer without azide groups as negative control. An Alexa Fluor 488-alkyne dye was coupled via copper(I)-catalyzed alkyne-azole cycloaddition (CuAAC) to the available azide groups for each sample. Afterwards, all samples were dissolved in THF to destroy the vesicle structures. The fluorescence signal of each sample was measured and is depicted in Fig. 2c. From the peak signals at 540 nm was calculated that around 45% azide groups remained after the reduction with the TCEP beads, indicating that only the outside azide groups were reduced. The reduction with free TCEP resulted in a fluorescence intensity of 8%, suggesting that most of the azide groups were reduced.

To confirm spatial control over the reduction and thereby the localization of the catalyst, gold nanoparticles (20 nm) with NHS linkers were coupled to the reduced stomatocytes and visualized with cryo-TEM. The gold nanoparticles coupled to the outside of reduced stomatocytes, showing partial reduction, as visualized in Fig. 2b. The control experiment with non-reduced stomatocytes did not show any binding (Supplementary Fig. 4). Important to note is that any molecule containing a carboxylic acid group could be attached, such as receptors, peptides, or sugar groups, depending on the desired application. Therefore, to proof this concept, an Alexa Fluor 647-NHS dye was attached to the amine groups on the outer surface of reduced stomatocytes via EDC coupling. With confocal microscopy the attachment of the dye was visualized (Supplementary Fig. 5).

**Fig. 1** Universal method to form polymeric nanomotors with spatial control over catalyst. (1) Formation of amphiphilic poly(ethylene glycol)-b-poly(D,L-lactide) (PEG-PDLLA) block copolymers, including an azide terminated block copolymer to act as functional handle. (2) Self-assembly of block copolymers with 5 wt% azide polymer into spherical polymersomes. (3) Shape transformation of the polymersomes into stomatocytes. Azide handles in the inner membrane of the stomatocyte are not shown for clarity. (4) Reduction of azide groups to amine groups on the outer surface by immobilized TCEP beads that are too large to enter the cavity. (5) Attachment of a dye to the amine groups on the outside via EDC coupling. (6) Covalent coupling of enzymes into the stomatocyte cavity via SPAAC. (7) Addition of physiological amounts of glucose activates the enzyme cascade to produce water and oxygen that propel the system forward.

**Coupling enzymes inside stomatocytes to form nanomotors.** Finally, enzymes were covalently coupled inside the stomatocyte cavity. Two different samples were made, one containing the enzyme catalase (Cat) and the other one containing two enzymes, catalase (Cat) and glucose oxidase (GOx). For both enzyme systems, the enzymes were coupled to DBCO-NHS, which was visualized with UV–Vis measurements, showing additional absorbance peaks at 310 nm, specific for the DBCO molecule (Supplementary Fig. 6a). A ratio of ten linker molecules per enzyme was added, which was verified by the ultraviolet (UV)–visible (Vis) experiments (Supplementary Fig. 6b). The activity of the enzymes after binding with DBCO-NHS was tested via an Amplex red assay. Two ratios of 10:1 and 30:1 DBCO linker molecules per catalase were tested and compared to unmodified catalase, they retained 98.9 and 96.2% activity, respectively, showing no significant loss in activity after binding (Supplementary Fig. 7). After washing the enzymes to remove the non-reacted DBCO linker, they were added to the stomatocyte sample for the coupling. The coupling of the enzymes to the azide functionalized polymer was visualized with sodium dodecyl
sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3a). For this experiment, catalase functionalized stomatocytes were dissolved in dioxane to precipitate the coupled enzymes and to remove the unreacted block copolymers. The cat-polymer conjugates were isolated and run on a 4–20% gel, together with a catalase concentration series to estimate the enzyme loading. The gel showed the bands around 60 kDa corresponding to the catalase subunit. For the conjugate sample an extra band was expected around 70 kDa, due to the attachment of the polymer. As catalase consists of 4 subunits, a ratio of around 3:1 in intensity was expected for the bands corresponding to the unbound subunits and the enzyme-polymer conjugates, respectively, which was visible on the gel (Fig. 3a). After formation of the nanomotors, their movement speed was tested in different amounts of fuel. The motors containing both GOx and Cat were tested with fuel concentrations of 10 and 30 mM glucose, whereas the motors containing only Cat were tested at 5 and 10 mM H2O2 (Fig. 3b). For each sample videos of 60 s were recorded and analyzed using Nanosight NTA2.2 software (Video S1-3). After tracking, the x and y coordinates of at least 100 particles per group were used to calculate their mean-squared displacement (MSD). These MSD values were plotted against Δt, showing increased movement speeds at higher fuel concentrations (Fig. 3b). From their curves, the average speeds were calculated and are depicted in Fig. 3c. The motion of enzyme functionalized stomatocytes show a parabolic fit, typical for bubble propulsion. Recently, our group has shown that bowl-shaped micromotors can help in confinement and nucleation of the bubbles and play a templating role for the formation of the bubbles, which collapse at regular times due to the filling of the cavity32. We think in the same way, oxygen nanobubbles are formed inside of the cavity and the regular collapse is responsible for the ballistic motion observed. In a control experiment, the motion of the enzyme loaded stomatocytes was compared with enzyme functionalized spherical polymersomes. First, spherical polymersomes with 5 wt% azide handles were created by dialysis against water instead of salt (Supplementary Fig. 8). To these polymersomes, catalase with the DBCO linker was added resulting in the random attachment of catalase on the spherical surface. The movement speed was tested at different fuel concentrations and the spherical polymersomes showed also increased movement speed, but with a linear fit, indicating enhanced diffusion (Supplementary Fig. 9).
This behavior is explained for the random attachment of catalase on the surface. This is comparable to previous studies with attachment of catalase to rod shaped nanomotors.20

In conclusion, we have shown the formation of a biodegradable supramolecular nanomotor with spatial control over the catalyst attachment and with a multivalent design. Well-defined stomatocyte structures were generated with the biodegradable PEG-PDLLA block copolymer, containing functional azide handles. Selective reduction on the outside surface of the stomatocytes with TCEP immobilized beads provided the structures with a secondary handle for coupling another functional group. Attachment of fluorescent dyes and functionalized gold nanoparticles showed the selective reduction of the outer azide groups, allowing the binding of enzymes inside the stomatocytes via click-reactions. SDS-PAGE and NTA experiments proved the formation of the nanomotor, which showed an increase in MSD after the addition of fuel.

**Methods**

**Synthesis of poly(ethylene glycol)-b-poly(D,L-lactide) (PEG-PDLLA).** Poly(ethylene glycol)-b-poly(D,L-lactide) (PEG-PDLLA) was synthesized by ring opening polymerization (ROP). For the formation of PEG$_2$-PDLLA$_{100}$, 0.2 mmol methoxy-PEG-OH macrorinitiator (194 mg) was mixed with 18 mmol D,L-Lactide (2.6 g, 13 wt% PEG in total). For the other polymer compositions the amounts were adjusted to obtain PEG$_{18}$-PDLLA$_{100}$ (397 mg methoxy-PEG-OH, 2.6 g D,L-Lactide) and N$_2$-PEG$_{97}$-PDLLA$_{15}$ (150 mg N$_2$-PEG-OH, 634 mg D,L-Lactide). First, the reagents were dried by adding dry toluene and removing the solvent under reduced pressure. Then, 15 mL dry DCM with 0.1 mmol DBU (15 mL, 0.1 mmol) was added to the dried material under argon. The reaction was left to proceed for 3–4 h at 30 °C. After finishing the polymerization, the mixture was washed twice with 1 M KH$_2$SO$_4$, dried with Na$_2$SO$_4$ and filtered off. The polymer was concentrated by evaporating most solvent (~4 mL) and then precipitated in ice cold diethyl ether (100 mL). The waxy substance was partly dried under nitrogen, dissolved in 1.4 dioxane (5 mL) and lyophilized to yield a white powder (~80% yield). Polymerization was checked with NMR and GPC (Supplementary Table 1 and Supplementary Fig. 1).

**Preparation of PEG-PDLLA stomatocytes with 5% azide handles.** In total 10 mg PEG-PDLLA polymer was weighed in a glass vial with stirring bar, 0.5 mg N$_2$-PEG$_{97}$-PDLLA$_{15}$, 4.5 mg PEG$_{18}$-PDLLA$_{100}$, and 5 mg PEG$_{44}$-PDLLA$_{100}$. The mixture was dissolved in 1 mL of organic solvent (1:1 THF:dioxane v/v). The vial was closed with a rubber septum and the mixture was stirred for 30 min at 800 rpm. Subsequently, 1 mL Milli-Q water (50 wt%) was added via a syringe pump at 1 mL per hour until a cloudy suspension was obtained. The suspension was transferred to a pre-hydrated membrane (Spectra/Por, molecular weight cutoff 12–14 kDa) and dialyzed against 1 L of 10 mM NaCl for 24 h in a fridge at 4 °C, with a solution change after 1 h to obtain the stomatocyte shape. Samples were stored in the fridge at 4 °C.

**Reduction of the outer azide handles.** For reduction of only outer azide handles: 200 μL of immobilized tris(2-carboxyethyl)phosphine (TCEP) immobilized on silica beads was centrifuged at 106 rcf to remove all liquid. To the beads, a suspension of azide functionalized stomatocyte vesicles (200 μL of 5.0 mmol/mL polymersomes) was added. The reaction mixture was stirred at 100 rpm for 36 h at room temperature, with halfway a change with fresh TCEP beads. After reduction, the stomatocyte solution was diluted with Milli-Q to 3 mL and centrifuged two times for 1 min at 106 rcf to remove the TCEP beads. The supernatant was concentrated by centrifuging for 15 min at 2655 rcf and dissolved up to 5.0 mg/mL polymersomes in NaHCO$_3$ buffer (0.1 M, pH 8.3).

**Attachment of Alexa Fluor 647-NHS to amide handles.** In all, 0.02 mg of Alexa Fluor 647-NHS ester was dissolved in 3 mL DMSO and added to 200 μL reduced stomatocytes, the reaction mixture was left stirring for 16 h in the dark at room temperature. The unreacted compounds were removed by spin filtration with 0.22 μm spin filters at 2655 rcf for 10 min. The binding was visualized with confocal and with fluorescence measurements.

**Attachment of gold nanoparticles to amide handles.** The conjugation protocol was performed as described in the assay provided in the kit. The stomatocytes were first concentrated by centrifuging for 15 min at 2655 rcf and the supernatant was removed. Afterwards, the stomatocytes were resuspended in reaction buffer and mixed with the NHS functionalized gold nanoparticles. The solution was mixed on the roller bench for 24 h at room temperature. Stomatocytes were collected by centrifugation and washing several times for 15 min at 2655 rcf.

**Coulping of DBCO-NHS to enzymes.** The enzyme of choice is dissolved in 500 μL PBS buffer (catalase 2 mg, 8.3 × 10$^{-5}$ mmol glucose oxidase 5 mg, 3.3 × 10$^{-5}$ mmol). To each enzyme solution a tenfold excess of DBCO-Sulfo-NHS was added and mixed for 24 h. Binding was verified by measuring absorbance using a UV–Vis spectrophotometer. The data and calculations are available in the Source Data file.
Quantification of azide reduction. The reduction was quantified by measuring fluorescence intensity after binding an Alexa Fluor 488-alkyne with click reaction. To collect fluorescence, non-reduced, TCEP beads reduced and free TCEP reduced 0.05 mg of Alexa Fluor 488-Alkyne, 0.25 mg of CuSO4·5H2O and 0.16 mg of (+)-Sodium 1-ascorbate were added and stirred for 12 h in the dark at room temperature. Unreacted compounds were removed by spin filtration with 0.22 μm spin filters at 2655 rcf for 10 min. The stomatocytes were dissolved in 2 mL THF, so that the vesicles were destroyed completely. Fluorescence intensities were measured with a Spectrofluorometer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Stomatocyte motors with catalase attached (400 μL) were centrifuged at 10,621 rcf for 10 min for collection. The supernatant was discarded and the motors were dissolved in 1 mL of dioxane to dissolve the polymers. The sample was again centrifuged and washed twice with dioxane to collect the clustered enzymes for 2 min at 10,621 rcf. Finally, the enzymes were dissolved in 50 μL Milli-Q water. The sample and a 20x dilution were loaded in a 4–20% SDS-precast protein gel (Bio-Rad), together with several concentrations of catalase (7.5, 3.75 and 1.87 μg) and the Precision Plus Unstained marker. The unprocessed scan is supplied in the Source Data file.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The experiment data that support the findings of this study are available from the corresponding author upon reasonable request. The source data underlying Figs. 2c, 3a-c and Supplementary Figs. 1b, 3a, c, 6a, b, 7, 9 are provided as a Source Data file.

Received: 7 June 2019; Accepted: 22 October 2019; Published online: 22 November 2019

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Acknowledgements
We acknowledge the NWO Chemische Wetenschappen VIDI Grant 723.015.001 for financial support. We also acknowledge support from the Ministry of Education, Culture and Science (Gravitation program 024.001.035).

Author contributions
J.T. and D.A.W. conceived and designed the experiments. J.T. and F.C. performed the experiments.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-019-13288-x.

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