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Improving catalase-based propelled motors endurance by enzyme encapsulation

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Abstract

Biocatalytic propulsion is expected to play an important role in the future of micromotors as it might drastically increase the number of available fuelling reactions. Though, most of the enzyme-propelled micromotors so far reported still rely on the degradation of peroxide by catalase, in spite being vulnerable to relatively high peroxide concentrations. To overcome this limitation herein we present a strategy to encapsulate the catalase and to graft those enzyme capsules on motor particles. Significant improvement of the stability in the presence of peroxide and other aggressive agents has been observed.

Introduction

The Whitesides research group pioneered the use of a Platinum layer to decompose hydrogen peroxide and generate autonomous movement in 2002.¹ Since then, the fabrication of novel and more efficient nano- and micromotors has rapidly turned into a growing field of research. Diverse mechanisms to transform energy into movement as: i) water decomposition by an Al-Ga alloy,² ii) use of hybrid systems containing living organism for propulsion,^{3, 4} and iii) fuel free approaches as UV light-induced molecular movement,⁵⁻⁷ electric fields,^{8, 9} magnetic fields¹⁰⁻¹⁴ or ultra sounds,^{15, 16} have been explored. Most of the examples so far reported are based on Platinum,¹⁷⁻²¹ which mimics the function of the biological enzyme catalase. However, its catalytic activities is not reaction-specific, one of the reasons that explains its rapid passivation²² while favouring side reactions. As the nano-/micromotors field is moving towards real life applications overcoming such limitations represents a challenge. Therefore the use of enzymes represents a promising alternative. The highly specific reactions catalyzed by enzymes avoid side reactions and undesired side products. Another advantage is that no heavy metals as Pt or Mn need to be discharged or recovered after their use, making the use of enzymes not only cheaper but also environmentally friendly. Moreover, due to the high flexibility of enzymes - over 4000 biochemical reactions are known to be enzyme catalysed- highly specific biocatalytic self-propelled movement could be induced in the near future. First successful approaches to integrate enzyme-catalyzed motors have already been done. Recently Sanchez *et al.* presented a tubular motor based on

catalase immobilization, the same enzyme that has been used in our group for the propulsion of an asymmetric hybrid motor.^{23, 24} Carbon nanotubes movement induced by enzyme tandems consuming biocompatible glucose fuel have been shown by Feringa *et al.*²⁵ Moreover, Mano and Heller demonstrated rapid propulsion of electron conducting carbon fibers containing a redox-polymer wired glucose oxidase and an oxygen-reducing bilirubin oxidase.²⁶ The main limitation for the application of enzymes is their immobilization. Even though several techniques for enzyme immobilizations are available,^{27, 28} chemical treatment is expected to damage it and consequently decrease their activity. To overcome this problem, we were inspired by a recently reported work of Lu and co-workers, who improved the enzyme performance upon encapsulation.²⁹ This method has been extended to several proteins, as for example enhanced green fluorescent protein, bovine serum albumin, superoxide dismutase and caspase-3.³⁰⁻³² Herein we apply the concept of enzyme encapsulation by a polymeric shell with the aim to improve the performance of biocatalytic micromotors (Fig. 1). For this, a modification of the methodology described by Lu is applied consisting on the introduction of amino groups on the capsule by proper selection of the monomer reactants. Such groups allow grafting of the encapsulated enzyme on the swimmers surface while favouring their stability.

Experimental section

Catalase was bought in Aldrich as a lyophilized powder. Commercially available enzymes mostly contain (NH₄)₂SO₄ and

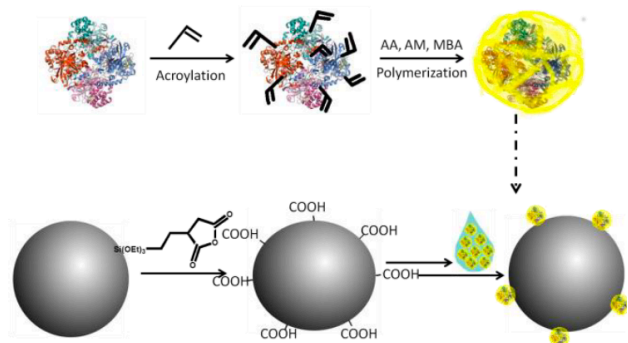


Fig.1. Schematic principle of enzyme encapsulation and covalent grafting of the capsules on the silica surface (AA -acrylamide, AM -hydrochlorid salt of 2-aminoethylmethacrylate, MBA - *N,N'*-methylene bisacrylamide)

smaller protein fragments. Therefore a purification step previous to use is recommendable. Thus, the enzymes were purified following a previously described method³² which briefly consist on the filtration of the commercial enzymes dissolved in 0.01 M sodium carbonate buffer pH 8.5 through Amicon filters. This way, the removal of smaller protein fragments, ammonium sulfate and chemical impurities are achieved.

Using a BCA assay the recovered protein after filtration was quantified to be around 63% using 100 kDa filter and 93% using a 10 kDa filter. In order to maintain more defined conditions in the polymerization the 10 kDa filter was defined as a standard pre-purification step. After this purification step only a slight lose in catalytic activity lower than 5% was documented (Fig ESI 1). The washing solution showed some remaining activity, which was insignificant compared to the remaining protein. Therefore it can be assumed that the mayor part of smaller protein fragments are not catalytically active and can be removed without losing significant catalytic activity.

Acryloylation. Approx. 5 mg of purified enzyme were dissolved in 1 ml, 0.01M sodium carbonate buffer (pH 8.5) and reacted with a 20 fold excess of *N*-acryloxysuccinimide, dissolved in 10 μ l DMSO during 30 min at room temperature. Finally, the reaction solution was filtered through Amicon filters of 50kDa and collected in 100 μ l 0.01M sodium carbonate buffer (pH 8.5). The modification was monitored by DLS and Zetapotential, as well as the protein content and the relative amino-content is compared.

Polymerization on acryloyl groups. Diluting the acryloylated enzyme in 1000 μ l 0.01M deoxygenated sodium carbonate buffer (pH 8.5), radical polymerization was performed. A ratio of 450-9000 eq. monomer/enzyme, aminoethyl methacrylate (positively charged monomer), 1200-12000 eq. acrylamide (neutral monomer) and 50-2000 eq. *N,N'*-methylene bisacrylamide as crosslinker were added in 2 ml 0.01M phosphate buffer (PB) (pH 7.4). The polymerization was initiated by adding 500 eq of ammonium persulfate and 1000 eq. *N,N,N',N'*-tetramethylethylenediamine dissolved in 1 ml deoxygenated PB buffer into the reaction media. The mixture was allowed to react at room temperature during 90 min in nitrogen atmosphere. Unreacted monomers and initiators were removed by filtration and subsequent washing steps applying Amicon filters 50KDa. The success of capsule formation was monitored by DLS where an increase in size from 7,2 nm (native catalase) to a range

from 9.5 up to 60 nm for encapsulated enzyme was observed. These results were confirmed by TEM using phosphotungstic acid as staining agent in order to visualize properly the enzyme-polymer capsules. Zeta potential measurements did not show significative changes. In order to confirm the presence of aminogroups in the capsule the fluorescamine test was employed.

Enzymatic activity assay. The enzymatic activity was determined spectrophotometrically by using H_2O_2 as a substrate following a procedure published elsewhere³³. Briefly, 25 μ L of a solution of enzyme in 50 mM PB pH 7.0 with a concentration of 1mg ml^{-1} was added to 11 mM H_2O_2 in 50 mM PB solution with rapid stirring. The decrease in absorbance at 240 nm (with an extinction coefficient of 0.041 $mmol^{-1} cm^{-1}$) was recorded after 30 seconds at 20 $^{\circ}C$. One unit of catalase will decompose 1 μ mol of H_2O_2 per minute at pH 7.0 and 20 $^{\circ}C$. The enzymatic activity was expressed in Units per milligram of dry solid.

Enzymatic stability evaluation.

Against enzymatic degradation by protease: 1 mg of the catalase capsules or native catalase was placed on 1 mL of a solution of 5 mg \cdot ml $^{-1}$ of protease in PB 0.1 M (pH 7.2). The mixture was incubated during 2 h at 37 $^{\circ}C$ and 100 rpm. After this time, the mixture was placed in a dialysis sack of Pure-A-LyzerTM immersed in 8 mL of H_2O_2 1.5%. The amount of H_2O_2 was determined by UV-absorption at 240 nm after 30 min.

Temperature stability: 50 μ l of encapsulated or native enzymes solution in PBS 0.1 M (pH 7.2) were added to 450 μ L of the same buffer. Then, the solution was exposed to high temperature (60 $^{\circ}C$ during 1h) under constant shaking and protected from light. After this time, the enzymatic activity of the enzymes was determined following the degradation of H_2O_2 by UV-absorption at 240 nm.

Surface modification of silica particles. 40 mg of commercial Silica particles of approx. 10 μ m were dried during 2h at 80 $^{\circ}C$. 5ml of dry toluene and a 5-fold (10mg) excess of (3-succinimylpropyl)-triethoxysilane regarding to silanol groups on the particle surface were added and reacted overnight. 1 nm^2 silica bears approximately 4.9 OH-groups^{34, 35}. After the reaction time, particles were washed with toluene and EtOH and dried subsequently.

Enzyme anchoring on silica particles. 2 mg particles were dispersed in 0.1 M PB with 1 mg *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and 2 mg *N*-hydroxysuccinimide (NHS) in order to activate COOH groups. After 30 min of stirring, 3 mg of native or encapsulated catalase respectively were added. Further stirring during 2h at room temperature let to covalent immobilization of the enzyme on the particle surface. After this time, the particles were extensively washed with 0.1 M PB in order to remove the physically absorbed proteins. The whole process was monitored by FTIR spectroscopy. (see Fig. 3a) and other analytical techniques such as TGA (see Fig. 3b), elemental analysis and amino assay as shown in table 2

Turn over evaluation of enzyme-silica motors. 1 mg of particles bearing the native or encapsulated catalase respectively were suspended in 0.2 mL of PB 0.1M pH 7.2, placed on the membrane of a Corning® Transwell® with 0.4 μ m of pore and immersed in 1.5 mL of a solution of 1.5% of H_2O_2 . After 30 min, the amount of H_2O_2 was determined by UV-absorption at 240 nm. Then, the liquid was

removed and replaced by 1.5 mL of fresh H₂O₂ 1.5% and the procedure was repeated ten times.

Enzyme anchoring on aminated Polystyrene particles. 10 µl of a commercial amine-modified polystyrene fluorescent blue beads aqueous solution 2.5% was diluted in 0.5 mL PB 0.1 M pH 7.2. Then, 0.5 mg of Suberic acid bis(*N*-hydroxysuccinimide ester) (DSS) as crosslinker solved in 10 µL of DMSO was added. After 30 min of stirring, 1 mg of native or encapsulated catalase was added. Further stirring at room temperature overnight led to covalently anchored encapsulated enzyme on the particle surface, while the grafting of native catalase was only successful to a very low extend.

Motion evaluation. The evaluation of the capacity to perform motion was performed on microscopy glass slides placed in a 6 well plate. Motor particles were placed in distilled water containing 2.5% of H₂O₂ and 10% glycerine to diminish media movement, to which 1 µl particles dispersed in distilled water were added. The movement was observed in an EVOS xl digital inverted microscope and videos were recorded with a EASTMAN KODAK EASYSHARE Z1015 IS digital camera at 29,97 fps. Obtained videos are available in the supporting information.

Results and discussion

Enzyme encapsulation. The first step for the enzyme encapsulation is the introduction of polymerizable groups on the enzyme surface. This reaction was performed using acrylic acid *N*-hydroxysuccinimide ester in pH 8.5 Na₂CO₃ 50mM buffer, which reacts with the free amino groups present in the protein. After purification, radical polymerization was carried out using a mixture of acrylamide (AA) as neutral monomer, *N,N'*-methylene bisacrylamide (MBA) as crosslinker and hydrochlorid salt of 2-aminoethylmethacrylate (AM) as monomer responsible for the introduction of amino groups in the presence of APS/TMEDA. To optimize the capsule formation, the monomer ratio (AA:AM:MBA) was initially fixed to 6.0:4.5:1.0 and different conditions varying the acrylamide:enzyme ratio between 1200:1 and 12000:1 were tested. Optimal conditions were found for low monomer contents (Table 1a,

maintained and the effect of the modification of the monomer-crosslinker relation was determined.

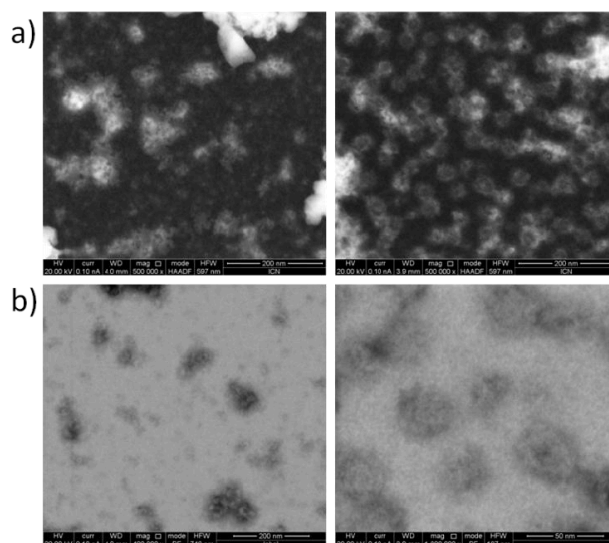


Fig. 2. a) Scanning transmission electron microscope (STEM) image in high angle annular dark field mode of native vs encapsulated enzyme solution. b): bright field STEM of native vs encapsulated enzyme solution magnification left: 400000x, right 1600000x. All samples were prepared by staining with 1% phosphotungstic acid.

A general trend that can be observed is a gelification process upon crosslinker increase, followed by entrapment of the enzyme capsules and low recovery ratio (Table 1a). Moreover, as expected, lower ratios of AM led to lower superficial amino contents. Interestingly, similar results are obtained upon increasing the crosslinker since it is capable to react with free amine-side chains in the polymer (Table 1b). Finally, optimal conditions for the fabrication of capsules with high recovery, defined size and high grade of accessible amino groups on the surface were those defined in entry d of Table 2. Fabrication of capsules with average size of 30 nm was confirmed by TEM using phosphotungstic acid as staining agent (Fig. 2). The

Table 1. Evaluation of conditions for capsule synthesis (a) variation of the monomer/enzyme ratio. (b) variation of relations between the monomers maintaining fixed the monomer enzyme ratio. Optimal conditions were marked grey in both tables.

Table1a	Monomer ratio			Capsules	
Entry	AA	AM	MBA	Recovered Mass [mg]	DLS [nm]
a	1200	900	200	3,71	9,57
b	3000	2250	900	< 1 mg	19,3
c	6000	4500	1000	< 1 mg	60,6
d	12000	9000	2000	Gel formation	

Table1b	Monomer ratio			Capsules		
Entry	AA	AM	MBA	Recovered Mass [mg]	DLS [nm]	NH ₂ [%]*
a	1200	900	200	3,71	9,57	87,5
b	1200	450	200	2,55	9,93	65,6
c	1200	900	300	< 1 mg	64,6	×
d	1200	900	100	3,77	14,2	175

entry a), as higher monomer content led to formation of bulk polymer. In the following, the optimal enzyme:AA ratio was

existence of superficial amino-groups has been proven by a fluorescamine assay; overall capsules present approximately 75% more accessible amino-groups than native catalase.

The enzymatic activity of each step was spectrophotometrically measured following the decomposition of H_2O_2 at 240 nm.³³ The acroylation step scarcely modified the activity retaining more than 95% of the activity. The encapsulated enzymes obtained after the polymerization process lost almost 50% of the initial activity (Fig ESI 1). The enzymatic activity was expressed in Units per mg of dry solid residue. Obviously, the presence of the polymer coat increases the weight of the enzyme in a proportion difficult to determine with precision and this fact is the main reason for this activity lost. Other reasons that can diminish the activity of the encapsulated enzyme can be active site blockage and lower peroxide diffusion into the polymer shell. Despite the decrease in the activity, the enzyme still retains around 50% of the initial activity after the acroylation and polymerization processes while exhibit a significant increase in its thermal stability and resistance to protease degradation. After 1 hour at 60 °C, the enzyme capsules retain more than 50% of their initial activity whereas the native enzyme loses almost 80% of its activity. (Fig. ESI 2)

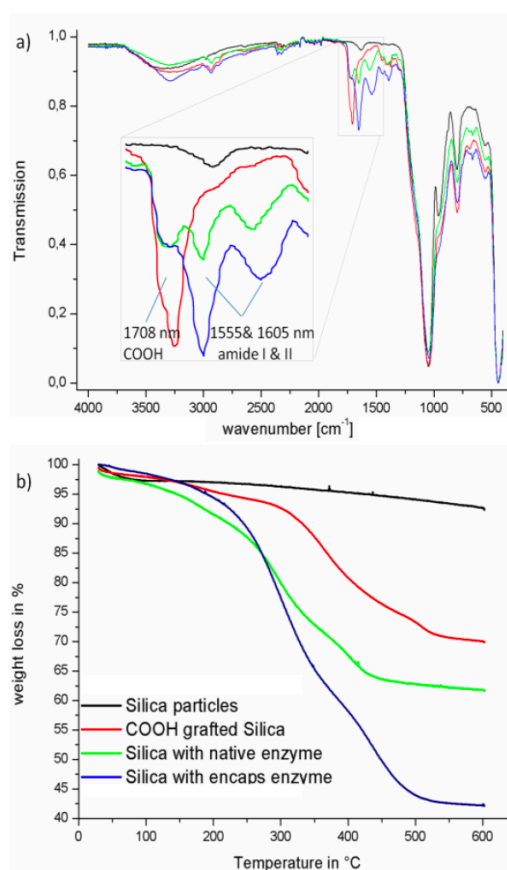


Fig. 3. a) Surface functionalization of Silica particles monitored by FTIR, where typical bands for grafted COOH groups and amide groups from the enzyme binding can be observed. b) Thermogravimetric analysis of each step of the process.

The stability of the capsules against enzymatic degradation was also tested incubating them in a solution of 5 mg mL⁻¹ of protease at 37 °C during 2 h. After this time, the remaining activity was evaluated following the decomposition of H_2O_2 as in the previous cases. The encapsulated enzyme retains more than 90% of its initial activity

whereas the native enzyme completely loses its activity. These results are of great relevance for potential applications of these motors in biological fluids where proteolytic enzymes can be present.

Silica functionalization and enzyme attachment. Carboxylic groups are introduced in the silica surface upon reaction with (3-succinimylpropyl)-triethoxysilane in toluene at 80 °C under nitrogen atmosphere. Once functionalized, the enzymatic capsules were covalently attached by coupling with EDC and NHS. The whole process was followed by FTIR; I) initially the spectrum is typical for naked silica (Fig 3a), II) after COOH-grafting C=O band of the carboxylic group is clearly visible at 1708 cm⁻¹ and III) during the conjugation with the enzyme capsules this band suffer a strong intensity decrease. This last observation also confirms that the enzymes are covalently bond and not only physically absorbed. Enzyme attachment was also confirmed by thermogravimetric (TGA) and elemental analysis. As can be observed in Figure 3b, COOH grafted silica particles exhibit a gradual mass decrease up to 22% in weight upon increase of the temperature. By comparison with the same temperature behaviour of bare silica particles, the weight loss has been tentatively attributed to the surface COOH groups. This value is considerably higher than that expected for a monolayer of carboxylgroups (approx. 6,5 wt%) and therefore, it can indicate that polycondensation is taking place. The higher amount of carboxylic groups on the surface could facilitate even more the enzyme attachment. According with the TGA experiments (Fig. 3b), the amount of native enzyme bounded to the silica surface was 8 %, whereas the encapsulated catalase reached 25%. The higher grafting amount for the encapsulated enzyme may account for: I) the polymer capsules increase the average weight of each enzyme unit and/or II) the capsules present a higher number of amino groups on their surface compared to the native catalase. Amino assay through fluorescence measurement after reaction with fluorescamine resulted in an approximately 75% higher amine content in the polymer shell than in the native catalase. The grafting reaction activated by EDC/NHS benefits from those aminogroups and the protein grafting is more efficient. The higher amount of protein bounded to the silica surface was confirmed by S-selective elemental analysis, which is completely selective to the enzyme, as neither the silica particles nor the methacrylate capsules contain Sulfur. The S-content in a sample of particles bearing encapsulated enzyme was approx. 5 times higher than in particles with native enzyme on their surface.

Finally, stability experiments of the catalase enzyme against the oxidation by the peroxide were done. Even though this enzyme contains a NADH factor in each subunit to protect itself from oxidation, it is well known that exposure to peroxide is critical for the enzyme stability, being rapidly deactivated. In order to study the stability of the attached enzymes against oxidation in the presence of hydrogen peroxide, the enzymatic activity of silica particles bearing native or encapsulated catalase respectively on their surface was determined after several work cycles exposing the particles to a fresh H_2O_2 solution each half hour. As it can be observed in Figure 4, silica particles conjugated with encapsulated catalase are able to decompose practically the same amount of hydrogen peroxide during more than 10 cycles losing only less than 40% of their initial activity at the end of the whole process. The silica particles covered by

native enzymes show a significant activity decrease after only 2 or 3 cycles.

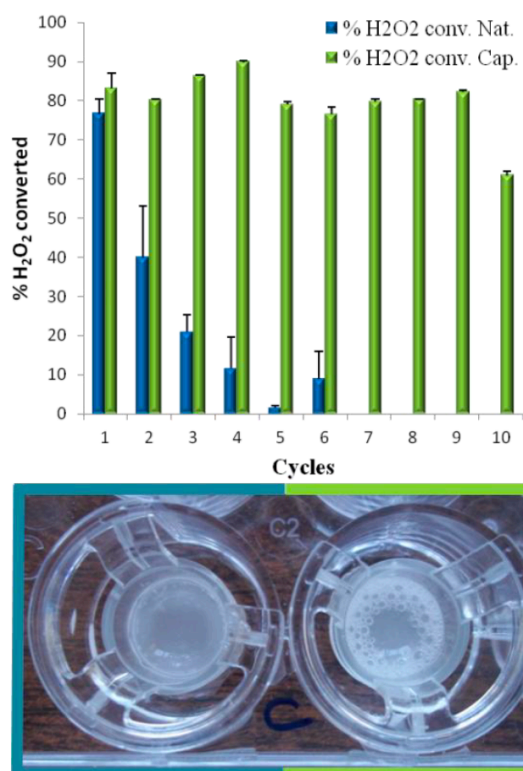


Fig. 4. Top: % H₂O₂ conversion by native enzyme vs encapsulated enzyme grafted on Silica particles. Bottom: Images taken after 10 cycles, left well correspond to native catalase-silica particles and right well to encapsulated enzyme-silica particles.

Motion experiments. The silica-enzyme particles were placed in a 2.5% peroxide solution whereupon immediate bubble production at the surface of the microparticles is visible. Fast bubble production leads to a “jet propulsion” behaviour, i.e. the fast generation of bubbles propel the movement. As a representative example Fig. 5a-b shows a circular trajectory of silica microparticles (Videos 1, and 2 ESI shows more examples of particle motion). No further mechanisms beyond bubble formation were detected in spite of most particle shaped motors are also strongly influenced by gradient driven factors (by comparison with other systems as tube-shaped motors that favour bubble driven mechanisms).^{17, 21} This fact has been tentatively attributed to the high reaction rates of our enzyme that leads to strong accumulation of oxygen bubbles. These results are similar to those already reported by us²³ and by Gibbs and Zhao.³⁶

Beyond the self-propelled movement, a remarkable stability enhancement is achieved upon encapsulation. Movement is ceased for native catalase after a short period of time as the enzyme gets oxidized by the fuel. On the contrary, in our experiments stalling takes far longer (depending on

experimental conditions in the range of 30 min) and is reversible by adding more peroxide fuel. This indicates that the stalling is not due to degradation of the enzyme but caused by the consumption of the fuel.²²

Finally, to demonstrate that amino coupling is not limited to carboxyl groups and/or silica particles or to discard non-specific linkage through hydrogen bonding, we coupled these encapsulated enzymes to fluorescent polystyrene (PS) particles which bear surface amino groups. For this, we used a Suberic acid bis(*N*-hydroxysuccinimide ester) crosslinker (DSS), able to connect via covalent bond formation amino to amino groups.²⁸ In this case, the enzymatic activity of the resulting particles was very low and difficult to measure with enough precision. However, the movement could be mainly observed in particle agglomerations (Fig 5c-d), which might be due to several factors. In the first instance due to the particle size of 1 μm the observation of single particles is more difficult, so bigger agglomerates easier catch the attention of the experimenter. On the other hand the anchoring of enzymes might be more favoured in agglomerations due to increased van der Waals forces. Bubble formation by catalase between fluorescent particles can be seen in video 3 ESI. Analogous observations as in silica motors confirm that the movement last longer and after stalling the movement could be reactivated by addition of new fuel.

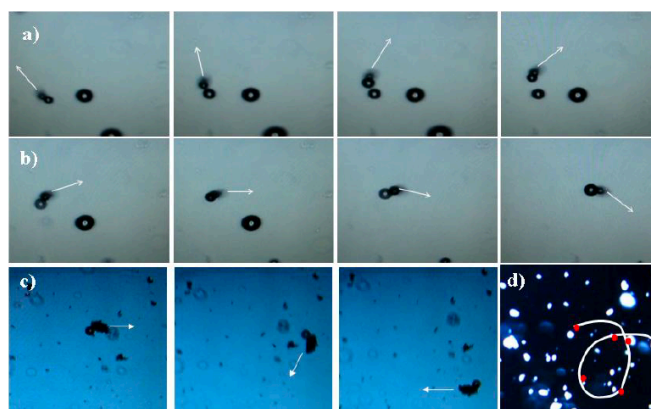


Fig. 5 a-b) Sequence of pictures showing a circular movement of a Silica motor caused by jet-propulsion mechanism. c) Sequence of pictures showing a circular movement of a PS particles motor and d) Trajectory followed by PS aggregate by fluorescence microscopy.

Conclusions

In this paper we present a method to encapsulate catalase in a novel polymer shell bearing amine groups which allows further attachment on different surfaces. The enzyme-capsules were characterized regarding their appearance, surface properties and their catalytic efficacy. Afterwards, a proper methodology for their grafting on motor particles was established and the improved performance of those motors evaluated. We found that the durability of our micromotors bearing enzyme capsules was manifold increased compared to motors with native enzyme. The reusability of both types was compared and the capsules were more stable after thermal

and enzymatic treatment, as well as in peroxide rich environment. Concluding, we can say that encapsulation of catalase strongly improved the resistance and reusability of catalase based micromotors. This result could pave the way to employ other enzymes, making accessible the use of other biologically relevant fuels.

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Notes and references

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