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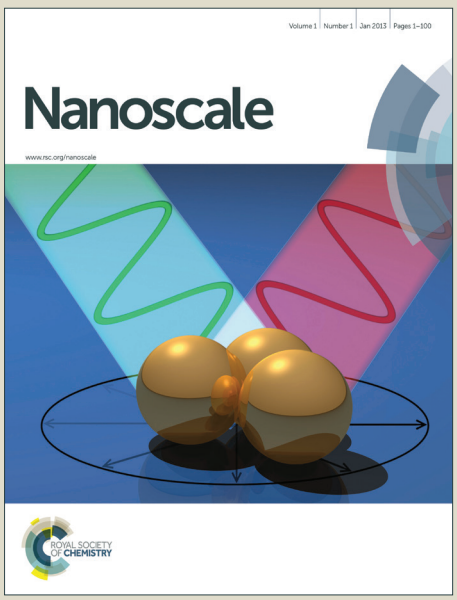

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Paper

Cross-linked polymersomes as nanoreactors for controlled and stabilized single and cascade enzymatic reactions

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Polymeric vesicles or polymersomes are one of the supramolecular entities at the leading edge of synthetic biology. These small compartments have shown to be feasible candidates as nanoreactors, especially for enzymatic reactions. Once cross-linked and equipped with pH sensitive material, the reaction can be switched off (pH 8) and on (pH 6) in accordance with the increased permeability of the polymersomes´ membranes under acidic conditions. Thus cross-linked and pH sensitive polymersomes provide a basis for pH controlled enzymatic reactions where no integrated transmembrane protein is needed for regulating the uptake and release of educts and products in polymersomes´ lumen. This pH-tunable working tool was further used to investigate their use in sequential enzymatic reactions (glucose oxidase and myoglobin) where enzymes are loaded in one common polymersome or in two different polymersomes. Crossing membranes and overcoming space distance between polymersomes was shown successfully, meaning that educts and products can be exchanged between enzyme´s compartments for successful enzymatic cascade reactions. Moreover the stabilizing effect of polymersomes is also observable by single enzymatic reactions as well as a sequence. This study is directed to establish robust and controllable polymersome nanoreactors for enzymatic reactions, describing a switch between an off (pH 8) and on (pH 6) state of polymersomes´ membrane permeability with no transmembrane protein needed for transmembrane exchange.

1. Introduction

Nature or natural processes have always been an inspiration for scientists, who want to rebuild them by synthetic means. Along with the development of tools to analyze cells and single cellular processes, synthetic biology arose in order to mimic cellular processes or cellular compartments.[1-3] The latter ones, called liposomes, have found their synthetic counterpart in polymersomes.[4, 5] Just like their biological equal, these polymeric vesicles are made up of a bilayer structure, which is now created of amphiphilic block copolymers instead of lipids.[4, 6-8] Their biomimetic structure makes them ideal, versatile research objects in synthetic biology,[1, 3, 9] for example in biomedical applications (drug-delivery systems),[10-13] as well as nanoreactors,[14-17] when encapsulating enzymes. Moreover, alternatives to polymeric vesicles are hollow capsules, e.g. using a layer-by-layer approach,[18-20] composed of polyelectrolyte multilayers in synthetic biology. Those supramolecular entities have been used for enzymatic conversions too, but without any further control over enzymatic activity and diffusion processes of educts and products.[21, 22]
In order to establish nanoreactors with no transmembrane proteins or protein channels for synthetic biology, key properties would be the following: (a) polymersome-forming block copolymers containing a stimuli-responsive component in the hydrophobic block,[23-25] (b) permanent encapsulation of the catalyst within the nanoreactor, (c) controllable diffusion processes through the nanoreactors wall from inside to outside and vice versa,[26-28] and (d) switching the catalytic activity on and off. Moreover, the most challenging point would be to control enzymatic cascade reactions with two (or more) catalysts encapsulated in separate nanoreactors.
Several reports exist on a variety of block copolymers in which the hydrophobic block turns (partially) into a hydrophilic one upon a specific external trigger. Tailoring polymersomes´ membrane disintegration by external stimuli consequently results in cargo release into the external matrix of the polymersomes.[29, 30] Besides various amine-based pH sensitive polymers,[31-34] temperature-[35] and redox sensitive[10, 36, 37] macromolecules have shown to be feasible candidates for this purpose. The advantage of pH sensitive systems is their release mechanism, which can exploit the pH decrease during the endolysosomal uptake path into cells.[38, 39]

The permanent encapsulation of the catalyst within the nanoreactor has been realized, while the controlled transmembrane diffusion needs to be allowed for substrates and reaction products.[13, 15] Enclosure of the enzymes (or other nanoparticles) is usually realized during the self-assembly process of the

polymersomes.[17] If internalized afterwards, methods like electroporation[40] or an endocytosis-like process[41] have shown to be feasible. For transmembrane diffusion of the substrates (Key property c) and products in single and cascade reactions on the other hand, transmembrane proteins are often incorporated to ensure an efficient exchange of molecules across the membrane.[23, 42, 43] These proteins can be simple channel proteins[3, 44] or specific enzymes to gate targeted molecules only, including a transformation of the molecules.[45-46] Another method to control the permeability of the membrane is CO_2,[47] pH itself[48] or a combination of cross-linking and a sensitive polymer within the hydrophobic part of the amphiphilic block copolymer.[8, 49, 50] Here, the cross-linking can either happen physically[51] or chemically using a photochemical reaction.[35, 49, 50, 52] As we have shown previously, photo-cross-linking leads to enhanced mechanical strength, a reversibly swelling membrane and allows for efficient control of transmembrane traffic.[17, 49, 50] Hence, a simple and non-toxic nanoreactor could be constructed and the desired pH control was shown for one cycle[17, 53] (referring to key property "d"). These initial results demonstrated that these photo-crosslinked polymersomes in principle cover all prerequisites outlined above for a nanoreactor, however, transmembrane diffusion of the substratewas proven only one way and just once. This motivated us to explore their potential in enzymatic reaction cycles and sequences for the establishment of artificial nanoreactors without any transmembrane proteins. Very recently, a new approach in enzymatic cascade reactions has been established by using multicompartmentalized polymersomes. However, there, the enzyme activity was triggered by passive transmembrane transport and could not be stopped once started.[54]

For a synthetic, microfluidic or industrial relevant application, a multiple controlled membrane crossing is necessary and reaction sequences in separate compartments must be possible. Consequently, we are interested in the robustness of our system by implementing various enzymatic reactions over multiple cycles and a number of days. On top of that, a reaction sequence between different enzymes in different polymersomes is aimed for to finally prove the technical relevance of our polymersome system. This would be the first time to show a pH controlled reaction sequence in polymersomes with separated reaction compartments. This study is directed to establish alternative nanoreactors without using any transmembrane proteins for transmembrane traffic but by tailoring the exchange of educts and products between different enzyme-loaded polymersomes by deploying a pH-driven permeability switch (pH 8 - off vs pH 6 - on) in the polymersomes' membrane (Figure 1B).

2. Experimental Section

Materials: If not stated otherwise, all chemicals were used as received. All chemicals, anhydrous tetrahydrofurane (THF, Sigma-Aldrich), anhydrous 2-butanone (Fluka) and triethylamine (Fluka) were stored over molecular sieve. Poly(ethylene glycol) methyl ether (MeO-PEG-OH; M_n ca. 2000 g/mol; Mw/Mn = 1.05), diethylaminoethyl methacrylate (DEAEM), 2,2'-bipyridine, 2-bromoisobutyryl bromide, 2-aminoethanol, 4-aminobutanol, methacryloylic chloride, copper-I-bromide, aluminium oxide (neutral, activated), 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, peroxidase from horseradish (HRP, essentially salt-free, lyophilized powder), 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), glucose oxidase from aspergillus niger (GOx, lyophilized powder), myoglobin from equine skeletal muscle (Myo, essentially salt-free, lyophilized powder), guaiacol, phosphate buffered saline (tablet), glucose, and magnesium sulfate were purchased from Sigma-Aldrich. 3,4-Dimethylmaleic acid anhydride, THF, toluene, chloroform-*d* and ethyl acetate were purchased from Acros Organics. From Merck (Germany) *n*-hexane, hydrochloric acid (37 %) and silica gel were purchased. Sodium hydroxide was purchased from Riedel-de-Haën.

Methods: The molecular weight distributions of the copolymers were assessed at 40 °C using a Polymer Laboratories PL-GPC50 Plus Integrated GPC system (Agilent Technologies, USA) equipped with a Polymer Laboratories pump, a PL ResiPore column (300 × 7.5 mm), a PL data stream refractive index detector and a PL-AS-RT Autosampler. The calibration was carried out using twelve polystyrene standards with M_n values ranging from 162 to 371,100 (Agilent Technologies, USA). The eluent was THF and the flow rate was 1.0 mL/min. The data were processed using Cirrus GPC offline GPC/SEC software (version 2.0).

^{1}H and ^{13}C NMR spectra were recorded using Bruker Avance III 500 spectrometer operating at 500.13 MHz (^{1}H) and 125.77 MHz (^{13}C), with $CDCl_3$ as solvent at room temperature. The copolymer compositions were determined from ^{1}H NMR spectra in dry $CDCl_3$, using the integrated signal assigned to the PEG block as an internal standard.

DLS studies of 2 g/L aqueous vesicle solutions were carried out over a range of pH at 25 °C using a ZETASIZER Nano series instrument (Malvern Instruments, UK) equipped with a multi-purpose autotitrator (MPT-2) and Dispersion Technology Software (version 5.00). The data were collected by the NIBS (non-invasive back-scatter) method using a Helium-Neon laser (4 mW, λ = 632.8 nm) and a fixed angle of 173°. All data were obtained using vol-% evaluation, assuming an RI of 1,5 for the polymer. The peak size given is the z-average within the measurements, except for radiation dependent measurements (Figure 7-SI), where the peak maximum was used.

The UV irradiation was carried out within a EXFO Omnicure 1000 (Lumen Dynamics Group Inc., Canada), equipped with a high pressure mercury lamp as UV source.

The hollow fibre filtration (HFF) was performed using a KrosFlo-Research-IIi (SpectrumLabs, USA), equipped with a polysulfone-based separation module (MWCO: 500 kDa, SpectrumLabs, USA). The actual cleaning procedure is described in the Electronic Supplementary Information (ESI).

The UV-Vis absorption spectra were recorded on a SPECORD 210 Plus (Analytic Jena, Germany). All investigations were performed in 1.5 mL semi-micro cuvettes of PMMA (Brand, Germany).

The pH value of the solutions were determined with a HI 221 Calibration Check Microprocessor pH Meters (Hanna Instruments, USA) after calibration of the electrode system with aqueous buffers (pH value 4.01 and 10.04).

Enzyme-filled polymersome preparation: 10 mg of Polymer was dissolved in 2 ml water at pH 1.5, while 1 mg of the enzyme was dissolved in 4 ml of 0.1 M PBS (pH 7.4). Both solutions were combined and the pH adjusted to pH 8 by adding 0.5 M NaOH slowly. The solution was stirred for 3 days. To receive cross-linked enzyme-filled polymersomes, 2 mL of the solution were placed in the UV-chamber and was irradiated for 40 seconds. The resulting two mixtures (cross-linked and non cross-linked enzyme-filled polymersomes) were

cleaned from non-enclosed enzyme using HFF (description in SI).

Enzymatic study: The enzymatic stock solutions were prepared and treated as described in SI. For activity experiments without polymersomes an aliquot of 300 µL of the prepared solutions (Myo or HRP, respectively) was used. The sample was treated with 8 µl of 0.1 M substrate solution in 0.1 M PBS at pH 7.4 (guaiacol for Myo and ABTS for HRP) and 8 µl of 1 M H_2O_2 (solution in 0.1 M PBS at pH 7.4). After the preparation the UV monitoring started (abs = 470 nm) and data points were recorded every second. The relative activity was determined after 300 seconds for Myo and 100 seconds for HRP. For normalized activity of enzyme activity presented in the relevant figures, one value, in most cases the highest value, was fixed as 100 % to normalize the other determined enzyme activities within one experiment series or repeating experiment series. Free GOx was examined as follows: An aliquot of 250 µL of the prepared solutions was used. The sample was treated with 8 µl of 0.1 M guaiacol (solution in 0.1 M PBS at pH 7.4), 8 µl of 1 M glucose (solution in 0.1 M PBS at pH 7.4) and 50 µl of 1 M Myo (solution in 0.1 M PBS at pH 6 or 8, respectively). After the preparation the UV monitoring started (abs = 470 nm) and data points were recorded every second. The relative activity was determined after 200 seconds.

Enzymatic studies in polymersomes: The filled polymersomes were prepared as described above (details in SI). For the activity experiments within one polymersome an aliquot of 300 µL was used. One aliquot was taken at once, while two next ones were taken after a pH switch to pH 6 (1 aliquot) and back to pH 8 (1 aliquot) was conducted. For an activity experiment, the sample was treated with 8 µL of 0.1 M guaiacol (solution in 0.1 M PBS, pH 7.4) and 8 µL of 1 M glucose (solution in 0.1 M PBS, pH 7.4). The sample was stirred for 5 minute and the UV monitoring (abs = 470 nm) started afterwards, data points were recorded every second. The activity experiments between two cross-linked polymersomes, first the prepared solutions of Myo- and GOx-filled cross-linked polymersomes were combined in a ratio of 1:1. An aliquot of 300 µL was used for the following activity experiments. One aliquot was taken at once, while two next ones were taken after a pH switch to pH 6 (1 aliquot) and back to pH 8 (1 aliquot) was conducted. For an activity experiment, the sample was treated with 8 µL of 0.1 M guaiacol (solution in 0.1 M PBS, pH 7.4) and 8 µL of 1 M glucose (solution in 0.1 M PBS, pH 7.4). The sample was stirred for 5 minute and the UV monitoring (abs = 470 nm) started afterwards, data points were recorded every second.

3. Results and Discussion

Our pH sensitive and photo cross-linked polymersomes consist of a multifunctional amphiphilic block copolymer, which was synthesized using a standard ATRP approach.[34, 55] It consists of the well-known biocompatible poly(ethylene glycol) (PEG)[37, 56, 57] as the hydrophilic part and a statistical mix of two components in the hydrophobic part providing the desired functionalities.

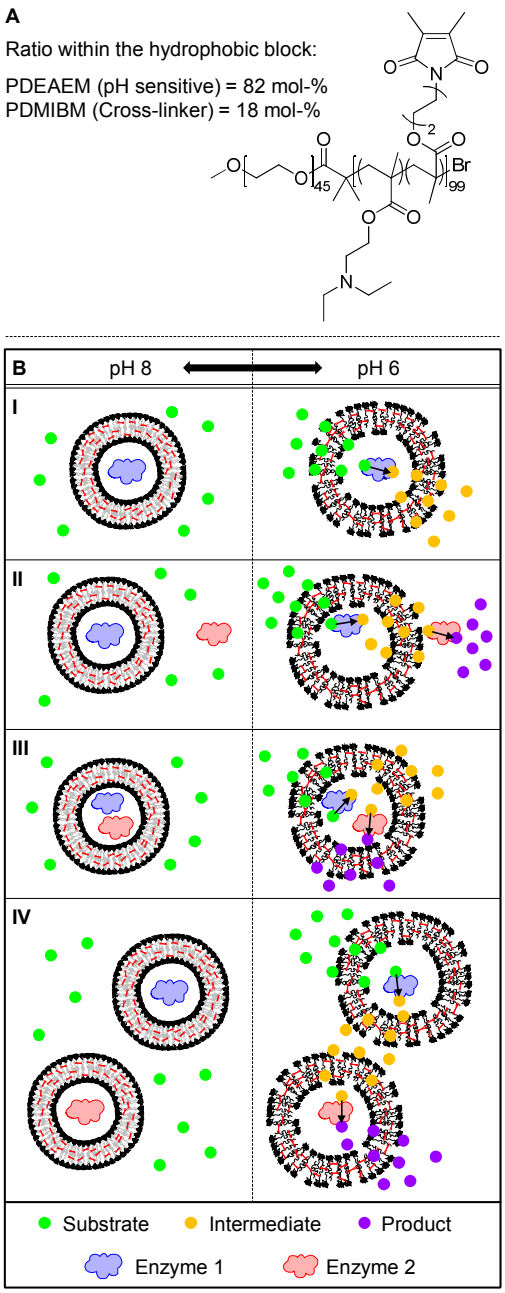

Fig. 1 (A) Chemical structure of the polymer used. (B) Enzymatic reactions were conducted for a single enzyme (I) and for a sequence of two enzymes (II-IV) where enzymes are enclosed in one or two polymersome(s): (II) Only enzyme 1 enclosed in the vesicle; (III) Enzymes 1 and 2 statistically enclosed in one vesicle; (IV) Enzymes 1 and 2 enclosed in separate vesicles. For all, reactions were monitored exclusively at pH 6. Further details for supporting sequential enzymatic reactions presented in Figures 2-8.

While pH sensitivity is provided by poly(diethylaminoethyl methacrylate) (PDEAEM, 82 mol-%), the photo cross-linkable poly(3,4-dimethyl maleic imido butyl methacrylate) (PDMIBM, 18 mol-%) (Figure 1A) provides stability towards disassembly upon UV irradiation. Similar to our previously reported results, the polymersomes experienced sufficient cross-linking after 40 s of UV irradiation (see ESI).

In an initial study we had demonstrated that controlled transmembrane traffic of photo cross-linked polymersomes is possible by pH change to yield a bionanoreactor with myoglobin as an incorporated enzyme.[17] Swelling behaviour of the vesicles is explainable by the protonation of the pH sensitive amino units in the hydrophobic block upon acidification.

However, due to the crosslinking, this protonation does not lead to polymersome disassembly as in non cross-linked vesicles.[17] Thus, the repelling force between the positive charges due to amine protonation is now counter-balanced by the cross-linking bonds created. As a result, the polymersomes show a reversible swelling upon acidification.[17] Once swollen, the membrane is hydrophilic and permeable, allowing small molecules to pass it via diffusion. This way, a previously encapsulated enzyme is only fed with substrate in an acidic pH value (pH 6) (Figure 1B, scheme I). Enzymatic conversion is stopped by changing the pH to basic values (pH 8).[17] In order to prove the technical relevance of our enzymes-encapsulated polymersomes we decided to address the challenge of an enzymatic cascade reaction. As a first step enzyme 1 is integrated in the polymersomes and an excess of free enzyme 2 is added to the solution of enzyme 1 loaded polymersomes (Figure 1B, scheme II). Thus, the reaction product from enzyme 1 has to cross the swollen polymersome membrane to initialize the reaction of enzyme 2 (Figure 1B, scheme II). In the next experiment (Figure 1B, scheme III), a mixture of both enzymes was encapsulated in the polymersomes. Since polymersomes are roughly 100 nm in diameter and the enzymes about 4-7 nm, there is a high chance that both types of enzymes are encapsulated within the same polymersome. Finally, just governed by statistics, polymersomes with mixed enzymes will be created to a larger extent.

Now, the product of enzyme 1 has to diffuse within one polymersome. Consequently, a short way is needed to initialize the second reaction. Hence, the cascade reaction between the two enzymes might be facilitated compared to the first experiment (Figure 1B, scheme II). In a final approach (Figure 1B, scheme IV) each type of enzyme is encapsulated separately into the polymersomes and the two polymersome solutions are mixed afterwards. In that case, the intermediate reaction product from enzyme 1 has to leave the first polymersome and then enter another polymersome through the acidified membrane in order to initialize the reaction of enzyme 2 (Figure 1B, scheme IV). Ideally, all enzymatic reactions take place exclusively at acidic conditions when the polymersome membranes are swollen (pH 6) and do not occur at a basic pH value when tight polymersome membranes are present (pH 8).

From the variety of enzymes available, we chose glucose oxidase (GOx) as enzyme 1, which turns D-glucose into D-glucono-δ-lactone (Figure 2C) and hydrogen peroxide.[58-60] Amongst many, the latter also acts as a cosubstrate for myoglobin (Myo) to oxidize guaiacol[58, 59, 61, 62] and for horse radish peroxidase (HRP) to oxidize ABTS (Figure 2).[63, 64] These enzymes (Myo and HRP) were chosen, since their corresponding reaction products are detectable using UV/Vis spectroscopy. In order to choose the better one as enzyme 2, Myo and HRP were analyzed separately (details presented below).

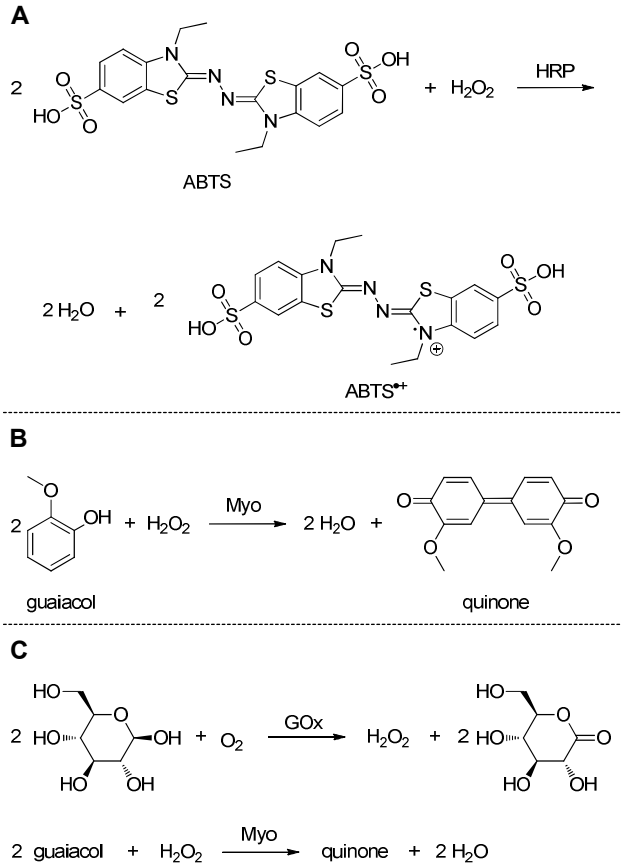

Fig. 2 Enzymatic reactions carried out: (A) enzymatic reaction for horse radish peroxidase (HRP),[63, 64] (B) enzymatic reaction for myoglobin (Myo),[62] (C) enzymatic cascade reaction for glucose oxidase (GOx)[60] and Myo used as example for the schemes II-IV in Figure 1.

In order to know the final amount of encapsulated enzymes, we checked the activity of the free ones at different concentrations yielding a standard calibration. The activity of the enclosed enzymes can therefore yield the effective concentration enclosed in the polymersomes. As we know the initial amount of enzyme added, this can give us an encapsulation efficiency (Figure 3A and ESI for details). Although only low amounts could be encapsulated (below 1 %), the amount was sufficient to perform enzymatic studies. Also, we were able to show that each and every single component mentioned in conjunction with Figure 2C (GOx cascade) is necessary for a complete reaction sequence, as this was the only circumstance where a reaction could be observed. As soon as one component is missing, no reaction can be observed (Figure 3B and ESI for details).

Independent of their concentration and final use, all three free enzymes were investigated under non-irradiated and irradiated conditions at pH 6 and 8 (Figure 4A). This was to ensure that the cross-linking process of the polymersomes and the pH switch would not affect them. It became quite obvious that the enzymes respond quite differently towards the UV irradiation applied (t = 40 s). In detail, GOx (enzyme 1) possesses the required high enzymatic activity at pH 6 before and after UV irradiation, but a slightly lower one at pH 8 for both conditions. For Myo and HRP (both candidates for enzyme 2), HRP, surprisingly, reveals a slightly higher

enzymatic activity if UV-irradiated, regardless of the pH value. Myo, however, shows a significant decrease in enzymatic activity (<40 %) if switched from pH 6 to pH 8, regardless of the irradiation state. In summary, all enzymes show a similar activity after 40 s of UV irradiation and only Myo shows a significant decrease in activity going from pH 6 to pH 8. It is therefore reasonable to use all enzymes tested for further investigations and rule out none at this stage.

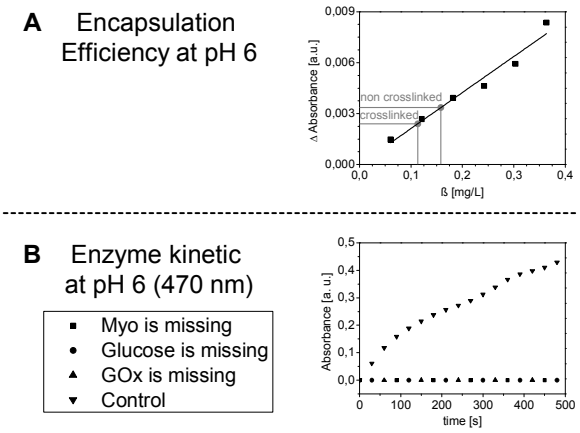

Fig. 3 (A) Calculation of internalized enzymes via standard calibration – Regression line f(x) = 0.0212x – 0.0000013 with R^2 = 0.968. (B) enzyme kinetics proving that all ingredients (Myo, GOx, Glucose) are necessary for a full conversion. A lack of substrates and enzymes result in no absorbing properties of enzymatic cascade reaction (Figure 2C).

The next fundamental step was to distinguish between pH dependent enzyme activity in cross-linked and non cross-linked polymersomes (Figure 4B) using HRP and Myo. During the encapsulation process of HRP and Myo into the polymersomes, obviously, not every enzyme was successfully enclosed and had to be removed afterwards. As before, we used a pressure-dependent separation system (hollow fibre filtration, HFF) to separate polymersomes from remaining free enzymes (see ESI).[17, 53] The successful enclosure of the enzyme needed to be proven and was achieved by monitoring the enzyme activity at pH 8. At this pH value, the polymersomes are at a non-swollen state and the membrane is not permeable, regardless of its cross-linking state. Consequently, no activity could be monitored for HRP as well as for Myo (Figure 4B, state b) proving that no free enzyme was present after the purification process. In contrast, large activity could be monitored at pH 6 for both enzymes tested in the cross-linked and non cross-linked state. At pH 6 the non cross-linked polymersomes disassemble, liberating the enzyme, while the acidified cross-linked membrane is now allowing for diffusion of the substrate across the membrane (Figure 4B, state b-a). This clearly proves a switch in membrane permeability upon membrane acidification. Furthermore, it could be proven that the membrane becomes leaky for the substrate only and not for the enzyme. This was shown by switching back to pH 8 afterwards (Figure 4B, state b-a-b), showing no enzyme activity again for the cross-linked polymersomes. In contrast, if the same pH sequence is applied on non cross-linked polymersomes (Figure 4B, from state b-a to b-a-b), a significantly higher residual activity was monitored, once a basic pH was reached again. This is due to the enzymes being not encapsulated into the polymersome anymore. Still, also for the non cross-linked system, compared to the free enzyme (Figure 4B), the decrease in activity is larger than just a result from the pH switch (Figure 4B). Here, we suspect that a substantial number of enzymes is covered by polymer agglomerates which form upon the switch back into the basic region and those agglomerates hinder diffusion of the substrate to the enzyme. Such agglomerates can be proven by DLS analysis after switching back to a basic state as there is a clear difference with and without enzymes by the additional peak observed once enzymes are present (ESI). These results prove that our concept of a pH controlled bionanoreactor based on two different enzymes encapsulated in cross-linked polymersomes (Figure 4B) works reliably. This is in great contrast to polymersome nanoreactors based on transmembrane proteins,[3, 39] which do not allow for a pH controlled reaction scheme but provide a generally constant permeability of the polymersome membrane under physiological conditions.

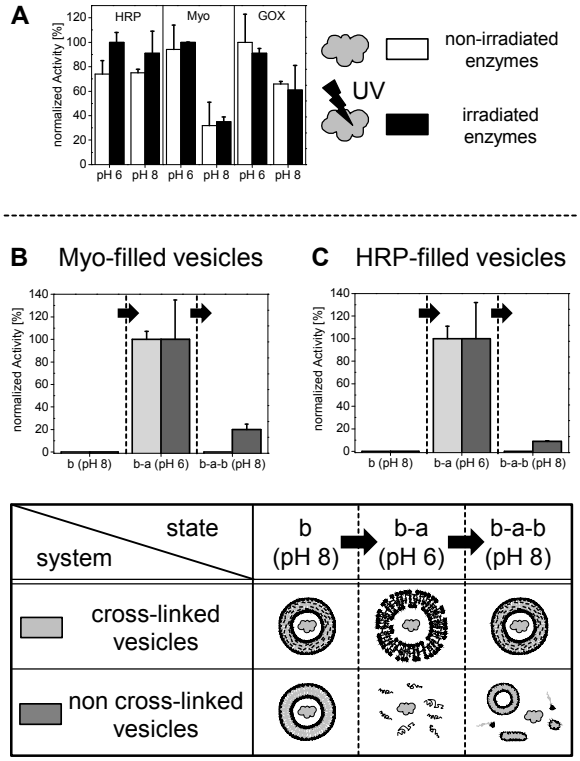

Fig. 4 Investigation of enzymatic activities in cascade reaction: (A) Enzyme activity of free enzyme in a non-irradiated state as well as an irradiated one, using 40 s of UV irradiation, for HRP, Myo and GOx. (B) pH dependent enzyme activity in cross-linked (40 s of UV irradiation) and non cross-linked polymersomes using Myo and HRP. Non cross-linked polymersomes disassembling at b-a state (pH 6) followed up by generating agglomerates with encapsulated enzymes besides partly free enzymes at b-a-b state (pH 8). Average data of three experiments are presented.

Since one swelling-deswelling cycle provided the reaction control desired, multiple cycles were to be tested in a next step (Figure 5). It was already known that the swelling-deswelling cycles of the polymersomes were stable over at least 5 cycles[17] and a continuous control over the enzymatic reactions over the same amount of cycles was therefore expected. It was unknown though, whether these repeated changes in pH might affect the enclosed enzymes in polymersomes. Thus, in control experiments HRP and myoglobin, in the free state and enclosed

in polymersomes, were subjected to several pH switches between pH 8 and 6. In order to have full experimental proof, this was monitored by UV-Vis spectrometry as well as visual analysis (Graphs and images in Figure 5). As expected, enzymatic reactions could be monitored exclusively at pH 6 for enclosed enzymes, due to the polymersomes being swollen at that pH as it was indicated by the strong green coloring of these samples. Furthermore, the enzymes encapsulated in the polymersomes show no residual activity at each time when pH 8 is reached (Figure 5, filled vesicles). Thus, enzymes are completely shielded in polymersomes at pH 8, causing a total loss in activity. In contrast to this, some residual activity was always monitored for the free enzyme solutions at pH 8 (Figure 5) not enclosed or free enzymes. This means that a basic pH hampers the activity of a free enzyme but totally blocks it for an encapsulated one. It can be noted, however, that the activity of Myo is in general more susceptible to the surrounding leading to a scattering in activity. Apart from that, full reaction control over the five cycles examined is given exclusively for enzymes in vesicles due to the reproducible change in polymersome membrane permeability.

However, it appears that increasing salt concentration, resulting in an increase in ionic strength during the cycle process, affects the enzyme activity as presented in Figure 5. This occurs independently whether the enzymatic conversion is carried out in free solution or in the cavity of the polymersomes. This may be a reason why Myo outlines high scattering in enzymatic activity throughout the cycle process.

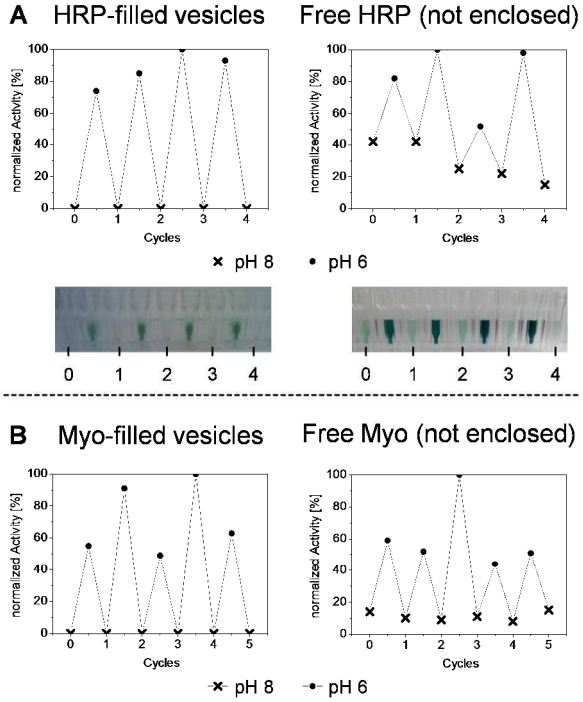

Fig. 5 Investigation of enzymatic activities in cascade reaction - Enzyme activity recorded for repeated pH changes for (A) HRP and (B) Myo. The polymersome-enclosed enzymes always have no residual activity at high pH values (pH 8), while the free ones do. This proves the stability of the vesicle membrane against successful educt and product transmembrane diffusion to enzymes in polymersomes at pH 6. The pictures included underline that the reactions only occur at an acidic pH (green colouring). Only one cycle process of three cycles is presented here.

After the investigation of the single enzymes, we now moved on the challenge of a cascade reaction and investigated the first cascade reaction presented as scheme II in Figure 1B. For that GOx as enzyme 1 was used. While no advantage of either Myo or HRP could be determined from their enzymatic activity, Myo was chosen as enzyme 2 due to its reaction approach being easier to handle. Initially, GOx is enclosed in the polymersomes and Myo was added together with D-Glucose and guaiacol (Figure 1B scheme II) after non enclosed GOx was removed via HFF purification step (ESI). We now had an additional diffusion barrier within the system. As previously mentioned GOx catalyzes the transformation of added glucose and produces hydrogen peroxide as a side product. Once formed, hydrogen peroxide can now leave the vesicle through the acidified membrane to reach the myoglobin and guaiacol, both added to the GOx-enclosed polymersome solution. Myo then catalyzes the oxidation of guaiacol by means of hydrogen peroxide.[58, 59] Then, the production of the final product, oxidized guaiacol, was monitored by UV-Vis spectroscopy. The results of this cascade reaction (Figure 1B, scheme II) are presented in Figure 6.

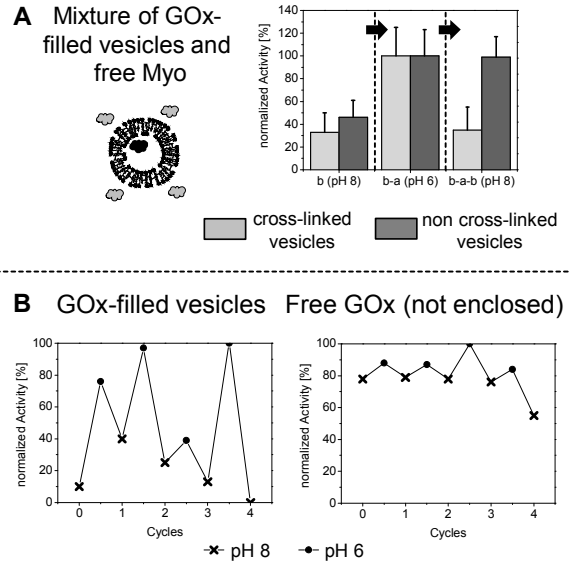

Fig. 6 Encapsulated and free GOx in the cascade reaction with free Myo added later on (example for scheme II in Figure 1B). Only when encapsulated in polymersomes the pH control of the GOx activity is obvious over various cycles. The free enzyme shows little pH dependency in its activity, while the GOx in polymersomes is far more active in an acidic state. Only one cycle process of three cycles is presented here.

In this experiment (Figure 6) we detected enzymatic activity already at pH 8, which is most probably due to some initial activity of myoglobin. The guaiacol added can already be oxidized by traces of hydrogen peroxide or other oxidizing agent, which are always present in aqueous solutions. However, once an acidic pH value (pH 6) was reached (Figure 6A), the activity was greatly enhanced due to the production of hydrogen peroxide by GOx, which was now produced due to the glucose added. As in the previous experiments, the activity went down to its previous level once pH 8 was reinstated, indicating the same control over the reaction as in all other

experiments (Figure 6A). However, the residual and initial activity suggests that not all non-enclosed GOx was removed, which is quite reasonable due to the bulky structure of the enzyme.[59] The principle of the pH control nanoreactor is still proven due to the results with the non cross-linked vesicles. Here, the level of enzymatic activity also rose upon acidification, but did not return to its original value when the solution was turned back to pH 8 again (Figure 6A). After acidification, the polymersomes disassemble and the free GOx is now still available after returning to pH 8, leaving enzymatic activity at a high level. This shows us that GOx is safely enclosed in the cross-linked polymersomes and does not pass the membrane at acidic conditions.

The switching process for this cascade reaction (scheme II in Figure 1B) was now repeated four times to evaluate, whether the reaction control remains over repeated pH changes (Figure 6B). As it could be expected, the detected activity never reached zero due to the reasons mentioned previously for one cycle with GOx containing polymersomes (Figure 6A). However, a substantial difference between the activity at acidic and basic conditions could be monitored at each time (Figure 6B). In contrast, the control with a pure enzyme showed higher activity levels at basic conditions in comparison to those of GOx-filled polymersomes and only small changes upon switches in the pH value. Hence, the control of the reaction sequence across the polymersome membrane remains over various cycles. This indicates that the polymersome membrane always goes back to its native state, where it is virtually closed for transmembrane diffusion processes and opens up completely upon swelling. This highly reproducible behavior was now proven with 2 single enzymes and one reaction cascade and may be taken as a general concept for this kind of polymersome.

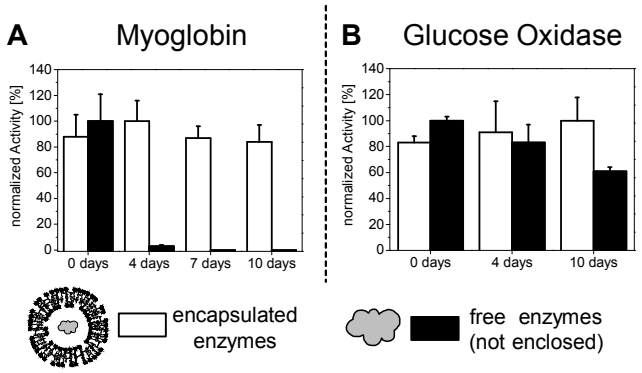

Fig. 7 Activity of enzymes (free and enclosed) over various days. (A) While free myoglobin shows a quick and large loss in activity, encapsulated enzyme remains stable. (B) For free GOx, a slight decay in activity could be observed, while the encapsulated one remained fully stable. Average data of three experiments are presented.

For technical use, for example, as "Lap-on-Chip device", the traffic across the membrane should not decay over time and the polymersome cavity should protect the enclosed enzyme (stabilization effect of polymersomes). The previously mentioned theory of the stabilization or stealth effect[53, 65, 66] was now to be proven by comparing enzyme activity within polymersomes and a solution of free enzyme (Figure 7). Thus, previously used enzymes, Myo and GOx, of the first cascade reaction were also used for long-term activity study. Consequently, free Myo and polymersomes-encapsulated Myo were studied over various days. Initially both solutions showed high levels of activity, but after 4 days almost no reaction product could be detected for free myoglobin and activity ceased completely after 7 days of stirring at pH 8. In contrast, the Myo encapsulated in the polymersomes showed no loss in activity even after 10 days (Figure 7). Hence, our encapsulation into polymersomes induces a long-term stabilizing effect on Myo, proving the stealth effect. In contrast to Myo, free GOx shows substantial enzymatic activity even after ten days (Figure 7) but still a slight gradual decay in monitored activity over time was noted. As with myoglobin, no decay at all in enzyme activity was observed when GOx was enclosed into our cross-linked vesicles proving the general stealth effect of our polymersome system. Additionally, transmembrane diffusion at swollen state is not hampered over time but remains at a constant level allowing small molecules entering and leaving the polymersome lumen.

Next we included both, GOx and Myo, in the same polymersome and cross-linked the system afterwards (Figure 1B, scheme III) to investigate the second possibility for carrying out a cascade reaction. In contrast to the previous system (scheme I in Figure 1B), both enzymes are now protected against degradation because of their encapsulation in the polymersomes. Logically, this system was now treated with glucose and guaiacol at the same time to monitor the reaction sequence. As with the pure enzymes, no activity was monitored at pH 8, but the system started working nicely after pH 6 was reached (Figure 8A). In this swollen state, both, glucose and guaiacol can enter the polymersomes readily. Once glucose is processed by GOx and hydrogen peroxide present, the small molecule can diffuse fast within the polymersomes to an adjacent Myo molecule. Now the second enzyme (Myo) starts to work and produces the oxidized guaiacol, which could be detected right after the substrates were added. When the system was brought back to pH 8 the reaction stopped. Hence, also this short reaction sequence within one polymersome can be controlled using the pH value of the hosting solution. Under the assumption that the uptake process of both enzymes into the polymersomes runs statistically, polymersomes with both enzymes are created and will be present, but polymersomes with just one kind of enzyme cannot be avoided completely.

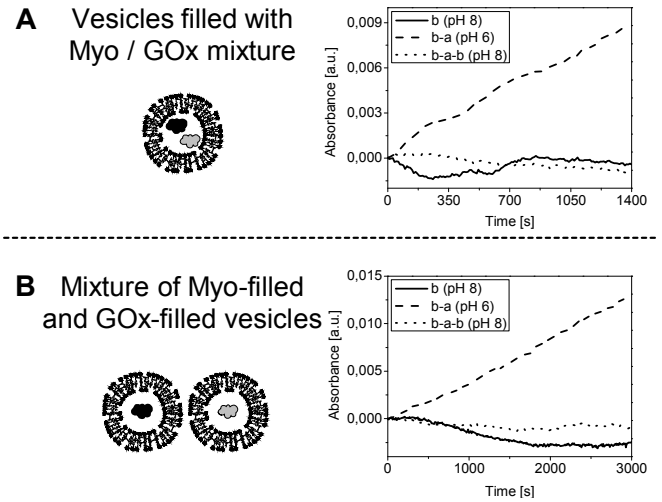

Fig. 8 (A) GOx and Myo encapsulated in one polymersome (example for scheme III in Figure 1B) and (B) in different polymersomes (example for scheme IV in Figure 1B). Both systems show reactivity at pH 6 only and none at before and

after switching to these conditions. This shows the complete control over transmembrane traffic, also various membranes and substrates are involved.

In the final experiment for potential cascade reactions two different sets of polymersomes were created separately: the first one was filled with GOx and the second one with Myo (Figure 1B, scheme IV). In order to observe the final reaction product of the enzymatic cascade reaction, the oxidized guaiacol, by UV-Vis spectroscopy (Figure 8B), all oxidation steps for enzyme 1, GOx, and enzyme 2, Myo, have to work smoothly (Figure 3). During these oxidation steps, the cross-linked polymersome membrane has to be crossed twice by hydrogen peroxide. For once, it has to leave the GOx filled polymersomes and then secondly enter a myoglobin filled polymersome. As it could be expected from previous results, no reaction was monitored (Figure 8B, state b) after the initial addition of the substrates glucose and guaiacol at pH 8 to the polymersome mixture, assuming that under those conditions both substrates could not reach their corresponding enzyme due to the tight polymersome membrane. In contrast, both enzymes worked at pH 6, since the oxidized guaiacol could be observed soon after both substrates were added (Figure 8B, b-a). Like in the previous experiments, the catalytic activity stopped again upon returning to pH 8 (Figure 8B). These results impressively outline how transmembrane diffusion across two membranes can be controlled effectively using our pH sensitive and cross-linked polymersomes. While diffusion is possible in acidic state also across multiple bilayers, it is knocked out completely at basic conditions.

Thus, it gives a promising way to switch enzymatic cascade reactions off and on in totally artificial nanoreactors under non-physiological conditions. These conditions resemble preferentially around physiological ones at pH 7.4 and will not harm enzymes´ activity in polymersomes´ lumen, also not over a longer period of time. These artificial nanoreactors can be considered as an alternative working tool in synthetic biology to establish more complex cascade reactions in terms of sequential and/or parallel reaction sequences. In contrast, previously described nanoreactors can draw their attention to strong basic pH[47] or to permanently permeable membranes triggered by various diffusion mechanisms.[42, 44, 46, 52]

4. Conclusion

We studied the use of our cross-linked and pH sensitive polymersomes as nanoreactors for enzymes. These polymersomes show a characteristic definite and reproducible swelling-deswelling of the bilayer membrane upon repeated pH switches. We demonstrated in multiple ways that effective control over transmembrane diffusion, and thus of enzyme activity is reached via the pH value of the solution. Regardless, whether a single enzyme or a group of enzymes are enclosed in one or even in different polymersomes, enzyme substrate can only diffuse through the membrane of the polymersomes at an acidic, e.g. swollen state. Similar to the swelling-deswelling cycle of the pure polymersomes,[17] the control over reactivity lasts over repeated pH changes. At pH 8 no transmembrane diffusion occurs and hence, no reaction can be observed. Besides their ability to regulate enzyme reactivity, the polymersomes also protect the enzymes from loss of activity. Our studies show that a free enzyme in solution rapidly loses its catalytic activity, while polymersome-encapsulated enzymes retain their ability to catalyze reactions at the same level also after at least 10 days. Hence, our polymersomes have a considerable stabilization effect on enzymes.

We believe that these results show the great potential of our polymersomes to work as industrial nanoreactors due to their specific ability to protect, separate and control enzymatic activity in one solution. This finding is also emphasizing the demand to develop artificial supramolecular entities as nanoreactors without any transmembrane proteins for controlling membrane transport in synthetic biology. Moreover, our group is interested in establishing enzyme-enclosed polymersomes in microfluidic devices in the future where those enzymatic entities can couple and uncouple on surfaces by non-covalent interaction features to vary potential enzymatic cascade reaction for Lap-on-Chip device.

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Notes and references

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