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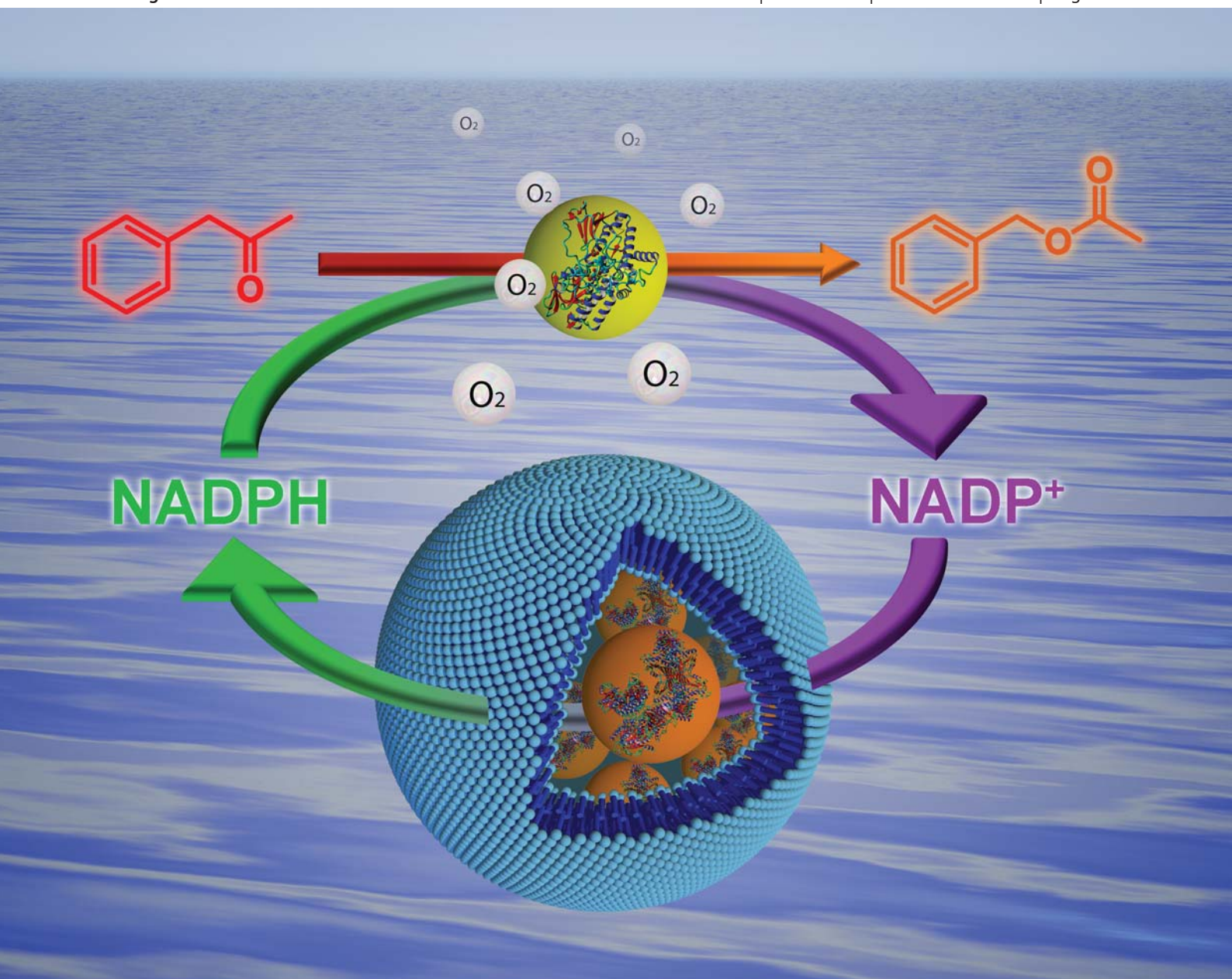

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Jan van Hest *et al.*

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COMMUNICATION

Cofactor regeneration in polymersome nanoreactors: enzymatically catalysed Baeyer–Villiger reactions†

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Enzymatically catalysed Baeyer–Villiger reactions were successfully performed making use of our novel cofactor regenerating nanoreactors. The system is based on intrinsically porous polymeric vesicles loaded with enzymes that are able to regenerate the cofactor NADPH.

The increasing interest in "green chemistry" processes has lately afforded an exponential growth of biocatalytic methodologies, in which enzymes are employed to perform organic reactions.[1,2] For instance, oxidoreductases offer the possibility to perform oxidative processes with a high level of chemo-, regio- and/or enantioselectivity under mild and sustainable conditions.[3]

Baeyer–Villiger monooxygenases (BVMOs) belong to a class of oxidative enzymes that can selectively catalyse the insertion of an oxygen atom adjacent to a carbonyl functional group, as well as the oxygenation of different heteroatoms.[4–6] To sustain their activity, the flavin present in the BVMO's active site has to be reduced after each catalytic cycle. Natural nicotinamide cofactors, such as nicotinamide adenine dinucleotide (phosphate) NAD(P)H, can be used as the electron supply for this purpose. However, stoichiometric use of this cofactor will not only severely hamper the efficiency of the biocatalytic reaction, it will also generate high costs when applied on a large scale.[7] In order to develop an employable, cell-free BVMO-catalysed process, an efficient cofactor recycling system is required. Chemical, electrochemical, and photochemical techniques have been developed to overcome this problem, but still are at an early stage.[8–10] In a more biomimetic approach, cofactor regeneration with isolated enzymes has been studied extensively, whereby glucose-6-phosphate dehydrogenase (G6PDH), alcohol dehydrogenase (ADH) and phosphite dehydrogenase (PTDH) have shown promising prospects.[11–14] In addition, the "self-sufficient" biocatalyst CRE2-PAMO was recently constructed by covalently fusing the thermostable BVMO phenylacetone monooxygenase (PAMO) from *Thermobifida fusca*[15] with PTDH.[16,17]

Polymersomes, assemblies of amphiphilic block copolymers in aqueous medium, have drawn particular attention in the last few years due to their resemblance to liposomes and cell membranes;[18] they are increasingly studied as model-templates for cell-like constructs and thereby of importance for both the field of biology and chemistry.[19] Compared with liposomes that are composed of small phospholipids or surfactants, high molecular weight polymer-based bilayer membranes exhibit superior chemical and physical stability.[20,21] Also, the chemical structure of the amphiphilic block copolymers can easily be modified, which provides polymersomes with properties suited for a range of applications.[22–25] The main drawback of a physically robust vesicle is the drastic decrease in permeability, which impedes diffusion of molecules both in and out of the polymersome. Block copolymers that self-assemble into an intrinsically porous bilayer can offer a solution to this problem, as has been demonstrated for the amphiphilic polystyrene-*b*-poly(3-(isocyano-L-alanyl-amino-ethyl)-thiophene) (PS-*b*-PIAT) block copolymer.[26,27] PS-*b*-PIAT vesicles proved to be permeable for small molecules but not for large biomolecules such as enzymes. For that reason, these polymersomes are greatly beneficial for biocatalytic transformations since they protect the enzymatic activity from harmful environmental effects while confining the reaction within the nanospace.[27,28] We envisioned that semipermeable vesicles should also be applicable for enzymatic cofactor regeneration. However, at the beginning of this investigation, it was not clear whether the (rather large) size of the cofactor would pose any limitations with respect to diffusion across the polymersome bilayer.

Herein, we report a novel cofactor regeneration system for BVMO-catalysed reactions with PS-*b*-PIAT polymersomes as biomimetic scaffolds for positional assembly of the essential enzymes (Fig. 1). Efficient cofactor regeneration was performed when the fusion protein CRE2-PAMO or G6PDH was encapsulated in the lumen of the vesicles, while in the latter case PAMO was separately added to the polymersome dispersion. Covalent immobilisation of PAMO onto G6PDH-loaded vesicles[29,30] yielded a less efficient reaction.

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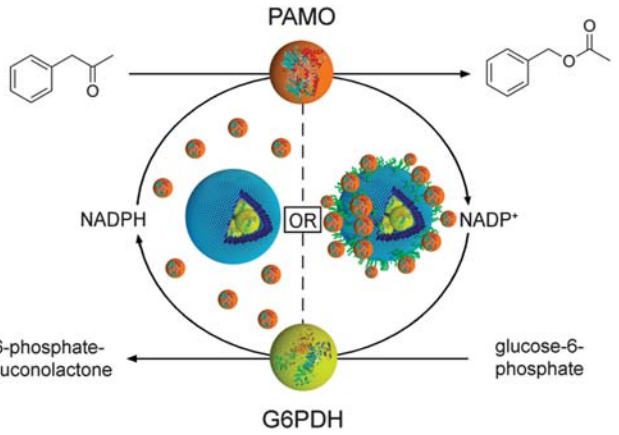

Fig. 1 Diagram depicting the regeneration of cofactor $NADP^+$ by G6PDH inside PS-*b*-PIAT polymersomes to sustain the conversion of phenylacetone catalyzed by PAMO. Two systems with encapsulated G6PDH were separately examined: PAMO in solution and PAMO covalently immobilized on the surface. (The CRE2-PAMO system is not shown here.)

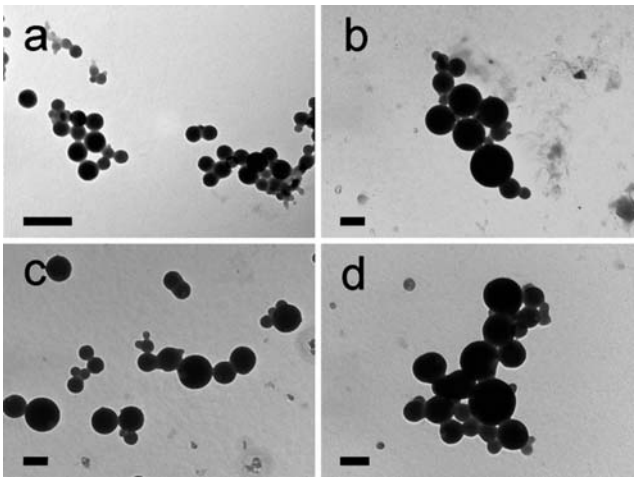

Fig. 2 TEM micrographs of PS_{40}-*b*-$PIAT_{50}$ polymersomes containing (a) CRE2-PAMO or (b) G6PDH in their lumen. PS_{40}-*b*-$PIAT_{50}$ polymersomes with 10 wt% PS_{52}-*b*-PEG_{66}-N_3 anchor are shown in (c) with G6PDH in the lumen and (d) G6PDH inside while PAMO is immobilised on the surface. Due to the high enzyme density in the lumen of the polymersomes, the membrane cannot be clearly distinguished. Scale bars: 200 nm.

The first objective of our study was to evaluate whether the cofactor NADPH and its oxidised $NADP^+$ analogue could penetrate the PS-*b*-PIAT bilayer membrane, despite their reasonably large (M_w: ~750) and multiply charged appearance. To this end, we selected the self-sufficient Baeyer–Villiger monooxygenase CRE2-PAMO for encapsulation in PS_{40}-*b*-$PIAT_{50}$ polymersomes; once NADPH enters, it can be oxidized and reduced in the same confined space. The polymers were self-assembled *via* the cosolvent method using 1,4-dioxane (dioxane, 16.6 vol%).[31] The choice for dioxane instead of the commonly applied THF was motivated by the drastic decrease in enzymatic activity of CRE2-PAMO when in the presence of THF, while addition of dioxane to the reaction mixture still yielded quantitative conversion of phenylacetone in 5 h. Polymersomes with CRE2-PAMO in their lumen were therefore obtained by gentle injection of PS_{40}-*b*-$PIAT_{50}$ in dioxane to Tris/HCl buffer (50 mM, pH 9.0) containing 1.56 μM of the biocatalyst. After 30 min of equilibration, the organic solvent and non-encapsulated enzyme were removed *via* size exclusion chromatography and filtration using centrifugation. The integrity of the polymersomes of ~90 nm in diameter was corroborated with transmission electron microscopy (TEM, Fig. 2a).

Prior to the enzymatic activity studies with CRE2-PAMO polymersomes, the incorporation efficiency of the enzyme was determined. Van Dongen *et al.* affirmed that encapsulation efficiencies lie around 20%;[30] a number much higher than expected based on statistical inclusion. In order to evaluate whether this high incorporation efficiency also occurred for our fused enzyme, we covalently linked a commercially available ruthenium complex with an isothiocyanate moiety to the free amines—of the lysines and *N*-terminus—of CRE2-PAMO. After encapsulation of the Ru-labelled enzyme in polymersomes, inductively coupled plasma-mass spectrometry (ICP-MS) was utilised to determine the amount of ruthenium, and hence the number of CRE2-PAMO, in the sample. As demonstrated in Table 1, the encapsulation efficiencies of 18% and 20% indicate reproducibility and are in correspondence to the literature.[30]

The CRE2-PAMO containing nanoreactors were mixed with phenylacetone (2.5 mM), NADPH and the cosubstrate phosphite in Tris/HCl buffer (50 mM, pH 9.0). The reaction mixture was shaken at 30 °C and the conversion was monitored with gas chromatography (GC) using extracts of reaction aliquots which were withdrawn in time. The results of the experiment, performed *in duplo*, are presented in Fig. 3a. Complete conversion of phenylacetone into phenylacetate within 15 h demonstrated that NADPH experienced no difficulties in penetrating the polymersome membrane. However, because of a decrease in the reaction rate compared with CRE2-PAMO in solution, we assume that the diffusion of NADPH and other components through the membrane had a significant effect on the substrate consumption. The reaction curve indicates furthermore steady-state kinetics, suggesting that the cofactor and (co-)substrate entered the nanoreactors with comparable ease, and that both parts of the fused enzyme remained active during the whole process.[16]

In the next step, we examined the efficiency of the Baeyer–Villiger oxidation using vesicles with the cofactor regeneration enzyme and monooxygenase located at different positions. In this set-up, the cofactor has to travel back and forward through the polymer membrane to be converted into the form that is of use for the other enzyme located outside the polymersome. Hereto, the more THF-tolerant G6PDH was encapsulated in the lumen by addition of PS_{40}-*b*-$PIAT_{50}$ in THF to a solution of the enzyme (12.4 U mL^{-1}) in phosphate buffer (20 mM, pH 7.4). After removal of the non-encapsulated G6PDH and the organic solvent molecules, the polymersomes were analysed with TEM (Fig. 2b). To assess the amount of cofactor regenerating enzyme inside the polymersomes, G6PDH was labelled with the previously mentioned Ru complex. Analysis of the Ru-G6PDH containing vesicles with ICP-MS revealed an encapsulation efficiency of 21%, which is comparable with results obtained for CRE2-PAMO (Table 1).

To investigate the effects of a PS-*b*-PIAT bilayer between G6PDH and PAMO on the biooxidation of phenylacetone, G6PDH-filled polymersomes were mixed with the substrate, NADPH and co-

Table 1 Encapsulation and conjugation efficiencies of enzymes in PS_{40}-*b*-$PIAT_{50}$ polymersomes

Labelled enzyme	Location	Initial quantity/nmol	Immobilised quantity/nmol	Efficiency (%)
CRE2-PAMO	Lumen	3.90	0.683	18
CRE2-PAMO	Lumen	3.90	0.794	20
G6PDH	Lumen	0.33 (31.0 U)	0.0699 (6.53 U)	21
G6PDH	Lumen (10 wt% anchor)[a]	0.33 (31.0 U)	0.0824 (7.70 U)	25
PAMO	Surface	4.64	1.05	30[b]

[a] G6PDH was encapsulated in polymersomes composed of PS_{40}-*b*-$PIAT_{50}$ and 10 wt% PS_{52}-*b*-PEG_{66}-N_3. [b] Measured immobilisation efficiency based on the absolute values is 23%; the given efficiency of 30% is dictated by the theoretical amount of azides introduced *via* the anchor.

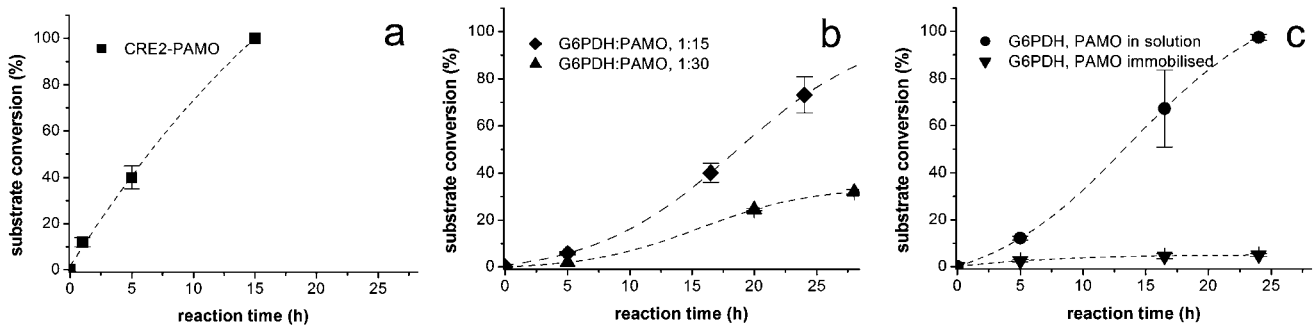

Fig. 3 Biooxygenation of phenylacetone into phenylacetate in time by (a) CRE2-PAMO in the lumen of PS-*b*-PIAT polymersomes, (b) G6PDH in the aqueous compartment while PAMO is dissolved in the surrounding solution (the concentration of PAMO and all reagents was kept the same while the concentration of G6PDH loaded polymersomes was varied) and (c) G6PDH inside PS-*b*-PIAT polymersomes containing 10 wt% PS-*b*-PEG-N_3 with PAMO either in solution or immobilised onto the membrane surface. All reactions were performed *in duplo*, the variation is indicated by the error bars.

substrate glucose-6-phosphate in Tris/HCl buffer at pH 9.0. PAMO was finally added separately to the vesicle dispersion in a—commonly employed—ratio of 30 : 1, PAMO : G6PDH. The reaction progress at 30 °C was monitored with GC, as given in Fig. 3b. In 28 h, the substrate was converted for 32% into phenylacetate, which clearly verifies that the cofactor regeneration can occur in a compartment separated from the Baeyer–Villiger reaction. Nevertheless, the polymer membrane between the monooxygenase and cofactor regenerator is significantly decelerating the reaction rate; a comparable reaction with the two essential enzymes in solution yielded full conversion in 30 min (see ESI† for further details). The ostensibly S-shaped curve designates a two-enzyme reaction, in contrast to the steady-state kinetics when both enzymes are in solution[32] and only PAMO is involved in the rate determining step. Hence, capsulation of G6PDH affected the overall reaction rate due to the more sluggish conversion of glucose-6-phosphate and thereby less NADPH production which is required by PAMO. As comparison, additional activity measurements were carried out in which the concentration of G6PDH-filled vesicles was doubled while the concentration of all other components remained the same (ratio 15 : 1 of PAMO : G6 PDH). As can be seen in Fig. 3b, the substrate was consumed approximately twice as fast compared with the reaction in which the ratio PAMO : G6PDH was 30 : 1.

A high level of control over biochemical processes in nature is gained through specific positional assembly of enzymes. Analogous to cells, polymersomes can serve as scaffolds to position enzymes not only in the lumen but also in the membrane and onto the surface.[30] To expand our regeneration system to a situation where all involved enzymes are spatially associated with only one polymersome, PAMO was covalently immobilised on PS_{40}-*b*-$PIAT_{50}$ vesicles loaded with G6PDH. Hereto, the polymersomes were equipped with a functional handle for a strain-promoted alkyne–azide cycloaddition reaction (SPAAC)[33,34] *via* coassembly of the azide-containing anchor polymer PS_{52}-*b*-PEG_{66}-N_3 (**3**) (see ESI† for further details).

In order to immobilise PAMO using the SPAAC reaction, introduction of a reactive moiety complementary to azides was required. For this purpose, we selected the probe bicyclo[6.1.0]nonyne (BCN) owing to its combination of accessibility and high reactivity.[35] BCN with a succinimidyl carbonate group was linked to the free, approachable amine of PAMO[36] under slightly basic conditions. The efficiency of the coupling was subsequently verified through conjugation of the azide-containing fluorescein derivative ClickGreen (see ESI† for further details),[37] which yielded 97% assuming that only one amine per enzyme was functionalized. Evaluation of the enzymatic activity of BCN-functionalised PAMO revealed that no noticeable difference in catalytic properties could be observed; the substrate was quantitatively converted in 5 h.

Prior to immobilisation of BCN-PAMO, G6PDH was encapsulated in polymersomes composed of PS_{40}-*b*-$PIAT_{50}$ and 10 wt% of anchor PS_{52}-*b*-PEG_{66}-N_3. TEM showed the formation of robust, spherical vesicles (Fig. 2c). The obtained encapsulation efficiency was again analysed with ICP-MS, and amounted to 25% (Table 1). To preclude the possibility that coassembly of the anchor had an influence on the porosity of the polymersomes and thus the enzymatic activity of G6PDH, a Baeyer–Villiger reaction was performed with unmodified PAMO added to the dispersion in a ratio PAMO : G6PDH of 15 : 1. The progress curve in Fig. 3c indicates that the consumption rate of phenylacetone is even slightly higher compared with plain, G6PDH-filled PS-*b*-PIAT vesicles, probably as a result of the somewhat better encapsulation efficiency.

Finally, BCN-functionalised PAMO was conjugated to the azido-anchors that were incorporated in the G6PDH-filled PS_{40}-*b*-$PIAT_{50}$ polymersomes by simply mixing the two in phosphate buffer. After 18 h of reaction time at 4 °C, the uncoupled PAMO was thoroughly removed by filtration using centrifugation. TEM analysis confirmed preservation of the integrity of the biohybrid polymersomes, as demonstrated in Fig. 2d. The conjugation efficiency was examined with PAMO that was labelled with the Ru-complex (0.1 eq. per free amine) prior to BCN-functionalisation. Table 1 shows that 1.05 nmol PAMO was immobilised on 10 wt% of anchor **3**. Assuming that the amount of PS_{52}-*b*-PEG_{66}-N_3 was equally distributed over the two membrane layers, 3.5 nmol of azido-moieties were available for conjugation to the enzyme. This suggests that 30% of the azides on the polymersome surface were occupied by PAMO. Since our monooxygenase is relatively bulky (65 kDa), it is likely that a certain percentage of the anchors became inaccessible by coverage of enzymes that were ligated to other azido-polymers.

To evaluate the enzymatic activity of our biohybrid nanoreactors, they were incubated with phenylacetone, glucose-6-phosphate and NADPH in Tris/HCl buffer (pH 9.0) at 30 °C. After 5 h of reaction time, 3% of the phenylacetone was consumed, which reached a conversion of 5% within 24 h (Fig. 3c). Although the reason for this lack of reactivity is not yet clearly defined, we consider either inaccessibility of the active site or deformation of the enzyme as a result of the immobilisation as plausible hypotheses, as well as the possibility that diffusion of NADPH through the membrane was hampered due to a surface covered in enzymes.

In conclusion, we have designed polymersome nanoreactors capable of performing enzymatic Baeyer–Villiger oxidations. With this, we demonstrate for the first time that polymeric vesicles can be employed as cofactor regeneration reactors. Most efficient substrate conversions have been observed when the monooxygenase fused to the cofactor regeneration enzyme was encapsulated in the aqueous compartment of the vesicle. Encapsulation of the $NADP^+$-regenerating G6PDH while PAMO was in solution also exhibited good performance. Although the functionalisation of PAMO with a BCN-carbamate moiety did not affect its reactivity towards phenylacetone, immobilisation of the enzyme onto azide-bearing polymersomes *via* the SPAAC reaction unfortunately afforded less active BVMO nanoreactors.

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