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Bio-catalytic nanocompartments for *in situ* production of glucose-6-phosphate

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Cells are sophisticated biocatalytic systems driving a complex network of biochemical reactions. A bioinspired strategy to create advanced functional systems is to design confined spaces for complex enzymatic reactions by using a combination of synthetic polymer assemblies and natural cell components. Here, we developed bio-catalytic nanocompartments that contain phosphoglucomutase protected by a biomimetic polymer membrane, which was permeabilized for reactants through insertion of an engineered α-hemolysin pore protein. These bio-catalytic nanocompartments serve for production of glucose-6-phosphate, and thus possess great potential for applications in an incomplete glycolysis, pentose phosphate pathway, or in plant biological reactions.

Mimicking natural cellular compartments and their function is a very promising bio-inspired strategy to develop multifunctional systems. Particularly, synthetic bio-catalytic nanocompartments (CatCs) based on polymer membrane enclosed cavities (polymer vesicles or polymersomes) are of interest. These can contain active compounds (enzymes, proteins, mimics) performing reactions in situ.[1,2] The reaction nanospaces inside CatCs are ideal for the study of sophisticated enzyme-based biocatalytic processes, such as energy production, creation of artificial organelles, or minimal protocells with wide application potential.[1,3] This can be realized through appropriate selection of active compounds to serve the desired application through their intrinsic functionality and use of polymersomes with mechanical stability and tunable properties (compartment size and morphology, membrane thickness, permeability, flexibility, and/or stimuli-responsiveness). The polymer membrane offers protection of encapsulated active compounds (enzymes), e.g. against degradation, hence it needs to preserve its architecture and at the same time allow passage of reaction substrates and products.[4]

Different strategies have been introduced to obtain permeable synthetic membranes:[5,6] polymers with intrinsically porous membranes,[7] stimuli-triggered transition of the membrane permeability[8] and insertion of natural membrane proteins similarly to permeabilization of cell membranes.[9] For instance, CatCs from carbohydrate-*b*-poly(propylene glycol) possess tunable permeability owing to the membrane hydrophobicity.[10] Alternatively, UV-crosslinked CatCs can undergo a controllable swelling (permeable state) – deswelling transition.[11] The biomimetic approach that stands out is the permeabilisation of the CatCs through insertion of biopores, as for example gramicidin[12] and natural membrane proteins (MPs) such as outer membrane protein (OmpF)[13] or FhuA[14] in the polymer membrane. Research has shown that a combination of flexible polymers and appropriate membrane thickness are necessary for a successful insertion of biopores and membrane proteins in synthetic membranes. Flexible polymers allow protein insertion also in membranes that are significantly thicker than lipid bilayers and cell membranes.[15,16] Therefore, if the synthetic membrane is not sufficiently flexible, MPs can only be inserted in membranes that are very similar to a phospholipid bilayer (approximately 5 nm thickness),[16,17] as it has been reported for α-hemolysin insertion in the membrane of polybutadiene-*block*-polyethyleneoxide (PEO_{12}-*b*-PBD_{21}) based polymer vesicles.[18] Among synthetic block-copolymers, polydimethylsiloxane-based polymers (e.g. PMOXA-*b*-PDMS-*b*-PMOXA) satisfy these key requirements well, as has been demonstrated by successful insertion of various biopores and MPs in polymersome membranes.[5,16]

This study focuses on PMOXA-*b*-PDMS-*b*-PMOXA CatCs that are permeabilized through insertion of a genetically engineered α-hemolysin (α-HL) (see ref.[19] for details on the membrane protein) to allow substrate entry and product exit through the CatC polymer membrane. The pore of engineered

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Electronic Supplementary Information (ESI) available: experimental details and supporting results (TEM, DLS, SDS PAGE, additional fluorescence spectra). See DOI: 10.1039/x0xx00000x

α-HL allows passage of molecules of up to 4 kDa in molecular weight,[20] whilst keeping an enzyme-based catalyst encapsulated within the CatCs (Fig. 1 and Fig. S1 ESI). Importantly, insertion of this genetically engineered α-HL was shown in liposomes and planar lipid membranes only,[19] its insertion in significantly larger synthetic polymer membranes is proven for the first time here. The CatCs prepared serve for production of glucose-6-phosphate (G6P), as for the first time phosphoglucomutase (PGM) was encapsulated inside a polymeric nanocompartment's cavity, thereby providing essential protection of the enzyme from degradation. PGM is an essential enzyme in the carbohydrate metabolism, which catalyses conversion of glucose-1-phosphate (G1P) to the product glucose-6-phosphate (G6P). Lack or reduced activity of PGM has been attributed to impaired plant growth and development.[21,22] In addition, PGM along with glucose-6-phosphate dehydrogenase (G6PDH) is crucial for the generation of energy and formation of nicotinamide adenine dinucleotide phosphate (NADPH), a reducing agent with a vital role in biological reactions. PGM is also an essential enzyme with major implications in metabolic pathways. In human disorders, the lack of PGM or its reduced activity has been attributed to glycogen storage diseases.[21,22]

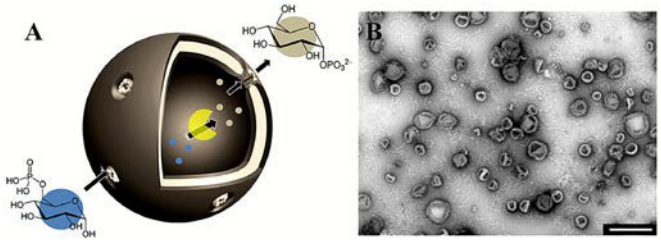

Fig. 1 Schematic illustration of the bio-catalytic nanocompartment with encapsulated phosphoglucomutase (yellow) for the conversion of glucose-1-phosphate (blue filled circle) to the product glucose-6-phosphate (grey filled circle) (A). Transmission electron microscopy (TEM) of the CatCs made by rehydration of $PMOXA_6PDMS_{34}PMOXA_6$ polymer film with 55 μg/mL phosphoglucomutase (PGM). Scale bar: 200 nm (B).

CatCs were formed via self-assembly of poly(2-methyloxazoline)$_6$-*block*-poly(dimethylsiloxane)$_{34}$-*block*-poly(2-methyloxazoline)$_6$, designated as $PMOXA_6$-*b*-$PDMS_{34}$-*b*-$PMOXA_6$ triblock-copolymer (molecular weight: M_w = 3.8 kg/mol, dispersity: Đ = 2.3, hydrophilic weight fraction: $f_{hydrophilic} = M_{PMOXA}/M_w$ = 32% - suitable to form vesicular structures in aqueous solutions),[15] using a typical film rehydration method.[12] Specifically, they were produced by rehydrating a block copolymer thin film with the enzyme cocktail mixture overnight (see experimental details in ESI), followed by extrusion through 200 nm pore filters and separation of the CatCs from the non-encapsulated PGM via dialysis.

Transmission electron micrographs (TEM) confirmed formation of spherical structures with a diameter of 100-200 nm in the presence of PGM, similar to the self-assembly observed of pure polymersomes (Fig. 1 B and S2 ESI). Additionally, light scattering data confirmed the size of around 160 nm of the spherical structures observed in TEM (Fig. S3 ESI). Moreover, no significant change in size was observed in the CatCs containing PGM with $6.12x10^{-4}$% sodium dodecyl sulphate (SDS) or with 0.7 μg/mL engineered α-HL, confirming the stability of this bio-catalytic nanocompartment. SDS used to solubilize the engineered α-HL neither affected the function of engineered α-HL[19] nor the architecture of the polymersomes.

PGM encapsulation in the cavity of the CatCs was determined by fluorescence correlation (FCS) spectroscopy[12,23] using PGM labelled with ATTO488 (Fig. S4 ESI). A diffusion time of τ ≈ 29 μs for the ATTO488 free dye in solution (Fig. 2, blue curve) was obtained from analysis of the FCS autocorrelation curves. Labelling PGM with the ATTO488 fluorophore (PGM-ATTO488), leads to an increase in diffusion time to 153 μs (Fig. 2, green curve), which indicated the presence of labelled enzymes. When the dye-labelled enzyme was encapsulated within CatCs (PGM-ATTO488 NRs), the architecture of CatCs was not affected (Fig. S5 ESI) and a diffusion time of ≈3 ms was obtained (Fig. 2, grey curve) indicating that the labelled enzyme is indeed encapsulated within the CatCs.

During encapsulation of PGM-ATTO488 inside CatCs (see Methods in ESI) some free PGM-ATTO488 molecules remain outside of the CatCs that are subsequently removed by dialysis. To confirm removal of non-encapsulated PGM-ATTO488, FCS autocorrelation curves were fitted using a two component model, which allowed to identify the percentage of species with fast diffusion time (non-encapsulated PGM-ATTO488) and the percentage of species with a slow diffusion time (PGM-ATTO488 encapsulated within the CatCs).[24] These FCS measurements confirmed that non-encapsulated PGM-ATTO488 was completely removed via dialysis: >99.99% of the diffusive fraction correlates with the diffusion time of τ ≈ 3018 μs (PGM-ATTO488 CatCs), while only a negligible remainder fraction correlates with the diffusion time of τ ≈ 153 μs (PGM-ATTO488).

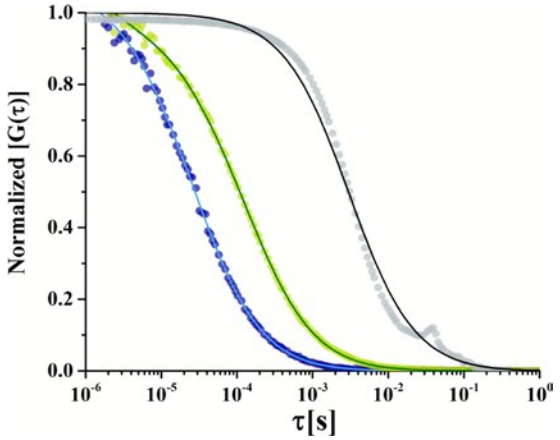

Fig. 2 FCS auto-correlation curves of free dye ATTO-488 (blue curve), PGM-ATTO488 conjugate (green curve), CatCs loaded with PGM-ATTO488 conjugate (grey curve), pH = 7.5, RT.

The $PMOXA_6$-*b*-$PDMS_{34}$-*b*-$PMOXA_6$ membrane itself is not permeable to the substrates and products of this reaction, it is only permeable to reactive oxygen species.[25] Hence, the membrane was permeabilised with an engineered α-HL (SDS page characterisation in Fig. S6 ESI). The activity of α-HL was tested using synthetic liposomes as a model system and a dye

leakage assay.[26] Carboxyfluorescein (CF, λ_{exc} = 495 nm/λ_{em} = 517 nm) was encapsulated in liposomes at a concentration of 50 mM where its fluorescence is quenched. Upon addition of α-HL, the release and thus dilution of CF led to a colour change from brown to green-yellow, confirming permeabilization (full characterisation of the liposomes can be found in the Fig. S7 ESI).

Subsequently, permeabilization of $PMOXA_6$-*b*-$PDMS_{34}$-*b*-$PMOXA_6$ polymersomes containing CF was performed under similar conditions to identify whether the engineered α-HL is able to insert into polymer membranes as well (Fig. S8 ESI). We observed that α-HL insertion was considerably more difficult in the case of synthetic membranes because they are significantly thicker (9.2 nm)[15] than the liposome lipid bilayer (5 nm). It has been reported that a high flexibility of the membrane is necessary to overcome the hydrophobic mismatch between the pore size and the membrane thickness and allow functional insertion of membrane pores, hence this particular copolymer was selected.[16] Again, a colour change from light brown to light green-yellow was observed after addition of the engineered α-HL as for liposomes, indicating successful insertion and membrane permeabilisation (Fig. S8A ESI). Upon the addition of the 1.3 μg/mL α-HL, TEM revealed similar vesicular structures with a diameter of ca. 100-200 nm as before (Fig. S8B ESI). After α-HL addition (at around 150-200 s, indicated by the orange arrow), dye leakage and increase in fluorescence upon release from the polymersomes was observed after 100-200 s (light blue curve in Fig. 3A). This time delay can be explained, because of the increased membrane thickness of the polymer membrane.[15] The results showed that the engineered α-HL is able to successfully insert in the polymer membrane of our nanocompartments. Additional experiments proved that membrane integrity of the nanocompartments is also maintained in presence of solubilisation buffer for engineered α-HL, which contained a low concentration of SDS (red curve in Fig. 3A and Figs. S9-11 ESI).

Further insight into the insertion of engineered α-HL in the membrane of the polymersomes was obtained with fluorinated-amphiphiles (F-amphiphiles), which hinder α-HL pore insertion into lipid membranes when used at concentrations above their CMC.[26] Commercially available FOS-choline (FFC) with a $C_6F_{13}C_2H_4$ single chain was shown not to interfere with lipid membrane integrity.[26] When FFC molecules are used at approximately 5 times their CMC, they stop further insertion of α-HL in liposome membranes without affecting the bilayer stability or functionality of the already inserted α-HL.

Similar experiments with $PMOXA_6$-*b*-$PDMS_{34}$-*b*-$PMOXA_6$ polymersomes revealed that α-HL insertion in the polymer membranes is also impeded when 10 mM fluorinated FOS-choline (FFC) is added to the polymersomes before addition of the engineered α-HL (Fig. 3B, blue curve). If the engineered α-HL is added first, it inserts and encapsulated dye is released until the F-amphiphile is added (indicated by an arrow in Fig. 3B green curve). 10 mM FFC aggregates (visualised by TEM in Fig. S12 ESI) blocked further insertion of engineered α-HL into polymersome membrane. Moreover, these exciting results provide a platform to control the number of functional engineered α-HL pores in more stable polymer membranes.

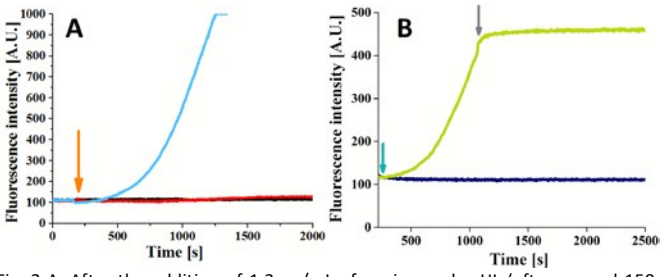

Fig. 3 A. After the addition of 1.3 μg/mL of engineered α-HL (after around 150-200 s, as indicated by the orange arrow), dye release and increase in fluorescence upon dilution from the polymersomes was observed after 100-200 s (light blue curve). No dye was released upon addition of SDS only (without engineered α-HL) during 2000 s (red curve). The black curve represents only the polymersomes, without any SDS or engineered α-HL, to identify the initial fluorescence of polymersomes due to the encapsulated dye.*

B. Addition of 10 mM F-amphiphile before (blue curve) and after insertion (as indicated by arrows) of engineered α-HL in the membrane of $A_6B_{34}A_6$ polymersomes containing the encapsulated CF.*

**Experiments were carried out in 300 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 (Buffer C, see ESI for details) at pH = 8.0, RT.*

Production of G6P was detected using an established glucose-6-dehydrogenase (G6PDH) enzyme coupled assay. Enzymatic activity of free and encapsulated PGM was determined by spectrophotometric monitoring of the production of NADPH (λ_{exc} = 340 nm/λ_{em} = 460 nm, for complete enzymatic conversion steps see Fig. S1).[27] NADPH was produced only when the G1P substrate was present in the enzyme cocktail mixture (Fig. S13 ESI). Typically, after addition of G1P an initial sharp and then gradual increase of fluorescence intensity due to formation of NADPH was observed. This confirms successful permeabilization of the membrane with α-HL and activity of the CatC (Fig. 4, purple curve).

Importantly, when just SDS and no α-HL was used, only the initial increase was observed when G1P was added followed by steady fluorescence intensity (Fig. 4, black curve). The fluorescence remained stable for up to 5000 s, showing that the G1P substrate cannot cross the CatC membrane without presence of α-HL and thus cannot initiate the reaction. Furthermore, CatCs containing 10 times higher amount of PGM showed activity as well (Fig. 14 ESI).

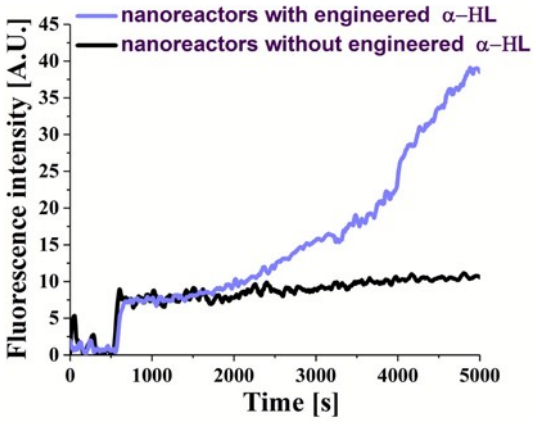

Fig. 4 Activity of the CatCs with encapsulated phosphoglucomutase in the absence (black curve) and presence (blue curve) of engineered α-hemolysins (pH = 7.5, RT). CatCs are successfully permeabilised due to the higher increase in fluorescence intensity in the presence of engineered α-HL (blue curve), as compared with the pore absence (black curve).

We successfully designed polymer CatCs by encapsulation of PGM and permeabilization of the polymer membrane using genetically engineered α-HL pore protein. Importantly, the pore protein only facilitates transport of small molecules like G1P and G6P through the membrane, while the enzyme remains entrapped and protected. The product G6P was detected with a G6PDH-coupled enzyme assay via the formation of fluorescent NADPH. G6P is a relevant metabolic compound, which may aid in an incomplete glycolysis, pentose phosphate pathway, or in plant biological reactions.

The authors would like to acknowledge the generous financial support from the SNSF and University of Basel. Samuel Lörcher is thanked for the polymer synthesis and Prof. Wolfgang Meier (University of Basel) for providing the polymer. Gabriele Persy and Christina Zelmer are acknowledged for TEM measurements. We are very grateful to Prof. Hagan Bayley (University of Oxford), as without him this fruitful collaboration with Dr. Shiksha Mantri would not have started. G. G.-G. gratefully acknowledges a postdoctoral research fellowship from the German Academic Exchange Service (DAAD).

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