Biomimetic artificial organelles with in vitro and in vivo activity triggered by reduction in microenvironment

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Despite tremendous efforts to develop stimuli-responsive enzyme delivery systems, their efficacy has been mostly limited to in vitro applications. Here we introduce, by using an approach of combining biomolecules with artificial compartments, a biomimetic strategy to create artificial organelles (AOs) as cellular implants, with endogenous stimuli-triggered enzymatic activity. AOs are produced by inserting protein gates in the membrane of polymersomes containing horseradish peroxidase enzymes selected as a model for nature’s own enzymes involved in the redox homeostasis. The inserted protein gates are engineered by attaching molecular caps to genetically modified channel porins in order to induce redox-responsive control of the molecular flow through the membrane. AOs preserve their structure and are activated by intracellular glutathione levels in vitro. Importantly, our biomimetic AOs are functional in vivo in zebrafish embryos, which demonstrates the feasibility of using AOs as cellular implants in living organisms. This opens new perspectives for patient-oriented protein therapy.
Mimicking biological processes by engineering biomimetic nanostructures represents an elegant strategy for addressing problems in various scientific fields, including materials science, chemistry, electronics and medicine1–3. By applying a bottom-up biomimetic design (i.e. arranging molecules at the nanoscale via self-assembly), it is possible to combine individual biological units, known for their sophisticated structure and activity (e.g. proteins, lipids, DNA), with robust synthetic materials (e.g. polymers, porous silica surfaces, nanoparticles). This serves to develop nanoscale biomimics with enhanced properties and functionalities4–8 with potential for a wide range of applications (sensitive biosensing, patient tailored therapeutics, detoxification of environmental pollutants, etc.)6,9–12.

Of particular interest are two different concepts that are currently the main focus in this research field: (i) artificial organelles (AOs) based on an essential need to offer efficient solutions for improved therapy and diagnostics13 and (ii) protocol systems intended to provide simple models of cells for understanding various internal processes12,14. These concepts are complementary, one is essential for advancing medical applications (AOs) whereas the second concept mimics cell behaviour based on very simple systems (protocols). Similarly, as in nature, the sizes of the compartments are completely different: while AOs have nanometre range sizes, protocols reach the micrometre range. Even though protocols represent the first archetypes of an artificial cell, they still inherently lack the material variety and responsiveness found in the most basic cellular structures, and have not yet been investigated in vivo to determine whether they preserve their functionality. AOs are particularly attractive nanoscale biomimics because they can provide a required compound/signal, detoxify harmful compounds, or change cellular conditions and reactions. AOs are based on compartmentalisation of active compounds (enzymes, proteins, catalysts, mimics) within artificial nanoassemblies that reach and function in the intracellular environment, and thus serve as simplified mimics of nature’s own organelles. Various examples of systems with potential to act as AOs have been developed based on liposomes, porous silica nanoparticles and polymer compartments (polymersomes) in combination with biomacromolecules13–16. However, very few have been evaluated in vitro to assess their in situ cellular functionality,6,15–18 and to the best of our knowledge, none has been assessed in vivo. In vivo functionality of such AOs is a crucial factor that is necessary to demonstrate that the concept of AOs is feasible in living organisms, and thus AOs can act as cellular implants.

Notably, natural organelles have membranes, since inside cells compartmentalisation is essential to provide confined reaction spaces for complex metabolic reactions. Therefore, an AO should preserve the compartmentalisation as a key factor in mimicking natural organelles. In this respect, polymer compartments, named polymersomes, are ideal candidates for the creation of AOs, because of their hollow spherical structure with a membrane serving as a border for an inner cavity and their greater mechanical stability than lipid-based compartments, i.e. liposomes19,20. In addition, the chemical nature of the copolymers provides the possibility of controlling their properties (e.g. size, stability biocompatibility, flexibility, stimuli-responsiveness)2,21. Polymersomes have been shown to serve either as carriers for biomolecules and mimics1,2,21,22, or more recently for development of nanoreactors and even the generation of AOs6,11,23. A key factor for supporting in situ reactions24,25 is to render the polymersome membranes permeable for substrates and products. An elegant approach bioinspired from the cell membrane is to incorporate biopores and membrane proteins25–27. Selective membrane permeability towards protons and ions is achieved by inserting small pore forming peptides27, while membrane proteins induce size-dependent cut-off permeability26,28–30 or even mediate the diffusion of specific molecules31,32. The few reported AOs exhibit enzymatic reactions either inside porous polymersomes6,32 or inside polymersomes equipped with channel porins27, with the aim of emulating cellular pathways (e.g. reactive oxygen species detoxification or glucose oxidation).

Another essential factor for tuning AO functionality is a triggered response to its environment, as, for example, the redox state of the cell, which regulates various processes involved in cellular signalling pathways33–35. While there are a few reported examples of polymersomes with a stimuli-responsive permeable membrane based on the incorporation of genetically or chemically modified membrane proteins36, only two of them have served for the design of catalytic nanocompartments36,37, and none has been used to control reactions inside AOs. Activation of the AO by a specific endogenous stimulus inside cells represents a challenging step in development of functional AOs in vivo. The design of AOs with triggered activity and the demonstration of their in vivo functionality represent necessary steps towards the creation of cell implants, and the provision of smart solutions for personalised medicine by a straightforward change of the biomolecules inside the AOs.

Here, we present a strategy for designing AOs with an in situ enzymatic reaction that is triggered by the presence of an intra-cellular stimulus, and demonstrate in vitro and in vivo functionality. Genetically modified outer membrane protein F (OmpF) porins were incorporated into polymersomes to induce redox responsiveness to the membrane, and horseradish peroxidase (HRP) simultaneously encapsulated inside their cavity to provide a source of the AO functionality. Such AOs with functionality triggered by intracellular changes represent an advance in mimicking that of nature’s own organelles, especially those that are involved in the redox equilibrium of the cellular homoeostasis. Amphiphilic block copolymers poly(2-methyloxazoline)-block-poly(dimethylsiloxane)-block-poly(2-methyloxazoline) (PMOXA–PDMS–PMOXA) were used to self-assemble into polymersomes, because such copolymers have already been shown to form membranes in which biopores and membrane proteins can be successfully inserted36–38, and to be taken up and to be non-toxic to various cell lines17. Once inserted in the polymersome membrane, the modified OmpF porins act as protein gates independent of the insertion direction, i.e. orientation in the membrane36,37,39,40, and trigger the in situ HRP enzymatic reaction when a stimulus is present in the cellular environment. HRP was selected as model enzyme, because peroxidases play a significant role in the redox homoeostasis of cells and cell apoptosis41. This strategy of providing stimuli-responsiveness to polymersome membranes neither affects the membrane integrity, as for stimuli-responsive synthetic membranes of compartments42, nor the size and structure of the polymersomes. Crucial steps were the evaluation of AO toxicity and functionality in human epithelial tumour cells (HeLa cells), and once these were established in vivo tolerability, preservation of the AO structure, and in situ regulation of the activity of the encapsulated enzyme in the vertebrate zebrafish embryo (ZFE) model.

Results
Bioengineering protein gates by modification of channel porins. The key factors in the design of AOs with activity triggered by changes in environmental conditions are on-demand permeability of the compartment towards enzymatic substrates/products and structural integrity of the polymersome, which mimics that of natural organelles. Therefore, our biomimetic strategy
aimed to equip PMOXA₆-PDMS₄₄-PMOXA₆ polymersome membranes with protein gates that are responsive to changes in glutathione (GSH) concentrations in intracellular environments, while preserving the structure of the nanocompartment (Fig. 1a).

It has been shown very recently that chemical modifications of amino acid residues at key locations of the OmpF porin backbone influence the translocation of substrates through the pore in a pH-responsive manner⁴⁶. Here we go one step further by using a double mutant of OmpF⁴⁷ to attach molecular caps to genetically introduced cysteine residues that serve to block/unblock the OmpF pore upon changes in redox potential, which occur when the system enters the intracellular microenvironment (Fig. 1b). In contrast to polymersomes with membranes containing OmpF genetically modified to release a payload in reductive conditions⁴⁸, our system controls the overall functionality of the AOs. We chose a cysteine double mutant of OmpF (OmpF-M) because cysteine residues, replacing the amino acids K89 and R270, were expected to form reduction-sensitive disulphide bonds with molecules selected to serve as molecular caps. These molecular caps remain attached in mildly oxidising environments and block substrate diffusion through the pore, whereas in the presence of reducing agents, such as intracellular GSH, their cleavage restores normal passage of small molecular weight molecules (<600 Da) through the OmpF pores. This approach mimics pathways of metabolism regulation, where proteins within the membranes of natural cell organelles are irreversibly activated or deactivated on demand⁴³,⁴⁴. In addition, we were interested in developing an irreversible protein gate in order to be able to rapidly evaluate the functionality of the organelle in vivo.

The ability of the cysteine residues of OmpF-M to form disulphide bonds with thiol groups of small molecular weight molecules was examined by two complementary assays, one using a suitable spin probe (bis-(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-yl) disulphide) and the second using the fluorescent dye SAMSA fluorescein (SAMSA-CF) (Fig. 1c, d).

Coupling reaction of the molecular caps with the cysteine residues of OmpF-M resulted in the formation of OmpF conjugates (OmpF-S-S-CF for OmpF conjugated with SAMSA-CF, and OmpF-S-S-NO for OmpF conjugated with bis-(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-yl) disulphide), respectively.

Binding of the thiol reactive spin probe to the protein was evaluated by a combination of LC-MS-MS and electron paramagnetic resonance (EPR). Upon in-gel digestion of the porin⁴⁵, LC-MS-MS analysis of the peptide fragments indicated a very high labelling efficiency of the spin probe to cysteine residues of the OmpF-M (96 ± 4%). Standard deviation is based on three measurements. The EPR spectrum of the bis-(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-yl) disulphide in phosphate-buffered saline (PBS) at 298 K consists of an isotropic triplet pattern (Supplementary Figure 1) with a hyperfine coupling $a_N$ value of 15.8 G that is similar to reported values for analogous nitroxide probes where no aggregation was present⁴⁶,⁴⁷. In contrast, OmpF-S-S-NO gave a broad anisotropic EPR spectrum with no isotropic component, and is similar to that reported for 5-DSA in lipid bilayers or cholesterol aqueous solutions⁴⁸. This EPR spectrum indicates hindered rotation of the nitroxide probe⁴⁹ after binding to the OmpF mutant (OmpF-S-S-NO), and demonstrates successful binding of the bis-(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-yl) disulphide to the modified OmpF mutant (Fig. 2a).

After exposure of OmpF-S-S-NO to 10 mM DTT an isotropic EPR spectrum ($a_N$ value of 15.9 G) characteristic of the freely rotating spin probe was observed (Fig. 2b). This clearly demonstrates that the nitroxide spin probe that is bound to thiol groups of the OmpF-M under oxidative conditions is cleaved in a reductive environment.

SAMSA-CF (Thermo Fischer Scientific) was selected as a molecular cap because its size (molecular weight 521.49 Da) was expected to block the OmpF-M pore, and because of its ability
to form cleavable disulphide bonds. Thus, attachment of SAMSA-CF to OmpF-M introduces a stimuli-responsiveness to the pore, and therefore to the polymersome membrane when OmpF-S-S-CF is inserted. In addition, the fluorescent properties of SAMSA-CF allow pore modification to be analysed by a combination of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorescence correlation spectroscopy (FCS).

LC-MS-MS analysis of the peptide fragments indicated a high labelling degree of OmpF-M (81 ± 31%). In addition, a fluorescent band appeared in the SDS-PAGE gel when SAMSA-CF was conjugated to OmpF-M, whereas the OmpF wild type did not interact with the fluorophore; this fluorescent band supports the formation of OmpF-S-S-CF (Supplementary Figure 2). To mimic the intracellular reductive environment, where the GSH concentration is kept at a constantly high level (10 mM GSH) by cytosolic enzymes, we studied the behaviour of the reduction-responsive molecular cap under reductive conditions (Supplementary Figure 2).

The binding of SAMSA-CF to OmpF-M cysteine residues was also evaluated by FCS, because it allows the determination of diffusion coefficients, which are correlated to possible interactions of the fluorescent molecules with supramolecular assemblies, such as polymersomes, liposomes and nanoparticles in the pico- to nanomolar concentration region.

![Fig. 2 Characterisation of stimuli-responsive OmpF Panel. a EPR spectra of bis-(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-yl) disulphide-labelled OmpF-M experimental (black) and simulated (blue) and b bis-(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-yl) disulphide-labelled OmpF-M in 1% OG incubated with 10 mM DTT experimental (black) and simulated (blue). c Normalised FCS autocorrelation curves for SAMSA-CF in PBS (black), SAMSA-CF in 1% OG (blue) and OmpF-S-S-CF in 1% OG (Red). Dotted line—experimental autocorrelation curves, full line—fit. d SAMSA-CF release kinetics from OmpF-M in 30 mM GSH, 1% OG, as measured by FCS and analysed with a two-component fit. Error bars show standard deviations from 60 measurements.](image-url)
7.4 by FCS, and analysed the results by a two-component fit. Due to cleavage of the disulphide bonds between the dye and the OmpF-M, the percentage of the free dye increased over time to 85 ± 9%, with a plateau after 1 h (Fig. 2d, Supplementary Figure 3). Standard deviations in the percentage of the free dye are based on individual measurements of the same probe (n = 60) during a set time point.

Catalytic enzyme-polymersome nanocompartments with protein gates. The effect of different concentrations of individual components on the functionality of the final system has already been reported for the insertion of OmpF wild type into PMOXA-PDMS-PMOXA polymersomes and enzyme encapsulation within the inner cavity. Here we used the optimised conditions and adapted them for the modified OmpF and our AOs. PMOXA6-PDMS44-PMOXA6 copolymers spontaneously self-assembled in the presence of HRP, HRP and OmpF-S-S-CF, or HRP and OmpF-S-S-NO, and hollow spherical compartments were identified by cryo-TEM (Fig. 3a, Supplementary Figure 4). These spherical polymer assemblies were demonstrated by light scattering to be polymersomes with: RH of 99 ± 2 nm for HRP-loaded polymersomes containing OmpF-S-S-CF, RH of 89 ± 4 nm for polymersomes loaded with HRP and equipped with OmpF-SH, and RH of 101 ± 1 nm for HRP-loaded polymersomes (Supplementary Tables 1 and 2). Standard deviations were determined based on Pearson’s coefficient of the correlation function and the Guinier fitted one. The polymersome architecture was not affected by 30 mM GSH, with structural parameters ρ (ρ = R_H/R_G) values in the range 0.90–0.96, which confirmed a hollow sphere morphology (Supplementary Tables 1 and 2). HRP-loaded polymersomes, HRP-loaded polymersomes equipped with OmpF-SH, and HRP-loaded polymersomes equipped with OmpF-S-S-CF all preserved their size and did not aggregate after 2 weeks storage at 4 °C in the dark (Supplementary Figures 5–7).

Insertion of channel proteins into enzyme-loaded PMOXA6-PDMS44-PMOXA6 polymersomes is critical for in situ activity of the encapsulated enzyme, because the channels allow substrates and products of the enzymatic reaction to pass through the membrane. As OmpF is a pore protein, its functionality is independent of its orientation inside the membrane, and the channel porin mediates the flow of molecules up to 600 Da.

We evaluated OmpF-S-S-CF and OmpF-S-S-NO insertion into the polymersome membrane using FCS and EPR, respectively. A diffusion time of τ_d = 2573 ± 960 μs was obtained by FCS for polymersomes with reconstituted OmpF-S-S-CF, indicating that the modified protein gates were successfully inserted into the polymer membranes (free OmpF-S-S-CF in 1% OG has τ_d = 588 ± 261 μs). Standard deviation of the diffusion times is acquired from individual measurements (n = 60). By comparing the molecular brightness of the free fluorophore (CPM = 2.2 ± 0.7 kHz) and the OmpF-S-S-CF equipped polymersomes (CPM = 18.9 ± 11.1 kHz), it was calculated that there were five OmpF-S-S-CF porins/polymersome; these values are similar to those reported previously for wild-type OmpF36 (Fig. 3b).
HRP-loaded polymersomes containing OmpF-S-S-NO produced a broad EPR spectrum (Fig. 3c), indicative of low mobility, a result similar to that reported for 5-DSA and 16-DSA inserted in polymersomes membranes.55 However, when these HRP-loaded polymersomes containing OmpF-S-S-NO were exposed to reductive conditions (10 mM DTT), an isotropic EPR spectrum \((a_N = 15.9 \text{ G})\) was observed superimposed on the broad peak, indicating successful cleavage of some of the nitroxide spin probe from the OmpF (Fig. 3c).

**Stimuli-responsiveness of the catalytic nanocompartments.** The effect of an external stimulus on the functionality of the HRP-loaded polymersomes equipped with OmpF-S-S-CF was evaluated by their response to the addition of 30 mM GSH. The fluorescent signal associated with formation of a resorufin-like product (RLP) during the in situ enzymatic reaction in the presence of Amplex Ultra Red (AR) as a substrate for HRP was measured spectroscopically.56 Enzymatic turnover of the AR substrate was significantly lower with HRP-loaded polymersomes equipped with OmpF-S-S-CF (by up to 36±4%) compared to HRP-loaded polymersomes equipped with OmpF-SH, suggesting that the molecular cap is sufficient to reduce the passage of small molecules through the pore. Note that the very low activity of HRP-loaded polymersomes without inserted OmpF was taken into account for background correction. Standard deviation is based on three measurements of separately prepared catalytic nanocompartments. Addition of 30 mM GSH to the system increased the activity of HRP-loaded polymersomes equipped with OmpF-S-S-CF up to that of HRP-loaded polymersomes equipped with OmpF-SH. This indicates that reduction of the disulphide bridge between the attached SAMSA-CF cap and cysteine residues of the OmpF-M successfully restored the OmpF-M pore permeability for the substrate of the enzyme by releasing the molecular cap (Fig. 3d, Supplementary Figures 8 and 9).

**Nanocompartments as stimuli-responsive AOs.** Here we have gone a step further by developing stimulus-triggered AOs, whose functionality is modulated by the responsiveness of modified OmpF porins inserted in the membrane of the catalytic nanocompartments. Previously designed AOs successfully overcame the first barrier of cell membranes and escaped from endosomes.17 As PMOXA-PDMS-PMOXA polymersomes are stable at acidic pH,36, we consider that this will favour a successful lysosomal/endoosomal escape during the recycling of lysosomes and endosomes.

Possible internalisation mechanisms of various PMOXA-PDMS-PMOXA-based polymersomes and their high cytocompatibility in various cell lines have already been reported.17,57–59 Here, we evaluated the cytocompatibility of the biomimetic AOs by testing their cellular toxicity using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulphophenyl)-2H-tetrazolium (MTS) assay before studying their intracellular activation and enzymatic activity. Notably, their biocompatibility at the cellular level was shown by the absence of any decrease in viability in HeLa cells even after 48 h (i.e. polymer concentration ranging from 0.25 to 0.75 mg ml\(^{-1}\)) (Supplementary Figure 10).

In order to study cellular internalisation and intracellular localisation, we first conjugated HRP with Atto488 (HRP-Atto488) and Atto647 (HRP-Atto647), respectively (Supplementary Figure 11). Then we encapsulated labelled-HRP inside the cavity of polymersomes, polymersomes equipped with OmpF-S-S-CF, and polymersomes equipped with OmpF-SH. Cellular uptake assays in HeLa cells indicated successful internalisation resulting in a particulate intracellular staining pattern with increasing intensity in a time-dependent manner from 8 to 24 h (Fig. 4a, Supplementary Figures 12 and 13). The quantitative analysis indicates that after 24 h AOs did not co-localise with early endosomes or lysosomes, confirming successful intracellular endosomal escape (Supplementary Figure 14).17 Localised HRP-Atto488 signals confirmed the intracellular integrity of the polymersomes. In sharp contrast, if cells were treated with a
membrane disrupting agent (i.e. 0.1% saponin) (Supplementary Figure 15), polymersome membranes were affected and resulted in an intracellular cytoplasmic distribution of HRP-Atto488.

The capacity of the AOs to act within target cells in a stimulus-responsive manner was investigated by using a combination of confocal laser scanning microscopy (CLSM) and flow cytometry to evaluate their potential to respond to increased intracellular GSH levels. HeLa cells were incubated with HRP-loaded polymersomes without OmpF or with HRP-loaded polymersomes equipped with either OmpF-S-S-CF (AOs) or with OmpF-SH. Extracellular polymersomes were removed by washing before imaging the intracellular activity of AOs. Cells were incubated with a 1:1 substrate mixture of H₂O₂ and AR to allow the intracellular deposition, and finally conversion of AR into its RLP by AOs. Note that both hydrogen peroxide and AR pass through the cellular membrane via passive partitioning, while they do not penetrate the membrane of polymersomes (Supplementary Figure 9). In contrast to untreated cells, or those incubated with HRP-loaded polymersomes without OmpF, a significant increase of intracellular fluorescence was observed with AOs equipped with OmpF-S-S-CF or OmpF-SH (Fig. 4b, Supplementary Figure 16). A similar trend was observed when AR turnover was quantified by flow cytometry (Supplementary Figure 17). The strong fluorescent signal for AOs based on HRP-loaded polymersomes equipped with OmpF-S-S-CF confirmed successful intracellular cleavage of the molecular cap attached to OmpF-M, and subsequent activation of the AOs within the intracellular environment of the HeLa cells (Supplementary Figure 17).

**In vivo activity of stimuli-responsive AOs.** As a step further to obtaining insight into their safety, tolerability and performance in vivo, AOs were studied in a ZFE model. ZFEs were selected, because of their recognition as a complementary vertebrate animal model for applications, such as compound screening in drug discovery, toxicological studies and recombinant disease animal model for applications, such as compound screening in drug discovery, toxicological studies and recombinant disease models. Compared to rodent in vivo models, the ZFE offers unique advantages: (i) high reproducibility, (ii) low costs, (iii) high level of genetic homology to humans, (iv) availability of transgenic lines and (v) most importantly for the evaluation of AO, optical transparency. Due to their optical transparency, ZFE provide the possibility of imaging fluorescently-tagged objects and fluorescent processes in vivo at a high resolution over time. Compared to rodent in vivo models, the ZFE offers unique advantages: (i) high reproducibility, (ii) low costs, (iii) high level of genetic homology to humans, (iv) availability of transgenic lines and (v) most importantly for the evaluation of AO, optical transparency. Due to their optical transparency, ZFE provide the possibility of imaging fluorescently-tagged objects and fluorescent processes in vivo at a high resolution over time. Compared to rodent in vivo models, the ZFE offers unique advantages: (i) high reproducibility, (ii) low costs, (iii) high level of genetic homology to humans, (iv) availability of transgenic lines and (v) most importantly for the evaluation of AO, optical transparency. Due to their optical transparency, ZFE provide the possibility of imaging fluorescently-tagged objects and fluorescent processes in vivo at a high resolution over time. Compared to rodent in vivo models, the ZFE offers unique advantages: (i) high reproducibility, (ii) low costs, (iii) high level of genetic homology to humans, (iv) availability of transgenic lines and (v) most importantly for the evaluation of AO, optical transparency. Due to their optical transparency, ZFE provide the possibility of imaging fluorescently-tagged objects and fluorescent processes in vivo at a high resolution over time. Compared to rodent in vivo models, the ZFE offers unique advantages: (i) high reproducibility, (ii) low costs, (iii) high level of genetic homology to humans, (iv) availability of transgenic lines and (v) most importantly for the evaluation of AO, optical transparency. Due to their optical transparency, ZFE provide the possibility of imaging fluorescently-tagged objects and fluorescent processes in vivo at a high resolution over time. Compared to rodent in vivo models, the ZFE offers unique advantages: (i) high reproducibility, (ii) low costs, (iii) high level of genetic homology to humans, (iv) availability of transgenic lines and (v) most importantly for the evaluation of AO, optical transparency. Due to their optical transparency, ZFE provide the possibility of imaging fluorescently-tagged objects and fluorescent processes in vivo at a high resolution over time. Compared to rodent in vivo models, the ZFE offers unique advantages: (i) high reproducibility, (ii) low costs, (iii) high level of genetic homology to humans, (iv) availability of transgenic lines and (v) most importantly for the evaluation of AO, optical transparency. Due to their optical transparency, ZFE provide the possibility of imaging fluorescently-tagged objects and fluorescent processes in vivo at a high resolution over time. Compared to rodent in vivo models, the ZFE offers unique advantages: (i) high reproducibility, (ii) low costs, (iii) high level of genetic homology to humans, (iv) availability of transgenic lines and (v) most importantly for the evaluation of AO, optical transparency. Due to their optical transparency, ZFE provide the possibility of imaging fluorescently-tagged objects and fluorescent processes in vivo at a high resolution over time.
Atto647-HRP was injected at concentrations of 0.2 mg ml−1. With Atto647-HRP, the free Atto647-HRP enzyme did not show significant macrophage colocalisation after 24 h, even when Atto647-HRP was injected at concentrations of 0.2 mg ml−1 (Supplementary Figure 21). Notably, only macrophages in circulation were targeted and not tissue resident macrophages (i.e. star shaped).

Once cellular internalisation of AOs by the early immune system of ZFE was successful in vivo, we explored the uptake rate, exact intracellular localisation and internalisation mechanisms of AOs in immune cells in vitro by using human macrophage differentiated THP-1 cells. AOs internalisation started as early as 30 min, and a strong internalisation by immune cells was achieved after 3 h (Supplementary Figure 22), with increasing uptake rates at higher time points. As THP-1 cells are immature macrophages with reduced phagocytic capacity, a higher uptake rate of AOs is possible for mature (primary) macrophages in vitro and in vivo. Importantly, all macrophage uptake studies were performed in the presence of serum proteins to mimic physiological conditions in vivo because opsonisation of nanoparticles by serum proteins can highly influence their interaction with cells. To obtain a mechanistic understanding of the internalisation process, THP-1 macrophages were pre-treated with different pharmacological pathway inhibitors. We used inhibitors with specific inhibition profiles: (i) polyinosinic acid to block scavenger receptors, (ii) colchicine to inhibit pinocytosis, (iii) cytochalasin B as phagocytosis inhibitor and (iv) sodium azide to inhibit all energy-dependent uptake processes. Cells not incubated with Atto488 HRP-loaded AOs served as a control. A 1.28-fold increase in the mean fluorescence intensity (MFI) was observed by flow cytometry analysis of the cells incubated with Atto488 HRP-loaded AOs for 6 h, which indicates internalisation of AOs by THP-1 macrophages. The uptake of AOs by macrophages was significantly inhibited by cytochalasin B (a 0.13-fold increase in MFI) and in a lower degree by sodium azide (0.43-fold increase in MFI), which indicates an energy-dependent phagocytic internalisation process (Fig. 5b, c). On the contrary, polyinosinic acid did not inhibit the AOs uptake, suggesting little or no involvement of the scavenger receptor in the internalisation mechanism of AOs (Fig. 5c).

The internalisation process analysed by CLSM using Lyso-Tracker™ Red DND-99 as a reporter for the lysosomal compartments indicates that AOs co-localise with lysosomal compartments during their internalisation process (Supplementary Figure 23). Interestingly, we could not detect a lysosome signal (lysoTracker) 24 h after incubation of macrophages with AOs, suggesting the presence of an intracellular lysosomal escape mechanism once the AOs are taken-up by macrophages (Supplementary Figure 23). After internalisation in macrophages, the signals associated with Atto488-HRP-loaded AOs in lysosomal compartments changed to larger intracellular vesicular signals. This suggests an expansion of the AO-bearing lysosomal compartments before the AOs are released into the cytosol. For an exact mechanism by which AOs escape the lysosomal compartment and interact with cellular membranes, further investigations are planned but they are beyond the scope of this study.

In order to assess in vivo stability, integrity and functionality of AOs when exposed to the conditions in the macrophage microenvironment, we performed a second injection of AOs together with the enzyme substrate AR. Injection of the co-substrate H2O2 in combination with AR was not necessary, since macrophages have the ability to produce H2O2. In addition, co-injection of H2O2 resulted in a red colouring of the whole blood volume, presumably due to haemolysis and thus interaction of AR with erythrocyte enzymes or haemoglobin (Supplementary Figure 24). Distinct colocalisation within macrophages of the converted AR oxidation product was found only for HRP-Atto488-loaded AOs equipped with either OmpF-SH or OmpF-S-S-CF: the molecular cap of OmpF-S-S-CF was cleaved in vivo leading to activation of the AOs. In sharp contrast, HRP-Atto488-loaded polymersomes without OmpF remained inactive, demonstrating that the polymersome membrane is sufficiently robust to remain intact in ZFE macrophages (Fig. 5d).

Discussion

Design and development of AOs able to function inside cells and support the natural organelles is a necessary step towards the creation of cellular implants. Complementary as a concept to that of protocols, AOs respond to an essential need to offer efficient solutions for improved therapeutic and diagnostic options. Previously reported examples of AOs were based on confined spaces for reactions by compartmentalisation of enzymes inside nanoscale assemblies, but were not able to function in a stimuli-responsive manner. Here, we introduce a strategy to develop AOs with functionality that can be switched on by changes in the cellular microenvironment. These stimuli-responsive AOs are created by simultaneous encapsulation of an enzyme involved in the cellular redox homeostasis and insertion of a genetically engineered channel porin to serve as a protein gate that triggers the enzymatic activity inside AOs.

Our AOs preserved their architecture and were activated after reaching the cellular microenvironment. More exciting, they are functional in a vertebrate ZFE model, which proves that the concept of AOs as cellular implants is feasible in vivo. Furthermore, stability, biocompatibility and low toxicity of AOs represent real advantages for medical applications compared to existing solutions for enzyme replacement, such as direct enzyme delivery and transfection.

We believe that in the future, the high versatility of our strategy will allow straightforward development of a large variety of AOs for specific medical applications by changing the encapsulated enzymes and/or of the stimuli-responsive property of the protein gates. However, a careful selection of substrates is required to overcome the limited ability to transit through the plasma membrane of specific substrates, which are commonly used in bulk enzymatic reactions.

This example of AOs activated by changes in cellular microenvironment and that remains functional in vivo, opens the perspective of complex in situ reactions inside AOs, and represents an important advance towards the generation of multifunctional systems that will support the development of personalised medicine.

Methods

OmpF expression and extraction. The OmpF K89 R270 cysteine mutant and the OmpF wild type were expressed in BL21 (DE3) OmpF Esherichia coli: detailed procedure is described in Supplementary Methods. The extracted fraction was analysed by a 4–15% Mini-PROTEAN® TGX™ Precast SDS (Bio-Rad Laboratories, USA) gel to confirm the protein purity and the protein concentration was determined using a BCA assay kit (Pierce Chemical Co, Rockford, USA). OmpF was stored at 4°C in 3% OG at a concentration of 1.2 mg ml−1 for several weeks.

OmpF modification with SAMSA fluorescein (OmpF-S-S-CF). The OmpF K89 R270 double cysteine mutant was modified by disulphide binding of SAMSA fluorescein to the free cysteine residues. The same reaction was also performed in
the presence of OmpF wild type in 3% octyl-glucopyranoside (OG) (Anatrace, USA) and 3% OG in order to serve as controls. Twenty microlitres of 959 µM SAMA-CF was added to 25 µl of OmpF wild type in 3% OG PBS and 3% OG PBS, followed by staining with the secondary goat anti-rabbit polyclonal antibody (ab2900, Abcam) (1:1000) or anti-LAMP1 (ab24170, Abcam) antibody (1:1000) for 30 min, when deprotection of SAMSA-CF was initiated by adjusting the pH of 5.5. HeLa cells were seeded in a well at a density of 5 x 10⁴ cells per well in a 24-well plate (8 x 10⁴ cells per well) and cultured in DMEM medium containing 10% foetal calf serum, penicillin (100 units ml⁻¹) and streptomycin (100 µg ml⁻¹) to allow attachment to the surface. After attachment, the medium was removed and catalytic nanocompartments were added to a final polymer concentration of 0.5 mg ml⁻¹. Cells were washed twice before being imaged at the respective time points.

Flow cytometry analysis of AO activity. HeLa (epitheloid cervix carcinoma, human; ATCC, CCL-2) cells were seeded at a density of 3 x 10⁴ cells per well and cultured in DMEM containing 10% foetal calf serum, penicillin (100 units ml⁻¹) and streptomycin (100 µg ml⁻¹) for 24 h at 37 °C in a humidified CO₂ incubator. Then the medium was exchanged and polymer solution was added in a final concentration of 0.5 mg ml⁻¹ for an additional 3 h, two washes and three times with PBS, trypsinised, centrifuged, washed and then suspended in 1 ml PBS. AR/H₂O₂ was added to a final concentration of 10 µM, and after 2 h, flow cytometry analysis was performed using a BD FACSAnCanto II flow cytometer (BD Bioscience, USA). Doublets were excluded using FSC and SSC detectors, single cells were counted and the number of live cells was detected in FLS (586/15; Resorufin Channel). A total of 10,000 single cells for each sample were analysed, and data processed using Flow Jo VX software (TreeStar, Ashland, OR).

Intracellular stability of AO. HeLa (epitheloid cervix carcinoma, human; ATCC, CCL-2) cells were seeded at a density of 3 x 10⁴ cells ml⁻¹ onto poly-o-lysine-coated glass coverslips. Cells were cultured for 24 h in DMEM growth medium (supplemented with 10% foetal calf serum, penicillin (100 units ml⁻¹) and streptomycin (100 µg ml⁻¹)). After 24 h, the medium was replaced with fresh growth medium (supplemented with 10% foetal calf serum, penicillin (100 units ml⁻¹) and streptomycin (100 µg ml⁻¹)) to allow attachment to the surface. After attachment, the medium was removed and catalytic nanocompartments were added to a final polymer concentration of 0.5 mg ml⁻¹. Cells were incubated for an additional 24 h in the medium, then washed three times with PBS and fixed with 4% PFA for 15 min. After a neutralisation step using 50 mM NH₄Cl, cells were either treated with PBS (control) or 0.1% saponin for 10 min at room temperature. Additional washing steps, cell nuclei were counterstained for 10 min using Hoechst 33342 (0.5 µg ml⁻¹). Finally, cells were embedded in Vectashield antifade mounting media. CLSM was performed using an Olympus FV-1000 inverted microscope (Olympus Ltd, Tokyo, Japan) equipped with 150 μm UPlanFL.N oil-immersion objective (numerical aperture 1.40). Cells were excited at 405 nm (Hoechst 33342) and 488 nm (Atto488-HRP), and the fluorescence signal was collected using Kalman modus between 425 and 475 nm and 500 and 600 nm, respectively. To minimise spectral cross talk, the samples were scanned using sequential mode. The laser settings were adjusted depending on the treatment. Images were processed using the Fiji open source image processing package of ImageJ.

Intracellular localisation of AO. HeLa (epitheloid cervix carcinoma, human; ATCC, CCL-2) cells were seeded at a density of 3 x 10⁴ cells per well onto poly-o-lysine-coated glass coverslips. Cells were cultured for 24 h in DMEM growth medium (supplemented with 10% foetal calf serum, penicillin (100 units ml⁻¹) and streptomycin (100 µg ml⁻¹)), then fixed for 10 min in 3.7% formaldehyde, pH 7.4, and the protein was kept at this value. For the measurement, 10 µl of the samples mixed with GSH were transferred to 220 µl of reaction mixture containing 250 µM 4-sulphophenyl)-2H-tetrazolium (MTS) assay (Promega) and used to determine cell viability. HeLa cells were seeded in a triplicate at a density of 2.5 x 10⁵ cells per well in a 96-well plate. Cells were cultured for 24 h in Dulbecco’s modified Eagle’s medium (DMEM) growth medium (supplemented with 10% foetal calf serum, penicillin (100 units ml⁻¹) and streptomycin (100 µg ml⁻¹)). After 24 h, the medium was removed and catalytic nanocompartments were added to a final polymer concentration of 0.5 mg ml⁻¹. Cells were washed twice before being imaged at the respective time points.

Flow cytometry analysis of AO activity. HeLa (epitheloid cervix carcinoma, human; ATCC, CCL-2) cells were seeded in a well of a 24-well plate (8 x 10⁴ cells per well) and cultured in DMEM containing 10% foetal calf serum, penicillin (100 units ml⁻¹) and streptomycin (100 µg ml⁻¹) for 24 h at 37 °C in a humidified CO₂ incubator. Then the medium was exchanged and polymer solution was added in a final concentration of 0.5 mg ml⁻¹ for an additional 3 h, two washes and three times with PBS, trypsinised, centrifuged, washed and then suspended in 1 ml PBS. AR/H₂O₂ was added to a final concentration of 10 µM, and after 2 h, flow cytometry analysis was performed using a BD FACSAnCanto II flow cytometer (BD Bioscience, USA). Doublets were excluded using FSC and SSC detectors, single cells were counted and the number of live cells was detected in FLS (586/15; Resorufin Channel). A total of 10,000 single cells for each sample were analysed, and data processed using Flow Jo VX software (TreeStar, Ashland, OR).
Dylight633-labelled antibody (1:1000; #35562, Thermo Fisher Scientific). Cell nuclei were counterstained for 10 min using Hoechst 33342 (0.5 μg ml⁻¹). Finally, cells were embedded in Vectashield antifade medium (Hoechst 33342 (2.5 μg ml⁻¹). Cell membranes were stained using CellMask Deep Red Plasma membrane stain (0.5 μM ml⁻¹) when indicated directly before imaging. Live cell imaging was performed as described in the previous section using an Olympus FV-1000 inverted microscope (Olympus Ltd, Tokyo, Japan) equipped with a ×60 UPlanFL N oil-immersion objective (numerical aperture 1.4). Orange colour indicated colocalisation of polymersomes with lysosomes (LysoTracker Red DND-99).

Quantitative uptake studies by flow cytometry. Differentiated THP-1 cells were incubated with Atto488-HRP-loaded polymersomes equipped with OmpF-S-S-CF at a final polymer concentration of 0.25 mg ml⁻¹ for specific time points as indicated. LysoTracker Red DND-99 (Invitrogen) was added to cells 1 h before imaging at a concentration of 50 nM when indicated. Cell nuclei were counterstained using Hoechst 33342 (2.5 μg ml⁻¹). Cells were then incubated using CellMask Deep Red Plasma membrane stain (0.5 μM ml⁻¹) when indicated directly before imaging. Flow cytometry analysis was performed using a BD FACSCanto II flow cytometer (BD Bioscience, USA) as described in the previous section.

Ethical regulations. All procedures on live zebrafish embryos (Danio rerio) were carried out following the Swiss legislation on animal welfare.

Data availability. The data that support the findings of this study are included in the Supplementary Information; the remaining data are available from the corresponding author upon reasonable request.

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Author contributions
T.E. contributed to the OmpF modification and characterisation, AOs production and characterisation, in vitro and in vivo assays, and the writing of the manuscript; C.E. contributed to the double mutant of OmpF and characterisation of the OmpF modification; D.W. contributed to the in vitro and in vivo assays; S.S. contributed to the in vivo assays; R.G. contributed to characterising the enzymatic reactions; A.N. contributed to the FCS experiments; M.S. contributed to the EPR experiments; O.O.-F. contributed to the OmpF modification and characterisation; J.H. contributed to the in vitro and in vivo experiments and writing of the manuscript; and C.G.P. contributed to the AOs concept and writing of the manuscript.

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