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## Biomimetic strategy to reversibly trigger functionality of catalytic nano-compartments by insertion of pH-responsive bio-valves

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# Biomimetic strategy to reversibly trigger functionality of catalytic nano-compartments by insertion of pH-responsive bio-valves

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3 **Abstract:** We describe an innovative strategy to generate catalytic compartments with  
4 triggered functionality at the nanoscale level by combining pH-reversible bio-valves and  
5 enzyme-loaded synthetic compartments. The bio-valve has been engineered by attachment of  
6 stimuli-responsive peptides to a genetically modified channel porin, enabling a reversible  
7 change of the molecular flow through the pores of the porin in response to a pH change in the  
8 local environment. The bio-valve functionality triggers the reaction inside the cavity of the  
9 enzyme-loaded compartments by switching the *in situ* activity of the enzymes on/off based on  
10 a reversible change of the permeability of the membrane, which blocks or allows the passage  
11 of substrates and products. The complex functionality of our catalytic compartments is based  
12 on preservation of the integrity of the compartments to protect encapsulated enzymes. An  
13 increase of the *in situ* activity compared to that of the free enzyme, and a reversible on/off  
14 switch of the activity upon the presence of a specific stimulus is achieved. This strategy  
15 provides straightforward solutions for development of catalytic nanocompartments efficiently  
16 producing desired molecules in a controlled, stimuli-responsive manner with high potential in  
17 areas, such as medicine, analytical chemistry, and catalysis.  
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40 **Keywords:** stimuli-responsiveness; catalytic nanocompartment; membrane-protein; triggered  
41 bio-activity; reversible permeability  
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3 In nature, controlled transport of molecules between cellular and subcellular compartments is  
4 vital for functional cellular metabolism and is achieved via delicate molecular mechanisms  
5 involving membrane proteins (MPs). A plethora of MPs located in cellular phospholipid  
6 bilayers allow active or passive transport of molecules through the compartment membrane.  
7 Such molecular transport is frequently triggered by stimuli such as pH or temperature change,  
8 transmembrane potential, light, or the presence of specific molecules.<sup>1-3</sup> A bioinspired  
9 strategy on focus today for development of new materials is based on interfacing  
10 biomolecules such as MPs, enzymes, and proteins with synthetic membranes (planar  
11 membranes and compartments).<sup>4, 5</sup> Such bio-nanoassemblies combine the activity of  
12 biomolecules with the architecture of a synthetic assembly, generating new materials with  
13 emergent properties and functionality that have high potential in various domains of  
14 technology, medicine and environmental sciences.<sup>5-7</sup> Bio-nanoassemblies have been designed  
15 as models in order to understand the activity of biomolecules in synthetic matrixes proposed  
16 as artificial organelles,<sup>8, 9</sup> molecular motors,<sup>10</sup> and simple models of artificial cells.<sup>11</sup> In  
17 addition, bio-nanoassemblies have been developed for applications, such as local synthesis of  
18 polymers,<sup>12</sup> active surfaces serving for production of antibiotics<sup>13</sup> or sensitive biosensing,<sup>14</sup>  
19 and membranes for water purification.<sup>15</sup>

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42 Particularly interesting are catalytic compartments named nanoreactors which are based on  
43 encapsulation of active compounds (proteins, enzymes, mimics) in the cavity of  
44 compartments where they are able to fulfil their function of protected reactions at the  
45 nanoscale.<sup>5, 16</sup> These catalytic compartments can be used to study enzymatic reactions in  
46 confined spaces, or for specific applications such as detoxification of reactive oxygen species  
47 and storage of oxygen,<sup>17</sup> enzyme-mediated biotransformation,<sup>18</sup> synthesis of polymers,<sup>12</sup> or  
48 even as simple mimics of cell organelles.<sup>19-21</sup> Polymer vesicles are particularly appealing  
49 compartments, with sizes ranging from nanometers (polymersomes) up to micrometers  
50 (synthetic giant unilamellar vesicles, synthetic GUVs). In comparison to liposomes (lipid  
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3 based compartments closely related to cell membranes), polymer compartments have specific  
4 advantages arising from the intrinsic chemical nature of the polymers: i) greater mechanical  
5 stability, ii) tunable membrane properties (thickness, flexibility, composition), and iii) ability  
6 to be functionalized with different molecular groups.<sup>22-24</sup> Appropriately selected amphiphilic  
7 copolymers that self-assemble into polymersomes or GUVs can be both nontoxic and  
8 responsive, changing their architecture in the presence of a specific stimulus, properties  
9 largely exploited for development of drug delivery systems.<sup>25</sup> However in the case of the  
10 catalytic compartments, it is not possible to use stimuli-responsive polymers that change their  
11 architecture, because their irreversible disassembly would lead to the release of the active  
12 compounds.<sup>26, 27</sup>

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26 Membrane permeability is an essential property for *in situ* reactions inside catalytic  
27 compartments, to allow the reaction substrates to pass inside and products to be released.  
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30 Polymer membranes have been rendered permeable by various approaches: i) using  
31 copolymers which generate porous membranes,<sup>28-30</sup> ii) inducing pores by chemical  
32 treatment,<sup>12, 18, 31</sup> or iii) mimicking cell membranes by insertion of biopores and MPs.<sup>6</sup> Once  
33 reconstituted in polymersome membranes, MPs such as AqpZ, LamB, OmpF, Tsx and FhuA<sup>6,</sup>  
34<sup>32, 33</sup> and membrane penetrating peptides such as gramicidin<sup>34, 35</sup> allow diffusion of ions and  
35 small molecular weight molecules with a selectivity based on their intrinsic properties. In  
36 order to develop stimuli-responsive compartments that preserve their architecture as well as  
37 support *in situ* reactions, a biomimetic approach is to use appropriate MPs to induce  
38 responsiveness. To date only a few liposome and polymersome membranes have been  
39 permeabilized by insertion of modified channel proteins that possess stimuli-  
40 responsiveness.<sup>36, 37</sup> The membrane proteins have been modified to equip them with a  
41 cleavable responsive molecular cap, which is cleaved in the presence of a specific stimulus,  
42 and the molecular flow through the pore is restored. Stimuli responsive protein pores were  
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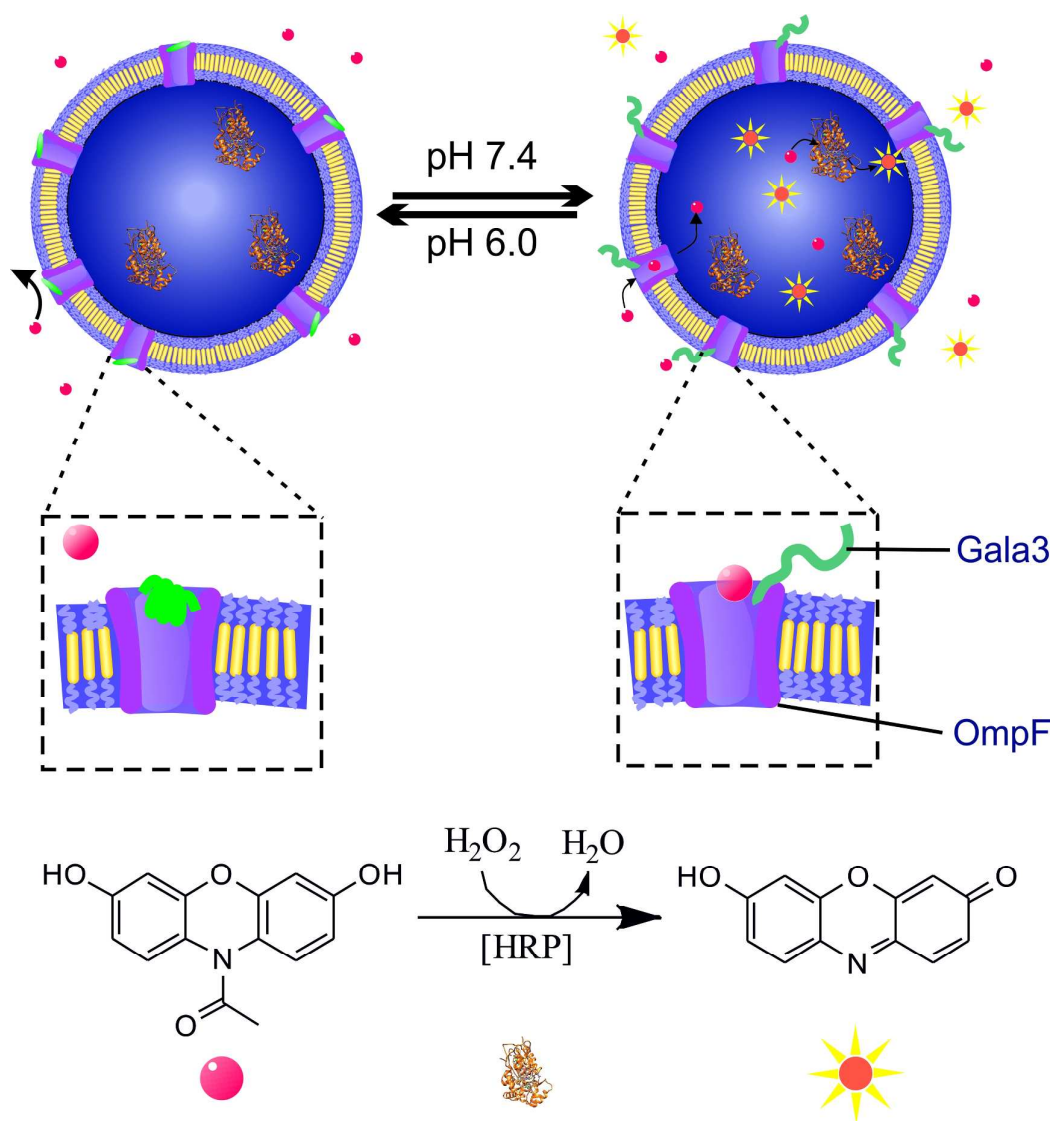
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3 designed to react to external changes in pH (OmpF,<sup>36, 38</sup> MS-CL<sup>39</sup>) or reduction potential  
4 (FhuA<sup>40, 41</sup>). Another approach to obtain stimuli responsive MPs is based on using amino acid  
5 mutations, to create charge repulsion in the channel interior (OmpF),<sup>38</sup> or introduce an elastin-  
6 like polypeptide loop with temperature triggered solubility.<sup>42</sup> A more appealing approach for  
7 practical applications, which involves a permanent attachment of a stimuli responsive  
8 molecular group, has been applied only to ion channels.<sup>43, 44</sup> Unfortunately, such engineered  
9 ion channels cannot be used as “gates” for catalytic compartments, because they do not allow  
10 the diffusion of small molecular weight enzymatic substrates.  
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21 We introduce here a biomimetic strategy to develop catalytic compartments with controlled  
22 functionality triggered in a stimuli-responsive manner by changes in the compartments  
23 environment. The catalytic compartments with reversibly triggered activity are obtained by  
24 introducing into the synthetic membrane of enzyme-loaded polymersomes bio-valves based  
25 on modified membrane proteins. Bio-valves were engineered by attaching molecules able to  
26 change the conformation and properties of channel porins in specific conditions. These  
27 attached molecules enable reversible opening and closing of the porin pores, depending on  
28 environmental conditions. Such bio-valves can efficiently switch on/off *in situ* activity when  
29 inserted in the membrane of the compartments (Figure 1). We selected OmpF as a model  
30 channel porin, because of its well characterised crystal structure, remarkable stability  
31 (temperature, solvents, detergents and pH),<sup>38, 45, 46</sup> and moderate pore size, allowing molecules  
32 of up to 600 Da to pass through.<sup>38, 46</sup> In order to favour the attachment of a molecular cap, we  
33 genetically engineered OmpF through point mutation to feature two cysteine residues in  
34 specific positions on the channel interior (OmpF-M), and then attached a peptide to those  
35 cysteines. Changes in pH induce this peptide to make reversible conformational changes and  
36 thus block (closed state) or unblock (open state) the pore of the peptide-OmpF-M conjugate  
37 (OmpF-C). OmpF-C was inserted into the membrane of polymersomes based on poly(2-  
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3 methyl-2-oxazoline)-block- poly(dimethylsiloxane)-block poly(2-methyl-2-oxazoline)  
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5 (PMOXA-*b*-PDMS-*b*-PMOXA) loaded with Horseradish Peroxidase (HRP). We selected  
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7 PMOXA- *b*-PDMS- *b*-PMOXA amphiphilic triblock copolymers for self-assembly into  
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9 polymersomes because of their flexible membranes and previously reported ability to  
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11 successfully incorporate MPs.<sup>47</sup> *In situ* activity of HRP was followed during reversible  
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13 changes in pH to evaluate the open/close functionality of OmpF-C.  
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17 Our approach is a resourceful strategy to design a reversible gating system that it is not  
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19 restricted to a single “activation” of the catalytic compartment by opening the pores but can  
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21 be switched on and off over several cycles, potentially maintaining stimuli-responsiveness for  
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23 long-term utilization. Both, a reversible and precise response in time and space upon an  
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25 external stimulus and the large variety of possible applications by encapsulation of  
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27 appropriate active compounds (enzyme, proteins, mimics) highlight the potential of our  
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29 system in areas such as medicine, catalysis, and technology, where such materials will  
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31 provide desired processes/compounds “on demand”.  
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35 To induce stimuli responsiveness of the OmpF porin, we attached a pH-responsive peptide  
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37 capable of significant changes in charge and conformation upon changes of pH from 7.4 to  
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39 6.0. Each of the pH-responsive amino acid groups must be accompanied by hydrophobic  
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41 amino acids, in order to create a more pronounced change in solubility between different  
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43 protonation states, and to be obtained by a straightforward synthesis and purification  
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45 procedure. An example of a peptide sequence that meets these requirements is GALA  
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47 (WEAALAEALAEALAEHLAEALAEALEALAA), previously reported to change its  
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49 polarity and conformation producing two distinct states pH 5.5 and 7.5.<sup>48</sup> In addition, the  
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51 peptide sequence has to be adapted to the geometric constrains of the elliptical OmpF pore  
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53 with an inner diameter of 7-11 Å.<sup>46</sup>  
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**Figure 1.** Schematic representation of a bio-valve functioning by reversible pore opening and closing inside the membrane of nanocompartments to trigger an *in situ* reaction (left: closed state; right open state). Modified OmpF pore (purple; stimuli responsive group green) is inserted in the polymersome membrane that separates the encapsulated enzyme (HRP) from the environment. The *in situ* reaction is triggered by the bio-valve functionality, which allows the diffusion through the OmpF pores of the substrate Amplex UltraRed® (magenta spheres) and the subsequent release of the fluorescent products (yellow stars).

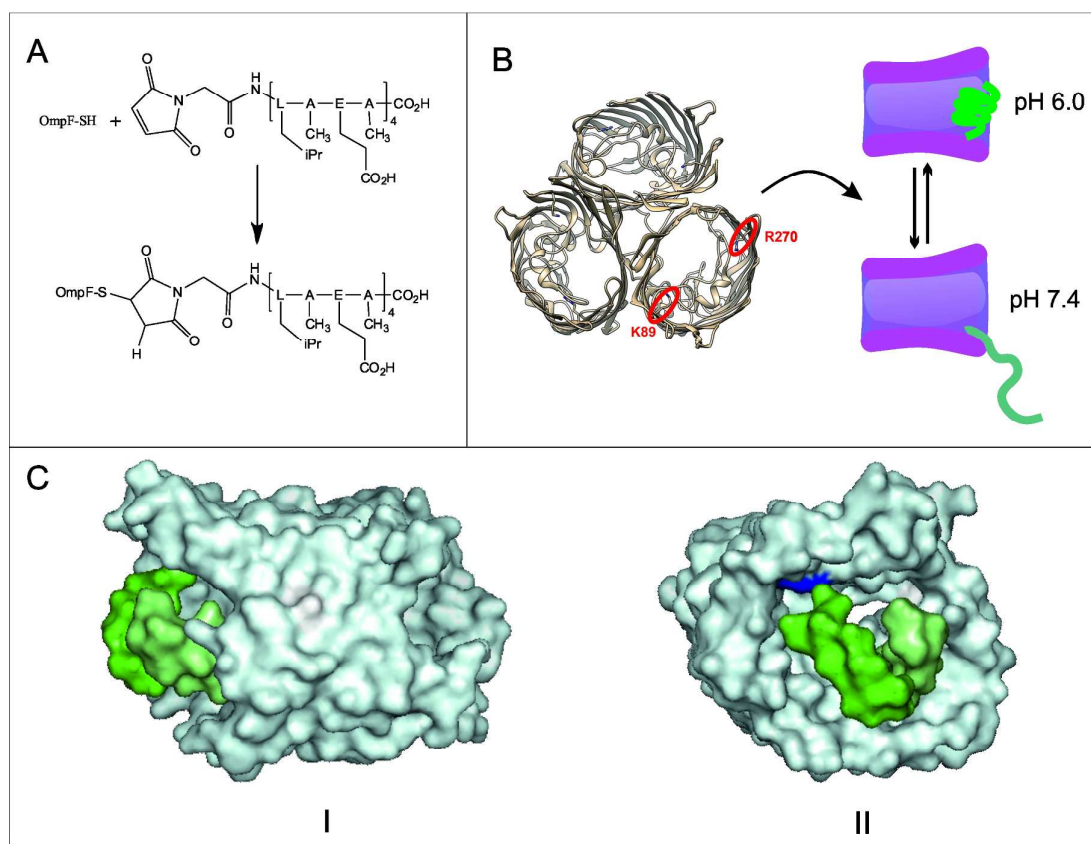


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3 Only peptides with a total length of 20-50 Å are suitable to block the pore, considering that  
4 both random coil and  $\alpha$ -helix structures are significantly shorter than the corresponding  
5 stretched peptide (Figure 2). In this respect, a GALA sequence shortened to  
6 LAEALAEALAEA (Gala3) is an optimum peptide sequence matching the requirement of  
7 closing the OmpF pore, and thus we selected it for this purpose. In order to allow a significant  
8 change in protonation, at least three carboxylic acids were incorporated in the peptide  
9 structure. Considering the neighbouring hydrophobic amino acids, the minimal length  
10 requirement for the peptide sequence is indeed obtained with Gala3.  
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21 In order to attach the responsive peptide to the channel protein, first OmpF was genetically  
22 modified to introducing cysteine residues at key locations in the OmpF amino acid sequence.  
23 The mutation sites K89C (lysine) and R270C (arginine) located inside the pore in the eyelet-  
24 region were replaced by cysteines (Figure 2) as anchoring points for the attachment of  
25 molecular groups that can block the molecular flow at the beginning of the pore or point  
26 outwards without obstructing the pore, in different conditions. (Figure 2B) 3D representations  
27 of OmpF-C (Figure 2C) were obtained using Pymol and the crystal structure of OmpF wild  
28 type from the RSCB Protein database with the cysteines residues attached. We used two  
29 mutations in order to increase the labelling efficiency of the porin, and to offer better  
30 conditions to block the OmpF pore because two peptides will occupy more space inside the  
31 pore.  
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46 The mutation was performed via polymerase chain reaction (PCR) of the entire plasmid. The  
47 K89C-mutation was introduced first, and then the R270C-mutation was introduced by the  
48 same procedure. The plasmid was shifted to higher masses than the template (unmodified  
49 plasmid), which remained supercoiled (Figure S1). After purification via gel-electrophoresis,  
50 the PCR product was isolated and sequenced, confirming the mutation (Table S2, Supporting  
51 Information). The double mutant of the porin, K89C-R270C-OmpF (OmpF-M) was then  
52 overexpressed in Omp8 E.coli. Its excess over naturally occurring OmpF-WT made further  
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3 separation unnecessary. OmpF-M could not be distinguished on gel from OmpF-WT or single  
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5 mutants K89C-OmpF and R270C-OmpF, indicating a successful expression and good purity,  
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7 because of the lack of additional bands in the Coomassie stained gel (Figure S2, S3,  
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9 Supporting Information). The occasionally observed splitting of the OmpF band (Figure S2) is  
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11 attributed to the presence of residual signalling peptide (2000 Da).<sup>49</sup> An OmpF-M  
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13 concentration of  $0.75 \pm 0.25$  mg/mL (extract without further concentration steps) was  
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15 determined by BCA total protein assay<sup>®</sup>. The presence of thiol groups and their accessibility  
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17 was evaluated by binding them with the thiol-reactive fluorophore acrylodane. As expected,  
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19 OmpF-WT did not reveal any fluorescence intensity upon addition of acrylodane, indicating  
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21 the lack of nonspecific binding of the fluorophore to the porin. On the contrary, the  
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23 fluorescence intensity of acrylodane when added to the thiol-bearing mutants (K89C-OmpF,  
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25 R270C-OmpF and OmpF-M), clearly indicates successful thio-functionalization of the porin.  
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27 Moreover, the fluorescence intensity of acrylodane when bound to the double mutant OmpF-  
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29 M was similar to that resulting from a double concentration of R270C-OmpF bound to  
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31 acrylodane (Figure S5, Supporting Information), indicating that both thiol groups are  
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33 accessible, and can bind to small molecular weight molecules. To attach Gala3 to the thiols of  
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35 the cysteine residues in the OmpF-M, Michael addition was chosen due to its straightforward  
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37 approach, fewer side-reactions compared to disulphide bonds formation with the cysteine  
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39 residues, or to direct alkylation of the cysteine residues with halogenoalkanes.<sup>50, 51</sup> In addition,  
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41 the chosen glycine maleic imide linker between OmpF-M and Gala3 is a small molecule (97  
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43 Da) and expected to have no impact on substrate translocation through the pore due to its  
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45 small size.  
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**Figure 2.** A. Michael addition of the Gala3 peptide to the cysteine side chains of the OmpF-M; B. Location of the mutation sites K89 and R270 (highlighted only in one of the three channels of the OmpF trimer) and a schematic representation of the cross-section of the pore in “closed state”, with Gala3 blocking the entrance of the pore (above) and in “open state”, with the attached Gala3 freely moving in its random-coil state (below). C. Three-dimensional representation of OmpF-C after binding of the pH responsive peptide Gala3 (green) attached in helical structure (compact state) to cysteine residues (blue) of OmpF-M showing closing of the pore: view from the side (I), and from the top (II).

The degree of labelling (DOL) of the double mutant OmpF-M with Gala3 (OmpF-C) was determined using a combination of mass spectrometry (MS), and the acrylodane assay. For

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3 the prior, OmpF-C and OmpF-M were purified via SDS-gel electrophoresis and digested  
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5 within the gel, resulting in various peptides. Mass spectroscopy is a direct and absolute  
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7 method for quantification, however, long hydrophobic peptides are particularly difficult to  
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9 ionize and transfer into the gas phase. Such peptide sequences are typical for membrane  
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11 proteins such as OmpF.<sup>52</sup> Thus it was necessary to perform an indirect measurement by  
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13 comparing the ratio between the intensities of the mass peaks of the cysteine-bearing OmpF  
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15 fragment without Gala3 with another peptide without cysteine, and calibrating it with OmpF-  
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17 M. (Equation 2; Supporting Information) This eliminated the dependency on the protein  
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19 concentration, as the other peptide served as an internal standard. The measurements indicate  
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21 that  $94\% \pm 5\%$  of the thiol groups within the double mutant OmpF-M bound Gala 3 ( $92\% \pm$   
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23  $5\%$  of K89C- and  $96\% \pm 5\%$  of R270C anchor points had the Gala3 peptide attached) (Table  
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25 S3). We used as a complementary qualitative assay, the acrylodane assay, which is based on  
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27 binding of acrylodane to the accessible thiol groups that remain unbound after the attachment  
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29 of Gala3. A DOL value of  $68\% \pm 9\%$  was obtained, indicating a successful binding of Gala3  
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31 to the modified OmpF (Figure S4, Supporting Information, Table S4). DOL value determined  
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33 with the acrylodane assay was lower than determined by mass spectroscopy due to  
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35 fluorophore interactions with aromatic amino acid residues.<sup>53, 54</sup> However, both MS and the  
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37 acrylodane assay indicate successful thio-functionalization of the OmpF-M, as an essential  
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39 step for the attachment of Gala3.  
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46 A second step in obtaining reversible pH-responsive catalytic compartments was to insert  
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48 OmpF-C into polymersome membranes together with simultaneous encapsulation of an  
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50 enzyme inside the cavity, and then to test the overall functionality. We used the rehydration  
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52 method to generate catalytic compartments,<sup>55</sup> NR, by self-assembly of PMOXA<sub>6</sub>-*b*-PDMS<sub>44</sub>-  
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54 *b*-PMOXA<sub>6</sub> amphiphilic block copolymers in the presence of: HRP (NR-), HRP and OmpF-C  
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56 (NRC), and HRP OmpF-M (NR+). OmpF-C/-M were directly added to an ethanolic  
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58 copolymer solution before drying until a thin polymer film was formed. Slow hydration with  
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3 biomolecule solutions in PBS induced the self-assembly process and formation of  
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5 polymersomes containing the biomolecules in specific locations depending of their intrinsic  
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7 character: OmpF-C/-M in the membrane, with HRP inside the cavity. In general, the  
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9 efficiency and homogeneity of polymersome formation, and OmpF insertion depend on  
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11 numerous factors, as for example the presence of surfactants, such as octylglucopyranoside  
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13 (OG), used in the membrane protein extraction and stabilisation.<sup>56</sup> (Figure S9, Supporting  
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15 Information). Since OG was only required for the initial steps of OmpF extraction and the  
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17 stabilisation in solution, its concentration was reduced to the minimal possible concentration  
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19 for storage (0.5 w/w %), and was further removed prior to mixing it with the copolymer  
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21 solution. Before dialysis, polymersome size was controlled by extrusion through 0.2  $\mu\text{m}$   
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23 polycarbonate filters. Polymersome solutions were diluted to the same concentration  
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25 (determined by their respective ability to scatter light). The final polymersome concentration  
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27 was considered as the lowest concentration determined for NR-, NRC and NR+. The  
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29 homogeneity of the polymersomes size was confirmed by dynamic light scattering as with a  
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31 PDI < 0.2.  
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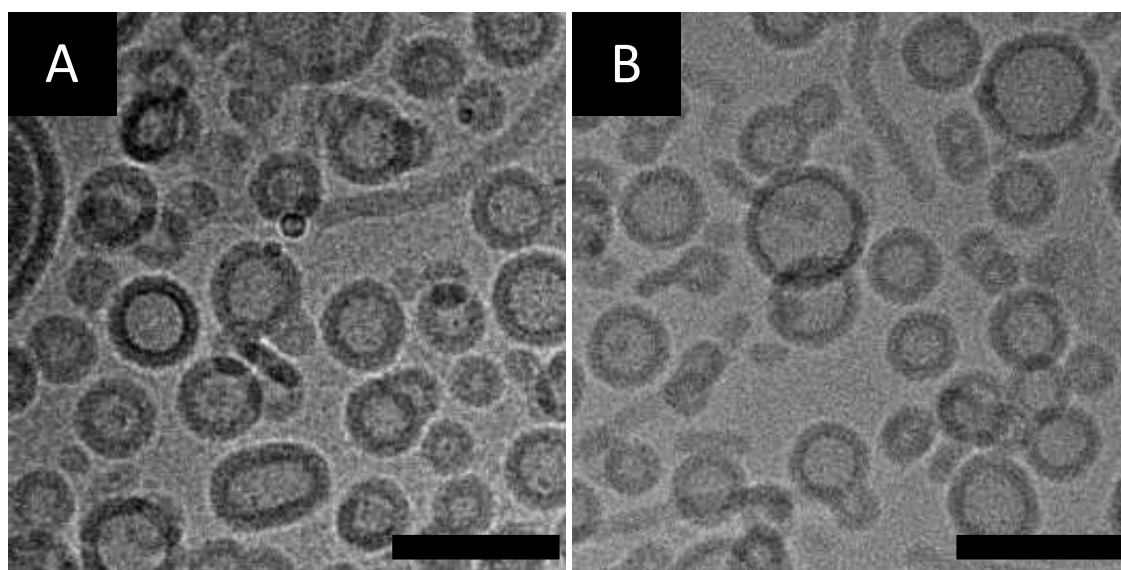
37 The size and the architecture of the polymer assemblies resulting from the self-assembly of  
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39 the copolymers without and with the biomolecules were determined by static and dynamic  
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41 light scattering, SLS and DLS (Table 1; Figure S6-S8, Supporting Information).  
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44 **Table 1:** Light scattering parameters of: HRP loaded polymersomes (NR-), HRP loaded  
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46 polymersomes with inserted OmpF-C (NRC), and HRP loaded polymersomes with inserted  
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48 OmpF-M (NR+).  
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	pH 7.4			pH 6.0		
	NR-	NRC	NR+	NR-	NRC	NR+
$R_g$ [nm]	61 $\pm$ 8	58 $\pm$ 4	61 $\pm$ 3	61 $\pm$ 1	62 $\pm$ 2	63 $\pm$ 2
$R_h$ [nm]	64 $\pm$ 8	61 $\pm$ 4	64 $\pm$ 3	66 $\pm$ 2	65 $\pm$ 2	67 $\pm$ 2
$\rho$ []	0.96	0.94	0.95	0.93	0.95	0.93

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3 The ratio ( $\rho$ ) between the hydrodynamic radius ( $R_h$ ) and radius of gyration ( $R_g$ ) was close to 1  
4 indicating that the polymer assemblies have hollow sphere architecture.<sup>57</sup>  
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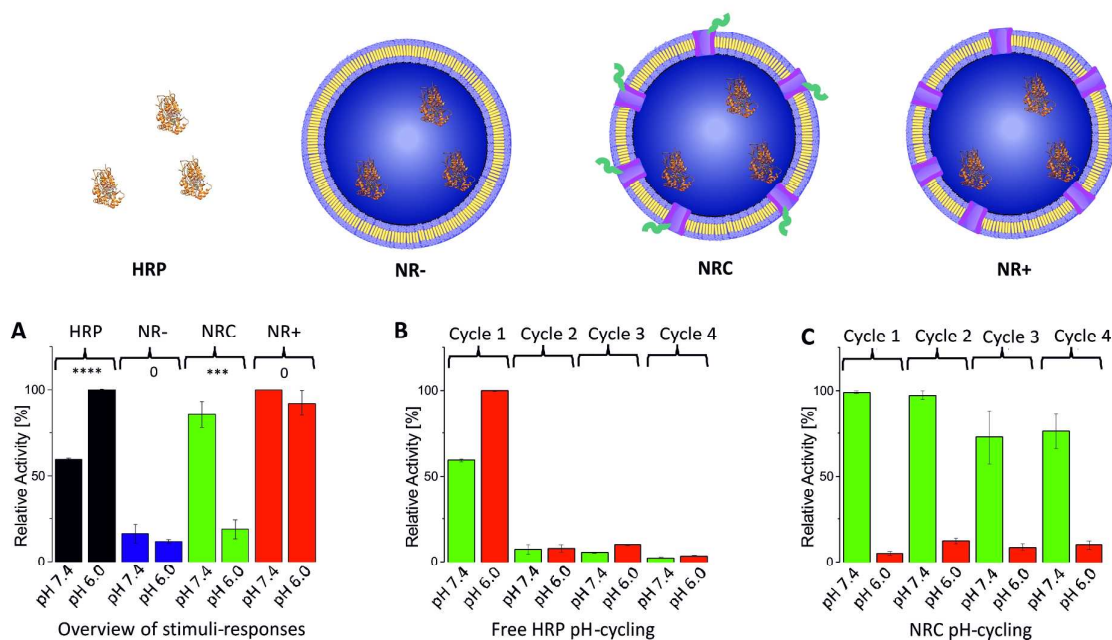
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8 TEM micrographs indicate that there were no significant differences in size and architecture  
9 between polymersomes loaded with HRP without OmpF (NR-) and those containing HRP  
10 with different OmpF mutants inserted (NR+ and NRC) (Figure S10-S12, Supporting  
11 Information). The diameter of the polymersomes obtained by TEM is between 50-120 nm, in  
12 agreement with the light scattering results (Table 2). In addition, polymersome NRs (without  
13 and with inserted OmpF-M/C) were stable at room temperature at both pH 7.4 and 6.0 for  
14 several days. Cryo-TEM micrographs clearly indicate that the membrane integrity is  
15 preserved upon insertion of OmpF-C, and confirm the size obtained by TEM analysis (Figure  
16 3). Unlike the other methods, cryo-TEM revealed a minor population of worms, but which is  
17 not interfering with the bio-valve insertion and functionality.  
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50 **Figure 3:** Cryo-TEM micrograph of A) HRP loaded PMOXA<sub>6</sub>-*b*-PDMS<sub>44</sub>-*b*-PMOXA<sub>6</sub>  
51 polymersomes equipped with OmpF-C, NRC, and B) HRP loaded PMOXA<sub>6</sub>-*b*-PDMS<sub>44</sub>-*b*-  
52 PMOXA<sub>6</sub> polymersomes without OmpF, NR-. Scale bar 100nm.  
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3 Before analysing the influence of the pH change on the activity of the catalytic compartments,  
4 the behaviour of free HRP was investigated, since enzymes have an optimal pH and a window  
5 of operation and stability. HRP presented a pH dependent activity, with an activity  
6 significantly higher at pH 6.0 than at pH 7.4 (Figure 4A), but becoming irreversibly  
7 deactivated below pH 5.0.<sup>58, 59</sup> Cycling pH changes between 7.4 and 6.0 revealed that the free  
8 enzyme lost its activity very fast. Already after the first full cycle of pH change, a drop in  
9 activity of the free HRP up to 12.5% of its initial value was observed, and it further decreased  
10 after the second complete pH change cycle. The decrease in enzymatic activity of the free  
11 HRP was mainly due to the adjustment of pH with small amount of concentrated HCl in order  
12 to avoid dilution. A temporary, local pH decrease below the final pH probably deactivated the  
13 enzyme<sup>58, 59</sup> (Figure 4C and S13 Supporting Information). There are other factors resulting in  
14 a drop of the perceived activity as well, as for example the accumulation of salt and  
15 fluorophores after each cycle that contributed to the reduction of the detected fluorescence  
16 intensity.  
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34 The low activity of the free HRP after the second pH change cycle remained higher at pH 6.0  
35 than at pH 7.4, but the enzyme did not recover (Figure 4B). This significant decrease in free  
36 HRP activity indicates the possible effect of harmful conditions in biosystems in the case of  
37 the free enzyme, as for example local changes in pH or a proteolytic attack. Therefore it  
38 induces the necessity to protect the enzyme when desired applications are planned, and  
39 supports our approach of development of catalytic nanocompartments. Catalytic  
40 nanocompartments play in biologic conditions a dual role: they shield the enzymes from local  
41 harmful conditions and proteolytic attack, whilst allowing them to perform their activity *in*  
42 *situ*.  
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**Figure 4.** A) Activity of HRP based on the increase in fluorescence intensity of HRP product, measured at pH 7.4 and pH 6: free HRP (black), NR- (blue), NRC (green), and NR+ (red). The stars indicate the statistical significance (P value; Table S6 Supporting Information). B) Free HRP activity after changing the pH from 7.4 to 6.0, and adding Amplex UltraRed® and H<sub>2</sub>O<sub>2</sub> in each cycle. Green: pH 7.4 and red: pH 6.0. C) Bio-valve functionality of OmpF-C when inserted into the membrane of HRP loaded polymersomes (NRC) after changing the pH from 7.4 to 6.0, and adding Amplex UltraRed® and H<sub>2</sub>O<sub>2</sub> in each cycle. Green: pH 7.4 and red: pH 6.0. The activities were corrected by taking the volume increase into account.

Stimuli responsiveness of the OmpF-C equipped membranes was evaluated by the *in situ* activity of encapsulated HRP at pH 6.0 and 7.4 both after the first pH cycle (Figure 4A), and after several cycles (Figure 4C). The increase of fluorescence intensity associated with the formation of resorufin-derivative inside the cavity of polymersome NRs represents a direct measure for the transmembrane diffusion of the HRP substrate. Polymersomes loaded with



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3 HRP, but without inserted OmpF, (NR-) had a very low residual activity after dialysis, size  
4 exclusion chromatography and treatment with proteinase K (Supporting Information  
5 polymersome preparation). In order to understand the cause of this residual HRP activity in  
6 NR-, interactions of residual HRP traces with the membrane during the polymersomes  
7 formation have been evaluated by addition of HRP in the environment of the polymersomes,  
8 and subsequent dialysis. Only HRP activity at the detection limit has been observed, which  
9 indicates no HRP bound on the external side of the membrane (Figure S14). Therefore, the  
10 residual activity of NR- is probably due to the partition of Amplex UltraRed between the  
11 aqueous phase and the hydrophobic membrane, resulting in a very small fraction of auto-  
12 oxidised Amplex UltraRed in the presence of H<sub>2</sub>O<sub>2</sub>.  
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26 The initial HRP activities of the NRs (NRC, NR-, NR+) were normalised for a simpler  
27 comparison, such that the highest activity of NR+ was set to 100%. (Figure 4, Figure S175-  
28 18, Table S5, Supporting Information). HRP activity values represent the mean value of three  
29 independent measurements. A remarkable stimuli-response of the catalytic compartment  
30 containing HRP was observed for a pH change of only 1.4 units: *in situ* HRP activity inside  
31 NRC decreased up to 20% of the activity at pH 7.4 upon the decrease of pH at 6.0, thereby  
32 changing from an activity close to NR+ to an activity close to the low residual activity of NR-  
33 (Figure 4A), completely overriding the pH-dependency of free HRP. This indicates, within  
34 the error limits, a change of the OmpF-C pore from an “open state” (pH 7.4) to a “closed  
35 state” (pH 6.0) due to a conformational change of Gala3 inside OmpF-C. The pH-responsive  
36 change in the accessibility of OmpF-C for molecular diffusion triggered *in situ* HRP activity,  
37 and induced a functionality change from an “active” catalytic compartment to a “non-active”  
38 one. This pH-dependent behaviour allows the catalytic nanocompartments to start their  
39 activity “on demand” by a simple pH change. At pH 6.0, where OmpF-C is in “closed” state,  
40 the catalytic compartment has low functionality, even if inside the cavity HRP is active, and  
41 expected to have a higher activity compared to that at pH 7.4 (Figure 4A, free HRP and  
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3 NRC). On the contrary, when OmpF-C is in an “open” state, substrates pass through the  
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5 membrane and the functionality of the catalytic compartment is fully restored due to the  
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7 intrinsic activity of HRP. Interestingly, both the enzyme activity of NRC and that of the  
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9 catalytic nanocompartment with OmpF-M, NR+, is significantly higher at pH 7.4 than that of  
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11 the free HRP at this pH, matching the activity value of the free HRP at pH 6.0 (Figure 4A).  
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13 Note that the increase of the enzyme activity inside the confined space of the polymersome  
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15 cavity compared with bulk assays was observed in similar conditions (amount of enzyme,  
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17 ratio of substrates). The increase in activity upon encapsulation of enzymes inside nano-  
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19 compartments is due to the effect of the confined nano-space, which enhances the interaction  
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21 of the enzymes with the substrates.<sup>60</sup> Inside the cavity of polymersomes there is an increase in  
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23 the probability of interaction between the enzyme molecules and their substrates due to an  
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25 increase of the duration the substrates are remaining in proximity of the enzymes. In addition,  
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27 the impermeable synthetic membrane allows molecular transport through and thus *in situ*  
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29 enzymatic reaction when the pores of OmpF-C are open, and serve for a rapid diffusion of  
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31 substrates/products. We estimated that for a 125nm diameter polymersome the mean number  
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33 of porin molecules per vesicle is around 11 OmpF molecules/vesicle have been inserted in the  
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35 conditions we used for OmpF insertion (Supporting Information).  
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42 The stimuli response behaviour of OmpF-C inserted in polymersome membranes was  
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44 reproducible (raw data: Figure S15-S19, Table S5 Supporting Information). The OmpF pore  
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46 with a 600 Da molecular weight cut-off allowed Amplex (300 Da) to pass through in its “open  
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48 state” despite being modified with a 1322.4 Da Gala3 peptide, which indicates that Gala3 is  
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50 extended outside of the pore at pH 7.4. Various molecular factors might be responsible for the  
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52 bio-valve functionality of Gala3 inside the OmpF pore: change in the peptide protonation  
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54 state, change in the peptide conformation, and different electrostatic interactions within the  
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56 pore. Based on average pKa-values, it can be estimated that at pH 6.0 only around 2% of the  
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58 glutamic acid groups would become protonated. Therefore, this change in the protonation  
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3 state of the Gala3-peptide is not the key parameter affecting the transport through the OmpF  
4 pore, being insufficient to explain the significant change in the *in situ* HRP activity (Figure  
5 S22, Table S8 Supporting Information). However, significant structural changes have been  
6 reported for the GALA-peptide: GALA is in a random coil at pH 7.4 and forms an  $\alpha$ -helix at  
7 pH 5.5.<sup>48</sup> We believe that even the short Gala3-peptide undergoes similar conformational  
8 changes in combination with interactions between GALA3 in the double mutant.  
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17 Catalytic compartments containing HRP and equipped with OmpF-M were still active after  
18 two weeks, which is a remarkable improvement in the stability of encapsulated HRP  
19 compared with the free enzyme (Figure S20, Supporting Information) The reversibility of the  
20 stimuli response of NRC was evaluated by cycling the pH change, and measuring the HRP  
21 activity triggered by these cycles. (Figure 4C). With each pH cycle, new Amplex UltraRed®  
22 and H<sub>2</sub>O<sub>2</sub> were added. Each time, upon a pH increase to 7.4, the NRC activity increased due  
23 to the opening of the OmpF-C pore, and inversely the activity decreased when the pH was  
24 decreased to 6.0, because the OmpF pore was closed. The activity of NRC at pH 7.4 on the  
25 fourth cycle slightly decreased to 75%, which can be explained by saturation effects resulting  
26 from successive additions of the substrate and salts<sup>61, 62</sup>. In comparison free HRP significantly  
27 decreased its activity in bulk after the first cycle (Fig S13). Therefore, our NRC is not only a  
28 reversible pH catalytic compartment, but also an ideal candidate to protect the enzymes and  
29 preserve their activity. Moreover, when compared to the time resolution of the enzyme  
30 kinetic, the change between open/closed states can be considered instantaneous.  
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49 We successfully developed catalytic compartments with reversible triggered activity by  
50 inserting bio-valves in the membrane of synthetic compartments loaded with enzymes. The  
51 role of the bio-valves is to control the *in situ* activity inside catalytic nanocompartments by  
52 changing in a reversible manner from an “open” to a “close” state that controls the molecular  
53 flow through the compartment membrane. The bio-valve resulted from attachment of stimuli-  
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3 responsive peptides to a genetically modified channel porin, OmpF. Changes in the  
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5 conformation inside the pores, upon a pH change of 1.4 units in the environment of the  
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7 nanocompartments block/unblock the pores of OmpF in a reversible manner. The pH-  
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9 reversible bio-valve functionality induced an *in situ* activity switch on/off by the molecular  
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11 flow of substrates/products through the polymersome membrane. Compared to all stimuli-  
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13 responsive compartments reported, which either change their integrity or do not allow a  
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15 controlled permeability, our synthetic compartments equipped with a genetically engineered  
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17 bio-valve represent a step beyond what has been achieved to date in complex functionality. In  
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19 addition, the encapsulation of the enzyme served both to increase the activity due to the  
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21 confined reaction space inside the polymersomes, and to increase its stability compared to the  
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23 free enzyme. We anticipate that further improvements to the system can be made by: i)  
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25 expanding it for other types of stimuli associated with attachment of corresponding molecular  
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27 groups, and ii) adjustment of the number and length of the stimuli responsive molecules to  
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29 modulate the molecular flow through the pores.  
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35 Our strategy to build catalytic compartments with reversible triggered functionality has the  
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37 potential to completely change the manner in which stimuli-responsive compartments are  
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39 developed in the future, especially but not limited to medical or catalysis applications. In  
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41 addition, the insertion of reversible bio-valves in synthetic membranes is not limited to  
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43 nanocompartments and can be easily expanded to planar membranes, resulting in a wide  
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45 range of applications ranging from analytical chemistry, nanofiltration, and up to medicine.  
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51 **Methods.** A detailed description of the reagents is found before each experimental section in  
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53 the Supporting Information. The double cysteine mutant K89C R270C of OmpF-WT (OmpF-  
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55 M) was created to introduce thiol groups as anchor points for the attachment of stimuli  
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57 responsive peptides. K89 C represents one lysine in 89<sup>th</sup> position, which is replaced by a  
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3 cysteine, whilst R270C represents one arginine replaced by a cysteine. The plasmid bearing  
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5 the OmpF-gene used in this work has been previously described.<sup>63</sup> Mutations were introduced  
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7 via site-directed mutation directly on the plasmid, by using a two-step PCR protocol<sup>64</sup> adapted  
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9 for the PfuTurbo DNA Polymerase (Agilent) and the corresponding primers (Table S1,  
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11 Supporting Information). The amplicon was then purified on a 1% agarose gel using a gel-  
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13 extraction kit from QIAGEN. The amplicon was first transfected to XL1-Blue ultra-  
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15 competent cells, then extracted and sequenced prior to transfection into BL21 (DE3)Omp8.<sup>63</sup>  
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17 This intermediate step over the ultracompetent cells was necessary to improve uptake because  
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19 the amplicon was significantly larger than the supercoiled plasmid.  
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23 Both OmpF-WT and the OmpF mutants (single mutants K89C-OmpF, R270C-OmpF, and the  
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25 double mutant K89C-R270C-OmpF) were expressed following a published procedure<sup>55</sup>, with  
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27 minor changes (see Supporting Information). The extraction of OmpF was made in PBS  
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29 buffer containing octylglucopyranoside (3 w/w % OG; Affymetrix). The purity of OmpF was  
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31 evaluated on a 12% SDS-PAGE gel, and the concentration of OmpF was measured  
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33 spectrometrically at  $\lambda = 280$  nm with Nanodrop (Witec Ag), and with a BCA-protein assay  
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35 (Thermo Fischer Scientific)  
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39 A DMSO solution of 20eq. Gala3 peptide-maleic imide per cysteine-group was slowly added  
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41 to 1mL OmpF-M (1g/L) in PBS with 3 w/w % OG at pH=7.4, and stirred overnight at room  
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43 temperature. OmpF-C was dialysed twice against 1L PBS containing 0.5 w/w % OG using  
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45 Midi GeBAflex-tube (50-800 $\mu$ L; 6-8kDa MWCO). The solution was concentrated 2x using  
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47 10kDa Amicon Centrifugal Filters (Milipore).  
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51 The protein concentration was measured spectrometrically at  $\lambda = 280$  nm with Nanodrop  
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53 (Witec Ag), and the labelling efficiency was evaluated by using the acrylodane assay (details  
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55 in Supporting Information), and mass spectrometry (applying SDS-gel for purification; in-gel  
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57 digestion and HPLC-ESI MS on the extract).<sup>65</sup>  
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3 Polymersomes either without and in the presence of biomolecules (OmpF and HRP) were  
4 prepared by the film rehydration method of PMOXA<sub>6</sub>-*b*-PDMS<sub>44</sub>-*b*-PMOXA<sub>6</sub> triblock  
5 copolymer (details in Supporting Information).<sup>66</sup> After confirming the purity and homogeneity  
6 of the polymersomes using a Malvern Zetasizer (PDI < 0.1), the polymersome concentration  
7 of different samples has been adjusted to avoid differences by comparing their ability to  
8 scatter light with a Spectramax M5 spectrophotometer in semi-micro PS cuvettes (VWR).  
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17 Light scattering experiments were performed using an ALV goniometer (ALV GmbH,  
18 Germany) equipped with an ALV He –Ne laser (JDS Uniphase, wavelength  $\lambda = 632.8$  nm).  
19 The solutions after film rehydration method (polymer concentration diluted to 1.25, 0.625,  
20 0.313 and 0.156 g/L) were measured in a 10mm cylindrical quartz cell at angles ranging from  
21 30° to 150° at 293 K with angular steps of 10°. ALV/Static & Dynamic FIT and PLOT  
22 program version 4.31 10/10 were used for data analysis. Static light scattering data were  
23 processed according to the Guinier-model.  
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33 Polymersome solutions were diluted to a polymer concentration of 0.05 g/L and 5  $\mu$ L were  
34 negatively stained with 2% uranyl acetate solution and deposited on a carbon-coated copper  
35 grid. The samples were examined with a transmission electron microscope (Philips CM-100)  
36 operated at 80 kV.  
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43 The activity of HRP (free and encapsulated into polymersomes equipped with or without  
44 OmpF-C or OmpF-M,) was measured using Amplex UltraRed® (Invitrogen) as substrate  
45 (500 $\mu$ M final concentration) and hydrogen peroxide as co-substrate (13.3 $\mu$ M final  
46 concentration). The HRP reaction was evaluated at pH 7.4 and 6.0. HRP activity was  
47 measured in: i) free conditions, ii) polymersomes equipped with peptide bearing OmpF-M,  
48 (NRC), iii) polymersome containing HRP without inserted OmpF, (NR-), and iv)  
49 polymersomes containing HRP and with OmpF-C inserted in the membrane, (NR+).  
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3 For each resulting kinetic curve based on the increase of the fluorescence intensity of the  
4 resorufin-derivative over time, the initial slope of the graph (0 to 200s) was taken as the initial  
5 activity and all values were normalised, such that the value corresponding to NR+ was set to  
6 100%. For the reversibility experiments, pH was adjusted with minimal amounts of  
7 concentrated hydrochloric acid or sodium hydroxide. HRP reaction was detected at pH 7.4,  
8 then the pH was decreased to 6.0 and another equivalent of Amplex UltraRed® and hydrogen  
9 peroxide were added and the fluorescence intensity of the resorufin-derivative was measured  
10 again. Then the pH was increased back to 7.4 to continue with the next cycle.  
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36 Hughes for editing the manuscript.  
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## 49 **ASSOCIATED CONTENT**

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51 The Supporting Information is available free of charge on the ACS Publications website at  
52 DOI:  
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56 Supporting information: detailed description of the experimental methods; additional data  
57 including DNA-sequencing; SDS-gels; DOL-measurements of the OmpF-conjugate; TEM-  
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and LS-data; enzyme and catalytic nanocompartments kinetics; ANOVA and calculations of number of OmpF/polymersome.

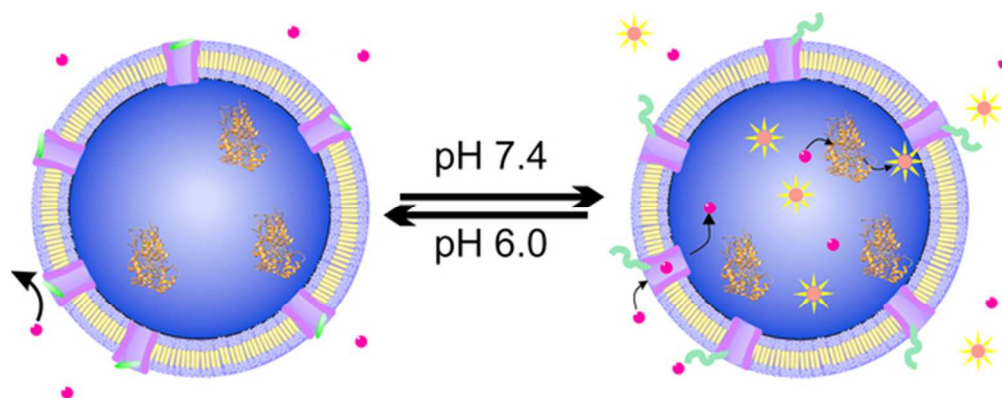
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