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Supporting Information

for *Small*, DOI 10.1002/smll.202303384

An Outer Membrane-Inspired Polymer Coating Protects and Endows *Escherichia coli* with Novel Functionalities

Andrea Belluati, Iain Harley, Ingo Lieberwirth and Nico Bruns**

An outer membrane-inspired polymer coating protects and endows *Escherichia coli* with novel functionalities

Andrea Belluati*[a][b], Iain Harley[c], Ingo Lieberwirth[c], Nico Bruns*[a][b]

[a] Dr. A. Belluati, Prof. Dr. N. Bruns.
Department of Chemistry
Technical University of Darmstadt
Alarich-Weiß-Straße 4, 64287 Darmstadt, Germany

Department of Pure and Applied Chemistry
University of Strathclyde
295 Cathedral Street, Glasgow G1 1XL, United Kingdom

E-mail: andrea.belluati@tu-darmstadt.de; nico.bruns@tu-darmstad.de

[c] I. Harley, Dr. I. Lieberwirth
Department of Physical Chemistry of Polymers
Max Planck Institute for Polymer Research
Ackermannweg 10, 55128 Mainz, Germany

Supporting information at the end of the document.

Experimental Section

Materials and strains

Unless otherwise specified, all reagents were purchased from Merck and used as received. Poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol) (Pluronic® L-121; average M_n ~4400, composition: 30% PEG) is a product of BASF SE and was purchased from Merck. The used strain was *E. coli* BL21 (DE3), which was purchased from Thermo Fisher and was used to transform all the plasmids. YFP was encoded in the plasmid pETTherm α/β,[1] sperm whale myoglobin (swMb, wild type) was in the plasmid pet-20b(+). The empty pet-26b(+) vector was a donation from Prof. Wolf-Dieter Fessner (Technical University of Darmstadt).

Preparation of GUVs

The GUVs were prepared according to a scaled-up published protocol.[2] Pluronic® L-121 was dissolved in toluene at 20 mg ml^{-1}. 20 µl of 380 mM sucrose were added to 150 µl of toluene, and mixed by vigorous pipetting for 1 minute (w/o emulsion). 175 µl of the w/o emulsion were then pipetted into 1.2 ml of outer water phase (either 380 mM sucrose solution, PBS or LB medium) and pipetted again for 1 minute. The solvent was left to evaporate under very mild air flow for 2 h.

Bacterial growth and protein expression

All strains were grown in 5-10 ml of LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl, 1 l ddH_2O) at 37 °C, supplemented with the antibiotic their plasmids encode the resistance for. Protein expression (YFP and myoglobin) was induced at OD_{600} 0.6 with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 0.1 mM 5-aminolevulinic acid.

Bacterial encapsulation

Bacteria were grown to OD_{600} 1. They were then diluted to OD_{600} 0.5 in fresh LB right before extrusion. Volumes of GUVs and bacterial suspensions were adjusted to obtain 1.1 ml of mixture at three different PL121:bacteria volume ratios (1:1, 3:1, 9:1), corresponding to the weight/volume% of 0.034%, 0.051%, 0.062% (SI Table 1). Then, 1 ml of these adjusted mixtures was pipetted into the syringe of a Mini Extruder (Avanti, USA) equipped with Whatman® Nuclepore® 1 µm track-etched membrane filters (VWR, Germany), and passed through for 12 times.[3] In this way, GUVs would break up passing through the pores, reforming around the bacteria extruded together with them.

The same ratios were used for mixed bacteria and GUVs, skipping the co-extrusion step. Naked bacteria were also diluted in LB to a final OD_{600} 0.05. We assumed the equivalence OD_{600} 1 = 8×10^8 cells.

Plasmid retention assay

Naked and encapsulated bacteria with pet-26b(+) were plated on LB-agar. The agar plates were incubated at 37 °C for 16 h. Then, colonies were counted. 100 colonies of each were picked and stabbed on new plates with kanamycin, incubated at 37 °C overnight, and the number of grown colonies was counted again. Bacteria grown without selective pressure (antibiotic) and with a damaged membrane lose more easily their plasmid. Transferring them

to a medium with the antibiotic will indicate what percentage lost their plasmid in the first step (*i.e.*, whether their membrane was damaged by the encapsulation).

Membrane labelling and CLSM imaging

1.6 µmol of cholesterol-PEG_4-N_3 were mixed with 4.8 µmol dibenzocyclooctyne-Cy5 (DBCO-Cy5) to 1 mL PBS and left to react at RT for 4 hours, then dialysed overnight (1 kDa MWCO) against PBS to remove unreacted dye. The intensity of the blue decrease only slightly after dialysis (visually confirmed), suggesting a good conjugation efficiency, and good retention by the dialysis membrane.

Samples were placed in Nunc® Lab-Tek® 8-well chamber slides (Thermo Fisher), 5 to 20 µl of sample in 200 µl PBS, with the addition of either 10 µl cholesterol- PEG_4-Cy5 or 10 µl 100 µM Nile Red (NR) in DMSO. To verify the membrane permeability, 10 µl of 1 mM sodium fluorescein in DMSO were added to the outside of the vesicles.

The imaging was done on a Leica SP8 CLSM, equipped with an HCX PL APO 63 × NA 1.2 W CORR CS2 objective (Fluorescein, YFP: ex. 488 nm, em. 505–525 nm; Nile Red: ex. 561 nm, em. 570–590 nm; Cy5: ex.635 nm, em. 660–690 nm, Propidium iodide: ex 488 nm, em 630-650 nm).

For the sizing of GUVs, between 30 to 60 GUVs per sample were imaged.

Images were optimized (brightness and contrast; applied evenly throughout a whole image, variance filter) and analysed via ImageJ.[4]

Multiple comparison one-way ANOVA with Tukey correction was used to compare the populations.

Fluorescein diacetate / propidium iodide staining

10 µL of FDA (100 µM) and 5 µL of PI (0.2 mg ml^{-1}) were added to 200 µL of suspension containing naked or encapsulated bacteria, and the resulting mixture was incubated for 10 min at RT. 10 µL of this solution were further diluted in 200 µL PBS and imaged with the CLSM. 3-4 random locations per frame were imaged and then the number of green-fluorescent (FDA, alive) or red-fluorescent (PI, dead) bacteria were counted.

CryoTEM

3 µl of the sample were placed onto a 400 mesh copper grid covered with lacey film (previously glow discharged). The grid was plunged into liquid ethane (automated plunging system, Vitrobot FEI) and transferred in liquid nitrogen to the TEM (Titan Krios G4,

Thermo-Fisher Scientific) and micrographs were acquired using a Ceta 4k camera (Thermo-Fisher Scientific). The TEM was operated at 300 kV. Membrane thickness was then measured via ImageJ,[4] n = 10 different measurements. Due to the dilution of the sample, 20 membrane segments were measured, using between 5 to 20 cells in total.

ζ-potential measurement

10 µl of the sample were diluted into 1 ml Tris HCl pH 7. Then, the ζ-potential was measured in a capillary flow cuvette with a Zetaview (Particlemetrix). 11 positions were imaged for all samples, and the average potential calculated. Samples were either incubated at 37 °C in LB or kept at 4 °C up to 24 h, and measured at given intervals. After the bacterial growth, an aliquot was diluted to OD_{600} 0.5 and re-encapsulated. Multiple comparison two-way ANOVA with Tukey correction was used to compare the populations.

Microplate measurements of bacterial cultures

Several biomarkers were measured on a Spark microplate reader (Tecan) and in a Clariostar Plus microplate reader (BMG Labtech), in Greiner transparent 96-well plates. Naked and encapsulated bacteria (10 µl) were diluted to final 200 µl in LB. Endpoint measurements were measure at RT, time runs were measured at 37 °C, 4 hours. OD_{600} was further measured for 24 h.

The absorbance of fluorescein (10 µL from 0.05 mM FDA) was measured at 490 nm, OD_{600} at 600 nm, and NADPH fluorescence at 340/460 (± 20) nm. Kinetic curves were integrated, and their values averaged and compared (two-way ANOVA with Tukey correction).

Cy5 and YFP fluorescence were measured at 620/682 nm and 485/535 nm (± 20 nm), respectively. Multiple comparison two-way ANOVA with Tukey correction was used to compare the measurements.

Bacterial stressors

Depending on the experiment, bacteria were incubated 20 min at 50 °C, 1 h at -20 °C, centrifuged (12000 RCF in an Eppendorf microgentrifuge) for 60 min, sonicated with a Fisherbrand ultrasound probe (70 s, 5 + 5 second pulse, amplitude 50%), incubated 3 h in MillIQ water or 1 M sucrose, 30 min in 70% EtOH and 10 min in 20% H_2O_2, or a combination thereof. After incubation with chemicals, bacteria were gently centrifuged and resuspended in LB before measurements. To test the bacterial resistance to stressors, fluorescein, normalized by the final cell density, OD_{600}, was used as a general indicator for

encapsulation performance, by comparing the stressed cells to the cells in the same encapsulation state, but with no treatment. After 3 hours and 50 minutes, FDA (10 µL from 1 µM in 200 µL) was added to each well and then incubated at RT. Fluorescence was measured at 485/535 nm (± 20 nm). Multiple comparison two-way ANOVA with Tukey correction was used to compare the measurements.

Activity of Mb-expressing *E. coli*

After 3 h of swMb expression, bacteria were subjected to temperature + centrifugation shocks, and then mixed with 2 µl 0.01% H_2O_2 and 2 µL 0.1 mM ABTS. The production of the colored ABTS radical cation was measured at 420 nm in the microplate reader. For the chemoluminescence, 100 µl 300 mM luminol substituted ABTS (whole luminescence spectrum recorded).

In the case of 2,4,6-TCP, 200 µl of bacteria were diluted to 1.5 ml LB with 50 mM 2,4,6-TCP and 1% H_2O_2 (H_2O_2 was otherwise substituted with 20 µl GOX 2 mg ml^{-1}, using the glucose present in LB) and incubated at 37 °C. Spectra from 240 to 700 nm were recorded (no replicates) at t = 0 h, 2 h and 4 h, in quartz cuvettes, in a Cary 60 UV-Vis spectrophotometer.

Lysozyme activity on bacteria

In plastic Eppendorf tubes, naked and encapsulated bacteria (final OD_{600} 0.1, various polymer w/v%) were mixed with lysozyme at 0.5, 1, 2 and 4 mg ml^{-1} for 2 h at 37 °C to a total volume 500 µl (with LB), supplemented with 2 mM EDTA. Afterwards, the samples were centrifuged (12000 x RCF) and washed 2 x to remove any free lysozyme, then resuspended and centrifuged at 1000 x g to remove any possible cell debris. 10 µL of the bacteria were diluted in 200 µL PBs with FDA (10 µL from 1 µM) and incubated for 10 min at RT. Fluorescence (485/535 nm (± 20 nm)) and OD_{600} were measured on a microplate reader. Viability was compared to that of naked, untreated bacteria at the same dilution, based to the FDA/OD_{600} ratio.

The selective killing of naked bacteria was assayed as follows: YFP-expressing bacteria were encapsulated according to the protocol described above. They were mixed with naked, non-fluorescent bacteria at two ratios (10:1 and 100:1 in favor of naked bacteria), and incubated with 4 mg ml^{-1} lysozyme for 4 h at 37 °C in LB, supplemented with 2 mM EDTA. As OD_{600} reflects all bacteria, but YFP fluorescence comes only from the encapsulated population, the YFP/ OD_{600} ratio indicates the relative ratios in the mixture. Unencapsulated bacteria have a

competitive advantage in growing, so without any selective killing, the ratio will decrease over time.

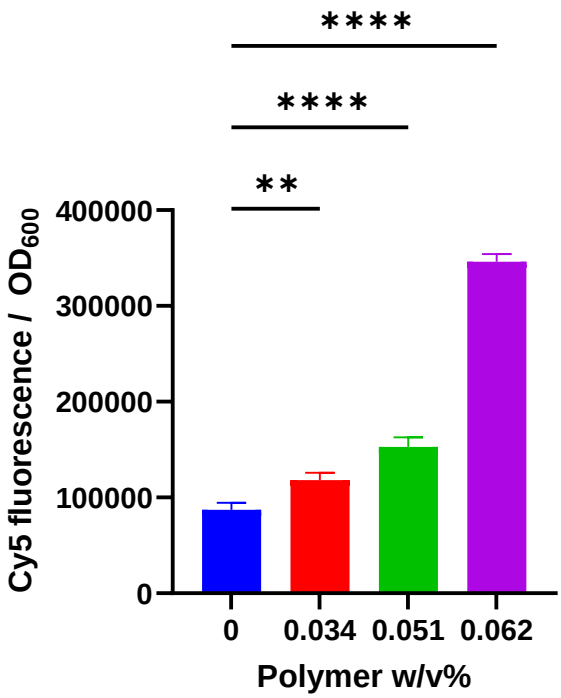
Surface functionalization with αAM

2 ml of a 2.5 mg ml^{-1} αAM solution in PBS was incubated with 45 µl of 10 mg ml^{-1} NHS-PEG_4-DBCO in PBS, for 4 hours at RT, then purified via a 40 kDa spin diafiltration device (Amicon, Merck) and further incubated overnight at 4 °C with 14.3 µl of 30 mg ml^{-1} cholesterol-PEG_4-N_3 in PBS, which was then spin filtered again. The concentration of αAM was measured by UV-vis spectroscopy at 280 nm using an extinction coefficient of 118 $mM^{-1}cm^{-1}$, calculated from the sequence as (N of tryptophan residues*5.5) + (N of tyrosine residues*1.5).[5] 100 µl of either PBS, αAM or αAM-PEG-cholesterol construct (at the same concentration) were mixed with 500 µl of bacteria. With or without a centrifugation step, 50 µl bacteria where then incubated for 4 h in 1 ml M9 minimal medium (Gibco) with 6.25 mg ml^{-1} of soluble starch, and OD_{600} was then measured. Multiple comparison two-way ANOVA with Tukey correction was used to compare the measurements.

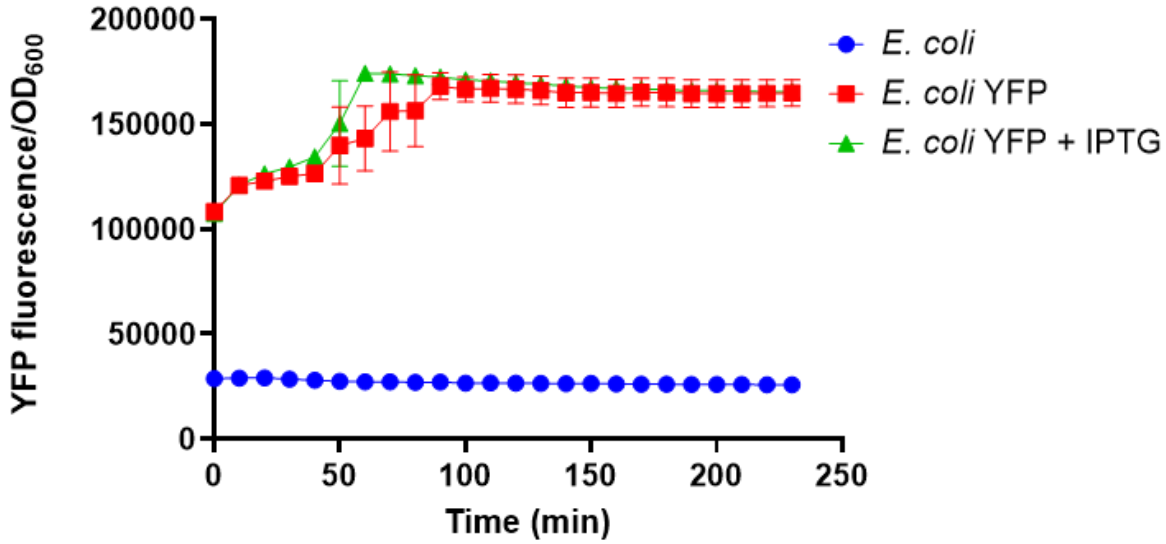
Surface functionalization with lysozyme

The functionalization with lysozyme was conducted in the same manner as with AAM (using 5 mg ml^{-1} lysozyme). Fluorescent, encapsulated bacteria with lysozyme-cholesterol (*E.coli*@0.62%) were mixed 1:10 in their favor with naked, non-fluorescent bacteria, and the YFP/ OD_{600} ratio was measured during the course of 4 h, for consistency with the previous assays. Multiple comparison two-way ANOVA with Tukey correction was used to compare the measurements.

Supporting Information



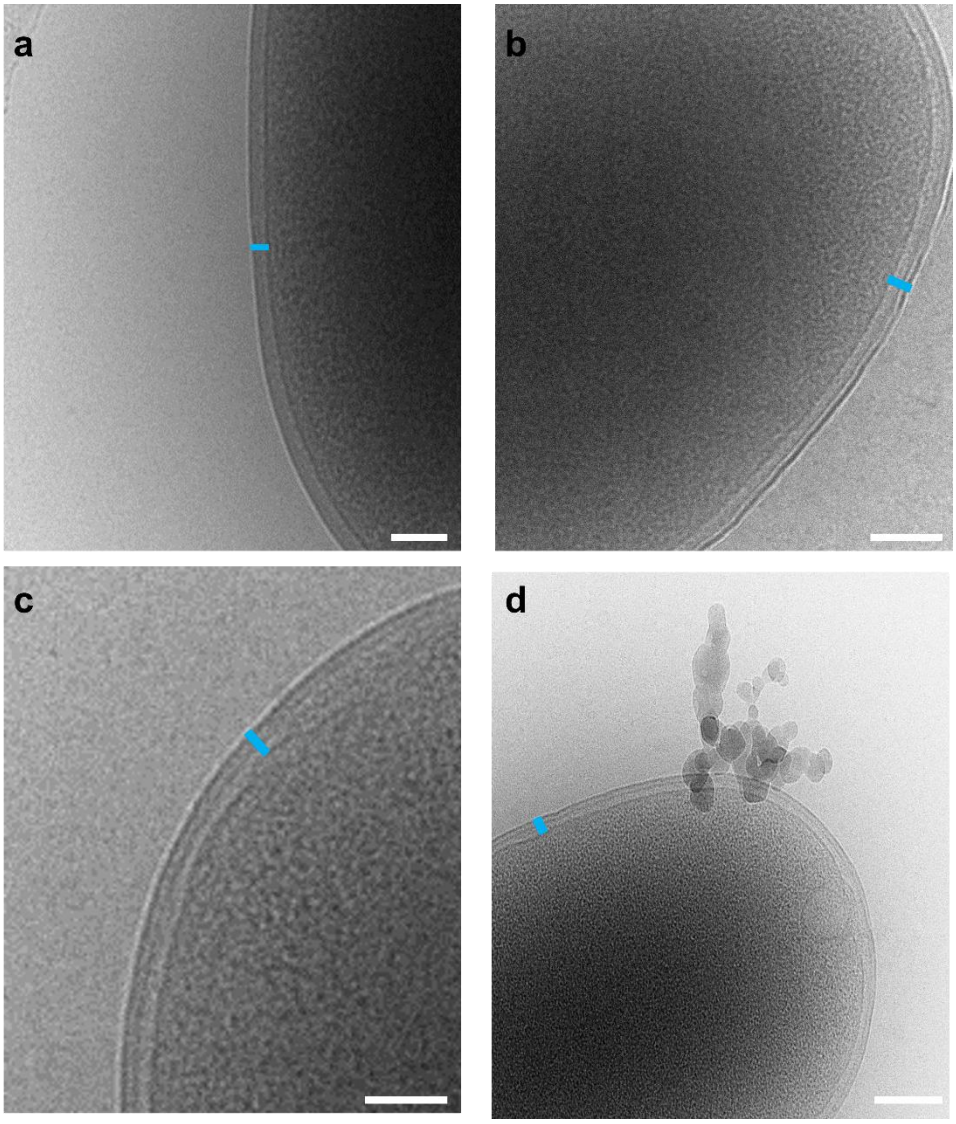
SI Figure 1. Fluorescence/OD_{600} after centrifugation. A higher value means that more of the membrane label Cy5-PEG_4-cholesterol is retained by each cell. Data displayed as mean ± S.D., n = 3. **: $p < 0.01$; **: $p < 0.001$ ****: $p < 0.0001$.



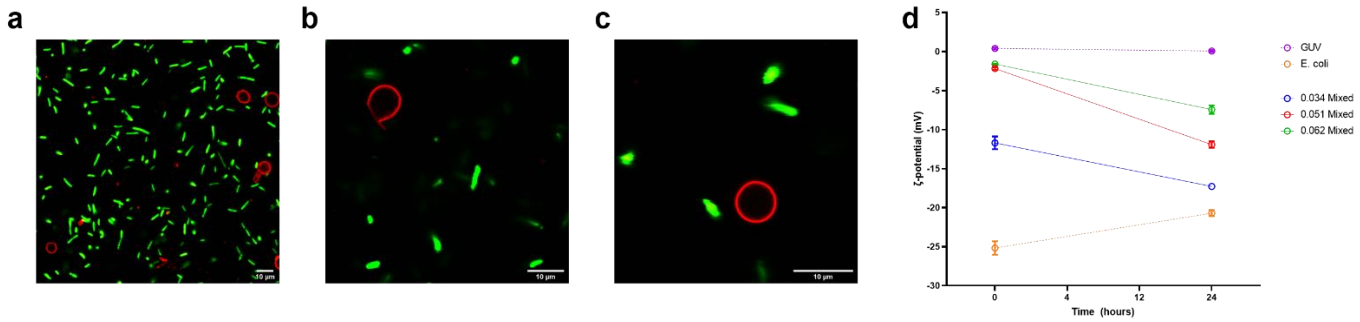
SI Figure 2. Fluorescence (normalized per cell) of plasmid-less *E. coli*, *E. coli* expressing YFP with and without induction, showing a detectablee fluorescence already before IPTG induction. Data displayed as mean ± S.D., n = 3.

SI Table 1. Overview of the weight/volume% ratios used in this study, and their equivalents in volumes weight/weight of cell and femtomoles per cell.

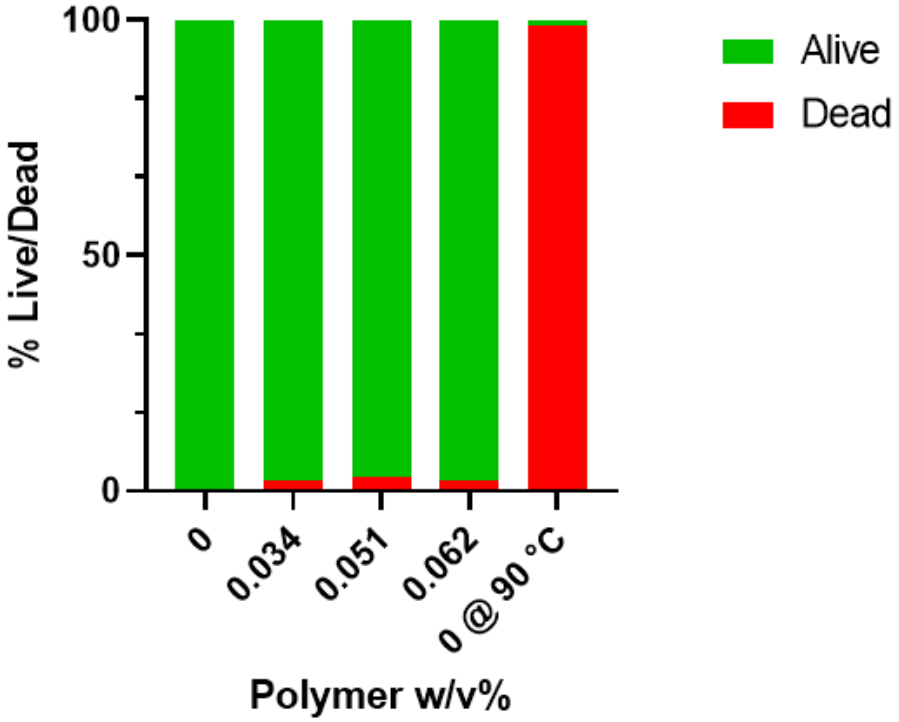
Polymer weight/volume%	**PL121:bacteria (final OD 0.05) (volume ratio)**	**Polymer:cell (mg/mg)**	**Polymer:cell (fmol/cell)**
0.034%	1:1	9	2
0.051%	3:1	12	3
0.062%	9:1	15	4



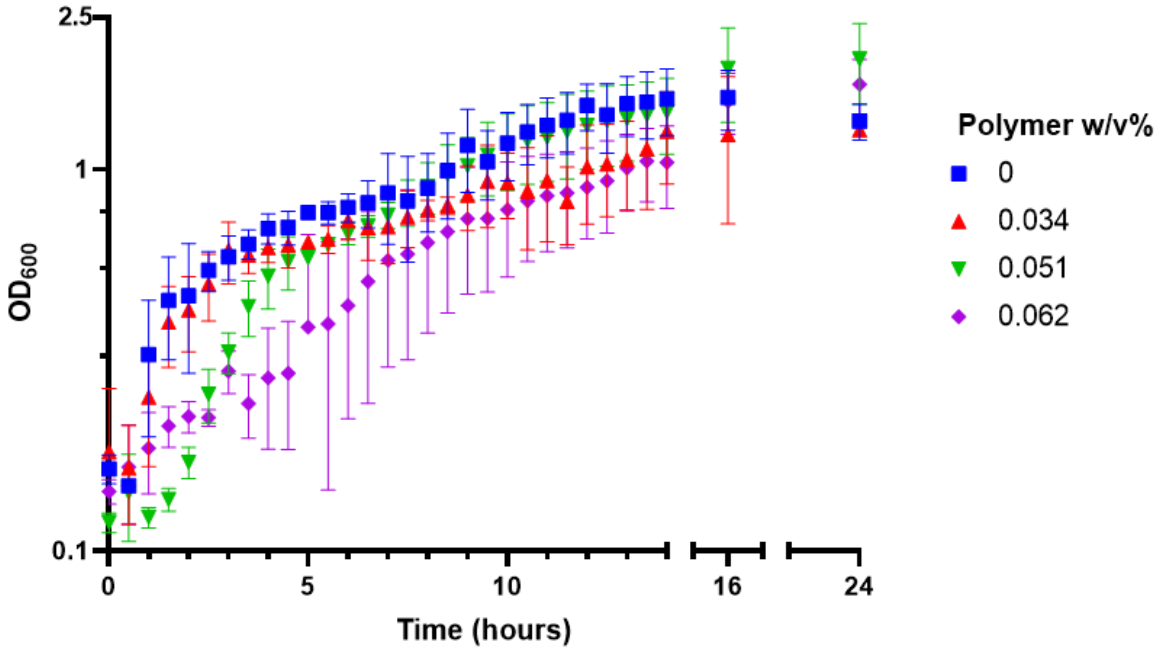
SI Figure 3. Cyro-TEM micrographs, showing membranes of *E. coli* and representative measured thicknesses**:** a) Membrane of a naked *E. coli*, the blue line is 33.2 nm. b) *E.coli*@0.034%, the blue line is 35.4 nm. c) *E.coli*@0.051%, the blue line is 39.1 nm. d) *E.coli*@0.062%, the blue line is 40.75 nm. A mass of additional polymer is visible on this bacterium. All scale bars are 100 nm.



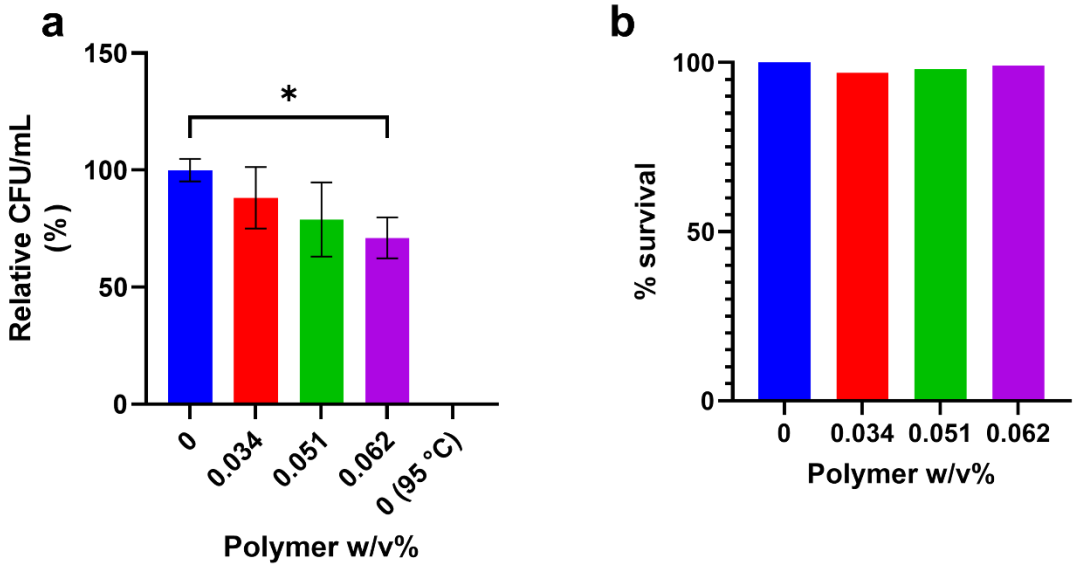
SI Figure 4. a-c) CLSM micrographs of YFP bacteria mixed with Cy5-presenting GUVs, showing the preferential insertion of the fluorophore in the polymersomes (overlays of YFP, green, and Cy5, red; scale bars = 10 µm). d) ζ-potential (mean, ± S.D., n = 3) of bacteria mixed with GUVs, without co-extrusion, right after sample preparation and 24 h later.



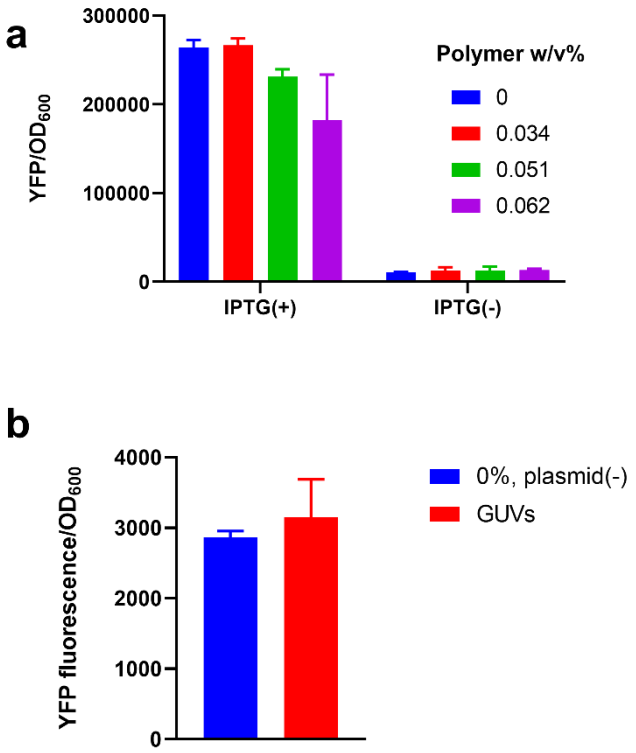
SI Figure 5. Live dead assay obtained by incubating cells with fluorescein diacetate, which stains alive cells, and propidium iodide, which stains dead cells (n = 120 cells). The control is naked cells incubated at 90°C for 10 minutes.



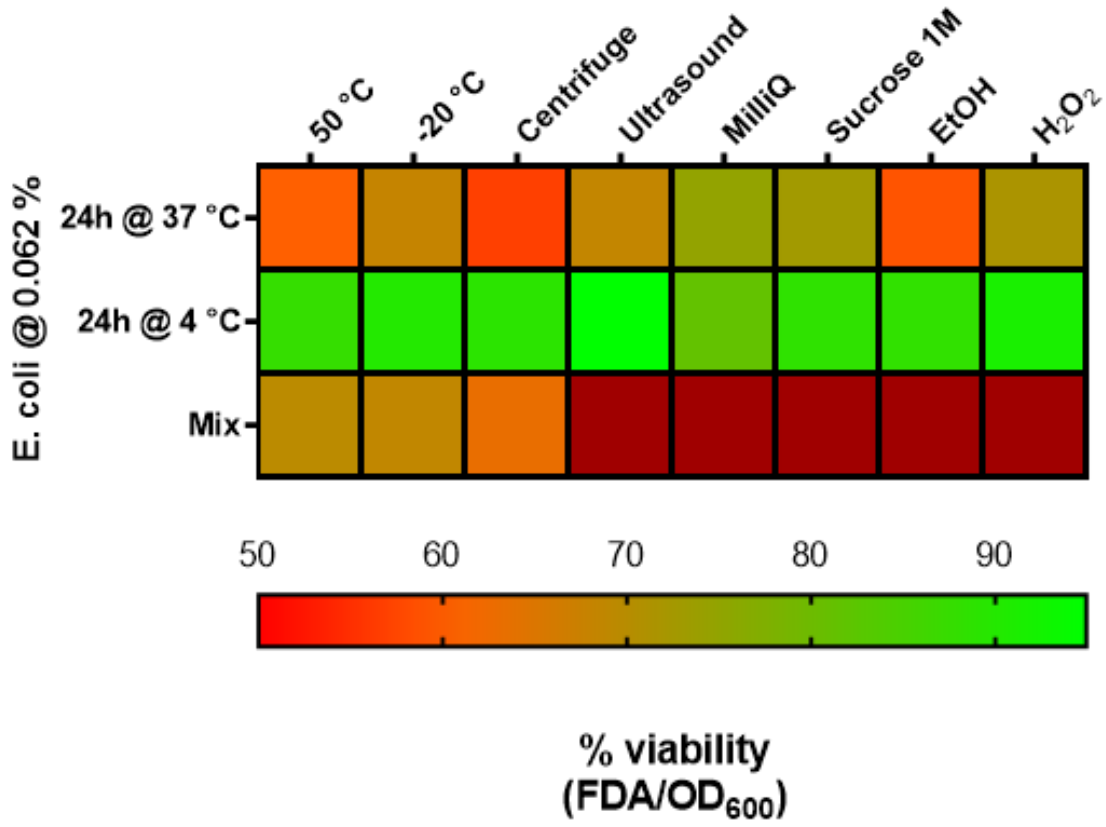
SI Figure 6. OD_{600} of naked and encapsulated cells (mean, ± S.D., n = 3) over 24 h. Beyond the 6-hour mark, the cell concentrations are quite similar regardless of encapsulation, and remain such up to the stationary phase.



SI Figure 7. a) Relative CFU/mL of naked and encapsulated bacteria, showing a small effect of encapsulation on cell proliferation (naked bacteria in hot water as negative control). Data displayed as mean ± S.D., n = 3 (a), n = 100 colonies (b). *: $p < 0.1$. b) Plasmid retention test, where bacteria with an intact membrane do not lose their plasmid and can thus survive on the corresponding selection antibiotic (single measurement).



SI Figure 8. a) YFP fluorescence/OD_{600} cells (naked and encapsulated) with and without IPTG, showing that encapsulation still allows induction of protein expression. b) Negative control of the signal by plasmid-less bacteria and pure GUVs. Data displayed as mean ± .D., n = 3.



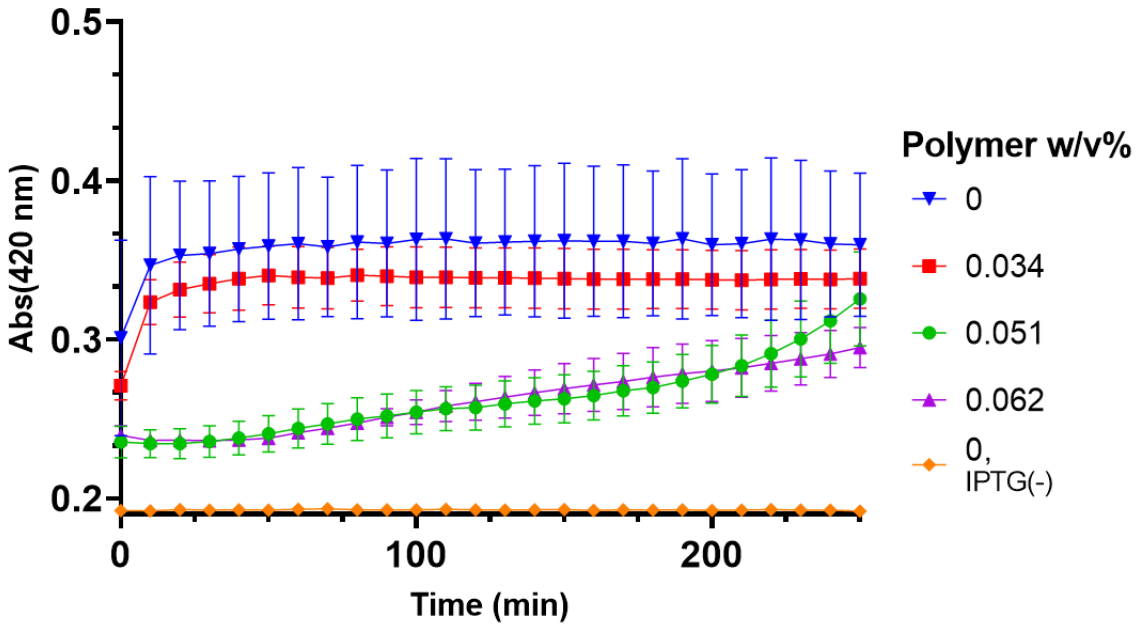
SI Figure 9: Heatmap of the % viability (expressed as FDA conversion / OD_{600}) of *E. coli* @0.062%, compared to the same bacteria, encapsulated but untreated, when subjected to a selection of harsh conditions, when either mixed with GUVs, or incubated (and allowed to grow, if possible) at 37°C or 4°C. Values below 50% were assigned dark red.

SI Table 2. % viability (FDA fluorescence / OD_{600}) of naked/encapsulated cells compared to the same conditions without treatment (mean ± S.D., n = 3).

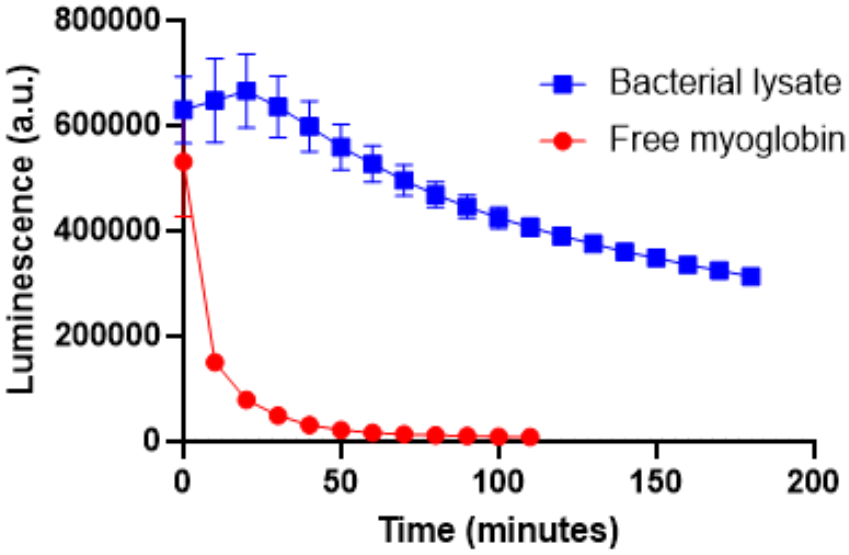
		50 °C	**-20 °C**	**Centrifuge**	**Ultrasound**	**MilliQ**	**Sucrose 1M**	**EtOH**	**H_2O_2**
Polymer w/v%	**0.062**	91.8 ± 1.6	98.6 ± 12.3	89.2 ± 4.4	75.9 ± 13.7	84.1 ± 11.1	66.4 ± 6.9	82.8 ± 7.7	65.4 ± 1.8
	0.051	86.9 ± 0.8	87.7 ± 2.8	83.8 ± 5.9	79.3 ± 8.4	74.6 ± 7.2	64.9 ± 0.8	68.7 ± 5.9	62.3 ± 2.8
	0.034	99.6 ± 9.1	99.2 ± 1.1	94.6 ± 8.8	86.1 ± 1.4	86.4 ± 10.4	58.6 ± 9.5	54.6 ± 7.2	31.5 ± 5.7
	0	65.5 ± 3.2	60.1 ± 4.7	72.7 ± 10.9	24.2 ± 2.1	38.3 ± 8.0	27.6 ± 9.1	38.2 ± 5.7	27.6 ± 4.1

SI Table 3. % viability (FDA fluorescence / OD_{600}) of *E. coli*@0.062% cells, compared to the same conditions without treatment, either mixed with GUVs, or encapsulated and stored 24 h at 4 °C or 37 °C, before being treated. (mean ± S.D., n = 3).

		50 °C	-20 °C	Centrifuge	Ultrasound	MilliQ	Sucrose 1M	EtOH	H_2O_2
***E. coli* @ 0.062%**	**24h @ 37 °C**	60.2 ± 12.3	68.0 ± 12.1	56.7 ± 8.7	68.3 ± 15.3	74.8± 14.3	72.9 ± 10.2	58.8 ± 13.4	71.5 ± 11.8
	24h @ 4 °C	87.7 ± 14.5	90.1 ±11.0	89.0 ± 15.3	94.9 ± 7.3	81.4 ± 11.8	88.5 ± 13.0	88.2 ± 12.9	91.5 ± 10.2
	Mixed	69.4 ± 13.3	68.6 ± 10.6	63.1± 8.3	37.3 ± 12.2	42 ± 6.4	25.5 ± 9.5	45.7 ± 5.9	33.4 ± 8.4



SI Figure 10. Absorbance of ABTS radical cations by Mb-harbouring bacteria, showing the slowing effect of the membrane for the external metabolites, and the absence of oxidation without Mb production. Data displayed as mean ± S.D., n = 3. Some error bars too small to be displayed.

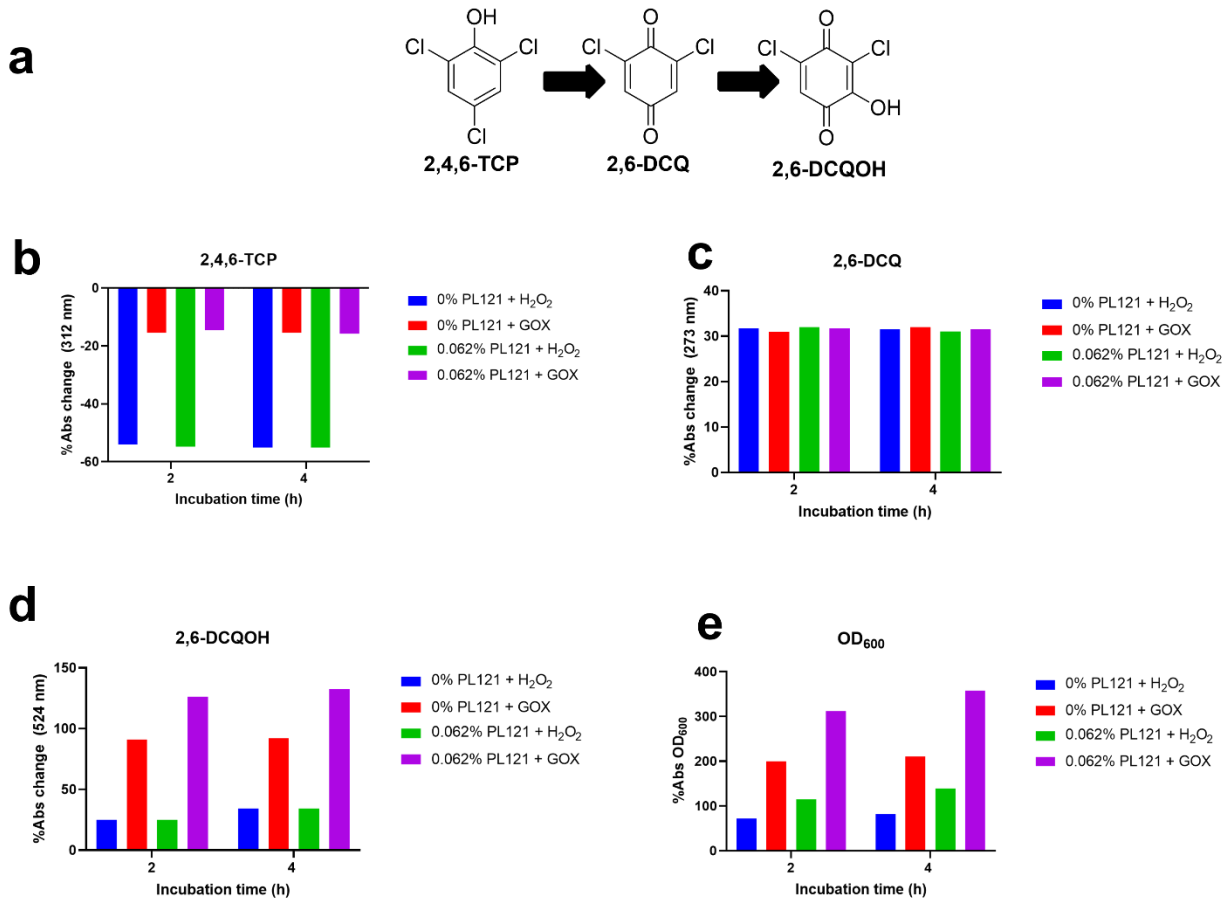


SI Figure 11. Luminescence profile derived from luminol oxidation in presence of free Mb and bacterial lysate after Mb expression, showing the high values quickly reached in the mixture and the decay coming from the peroxidation of the product, which does not happen when the diffusion is slower and controlled. Data displayed as mean ± S.D., n = 3. Some error bars too small to be displayed.

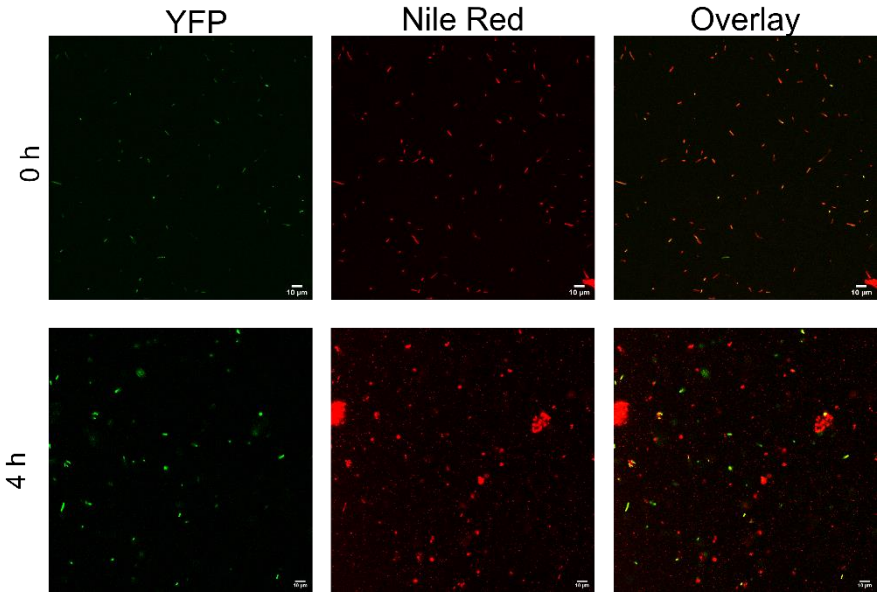
Whole-cell 2,4,6-trichlorochlorophenol detoxification

2,4,6-trichlorophenol (2,4,6-TCP) can be metabolized to the more electronegative product 2,6-dichloroquinone (2,6-DCQ), which can be further oxidized to its less toxic hydroxyquinone derivative (2,6-DCQOH) at high concentration of ROS (SI Figure 8a).[6] H_2O_2 was supplied to naked bacteria and E.coli@0.62% bacteria, either in one shot, or gradually via a glucose/glucose oxidase (GOX) feed. The absorbance of 2,4,6-TCP decreased quickly when the peroxide was directly supplied (SI Figure 8b), independent of the encapsulation, indicating that a high initial concentration of peroxide enhanced the activity of Mb. The amount of the intermediate 2,6-DCQ (SI Figure 8c) was not affected by either encapsulation or H_2O_2 source, suggesting that its production was independent of these two factors. Interestingly, the highest production of 2,6-DCQOH was detected when GOX provided the peroxide to E.coli@0.62%, and the second highest for GOX+naked bacteria (SI Figure 8d). From this result, we can infer that the rapid addition of H_2O_2 enhances the removal of 2,4,6-TCP, compared to the use of GOX. However, the resulting 2,6-DCQ is not oxidized further, as even the PL121 membrane fails to counter the combined toxicity of the

model pollutants and the peroxide. Instead, with a GOX-Mb cascade, 2,6-DCQ is transformed more easily in the less toxic 2,6-DCQOH, with E.coli@0.62% showing the highest transformation of all. The growth of bacteria, i.e. their survival, was correlated with the production of 2,6-DCQOH (SI Figure 8e). From the perspective of the membrane, it did not improve the outright removal of 2,4,6-TCP, but it acted as a barrier, allowing a slow and constant flow of both H_2O_2 and chlorophenols which, in conjunction with GOX, made the encapsulated bacteria capable of detoxifying 2,4,6-TCP until the end of the cascade, and the bacteria more able to increase their biomass.



SI Figure 12 a) Reaction scheme of the reaction leading from the very toxic 2,4,6-TCP to the less harmful 2,6-DCQOH b) % variation of the absorbance typical for 2,4,6-TCP over time. c) % variation of the absorbance typical for 2,6-DCQ over time. d) % variation of the absorbance typical for 2,6-DCQOH over time. e) % variation of the OD_{600}, showing how the slower production and diffusion of hydrogen peroxide by in-situ generation with glucose oxidase favors the production of 2,6-DCQOH and bacterial proliferation alike.



SI Figure 13. CLSM micrographs of YFP bacteria, with the lipid stain Nile Red, highlighting both fluorescent and non-fluorescent bacteria, showing the appearance of cell debris (and a higher frequency of fluorescent bacteria) after incubation with lysozyme for 4 h.

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