Supporting Information

Spectrophotometric Quantification of Horseradish Peroxidase (HRP) with *p*-Phenylenediamine for Analyzing Peroxidase-encapsulating Lipid Vesicles (Liposomes)

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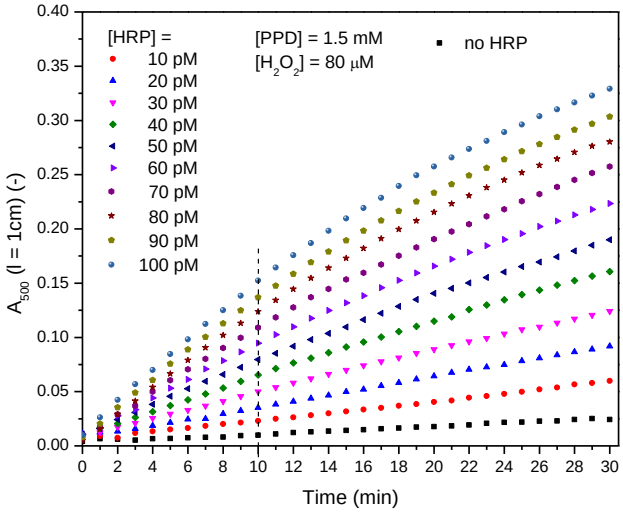
CONTENT INFORMATION: This Supporting Information contains additional experimental details and additional data for the assay of horseradish peroxidase (HRP) with *p*-phenylenediamine (PPD) and on the preparation and analysis of HRP-encapsulating lipid vesicles.

TABLE OF CONTENTS

Spectrophotometric Monitoring of the Reaction of PPD after Addition of HRP and H_2O_2

a



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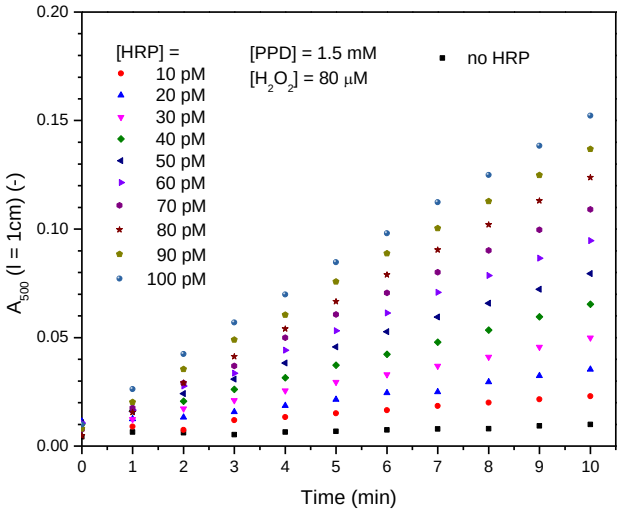


Figure S-1. Changes of A_{500}, the absorbance at λ_{iso} = 500 nm of the assay solutions containing different HRP concentrations during the first 30 min (a) and 10 min (b) after H_2O_2 addition with [PPD] = 1.5 mM and [H_2O_2] = 80 μM at pH 7.0 and ≈25 °C. The reaction solutions were incubated in a quartz cuvette with a path length of 1 cm. Buffer solution used: 0.1 M NaH_2PO_4/Na_2HPO_4 (buffer-1).

Preparation of HRP-encapsulating POPC LUV_{200} Suspensions

Dry POPC film formation. At first, 30 mg POPC (40 μmol), dissolved in 2 mL chloroform, were added to a 10 mL round bottom flask and dried by evaporating the solvent using a rotation evaporator with under-pressure (200 mbar) and a water bath warmed to 40 °C. The resulting lipid film was further dried *in vacuo* overnight at ~25 °C.

POPC film hydration with an aqueous HRP solution. HRP was dissolved in buffer-2 and diluted to a concentration of 62.6 μM (determined spectrophotometrically), from which 1 mL (containing 62.6 nmol HRP) was added to the flask to disperse the POPC film at room temperature. The swelling process was promoted by gentle swirling and shaking the flask (lowest power on a bench-top vortex). After 15 min, most of the POPC was dispersed, resulting in a reddish milky suspension (which contained mostly multilamellar vesicles, MLV).[S1,S2] An additional 1 mL of buffer-2 was added to the flask to dilute the dense liposome-HRP mixture. The final 2 mL suspension (containing 62.6 nmol HRP and 40 μmol POPC) was transferred into a 10 mL glass vial with screw-lid.

POPC vesicle extrusion. The 1.5 mL Extruder® from Lipex Biomembranes[S3] was used to decrease and homogenize the size and the lamellarity of the vesicles. The Extruder® was assembled with a 10 mm PE drain disc from Whatman and respective polycarbonate membranes. The vesicle suspension was first passed at room temperature 10 times through a 400 nm pore membrane, then 10 times through a 200 nm pore membrane (from Whatman). The obtained large unilamellar vesicles are abbreviated as LUV_{200}. This suspension contained a mixture of HRP-encapsulating LUV_{200} and free HRP in bulk solution.

Separation by size-exclusion chromatography. HRP-encapsulating POPC vesicles were separated from free HRP by size exclusion chromatography on Sepharose 4B, packed in a column of 35 cm in length and of an inner diameter of 1.1 cm. A volume of 0.5 mL of the LUV_{200}-HRP mixture (containing 15.7 nmol HRP and 10 μmol POPC) were applied onto the glass column and eluted with buffer-2, with a flow rate of 0.33 mL min^{-1}, collecting fractions of 0.78 mL volume. The UV/VIS spectrum of each fraction was recorded and the measured absorbance at 275 nm (A_{275}) and at 403 nm (A_{403}) were plotted for each fraction to localize the vesicle fractions (*fractions 15-21*) and the fractions containing free HRP (*fractions 32-43*). The vesicle fractions were then analyzed by dynamic light scattering, and the concentration (amount) of POPC was determined in each fraction(see **Figure 5** and EXPERIMENTAL SECTION of the main text). Furthermore, the activity of HRP was determined in each fraction with $ABTS^{2-}$ (1.0 mM)/H_2O_2 (0.2 mM) and PPD (1.0 mM)/H_2O_2 (0.2 mM), see **Figure 5**.

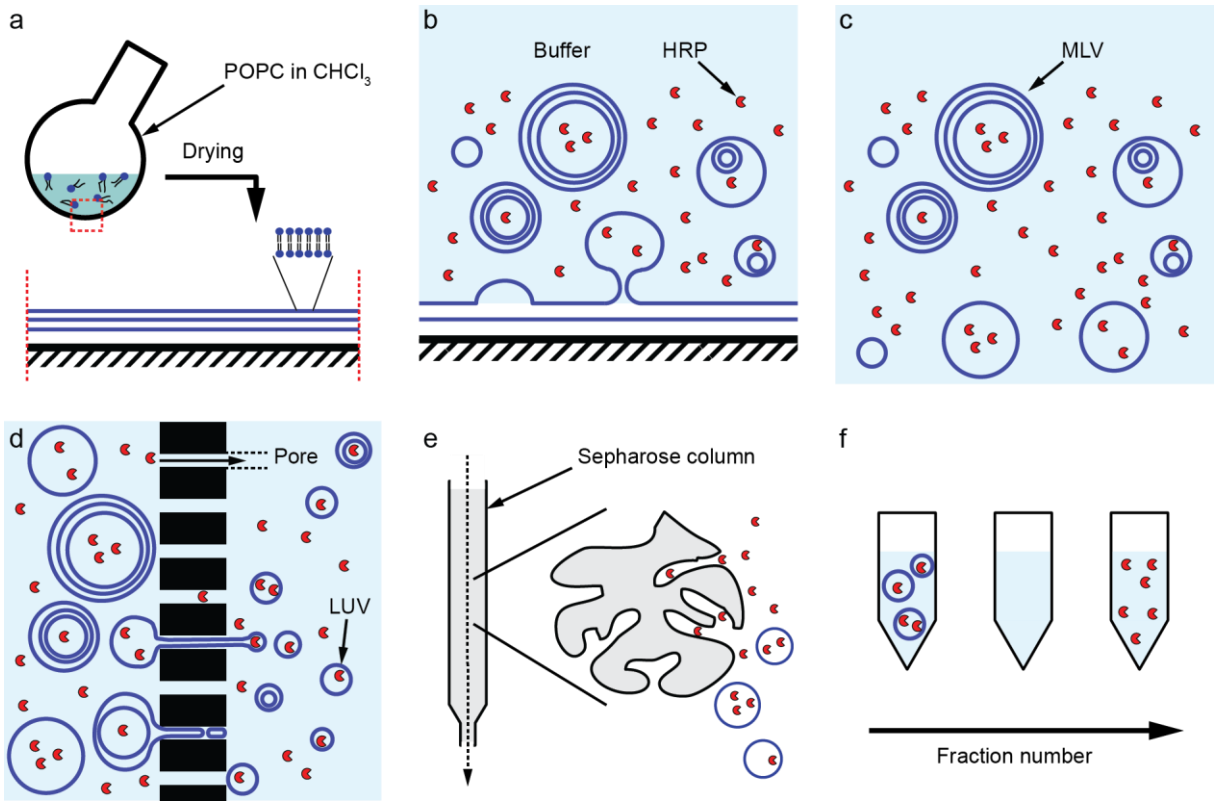


Figure S-2. Schematic of the preparation of HRP-encapsulating large unilamellar POPC vesicles: a) POPC was dried in a glass flask. b) The dried POPC film (40 μmol POPC) was dispersed with HRP containing buffer (1.0 mL). c) Vesicles, mainly multilamellar vesicles (MLV), were formed. Some of the HRP was encapsulated. d) After dilution with buffer (1.0 mL), the vesicles were extruded through polycarbonate membranes, first with 400 nm and then with 200 nm pore membranes, resulting in LUV_{200}. e) HRP-encapsulating LUV_{200} were separated from free HRP by size exclusion chromatography on a Sepharose 4B column. f) A particle size dependent separation occurred, whereby HRP-encapsulating LUV_{200} eluted before free HRP. Buffer solution used: 0.05 M KH_2PO_4/K_2HPO_4 (buffer-2).

Quantification of POPC in the Vesicle Fractions[S4,S5]

A 0.1 M ammonium ferrothiocyanate solution was prepared at room temperature by dissolving 27 g ferric chloride hexahydrate and 30.4 g ammonium thiocyanate in 1 L distilled water. For the calibration, a 0.1 mg mL^{-1} POPC standard solution in chloroform was prepared. For the assay, a known volume of standard solution or sample was diluted in chloroform, 2 mL thereof were transferred to a 15 mL centrifugation tube. A volume of 2 mL of the ammonium ferrothiocyanate solution was added on top and the tube was vortexed, then centrifuged for phase separation with a Hermle Z320 K centrifuge at room temperature for 5 min at 300 g. The lower chloroform phase contained complexes between POPC and spectrophotometrically detectable ferrothiocyanate. The absorbance of the chloroform phase was measured, and A_{485} was used to quantify POPC, see **Figure S-3**.

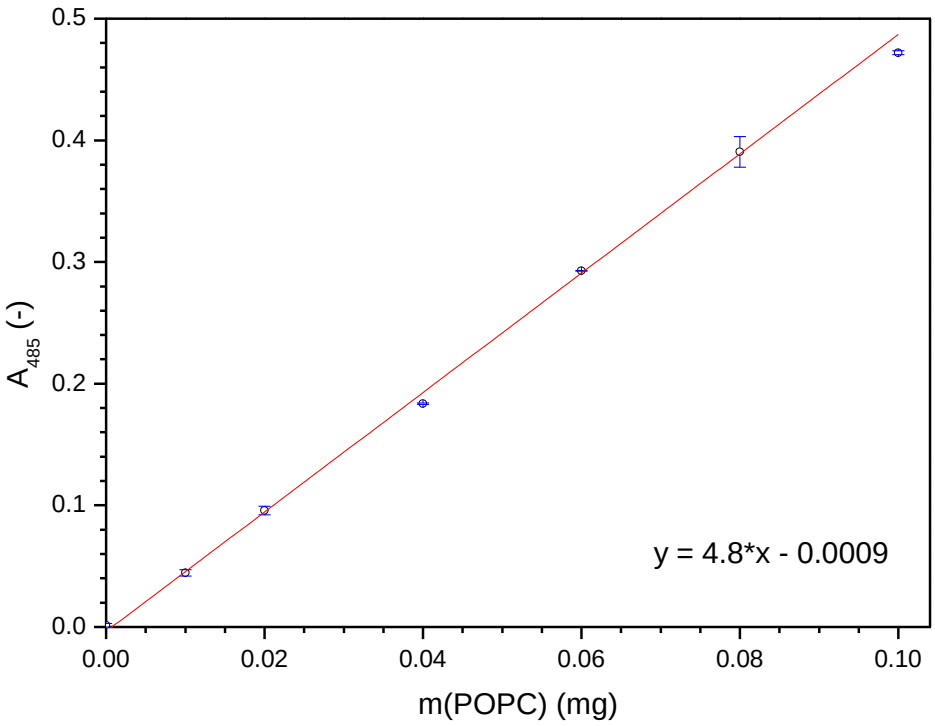


Figure S-3. Calibration curve for the quantification of POPC in the vesicle fractions. For the calibration, the mean absorbance at 485 nm (A_{485}) was measured for different amounts of POPC using the Stewart assay at 25 °C. The error bars represent the standard deviations (n = 2). Path length: 1.0 cm; r^2 = 0.9953. Molar mass of POPC, M(POPC) = 760 g mol^{-1}, *i.e.*, 0.02 mg POPC correspond to 13.16 nmol POPC.

Effect of Sodium Cholate on POPC Vesicle Turbidity and HRP Activity

At 8.0 mM cholate, the activity of 1.4 nM HRP was assessed with $ABTS^{2-}/H_2O_2$ by measuring the oxidation of $ABTS^{2-}$ (1.0 mM) in the presence of H_2O_2 (0.2 mM) for the initial 150 s (**Figure S-4b**), see EXPERIMENTAL SECTION for details of the activity measurements. Compared to the activity in the absence of cholate, there was a decrease of about 10 %.[S6] Therefore, in the presence of 8.0 mM sodium cholate (i) there was a complete destruction of the vesicles, which is in good agreement with previous findings,[S7] and (ii) HRP was inactivated by only about 10 %. As a conclusion, for the determination of the total activity of vesicle-entrapped HRP and leaked HRP, cholate was added to the vesicle suspension to yield a final concentration of 8.0 mM.

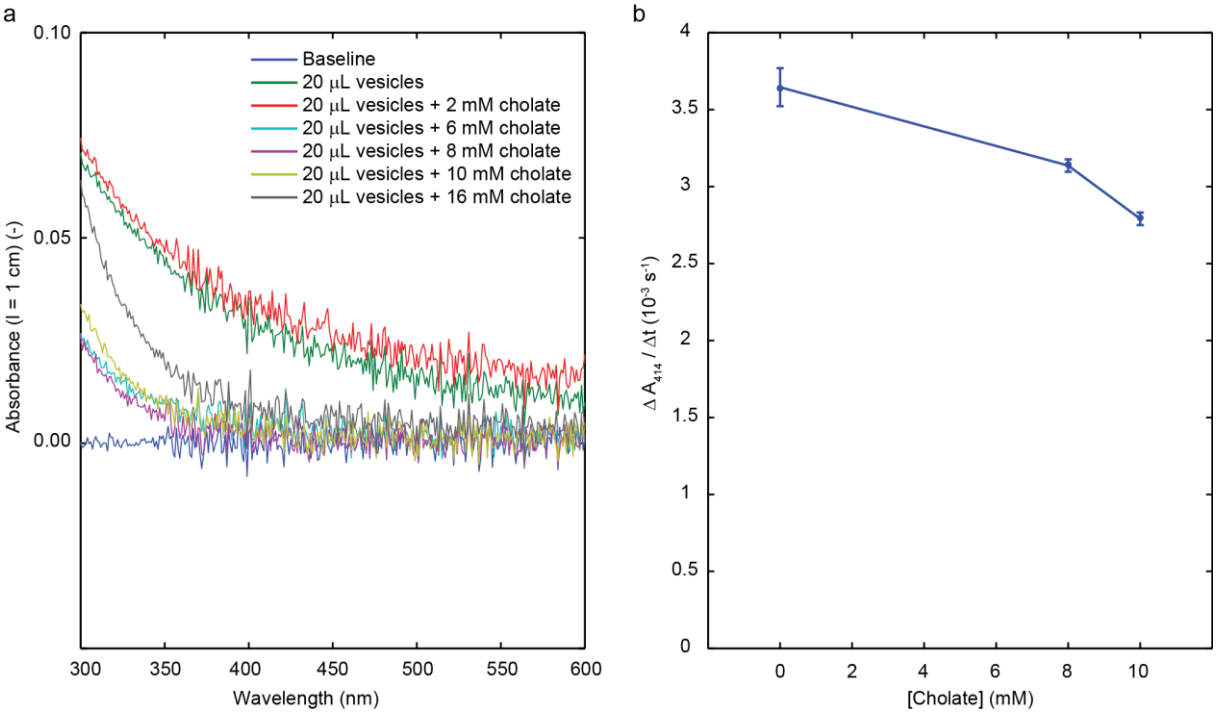


Figure S-4. Cholate effect on the turbidity of POPC LUV_{200} (a) and on the activity of HRP, as measured with $ABTS^{2-}$; path length: 1.0 cm (b).
For (a) 20 µL of the main fraction *(fraction 17)* of empty POPC LUV_{200} (2.0 mM POPC) was added to 1.0 mL buffer-2 (0.05 M KH_2PO_4/K_2HPO_4). "20 µL vesicles" added to 1.0 mL means that [POPC] = 20 µM. Therefore, the molar ratios of cholate to POPC in the experiments were 0, 100 (2 mM cholate), 300 (6 mM cholate), 400 (8 mM cholate), 500 (10 mM cholate), and 800 (16 mM cholate), respectively.

Calibration Curves for Free HRP Measured with $ABTS^{2-}$ / H_2O_2 without and with Cholate

Calibration curves with free HRP: (i) no sodium cholate, (ii) with 8.0 mM sodium cholate
The conversion of $ABTS^{2-}$ to the radical anion ($ABTS^{\bullet-}$) was measured as the increase in A_{414} with time in quartz cuvettes with a path length of 1.0 cm (ε_{414} = 36 000 M^{-1} cm^{-1}).[S8]

(i) 100 μL of the 10 mM $ABTS^{2-}$ stock solution was added to an appropriate volume of buffer-2, followed by the addition of a known volume of a 136 nM HRP solution (prepared with buffer-2) and 10 μL of the 20 mM H_2O_2 stock solution to yield a total reaction volume of 1.0 mL. The final HRP concentration was between 0.7 and 5.6 nM, [$ABTS^{2-}$] = 1.0 mM, and [H_2O_2] = 0.2 mM. The absorption spectra were recorded at 25 °C every 15 s during the initial 150 s after H_2O_2 addition, see the calibration curve in **Figure S-5a**.

(ii) 100 μL of the 10 mM $ABTS^{2-}$ stock solution was added to an appropriate volume of buffer-2 and 40 μL of the 200 mM sodium cholate stock solution, followed by the addition of a known volume of a 136 nM HRP solution (prepared with buffer-2) and 10 μL of the 20 mM H_2O_2 stock solution to yield a total reaction volume of 1.0 mL. The final HRP concentration was between 0.7 and 2.8 nM, [$ABTS^{2-}$] = 1.0 mM, [cholate] = 8.0 mM, and [H_2O_2] = 0.2 mM. The absorption spectra were recorded at 25 °C every 15 s during the initial 150 s after H_2O_2 addition, see the calibration curve in **Figure S-5b**.

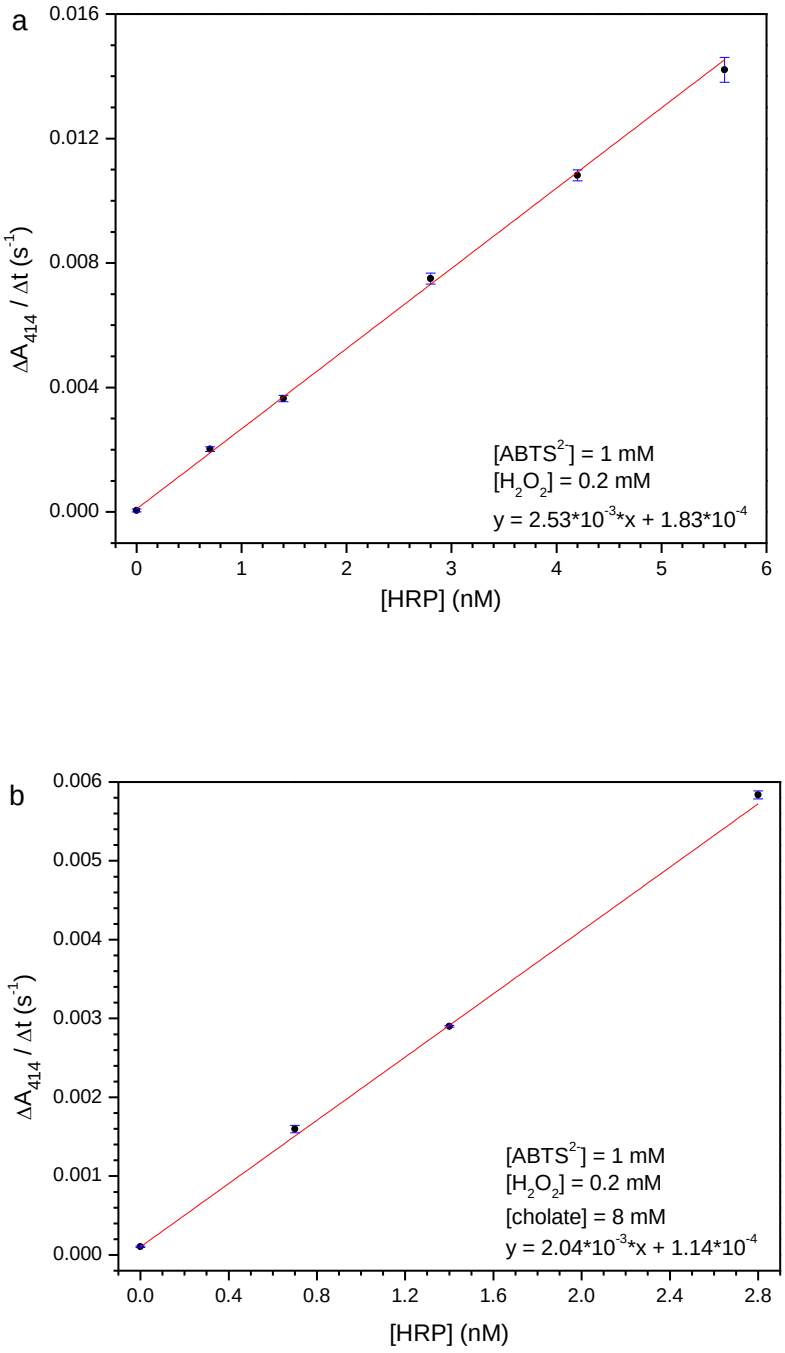


Figure S-5. Calibration curves for the activity of free HRP, as measured with $ABTS^{2-}/H_2O_2$ at pH 7.0 and 25 °C. (a) Measurements without cholate, (b) measurements in the presence of 8.0 mM cholate. The activity (in $\Delta A_{414}/\Delta t$, s^{-1}) was measured for 0.7 to 5.6 nM HRP (a), or for 0.7 to 2.8 nM HRP (b). $[ABTS^{2-}]$ = 1.0 mM and $[H_2O_2]$ = 0.2 mM were fix. The error bars represent the standard deviation for n = 3. For (a): r^2 = 0.9988; for (b): r^2 = 0.9997. Buffer solution used: 0.05 M KH_2PO_4/K_2HPO_4 (buffer-2).

Activity of *Fraction 16* Measured with $ABTS^{2-}$ / H_2O_2 without and with Cholate

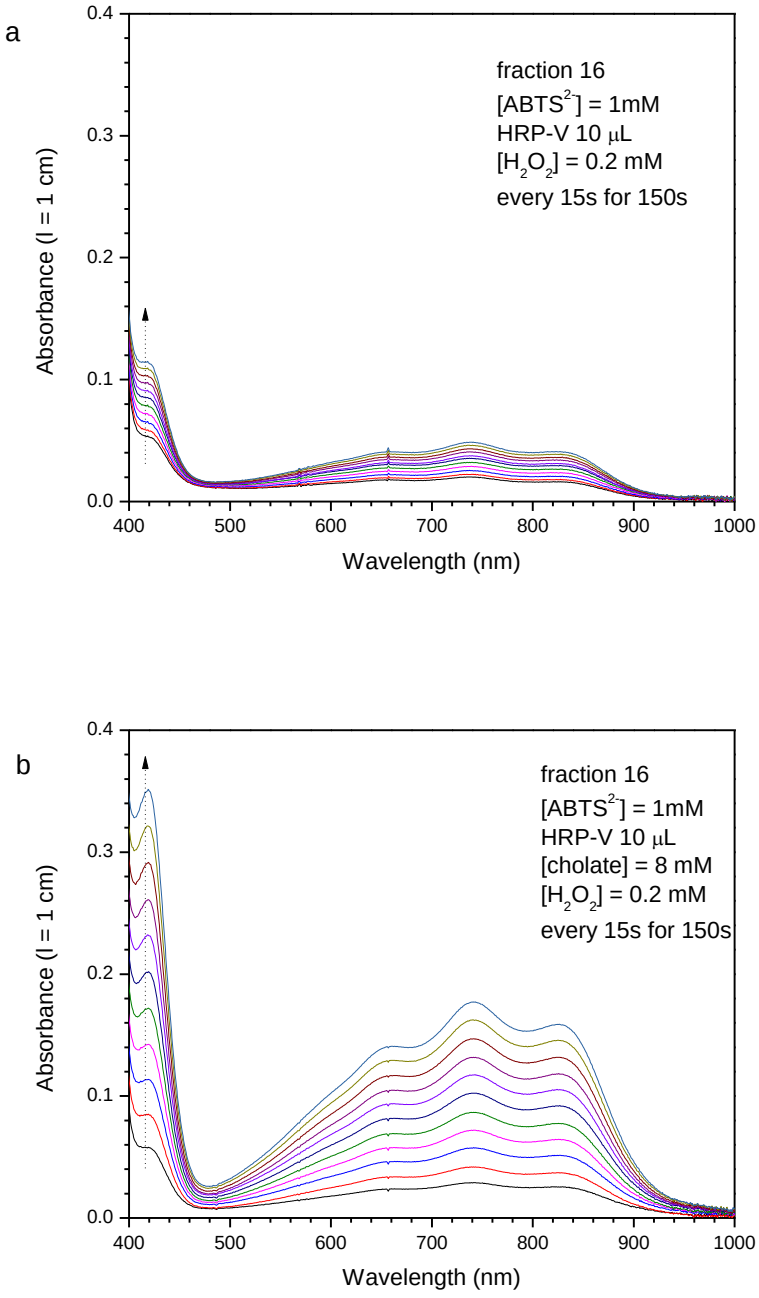


Figure S-6. Changes in the absorption spectrum of the $ABTS^{2-}$ assay solution, caused by the addition of *fraction 16* (see **Figure 5**) without cholate (a) and with 8 mM cholate (b). [$ABTS^{2-}$] = 1.0 mM, 10 uL vesicle *fraction 16* and [H_2O_2] = 0.2 mM at pH 7.0 and 25 °C. The spectrum was recorded every 15 s during the initial 2.5 min of the reaction in a quartz cuvette with a path length l = 1 cm. The dashed arrow indicates the change of the absorbance at λ = 414 nm over time. Buffer solution used: 0.05 M KH_2PO_4/K_2HPO_4 (buffer-2).

Michaelis-Menten Kinetics of Free HRP Measured with $ABTS^{2-}$ / H_2O_2

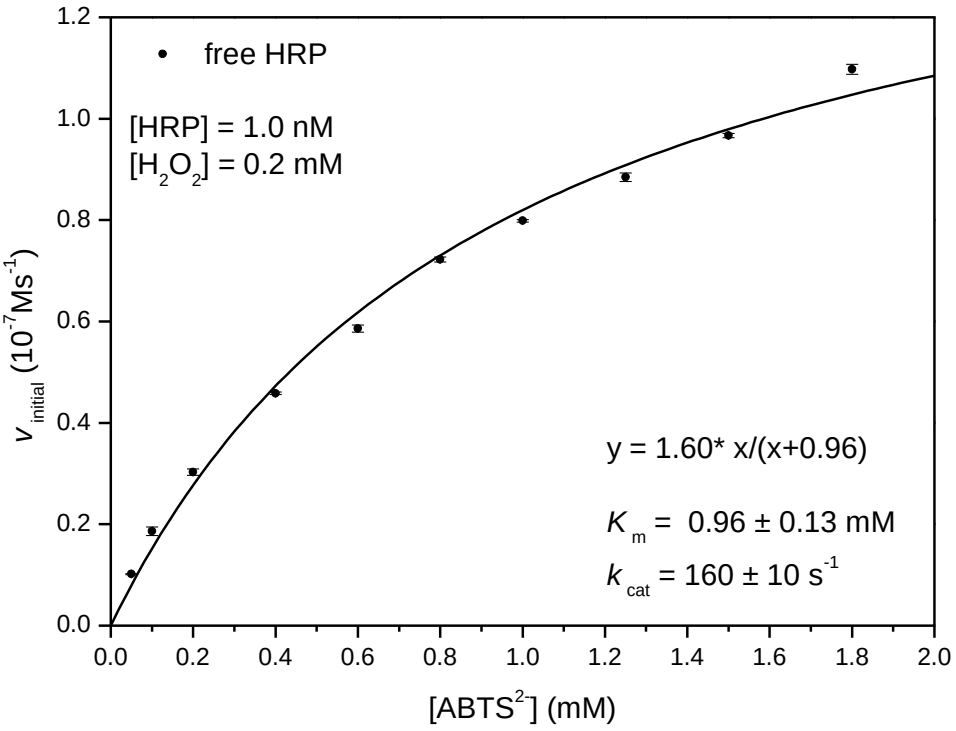


Figure S-7. Initial rate of $ABTS^{2-}$ oxidation at pH 7.0 and 25 °C for 1 nM HRP, 0.2 mM H_2O_2, and varying concentrations of $ABTS^{2-}$. Buffer solution used: 0.05 M KH_2PO_4/K_2HPO_4 (buffer-2). The data points are mean values with standard deviations (n = 3). The data points were fitted with the Michaelis-Menten equation, $v_{in} = ([HRP]\cdot k_{cat}\cdot[ABTS^{2-}])/(K_M + [ABTS^{2-}])$. The maximal velocity, $v_{max} = [HRP]\cdot k_{cat}$ and the Michaelis constant K_M obtained from the fit were 1.6±0.1 10^{-7} M s^{-1} and 0.96±0.13 mM, respectively; $r^2 = 0.9977$.

Calibration Curves for Free HRP Measured with PPD / H_2O_2 without and with Cholate

Calibration curves with free HRP: (i) no sodium cholate, (ii) with 8.0 mM sodium cholate
The conversion of PPD to Bandrowski's base was measured as the increase in A_{500} with time in quartz cuvettes with a path length of 1.0 cm ($\varepsilon_{500} = 1.109 \cdot 10^4\ M^{-1}cm^{-1}$).[S9]

(i) 100 μL of the 10 mM PPD stock solution was added to an appropriate volume of buffer-2, followed by the addition of a known volume of a 136 nM HRP solution (prepared with buffer-2) and 10 μL of the 20 mM H_2O_2 stock solution to yield a total reaction volume of 1.0 ml. The final HRP concentration was between 136 and 476 pM, [PPD] = 1.0 mM, and [H_2O_2] = 0.2 mM. The absorption spectra were recorded at 25 °C every 15 s during the initial 150 s after H_2O_2 addition, see the calibration curve in **Figure S-8a**.

(ii) 100 μL of the 10 mM PPD stock solution was added to an appropriate volume of buffer-2 and 40 μL of the 200 mM sodium cholate stock solution, followed by the addition of a known volume of a 136 nM HRP solution (prepared with buffer-2) and 10 μL of the 20 mM H_2O_2 stock solution to yield a total reaction volume of 1.0 ml. The final HRP concentration was between 136 and 476 pM, [PPD] = 1.0 mM, [cholate] = 8.0 mM, and [H_2O_2] = 0.2 mM. The absorption spectra were recorded at 25 °C every 15 s during the initial 150 s after H_2O_2 addition, see the calibration curve in **Figure S-8b**.

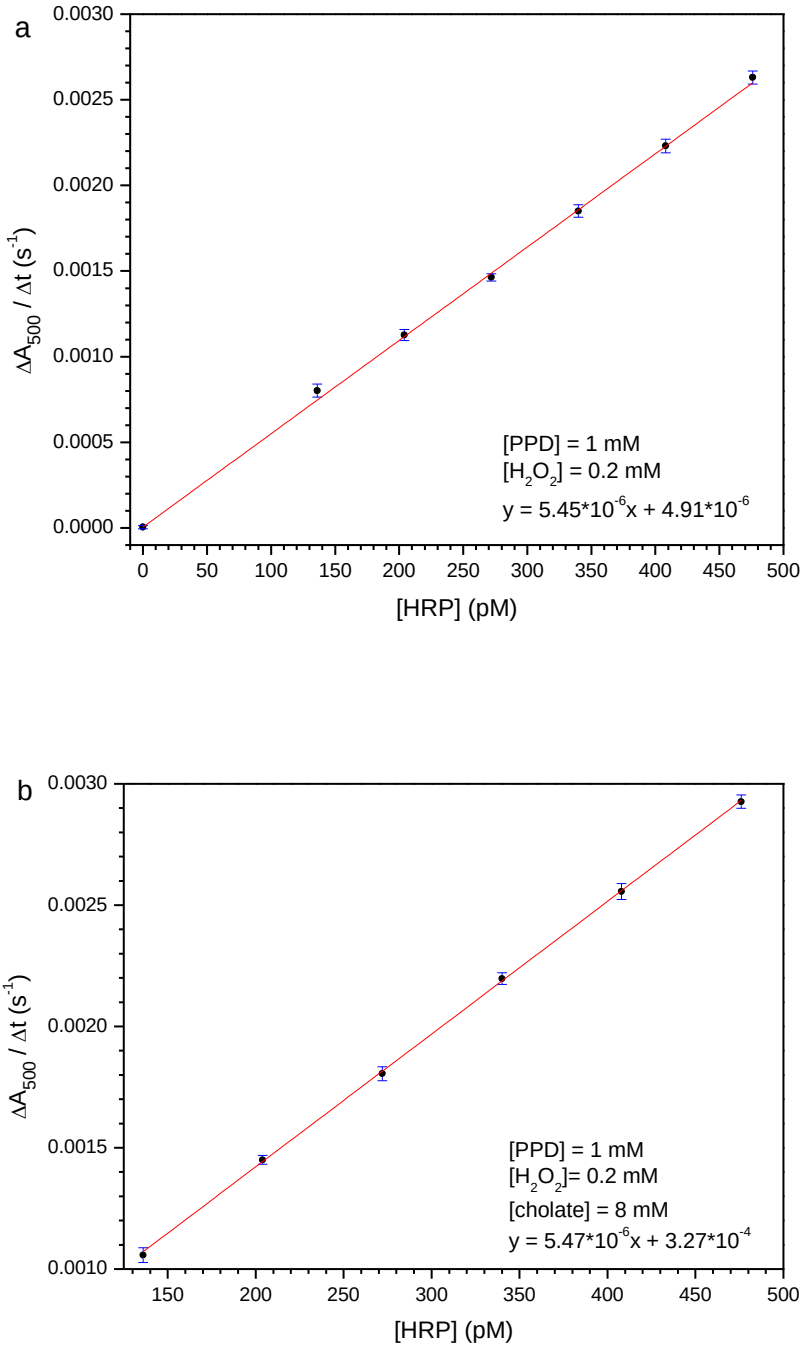


Figure S-8. Calibration curves for the activity of free HRP using PPD without (a) and with 8.0 mM cholate (b). [PPD] = 1.0 mM, [H_2O_2] = 0.2 mM at pH 7.0 and 25 °C. Buffer solution used: 0.05 M KH_2PO_4/K_2HPO_4 (buffer-2). For (a): r^2 = 0.9995; for (b): r^2 = 0.9998.

Activity of *Fraction 15* Measured with PPD / H_2O_2 without and with Cholate

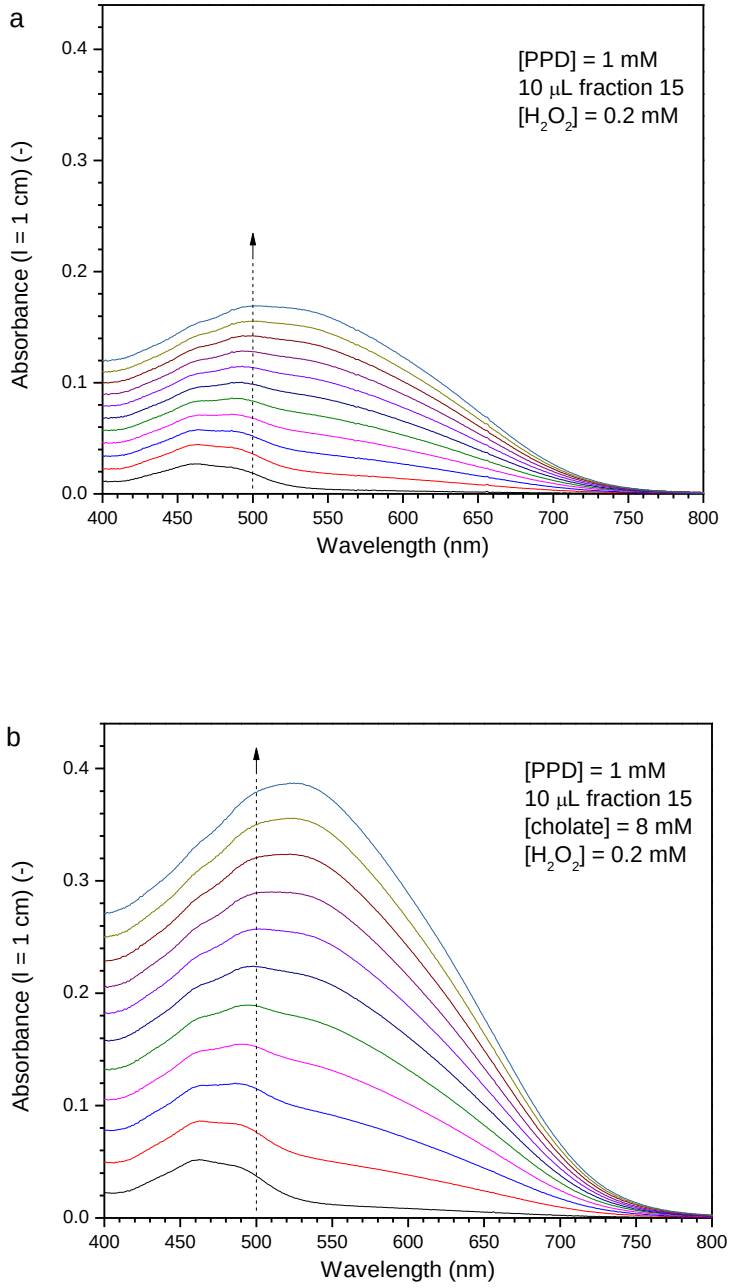
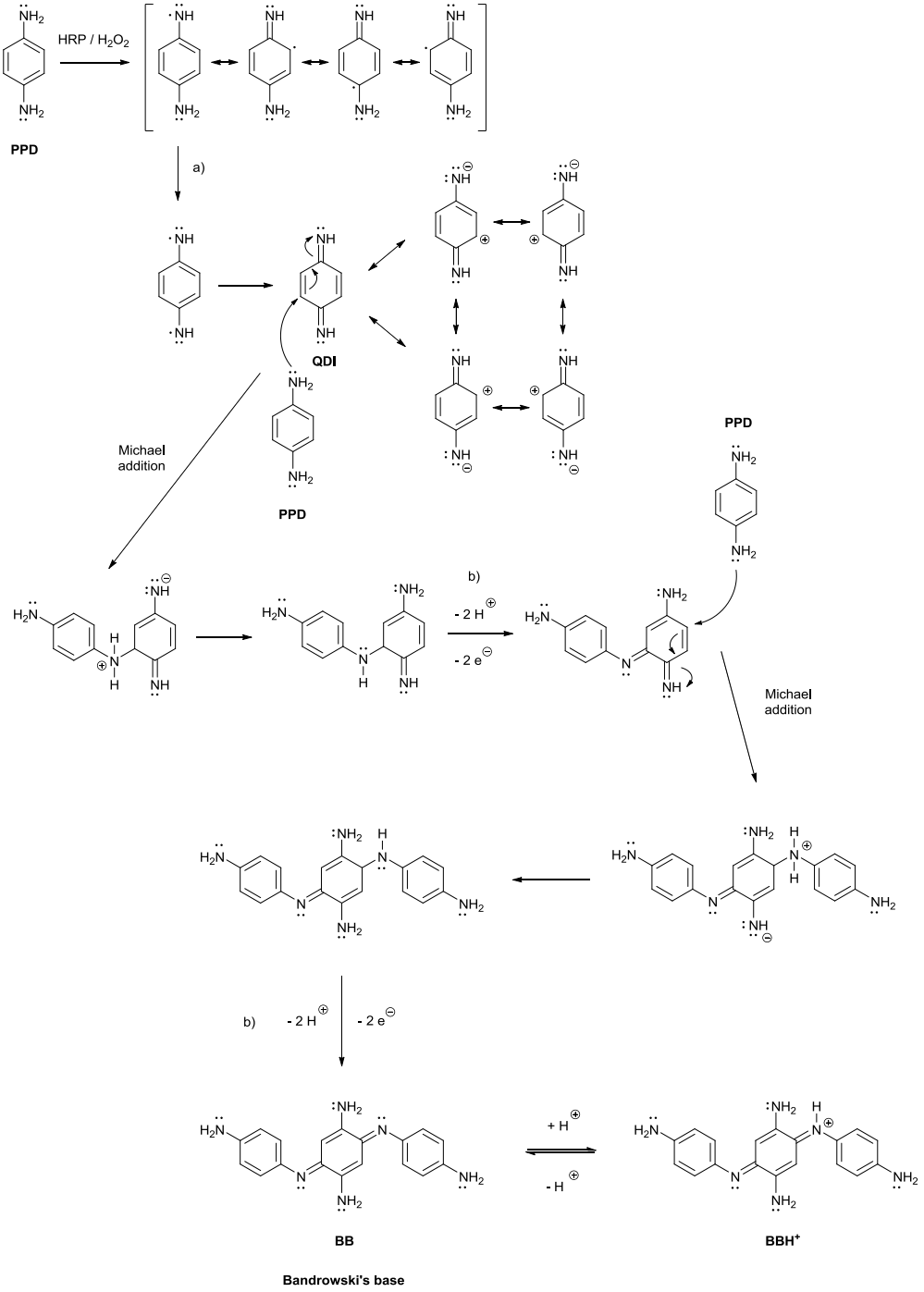


Figure S-9. Changes in the absorption spectrum of the PPD-assay solution, caused by the addition of *fraction 15* (see Figure 5) without (a) and with 8.0 mM cholate (b). [PPD] = 1.0 mM, 10 μL vesicle *fraction 15* and [H_2O_2] = 0.2 mM at pH 7.0 and 25 °C. The spectrum was recorded every 15 s during the initial 2.5 min of the reaction in a quartz cuvette with a path length of 1 cm. The dashed arrows indicate the changes in absorbance at λ = 500 nm over time. Buffer solution used: 0.05 M KH_2PO_4/K_2HPO_4 (buffer-2).

Suggested Mechanism for the Formation of Bandrowski's Base from PPD and H_2O_2



Scheme S-1. Suggested mechanism for the formation of Bandrowski's base from 3 molecules of PPD in the presence of H_2O_2 with HRP as catalyst. a): dispropotionation or with involvement of HRP; b) with H_2O_2 or with redox equilibria involving PPD$^{\bullet}$. See also the work of Sousa *et al.* (2013)[S10] with laccase/O_2, and Corbett's previous detailed studies on the non-enzymatic formation of Bandrowski's base from PPD.[S9, S11] **PPD**: *p*-phenylendediamine, pK_a (PPDH$^+$) ≈6.1-6.4.[S9, S12, S13] **QDI**: quinonediimine, pK_a (QDIH$^+$) =5.75.[S14] **Bandrowski's base**: pK_a (BBH$^+$) ≈7.4[S9]

MS Support for the Formation of Bandrowski's Base from PPD and H_2O_2

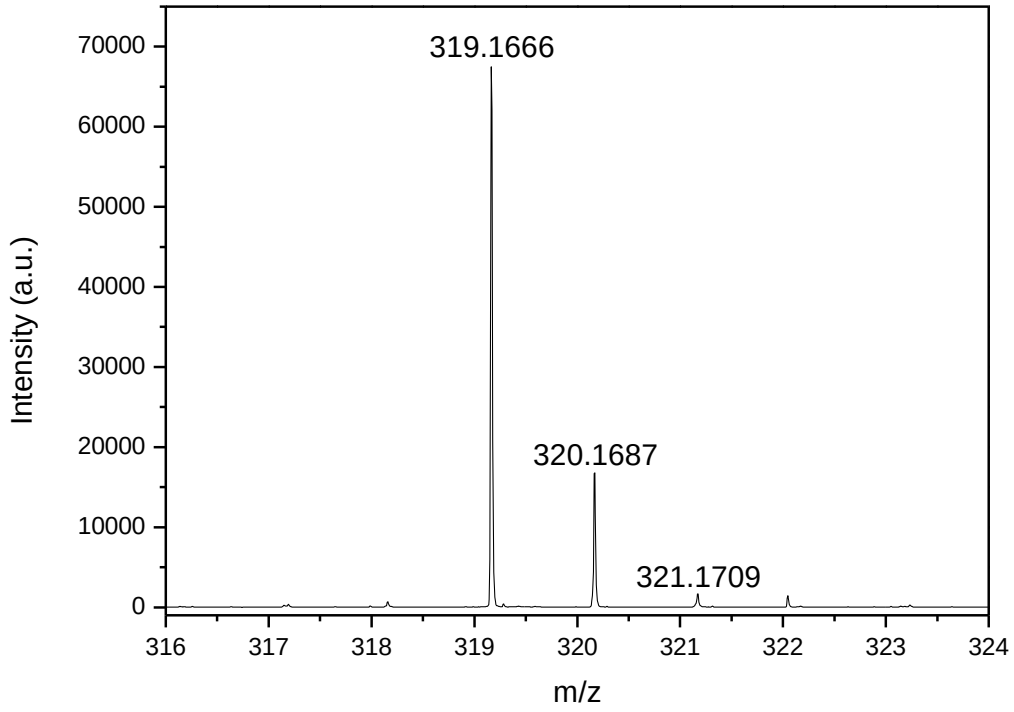


Figure S-10. Electrospray ionization mass spectrometry analysis of the extracted reaction products. For details, see Junker et al. (2014).[S15]
After a reaction time of 24 h at room temperature ([PPD] = 1.5 mM, [HRP] = 30 pM, [H_2O_2] = 80 μM) in buffer-1 (0.1 M NaH_2PO_4/Na_2HPO_4). For the extraction, the reaction solution was treated with *t*-butylmethylether (MTBE): 100 μL reaction solution, 400 μL MTBE.

Monoprotonated form of Bandrowski's base, BBH^+, $M(C_{18}H_{19}N_6)^+$; m/z = 319.1666 (found); 319.1671 (theoretical value).

HRP Activity Measurements of *Fractions 30-45* (Free HRP) with $ABTS^{2-}$

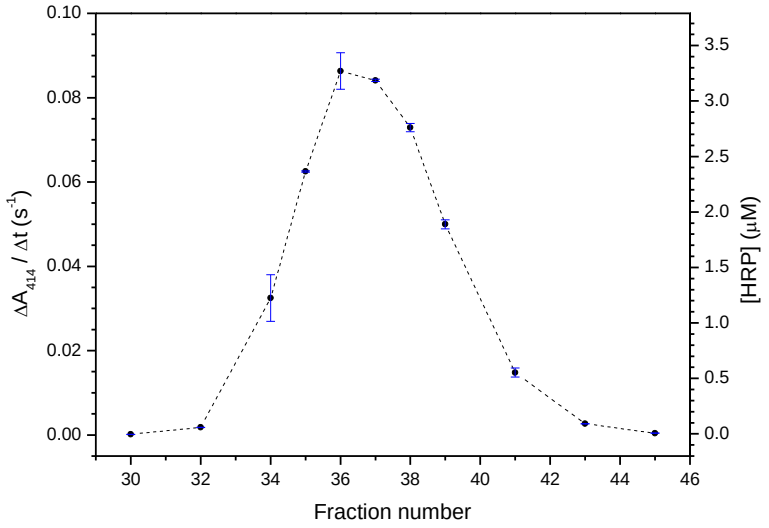


Figure S-11. Analysis of the fractions 30-45 (see **Figure 5**) for HRP activity by using the $ABTS^{2-}$ assay. The y-axis on the left hand side is the measured increase in A_{414} due to the formation of $ABTS^{\bullet-}$ (l = 1 cm); the axis on the right hand side refers to the HRP concentration in the fraction; fraction volume: 0.78 mL.

Applied onto the Sepharsoe 4B column: 16 nmol HRP; n.d.: not determined; est.: estimated

Fraction number	[HRP] (μM)	n(HRP) (pmol)		Fraction number	[HRP] (μM)	n(HRP) (pmol)
30	0	0		39	1.905±0.001	1486
31	n.d.	est.: ≈25		40	n.d.	est.: ≈540
32	0.064	50		41	0.514±0.001	401
33	n.d.	est.: ≈450		42	n.d.	est.: ≈330
34	1.212±0.006	945		43	0.098	76
35	2.402±0.000	1874		44	n.d.	est.: ≈30
36	3.272±0.004	2552		45	0.010	8
37	3.184±0.000	2483				
38	2.742±0.001	2139		**Total**	–	**≈13400**

Control Measurements of "Empty" POPC LUV_{200} with $ABTS^{2-}$ / H_2O_2

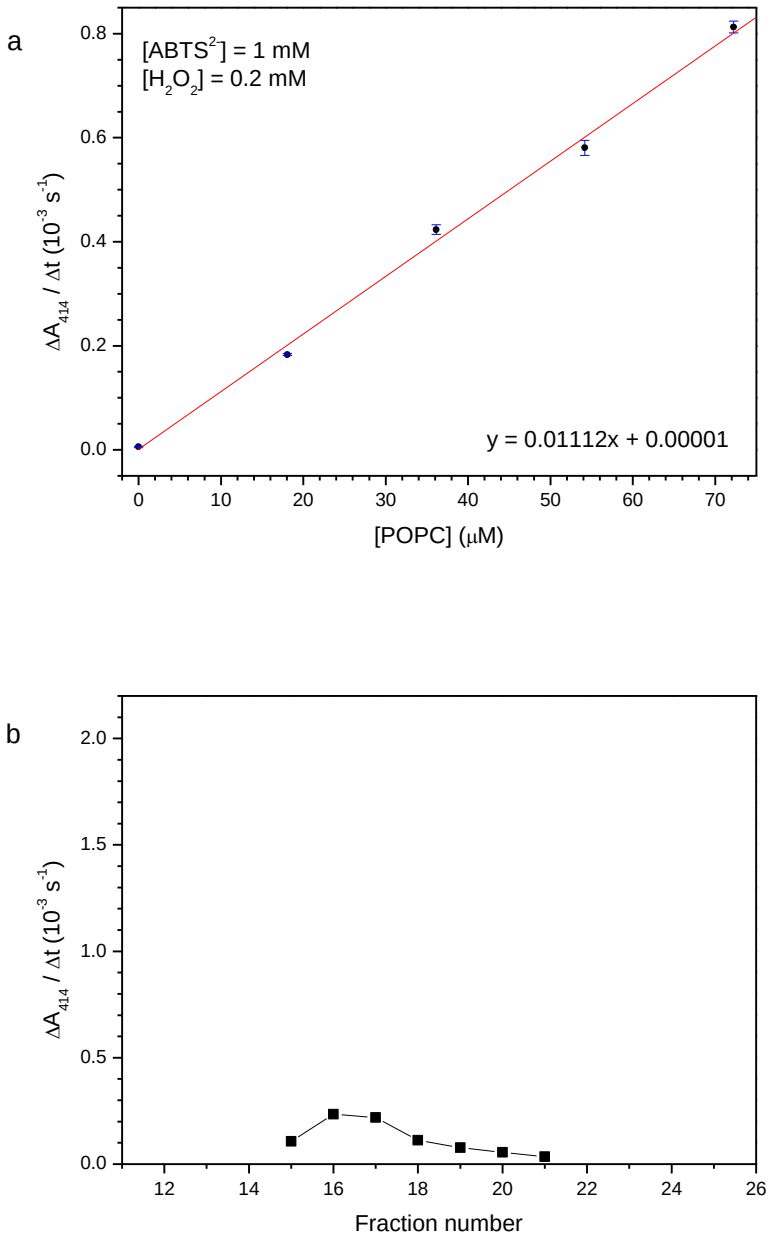


Figure S-12. Control measurements with "empty" POPC vesicles. (a): Effect of "empty" POPC vesicles on the stability of $ABTS^{2-}$. "Empty" POPC LUV_{200} vesicles were prepared in the same way as described for the HRP-encapsulating vesicles, but without using HRP. (b): The vesicles were then run through a Sepharose 4B column (l = 35 cm; d = 1.1 cm; applied volume: 0.5 mL 20 mM POPC). The "activities" of the vesicle fractions were measured with $ABTS^{2-}$ and H_2O_2 as substrates in the same way as described for the vesicle fractions with the HRP-containing vesicles; $r^2 = 0.9939$.

Control Measurements of "Empty" POPC LUV_{200} with PPD / H_2O_2

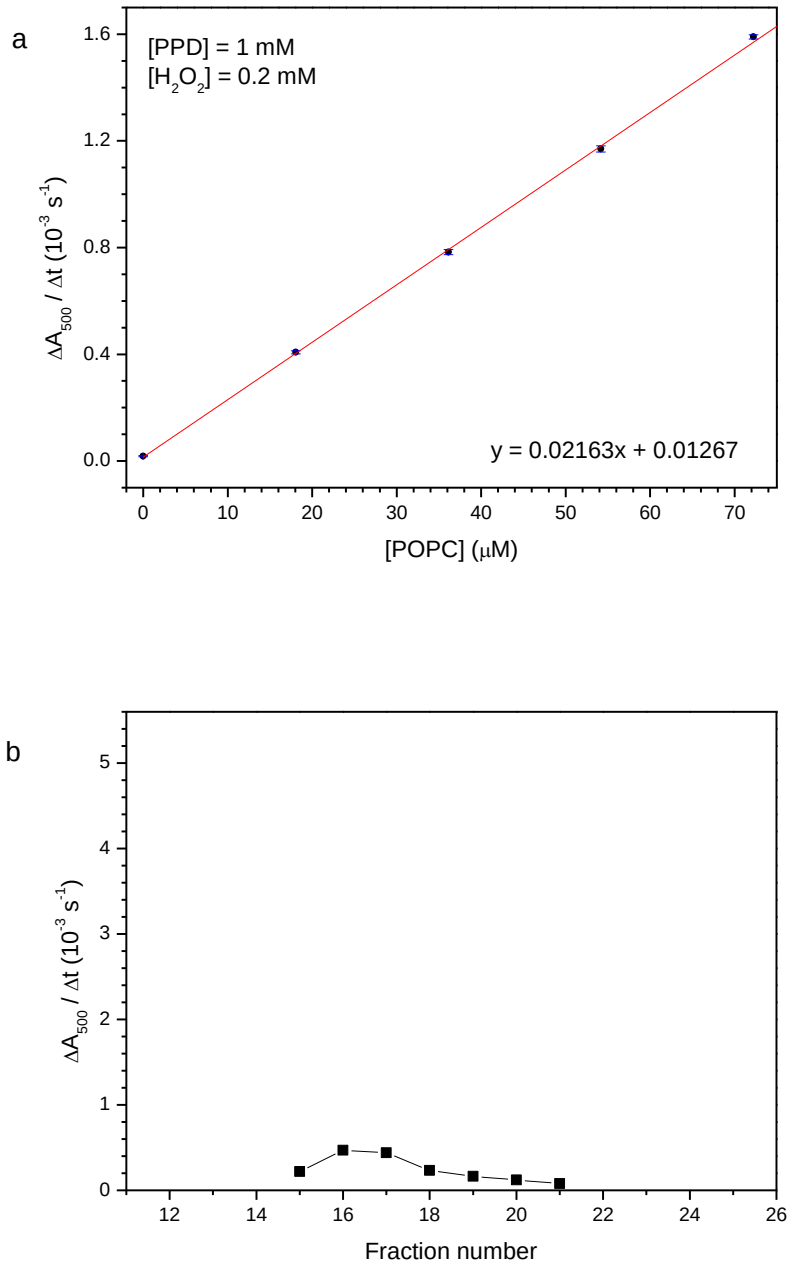


Figure S-13. Control measurements with "empty" POPC vesicles. (a): Effect of "empty" POPC vesicles on the stability of PPD. Effect of "empty" POPC vesicles on the stability of PPD. "Empty" POPC LUV_{200} vesicles were prepared in the same way as described for the HRP-encapsulating vesicles, but without using HRP. (b): The vesicles were then run through a Sepharose 4B column (l = 35 cm; d = 1.1 cm; applied volume: 0.5 mL 20 mM POPC). The "activities" of the vesicle fractions were measured with PPD and H_2O_2 as substrates in the same way as described for the vesicle fractions with the HRP-containing vesicles; $r^2 = 0.9995$.

Quantification of POPC and HRP in Each Vesicle Fraction

Table S-1. Quantification of POPC and active HRP in each vesicle fraction, as obtained by chromatographic separation of HRP-encapsulating LUV_{200} from free HRP, see **Figure 5** (fractions 15-21). A volume of 0.5 mL of the extruded vesicle suspension (20 mM POPC, 31 μM HRP) was applied onto the Sepharose 4B column (l = 35 cm; d = 1.1 cm). For the HRP activity measurements, 10 μL volumes of the vesicle fractions (0.78 mL each) were added to 1.0 mL of the assay solutions. The calibration curves used are the ones in **Figure S-3** (POPC), in **Figure S-5b** ($ABTS^{2-}$), and in **Figure S-8b** (PPD), see EXPERIMENTAL SECTION for details.

Fraction number	Stewart assay	$ABTS^{2-}$ assay		PPD assay	
	n(POPC) (nmol)	$\Delta A_{414}/\Delta t$ (10^{-3} s^{-1})	n (HRP) (pmol)	$\Delta A_{500}/\Delta t$ (10^{-3} s^{-1})	n (HRP) (pmol)
15	771	0.76	24.68	1.98	23.56
16	1685	2.00	72.11	5.30	70.89
17	1575	1.65	58.73	4.37	57.63
18	809	0.83	27.17	2.17	26.27
19	554	0.41	11.30	0.97	9.15
20	400	0.31	7.62	0.76	6.21
21	250	0.22	4.12	0.55	3.25
overall	6044	-	205.76	-	196.96

POPC concentration in the different fractions: As an example, the POPC concentration in *fraction 17* was n(POPC) / fraction volume = 1575 nmol / 0.78 mL = 2.0 mM.

The determined total amounts of POPC and HRP in the vesicle fractions were ≈6 μmol (6044 nmol) and ≈200 pmol, respectively.
Applied onto the column under the assumption that no POPC or HRP was lost during extrusion: $n(POPC) = 0.5 \cdot 10^{-3}$ L x $20 \cdot 10^{-3}$ mol $L^{-1} = 1.0 \cdot 10^{-5}$ mol = 10 μmol; $n(HRP) = 0.5 \cdot 10^{-3}$ L x $31 \cdot 10^{-6}$ mol $L^{-1} = 1.55 \cdot 10^{-8}$ mol ≈ 16 nmol.

DLS Measurements of Vesicle Fractions

Table S-2. DLS measurements of vesicle *fraction 16* and of the pooled vesicle fractions (15-21), see **Figure 5**). The measurements were performed at 21 °C. Each sample was measured three times with five sub-runs. The hydrodynamic diameter (D_h), as Z-average, and the polydispersity (PDI) are average values and standard deviations of three measurements (in bold).

Sample	Z-average (nm)	PDI (-)
HRP vesicles, *fraction 16* (1:10)	176.4	0.091
	180.0	0.057
	184.1	0.045
	180.2±3.9	**0.064±0.02**
HRP vesicles, *fraction 16* (1:20)	176.4	0.081
	182.9	0.048
	183.4	0.103
	180.9±3.9	**0.077±0.03**
HRP vesicles, pooled fractions (1:2)	176.2	0.092
	180.5	0.088
	180.8	0.109
	179.2±2.6	**0.096±0.01**
HRP vesicles, pooled fractions (1:4)	182.1	0.091
	181.0	0.087
	181.3	0.109
	181.5±0.57	**0.096±0.01**

The size stability of HRP-encapsulating POPC LUV_{200} was determined by DLS measurements of a vesicle fraction which was stored for 28 days at 4 °C. For these measurements, a separate preparation of HRP-encapsulating vesicles was carried out. The vesicle fraction was analyzed immediately after size exclusion chromatography and yielded a z-average (hydrodynamic diameter) of 190.4±1.3 nm with a PDI of 0.088±0.005. Upon storage at 4 °C for 13, 16, 23, and 28 days, the values were 187.8±1.6 nm (0.114±0.031), 189.0±0.6 nm (0.126±0.022); and 191.2±0.6 nm (0.136±0.017), respectively.

Simulation of the Uptake of PPD by POPC LUV_{200}

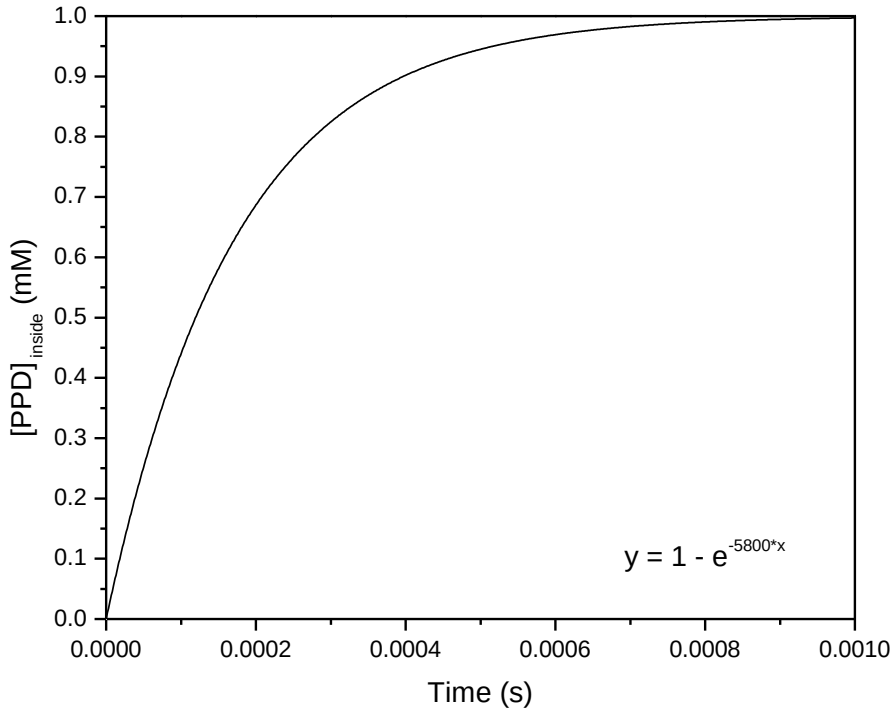


Figure S-14. Simulation of the time-dependent uptake of PPD by POPC LUV_{200}.

For the simulation, Fick's first law was considered: $J(t) = P \cdot \Delta[PPD(t)]$, with J(t) being the flux of PPD across the POPC bilayer at time t; $\Delta[PPD(t)]$ being the difference in the molar PPD concentrations outside and inside the vesicles at time t; and *P* being the permeability coefficient for PPD and the POPC bilayer. Assuming first order kinetics, the concentration of PPD inside the vesicles at time t, $[PPD]_{inside}$ (t), is [S16, S17]

$$[PPD]_{inside}(t) = [PPD]_{outside}(0)\,(1 - e^{-k \cdot t})$$

with $[PPD]_{outside}$ (0) being the PPD concentration in the bulk solution at t = 0; and *k* being the first order rate constant for the permeability of PPD.

The correlation between P and k is as follows:[S18]

$$k = 3P/R$$

with R being the radius of the vesicles.

The simulation was made with $[PPD]_{outside}$ (0) = 1.0 mM, R = 90 nm = $9 \cdot 10^{-6}$ cm (the determined radius of the LUV_{200}), and $P = 1.74 \cdot 10^{-2}$ cm s^{-1}.

Another Preparation of a HRP-encapsulating POPC LUV_{200} Suspension

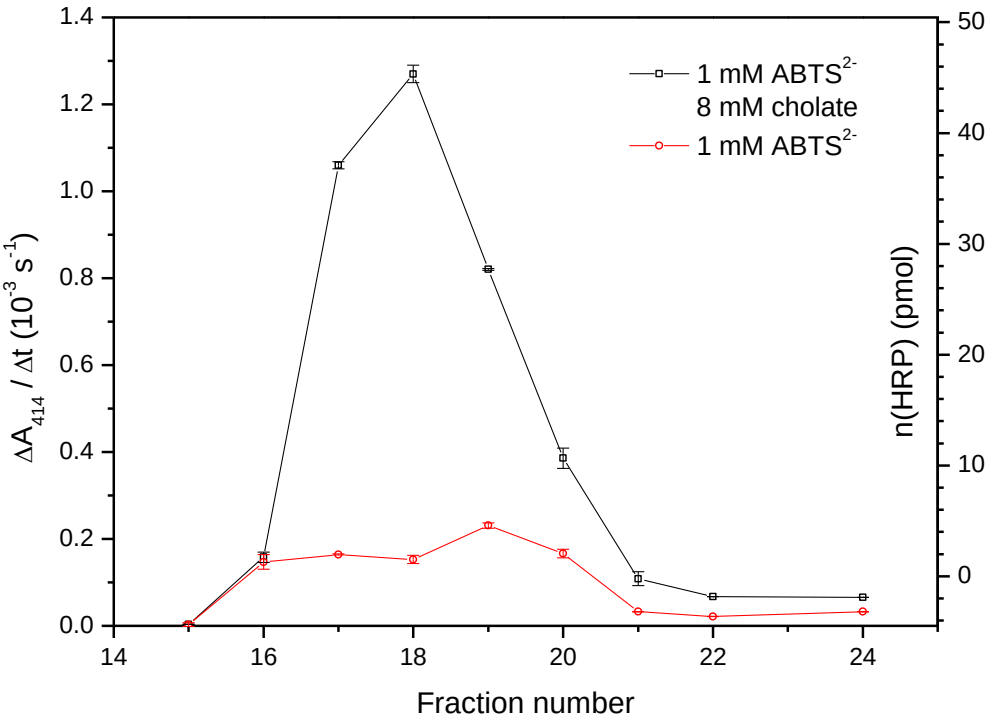


Figure S-15. Second preparation of HRP-encapsulating POPC LUV_{200}. Chromatogram for the separation of non-entrapped HRP from HRP-encapsulating POPC LUV_{200} by size exclusion chromatography with Sepharose 4B (l ≈ 35 cm; d = 1.1 cm; applied volume: 0.5 mL (20 mM POPC, 31 μM HRP); elution flow rate (buffer-2): 0.33 mL/min; fraction volume: 0.80 mL), see EXPERIMENTAL SECTION for details. The HRP activity in the vesicle fractions was measured with $ABTS^{2-}$ without (black, open squares) and with cholate (8.0 mM, red, open circles); the y-axis on the left hand side is the increase in A_{414} per s, caused by the addition of 10 μL of the fraction to 1 mL of the assay solution (l = 1 cm); the axis on the right hand side refers to the calculated amount of HRP for the measurements carried out in the presence of 8.0 mM cholate and taking into account the calibration curve in **Figure S-5b** (for $ABTS^{2-}$). Details for *fractions 17, 18,* and *20* are given in **Table S-3**.

Table S-3. Results from the analysis of *fractions 17, 18,* and *20* of the second preparation of HRP-encapsulating LUV_{200}, see the chromatogram in **Figure S-15**. The POPC concentration was determined with the Stewart assay.[S4, S5]

Fraction number	[POPC] (mM)	Number of vesicles, N_{ves}	[vesicle] (nM)	ABTS assay	
				n(HRP) (pmol)	Number of HRP molecules per vesicle, N_{HRP}
17	2.32	$4.02 \cdot 10^{12}$	8.35	37.10	6
18	2.15	$3.73 \cdot 10^{12}$	7.73	45.33	7
20	0.71	$1.23 \cdot 10^{12}$	2.55	10.65	5

For *fraction 18*:
$[HRP]_{vesicle} = n(HRP) / (V_{ves} \cdot N_{ves}) = 45.33 \cdot 10^{-12}$ mol / $(2.88 \cdot 10^{-18}$ L $\cdot\ 3.73 \cdot 10^{12}) = 4.2 \cdot 10^{-6}$ M

Michaelis-Menten Kinetics of HRP Inside POPC LUV_{200} Measured with PPD / H_2O_2

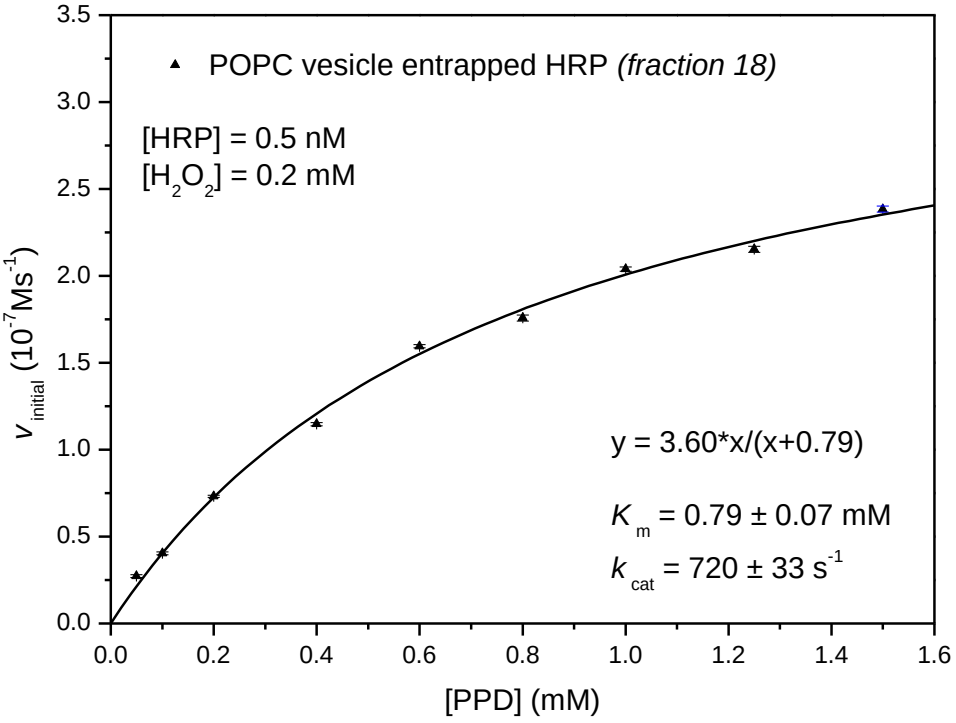


Figure S-16. Activity measurements with HRP-encapsulating POPC LUV_{200} with *p*-phenylenediamine (PPD) at 25 °C and pH = 7.0 (buffer-2), with 0.2 mM H_2O_2 and a total HRP concentration of 0.5 nM. $[HRP]_{vesicle}$ = 4.2 μM, N_{HRP} ≈7 (*fraction 18*), see **Table S-3**, [POPC] = 19 μM. The initial rate of product formation (Bandrowski's base) – determined from the increase in A_{500} during the first 100 s – is plotted as a function of PPD concentration. Each data point represents the mean and standard deviation from three measurements. The data points were fit with the Michaelis-Menten equation; r^2 = 0.9955. For details, see the EXPERIMENTAL SECTION and RESULTS AND DISCUSSION.

Please note that for the conversion of the measured initial change in A_{500} per time unit into initial velocity, it was assumed that ε_{500} (Bandrowski's base) was the same as in bulk solution (11 090 $M^{-1}cm^{-1}$).[S11]

Experiments Demonstrating the Release of Bandrowski's Base Formed Inside HRP-encapsulating POPC LUV_{200} from the Vesicles

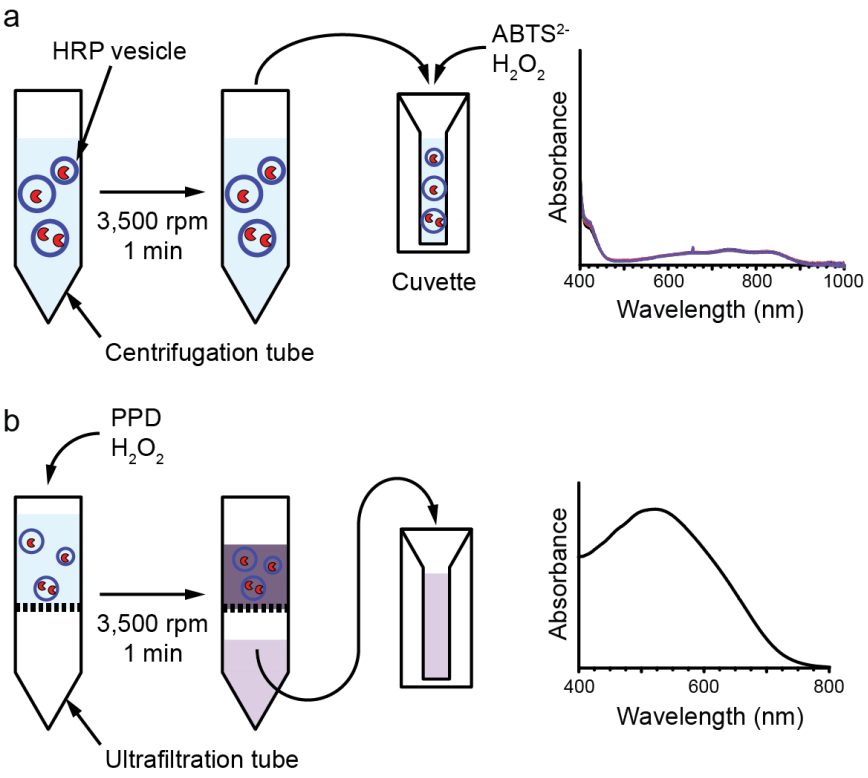


Figure S-17. Schematic of the experiments showing that Bandrowski's base, which was formed inside HRP-encapsulating LUV_{200} at pH 7.0 (buffer-2), leaked out from the vesicles.

(a) HRP-encapsulating POPC LUV_{200} were prepared (*fraction 16*, **Figure 5**, **Table 1**) and put into a centrifugation tube (100 µL *fraction* 16 + 900 µL buffer-2) and then centrifuged with a Hermle Z 320H centrifuge at 3500 rpm for 1 min. Afterwards, 100 µL of the vesicle suspension were added to 900 µL of an $ABTS^{2-}$ assay solution containing 0.5 mM $ABTS^{2-}$ and 40 µM H_2O_2. The UV/Vis spectrum was measured every minute for the initial 5 minutes, indicating the expected formation of small amounts of $ABTS^{\bullet-}$ only, see also **Figure S-6a** and **Figure S-12**. These measurements showed that centrifugation does not lead to a release of encapsulated HRP from the vesicles.

(b) 3 µL of a HRP-encapsulating POPC LUV_{200} suspension (*fraction 16*) were added to 1 mL PPD assay solution (0.5 mM PPD, 80 µM H_2O_2, buffer-2). The vesicle suspension was then added to an ultrafiltration device (from Millipore, 100 000 cutoff), incubated for 10 min and then centrifuged for 1 min at 3500 rpm. The UV/Vis spectrum of the ultrafiltrate was measured. The intensity of the absorption band around 500 nm was similar to what was expected for the type of measurements shown in **Figure S-9**. This indicates that at least some of Bandrowski's base formed inside the vesicles leaked out from the vesicles.

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