Lipid peroxidation of polyunsaturated fatty acid (PUFA) phospholipids induces necrotic cell death through compromised cell membrane integrity during ferroptosis. We established assays to investigate oxidoreductase-mediated oxidative rupture, specifically via NADPH-cytochrome P450 reductase (POR) and NADH-cytochrome b5 reductase (CYB5R1), of PUFA phospholipids in artificially generated protein-free liposomes. Liposome breakage was detected via Tb³⁺ liposome release and electron microscopy liposome morphology imaging. This protocol was also applied to other oxidoreductases with analogous functions and investigation of ferroptotic membrane damage in cell-free systems.
Protocol
Assessing POR and CYB5R1 oxidoreductase-mediated oxidative rupture of PUFA in liposomes

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https://doi.org/10.1016/j.xpro.2021.100360

SUMMARY
Lipid peroxidation of polyunsaturated fatty acid (PUFA) phospholipids induces necrotic cell death through compromised cell membrane integrity during ferroptosis. We established assays to investigate oxidoreductase-mediated oxidative rupture, specifically via NADPH-cytochrome P450 reductase (POR) and NADH-cytochrome b5 reductase (CYB5R1), of PUFA phospholipids in artificially generated protein-free liposomes. Liposome breakage was detected via Tb³⁺ liposome release and electron microscopy liposome morphology imaging. This protocol was also applied to other oxidoreductases with analogous functions and investigation of ferroptotic membrane damage in cell-free systems. For complete details on the use and execution of this protocol, please refer to Yan et al. (2020).

BEFORE YOU BEGIN
The protocol below describes a method for detecting lipid peroxidation induced liposome damage by oxidoreductases POR and CYB5R1, mediated through a series of redox reactions. Moreover, this method is applicable to liposome damage by other triggers, such as pore-forming mixed lineage kinase domain like pseudokinase (MLKL) proteins (Wang et al., 2014) and gasdermin proteins (Ding et al., 2016) induced membrane leakage without any chemical reactions.

Purification of recombinant POR and CYB5R1 from bacterium

© Timing: 2 days

1. Generate recombinant vectors for the expression of oxidoreductases POR and CYB5R1 in the bacterium pET28c vector. Delete the transmembrane domains of POR (aa 2–42) and CYB5R1 (aa 2–28) to facilitate purification. The vectors are termed hereafter as pET28a-D2-42 POR and pET28a-D2-28 CYB5R1. Generate specific point mutations of POR variants (Y178D; A284P; R454H; V489E; C566Y; V605F) by site-directed mutagenesis. All vectors are available upon request from Yan et al. (2020). Vectors were transfected into E.coli strain JM109 (DE3) and plated for the growth of single clones.

△ CRITICAL: The deletion of the transmembrane domain of POR/CYB5R1 is critical for high-efficiency expression and purification of both proteins.

2. Pick Single clones and culture in 50 mL of Luria-Bertani (LB) medium containing 50 µg/mL kanamycin for 16 h.
3. Inoculate 10 mL of cultured bacterium into 1 L of kanamycin resistant LB medium in 2.5 L flasks at 37°C to reach an optical density (OD) value of 0.8. Then, add 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 100 μM riboflavin into the LB media for further culture at 15°C for ~20 h. Handle a total of 5 L of LB side-by-side.

**CRITICAL:** Riboflavin is required for the purification of functional POR/CYB5R1, as it is essential for the enzymatic biosynthesis of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). Both POR and CYB5R1 are flavoproteins, and FAD and FMN are cofactors of POR and FAD is a cofactor of CYB5R1.

*Note:* The optimal OD value of bacterium is between 0.6 and 1.0 when adding IPTG in order to achieve higher protein expression.

4. Harvest the bacterium by centrifugation for 30 min at 4,000 × g and lysed in buffer I (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 15 mM imidazole) in the presence of Protease Inhibitor Cocktail (Roche) and Phenylmethylsulfonyl fluoride (PMSF). The solution is then French pressed at 60,000 psi, twice.

5. Discard cell debris by centrifugation for 30 min at 20,000 × g. Then load the soluble proteins on a Nickel column. Wash the non-specifically bound proteins extensively.
   a. Wash the column by 10× volume of buffer II (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 15 mM imidazole, 0.5% Triton X-100), twice.
   b. Second, wash the column by 10× volume of buffer III (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 15 mM imidazole, 0.5% Triton X-100), twice.
   c. Third, wash the column by 20× volume of buffer I to eliminate detergent.

6. Elute proteins with buffer IV (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 300 mM imidazole). Discard imidazole and any remaining detergent in a 30 kDa cut-off concentrate tube (for POR) or a 10 kDa cut-off concentrate tube (for CYB5R1). Store the proteins in buffer V (20 mM Tris-HCl, pH 8.0, 150 mM NaCl) and froze proteins under −80°C.

*Note:* The protein exhibits a yellow color due to the binding of FMN and/or FAD.

*Note:* The buffers were stored at 4°C until use.

**CRITICAL:** The proteins were aliquoted (10 μL per tube, 10 μM) to avoid repeated freezing/thawing cycles.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| JM109 (DE3) | TransGen Biotech | CD601 |
| **Biological samples** | | |
| Soy phospholipid mixture | Avanti | 690050P |
| Cardiolipin solution from bovine heart | Sigma-Aldrich | SRE0029 |
| 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (16:0 PC, DPPC) | Avanti | 850355P; CAS: 63-89-8 |
| 1-Stearoyl-2-Arachidonoyl-sn-glycero-3-phosphoethanolamine (18:0-20:4 PE, SAPE) | Avanti | 850804C; CAS: 61216-62-4 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Ferrostatin-1 (Fer-1) | Selleck | S7243; CAS: 347174-05-4 |
| Liproxstatin-1 (Lip-1) | Selleck | S7699; CAS: 950455-15-9 |
| Terbium (III) chloride | Sigma-Aldrich | 451304; CAS: 10042-88-3 |
| Dipicolinic acid (DPA) | Sigma-Aldrich | P63808; CAS: 499-83-2 |

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### Critical commercial assays

| Protocol                                      | Source                          | Identifier |
|------------------------------------------------|---------------------------------|------------|
| Fast Mutagenesis System                       | TransGen Biotech                | FM111-02   |

### Oligonucleotides

| Oligonucleotide                        | Sequences                                      |
|----------------------------------------|------------------------------------------------|
| 28c-Ncol(gi)-CYB5R1no28-F             | ACTTTAAGAAGGAGATATACCATGTTGGTTCGGAG            |
|                                        | GTCCCGCAGG                                    |
| 28c-Notl(gi)-CYB5R1-R                  | TCAGTGGTGGTGGTGGTGGTGGTGGTGAATTCTG            |
|                                        | CATCTTGT                                       |
| 28c-Ncol(gi)-PORno42-F                 | ACTTTAAAGGAGGATACCATGCTGTTCCGGAA              |
|                                        | GAAAAAAGAG                                    |
| 28c-Notl(gi)-POR-R                     | TCAGTGGTGGTGGTGGTGGTGGAAGCCACACGTC            |
|                                        | CAGGCAGG                                     |
| POR-Y178D-F                            | TGGGTCTGACAACCCGCCAGAGAGCAGAATCGGTC           |
| POR-Y178D-R                            | CCGTGTCTTGGCCAGGCGCAACACCGGGCAAACTTCCG        |
| POR-A284P-F                            | CCAAGAATCCCTTCTCCAGCCAGCGTGAACCACAA          |
| POR-A284P-R                            | TGCCAGGAGGATGATCTGCGGAGGAGGCGATGGAGGCTCT     |
| POR-R454H-F                            | CTGCTTACAGTGCAGCCACTACTCTACTTCCGCCGG         |
| POR-R454H-R                            | GTGGGCTGCACTTACAGGCGAGACAGCAGCTGGAGGCT       |
| POR-V489E-F                            | GGCGAATCCCAAGAAGCGAGGCGAGCCAAAAATTGCT         |
| POR-V489E-R                            | TCGTGATTGTCCTGAGGCGCCGCTGTTCTCGTATTCT        |
| POR-C566Y-F                            | CACGTCGTATCTAGCGTACAGAAGAAGCGAGGAG           |
| POR-C566Y-R                            | TAGCCGAATACAGCAGGAGATGTCCTTCAGGACTGCTGGAG    |
| POR-V605F-F                            | CAGAGGCACAAAGTATTTGATACCCAGCCTGCTGAG         |
| POR-V605F-R                            | GAAGTACACTTGTGCTGCGCTTGCCTCCGGGCCTGAG        |

### Recombinant DNA

| Recombinant DNA                        | Sequences                                      |
|----------------------------------------|------------------------------------------------|
| pET-28c-Δ2-42 POR-His                  | This paper                                     |
| pET-28c-Δ2-42 POR Y178D -His           | This paper                                     |
| pET-28c-Δ2-42 POR A284P -His           | This paper                                     |
| pET-28c-Δ2-42 POR R454H -His           | This paper                                     |
| pET-28c-Δ2-42 POR V489E -His           | This paper                                     |
| pET-28c-Δ2-42 POR C566Y -His           | This paper                                     |
| pET-28c-Δ2-42 POR V605F -His           | This paper                                     |
| pET-28c-Δ2-28 CYB5R1-His               | This paper                                     |

### Software and algorithms

| Software and algorithms              | Source                          |
|--------------------------------------|---------------------------------|
| GraphPad Prism 8                     | GraphPad Software               | https://www.graphpad.com/ |

### Other

| Buffer | Description                     | Identifier |
|--------|---------------------------------|------------|
| Buffer I | 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 15 mM imidazole | N/A        |
| Buffer II | 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 15 mM imidazole, 0.5% Triton X-100 | N/A        |
| Buffer III | 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 15 mM imidazole, 0.5% Triton X-100 | N/A        |

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**STEP-BY-STEP METHOD DETAILS**

**Preparation of liposomes for electron microscopy negative staining**

© Timing: 30 min

This section describes how to prepare liposome which do not enclose Tb$$^{3+}$$ ions. These liposome was used as a substrate for POR mediated lipid peroxidation and the damage was detected by electron microscopy.

1. Dissolve the soy phospholipid mixture and bovine cardiolipins from heart in chloroform (10 mg/mL stock solution) under nitrogen and store them at −20°C. Add 80 μL of soy phospholipids and 20 μL bovine cardiolipins into 500 μL of chloroform in a round-bottomed flask (10 mL).

   △ CRITICAL: The lipid should be carefully covered with nitrogen to protect from oxygen. Even a small amount of oxygen would cause oxidative damage to the lipids with time.

   Optional: Argon could also be used to protect phospholipids from oxygen.

   **Note:** As this protocol is used for testing lipid peroxidation of PUFA phospholipids mediated membrane damage, it is critical to make sure PUFA phospholipids are included in the lipid mixtures. In this protocol, PUFA phospholipids were included in the soy phospholipid mixture and bovine cardiolipins. However, when using other phospholipids for example PUFA contained phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine are all optional. According to our previous research, when approximately 2% of membrane lipids are oxidatively damaged, the liposome membrane becomes permeabilized. Therefore, at least 2% of PUFA phospholipids should be incorporated into the liposomes. The higher the concentration of PUFA phospholipids, the more easily lipid peroxidation-mediated membrane damage occurred.

2. Evaporate the chloroform using a vacuum rotary evaporator (BUCHI) using gentle and continuous rotation (150 rpm) under a water bath at 30°C to form an evenly spaced phospholipid film.

   △ CRITICAL: Gentle and continuous rotation of the flask is critical for the formation of an even lipid film.
3. Add 500 μL of buffer H (20 mM HEPES, pH 7.4, 150 mM NaCl) into the flask and the lipid film was detached by vortex.

   **Optional:** Sonication of the flasks also helps the detachment of phospholipids from the glass wall.

   **Note:** The buffer H was stored at 4°C until use.

4. The hydrated phospholipid was draw via syringe and subjected to extrusion through a polycarbonate membrane (100 nm) 30 times using a mini-extruder (Avanti) to generate morphologically uniform liposomes.

   △ **CRITICAL:** The extrusion process must be slow and gentle, with an approximate speed of 100 μL per second.

   **Pause point:** The liposomes can be stored at 4°C for approximately one week.

   **Note:** Pre-warming of the mini-extruder on a 65°C block greatly facilitates the solubility of some phospholipids.

**Inducing POR mediated oxidative breakage of liposomes**

   **Timing:** 2 h

This section describes how to perform POR catalyzed redox reactions with liposome as the substrate.

5. Add liposomes (100 μg/mL), POR (100 nM), and ferric chloride (120 μM) to buffer L (20 mM HEPES, pH 7.4, 100 mM NaCl) in 50 μL system in 1.5 mL tubes. Pass Oxygen (3 L/min) into the mixture via a thin needle for 10 s.

   △ **CRITICAL:** The NaCl concentration in buffer H (inside the liposomes) is higher than in buffer L (outside the liposomes), which is critical for maintaining the morphology of liposome under electron microscopy.

   **Note:** The addition of oxygen should be done carefully to avoid spilling the reaction mixture.

   **Note:** The buffer L was stored at 4°C until use.

   **Note:** Appropriate controls were made: a negative control group with eliminated POR protein, a group with an equivalent amount of the POR Y178D mutant variant, and a group including the lipid peroxidation inhibitor Fer-1 (10 μM).

6. Initiate the reaction with the addition of NADPH (50 μM) and reacted for 90 min at 15°C–25°C.

**Negative staining of liposomes and imaging under electron microscopy**

   **Timing:** 2 h

This section describes the method for negative staining liposomes for transmission electron microscopy.
7. EM grid pre-treatment: the EM grid (ultra-thin carbon supporting films, 300 mesh) was discharged in a plasma cleaner (PDC-32G) with a current intensity of “Low” gear for 30 s.

△ CRITICAL: The pre-treatment of EM grid should be performed right before use and the grids are ready by the time the reaction (step 6) is done.

Optional: Any plasma cleaner and glow discharge plasma would work as alternatives.

8. Liposome adsorption: 5 μL of liposomes were added to the grid for 1 min to allow attachment. The remaining liquid was carefully removed by filter paper dabbing.

△ CRITICAL: The liposomes in the POR-free group can be difficult to attach to the EM grid. Glycerol (1% v/v) facilitate this process.

Optional: An equivalent level of BSA (100 nM) may also facilitate liposome grid binding in the POR-free group.

9. Gently wash off Loosely attached liposomes, or other reaction system components with distilled water for 20 s, twice. Discard water via a filter paper.

10. Stain the bound liposomes with 5 μL 2% uranyl acetate for 30 s, 2 times. Discard the remaining uranyl acetate via filter paper, and the sample was allowed to dry at 15°C–25°C.

Pause point: The stained EM grid can be stored for an extended period (several weeks) before electron microscopy imaging.

Note: Liposomes were stained twice with uranyl acetate, because the remaining water on the sample would decrease concentration of uranyl acetate in the first staining process, and, further, to increase the staining depth.

11. Normal or oxidatively damaged liposomes were imaged on a Tecnai T12 microscope (Thermo Fisher Scientific) at 120 kV. Take the photographs on a Gatan 4k × 4k CCD camera (nominal magnification 26,000×).

Optional: Any transmission electron microscope with a similar capacity would be a suitable alternative for imaging.

Preparation of liposomes for leakage detection by spectrophotometry

© Timing: 3 h

This section describes how to prepare liposome enclosing Tb³⁺ ions. These liposomes were used as substrates for POR or CYB5R1 mediated lipid peroxidation, and the leakage of Tb³⁺ ions from the liposomes was detected via spectrophotometry.

12. Dissolve the soy phospholipid mixture, bovine cardiolipins, and synthesized PUFA SAPE (18:0–20:4 PE) or saturated fatty acid (SFA) DPPC (16:0 PC) in chloroform (10 mg/mL each) under nitrogen and stored at −20°C. Add 100 μL of individual phospholipid, or different ratios of SAPE and DPPC combined phospholipids, into 500 μL of chloroform in a 10 mL round-bottomed flask. The evaporation of chloroform was then performed as described in step 2.

13. To enclose Tb³⁺ ions, the phospholipid films were hydrated and detached in 500 μL of buffer TL (20 mM HEPES, pH 7.4, 100 mM NaCl, 50 mM sodium citrate, 15 mM TbCl₃). Liposomes were then prepared as described in step 4.
CRITICAL: Pre-warming of the mini-extruder on a 65°C block is required for the solubilization of phospholipids like DPPC.

Note: The buffer TL was freshly prepared and stored on ice until use.

14. Unencapsulated Tb$^{3+}$ ions were removed by extensive washing with 8 cycles of 15 mL buffer L in 100 KD cut-off ultrafiltration tubes (Millipore) and centrifugation at 4°C, after which they were re-suspended in 500 μL of buffer L.

CRITICAL: The extensive washing of external Tb$^{3+}$ ions are crucial to a low background in the following assays. The existence of external Tb$^{3+}$ ions can be detected by the addition 50 μM of DPA in the flowthrough buffer L and a subsequent increase in fluorescence signal ($\lambda_{ex}=270/\lambda_{em}=620$ nm) (see below).

Optional: Centrifugation of the liposomes at 100,000 x g for 30 min, then washing the pellet 3 times is also feasible for obtaining external Tb$^{3+}$ ions free liposomes.

Pause point: The liposomes may be stored at 4°C for approximately one week.

Inducing POR/CYB5R1 mediated oxidative breakage of liposomes encapsulating Tb$^{3+}$

Temporal: 1 h

This section describes how to perform the POR and CYB5R1 catalyzed redox reactions on liposomes encapsulating Tb$^{3+}$.

15. Dilute liposomes encapsulating Tb$^{3+}$ (100 μg/mL), oxidoreductase (100 nM), ferric chloride (120 μM), and DPA (50 μM) into 100 μL of buffer L. Pass oxygen (3 L/min) into the mixture via a thin needle for 10 s. Add nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH) (50 μM) into the mixture to initiate the reaction.

Note: In the negative control groups, no oxidoreductase, NADPH, or ferric chloride were added. In some groups, WT or mutant POR proteins were included. When analyzing the additive effect of POR and CYB5R1, 100 nM each of the protein was added. In some groups, Fer-1 (1 μM) or Lip-1 (1 μM) were added.

Note: NADPH is an electron donor for POR, and NADH is an electron donor for CYB5R1. When analyzing the additive effect of POR and CYB5R1, both electron donors were included in the mixtures.

16. As soon as NADPH and/or NADH was added, the fluorescence signal ($\lambda_{ex}=270/\lambda_{em}=620$ nm) was measured via microplate reader as Ft0. Then, the fluorescence signals were recorded at 20 s intervals for about 120 times (about 40 min), after which 1 μL of Triton X-100 (0.1%, v/v) was added to the liposomes to completely release the Tb$^{3+}$ ions. The fluorescence signal value achieved by Triton X-100 was recorded as F(t). At each time point (Ft), the percentage of liposome leakage was defined as: Leakage (t) % = [(Ft-Ft0) / (F(t)100-Ft0)] × 100.

CRITICAL: Previous research (Wang et al., 2014) used emission wavelength $\lambda_{em}=490$ nm to detect the fluorescence signal of Tb$^{3+}$/DPA chelates. NADPH has a strong signal under $\lambda_{em}$ 490 nm but is not detected under $\lambda_{em}$ 620 nm. Additionally, $\lambda_{em}$ 620 nm gives a higher signal for Tb$^{3+}$/DPA than $\lambda_{em}$ 490 nm. Therefore we use $\lambda_{em}$ 620 nm in this assay.
EXPECTED OUTCOMES

When observed by electron microscopy, liposomes will be a little heterogeneous, with sizes ranging from 50 to 200 nm in diameter. The morphology of liposomes will not always round, and “antenna” like structures are also common. The extent of “antenna” decorating liposomes varies depending on the different lipid compositions. In contrast with the intact liposomes in the control group (Figure 1A left), any liposomes with ruptured membranes (top right in Figure 1A right), fragmented morphology (lower left in Figure 1A right), or holes (lower right in Figure 1A right) were categorized as damaged liposomes. POR, but not POR Y178D mutant, mediated oxidative damage of liposomes will rupture and fragment the liposome membrane. Fer-1 will block the POR mediated oxidative damage of liposomes (Figures 1A and 1B).

When assessed by spectrophotometry, POR/CYB5R1 will induce liposome leakage by increasing Tb$^{3+}$/DPA fluorescence. POR mutants or Fer-1/Lip-1 will block these processes. POR mediated increase of Tb$^{3+}$/DPA fluorescence signal will occur when any PUFA are present (soy phospholipid mixture, bovine cardiolipins, or SAPE). POR will not cause leakage when liposomes are totally comprised from SFA (DPPC) (Yan et al., 2020) (Figure 2).

LIMITATIONS

- The proteins used in this protocol are purified from bacterium. It is unclear whether the enzymatic activity of POR/CYB5R1 would be different when they are purified from mammalian cells.
The proteins used in this protocol are deficient in their transmembrane domain. It is unclear whether the transmembrane domain of POR/CYB5R1 would affect their function in these in vitro assays.

Special instruments are required (i.e., PLASMA CLEANER and electron microscope) to observe POR mediated membrane damage.

The pore size formed by POR/CYB5R1 is not assessed. Fluorescent labeled dextran with different molecular weights could be used as an encapsulated substrate to reflect pore size during oxidative membrane damage.

The types, ratio, and composition of lipids in this assay may not fully reflect the membrane lipid composition.

The liposomes used in this protocol are membrane protein free. The potential impact of membrane proteins on lipid peroxidation-mediated membrane damage is an unknown.

TROUBLESHOOTING

Problem 1
Enzyme-independent damage of liposomes in the presence of ferric chloride (step 1).

Potential solution
PUFA phospholipids are easily oxidized when exposed to oxygen, and oxidative damage in phospholipids can be amplified by iron in a non-enzymatic catalyzed manner. Therefore, phospholipids...
resolved in chloroform need to be carefully protected from oxygen by inert gas, such as nitrogen or argon, to lower the background.

**Problem 2**  
Few liposomes attached into EM grid in the POR-free group (step 8).

**Potential solution**  
Proteins in the reaction mixture facilitate the attachment of liposome to EM grid. In the blank control group where no POR protein is added, glycerol (1% v/v) or similar concentration of BSA (100 nM) can be added to increase the binding between the liposome and EM grid.

**Problem 3**  
Insoluble of phospholipids during extrusion process (step 13).

**Potential solution**  
Higher concentrations of phospholipids are not easy to fully solubilibize. In particular, when only SFA DPPC is included it is extremely difficult to maintain solubility during extrusion. Pre-warming of the mini-extruder on a 65°C blocker greatly facilitates the solubility of DPPC. Moreover, lowering the concentration of DPPC (less than 2 mg/mL) facilitates the extrusion process.

**Problem 4**  
High fluorescence background in the spectrophotometry assay (step 14).

**Potential solution**  
Only a portion of the Tb3+ ions are encapsulated into liposomes during the extrusion processes, a great deal of Tb3+ ions remain outside of liposomes. It is important to ensure the external Tb3+ ions are absolutely washed out. Regularly checking for Tb3+ ions in the flowthrough fraction in the 100 KD cut-off concentrate tube by adding DPA is important. Ideally, the fluorescent signal of the flowthrough fraction is close to the value of buffer L plus DPA, indicating a low contamination of external Tb3+.

**Problem 5**  
High fluorescence background caused by NADPH (step 16).

**Potential solution**  
NADPH, or other components in your assay system, might give a high fluorescence background. Reducing the concentrations of contaminated fluorescence materials would decrease the background but sometimes this can cause inefficient enzymatic reactions. Another method is to re-scan the excitation and emission wavelength spectrum of the contaminated fluorescence material and the target, to achieve an optimal excitation and emission wavelength for the assay. A combination of these two methods is also viable.

**RESOURCE AVAILABILITY**

**Lead contact**  
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xiaodong Wang (wangxiaodong@nibs.ac.cn).

**Materials availability**  
All recombinant vectors expressing WT or mutant POR proteins and CYB5R1 are available upon reasonable request from the lead contact without restriction.

**Data and code availability**  
This study did not generate/analyze datasets/code.
ACKNOWLEDGMENTS

We thank Drs. Qi Sun, Jingjin Ding, Huan Zeng, and Yang She for sharing materials for the liposome and negative staining assays. We thank Miss. Hexia Luo for assisting in electron microscopy. Y.A. was supported by China Postdoctoral Innovation Talent Support Program. This work was supported by institutional grants from the Chinese Ministry of Science and Technology and the Beijing Municipal Commission of Science and Technology.

AUTHOR CONTRIBUTIONS

Conceptualization, B.Y., Y.A., and X.W.; Writing – Original Draft, B.Y. and Y.A.; Writing – Review & Editing, B.Y., Y.A., Z.Z., and X.W.; Funding Acquisition, Y.A., Z.Z., and X.W.; Supervision, Z.Z. and X.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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