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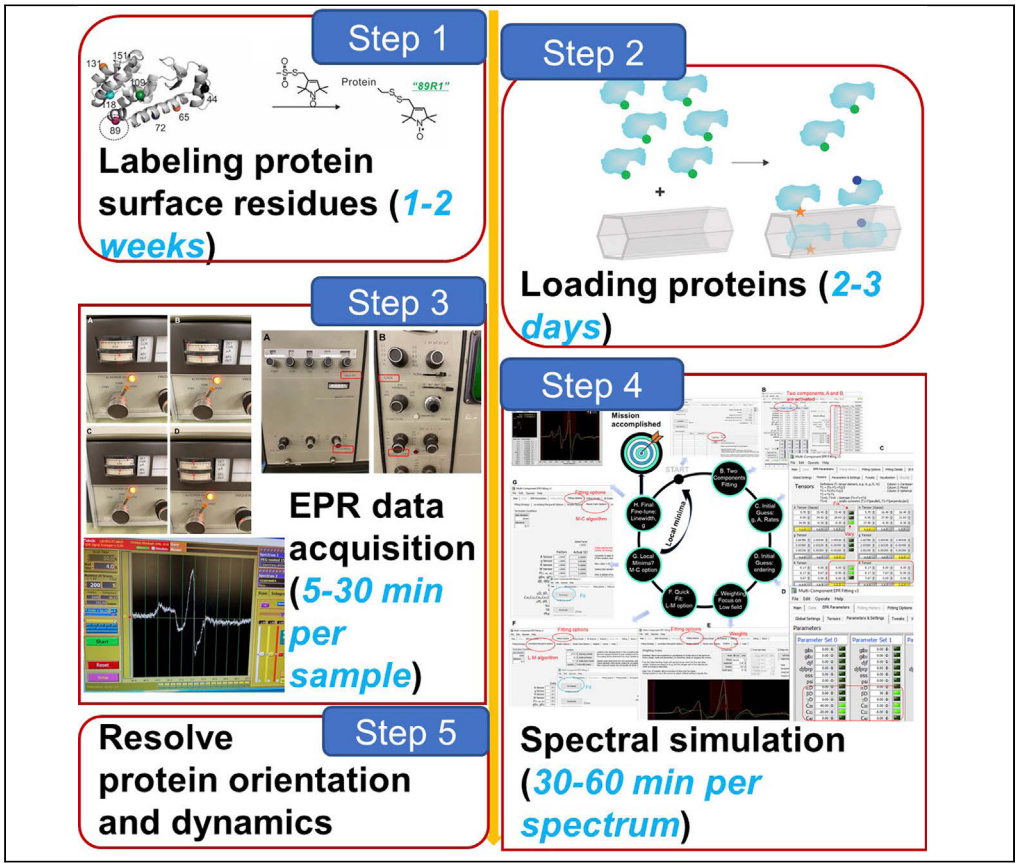
Protocol for resolving enzyme orientation and dynamics in advanced porous materials via SDSL-EPR



Enzyme encapsulation in metal-organic frameworks (MOFs)/covalent-organic frameworks (COFs) provides advancement in biocatalysis yet the structural basis underlying the catalytic performance is challenging to probe. Here, we present an effective protocol to determine the orientation and dynamics of enzymes in MOFs/COFs using site-directed spin labeling and electron paramagnetic resonance spectroscopy. The protocol is demonstrated using lysozyme and can be generalized to other enzymes.

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Highlights

A protocol to resolve protein orientation/ dynamics in porous materials is provided

Site-directed spin labeling is combined with electron paramagnetic resonance

Principles of protein labeling and key data acquisition steps are summarized

Spectral simulation details with troubleshooting procedures are detailed

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Protocol for resolving enzyme orientation and dynamics in advanced porous materials via SDSL-EPR

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SUMMARY

Enzyme encapsulation in metal-organic frameworks (MOFs)/covalent-organic frameworks (COFs) provides advancement in biocatalysis, yet the structural basis underlying the catalytic performance is challenging to probe. Here, we present an effective protocol to determine the orientation and dynamics of enzymes in MOFs/COFs using site-directed spin labeling and electron paramagnetic resonance spectroscopy. The protocol is demonstrated using lysozyme and can be generalized to other enzymes.

For complete information on the generation and use of this protocol, please refer to Pan et al. (2021a).

BEFORE YOU BEGIN

Enzyme encapsulation in mesoporous materials enhances enzyme stability and reusability (Nel et al., 2009, Küchler et al., 2016, Sheldon and Woodley, 2018, López-Gallego et al., 2017, Sheldon, 2012). The resultant enzymatic performance depends on the orientation and dynamics of the entrapped enzymes, which are often challenging to determine at the sufficiently high resolution, due to the interference of the porous background (Pan et al., 2021a). We recently demonstrated a unique approach to bridge this knowledge gap using site-directed spin labeling (SDSL) in combination with Electron Paramagnetic Resonance (EPR) spectroscopy (Pan et al., 2021a). SDSL-EPR has been proved powerful in determining protein and other biomacromolecule structural information in complex biological systems; our team recently extended it to the interface of enzyme and mesoporous materials, which led to meaningful structural information that explains the resultant enzyme activity (Pan et al., 2021b, Sun et al., 2019, Pan et al., 2018). Thus far, due to the uniqueness of EPR spectroscopy especially spectral simulation, there has been limited step-by-step procedure reported for utilizing SDSL-EPR on probing structural information at the enzyme-porous materials interface. Therefore, here we present a detailed protocol for using our strategy to fill in this gap. We particularly focus on determining the relative orientation of enzyme in respect to the pore surfaces as well as the backbone dynamics of enzyme upon encapsulation. The protocol was tested on a model enzyme, lysozyme, upon encapsulation in Metal-Organic Frameworks/Covalent-Organic Frameworks (MOFs/COFs). Lysozyme is a good model because both large (~μm) and small (~nm) size substrates can be catalyzed by this enzyme (Vocadlo et al., 2001, Kao et al., 2014). Meanwhile, MOFs/COFs are advanced porous platforms for enzyme encapsulation (Howarth et al., 2016, Majewski et al., 2017, Drout et al., 2019, Wang et al., 2020, Gkaniatsou et al., 2017, Lyu et al., 2014, Lian

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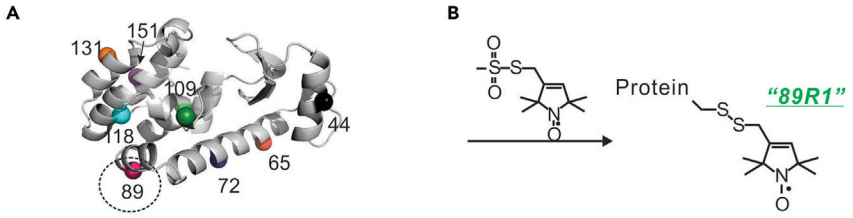

Figure 1. Example surface site selection and spin labeling of a protein's cysteine

(A) Eight sites are selected for spin labeling to cover most of the surface regions of our model enzyme, lysozyme.

(B) Using residue 89 as an example, a nitroxide spin labeling compound (HO-225) is attached to site 89 via a disulfide bond. Figure adapted from reference with permission (Pan et al., 2021b) This article was published in Chem Catalysis, 1, Y. Pan, Q. Li, H. Li, J. Farmakes, A. Ugrinov, X. Zhu, Z. Lai, B. Chen, and Z. Yang, A general Ca-MOM platform with enhanced acid-base stability for enzyme biocatalysis, 1–16, Copyright Elsevier (2021).

et al., 2017, Li et al., 2020, Farmakes et al., 2020, Neupane et al., 2019). Note that, a unique feature of SDSL-EPR is that it is immune of the background matrices (under low water volume; see below) (Pan et al., 2021a). Thus, with minimal modifications, the protocol can be used to determine the orientation and dynamics of other enzymes upon entrapping in other porous materials. As is evident from the name, SDSL-EPR requires both protein spin labeling and EPR measurement. Therefore, in this protocol, we strive our best to cover as many details as possible in both aspects, so that the protocol can be generalized to other enzymes/materials. Before one begins, the following Preparations need to be carried out.

Determine enzyme surface sites for spin labeling

The principle to determine enzyme orientation is to identify the surface residues that are in contact with the porous materials so that the enzyme "sits" on the pore surfaces with these residues while pointing the rest away (Pan et al., 2021a). Thus, it is critical to place spin labels on representative residues of the target enzyme to cover most of the protein surface.

Timing: varies

1. Obtain the crystal structure and full DNA sequence of the target enzyme and determine the sites to attach the EPR spin label.
   a. Identify representative surface residues of the enzyme. In our case, the crystal structure of T4 phage lysozyme is available at the Protein Data Bank (PDB: 3lzm). Based on this information, we found 44, 65, 72, 89, 109, 118, 131, and 151 residues are sufficient to cover most of the protein surface (Figure 1A).
   b. Acquire/collect available structural information of the spin label on proteins. This step is necessary because a spin label is essentially a protein sidechain. Knowing the structural basis of this sidechain is critical for revealing the dynamics of the local protein backbone motion. The most commonly used spin label is the HO-225 (also known as 1-oxyl-2,2,5,5-tetramethyl-Δ3-(methanesulfonyloxymethyl)pyrroline; see Figure 1B and key resources table. Alternative spin labels are also possible as summarized in our recent work(Pan et al., 2021a); however, HO-225 is the most optimal for extracting the local dynamics information), which reacts with a protein thiol (cysteine) and generates a nitroxide sidechain named as R1. There are a number of crystal structures of R1 attached on α helices or β strands, as well as extensive studies presented in pioneering EPR works that report the dynamics of the R1 sidechain (López et al., 2009, Columbus and Hubbell, 2004, Columbus et al., 2001, Altenbach et al., 2015). We suggest accumulate available information so that latter data analysis can be proceeded with minimal errors/concerns. In our case, we are aware of the anisotropy of the R1 motion in lysozyme as well as how protein local and global motion affect the resultant spectra.

⚠ CRITICAL: We suggest to label on protein α helices or βstrands, because the conformations of a number of spin labels on these secondary structures are well-understood (Altenbach et al., 2015, López et al., 2009, Zhang et al., 2010, Columbus et al., 2001, Columbus and Hubbell, 2004). Loop regions can also be labeled but require additional caution in data analysis. We also suggest avoiding labeling the beginning and the end of a helix or strand because mutating these sites may cause protein structural disturbance.

⚠ CRITICAL: For a protein/enzyme with DNA sequence but no structural information on the secondary structure or higher resolution, our strategy may still be applicable. The key would be to employ multiple EPR frequencies and viscous conditions to accurately determine the dynamics of protein motion (see section "EPR spectral simulation", steps 18–21). For a cysteine-rich protein, alternative strategies are available as well (see Section "protein spin labeling", step 1).

Prepare primers for cysteine mutation and spin labeling

Timing: 1 week

2. Basic rules.
   a. Primers should contain 20–40 base pairs with a GC content >40%
   b. Start and end a primer with a G or C.
   c. The melting temperature (T_m) should be ~ 70°C.
   d. Stack the 5′- (or, forward) and 3′- (backward) primers if possible to improve the mutation efficiency.
   e. Minimize the number of mismatches: the code of cysteine is TGC or TGT, whichever gives the minimal mismatch.
3. Design the 5′ primer.
   a. Copy the 5′ DNA sequence of 20–40 base pairs with the target mutation site in the middle.
   b. Change the mutation site to TGC or TGT, whichever gives the minimal mismatch.
   c. Calculate the GC content and T_m at http://www.biophp.org/minitools/melting_temperature/demo.php?primer=&basic=1&NearestNeighbor=1&cp=100&cs=30&cmg=2
   d. If all basic rules are satisfied, accept the primer sequence. Otherwise, adjust the sequence (length) slightly (while keeping the mutation site in the sequence).
4. Design the 3′ primer.
   a. Copy the 3′ DNA sequence of 20–40 base pairs with the target mutation site in the middle.
   b. Change the mutation site to ACG or ACA, whichever gives the minimal mismatch.
   c. Calculate the GC content and T_m at http://www.biophp.org/minitools/melting_temperature/demo.php?primer=&basic=1&NearestNeighbor=1&cp=100&cs=30&cmg=2
   d. If basic rules are satisfied, reverse the primer sequence. Otherwise, adjust the sequence slightly (with the mutation site in the sequence).
   e. Reverse the sequence because primers have to be ordered starting at the 5′ end from commercial sources (see below).
5. Order primers for each mutation from commercial resources. In our case, we order from IDT DNA technologies. https://www.idtdna.com/pages
6. Primers should be stored at −20°C.

Prepare buffers

Timing: 1–2 h

7. Prepare the lysate buffer (pH = 7.6): 3.04 g/L Tris-Base (25 mM), 5.23 g/L MOPS (25 mM), and 37 mg/L EDTA (0.1 mM)

8. Prepare the high salt buffer (pH = 7.6): 3.04 g/L Tris-Base (25 mM), 5.23 g/L MOPS (25 mM), 0.51 g/L DTT (5 mM), 58.4 g/L NaCl (1.0 M), and 37 mg/L EDTA (0.1 mM)
9. Prepare the low salt buffer (pH = 7.6): 3.04 g/L Tris-Base (25 mM), 5.23 g/L MOPS (25 mM), 0.51 g/L DTT (5 mM), and 37 mg/L EDTA (0.1 mM)
10. Prepare the spin labeling buffer (pH = 6.8): 10.46 g/L MOPS (50 mM) and 1.46 g/L NaCl (25 mM).

⚠ CRITICAL: Store buffers at 4°C or add DTT right before protein purification. Obviously, buffer preparation depends on the protein of interest. Buffers should be filtered (0.22 μm) before protein purification to avoid clogging the chromatographic columns.

Prepare medium for cell growth

Timing: 1 day

11. Prepare liquid LB medium.
    a. Prepare 1000 × ampicillin (AMP): dissolve 1 g AMP in 10 mL sterilized water. Filter with 0.22 μm sterilized filter to avoid large aggregates and remove potential bacteria (see key resources table). Store in 1 mL aliquots under −20°C.
    b. Dissolve 25 g of LB medium in 1 L ddH_2O in a 2-liter Erlenmeyer flask. Autoclave for 15–18 min. Let cool to room temperature. Add 1 mL of the 1000 × AMP for each liter of the LB medium. Store the medium at room temperature for a short period of time (a few days) or at 4°C.
12. Prepare solid LB medium.
    a. Dissolve 25 g of LB medium and 15 g agar in 1 L ddH_2O in a 2-liter Erlenmeyer flask. Autoclave for 15–18 min. Let cool to 65°C (higher than this temperature the AMP can be degraded. Lower than this temperature it would become difficult to pour the medium). Add 1 mL of the 1000 × AMP. Pour 25–30 mL of the warm medium into a plate. Let all plates cool at room temperature for a few hours. Store in a sterilized sleeve bag under 4°C.

Optional: We found it helpful to preset an oven at 65°C so that the medium can be accurately cooled to the desired temperature.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
MOPS	Sigma-Aldrich	M1254
Tris base	Sigma-Aldrich	10708976001
EDTA	Sigma-Aldrich	E9884-100G
DTT	Sigma-Aldrich	10708984001
NaCl	Sigma-Aldrich	S9888
Ampicillin	Gold Biotechnology	A-301-5
IPTG	Gold Biotechnology	I2481C
LB medium	MP Biomedicals	113002032
Agar	MP Biomedicals	02150178-CF
HEPES	Sigma-Aldrich	H3375
HO-225	Toronto Research Chemicals	O872400
3-(2-Iodoacetamido)-PROXYL -spin label	Sigma-Aldrich	253421
Critical commercial assays		
Lysozyme Activity Kit	Sigma-Aldrich	LY0100

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
PCR Kit	New England BioLabs	M0273
Mini-preparation	QIAGEN	27104
Chitosan	Sigma-Aldrich	CAS 577-76-4
Borosilicate tube with 0.6 I.D. × 0.8 O.D.	Wilmad Glass	712-SQ-250M
Software and algorithms		
Multi-component for CW EPR spectral simulation	https://sites.google.com/site/altenbach/labview-programs/epr-programs/multicomponent?authuser=0	N/A
Calculate the GC content and T_m	http://www.biophp.org/minitools/melting_temperature/demo.php?primer=&basic=1&NearestNeighbor=1&cp=100&cs=30&cmg=2	N/A
Confirm the DNA sequence	Molecular Cloning Laboratories (MCLab https://www.mclab.com/)	N/A
The standard Transformation Protocol	New England Biolabs: https://www.neb.com/protocols/2012/05/21/transformation-protocol	N/A
Other		
Syringes	Sigma-Aldrich	Z116866; Z116882
Filters	Fisher Scientific	09-719G
Plates	VWR	100217-280
Flasks	Thermo Scientific™	10-042-5N
Filtration (Amicon) concentrators	MilliporeSigma Amicon	UFC901008
Autoclave tape	Sigma-Aldrich	A2549
Critoseal	Fisher Scientific	#0267620
Commercial primer resource	https://www.idtdna.com/pages	N/A
Confirm the molecular weight and the yield of the spin-labeled protein by gel electrophoresis.	https://assets.thermofisher.com/TFS-Assets/BID/brochures/precast-protein-gels-brochure.pdf	N/A
Protein ladder	https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-gel-electrophoresis/protein-standards-ladders.html	26616

Optional: The resources listed below were only based on our experience. In principle, the chemicals and resources can be obtained from any reliable commercial sources and do not need to be limited to those listed in our table.

MATERIALS AND EQUIPMENT

Buffer for protein purification and spin labeling

Name	Composition/Concentration	pH
Lysate buffer	3.04 g/L Tris-Base (25 mM), 5.23 g/L MOPS (25 mM), 37 mg/L EDTA (0.1 mM)	7.6
Low salt buffer	3.04 g/L Tris-Base (25 mM), 5.23 g/L MOPS (25 mM), 0.51 g/L DTT (5 mM), 37 mg/L EDTA (0.1 mM)	7.6
High salt buffer	3.04 g/L Tris-Base (25 mM), 5.23 g/L MOPS (25 mM), 0.51 g/L DTT (5 mM), 58.4 g/L NaCl (1.0 M), 37 mg/L EDTA (0.1 mM)	7.6
Spin labeling buffer	10.46 g/L MOPS (50 mM), 1.46 g/L NaCl (25 mM).	6.8
HO-225 stock solution	200 mM in acetonitrile	
HEPES buffer	50 mM HEPES	7.0–7.4

Equipment for EPR spectral acquisition: CW-EPR spectrometer with a proper cavity. In our case, we used a Varian E-109 equipped with a standard cavity resonator.

Equipment for protein purification and SDSL		
Name	Function	Identifier
Water bath	Heat-shock	Isotemp FSGPD05
Shaker-incubator	Incubation	ThermoFisher MaxQ4000
Refrigerated centrifuge	Separation	Sorvall ST 8R
FPLC	Protein purification	Akta
UV spectrometer	Measure protein absorption	Nanodrop
Oven	Heating/maintain a warm temperature	Global Industrial, Item #: T9FB918772
Ion-exchange column	Protein purification	GE Healthcare, HiTrap SP HP 17115101
Desalting column	Protein purification	Sigma-Aldrich, HiTrap 26/10 GE17-5087-01

Alternatives: Other CW EPR spectrometers such as the Bruker EMXnano, microESR, and Magnettech ESR5000 can also be used.

STEP-BY-STEP METHOD DETAILS

Protein spin labeling

Timing: 1–2 weeks

This major step is to create the needed cysteine mutants and spin label each. The purification protocol is suitable for isolating charged proteins. Note that one cysteine mutation has to be placed in the protein at a time (to avoid spectral overlap). Therefore, usually multiple single cysteine mutants are needed. The following procedures are to label one cysteine mutant. Usually for 8–10 mutants, 1 week is sufficient to complete all procedures. For each additional 8–10 mutants, another week is needed.

⚠ CRITICAL: For other proteins, an appropriate purification protocol should replace this major step.

1. Create plasmids with the needed mutants.
   a. The ordered primers (see above Section "prepare primers for cysteine mutation and spin labeling", step 5) will be dissolved in sterilized water following the standard procedure of polymer chain reaction (PCR) available at New England Biolabs: https://www.neb.com/protocols/0001/01/01/taq-dna-polymerase-with-standard-taq-buffer-m0273
   b. Then, the cysteine-free, pseudo-wildtype plasmid of the protein will be applied to include the mutants in the plasmid following the PCR procedure above.

⚠ CRITICAL: Native free cysteines of the target protein have to be mutated to a serine or alanine (native cysteines forming disulfide bonds do not participate in spin labeling and do not need to be mutated). This is usually not a major hurdle given the low natural abundance of free cysteine in most proteins. For cysteine-rich proteins, unnatural amino acids can be employed (see below).

   c. Transform the plasmid into cells via mini-prep using the standard procedure and the commercial kit: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/plasmid-dna/qiaprep-spin-miniprep-kit/#orderinginformation
   d. Confirm the sequence is needed via commercial DNA sequencing service. We use Molecular Cloning Laboratories (MCLab https://www.mclab.com/) for sequencing.
   e. The resultant DNA can be stored at −20°C for further use.

2. Transformation of mutants.
   a. The standard transformation protocol that transform the DNA into solid LB medium (plates) available at New England Biolabs is usually applied with minor modifications: https://www.neb.com/protocols/2012/05/21/transformation-protocol

3. Protein expression.
   a. For each mutant, inoculate one colony from the plate and grow in 25 mL LB (1 × AMP) medium at 37°C for 12–16 h (205–225 rpm shaking rate).
   b. Add the 25 mL overnight culture into 1 L LB (1 × AMP) medium and incubate at 37°C under shaking for 4 h.
   c. Induce each liter of cells with 1 mL (1 M) IPTG at 37°C for 1.5 h under shaking.
   d. Immediately harvest the cells via centrifugation at 4000 rpm for 15–45 min.
   e. Resuspend the pellets with 25–50 mL of lysate buffer via gentle shaking.

   ⏸ **Pause point:** Cell pellets can be stored at −20°C for at least several months.
   f. Sonicate the pellet suspension on ice under a 50% duty cycle for 5 min. Place on ice for 5 min.

   Optional: If larger pellets are present after sonication, another round of sonication can be applied.
   g. Separate the supernatant from the cell debris via centrifugation at 15,000 rpm at 4°C for 1 h.
   h. Take the supernatant and filter with 0.22 μm to remove large particles/pellets.

4. Protein purification and spin labeling.
   a. Equilibrate the FPLC motors with lysate buffer.
   b. Load the supernatant into a GE ion-exchange (SP HP) column (see equipment above). Rinse the column with the lysate buffer until the UV absorbance is near zero. Collect flow-through.
   c. Elute the column with varied salt content: 0%–10% (high salt buffer content) for 2 min, 10%–30% for 10 min, 30%–100% for 0.5 min, and wash with 100% for 50 mL. The lysozyme protein should collect at 15%–25%.

   ⚠ CRITICAL: DTT is needed in the above steps (before running the desalting column) to avoid protein dimerization caused by disulfide formation between cysteines.
   d. Switch to the desalting column and equilibrate the column with the spin labeling buffer (100 mL; no DTT).
   e. Load the cysteine mutants prepared above into the desalting column. Collect the protein as the first peak appears (12 mL).
   f. Immediately add in HO-225 (10-fold excess) to react with the cysteine mutant.
   g. Cover the container with foil and leave under 4°C for overnight.
   h. Remove unreacted HO-225 via filtration-centrifugation using Amicon concentrators with a 10 kDa cutoff.
   i. Bring the protein concentration to ~0.2–0.5 mM using the Amicon concentrators for storage at 4°C.

5. Protein characterization
   a. Confirm the molecular weight and the yield of the spin labeled protein by gel-electrophoresis using the standard procedures and protein ladder at https://assets.thermofisher.com/TFS-Assets/BID/brochures/precast-protein-gels-brochure.pdf and https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-gel-electrophoresis.html. T4 lysozyme should appear at ~18.7 kDa.

   Note: Because protein gel experiment procedure and commercial kits are well-established, only links are provided here for the conciseness of this protocol.
   Troubleshooting 1
   b. Confirm the secondary structure of the spin labeled protein by Circular Dichroism. All mutants should show the expected CD spectrum as in the published work.(Pan et al., 2017, Yang et al., 2014)
   c. Confirm the activity of the spin labeled protein using the standard lysozyme activity kit available at Sigma-Aldrich (LY0100).

Troubleshooting 2

Optional: Right after step 3b, an aliquot of 0.5 mL cells can be mixed with 0.5 mL of sterilized glycerol uniformly and stored at −20 or −80°C for future expression. For long-term storage, the labeled proteins can be stored at −20°C.

Note: Protocols above uses T4 lysozyme as an example to highlight the needed steps. Protein purification clearly depends on the specific enzyme. Thus, this major step has to be dependent on the target enzyme. In addition to the kits listed above, other commercial kits for PCR, mini-prep, and protein activity assays can also be used.

Enzyme loading/encapsulation into porous materials

Timing: 2–3 days

Loading a spin labeled enzyme into mesoporous materials, especially MOFs/COFs, is no different from loading regular enzymes. The only caution would be to avoid reducing conditions, which cleaves the disulfide bonds between the label and the protein cysteine. Because enzymes can be loaded into pre-formed MOFs/COFs and via co-precipitation, we separately discuss these two loading events.

6. Protein loading
   a. To load into a pre-formed MOF/COF, the MOF/COF should be resuspended with a proper buffer (usually HEPES at pH 7.0–7.4) that does not disturb the MOF/COF scaffold. Then, the labeled protein is mixed with the materials in the HEPES buffer and shaken gently for overnight at room temperature. Usually for each 1 mg of MOF/COF, 1–2 mg of protein is needed to ensure protein is in excess to facilitate the loading. If needed, an additional day can be given.

   ⚠ CRITICAL: Depending on the enzyme, lower temperature may be needed for protein stability. If the HEPES buffer disturb a particular MOF/COF, other buffers such as acetate or MES buffer can be used.

   b. To encapsulate an enzyme in a MOF via co-precipitation, the needed metal ion (ca. Zn^{2+} or Ca^{2+} as in our recent studies) and ligand on the order of a few mM together with the enzyme (~mg/mL) are mixed in the appropriate medium (water or MeOH). The co-precipitation can go overnight with an option of gentle shaking.

7. Remove unwanted species.
   a. Remove free protein, metal, and ligand via centrifugation at 14,000 rpm for 5 min. Resuspend the enzyme@MOF/COF pellets with water, and centrifuge again. If needed, 3–5 cycles are needed (as long as there is no residual protein and ligand in the supernatant of the wash, which can be determined via UV-vis absorption spectroscopy). The resultant enzyme@MOF materials are in the pellets and can be stored at 4°C in water or MeOH.

8. Confirm the integrity of the materials and the function of the enzyme.
   a. Standard MOF/COF characterization (Sun et al., 2019; Wang et al., 2020; Majewski et al., 2017; Drout et al., 2019; Li et al., 2018; Li et al., 2016; Gkaniatsou et al., 2017; Lian et al., 2017; An et al., 2020) such as scanning electron microscope (SEM), powder X-ray diffraction (PXRD), thermalgravimetric analysis TGA, and N_2 absorption experiments should be carried out to confirm the loading of the enzyme as well as the structure of the MOF/COF scaffolds. Usually near-identical SEM and PXRD are expected before and after enzyme loading.

Note: Because MOF/COF characterization is well-known and standardized, no detail is provided here.

b. Confirm enzyme activity. Lysozyme is a unique case because it can catalyze both large and small size substrates. For the large size substrates, bacterial cell walls, which can be hydrolyzed by lysozyme encapsulated on the surface of MOFs via co-precipitation, the commercial kit described above (Sigma-Aldrich) can be used.
c. For smaller substrates that contain a 1,4-glycosidic bond, such as the 11-chitosan, which can diffuse into the MOF/COF channels, the following steps can be used to determine lysozyme activity following the literature:(Sun et al., 2019)
   i. The lysozyme loaded MOFs/COFs are mixed with chitosan solution (0.6 mg mL^{-1} in 0.1 M acetate solution, 4 mL, (CAS 577-76-4), buffer (0.1 M acetate solution, 1 mL), and a magnetic stirrer in a 10 mL Schlenk flask.
   ii. The container of the mixture is then sealed and heated in a preheated water bath (50°C) under constant magnetic stirring for 2 h.
   iii. The pellets are removed via filtration through a 0.45-μm membrane filter.
   iv. 1.5 mL of the flow-through was added into 2.0 mL of coloring reagent dropwise (0.05% potassium ferricyanide solution prepared by dissolving 0.5 g potassium ferricyanide in 1.0 L of 0.5 M sodium carbonate), followed by being heated at 100°C for 15 min.
   v. Upon cooling to room temperature, the optical intensity at 420 nm (OD420) was determined using a UV spectrometer. The decrease in OD420 is proportional to the amount of reducing groups generated when chitosan was hydrolyzed by lysozyme.

Troubleshooting 3

Optional: If a reducing condition is needed for enzyme function, the IAP-spin label based on the formation of a S-C bond (instead of S-S bond) can be used (Sahu and Lorigan, 2018).

Pause point: Enzyme@MOF/COF materials can be stored at 4°C for 2–4 weeks.

Acquire EPR spectra

Timing: 5–30 min per spectrum

Upon confirming the enzymes are functional on MOFs/COFs, continuous wave (CW) EPR spectrum for each mutant on each MOF/COF should be acquired, analysis of which (see latter steps) will result in enzyme orientation and dynamics information. The procedures below are based on our spectrometer, a Varian E-109 with a cavity resonator. Similar steps with minor alterations can be followed when using other commercially available CW EPR spectrometers. Depending on loading capacity of the specific materials, each CW EPR spectrum can take 5–30 min.

9. Load samples to EPR capillaries.
   a. For room temperature CW EPR measurement, usually minimal water volume is suggested to avoid the absorption of microwave power by the water molecular dipoles. A borosilicate tube with 0.6 I.D. × 0.8 O.D. is often sufficient with 1–2 cm sample height loaded.

⚠ CRITICAL: For most enzyme@MOF/COF materials, a relatively larger total volume, ca. 20–40 μL, is needed so that the effective volume of the pellets is sufficient for EPR measurement. A pipette is needed to load such a large volume.

⚠ CRITICAL: Due to the heterogeneous phase of the sample, we found it the best practice if both ends of the EPR tube is open, so that the sample can be loaded into one end by making the capillary part of the pipette tip. Both ends can then be sealed with a wax (ca. Critoseal from Fisher/McKesson; see above).

10. Turn on the CW EPR spectrometer.

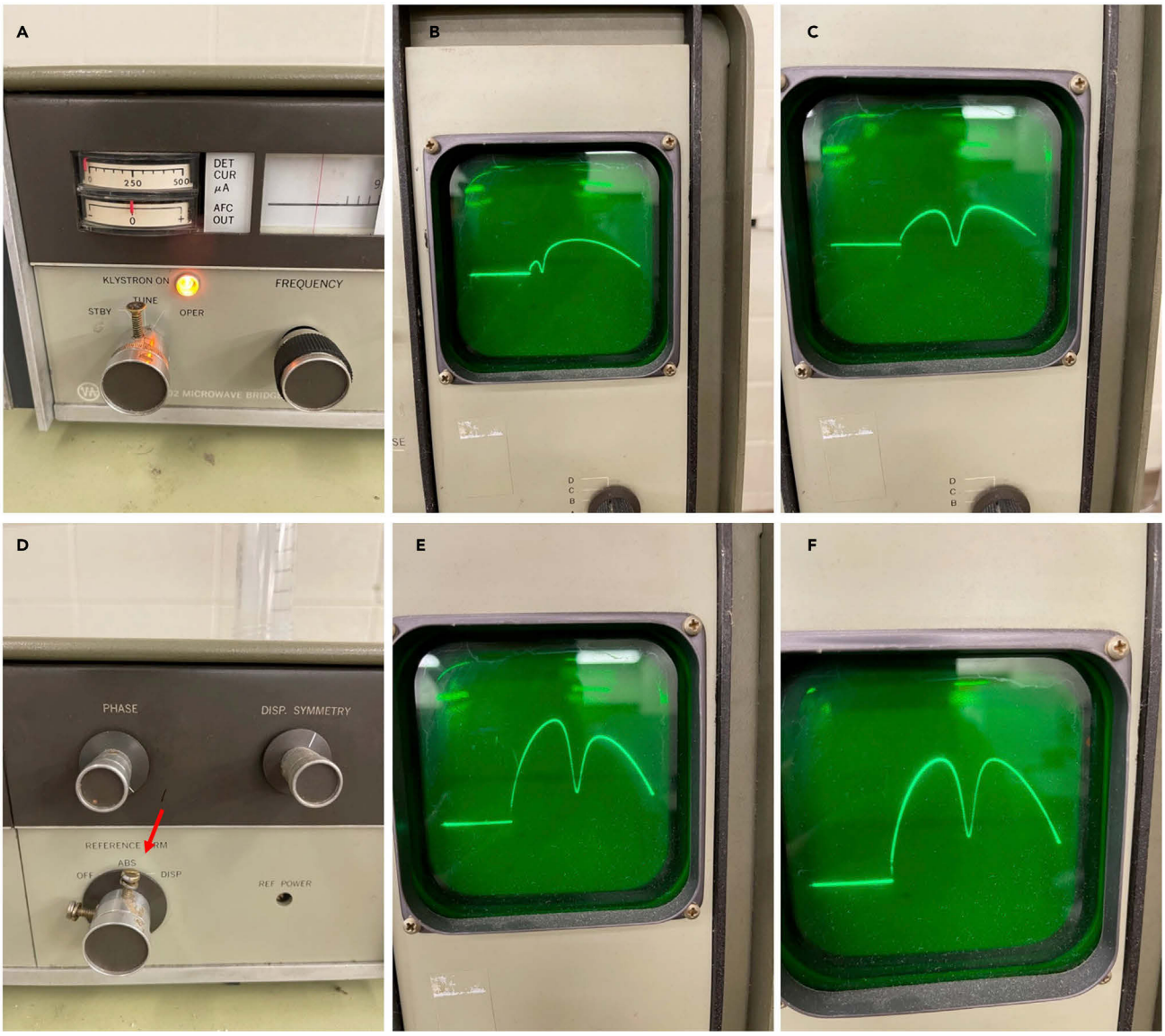

Figure 2. Tuning up the cavity

(A) Switch the bridge from the "STBY" (Stand by) mode to the "TUNE" mode (also see Figure 3).

(B and C) The typical tuning dip before and after loading a sample capillary.

(D) Switch Reference Arm from "OFF" to "ABS" (indicated by the arrow).

(E) The phase of the tuning dip under ABS Reference Arm is asymmetric.

(F) Adjust PHASE (see D) to make the tuning dip symmetric.

Note: It is always a good practice to give sufficient time (5–10 min in our case) for the electronics and cooling water to settle down/equilibrate.

11. Under the "Tune" mode (Figure 2A) and a low power (ca. 30 dB), insert the sample tube to the cavity. An indication of sample at the effective magnetic field region of the cavity is a "shift" in the tuning dip (Figure 2B *vs* 2C).

⚠ CRITICAL: No change in the tuning dip position usually indicates the wrong sample position. This can be corrected by optimizing the sample height and capillary position. It may

Figure 3. Optimize sample position and power

(A) Switch to "OPER" (operate) mode.

(B) Adjust FREQUENCY (the knob right next to the "OPER") to center "AFC OUT".

(C) The "DET CUR" (detection current) is usually not centered once the Reference Arm is switched on.

(D) Adjust sample position by the Iris to center the "DET CUR".

also be caused by a high water volume, which can be corrected by using a thinner capillary or increasing the porous materials content.

12. Switch the Reference Arm to ABS (Figure 2D), the tuning dip should be asymmetric (Figure 2E). Adjust the sample position by adjusting the Iris to make the tuning dip symmetric (Figure 2F). Turn off Reference Arm.
13. Switch to the "Operate" mode (Figure 3A). Adjust the main frequency to ensure the AFC OUT is centered (Figure 3B). Tune the Reference Arm to ABS. The Detection Current is usually asymmetric (Figure 3C). Adjust the Iris to center the DET CUR (Figure 3D).

Figure 4. Key EPR parameters setup

(A) Set up the scan range by setting the center field (upper panel) and the scan range (lower panel).

(B) The "GAIN" (video gain; upper) and "MODULATION" (modulation amplitude; lower) need to be pre-set (and can be further optimized).

⚠ CRITICAL: Failing to center both AFC and DET CUR usually indicates a high water volume, which should be corrected by reducing the sample volume (but not changing the sample position).

14. Apply microwave power to the sample/cavity.
    a. Slowly increase the power to 12 dB (in a min or two) while adjusting the frequency to maintain the AFC OUT centered and the Iris to maintain the DET CUR centered.

⚠ CRITICAL: The operation should be processed slowly.

15. Set up the EPR parameters.
    a. Set up the initial center magnetic field. For 9.6 GHz, usually ~3390 G is a good starting point (Figure 4A).

Note: The center field will be optimized in latter steps.

    b. Set the scan range to 100 G (Figure 4A), which is often sufficient for HO-225 spin label and protein dynamics measurement at room temperature.

Note: A wider scan range may be needed if dipolar broadening or exchange broadening occurs (typically 160 G or 200 G will be needed in these cases).

    c. The modulation frequency is 100 KHz. Video Gain starts at 160 and can be optimized based on the strength of the signal. Modulation depth of 1 G is usually good for nitroxide spin labels on protein studies at room temperature, which can be adjusted latter if needed (Figure 4B).

16. Run a test scan and optimize the EPR parameters.
    a. Adjust the center field and Video Gain based on the test run (Figure 5).
    b. Decrease the modulation depth to 0.5 G to ensure that the linewidth of each peak is not narrowed. Otherwise, keep reducing the modulation depth until the linewidth stops narrowing.

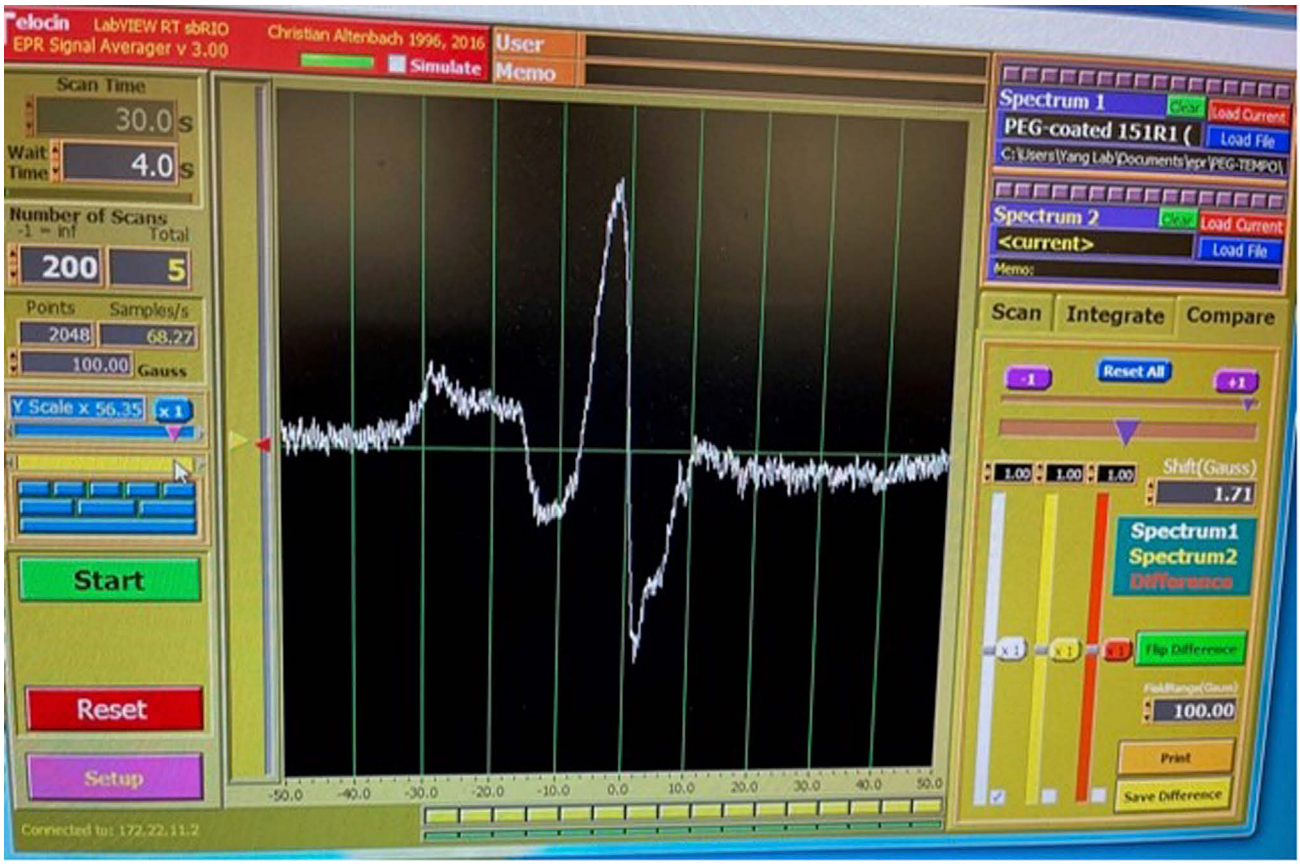

Figure 5. Example CW EPR spectrum
The user interface is generously provided by Dr. Altenbach and Prof. Hubbell at UCLA.

⚠ CRITICAL: Reducing the modulation depth would reduce the signal intensity. Therefore, the maximal modulation depth that does not broaden the spectrum should be used.

17. Acquire a CW EPR spectrum for each mutant via signal averaging until a satisfied signal-to-noise ratio (usually >30) is reached (usually 5–30 min are needed depending on sample concentration).

Troubleshooting 4

Optional: 12 dB power is ~200 mW, which is reasonable for R1 spin label and protein studies. However, lower power can be tested to ensure no spectral broadening.

Pause point: All spectra should be properly stored electronically with all parameters accurately documented for further use.

EPR spectral simulation

Timing: 30–60 min per spectrum

This major step is to extract dynamic information of the protein labeled site from the acquired CW EPR spectra (key scheme see Pan et al., 2021a). The motion of a spin label attached to an α helix (Figure 6A) has been extensively studied with sufficient knowledge obtained, which sets up the basis of the spectral simulation. For example, the motion of the R1 spin label can be considered as

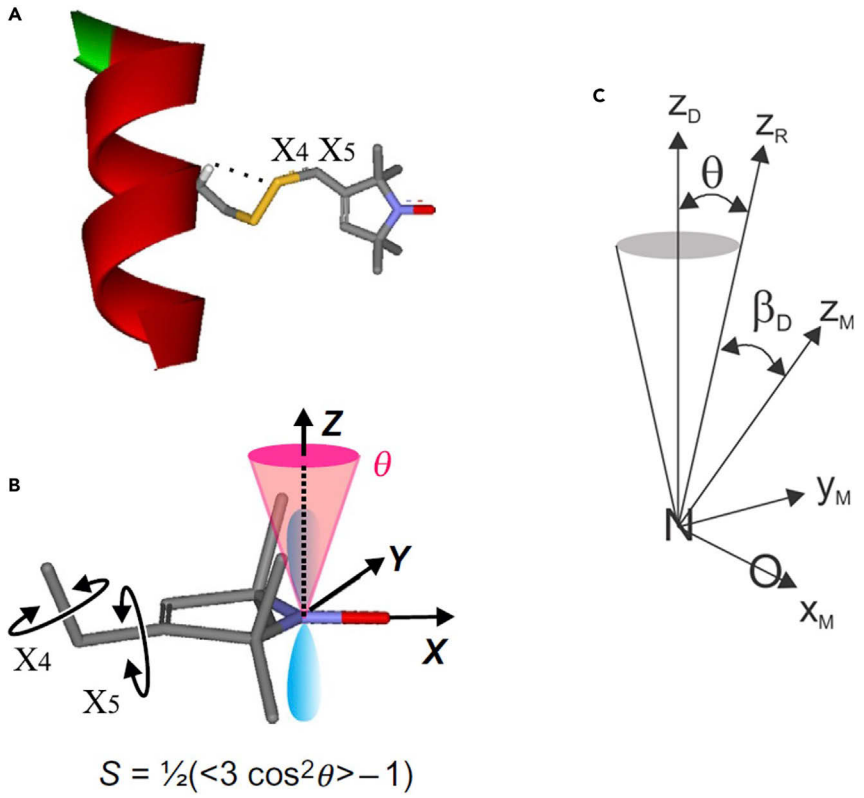

Figure 6. Crystal structure and mode of motion of the R1 spin label

(A) The 4th and 5th dihedral angles are critical to the motion of the sidechain.

(B) The coordination systems and key angles in the MOMD simulation model.

(C) Definition of the three coordinate systems related to the MOMD model. Figure adapted with permission (Altenbach et al., 2015)

"anisotropic" (Figure 6B); the typical ranges of the rate (τ) and order (S) and how these parameters alter upon protein rotational tumbling changes are also documented in the literature (Altenbach et al., 2015; Columbus et al., 2001; Gaponenko et al., 2000).

⚠ CRITICAL: For an unknown protein labeled at a non-helical region (ca. a loop region), X-band CW EPR together with multi-frequency EPR and varied viscosity conditions are suggested to accurately determine the rate and order parameters of the spin label, minimizing the potential errors (Pan et al., 2021a).

18. Pre-data processing to prepare for spectral simulation.
    a. Baseline correction. Usually for our spectra, a 2nd–3rd order polynomial baseline is sufficient. A baseline correction program by Dr. Christian Altenbach and Prof. Wayne Hubbell at UCLA written in LabView is employed in our baseline correction.

Optional: Any program that can correct spectral baseline can be applicable.

⚠ CRITICAL: A correct baseline is critical for the simulation as bumpy baseline can affect the fitting parameters.

    b. Normalize the spectra. Because a typical CW EPR spectrum is in the first-derivative format, normalizing the double-integration of the baseline-corrected spectra to 1 (or 100%) is the general operation, in order to facilitate the spectral simulation (which assumes the total spin density is 1 and provides the relative population of each component).

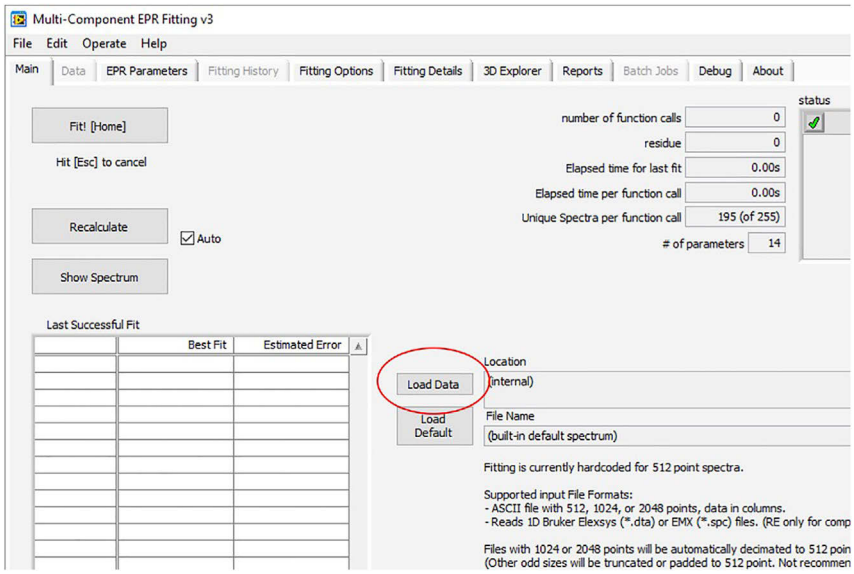

Figure 7. The user interface of Multi-component when loading an experimental data set

A window dialog will pop-up so that the experimental data can be selected from a file location (click the "Load Data" as highlighted by the red circle).

Optional: For most spectral simulation software, a normalized spectrum is ideal for obtaining the correct relative population in a multi-component spectrum as well as the precise dynamics parameters.

c. Center each spectrum. The center field of a spectrum is reset so that the integrated areas on both sides of the center field are equal.

19. Load a pre-processed spectrum into the "Multi-component" program (Figure 7) available at https://sites.google.com/site/altenbach/labview-programs/epr-programs?authuser=0.

Optional: EasySpin (Stoll) and the MOMD program (Freed; Figure 6C) can also be used for simulation since these programs share the same principle (Stoll and Schweiger, 2006; Zhang et al., 2010; Pan et al., 2021a).

20. Set up the initial fitting parameters. Usually, we encounter a relative mobile and an immobile spectral component for enzymes encapsulated in mesoporous materials. Therefore, at least two components are needed to fit the experimental data. In rare cases, 3–4 components are also possible.

a. Activate two components (out of the four components available in the program; Figure 8) by switching the "Component" under A and B to "Enabled" (while keeping components C and D "Disabled"). Usually we define component A as the spectrum contributed by enzyme in contact with (or immobilized on) the MOF/COF, while component B as the non-contact (or mobile) one.

Optional: In rare cases such as more than two "mobile" motions of the protein labeled site, three components can be used.

⚠ CRITICAL: Each tensor under columns A and B are defined by "set 0" to "set 3". "set 0" indicates the first set of a tensor in the "Tensors" Tab. "set 1" indicates the second set. "set 1" does not necessarily stand for component B. For example, for the W tensor (line-width tensor), both components A and B share "set 0", the same W tensor.

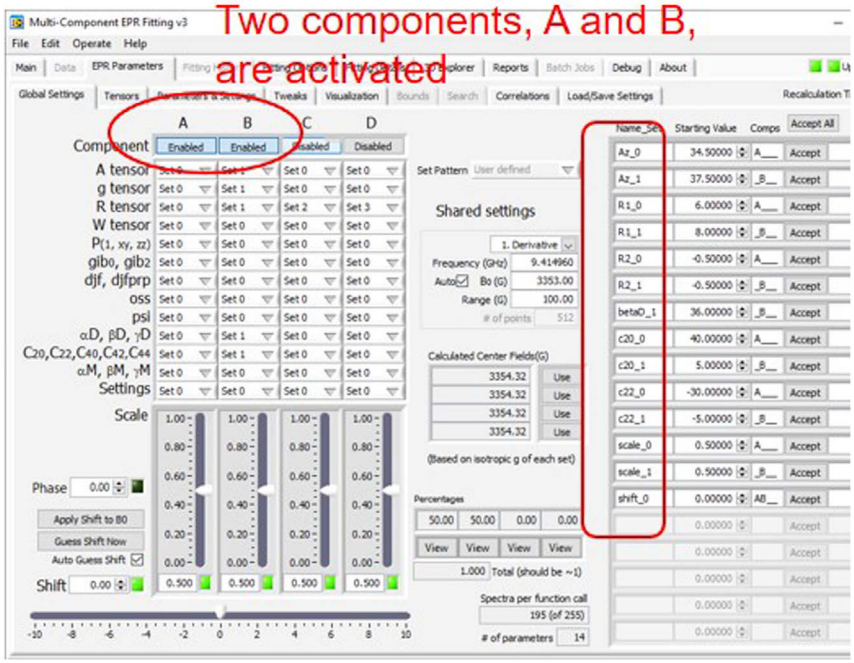

Figure 8. Activating two components while keeping the rest "disabled"

All parameters allowed to vary are highlighted by the red rectangle on the right.

b. Switch to the "Tensors" tab. Input the initial "guesses" of the g- and hyperfine-tensors: g_{xx}=2.0078, g_{yy}=2.0058, g_{zz}=2.0023 for both "im" and "m" components; A_{xx}= 5.7 G, A_{yy}=6.0 G, for both "im" and "m" components ("im"="immobilized" and "m"="mobilized"); A_{zz}=34.5 and 37.5 G, typical for hydrophobic and hydrophilic local environment, for the "im" and "m" components, respectively (Figure 9). These initial values are obtained from extensive simulations of many spectra and are usually good "guesses" to initiate the simulation. These variables will be optimized during the simulation. Allow A_{zz} to be varied during the simulation by activating the green light after each A_{zz}.

c. In the rate "R Tensor" (due to anisotropy, see above), the R values instead of the actual effective correlation time, τ_i, of R1 diffusion are the input, wherein $1/6R = \tau_i$. The R tensor is defined in Figure 6B where the average constant (third column of the R Tensor with "1,2,3" labeled), R1 is (Rx + Ry + Rz)/3, R2 is Rz – (Rx – Ry)/2, and R3 is Rx – Ry. For our case, where an axial symmetry is assumed, R1 and R2 are non-zero. The average rate constants, R1, of 6.00 and 8.00 are often input for the "im" and "m" components, respectively, with −0.5 to account for the anisotropic motion of both components, R2 (see Figure 6B). Allow R0 and R1 to vary during the simulation by activating the green light after each tensor component.

d. Next, key ordering parameters need to be put in (Figure 10). A restoring (ordering) potential (U) is used to calculate the spatial constraints of the spin label within the "cone". The potential $U(\theta) = -1/2kBTc_0^2 (3\cos^2\theta - 1) + H.O.T.$, where c_0^2 is a scaling coefficient and H.O.T. represents higher order terms. The spatial ordering of the diffusion tensor, S, can be computed following Figure 6B. In our simulations, only the dominant term and the first H.O.T. term were involved, leading to the order parameters, S_{20} and S_{22}, in our simulation, which can be computed from the C_{20} and C_{22} coefficients (Figure 10). For the "im" component, due to the strong contact of the label with some species (ca. surface of mesopores), typical initial C_{20} and C_{22} are ∼40 and ∼−40, respectively. For the "m" component, we find that 5 and −5 respectively are usually good initial guesses. Allow these C parameters to vary during the simulation by activating the green light after each.

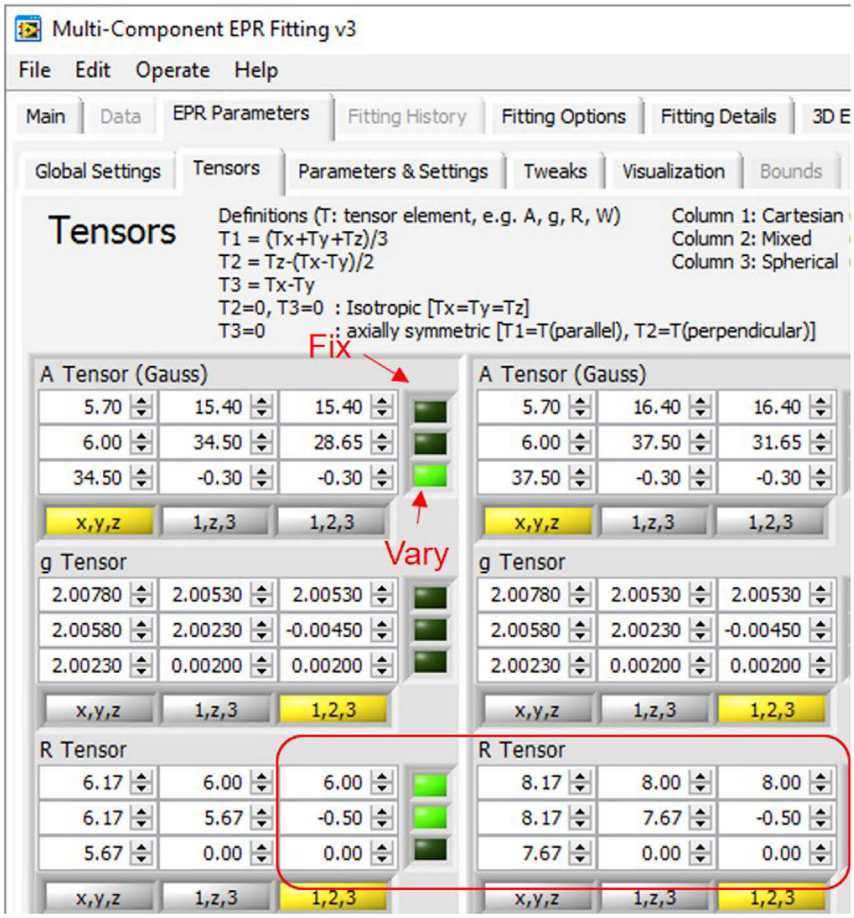

Figure 9. Setting up the g and A tensors

An activated green light after a variable means this variable is allowed to be varied during the fitting (see red arrow labeled by "Vary"). Inactivated green light (see red arrow labeled by "Fix") means the variable cannot be varied during the fitting.

e. The tilt of the diffusion tensor with respect to the molecular axis of the nitroxide is specified by the Euler angles (α_D, β_D, γ_D). For axially symmetric motion, only β_D and γ_D need be specified. For z-axis anisotropic motion, the diffusion tilt was at $\beta_D = 36°$, $\gamma_D = 0°$. β_D is allowed to be altered during the simulation. If $\beta_D = 36°$ does not provide a reasonable fit, switch β_D to 90° and initiate the fitting again.

21. Fit.
    a. Fitting emphasis. Usually the low-field region of the spectrum is the most informative area; thus, the central peak is weighted by 50% (Figure 11).
    b. The Levenberg-Marquardt fit is carried out to quickly determine the parameters that best fit the spectrum (finding a minimal χ^2; Figure 12).
    c. The Monte-Carlo fitting option in order to avoid falling into the local minima (Figure 13).
    d. Other parameters such as the linewidth tensor W are allowed to change to fine-tune the fitting. The χ^2 is reduced over time as shown in the upper left panel while the variables in real time during fitting is shown in the lower left panel of Figure 14. The raw data (white), simulated spectrum (green), and the difference between the two (red) are shown in the right panel of Figure 14.

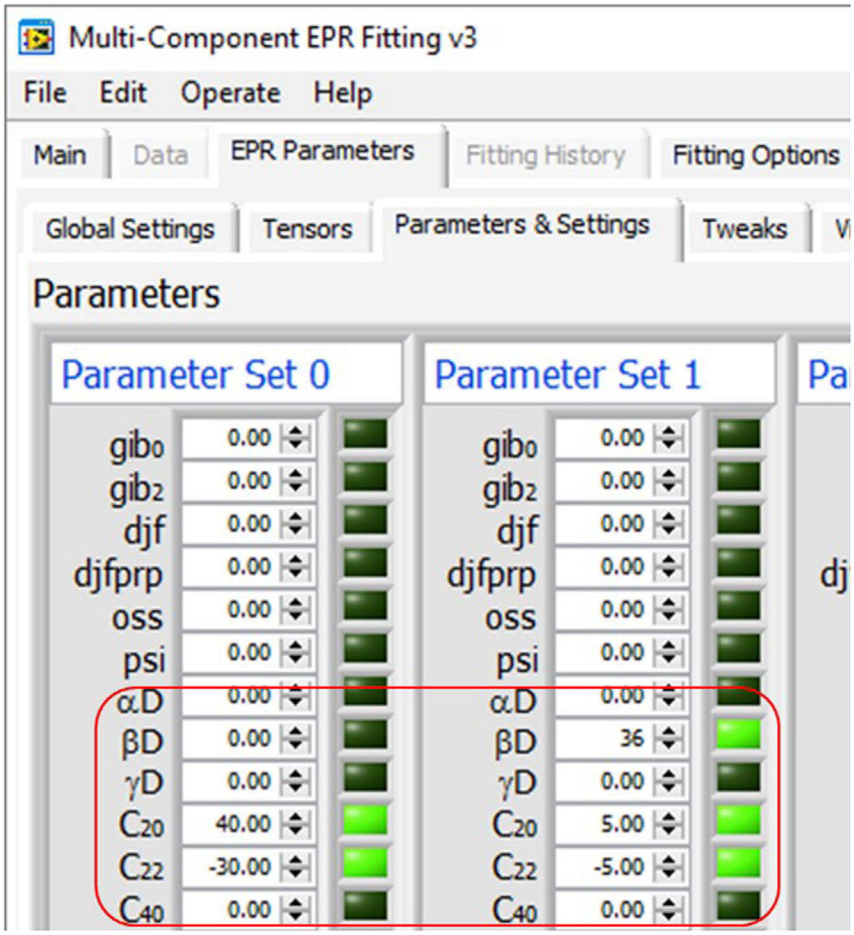

Figure 10. Setting up the parameters of spatial ordering and key Euler angels

Troubleshooting 5

Data interpretation: determine enzyme orientation

Timing: varies

This major step is to summarize the general concept of how to determine enzyme relative orientation in a porous material based on EPR simulation. The principle is applicable to enzymes adsorbed on other surfaces.

22. Determine the regions of an enzyme that contact a porous material.
    a. In most mesopores, enzymes tend to display a relatively random orientation with some preference (instead of a uniform orientation). The relative population of the contact or immobile spectral component with respect to the mobile one represents the chance for the labeled site to contact a solid surface.
    b. Scanning multiple enzyme surface sites can, therefore, lead to the regions of the enzyme that are favored to contact the solids.

Data interpretation: Determine enzyme dynamics

Timing: varies

Figure 11. Setting up the weighting of fitting

This major step is to extract additional dynamic information, in addition to the relative population, in order to provide deeper insights into enzyme behavior on MOFs/COFs.

23. Ordering and rate parameters of the contact component.
    a. The ordering parameters, C_{20} and C_{22}, of the immobile component indicate the relative spatial restriction of the spin label sidechain at the contact site. The smaller the ordering parameters, the less restricted the contact. This is an indication of the relative packing density of the MOF.

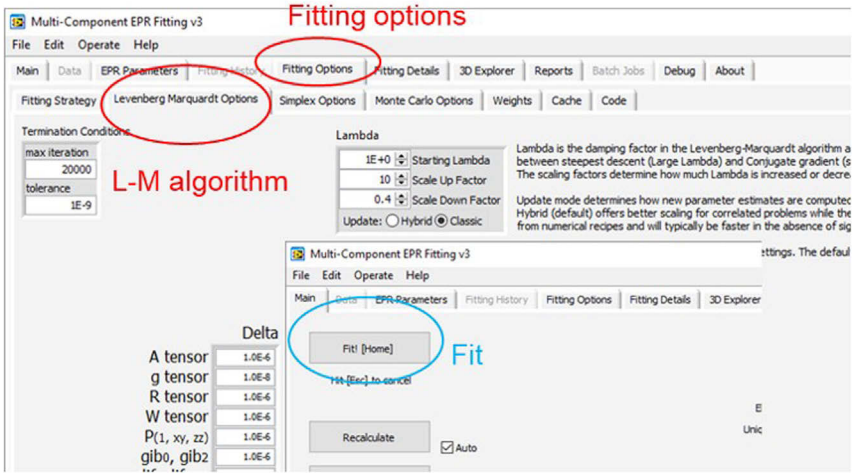

Figure 12. Selecting the Levenberg Marquardt fitting option and click "fit" in the main tab

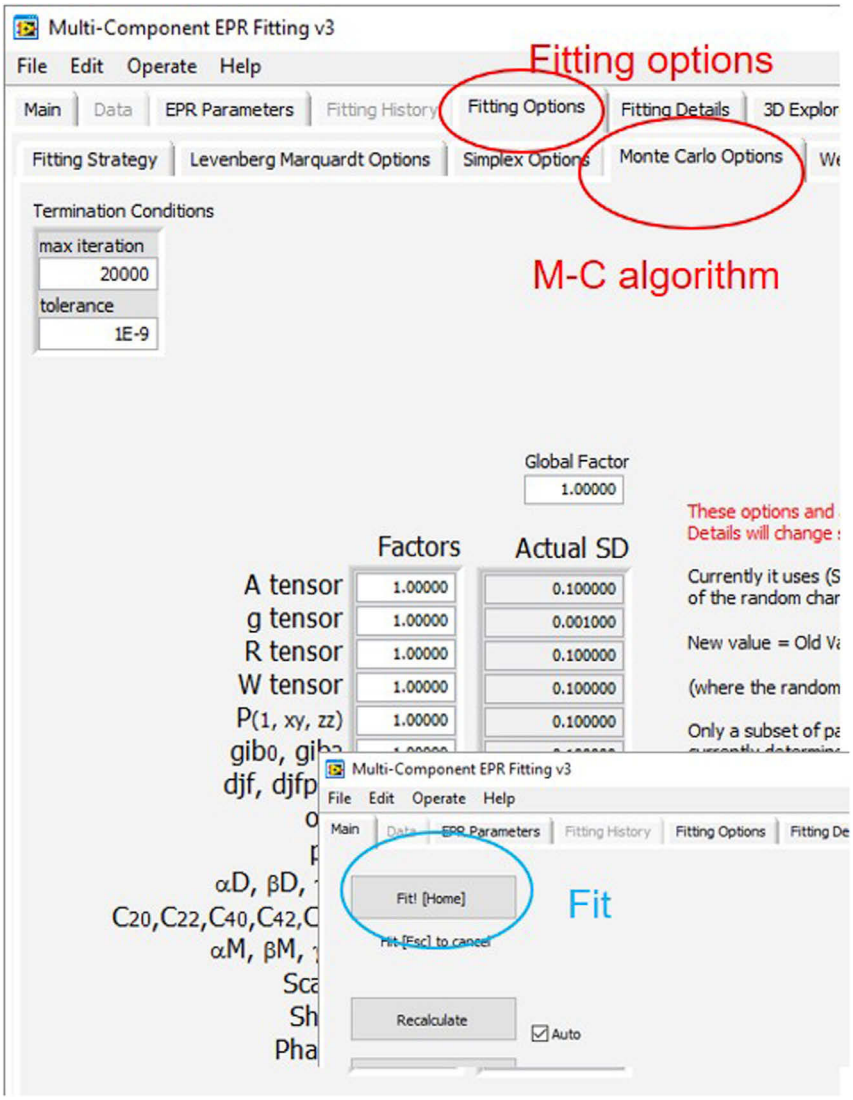

Figure 13. Selecting the Monte Carlo fitting option and click "fit" in the main tab

b. The rate parameter reflects the dynamics of the label at the contact site. The more dynamic the enzyme, and therefore, the more active likely for the enzyme.

24. Possible spin exchange interactions.
    a. Spin exchange interaction can occur due to the conjugated structure of the building blocks of the MOFs and COFs. A line broadening with reduced spectral intensity at the low field peak and enhanced/symmetric center peak is a clear indication of spin exchange interaction between the labeled enzyme and the MOF/COF materials.
    b. In the presence of spin exchange, the regular Multi-component spectral simulation cannot provide reasonable fits. Instead, a few spectral features can be used to quantify the exchange interaction.
    c. Example data with strong spin exchange are shown in Figure 15A middle and right panel.(-Sun et al., 2019) The relative peak intensity of the low-field peak versus the central line (I_0/(I_{cp}+I_{cn})) as well as the positive and negative peaks of the central peak (I_{cp}/I_{cn}; Figure 15B) can quantitatively assess the relative strength of the spin exchange interaction. The smaller the I_0/(I_{cp}+I_{cn}), the stronger the exchange; the closer the I_{cp}/I_{cn} to 1, the more symmetric the central peak, and the stronger the spin exchange.

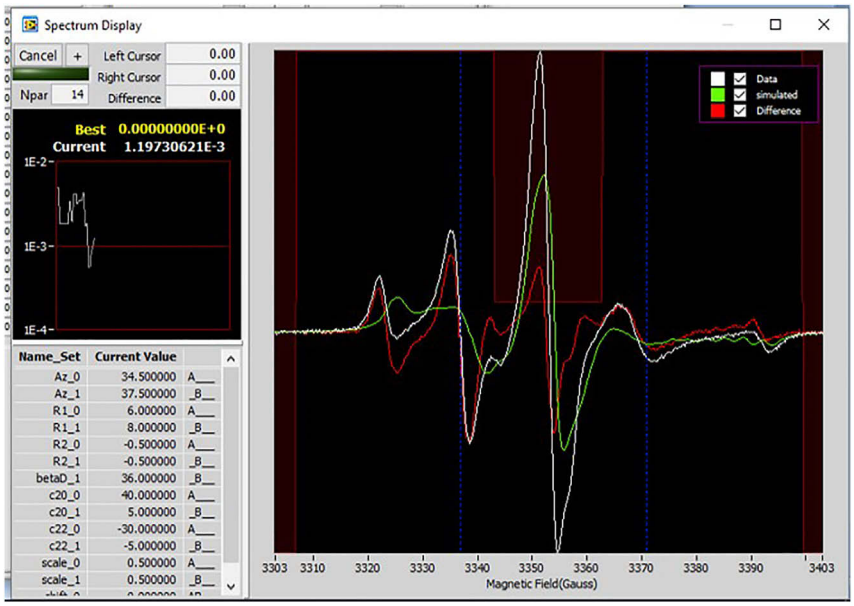

Figure 14. Typical interface of fitting

EXPECTED OUTCOMES

Typical outcome of the simulation is a table of the key parameters defined above. An example simulation result is shown in Table 1.(Pan et al., 2021b)

The relative population/probability of the mobile component (m%) of each labeled site on the target enzyme is determined. Based on the population, the orientation of enzyme in relative to the MOF/COF surface can be determined. The relative mobility in terms of the spatial restriction and rate of motion of each labeled site (see R and C parameters) are also determined. The combined orientation and dynamics information leads to the rationalization of the catalytic activity of the encapsulated enzyme in MOFs/COFs.

LIMITATIONS

It has to be noted that the protocol above cannot determine global structural changes of an enzyme upon encapsulation in MOF/COF, which is another important structural factor that determined the enzymatic performance. Other EPR techniques are needed (Pan et al., 2021a; Jeschke, 2012; Borbat and Freed, 2014; Yang et al., 2014)

The protein labeling procedures are for enzymes that can be expressed in E Coli. Other enzymes that require other cell lines can be labeled similarly.

Only R1 spin label has been extensively used for protein dynamics probing. If a different label is needed, the motion of the label sidechain needs to be determined using different sets of fitting parameters. In principle, determining the parameters of the spin label's motion would require CW EPR at multiple frequencies and/or under varied viscosity conditions, especially for labels different from R1 and/or on non-helical structures, to minimize the potential errors in the determined rate and order parameters of the spin label.

TROUBLESHOOTING

Problem 1

It is possible that for some protein mutants, the yields are not ideal (typically ~μg of protein is needed). This could be reflected by a relatively weak band as compared to the wildtype protein in the gel.

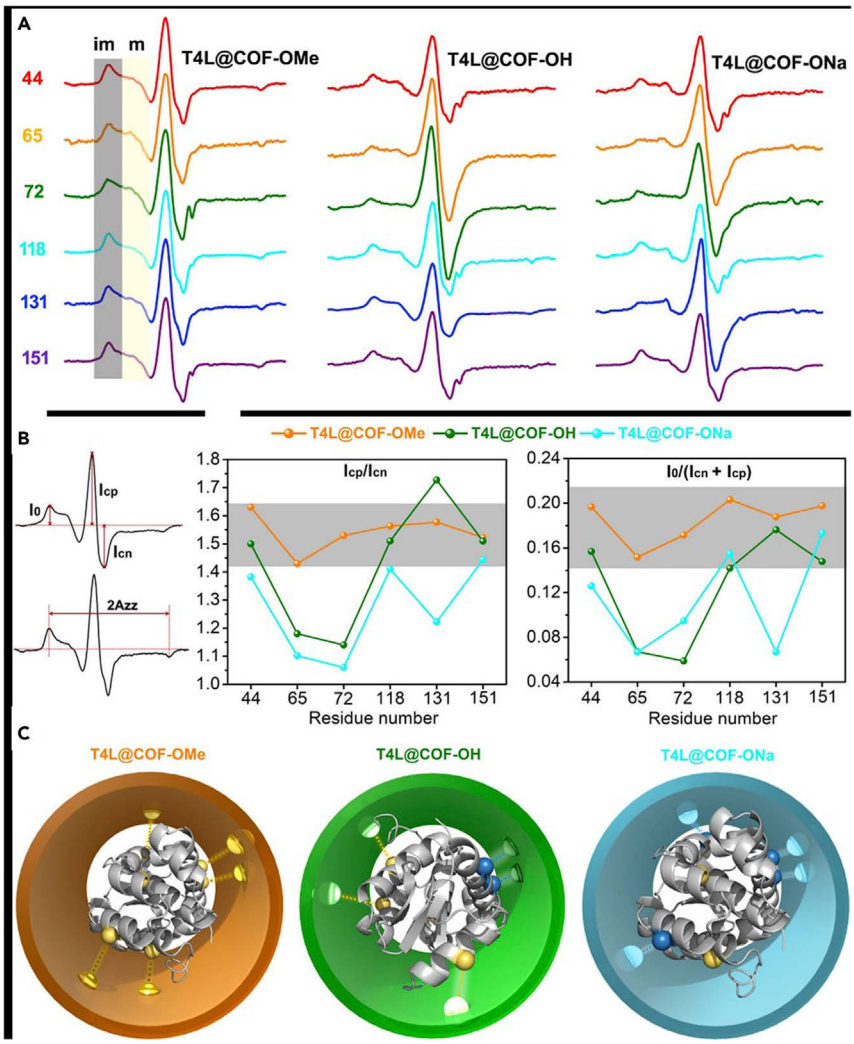

Figure 15. Example spin exchange interactions

(A) Typical EPR data for an enzyme entrapped in COFs. Strong spin exchange effects are observed in 65 and 72 of the middle panel and 44, 65, 72, and 131 of the right panel.

(B) Definitions of variables to quantitatively described the strength of the spin exchange interaction (left). The key variables of spin exchange for each residue is plotted in the middle and right panel. Figure adapted from reference with permission (Sun et al., 2019)

Potential solution

We suggested selecting a nearby residue that can also represent the corresponding secondary structure/protein surface to make the mutation.

Problem 2

For cysteine-rich proteins/enzymes, it is unrealistic to mutate all native cysteines to alanines or serines, which would seriously disturb protein structure and function.

Potential solution

The unnatural amino acids can be introduced to the plasmid/DNA of the protein, followed by spin labeling as described in a recent work. Detailed procedures to introduce unnatural amino acids into proteins are available in the literature and at several resources (Xiao and Schultz, 2016, Kim et al., 2013, Fleissner et al., 2009).

Table 1. Example outcome of EPR simulation for multiple labeled sites in the absence and presence of urea ([a]: 44R1_u indicates urea perturbation)

	$R_{z,\ im}$ [b]	$R_{z,m}$ [b]	$R_{2,im}$	$R_{2,m}$	$C_{20,im}$	$C_{22,im}$	$C_{20,m}$	$C_{22,m}$	m%	χ^2
44R1	6.13	7.17	0.87	−2.06	39.3	−39.7	8.63	−10.0	52.1	$1.83e^{-5}$
44R1_u[a]	6.23	8.10	1.21	−1.79	41.7	−45.0	7.88	−9.45	49.7	$2.57e^{-5}$
65R1	6.28	7.00	1.17	−2.00	42.1	−40.8	9.63	−11.6	29.2	$1.67e^{-5}$
65R1_u	6.07	8.02	0.93	−1.77	45.2	−45.5	8.22	−9.95	31.9	$2.75e^{-5}$
72R1	6.05	6.73	0.96	−3.16	45.6	−44.2	8.81	−10.4	35.4	$1.56e^{-5}$
72R1_u	6.13	8.03	0.87	−0.91	49.1	−45.5	9.93	−13.9	33.4	$4.00e^{-5}$
89R1	6.13	7.11	1.37	−1.6	45.0	−43.2	8.81	−10.8	41.2	$2.12e^{-5}$
89R1_u	6.17	8.01	0.74	−0.76	49.5	−44.5	8.72	−12.1	38.6	$1.57e^{-5}$
109R1	6.13	7.11	1.26	−1.80	46.4	−42.5	8.61	−10.5	36.9	$1.79e^{-5}$
109R1_u	6.10	8.05	1.33	−1.66	50.8	−45.5	9.67	−11.7	30.4	$1.06e^{-5}$
118R1	6.07	7.07	0.65	−2.15	44.6	−42.9	8.00	−9.84	33.5	$1.78e^{-5}$
118R1_u	6.09	8.03	1.16	−1.92	51.4	−44.8	8.67	−10.8	29.4	$2.06e^{-5}$
131R1	6.07	7.03	1.03	-1.89	45.4	−43.6	9.76	−12.1	49.6	$2.75e^{-5}$
131R1_u	6.00	8.07	0.91	−1.64	53.2	−48.7	11.2	−14.4	49.2	$3.60e^{-5}$
151R1	6.05	6.97	1.04	−2.43	45.7	−43.7	9.61	−11.3	47.5	$1.01e^{-5}$
151R1_u	5.98	8.05	1.15	−2.67	54.8	−50.2	9.05	−10.9	48.5	$2.30e^{-5}$

Data adapted from reference with permission (Pan et al., 2021b).

Problem 3

Some MOFs/COFs may be unstable during the activity test procedures so that the disassembled MOFs/COFs building blocks may interfere the activity assay.

Potential solution

We suggested selecting an alternative activity assay which does not disturb the scaffold of MOFs/COFs. For example, in our case, we can use the fluorescence-based lysozyme activity detection approach as detailed in the literature (Kao et al., 2014).

Problem 4

It is possible that for some MOFs/COFs the loading capacity of an enzyme is low, which affect the EPR signal intensity.

Potential solution

We suggested increasing the amount of MOFs/COFs during loading as well as the protein concentration. It is also possible to load more than 20 μL of samples by using a larger EPR tube. The total water volume may still be sufficiently low, given the fact that most volume is occupied by the MOFs/COFs.

Problem 5

It is possible that several combinations of fitting parameters provide the same quality of fit.

Potential solution

We suggested looking at the Correlation Table of the fitting program. A correlation factor of close to 1.0 indicates the close correlation between the two parameters. We usually fix the rate and order parameters among different mutations and vary the population.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Zhongyu Yang (zhongyu.yang@ndsu.edu).

Materials availability

The study did not generate new unique reagents.

Data and code availability

All data and code generated in this study are available from the lead contact without restriction.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Z.Y. and X.Z. designed the protocol. Y.P., H.L., Q.L., I.S., D.J., and M.L. assisted in writing, figure preparation, and literature search. H.L. and B.C. assisted in manuscript writing. Z.Y. and X.Z. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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