Tight and specific lanthanide binding in a de novo TIM barrel with a large internal cavity designed by symmetric domain fusion

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De novo protein design has succeeded in generating a large variety of globular proteins, but the construction of protein scaffolds with cavities that could accommodate large signaling molecules, cofactors, and substrates remains an outstanding challenge. The long, often flexible loops that form such cavities in many natural proteins are difficult to precisely program and thus challenging for computational protein design. Here we describe an alternative approach to this problem. We fused two stable scaffolds with C2 symmetry—a de novo designed dimeric ferredoxin fold and a de novo designed TIM barrel—such that their symmetry axes are aligned to create scaffolds with large cavities that can serve as binding pockets or enzymatic reaction chambers. The crystal structures of two such designs confirm the presence of a 420 cubic Ångström chamber defined by the top of the designed TIM barrel and the bottom of the ferredoxin dimer. We functionalized the scaffold by installing a metal-binding site consisting of four glutamate residues close to the symmetry axis. The protein binds lanthanide ions with very high affinity as demonstrated by the metalloprotein assemblies that enclose large internal chambers. The high stability of de novo scaffolds enables ready functionalization of these chambers; for instance, with specific metal-binding sites, as demonstrated here by generating a lanthanide-binding protein with ultra-high affinity.

Significance

Despite considerable advances in de novo protein design in recent years, it still remains challenging to engineer proteins with large internal cavities that can be functionalized to become biotechnological tools, such as specific binders, sensors, or catalysts. In this work, we outline a computational strategy to combine multiple de novo designed domains into symmetric protein assemblies that enclose large internal chambers. The high stability of de novo scaffolds enables ready functionalization of these chambers; for instance, with specific metal-binding sites, as demonstrated here by generating a lanthanide-binding protein with ultra-high affinity.

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enhanced through energy transfer from a close-by antenna molecule, an approach that is exploited in so-called lanthanide-binding tags (20, 21). De novo designed peptides were shown previously to form lanthanide-binding coiled coils with potential applications as paramagnetic MRI contrast agents (22, 23). Their strong Lewis acidity also makes lanthanides powerful catalysts for various synthetically useful reactions (24), although their biological relevance was only recently discovered, when lanthanide-dependent methanol dehydrogenases were isolated from certain methylotrophic and methanotrophic bacteria (25, 26). Here, we introduce a de novo lanthanide-binding protein with ultra-high affinity, which provides a starting point for the future engineering of lanthanide-dependent biotechnological tools and artificial enzymes.

**Results**

**DeNovoTIM15.** We combined stabilizing sequence changes (18) with circular permutation and substitution of residues at the top of the TIM barrel to produce stable and modular starting TIM barrel scaffolds (Fig. 1A). These proteins, dubbed DeNovoTIMs, were expressed at high levels in *Escherichia coli*. They are stable beyond 95 °C as assessed by circular dichroism (CD) and have a guanidinium chloride unfolding midpoint of ∼2M (Fig. 1B). One of the variants, DeNovoTIM15, was crystallized and the structure solved using the Rosetta model as input for molecular replacement at a resolution of 2.3 Å (Table 1). The design model and crystal structure were very similar, with an all-atom Ca root-mean-square deviation (rmsd) of 1.65 Å (Fig. 1C). Exploiting the symmetry of the de novo TIM system, we were able to express a half fragment of DeNovoTIM15, containing only the first two repeat units of the protein. This protein was expressed in *E. coli* and purified. It was shown to be a homodimer by size exclusion chromatography (SEC) (SI Appendix, Fig. S1), consistent with a C2-symmetric homodimeric assembly. Relocating the termini from the top to the bottom of the barrel by circular permutation provided positions at the top of the TIM barrel where loops could be substituted with inserted domains. We inserted domains in one of the loops that is structurally equivalent to the prepermutation termini: the β-α loop connecting β3 and α3.

**Symmetric Fusion Design.** To create large, engineerable reaction chambers at the top of the TIM barrel, we used a computational approach that takes advantage of the C2 symmetry of many de novo designed homodimers. C2-symmetric de novo designed homodimer proteins were fused to the top of the half-DeNovoTIM15 using a method that preserves symmetry of the resulting assembly. Two different symmetric homodimers were considered for the top of the reaction chamber: a de novo designed ferredoxin (FD) fold (27) and cFr (28), derived from Top7 (29), a de novo designed protein with a fold not previously seen in nature. Both of these dimers have continuous β-sheets extending across the dimer interface. The domain insertion function of RosettaRemodel (30) was used to connect each “lid” monomer to a monomer of the...
homodimeric TIM barrel. Linker loops between zero and six residues in length were generated to connect the domains (Fig. 2A), and C2 symmetry as well as distance constraints were imposed during design to arrive at fusion proteins in which both the TIM barrel dimer interface and lid dimer interface were intact. We discarded all models for which complete chain closure was unsuccessful. Limited sequence changes were made at the interface between domains to avoid generating structures with stability dependent on these interactions. Changes to the insert (four per monomer) and TIM (two per monomer) domains were solely made to reduce the charge lining the chamber between domains. However, all scaffolds also remain fully folded in substantial amounts of cosolvent, such as 50% acetonitrile (Fig. S3).

**Characterization of TFD Designs.** The most successful fusion contained a C2-symmetric FD atop DeNovoTIM15, with three-residue glycine linkers closing both junctions. This protein, dubbed TIM-FD dimer (TFD) is remarkably stable, soluble, and robust. Two variants of this scaffold were characterized. The first, TFD-HE, contains a histidine in position 31 and a glutamate substitution in position 154 of the full-length construct. In each case, there were four altered residues, corresponding to the C4 symmetry of the TIM barrel.

**Table 1. Data collection and refinement statistics**

| PDB ID     | DeNovoTIM15 | TFD-HE   | TFD-EE   | TFD-EE N6W + Tb(III)† |
|------------|-------------|----------|----------|-----------------------|
| Wavelength | 1.00        | 1.00     | 1.00     | 1.00                  |
| Resolution range | 37.44 to 2.20 (2.28 to 2.20) | 32.95 to 1.41 (1.46 to 1.41) | 46.44 to 2.5 (2.59 to 2.5) | 30.00 to 1.85 (1.95 to 1.85) |
| Space group | P 32       | P 21     | P 21     | P 21                  |
| Unit cell (Å) | 90.90 120 | 90.95 27.90 | 90.95 50 | 90.101.6 90 |
| Total reflections | 93,205 (7,829) | 202,977 (19,737) | 109,529 (10,001) | 172,450 (24,339) |
| Unique reflections | 10,220 (946) | 55,043 (5,447) | 34,814 (3,455) | 56,427 (8,189) |
| Multiplicity | 9.1 (8.2) | 3.7 (3.6) | 3.1 (2.9) | 3.1 (3.0) |
| Completeness (%) | 99.54 (97.44) | 97.03 (95.08) | 97.88 (96.05) | 98.4 (97.9) |
| CC1/2 | 0.988 (0.558) | 0.995 (0.220) | 0.999 (0.776) | 0.998 (0.836) |
| Mean I/sigma(I) | 8.32 (2.31) | 7.39 (0.77) | 14.17 (2.10) | 13.12 (1.94) |
| Wilson B-factor | 50.02 | 16.91 | 56.35 | 31.37 |
| R-merge | 0.204 (2.76) | 0.104 (1.79) | 0.0524 (0.505) | 0.034 (0.339) |
| Refinements used for R-free | 10,220 | 54,890 | 34,799 | 29,150 |
| R-work | 0.200 | 0.182 | 0.227 | 0.195 |
| R-fre | 0.216 | 0.212 | 0.281 | 0.229 |
| Number of nonhydrogen atoms | 1,310 | 2,847 | 7,319 | 2,649 |
| Macromolecules | 1,291 | 2,658 | 7,208 | 2,600 |
| Solvent | 19 | 21 | 51 | 5 |
| Protein residues | 182 | 168 | 60 | 44 |
| RMSD (bonds) | 0.004 | 0.012 | 0.002 | 0.003 |
| RMSD (angles) | 0.94 | 1.10 | 0.47 | 1.18 |
| Ramachandran favored (%) | 98.33 | 99.43 | 97.07 | 99.70 |
| Ramachandran allowed (%) | 1.67 | 0.57 | 2.93 | 0.30 |
| Ramachandran outliers (%) | 0.00 | 0.00 | 0.00 | 0.00 |
| Rotamer outliers (%) | 2.73 | 0.37 | 1.70 | 3.65 |
| Clashscore | 3.91 | 4.98 | 6.17 | 1.87 |
| Average B-factor | 55.91 | 23.29 | 85.40 | 50.40 |
| Macromolecules | 55.97 | 22.26 | 85.79 | 50.57 |
| Ligands | 56.27 | 63.68 | 30.28 | | |
| Solvent | 51.81 | 35.41 | 57.92 | 42.83 |

Statistics for the highest-resolution shell are shown in parentheses.†Friedel pairs were treated as different reflections.
We hypothesized that the four glutamate residues at the center of TFD-EE would provide a suitable coordination environment for large trivalent cations, such as lanthanides, as these are typically oxophilic and can interact directly with eight or more ligand atoms. Inspired by the design of lanthanide-binding tags (21), we installed an intrinsic antenna and observed electron density consistent with a bound metal ion, even though no specific metal was added to the protein sample (SI Appendix, Fig. S4). We believe this metal copurified with the protein from the intracellular environment or lysate. The coordination sphere resembles the situation in natural calcium-binding proteins, which led us to hypothesize that the bound metal is likely a Ca\(^{2+}\) ion. Microwave inductively coupled atmospheric pressure plasma mass spectrometry (MICAP-MS) experiments supported this hypothesis (SI Appendix, Tables S1 and S2), so a Ca\(^{2+}\) ion was modeled into the crystal structure at the center of each dimer.

Lanthanide Binding. We hypothesized that the four glutamate residues at the center of TFD-EE would provide a suitable coordination environment for large trivalent cations, such as lanthanides, as these are typically oxophilic and can interact directly with eight or more ligand atoms. Inspired by the design of lanthanide-binding tags (21), we installed an intrinsic antenna residue adjacent to the metal-binding site (N6W mutation); this tryptophan serves as a site-specific sensitizer for lanthanide luminescence (Fig. 4 A–C). When the protein was mixed with TbCl\(_3\), strong tryptophan-enhanced terbium(III) luminescence was observed upon excitation at 280 nm, enabling quantitative measurement of lanthanide binding through a specific spectroscopic readout. Furthermore, we determined the crystal structure of terbium(III)-bound TFD-EE N6W at 1.85 Å resolution and observed strong anomalous electron density at the metal-binding site originating from specific binding of the heavy ion (Fig. 4D). The C2 symmetry of the scaffold is lost in this structure due to a translation of the FD dimer with respect to the TIM barrel, further indicating the flexibility of the linkers that connect both domains.

We titrated metal-free protein with preincubated mixtures of TbCl\(_3\) and ethylenediaminetetraacetic acid (EDTA) and monitored the enhanced terbium signal over time (Fig. 4D). The protein slowly removes the metal from the EDTA complex, which has a binding constant of \(1.6 \times 10^{-18}\) M (31), suggesting ultra-high affinity. A binding curve based on the estimated free metal concentration in these mixtures indicates a binding affinity of TFD-EE for Tb(III) in the subfemtomolar range. Notably, when the Tb(III)/protein complex is formed first, even 100-fold excess EDTA does not quantitatively remove the metal from the scaffold after 1 wk of incubation. Displacement experiments with other lanthanides showed that Tb(III) bound to TFD-EE N6W can be exchanged on the timescale of hours. Eu(III) and Yb(III) bind with comparable affinity, while Ce(III) binds ~10-fold weaker (SI Appendix, Fig. S5).

Discussion

By fusing two de novo designed homodimeric proteins such that their C2 symmetry axes are preserved, we have created a homodimeric protein with a TIM barrel base and a dimeric FD lid surrounding an enclosed chamber. Fusion of de novo designed building blocks has been used previously to create proteins with extended beta sheets (32). We show here that extended enclosed cavities can be created by designed juxtaposition of de novo C2 building blocks. Some examples of similar fusions creating cavities exist in nature, such as AAA+-type molecular machines (33) or ATP-binding cassette (ABC) membrane transporters (34). However, to our knowledge, TFD is the most compact protein to enclose a central cavity in this manner.
Our TIM-FD design has attractive features as a scaffold for future de novo enzyme design. First, a large chamber is formed at the interface between two structured domains (Fig. 3 F and G). In TFD, there are $9 \times 2$ residues pointing into this chamber from the TIM barrel, and $9 \times 2$ from the FD, for a total of 36 functionalizable residues. Some of these residues could be substituted with hydrophobic amino acids to increase the hydrophobic character of the chamber. Second, the high stability of TFD suggests it should tolerate considerable modification of the residues surrounding the chamber. TFD and the derivative A

![Image](https://example.com/image.png)

Fig. 3. Stability and structure of TFD proteins. (A) Thermal and chemical denaturation profiles measured by circular dichroism for TFD-EE and TFD-HE. Both proteins are stable beyond 95 °C, and possess two inflection points upon chemical denaturation, reflecting the TIM and FD denaturation points at around 2 M and 7 M guanidinium chloride. A slight change in the TIM melting is observed due to the Glu-His substitution of TFD-HE relative to TFD-EE. (B–E) The alignment of TFD design model and crystal structures indicate the variability in interdomain orientation between the FD and TIM domain. (B) TFD design model. (C) TFD-HE structure, aligned to the TIM barrel of the design model in B. The upper FD domain carries a 10° twist relative to the TIM. (D) TFD-EE structure, aligned to the same TFD design model. This structure carries a 34° twist. (E) Superimposition of all three dimers of the asymmetric unit of the TFD-EE structure, and the dimer from the TFD-HE structure illustrates the different interdomain conformations accessed TFD. Helix 2 of the first FD colored red for emphasis. (F) TFD encloses a large internal cavity that is solvent accessible from both sides but enclosed between the top and bottom domains. TFD-HE surface displayed at 1.9 Å solvent radius, cross-section that includes molecular symmetry axis. (G) In the TFD-HE structure, this chamber encloses about 420 Å³. Rendered at 1.9 Å solvent radius cavity mode in PyMol.
designs presented here are homodimeric proteins, but the modular design makes it readily convertible to an asymmetric monomeric protein through the simple closure of the protein chain between two repeats (SI Appendix, Fig. S6). Control of the interdomain configuration of the TIM domain and FD remains a challenge. While the homodimeric domains were both internally unaffected by fusion into a two-domain homodimeric assembly, their relative orientations were not exactly as predicted. Controlling this relative position will be important for construction of binding sites that use elements from both domains. Future design will address the relative orientations through engineering of the contacting surfaces and linking loops. It should also be noted that this mobility, if controlled, could prove beneficial in the longer term as dynamics are often important to enzyme function.

In this work, the TIM barrel component of the highly stable TFD scaffold was equipped with a specific binding site for lanthanide ions. As lanthanides find applications in various technologies, including magnets, lasers, luminescent materials, and optoelectronics, there is an increasing demand in industry (35). However, conventional mining of these elements is problematic, and other sources of low lanthanide concentration must be considered. A biomacromolecular extraction concept has recently been suggested using lanmodulin, a natural lanthanide-binding protein (36). Our de novo lanthanide-binding scaffold, TFD-EE, exhibits even higher lanthanide affinity and tolerates heat and cosolvents to a remarkable extent. It may thus be utilized in biotechnological approaches to lanthanide recycling as well as lanthanide-dependent catalysis. While the FD lid domain of TFD-EE is not involved in metal binding, it will be crucial for further engineering; for example, providing residues for specific substrate recognition inside the cavity. We are currently pursuing the design of enzyme active sites in the lanthanide-bound TFD scaffolds—for both hydrolytic and carbon-carbon bond forming reactions that can capitalize on the strong Lewis acidity of lanthanide ions. Our approach can be extended to various other metals by changing the type and geometry of the coordinating

Fig. 4. Structure and lanthanide-binding properties of TFD-EE. (A) Crystal structure of the Tb(III)-bound TFD-EE N6W dimer at 1.85 Å resolution. The protein was equipped with an intrinsic tryptophan antenna (N6W, pink) to enhance Tb(III) luminescence through energy transfer. The initial C2 symmetry of the dimeric scaffold is lost, as the FD lid domain is both rotated and translated compared to the structures shown above. Each monomer exhibits a distinct conformation of the flexible linkers that connect both domains. (B) Close-up view of the metal-binding site, which consists of 2 × 2 glutamate residues (green and gray). 2Fo–Fc electron density is shown as a blue mesh contoured at 1.0 σ. Anomalous electron density is shown as a red mesh at 15.0 σ, indicating specific binding of the lanthanide ion. The antenna tryptophan (pink) is in close proximity (<5.0 Å), allowing for efficient energy transfer. The distance between Tb(III) and the coordinating carboxylate oxygen atoms is 2.3 to 2.5 Å and the distance between Tb(III) and the axial water molecule is 2.7 Å. (C) Tryptophan-enhanced Tb(III) luminescence upon excitation at 280 nm. Control proteins with either no tryptophan antenna (TFD-EE wild-type) or knocked-out binding site (Glu to Gln mutations, TFD-QQ N6W) show only background signal. (D) Lanthanide-binding measurements using the specific luminescence readout. Direct titrations were not possible due to the very high affinity and slow binding kinetics. However, titrating TFD-EE N6W with preincubated mixtures of EDTA and TbCl3 allows for the estimation of the Tb(III)-binding affinity of the protein scaffold in the subfemtomolar range, based on Kd(Tb3+/EDTA) = 1.6 × 10−18 M as the reference.
residues. Large cofactors may also be inserted through specific interactions with the TIM domain or FD domain. We believe the internal chamber-containing scaffolds will provide a modular platform for de novo enzyme engineering, contributing to the long-standing goal of designing novel enzymes from scratch.

Methods
Gene Synthesis, Protein Expression, and Purification. Genes were synthesized by commercial vendors and cloned into vectors for protein expression in E. coli. Protein was expressed in shaking liquid cultures using either isopropyl thiogalactoside or autoinduction methods. Cells were lysed using high-pressure homogenization or sonication and clarified by centrifugation. This lysate was purified by gravity-flow nickel-affinity chromatography and allowing the connecting loops to be designed. The inserted domain has both its termini connected within a loop of a hosting structure;RosettaRemodel was used to design the connections (or “linkers”) between DeNovoTIM15 and each inserted domain (Fig. 2). We used the design feature that facilitates the introduction of a domain as a rigid body insertion while allowing the connecting loops to be designed. The inserted domain has both its termini connected within a loop of a hosting structure; RosettaRemodel uses random fragments to sample the linkers and loop closure criteria to screen plausible fusions of the inserted domain and the host structure. By sampling the linker conformations, the relative position of the inserted domain with respect to the host structure is also sampled (Fig. 2). Linker lengths between zero and six amino acids were tested, though only a subset produced fully connected fusion proteins. For this project, we enabled symmetry with the domain insertion feature in RosettaRemodel, with the C2 symmetry of the host structure (TIM barrel) held constant while the rigid-body conformations of the FD or Cr domains move synchronously across the interface during sampling. To ensure that the dimer interfaces of the inserted domains are maintained, restraints corresponding to the FD and Cr dimers were used, defined as distance constraints harvested from atom-pairs from the dimer structures. The RosettaRemodel flags and the calculation setups as blueprint files are given in the SI Appendix. Methods. Thermal and Chemical Protein Denaturation Measured by CD Spectroscopy. CD experiments were performed in quartz cuvettes with 2 mm path length using a JASCO J-710 CD instrument. The proteins were diluted in pure water or 0.5 to 8.0 M guanidinium chloride solution to a final concentration of 20 μM TFD monomer, with a 30 min incubation time at room temperature prior to measurement. Thermal denaturation curves were measured in the absence of denaturing agent at 222 nm from 25 to 95 °C with a scan speed of 30 K/ h. Chemical denaturation curves were generated by measuring CD spectra (260 to 200 nm, averaging five scans) at various concentrations of guanidinium chloride at 25 °C. The signal at 222 nm was converted into mean residue ellipticity, and then to fraction of total protein unfolded. All points were measured in triplicates. The curves were fit with a sigmoidal equation to determine the transition points.

Protein stability in cosolvents was measured by recording CD spectra (260 to 200 nm, five averages) of TFD-EE (c = 20 μM monomer) after incubation in 0 to 90% methanol or acetonitrile for 20 min at 25 °C.

Crystallization and Structure Determination. All proteins were crystallized by vapor diffusion. The reengineered DeNovoTIM15 was crystallized in Mophus screen solution D9 (37): 10% wt/vol polyethylene glycol (PEG) 20000, 20% vol/vol PEG monomethyl ether 5500 0.2 M each of each of 1,6-hexanediol, 1-butanol, 1-propanediol, 2-propanol, 1,4-butanediol, 1,3-propanediol, 0.1 M bis(Tris)Tris base pH 8.5. TFD-HE was crystallized in 0.1 M citric acid pH 4.0 and 2.4 M ammonium sulfate. Large rod-shaped crystals were found after 6 mo of incubation. Crystals of TFD-EE were grown in a sitting drop vapor diffusion setup in 96-well plates at 20 °C. The protein solution (10 mg/mL in 25 mM Hepes pH 7.5) was mixed 1:1 with the reservoir solution, diluting 9.1% with DeNovoTIM15 and TFD-PE 4000, 5% to 10% isopropanol using microsyringe (38). Rod-shaped crystals grew within 7 d. TFD-EE N6W was mixed 1:1 with TbcI3 and concentrated to 8 mg/mL. Crystals grew in 0.1 M Hepes pH 7.5, 20% PEG 4000, and 10% isopropanol, containing Gly3 as an additive. The cryo-protectionant solutions for all TFD-EE crystals consisted of the reservoir solution supplemented with 20% ethylene glycol. Crystals were cryo-cooled in liquid nitrogen.

Diffraction data from TFD-EE and TFD-HE were collected at the Advanced Light Source (ALS) Beamline 8.2.1. TFD-EE data were collected at the synchrotron beamline X06SA (PHI) and Tbid-bound TFD-EE N6W at X06DA (PXIII) at the Swiss Light Source. In all cases, data collection was diffraction was collected at 1.0 A with the crystals kept at 100 K. The program XDS was used for initial data processing (39).

All structures were refined and built using the Phenix and CCP4 software suites (40, 41), using the program PHASER (42) to phase the structures by molecular replacement. DeNovoTIM15 was phased using the unaltered Rosetta design model. TFD-EE and TFD-HE structures were phased by searching separately for the TIM domain and FD domain. In the case of the third TFD-EE dimer and TFD-HE structure, the FD dimer could not be placed by phaser, but was manually built by modeling helices into Fpβ, difference density and iterative manual model building. Further model building and refinement were done in Coot, cycles using programs, and phenix.refine (40) or Refmac (44), including translation–libration–screw refinement (45) and reference model restraints to the Rosetta models. The model quality was validated using MolProbity (46). Structure illustrations were prepared with PyMOL (47), while estimates of volume of enclosed cavity were obtained using the CSTp server (48), based upon models with bulk solvent-accessible channels plugged with dummy atoms. Structures were deposited to the Protein Data Bank (PDB) with accession numbers 6WVS, 6WXP, 6WXO, and 6ZV9. Summary statistics are presented in Table 1.

Lanthanide-Binding Measurements. Tryptophan-enhanced terbium luminescence was measured in 96-well plates using a Varioskan Flash plate reader in time-resolved fluorescence mode (TRF, delay time: 50 μs, integration time: 1 ms). This mode allows for suppression of all background signals, collecting only the long-lived terbium luminescence in a pulsed experiment. All experiments were performed with 1 mM Hepes pH 7.5 using a PD10 desalting column. The same buffer was used as the reference sample. First, a MACP-MS element screen was performed at 45 μM TIM-EE monomer to generate semiquantitative estimates of element concentrations. Subsequently, the amount of calcium in the sample was determined quantitatively using a MACP-MS experiment, using an external calibration between 0 and 500 μg/L Ca, with 5c as an internal standard. Further experimental details are described in the SI Appendix.

ICP-MS Measurements. Element screening and Ca quantification was carried out using MICAP-MS systems. The instruments are prototype instruments developed in the Laboratory for Inorganic Chemistry at ETH Zürich specialized for measuring metal ions (49). A TFD-EE sample was buffer-exchanged with 1 mM Hepes pH 7.5 using a PD10 desalting column. The same buffer was used as the reference sample. First, a MACP-MS element screen was performed at 45 μM TIM-EE monomer to generate semiquantitative estimates of element concentrations. Subsequently, the amount of calcium in the sample was determined quantitatively using a MACP-MS experiment, using an external calibration between 0 and 500 μg/L Ca, with 5c as an internal standard. Further experimental details are described in the SI Appendix.
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