Coiled-coil (CC) dimers are versatile, customizable building modules for the design of diverse protein architectures unknown in nature. Incorporation of dynamic self-assembly, regulated by a selected chemical signal, represents an important challenge in the construction of functional polypeptide nanostructures. Here, we engineered metal binding sites to render an orthogonal set of CC heterodimers Zn(II)-responsive as a generally applicable principle. The designed peptides assemble into CC heterodimers only in the presence of Zn(II) ions, reversibly dissociate by metal ion sequestration, and additionally act as pH switches, with low pH triggering disassembly. The developed Zn(II)-responsive CC set is used to construct programmable folding of CC-based nanostructures, from protein triangles to a two-chain bipyrimal protein cage that closes and opens depending on the metal ion. This demonstrates that dynamic self-assembly can be designed into CC-based protein cages by incorporation of metal ion–responsive CC building modules that act as conformational switches and that could also be used in other contexts.

INTRODUCTION

Metal ion binding is integral for function, folding, and formation of quaternary complexes of many proteins (1, 2). Understanding metal–protein interactions is, therefore, an important piece of the protein folding puzzle. Because metal coordination can be easily regulated via changes in the concentration of a competing chelator or pH, metal ion binding and unbinding events often trigger conformational changes, a cornerstone of many natural regulatory mechanisms. Conformational changes induced by metal ion binding regulate muscle contraction (3), ion channel flux (4), DNA binding affinity of transcription factors (5–7), and mediate ubiquitination (8). Designing metal binding sites into protein scaffolds is, therefore, an attractive strategy toward achieving designed protein complexes with externally controllable assembly/disassembly, mimicking natural molecular machines.

Coiled coils (CCs) have been proven to be suitable building blocks for designing modular protein nanostructures (9–11). CC sequences are characterized by a heptad repeat pattern, commonly denoted as abcdefg, where hydrophobic residues are located at positions a and d, while other positions are predominantly occupied by polar amino acid residues (12). Assembly of individual peptide chains into a left-handed superhelix is mediated by hydrophobic interactions between amino acid residues at a and d positions, and salt bridging between residues at e and g positions. Residues at b, c, and f positions primarily do not affect oligomerization properties but contribute to the thermodynamic stability of the CC assembly (13). Thanks to the insights into the rules governing the sequence–structure relationship in CCs, it is feasible to precisely design the number and the orientation of α helices in a CC assembly by carefully selecting interacting residues (14).

By correctly combining CC-forming peptides or linking them to other oligomerizing units, diverse CC-based nanostructures can be created (9–11). We have previously shown that CC dimers can be used to design mono- and multimeric protein cages of different polyhedral shapes (15–19). This strategy, termed CC protein origami (CCPO), is based on the concatenation of orthogonal CC-forming peptides (20) into one or several polypeptide chains that self-assemble into the desired fold determined by the pattern of pairwise interactions between the CC-forming modules.

That CCs can also be engineered to bind metal ions with high selectivity and affinity has been first demonstrated more than three decades ago (21, 22). Initial design attempts focused on Zn(II) binding sites; however, subsequent work has shown CCs can be adapted to bind a diverse array of metal ions (23–27). Moreover, multiple structurally diverse binding sites could be incorporated into a single-peptide scaffold (28–30), with some designs mimicking natural metalloenzymes both in structure and function (31, 32). Because metal binding can be coupled to protein folding and assembly, many of the hitherto designed metal binding peptides assumed a well-folded structure only in the presence of metal ions (22, 33–35), in effect acting as metal-regulated conformational switches. The designs reported so far were based, with few exceptions (36, 37), not on CC dimers but on CC trimers and higher-order α-helical bundles with metal ions buried in the cavity between multiple helices. Recently, we demonstrated that a monomeric peptide could be transformed into a conformational switch by incorporating a His residue at the e position in its final heptad (34). The peptide, named SwitCCh, formed a parallel CC homodimer only in the presence of Zn(II) ions. While CCs and α-helical bundles can act as conformational switches individually, they could also be applied to program conformational flexibility and regulated self-assembly into larger protein nanostructures. For example, it has been recently shown in a study by Tezcan and co-workers that four-helix bundles can serve as building blocks for designing larger cage-like architectures with metal-mediated assembly (38). Similarly, by incorporating Zn(II)-dependent CC dimers into CCPO cages, it might be possible to reversibly regulate their folding and (dis)assembly.
Here, we use rational metal-site engineering to design a four-member set of orthogonal Zn(II)-dependent heterodimeric CCs. The designed set is applied to construct single-chain CCPO triangular folds with dynamic Zn(II)-dependent folding. Comparing different design variants, we show that the temperature where Zn(II) ions have a substantial effect on the secondary and tertiary structure can be fine-tuned by CC building block selection and rational mutation of residues at b, c, and f heptad positions. Last, we demonstrate the design of a metal ion–regulated self-assembly of a multimeric CC-based bipyramidal protein cage.

RESULTS
Design of Zn(II)- and pH-responsive CC modules
Most of the hitherto designed orthogonal CC sets are composed of heterodimeric pairs, due to clear design rules for their creation (39).

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

Fig. 1. Design and biophysical characterization of the Zn(II)-responsive orthogonal dimeric CC set. (A to D) Sequence and design models for CC34SHb3H3 (A), CC56SHb3H2 (B), CC56SHb3H3 (C), and CC78SHb3H2 (D). Heptads with negatively and positively charged residues at eg positions are colored red and blue, respectively, while heptads containing the ZnHis3 motif are shown in gray. Model CC structures were built with the ISAMBARD software package (51). (E to H) CD spectra in the absence and presence (200 μM) of Zn(II) ions for individual peptides and peptide pair mixtures (20 μM). CD signal is shown as mean residue ellipticity (MRE). (I to L) MRE at 222 nm as a function of temperature in the presence and absence of Zn(II) ions. Experimental data (dots) were fit with a thermodynamic two-state model (blue line) to determine the melting temperatures (T_m). (M to P) Normalized CD signal at 222 nm (gray bars) after consecutive additions of equimolar amounts of Zn(II) and EDTA for CC34SHb3H3 (M), CC56SHb3H2 (N), CC56SHb3H3 (O), and CC78SHb3H2 (P). The data are shown as means ± SD (n = 3). (Q) Protein-protein interaction matrix in the presence of Zn(II) ions. Propensity for interaction was estimated from MRE at 222 nm.
interaction in the absence of Zn(II) (fig. S1). In each pair, one of the peptides contained an additional His at a neighboring position to achieve tighter Zn(II) binding, because the homodimeric peptide S witCh, where coordination was mediated by only two His residues, displayed a relatively low Zn(II) binding affinity [dissociation constant ($K_d$) ~ 400 μM] (34). To probe the effect of binding site placement within the four-heptad peptide on conformational switching, we positioned the ZnHis$_3$ motif into the second [CC56$_{SHb}$3H2 (Fig. 1B) and CC78$_{SHb}$3H2 (Fig. 1D)], third [CC34$_{SHb}$3H3 (Fig. 1A) and CC56$_{SHb}$3H3 (Fig. 1C)], or fourth heptad [CC43$_{SHb}$3H4 (table S1)]. In addition, residues at the $b$, $c$, $f$ heptad positions were modified to promote salt bridging, thus increasing α-helical propensity and stability (13). Last, we created model structures of designed peptide pairs with ISAMBARD to confirm His residues were at an appropriate distance for Zn(II) coordination (Fig. 1, A to D).

The secondary structure of the designed peptide pairs was analyzed using circular dichroism (CD) spectroscopy (Fig. 1, E to H). In the absence of metal ions, the peptide pairs were predominantly unfolded with the exception of CC34$_{SHb}$3H4 that contained the appropriate distance for Zn(II) coordination (Fig. 1, A to D). Pairs with ISAMBARD to confirm His residues were at an appropriate distance for Zn(II) coordination (Fig. 1, A to D). In the absence of metal ions, the peptide pairs were predominantly unfolded with the exception of CC34$_{SHb}$3H4 that contained the Zn(II) binding site in the fourth heptad, close to the C-terminal end (fig. S2). Peptide pair CC34$_{SHb}$3H4 was, therefore, not examined further. For other peptide pairs, the addition of Zn(II) ions resulted in a large increase in ellipticity at 208 and 222 nm, characteristic for the formation of helical structures (Fig. 1, E to H). To confirm the observed increase in helicity was not due to the formation of homo-oligomeric species, individual peptides were investigated. No Zn(II)-dependent changes in secondary structure were observed, confirming that both peptide partners were needed for the formation of α-helical CCs. In addition, CD spectroscopy was used to monitor temperature-induced unfolding (Fig. 1, I to L). In the absence of Zn(II) ions, melting temperatures ($T_m$) were below 20°C. Addition of Zn(II) ions caused a large increase in $T_m$. Notably, peptide pairs CC34$_{SHb}$3H3 (Fig. 1I) and CC56$_{SHb}$3H3 (Fig. 1K) with the ZnHis$_3$ site in the third heptad were characterized by a higher $T_m$ (42° to 43°C) than peptide pairs CC56$_{SHb}$3H2 (Fig. 1J) and CC78$_{SHb}$3H2 (Fig. 1L; $T_m$ = 32° to 33°C), where the binding site was located in the second heptad in the center of the peptide.

Next, we used size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) to confirm that Zn(II) ions induced formation of CC dimers rather than higher-order species. In the absence of Zn(II) ions, we observed two peaks in the chromatograms of peptide mixtures, corresponding to individual peptides (fig. S3). Addition of Zn(II) ions to the mobile phase resulted in peptide pairs eluting in a single peak whose mass corresponded to a dimer as designed (fig. S4).

Zn(II) binding affinity was determined by monitoring ellipticity at 222 nm as a function of Zn(II) concentration (figs. S5 and S6). For all peptide pairs, ellipticity increased until Zn(II):CC molar ratio was approximately 1:1, after which saturation was observed. This indicated a single Zn(II) ion bound into each designed heterodimer. $K_{d58}$, calculated by fitting a two-state model to experimental data (see the Supplementary Methods), were in the low micromolar region with Zn(II) binding strongest to CC34$_{SHb}$3H3 ($K_d = 1 \pm 0.1 \mu M$). We observed CC formation also after the addition of other divalent metal ions [Ni(II), Co(II), and Cu(II)]; however, the $K_{d58}$ were 10- to 300-fold higher (figs. S7 to S9).

Next, we examined whether metal-induced self-assembly could be reversed either by the addition of a strong metal chelator such as EDTA or by decreasing the pH, which affects the protonation state and coordination ability of His residues. Upon EDTA addition, the CCs promptly disassembled, reflected in the decrease in ellipticity at 222 nm to levels observed before Zn(II) addition (Fig. 1, M to P). Moreover, switching between the monomer and dimer state could be repeated reversibly for several cycles, without loss in amplitude. In addition, all four peptide pairs exhibited pH-induced disassembly at low pH (fig. S10). For designs CC56$_{SHb}$3H2 and CC78$_{SHb}$3H2, the transition was highly cooperative with a midpoint at pH 6, while for CC34$_{SHb}$3H3 and CC56$_{SHb}$3H3, dissociation occurred over a broader range of pH values with a midpoint at pH ~5. The reduced cooperativity and lower transition pH values are most likely due to the stronger association between peptide chains as reflected in thermal stability, making His residues more resistant to protonation.

If the designed Zn(II)-dependent CCs were to be used for the construction of modular CC nanostructures, they ought to display high interaction specificity (Fig. 1Q). Pairwise all-by-all CD analysis revealed each peptide formed a CC preferentially with its peptide partner. Some cross-reactivity was observed at higher Zn(II) concentrations. While peptide P6$_{SHb}$3H3 still preferentially bound its partner P5$_{SHb}$3H3, it displayed a homo-oligomerizing tendency and weakly interacted with peptides P6$_{SHb}$3H2 and P4$_{SHb}$3H3 (fig. S11).

To further probe the modularity of the binding site design, we examined whether inserting the ZnHis$_3$ motif into the second and third heptads of CC56$_{SHb}$ would result in a CC able to bind two Zn(II) ions (table S1). While the resulting module CC56$_{SHb}$6H was unresponsive to Zn(II) ions, the individual peptide P6$_{SHb}$6H displayed homo-oligomerization in response to Zn(II) ions at high peptide concentrations (fig. S12), indicating that insertion of a larger number of mutations results in less predictable CC pairing.

**Tunable Zn(II)-driven regulation of CCPO self-assembly**

Because the development of synthetic protein cages and materials with controllable and reversible self-assembly is one of the current goals of protein design, we sought to use the Zn(II)-responsive CCs to construct single-chain CCPO cages with inducible folding and unfolding. As a case study, we used a triangular fold composed of six peptide modules (Fig. 2 and table S2). The control construct TR16-control (Fig. 2A), composed of peptide pairs CC34$_{SHb}$3H3, CC56$_{SHb}$3H3, and CC78$_{SHb}$3H2 without Zn(II) binding sites, showed high helicity (~75%; Fig. 2B) and high thermal stability ($T_m$ = 82°C; Fig. 2C). CC building blocks were then replaced with their Zn(II)-responsive counterparts: CC34$_{SHb}$3H3, CC56$_{SHb}$3H3, and CC78$_{SHb}$3H2. At 20°C, the resulting design TR16-1 (Fig. 2D) displayed a high α-helical content (82%) that was independent of Zn(II) ion concentration (Fig. 2, E and F, and fig. S13). Next, we used SEC-MALS to qualitatively evaluate the effect of Zn(II) ions on the tertiary structure. In the absence of metal species, TR16-1 eluted at approximately the same retention time as the control assembly, indicating that the design assumed a folded triangular state (Fig. 2G). Addition of Zn(II) had a minimal effect on the retention time. Nonetheless, thermal denaturation measurements revealed addition of Zn(II) ions significantly increased the protein’s thermal stability, with $T_m$ increasing from $37° \pm 1°C$ to $56° \pm 1°C$ (Fig. 2F). Zn(II) ions had the highest impact on secondary structure at ~50°C, triggering a transition from the unfolded to the folded state (Fig. 2H). Together, the results show that while the insertion of the ZnHis$_3$ binding site into each CC module did not prevent the formation of the triangular fold in the absence of Zn(II) ions, it did diminish its stability, which could however be partially recovered by the addition of Zn(II) ions.
To shift the temperature where Zn(II) ions control protein folding to a lower temperature range, we investigated several alternative CCPO triangle designs, differing in CC building block composition (Fig. 3, A and B, and figs. S13 and S14). After replacing the C-terminal pair CC34SHb3H3 with the homodimeric SwitCh (design TRI6-2) and the central building block with CC56SHb6H or the homo-oligomeric P6SHb6H (designs TRI6-3 and TRI6-4, respectively), Zn(II)-dependent folding was observed at 37°C (Fig. 3, C to E, and fig. S14). We further destabilized the triangular fold by modifying b, c, f residues in the N-terminal CC module CC78SHb3H2 (13). In the construct TRI6-5, we changed all b, c, f residues to Ala, whereas the design TRI6-6 contained in those positions polar negatively charged residues (Fig. 3B). At room temperature, TRI6-5 displayed only low helicity (36%), while TRI6-6 was unfolded (Fig. 3E). In both cases, the addition of Zn(II) led to an increase in helicity (59 and 42%, respectively) and a cooperative unfolding transition at 46° ± 1°C and 27° ± 1°C, respectively (Fig. 3, C and D). Greater degree of unfolding in the absence of Zn(II) ions was also confirmed by SEC-MALS measurements (fig. S14).

To study the effect of Zn(II) ions on the tertiary structure of selected triangular designs (fig. S15) in detail, we used small-angle x-ray scattering (SAXS), which is particularly suitable to study dynamic assemblies. The scattering curve of the control construct (TRI6-control) matched well to a triangular model structure and was characterized by a radius of gyration ($R_g$) of 2.85 ± 0.05 nm (Fig. 4, A and B), and a maximal particle distance ($D_{max}$) of 9.7 ± 0.5 nm, in agreement with the design (Fig. 4C). For TRI6-3, TRI6-4, TRI6-5, and TRI6-6, we observed higher $D_{max}$ and $R_g$ values in the absence of Zn(II) (Fig. 4C). In comparison to TRI6-control, TRI6-6 was characterized by a twofold higher $D_{max}$ (21 ± 1 nm), a size increase consistent with unfolding of a monomeric protein (42). Kratky analysis further corroborated TRI6-control was well folded and contained multiple domains connected with flexible linkers, while other proteins were either partially or fully unfolded in the absence of Zn(II) ions (Fig. 4D). We evaluated the degree of unfolding in the absence of Zn(II) ions by the ensemble optimization method (EOM) (43). Briefly, a large pool of unfolded random chain configurations was generated, after which a genetic algorithm was used to

Fig. 2. Biophysical characterization of a Zn(II)-responsive CCPO triangle. (A) Schematic representation of the CCPO triangle design TRI6-control along with the arrangement of CC building block composition. (B) MRE as a function of wavelength in the absence and presence of Zn(II) ions (yellow and red curves, respectively) observed for TRI6-control. (C) MRE at 222 nm as a function of temperature in the absence and presence of Zn(II) ions (gray and black dots, respectively) observed for TRI6-control. A two-state unfolding model was fit to experimental data (yellow and red curves, respectively) to determine the $T_m$. (D) Schematic representation of the CCPO triangle design TRI6-1. (E) MRE as a function of wavelength in the absence and presence of Zn(II) ions (yellow and red curves, respectively) observed for TRI6-1. (F) MRE at 222 nm as a function of temperature in the absence and presence of Zn(II) ions (gray and black dots, respectively) observed for TRI6-1. $T_m$ was determined by fitting a two-state unfolding model to experimental data (yellow and red curves, respectively). (G) SEC-MALS was used to analyze the effect of Zn(II) ions on the tertiary structure of TRI6-1. The dotted line marks the retention time observed for the control construct. (H) CD spectra of TRI6-1 at different Zn(II) ion concentrations observed at 50°C. CD profile before the addition of Zn(II) ions is shown in yellow, while the red curve corresponds to the CD profile observed at the highest Zn(II) concentration.
select the ensemble of structures best matching the observed SAXS profile (fig. S16). Comparison of $R_g$ distributions calculated from the optimized ensembles indicated only TR16-6 was completely unfolded in the absence of Zn(II) ions, while other designs were characterized by a more compact ensemble of structures (figs. S16 and S17). We repeated the SAXS measurements at different Zn(II) ion concentrations. Addition of Zn(II) ions led to a well-defined maximum at $qR_g = \sqrt{3}$ in the Kratky spectrum coupled with a decrease in the plateau at higher $qR_g$ values, which indicates that the proteins acquired a more compact fold (Fig. 4D) (44). Furthermore, $R_g$ values decreased as Zn(II) concentration was increased (Fig. 4E). At higher Zn(II) concentrations, $R_g$ for TR16-3, TR16-4, and TR16-6 showed a good agreement to the $R_g$ observed for the TR16-control. The most pronounced change was observed for TR16-6, which underwent a decrease in $R_g$ from 4.8 ± 0.2 nm to 3.0 ± 0.1 nm. Moreover, SAXS profiles at high Zn(II) concentration were similar to that observed for TR16-control (fig. S18), particularly in the case of TR16-4 and TR16-6, suggesting these constructs assumed a triangular shape (fig. S19).

To support the results of SAXS analysis, folding of TR16-5 and TR16-6 was additionally monitored by Förster resonance energy transfer (FRET). Because the N and C termini should come in close proximity as the proteins fold, a pair of cyanine dyes (sulfo-Cy3 and sulfo-Cy5) was attached at the termini, and the FRET ratio was monitored as a function of Zn(II) ion concentration (Fig. 4F). For both designs, increasing the concentration of Zn(II) ions led to a notable rise in the FRET signal, indicating the distance between the N and C termini decreased as the proteins folded, further corroborating the designs assumed a triangular structure in the folded state. To extract binding affinities for CC building blocks, we used to reversibly control the assembly process of multimeric CCPO cages. As a proof of concept, we used the recently described heterodimeric CCPO bipyramid SBP12a,b (17), composed of two tetrahedrally shaped domains that interact via a triangular interface encompassing three CC dimer–forming peptide segments. We have previously shown that the assembly of the two-chain protein bipyramid could be regulated by proteolysis; however, that process was unidirectional. Aiming to achieve a reversible, Zn(II)-regulated self-assembly, we replaced the interfacing CCs with CC56 SHb 3H2, CC56 SHb 3H2, and CC78 SHb 3H2. In addition, we showed the triangular structures could be reversibly folded and unfolded by sequestering Zn(II) ions from solution by EDTA addition (fig. S20). Moreover, the addition of other divalent metal ions did not result in a FRET increase, indicating the designs selectively bind Zn(II) ions (fig. S21).

Metal ion–regulated assembly of a heterodimeric CCPO bipyramid

Last, we examined whether the Zn(II)-dependent CCs could be used to reversibly control the assembly process of multimeric CCPO cages. As a proof of concept, we used the recently described heterodimeric CCPO bipyramid SBP12a,b (17), composed of two tetrahedrally shaped domains that interact via a triangular interface encompassing three CC dimer–forming peptide segments. We have previously shown that the assembly of the two-chain protein bipyramid could be regulated by proteolysis; however, that process was unidirectional. Aiming to achieve a reversible, Zn(II)-regulated self-assembly, we replaced the interfacing CCs with CC56 SHb 3H2, CC56 SHb 3H2, and CC78 SHb 3H2, while keeping the rest of the modules unchanged (Fig. 5A and table S2). We named the resulting proteins SBP$^{Zn}$1 and SBP$^{Zn}$2 and produced them in Escherichia coli (fig. S22).

We first analyzed the assembly of the two tetrahedral domains by CD spectroscopy. Adding Zn(II) ions to an equimolar mixture of SBP$^{Zn}$1 and SBP$^{Zn}$2 increased the $\alpha$-helical content by 13% (Fig. 5B) and the $T_m$ of the first unfolding transition by 5°C (Fig. 5C). This suggests Zn(II) ions provided additional structural stabilization, most likely to the interfacial segments. SEC-MALS measurements...
confirmed the tetrahedral domains assembled into a heterodimer only in the presence of Zn(II) ions and were otherwise monomeric (Fig. 5D). On the other hand, secondary structure, thermal stability, and oligomerization state of each individual domain were not significantly affected by Zn(II) ion addition (fig. S23). Next, we labeled the tetrahedral domains at the interface with fluorescent dyes sulfo-Cy3 and sulfo-Cy5 and monitored their association through FRET (Fig. 5E). The addition of Zn(II) caused a strong increase in the FRET signal. Although three binding sites were present at the interacting interface, the plateau was observed after the addition of two equivalents of Zn(II), indicating the binding of two metal ions was sufficient to assemble the bipyramidal cage, as would be expected for two rigidly interacting tetrahedra. Experimental data were fit to a model that described the binding sites as identical (to avoid over-fitting) but allowed for cooperativity (see the Supplementary Methods). The determined $K_d$s were in the nanomolar range ($K_d^1 = 90 \pm 10$ nM, $K_d^2 = 10 \pm 2$ nM). Last, we examined the reversibility of the self-assembly (Fig. 5F). The addition of EDTA to the solution of the SBP-Zn1:SBP-Zn2-Zn(II) complex led to a decrease in the FRET signal, indicating a disassembly of the bipyramidal cage. Moreover, Zn(II)/EDTA-triggered cycling between the assembled and disassembled state could be performed several times without a substantial loss in the amplitude of the FRET signal. Only weak association was observed in the presence of other divalent metal ions (fig. S24).

To confirm the heterodimer assumed a bipyramidal shape in solution, we analyzed it with SEC-SAXS (Fig. 5G and fig. S25). $R_g$ and $D_{\text{max}}$ were 4.2 ± 0.05 nm and 12.3 ± 0.5 nm, respectively, in agreement with values observed for the previously designed two-chain CCPO bipyramid (4.0 and 11.8 nm, respectively) (17). To investigate the possible conformational heterogeneity, an ensemble of bipyramidal cage models was compared to the scattering profile. A good fit was obtained with a single bipyramidal cage model,
suggesting that the tetrahedral domains assembled into a bipyramidal shape as designed (Fig. 5, G and H, and fig. S25). Moreover, theoretical scattering curves calculated for tetrahedral models of individual subunits or open conformations of the bipyramidal cage did not match the observed SAXS scattering curve (fig. S25). The molecular envelope obtained with an ab initio reconstruction from SAXS data overlapped reasonably well with the best-fit cage model (Fig. 5H and fig. S26). The slight deviation between the envelope and best-fit model might be due to the presence of an exposed central cavity, a characteristic feature of CCPO cages or the partial flexibility of the bipyramidal cage detected also in the Kratky plot (fig. S25).

**DISCUSSION**

One of the reasons the design of bridging metal centers has been such a successful strategy for generating supramolecular complexes is the relative strength of the metal-ligand bond driving the assembly, resulting in a robust design (45, 46). However, the oligomerization state of the self-assembled structure does not depend only on the preferred coordination geometry of the metal ion but also on non-covalent interactions between residues that come in close proximity as a result of the metal ion-mediated assembly, rendering the design of supramolecular assemblies via metal coordination more difficult (46). By incorporating ZnHis3, a frequently used motif in metalloprotein design, into an orthogonal CC set, we harnessed the importance of noncovalent interactions to design a set of four orthogonal Zn(II)-dependent heterodimeric CCs that differ in the pattern of electrostatic, hydrophobic, and metal chelating interactions.

While our modular design strategy highlights how metal sites can be used to guide CC self-assembly, the position of the binding site in the peptide sequence importantly affected the Zn(II)-dependent...
self-assembly. Terminally positioned binding site did not provide a sufficient perturbation to the hydrophobic seam to prevent CC formation in the absence of metal ions, while placing the ZnHis3 motif in the second or third heptad, located centrally, resulted in Zn(II)-controlled self-assembly. This suggests that the cluster of hydrophobic interactions is more potently disrupted at its center. CCs with the ZnHis3 motif in the second heptad versus the third heptad behaved somewhat differently. α-Helical content was higher for CC pairs containing ZnHis3 motif in the second heptad (62 and 66% for CC56SHb3H2 and CC78SHb3H2; Fig. 1, F and H, respectively) than if the binding site was present in the third heptad (55 and 50% for CC34SHb3H3 and CC56SHb3H3; Fig. 1, E and G, respectively). Conversely, thermal stability was lower if ZnHis3 was positioned in the second heptad. In addition, peptide pairs that contained the binding site in the second heptad displayed a somewhat weaker Zn(II) binding affinity in comparison to peptide pairs with His residues in the third heptad. We posit that the Zn(II) coordination site to a certain degree represents a disruption in the CC motif. Consequently, this affects the balance between binding affinity and helical content, with more rigidly organized coordination sites leading to higher Zn(II) affinity and thermal stability, but less helical assemblies. It is possible that the position of the ZnHis3 motif in the peptide sequence also affects the precise metal coordination geometry. High-resolution structural characterization of the peptides in complex with Zn(II) ions aimed at understanding the origins of these differences is ongoing.

Successfully designed peptide pairs displayed a high degree of orthogonality. Although CC56SHb3H2 and CC56SHb3H3 displayed some degree of cross-reactivity, on-target pairing was still preferred, even though the two peptide pairs shared the same hydrophobic and electrostatic pattern but differed in the position of the metal binding site. This suggests the possibility of combining standard CC noncovalent interactions with engineered metal sites to expand the size of available orthogonal CC pairs, allowing for the design of more complex CC-based nanostructures. However, insertion of several mutations in a CC pair can disrupt the pairing specificity imparted by the hydrophobic and electrostatic interaction pattern, as in the case of CC56SHb6H.

To explore the potential of the developed CCs as building blocks for designing dynamic protein assemblies, the set was used to construct a series of CCPO polyhedral cages. The initially designed single-chain CCPO triangle composed of validated Zn(II)-responsive CCs (CC78SHb3H2, CC34SHb3H3, and CC56SHb3H3) displayed Zn(II)-dependent folding only at elevated temperatures. This reflects the strong effect that connecting peptide pairs into a single chain has on the thermodynamics of the assembly by reducing the loss in entropy normally associated with dimerization. Because of the modular nature of CCPO cages, we were able to reduce the temperature range where Zn(II) ions controlled the folding equilibrium of the CCPO triangle by modulating its stability at the level of individual CC pairs. We demonstrated that several strategies can be used to manipulate the stability of the triangular fold: building block swapping, engineering of additional metal binding sites, or mutation of solvent exposed b, c, f residues. To achieve Zn(II)-induced folding of single-chain structures at room temperature, it was necessary to destabilize all the building modules. This highlights the strength of the CCPO design strategy, namely, that the properties of individual CC modules can be tuned in a wide range; however, the behavior of CCPO cages depends on the combination of selected CC modules and their cumulative contributions. Conversely, in the case of the CCPO bipyramid, adjusting the stability of the Zn(II)-responsive CC elements was not required.

Together, our results show the developed CC toolbox can be used to regulate the assembly of both multimeric and monomeric CCPO structures. The ability to reliably and reversibly control the quaternary structure of CC-based cages will facilitate the design of molecular machines for various applications, e.g., CC-based cages for delivery and release of cargo in cellular environments with low Zn(II) concentration or low pH [e.g., in the nucleus (47) or endosomes (48)]. In addition, because the N and C termini of CCPO cages come into close proximity in the folded state, Zn(II)-dependent CCPO triangles could be used to control the activity of split proteins, whose function requires interaction between separate protein halves, or as protein sensors of cellular Zn(II), where the ability to modulate Zn(II) affinity could be particularly advantageous. Last, we believe the developed set of CC conformational switches could also be used to program dynamic properties into other previously reported CC-based nanostructures (9, 11, 49) with minimal adaptation and likely also in other contexts with CC modules serving as the interaction interface. Because of their hetero-specificity, they are particularly suited for labeling applications and as scaffolds to govern intracellular trafficking, bring distinct protein domains in close proximity, for example, to create biocatalytic cascades, or perform information processing within cells (50).

**MATERIALS AND METHODS**

**Materials**

Chemicals were purchased from Sigma-Aldrich (Merck, Germany). The peptides were purchased from Proteogenix (France). The peptides’ N termini were protected by the acetyl group, while the C termini were protected by amidation. Synthetic genes were ordered from Twist Bioscience (CA, USA).

**Design and modeling of CCPO structures**

Models of CC pairs were generated with ISAMBARD CC modeling package written in Python (51). Optimal structural parameters (superhelix radius, pitch, and Crick angle) were found with a genetic algorithm. BUDE force field was used to calculate the internal energy. CCPO design strategy consists of covalently linking CC-forming peptides into a single polypeptide chain that can fold into the desired structure. For each new polyhedral shape, a topological analysis has to be performed to find all Eulerian double paths, i.e., paths that visit each polyhedral edge exactly twice, providing a blueprint for connecting the CC-forming peptides. In the case of two-dimensional objects such as triangles, the solution is trivial. There is a single possible double path, which circumvents the shape twice. After choosing the appropriate building blocks as described in the Results section, molecular models of CCPO triangles were built using the CoCoPOD computational platform (16) available at https://github.com/NIC-SBI/CC_protein_origami. CCPO bipyramid with Zn(II)-dependent assembly was designed on the basis of the previously reported bipyramidal cage SBP125a (17). The latter was composed from two tetrahedral domains interacting via a triangular interface, encompassing three CC pairs. To achieve Zn(II)-directed assembly, the interfacial peptides were exchanged for Zn(II)-responsive CC pairs. Bipyramid models were built using the CoCoPOD computational platform (16, 17).
Molecular cloning

Passages of plasmid propagation were carried out using the *E. coli* strain DH5α [F−*q80lacZΔM15 ΔlacZYA−argF*] U169 *recA1 endA1 hsdR17 (rK-, mK-)* *phoA* supE44 Δ thi-1 gyr96 relA1 (NEB, MA, USA). Transformation was carried out via heat shock with competent *E. coli* cells. Single colonies were propagated in the presence of the antibiotic kanamycin (Goldbio, MO, USA), added to Lysogeny broth (LB) media to reach 50 μg/ml.

All the proteins characterized in vitro were cloned in the expression vector pET41a(+) (Merck, Germany) between the restriction sites Nde I and Xho I. The affinity tags at either the 5’ or 3’ end were cloned between the restriction sites Xba I and Nde I or Xho I and Avr II, respectively. The DNA sequences coding for the protein constructs were optimized for *E. coli* codon usage and subsequently constructs were optimized for *E. coli* cells. Transformation was carried out via heat shock with competent *E. coli* cells. Single colonies were propagated in the presence of the antibiotic kanamycin (Goldbio, MO, USA), added to Lysogeny broth (LB) media to reach 50 μg/ml.

For SEC, we used HiLoad Superdex 200 resin (Cytiva, MA, USA), packed in a 26/600 XK column (Cytiva, MA, USA) equilibrated with filtered and degassed SEC buffer [20 mM tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, v/v, and 1 mM TCEP]. Samples were injected into the column after concentration with centrifugal filters (3K or 10K; Amicon-ultra, Millex Sigma-Aldrich, MO, USA) and filtration in 0.22-μm syringe filters (Millex Sigma-Aldrich, MO, USA). The chromatography was run with an AKTA pure FPLC system (Cytiva, MA, USA) in SEC buffer with a linear flow rate of 2.6 ml/min.

To remove the residual uncleaved protein, the sample was flown through 2.5 ml of Ni-NTA resin (Goldbio, MO, USA) previously conditioned in buffer A and eluted with buffer D [50 mM tris-HCl (pH 8.0), 150 mM NaCl, 50 mM imidazole, and 1 mM TCEP]. Samples were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (54) in a Bio-Rad (CA, USA) mini-PROTEAN apparatus in 12% or 15% discontinuous polyacrylamide gels containing SDS, next to prestained molecular markers (Thermo Fisher Scientific, MA, USA). Afterward, the gels were dyed with InstantBlue (Millex Sigma-Aldrich, MO, USA). SDS-PAGE confirmed the uncleaved protein remained bound to the Ni-NTA resin.

After the isolation process, any bound metal ions were removed with dialysis. First, EDTA was added to the samples to reach 5 mM final concentration. The sample was then dialysed against 1 liter of tris-based buffer (50 mM tris, 150 mM NaCl, and 1 mM TCEP) at pH 7.5) containing 5 mM EDTA. The dialysis proceeded for at least 24 hours, with a buffer change after the first 6 hours. To remove EDTA, the dialysis procedure was repeated, but without including EDTA in the dialysis buffer (50 mM tris, 150 mM NaCl, and 1 mM TCEP at pH 7.5).

The proteins were stored at −80°C after being flash frozen in liquid nitrogen. Protein concentrations were determined measuring absorbance at 280 nm using the extinction coefficients calculated with ProtParam (55).

FRET experiments

Cysteine residues in selected protein constructs were fluorescently labeled with Sulfo-cy3 and Sulfo-cy5 (Lumiprobe, MD, USA) via thiol-maleimide coupling. Dyes were dissolved in 100 μM dimethyl sulfoxide to approximately 5 mM final concentration. Dyes were added to 1 ml of protein solution at 1 mg/ml in 10-fold molar excess. SBPZn1 was labeled with Sulfo-cy3, while SBPZn2 was labeled

Aupić et al., *Sci. Adv.*, 8, eabm8243 (2022) 17 June 2022

9 of 12
with Sulfo-cy5. In the case of triangular designs, the dyes were first mixed together and then added to the protein solution. The reaction mixture was left overnight at 4°C. The labeled proteins were purified on PD 10 disposable desalting column (Cytiva, MA, USA) with tris buffer (50 mM tris and 150 mM NaCl, pH 7) used as the mobile phase.

Fluorescent spectra were measured with multiplate fluorescence reader Synergy Mx (Bio TeK, VT, USA) in 96-well plates. For triangular constructs, 80 µl of protein solution (6.25 mM) was placed in each well. Stock ZnCl₂ solution (30 mM ZnCl₂, 50 mM tris, and 150 mM NaCl, pH 6) was added to the wells to reach target Zn(II) concentration. Sample volume was adjusted to 100 µl by addition of tris buffer. For the complex SBP[Zn]₁:SBP[Zn]₂, the proteins were mixed in equimolar ratio in a final volume of 50 or 100 µl in 96-well plates at a final concentration of 1.5 µM. The protein was titrated with ZnCl₂ or other metals in a range of concentrations between 0.1 and 7 µM. Three independent samples were measured for each concentration. FRET ratio was calculated as

\[
FRET = \frac{I_A}{I_A + I_D}
\]

where \(I_A\) is acceptor emission at 668 nm, and \(I_D\) is the donor emission at 563 nm upon excitation at 528 nm. The model fitting was performed with Python package SciPy (56) and is described in detail in the Supplementary Materials.

**CD spectroscopy**

CD experiments were performed on a ChiraScan instrument (Applied Photophysics, UK). Temperature was controlled with a Peltier thermal control block (Melcor, NJ, USA, now part of Laird Technologies). For individual peptides and CC peptide pairs, CD measurements were performed at 20 µM concentration. Samples of CCPO triangles were measured at approximately 6 µM concentration. The proteins SBP[Zn]₁ and SBP[Zn]₂ were measured at approximatively 1 mg/ml, while the proteins SBP[Zn]₁ and SBP[Zn]₂ were injected at a concentration of approximately 0.5 mg/ml. Accordingly, a different concentration of ZnCl₂ was added to the mobile phase, 500 µM for triangular proteins and 20 µM for proteins SBP[Zn]₁ and SBP[Zn]₂. Analysis of the peaks of interest was conducted with Astra 7.0 software (Wyatt, CA, USA).

**Small-angle x-ray scattering**

SAXS experiments were performed at the P12 beamline that is part of the PETRA-III synchrotron (DESY, Hamburg, Germany). To avoid absorption by Zn(II), x-ray wavelength was 1.38 Å. Scattering intensity was recorded in the range from 0.028 to 6.6 nm⁻¹ with the Pilatus 6M detector, placed 3 m from the sample. Batch measurements were performed with the robotic sample handler (58) in flow-through mode to avoid radiation damage. Sample volume was 40 µl. Buffer scattering was collected before and after each sample and used for background subtraction. For each sample and buffer, scattering was recorded over 40 frames lasting 0.05 s. Frames without any radiation damage were automatically averaged (59). To determine the structure of CCPO triangles in the absence of Zn(II) ions, a dilution series consisting of at least four concentrations (~1, 2, 4, and 8 mg/ml) was prepared and measured for each protein variant. In addition, scattering was recorded at different Zn(II) concentrations (0, 30, 100, 300, and 500 µM). Before SAXS measurements, 100 µl of protein sample (1 mg/ml) was dialyzed against 1 liter of Hepes-based buffer (50 mM Hepes, 150 mM NaCl, 1 mM TCEP, and 10% glycerol, pH 7) at target ZnCl₂ concentration. The structure of the complex SBP[Zn]₁:SBP[Zn]₂ was analyzed with SEC-SAXS. Mobile phase (50 mM Hepes, 150 mM NaCl, 1 mM TCEP, 100 µM ZnCl₂, and 10% glycerol, pH 7.5) was flowed through the column (Superdex 200 increase 10/300; GE Healthcare, IL, USA) at a flow of 0.4 ml/min. A total of 3600 scattering frames were collected with exposure time of 0.995 s. ATSAS software (60) was used for merging and data analysis. Ab initio modeling of molecular envelopes was performed in two steps. First, 20 structures were generated with DAMMIF and were subsequently averaged with DAMMCTR. The averaged model was lastly refined with DAMMIN. PepSiSAXS (61) was used to compare experimental scattering profiles to model structures generated with the CoCoPOD software.
EOM 2.0 was used to analyze the conformational flexibility of triangular constructs in the absence of Zn(II) ions. A total of 10,000 random chain configurations were generated, and genetic algorithm was run 50 times to obtain a good ensemble fit. The pool of partially folded triangular structures, differing in the number of assembled CC pairs, was generated with the CoCoP0D software. Because the unpaired peptides likely do not have helical secondary structure, the models were subjected to a short molecular dynamics (MD) optimization, run at an elevated temperature (500 K) to unfold the unpaired peptides. The assembled CCs were treated as rigid bodies. Models both before and after MD optimization, totaling 210 structures, were fit to experimental scattering profiles to identify a single best matching structure.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at https://science.org/doi/10.1126/supplementary-materials.

REFERENCES AND NOTES
View/request a protocol for this paper from Supplementary material for this article is available at https://science.org/doi/10.1126/supplementary-materials.

5. J. H. Shin, H. J. Jung, Y. J. An, Y. B. Cho, S. S. Cha, J. H. Roe, Graded expression of zinc-responsive genes through two regulatory zinc-binding sites in Zur. Proc. Natl. Acad. Sci. U.S.A. 108, 10504–10509 (2011).

6. W. Qiao, M. Mooney, A. J. Bird, D. R. Winge, D. J. Eide, Zinc binding to a regulatory zinc-sensing domain monitored in vivo by using FRET. Proc. Natl. Acad. Sci. U.S.A. 103, 8674–8679 (2006).

7. C. Eicken, M. A. Pennella, X. Chen, K. M. Koshlap, L. M. VanZile, J. C. Sacchettini, D. P. Giedroc, A metal-ligand-mediated intersubunit allosteric switch in related SmtB/Arsl zinc sensor proteins. J. Biol. Chem. 333, 683–695 (2003).

8. M. McMahan, S. R. Swift, J. D. Haynes, Zinc binding triggers a conformational-switch in the cullin-3 substrate adapter protein KEAP1 that controls transcription factor Nrf2. J. Biol. Chem. 283, 45–57 (2018).

9. J. M. Fletcher, R. L. Harniman, F. R. H. Barnes, A. L. Boyle, A. Collins, J. Mantell, T. H. Sharp, M. Antogniazi, P. J. Booth, N. Linden, M. J. Miles, R. B. Sessions, P. Verkade, D. N. Woolfson, Self-assembly cages from coiled-coil peptide modules. Science 340, 595–599 (2013).

10. Z. Chen, M. C. Johnson, J. Chen, M. J. Bick, S. E. Boyken, B. Lin, J. J. de Yoreo, J. M. Kollman, D. Baker, F. DiMaio, Self-assembly 2D arrays with de novo protein building blocks. J. Am. Chem. Soc. 141, 8891–8895 (2019).

11. J. P. M. Vrancken, J. P. M. Vrancken, J. Aupič, A. Caddy, R. Jerela, J. R. H. Tanre, A. R. D. Voet, Molecular assemblies built with the artificial protein Pizza. J. Struct. Biol. X 4, 100027 (2020).

12. D. N. Woolfson, The design of coiled-coil structures and assemblies. Adv. Protein Chem. 79, 70–112 (2015).

13. I. Drobnak, H. Gradlari, A. Ljubetić, E. Merljak, R. Jerela, Modulation of coiled-coil dimer stability through surface residues while preserving pairing specificity. J. Am. Chem. Soc. 139, 8229–8236 (2017).

14. D. N. Woolfson, Coiled-coil design: Updated and upgraded. Subcell. Biochem. 82, 35–61 (2017).

15. H. Gradlari, S. Bodić, T. Doles, D. Vengust, I. Hafner-Bratkovič, A. Mertelj, B. Webb, A. Šali, S. Klavžar, R. Jerela, Design of a single-chain polypeptide tetrahedron assembly from coiled-coil segments. Nat. Chem. Biol. 9, 362–366 (2013).

16. A. Ljubetić, F. Lapenta, H. Gradlari, I. Drobnak, J. Aupič, Ž. Strmšek, D. Lainšček, I. Hafner-Bratkovič, A. Majerle, N. Krivec, M. Benčina, T. Pisanc, T. C. Velčišek, A. Round, J. M. Carazo, R. Melero, R. Jerela, Design of coiled-coil protein-origami cages that self-assemble in vitro and in vivo. Nat. Biotechnol. 35, 1094–1101 (2017).

17. F. Lapenta, J. Aupič, M. Mezzioli, Ž. Strmšek, S. D. Vela, D. I. Svergun, J. M. Carazo, R. Melero, R. Jerela, Self-assembly and regulation of protein cages from pre-organised coiled-coil modules. Nat. Commun. 12, 939 (2021).
48. J. R. Casey, S. Grinstein, J. Orłowski, Sensors and regulators of intracellular pH. *Nat. Rev. Mol. Cell Biol.* 11, 50–61 (2010).

49. E. De Santis, M. G. Ryadnov, Peptide self-assembly for nanomaterials: The old kid on the block. *Chem. Soc. Rev.* 44, 8288–8300 (2015).

50. T. Fink, J. Lonzaric, A. Pnaznik, T. Plaper, E. Merljak, K. Leben, N. Jerala, T. Lebar, Ž. Strmšek, F. Lapenta, M. Benčina, R. Jerala, Design of fast proteolysis-based signaling and logic circuits in mammalian cells. *Nat. Chem. Biol.* 15, 115–122 (2019).

51. C. W. Wood, J. W. Heal, A. R. Thomson, G. J. Bartlett, A. Á. Ibarra, R. L. Brady, R. B. Sessions, D. N. Woolfson, ISAMBARD: An open-source computational environment for biomolecular analysis, modelling and design. *Bioinformatics* 33, 3043–3050 (2017).

52. D. G. Gibson, L. Young, R. Y. Chuang, J. C. Venter, C. A. Hutchison III, H. O. Smith, Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* 6, 343–345 (2009).

53. C. Robichon, J. Luo, T. B. Causey, J. S. Benner, J. C. Samuelson, Engineering Escherichia coli BL21(DE3) derivative strains to minimize E. coli protein contamination after purification by immobilized metal affinity chromatography. *Appl. Environ. Microbiol.* 77, 4634–4646 (2011).

54. U. K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685 (1970).

55. M. R. Wilkins, E. Gasteiger, A. Bairoch, J. C. Sanchez, K. L. Williams, R. D. Appel, D. F. Hochstrasser, Protein identification and analysis tools in the ExPASy server. *Methods Mol. Biol.* 112, 531–552 (1999).

56. P. Virtanen, R. Gommers, T. E. Oliphant, M. Haberland, T. Reddy, D. Cournapeau, E. A. Quintero, C. R. Harris, A. M. Archibald, A. H. Ribeiro, F. Pedregosa, P. van Mulbregt; Y. Feng, E. W. Moore, J. V. Plas, D. Laxalde, J. Perktold, R. Cimrman, I. Henriksen, K. J. Millman, N. Mayorov, A. R. J. Nelson, E. Jones, R. Kern, E. Larson, C. J. Carey, İ. Polat, D. F. Hochstrasser, Protein identification and analysis tools in the ExPASy server. *Nat. Methods* 112, 531–552 (1999).

57. Y. H. Chen, J. T. Yang, K. H. Chau, Determination of the helix and β form of proteins in aqueous solution by circular dichroism. *Biochemistry* 13, 3350–3359 (1974).

58. A. Round, F. Felisaz, L. Fodinger, A. Gobbo, J. Huet, C. Villard, E. A. Blanchet, P. Pernot, S. McSweeney, M. Roessle, D. I. Svergun, BioSAXS Sample Changer: A robotic sample changer for rapid and reliable high-throughput x-ray solution scattering experiments. *Acta Crystallogr. D Struct. Biol.* 71, 67–75 (2015).

59. D. Franke, A. G. Kikhney, D. I. Svergun, Automated acquisition and analysis of small angle x-ray scattering data. *Nucl. Instrum. Methods Phys. Res. A* 689, 52–59 (2012).

60. D. Franke, M. V. Petoukhov, P. V. Konarev, A. Panjkovich, A. Tuukkanen, H. D. T. Mertens, A. G. Kikhney, N. R. Hajizadeh, J. M. Franklin, C. M. Jeffries, D. I. Svergun, ATSAS 2.0: A comprehensive data analysis suite for small-angle scattering from macromolecular solutions. *J. Appl. Cryst.* 50, 1212–1225 (2017).

61. S. Grudinin, M. Garkavenko, A. Kazennov, Pepsi-SAXS: An adaptive method for rapid and accurate computation of small-angle x-ray scattering profiles. *Acta Crystallogr. D Struct. Biol.* 73, 449–464 (2017).

62. J. A. Nelder, R. Mead, A simplex method for function minimization. *Comput. J.* 7, 308–313 (1965).

63. C. Zhu, R. H. Byrd, P. Lu, J. Nocedal, Algorithm 778: L-BFGS-B: Fortran subroutines for large-scale bound-constrained optimization. *ACM Trans. Math. Softw.* 23, 550–560 (1997).

64. E. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng, T. E. Ferrin, UCSF Chimera—A visualization system for exploratory research and analysis. *J. Comput. Chem.* 25, 1605–1612 (2004).

Acknowledgments: The synchrotron SAXS data were collected at beamline P12 operated by EMBL Hamburg at the PETRA III storage ring (DESY, Hamburg, Germany). We would like to thank S. Da Vela for the assistance in using the beamline. Funding: This research was supported by Slovenian Research Agency projects P4-0176, J1-1711, J1-2481, and J1-9173, European Research Council (ERC) Advanced Grant project MaCCChines grant agreement ID: 899259 (R.J.), INExT, grand number 653706 (PID: 2437, 2706), and ONR, grant number N62909-20-1-2090. Author contributions: Conceptualization: J.A., F.L., Ž.S., E.M., and T.P. Investigation: J.A., F.L., Ž.S., E.M., and T.P. Visualization: J.A. Supervision: R.J. Writing—original draft: J.A. and R.J. Writing—review and editing: J.A., F.L., Ž.S., E.M., and R.J. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.