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Research Article

Cell-free protein synthesis enables high yielding synthesis of an active multicopper oxidase

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Multicopper oxidases (MCOs) are broadly distributed in all kingdoms of life and perform a variety of important oxidative reactions. These enzymes have potential biotechnological applications; however, the applications are impeded by low expression yields in traditional recombinant hosts, solubility issues, and poor copper cofactor assembly. As an alternative to traditional recombinant protein expression, we show the ability to use cell-free protein synthesis (CFPS) to produce complex MCO proteins with high soluble titers. Specifically, we report the production of MCOs in an *Escherichia coli*-based cell-free transcription-translation system. Total yields as high as 1.2 mg mL^{-1} were observed after a 20-h batch reaction. More than 95% of the protein was soluble and activity was obtained by simple post-CFPS addition of copper ions in the form of $CuSO_4$. Scale-up reactions were achieved from 15 to 100 µL without a decrease in productivity and solubility. CFPS titers were higher than in vivo expression titers and more soluble, avoiding the formation of inclusion bodies. Our work extends the utility of the cell-free platform to the production of active proteins containing copper cofactors and demonstrates a simple method for producing MCOs.

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1 Introduction

Cell-free protein synthesis (CFPS) is emerging as a robust platform for fundamental and applied research in the areas of synthetic biology and biotechnology [1, 2]. CFPS has been developed with several different organisms (e.g. *E. coli*, wheat germ, rabbit reticulocytes, CHO cells, yeast, and insect cells) to produce various proteins including therapeutic vaccines, virus-like particles, and membrane proteins [3–26]. CFPS is also being used for high-throughput functional and structural studies of a wide variety of proteins [27, 28], as well as more recently for rapid prototyping of biological circuits [29–35]. Although different CFPS systems have their own advantages, the *E. coli*-based system is the most commonly used due to fast cell growth of the source strain, inexpensive and streamlined extract preparation, and generally high protein yields. Because of the absence of cell walls in cell-free systems, cofactors (e.g. metal ions) and substrates are easily added to the reaction and the expression environment is highly controlled. This enables the production of fully active complex biocatalysts. The *E. coli*-based platform has successfully been used to produce enzymes with metal cofactors such as [FeFe] hydrogenases [36–40] and P450 monooxygenase [41]. Using multicopper oxidases as a

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Abbreviations: CFPS, Cell-free protein synthesis; **MCO**, Multicopper oxidase

model, we investigated the use of cell-free biosynthesis to produce copper enzymes.

Multicopper oxidases (MCOs) are a well-characterized group of enzymes that couple the reduction of dioxygen to water with the oxidation of a broad range of substrates [42]. Because of their broad activity, they have attracted significant attention for use in a variety of biotechnological applications including wastewater decolorization, pulp delignification, and enzymatic fuel cells [43]. Natively, MCOs are found in all kingdoms of life and are known to function in plant development, metal homeostasis, and fungal delignification [44]. To perform their function, MCOs require four copper atoms arranged in two distinct metal centers, a mononuclear type 1 copper and a trinuclear copper cluster formed by a type 2 copper and a pair of type 3 coppers [45]. Substrates are oxidized at the type 1 copper center and electrons are then transferred to the trinuclear copper cluster where oxygen is reduced to water [45].

MCOs were chosen for this study because their reported in vivo expression yields tend to be low and copper incorporation can be challenging [46]. In the present work, two bacterial MCOs from *Nitrosomonas europaea* (NeMCO) and *Streptomyces coelicolor* (ScMCO) and two archaeal MCOs from *Haloarcula marismortui* (HmMCO) and *Nitrosopumilus maritimus* (NmMCO) were synthesized. Using the *E. coli* CFPS platform, three of the four MCOs had final yields of more than 1 mg mL^{-1}. Significant amounts of copper (100 μM) can be present during synthesis without impacting expression levels; however, given the high expression levels of the MCO this is not sufficient to completely metal load the protein. We found that $CuSO_4$ stimulates MCO activity by post-translational addition after CFPS. To the best of our knowledge, this is the first report on cell-free synthesis of an active MCO in the *E. coli*-based system. Our results, therefore, expand the ever-growing utility of CFPS techniques to include the production of MCOs and proteins with copper cofactors.

2 Materials and methods

2.1 Bacterial strains and plasmids

Highly active S30 crude extracts were generated from a genomically recoded release factor 1 (RF1) deficient *E. coli* strain (*E. coli* C321.ΔA.705) [47]. Multicopper oxidase genes from *Haloarcula marismortui* (HmMCO, accession no. WP_004594571), *Nitrosopumilus maritimus* (NmMCO, accession no. WP_012215737) and *Streptomyces coelicolor* (ScMCO, accession no. WP_003972284) were codon optimized with *E. coli* class 2 codons, native secretion signals were omitted, and synthesized as gBlocks® (IDT Inc., Coralville, IA, USA). The *Nitrosomonas europaea* gene (NeMCO accession no. WP_011111538) was not codon optimized, but it was also synthesized using gBlocks®. The gBlocks® were incorporated into the vector pY71 using Gibson Assembly [48] and the DNA sequence verified (Eurofins Genomics, Huntsville, AL, USA). The four MCO genes were cloned in frame with a C-terminal tag comprised sequentially of a TEV cleavage site, $(Gly_3Ser)_2$ linker, β-strand 11 of GFP [49], a Gly_3Ser linker and a Strep-tag. The GFP11 sequence was inserted before the Strep-tag to facilitate a future study for high-throughput screening soluble expression of MCO variants. The following plasmids were tested in in vitro CFPS assays: pY71-NeMCO-TGS, pY71-HmMCO-TGS, pY71-NmMCO-TGS and pY71-ScMCO-TGS.

2.2 Preparation of cell extracts

E. coli cells were grown in 1 L of 2xYTPG (yeast extract 10 g L^{-1}, tryptone 16 g L^{-1}, NaCl 5 g L^{-1}, K_2HPO_4 7 g L^{-1}, KH_2PO_4 3 g L^{-1} and glucose 18 g L^{-1}, pH 7.2) in a 2.5-L Tunair flask (IBI Scientific, Peosta, IA) at 34°C and 220 rpm with inoculation of 20 mL overnight cultures (initial OD_{600} of ~0.05). Cell growth, collection and extracts were prepared as described by Kwon and Jewett [50].

2.3 CFPS reactions

Standard CFPS reactions were performed using the PANOx-SP system as previously described [51]. For copper loading, $CuSO_4$ was added to the standard system with final concentrations of 10, 20, 50, 100, 200 and 500 μM. For scale-up reactions with 50 and 100 μL mixture, the reactions were carried out in 2 mL microcentrifuge tubes. All CFPS reactions were incubated for 20 h at 30°C.

2.4 Quantification of synthesized MCOs

The yields of synthesized MCOs were quantified by the incorporation of ^{14}C-leucine into trichloroacetic acid-precipitable radioactivity using a liquid scintillation counter (MicroBeta2, PerkinElmer, Waltham, MA) as described previously [52]. Total protein yields were measured directly after cell-free reactions with the 15 μL mixture. To determine the soluble protein yield, 15 μL of the reaction mixture was centrifuged at 12 000 ×*g* and 4°C for 15 min [53]. 10 μL of the supernatant were taken out for soluble MCO quantification.

2.5 Autoradiography analysis

After the cell-free reaction, 3 μL of each sample was loaded on a 4–12% NuPAGE SDS-PAGE gel (Invitrogen). The gel was stained using SimplyBlue™ SafeStain solution (Invitrogen) and destained in water. Then, the gel was fixed with cellophane films (Bio-Rad), dried overnight in a GelAir Dryer (Bio-Rad) without heating, and exposed for 48 h on a Storage Phosphor Screen (GE Healthcare

Biosciences, Pittsburgh, PA). The autoradiogram was scanned using a Storm Imager (GE Healthcare Biosciences, Pittsburgh, PA) and analyzed using Quantity One software (Bio-Rad, Hercules, CA).

2.6 MCO activity assay

MCO activity was measured colorimetrically by following oxidation of ABTS (ε = 36.8 $mM^{-1}cm^{-1}$ at 405 nm), which turns blue upon oxidation [54]. Colorimetric assays were performed in 1 mL of 100 mM Britton-Robinson buffer at pH 4.0 containing 4 mM ABTS using an Agilent 4853 spectrophotometer. All cell-free reactions were diluted 10-fold and incubated with 0 to 5 mM $CuSO_4$ or 0 to 500 µM $Cu(CH_3CN)_4PF_6$ and at room temperature for 2 h. In cases where $Cu(CH_3CN)_4PF_6$ was added, the reaction was transferred into an anaerobic Coy chamber and allowed to equilibrate for 2 h prior to addition of copper. All reactions were initiated by addition of 40 to 80 µL of diluted cell-free samples and reported values are averaged from at least three replicates. All reactions were performed at room temperature.

2.7 In vivo expression of MCOs

MCOs were expressed in *E. coli* strain BL21(DE3) and grown in homemade autoinduction media [55]. Cells were grown at 37°C to an OD_{600} of ~0.6 and then moved to 20°C for 16 h. Prior to harvest cells were incubated with 5 mM $CuSO_4$ for 2 h and then centrifuged for 10 min at 6 400 ×*g* and 4°C. Cells were suspended in 50 mM TRIS pH 8.0 and 500 mM NaCl and lysed by sonication for 8 min with 1 s pulses and 3 s resting time. Lysates were centrifuged for 1 h at 185 000 ×g and 4°C and the supernatants applied to a streptactin column (IBA Life Sciences). MCOs were eluted with 50 mM TRIS pH 8.0, 500 mM NaCl, and 5 mM *d*-desthiobiotin and then concentrated in Amicon centrifugal devices with a 30 kDa nominal molecular weight cutoff (EMD Millipore). The concentrated MCOs were then further purified on a Superdex 200 Increase (GE Healthcare Life Sciences) size exclusion column equilibrated with 50 mM TRIS pH 8.0 and 500 mM NaCl. Final yields of the MCOs were determined using their extinction coefficients at 280 nm.

3 Results and discussion

3.1 CFPS enables soluble high-level expression of four MCOs

The *E. coli*-based cell-free system used in this work is similar to a previously published report [51], with only minor modifications (see above). The reactions were allowed to run for 20 h in batch operation and the yields of cell-free synthesized MCOs were quantified by monitoring ^{14}C-leucine incorporation. As shown in Fig. 1A, the best expressing variant, NeMCO, gave a protein yield of 1.2 ± 0.1 mg mL^{-1} (>95% soluble), while ScMCO yields reached less than 0.2 mg mL^{-1}. HmMCO and NmMCO also reached high final yields of ~1 mg mL^{-1}; however, their soluble fractions were lower than NeMCO, constituting 36 and 70% of the total protein expression, respectively. These yields are higher than those typically

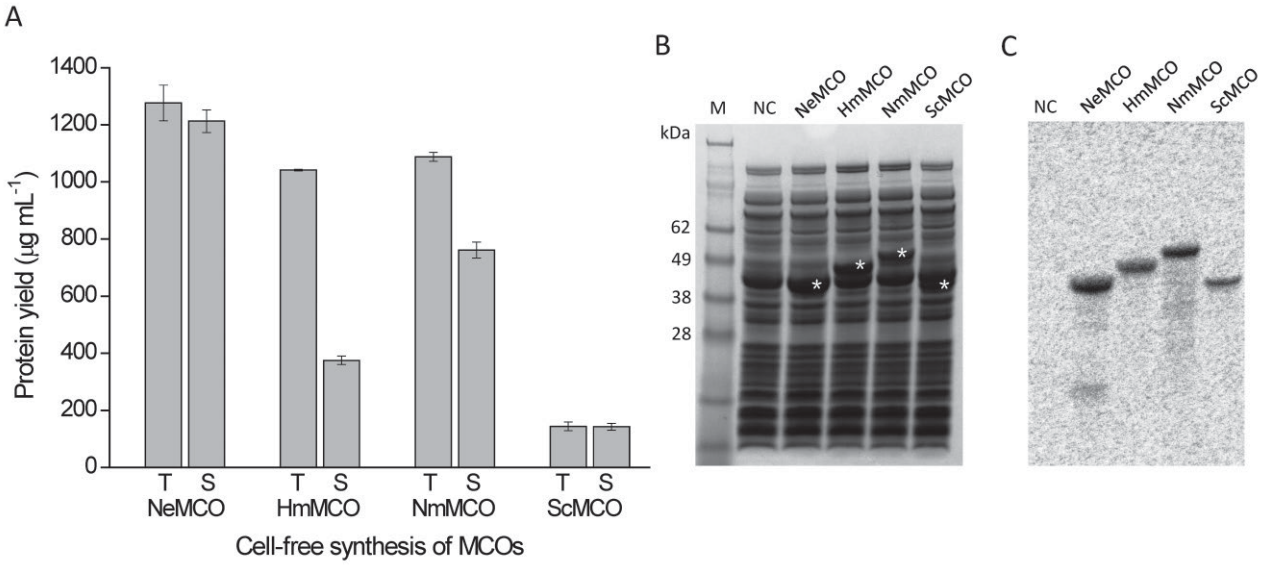

Figure 1. Cell-free protein synthesis of two bacterial and two archaeal MCOs. (**A**) Protein yields of MCOs. T, total protein; S, soluble protein. Error bars represent standard deviations from three independent samples. (**B**) SDS-PAGE analysis of cell-free produced MCOs. Each MCO is indicated by an asterisk. Expected molecular weight: NeMCO (41.1 kDa), HmMCO (43.5 kDa), NmMCO (51.6 kDa), ScMCO (39.4 kDa). M, protein molecular weight marker; NC, negative control without plasmid in the reaction. (**C**) Autoradiogram of radioactive labeled proteins.

Table 1. Examples of MCOs heterologously expressed in vivo

MCO[a)]	Source	Expression host	Titer (mg L^{-1})	Reference
NeMCO	*Nitrosomonas europaea*	*E. coli*	42.0 ± 14[b)]	This Study
HmMCO	*Haloarcula marismortui*	*E. coli*	Inclusion bodies[b)]	This Study
NmMCO	*Nitrosopumilus maritimus*	*E. coli*	Inclusion bodies[b)]	This Study
ScMCO	*Streptomyces coelicolor*	*E. coli*	11.0 ± 4.1[b)]	This Study
Laccase	*Bacillus subtilis*	*E. coli*	20	[56]
Laccase	*Aeromonas hydrophila*	*E. coli*	10	[57]
Laccase	*Streptomyces coelicolor*	*Streptomyces lividans*	350	[63]
Laccase	*Myceliophthora thermophila*	*Saccharomyces cerevisiae*	18	[64]
Laccase	*Schizophyllum commune*	*Pycnoporus cinnabarinus*	1200	[65]

a) Laccase belongs to the multicopper oxidase family (MCO).
b) Yield after metal loading and growth in autoinduction media. In all cases significant quantities of inclusion bodies were observed in addition to the reported soluble expression yields.

observed in vivo. For example, the expression levels of laccases (belonging to the MCO family) from *Bacillus subtilis* and *Aeromonas hydrophila* in *E. coli* are ~20 mg L^{-1} and ~10 mg L^{-1}, respectively [56, 57]. The fact that different MCOs from different organisms expressed at different titers was not surprising, as expression levels are known to be highly dependent on several gene sequence features, such as the position dependent codon usage, ribosome binding site, mRNA structure, etc. [58]. Even with different titers, expressed MCOs could be identified from the *E. coli* endogenous proteins using SDS-PAGE gels. These results show that the correct molecular weights were obtained (Fig. 1B) and were further confirmed by autoradiogram analysis (Fig. 1C). Because NeMCO produced the highest soluble yields, subsequent analysis focused on it as a target.

To directly compare our CFPS expression system to conventional in vivo strategies, the four MCO constructs were also expressed in living *E. coli* (Table 1). In vivo expression yields for all constructs were less than 50 mg L^{-1}, which is lower than that obtained with the *E. coli*-based CFPS system. Notably, two of the constructs, HmMCO and NmMCO, form only inclusion bodies in vivo, whereas they are soluble in vitro. This highlights a potential advantage for CFPS. We believe the increased solubility in vitro may arise from the dilute total protein environment in the extract (~10 mg mL^{-1} total *E. coli* protein) as compared to the cytoplasm (~200–300 mg mL^{-1} total *E. coli* protein), which helps favor protein folding over aggregation.

3.2 Effect of scalability and copper concentration on CFPS yields of NeMCO

To demonstrate the potential application of CFPS of MCOs, the reaction volume of NeMCO was scaled from 15 to 100 µL. While the reaction volume increased more than six times compared to the standard reaction, NeMCO expression levels were similar to small scale reactions with total yields of ~1.2 mg mL^{-1} (Fig. 2A). Importantly, approximately 95% of the expressed protein remained soluble.

Copper is necessary for reconstitution of the MCO's activity. To this end, the effect of copper concentration

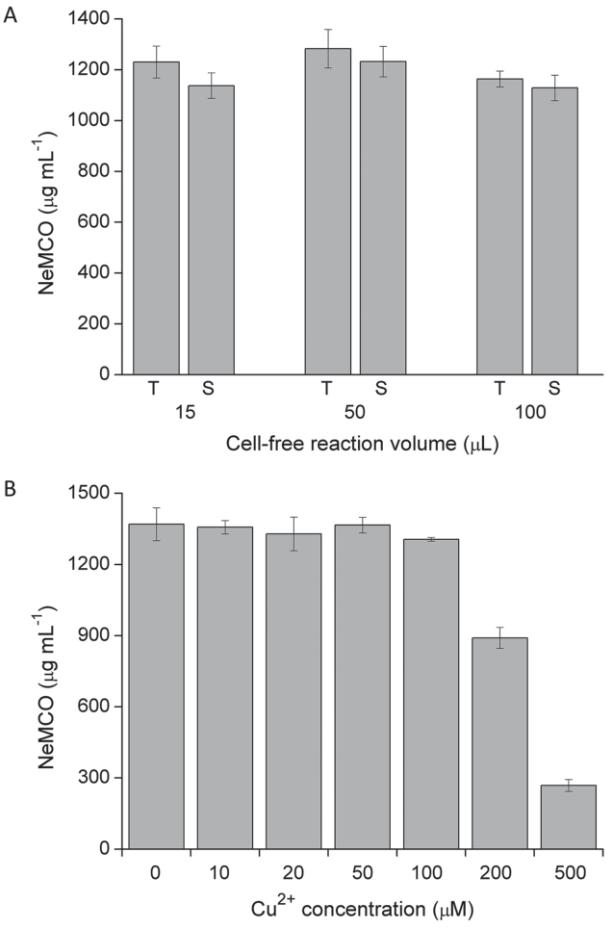

Figure 2. (**A**) Cell-free protein synthesis of NeMCO with scale-up systems. T, total NeMCO; S, soluble NeMCO. (**B**) Effect of Cu^{2+} on NeMCO synthesis in the cell-free system. Error bars represent standard deviations from three independent samples.

on expression levels was studied using NeMCO as copper is toxic for many bacteria. At moderate concentrations, 0 to 100 µM, there is no obvious decrease in protein yield (Fig. 2B), suggesting some benefits for CFPS avoiding toxicity that occurs in cells. However, at high concentrations of copper, 200 µM and above, expression drops significantly and copper appears to be toxic to the combined transcription-translation system. Consistent with the hypothesis that copper may be toxic to CFPS, we found that copper concentrations of 100 µM reduced expression of superfolder green fluorescent protein (which does not have copper cofactors) four-fold as compared to reactions without supplemented copper (data not shown). Our results suggest that copper appears to be toxic to CFPS at high concentrations, but less toxic when manufacturing copper-containing proteins, which likely sequester the copper.

3.3 Cell-free synthesized NeMCO is active, and stimulated by post-translational copper addition

To verify that the cell-free synthesized NeMCO was active, assays were performed by following the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), a non-native substrate, which is blue in its oxidized form. Initially these experiments focused on NeMCO that was synthesized in the presence of 100 µM copper. Our results indicate that synthesis in 100 µM copper is not sufficient to obtain active protein but post-translational addition of $CuSO_4$ stimulates activity (Fig. 3). In cell-free reactions the final concentration of NeMCO is ~30 µM and each monomer requires at least four coppers. It is therefore not unexpected that NeMCO is not active immediately following the cell-free reaction since there is not enough copper in solution to fully load the enzyme. To test if post-translational addition of copper stimulates activity, reactions were diluted and Cu(II) in the form of $CuSO_4$ was added (Fig. 3). Our data show that activity is stimulated by relatively low concentrations of copper and is diminished if too much copper is added. Diminished activity is likely due to protein precipitation caused by excess copper ions. Importantly, we do not observe copper acting as a catalyst alone to oxidize the substrate. Negative controls with similarly prepared CFPS reactions without plasmids were performed with and without copper and oxidation of the dye was not observed.

Previous reports suggest that maximal activity of MCOs is obtained when copper is loaded as Cu(I) [56]. To test this possibility, cell-free reactions were taken into an anaerobic Coy chamber and incubated with a Cu(I) compound, $Cu(CH_3CN)_4PF_6$, in a similar fashion as the $CuSO_4$ experiments. These data show that incubating with Cu(I) versus Cu(II) results in similar levels of activity. Cu(I) could not be tested at concentrations above 500 µM because at these concentrations direct reduction of ABTS by unbound Cu(I) is observed preventing reliable data collection. Unfortunately, the current specific activity of NeMCO is ~10% the rate of the same protein produced in vivo lacking a C-terminal tag [59]. We attribute this diminished rate to the large C-terminally encoded tag, which has been previously shown to greatly reduce MCO activity [60]. Indeed, the lower specific activity in the CFPS system matches the activity of in vivo expressed NeMCO in *E. coli* with the same C-terminal tag (data not shown). Thus, our work provides a promising route to discovery-based applications that require rapid, low-cost protein expression, such as high-throughput screening of soluble MCO variants.

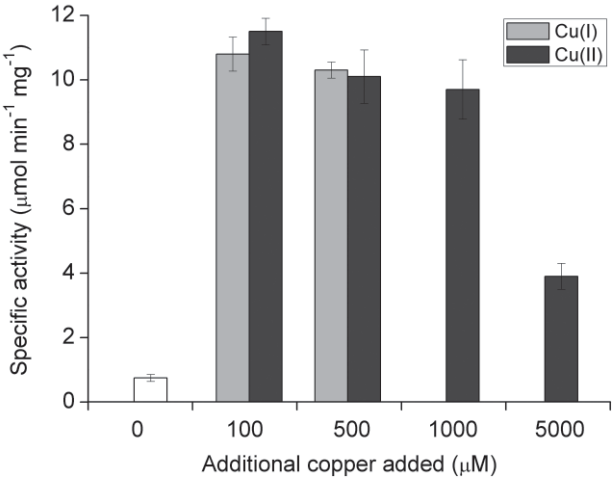

Figure 3. Effect of copper concentration on specific activity. White, no additional copper added; light gray, addition of Cu(I) as $Cu(CH_3CN)_4PF_6$; dark gray, addition of Cu(II) as $CuSO_4$. Error bars represent standard deviations from three independent samples.

4 Concluding remarks: CFPS offers a new approach for producing MCOs at high titers

In conclusion, multiple MCOs were expressed in an *E. coli*-based CFPS system with high yields (>1 mg mL^{-1}). Scale-up reactions were achieved from 15 to 100 µL without decreasing productivity and solubility. Importantly, MCO activity can be obtained by simple addition of copper ions post-translation and our work also establishes a threshold for copper tolerance in *E. coli*-based CFPS. Our work is important because it allows for expression levels that are adequate for testing MCO activity, setting the stage for a high-throughput expression platform that can be executed in a day through the use of PCR templates, as compared to days to weeks in vivo [6].

Indeed, our approach may provide some potential advantages for high yielding expression in high-throughput as compared to existing state of the art. As summarized in the review paper [61], different heterologous

hosts have been used to express MCOs (mostly laccases) including bacteria systems (e.g. *E. coli* and *Streptomyces lividans*), yeast systems (e.g. *Saccharomyces cerevisiae, Pichia pastoris*, etc.), and filamentous fungi systems (e.g. *Aspergillus niger*, *Trichoderma reesei*, etc.). Previous expression titers are typically lower than those observed here (~10–50 mg L^{-1}). A summary of these results, including the in vivo expression of the constructs expressed in vitro in this study, can be found in Table 1.

Looking forward, opportunities to improve the CFPS system include gaining a better understanding of the copper loading conditions, enabling efficient glycosylation that is required for some archaeal enzymes [62], and demonstrating higher preparative reaction scales of active MCOs. However, we note that the *E. coli* CFPS system has already been shown to scale linearly from µL reactions to the 100 L scale, an expansion factor of 10^6, to enable manufacturing scale synthesis of complex proteins [26]. We therefore anticipate that CFPS systems will provide a new avenue for producing MCOs in the future.

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The authors declare no financial or commercial conflict of interest.

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