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Development of a CHO-Based Cell-Free Platform for Synthesis of Active Monoclonal Antibodies

Rey W. Martin,[†,‡] Natalia I. Majewska,[†] Cindy X. Chen,[†] Thomas E. Albanetti,[∥] Rod Brian C. Jimenez,[⊥] Albert E. Schmelzer,[∥] Michael C. Jewett,*[,†,‡,§,#,▽] and Varnika Roy*[,∥]

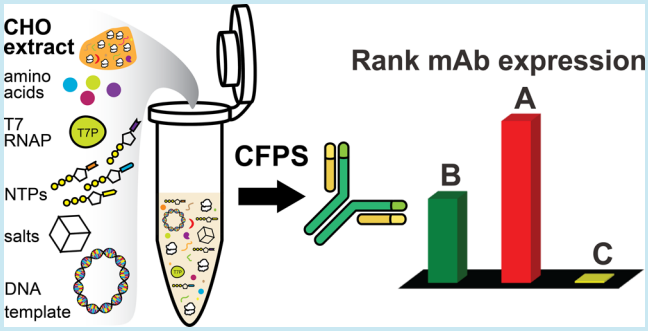
[†]Department of Chemical and Biological Engineering, [‡]Chemistry of Life Processes Institute, and [§]Interdisciplinary Biological Sciences Program, Northwestern University, Evanston, Illinois 60208, United States

[∥]Cell Culture and Fermentation Sciences and [⊥]Analytical Sciences, MedImmune LLC, Gaithersburg, Maryland 20878, United States

[#]Member, Robert H. Lurie Comprehensive Cancer Center and [▽]Member, Simpson Querrey Institute, Northwestern University, Chicago, Illinois 60611, United States

Supporting Information

ABSTRACT: Chinese Hamster Ovary (CHO) cells are routinely optimized to stably express monoclonal antibodies (mAbs) at high titers. At the early stages of lead isolation and optimization, hundreds of sequences for the target protein of interest are screened. Typically, cell-based transient expression technology platforms are used for expression screening, but these can be time- and resource-intensive. Here, we have developed a cell-free protein synthesis (CFPS) platform utilizing a commercially available CHO extract for the rapid *in vitro* synthesis of active, aglycosylated mAbs. Specifically, we optimized reaction conditions to maximize protein yields, established an oxidizing environment to enable disulfide bond formation, and demonstrated the importance of temporal addition of heavy chain and light chain plasmids for intact mAb production. Using our optimized platform, we demonstrate for the first time to our knowledge the cell-free synthesis of biologically active, intact mAb at >100 mg/L using a eukaryotic-based extract. We then explored the utility of our system as a tool for ranking yields of candidate antibodies. Unlike stable or transient transfection-based screening, which requires a minimum of 7 days for setup and execution, results using our CHO-based CFPS platform are attained within 2 days and it is well-suited for automation. Further development would provide a tool for rapid, high-throughput prediction of mAb expression ranking to accelerate design−build−test cycles required for antibody expression and engineering. Looking forward, the CHO-based CFPS platform could facilitate the synthesis of toxic proteins as well.



KEYWORDS: *Chinese Hamster Ovary, monoclonal antibody, antibody ranking tool, cell-free protein synthesis, synthetic biology*

High yielding therapeutic protein production has transformed the biopharmaceutical industry and our ability to treat illnesses including cancer. Despite the prevalence of a variety of mammalian cell lines available for recombinant protein production, Chinese Hamster Ovary (CHO) cells dominate the industry for monoclonal antibody (mAb) production.[1] Nearly 70% of all therapeutic proteins are produced in CHO cells, as production can be easily scaled up with protein titers reaching over 5 g/L,[2] and many of these proteins have been awarded regulatory approval over the past 3 decades.[2,3] *In vivo* CHO-based (herein referring to intact, viable cells) production technologies have shown great success for the production of therapeutic proteins; however, these methodologies are unwieldy, and construction and expression of stable cells lines expressing recombinant mAbs can take several weeks to execute.[4] With the increase in demand of protein-based biologics, high-yielding screening technologies for the rapid production of new kinds of therapeutic proteins will prove valuable.[5] Cell-based mAb bioprocessing timelines, including those leveraging CHO cells, would greatly benefit from a technology that could expedite synthesis and screening efforts for mAb characterization.[6]

As a complementary approach to *in vivo* CHO-based technologies for mAb screening, we propose that cell-free protein synthesis (CFPS) could help alleviate the screening bottleneck for producing new mAb therapeutics. We feel that, given the use of CHO cells for the synthesis of mAbs and biologics in an industrial setting, a CHO-based cell-free platform would best mimic the biological context in which these biologics would be synthesized as opposed to other commercially available systems (*e.g.*, *E. coli*-based cell-free system and Human Coupled IVT Kit). The key idea is that one could use cell-free systems to prototype genetic design

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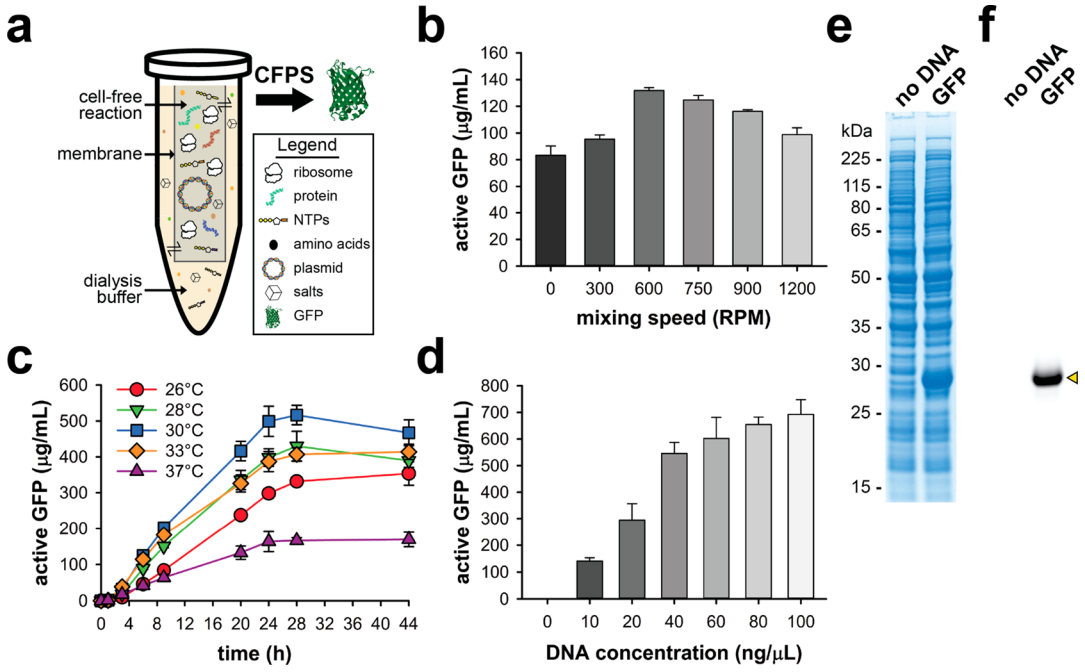

Figure 1. Optimizing CHO-based semicontinuous cell-free reaction parameters. (a) Schematic of CHO-based cell-free protein synthesis (CFPS) reactions performed in a semicontinuous format where the reaction chamber includes plasmid DNA to direct the transcription and translation of a desired protein. The dialysis buffer solution is mixed to ensure homogeneity of solutes. (b) Active green fluorescent protein (GFP) synthesized from cell-free reactions incubated for 6 h at 30 °C under a range of mixing speeds (0 to 1200 rpm). (c) Time course of active GFP synthesis from CFPS reactions performed under a range of temperatures (26 to 37 °C) over 44 h at 600 rpm. (d) Effect of DNA concentration (0 to 100 ng/μL) on active GFP synthesis measured after 24 h of incubation, at 30 °C, and at 600 rpm. Three independent CFPS reactions were performed, and one standard deviation is shown. (e) SDS PAGE gel and (f) autoradiogram analysis of cell-free reactions supplemented with ^{14}C-Leucine, with and without plasmid DNA under optimized conditions. Yellow arrow indicates GFP (MW ~ 25 kDa) position.

performance and expression titers before fully evaluating smaller design sets in cells. In other words, the CFPS platform would act as a screening step during lead identification and optimization, which may speed-up the design-build-test cycles required for antibody engineering. The advantages of CFPS for this application are 3-fold: (i) processes that take days or weeks to design, prepare, and execute *in vivo* can be done more rapidly in a cell-free system, since no time-consuming culturing steps are needed, (ii) the acellular nature allows for facile manipulation of the reaction environment, direct monitoring, and rapid sampling, and (iii) PCR template driven expression coupled with liquid handling allows for cost-effective, high-throughput screening.[7−9]

To support our proposed strategy, recent efforts in CFPS suggest the possibility of using cell-free environments for rapid prototyping.[10] For example, multiple reports have shown the ability to use cell-free systems for rapidly characterizing DNA- and/or RNA-based genetic circuits.[11,12] Other studies have shown the ability to use cell-free systems to characterize genetic part libraries to inform experimentalists how to best tune protein expression in cells.[13]

Toward the long-term goal of developing a tailor-made cell-free platform for rapid, high-throughput prediction and expression ranking of mAbs, our paper had two objectives. First, we aimed to demonstrate the ability to use a commercially available CHO-based CFPS kit for synthesis of full-length mAbs. Second, we sought to use our system to rank mAb expression of three distinct and pharmaceutically relevant mAb constructs. Development and application of this technology involved three steps. First, we established and validated oxidizing reaction conditions for cell-free mAb synthesis using the 1-step CHO High Yield In Vitro Translation (IVT) kit. We built on the pioneering work by Sutro Biopharma that has previously shown the synthesis of an aglycosylated intact mAb in an *Escherichia coli* based CFPS system at yields >1 g/L.[14,15] While their accomplishments have provided a large wealth of knowledge to better understand mAb synthesis and folding in cell-free systems, it was not clear *a priori* how the results using a bacterial system would translate toward an *in vitro* CHO-based mAb expression system. To our knowledge, there is no report documenting the synthesis of a full-length intact mAb using a eukaryotic-based CFPS platform, even though expression of antibody fragments has been accomplished.[16−20] Second, we optimized heavy and light chain plasmid expression time to minimize aggregation by-products. Third, we demonstrated the ability to rank mAb variant expression profiles in a manner that matches well with previous CHO *in vivo* data. Finally, in order to demonstrate that our *in vitro* CHO-based mAb cell-free expression platform holds potential for future mAb activity screens, we showed that the cell-free synthesized mAbs were active. Looking forward, we expect that the CHO-based CFPS platform for synthesis of active mAbs will enable accelerated screening pipelines for identifying robust and reliable high expressing genetic constructs prior to putting them into a CHO cell. This work has implications for bioengineering, protein expression, and synthetic biology projects.

RESULTS AND DISCUSSION

Optimizing CHO-Based Cell-Free Reaction Parameters. With the goal of being able to synthesize active mAbs, we first set out to optimize protein expression in the 1-step CHO

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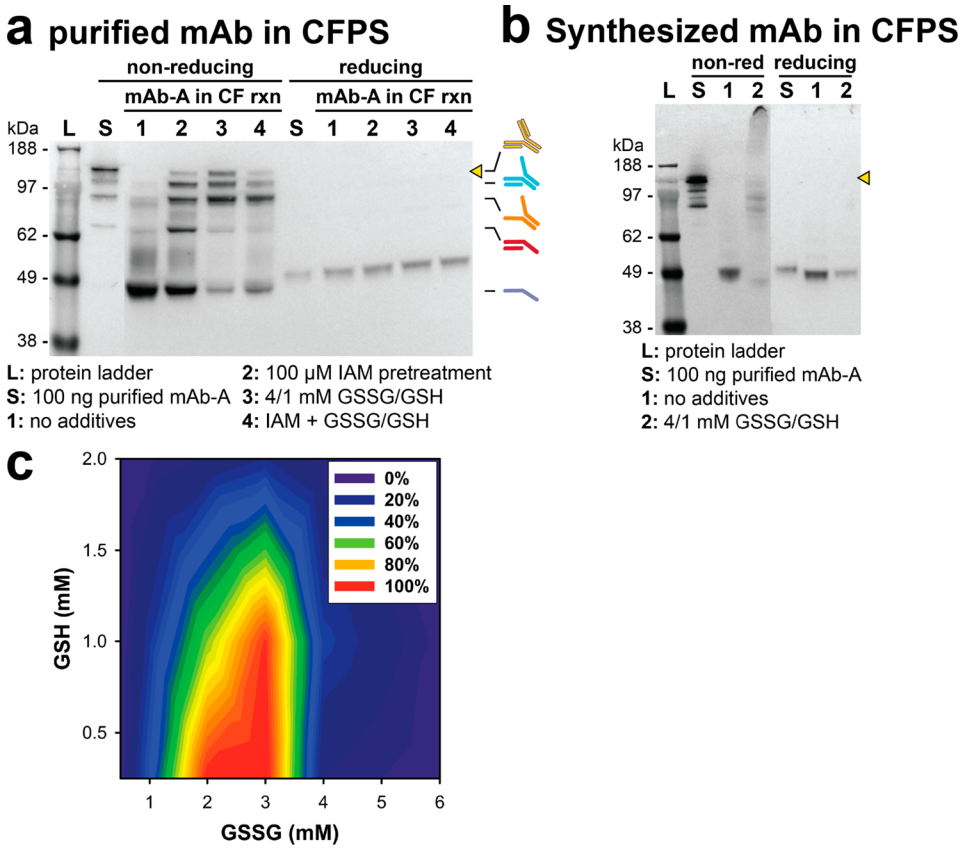

Figure 2. Impact of oxidizing environment on mAb stability and synthesis. (a) Western Blot analysis of 200 ng of purified mAb-A spiked into cell-free reactions with the addition of iodoacetamide (IAM) or a 4/1 mM ratio of oxidized to reduced glutathione (GSSG/GSH). (b) Western Blot analysis of cell-free synthesized mAb-A in the presence or absence of 4/1 mM GSSG/GSH buffer. Additionally, a small increase in molecular weight (~1.5 kDa) due to the presence of glycosylation motifs on reduced HC derived from purified mAb is observed relative to cell-free synthesized HC. (c) Heat map detailing the percent values of cell-free synthesized HC-HC-LC relative to the 2/0.25 mM GSSG/GSH data point. Western Blots are probed for HC protein and the data used for the heat map analysis are shown in Supplementary Figure S2. Yellow arrow indicates intact mAb (~150 kDa) position.

High Yield IVT kit using a 100 μL semicontinuous CFPS reaction setup (Figure 1a). This setup lends itself well to medium-throughput protein expression and provides "high yield" due to substrate replenishment by the dialysis buffer and toxic byproduct removal from the cell-free environment.[21] For ease of initial analysis, green fluorescent protein (GFP) was used as a reporter protein to analyze CFPS productivity under a variety of reaction parameters. Specifically, we investigated mixing speed, reaction temperature, reaction time, and plasmid DNA concentration. First we tested the impact of mixing speed on CFPS yields. We hypothesized that efficient mixing would be important for enhanced small-molecule exchange to fuel high level protein synthesis. As such, the effect of mixing speed (0−1200 rpm) on GFP yield was investigated using a thermomixer. After 6 h of expression, ~130 μg/mL of GFP was produced at the optimal mixing speed of 600 rpm, which was a 58% increase in GFP production relative to no mixing (0 rpm) (Figure 1b).

To further enhance yields and study the kinetics of the reactions, incubation times and temperatures were varied while maintaining optimal mixing speeds (Figure 1c). The highest protein yield (~510 μg/mL) was observed between 24 to 28 h of expression at 30 °C. In general, GFP synthesis at higher temperatures had slightly faster initial kinetics, but was overall less productive. CFPS reactions maintained productivity for the first 24 h, after which protein synthesis terminated. Interestingly, appreciable amounts of protein product are not observed until 3 h after the start of the reaction. While we do not understand this phenomenon, we reasoned it could have implications for optimizing expression times of heavy chain (HC) and light chain (LC) plasmids when synthesizing mAbs, which we study below.

DNA concentration is a key factor that influences the amount of protein produced in CFPS reactions.[22] Because CHO extracts may contain endogenous DNases that can degrade DNA over time, we next sought to optimize protein expression by tuning the plasmid concentration in cell-free reactions. We hypothesized that an increase in plasmid concentration would lead to an increase in protein production. We observed a ~20% improvement in protein synthesis by doubling the plasmid concentration from the baseline, 40 ng/μL, to 80 ng/μL (Figure 1d). When combined, our systematically optimized conditions (600 rpm mixing speed, 24 h, 30 °C, and ~80 ng/μL of total plasmid DNA) enabled synthesis of ~700 μg/mL of GFP, which was visible on a SimplyBlue SafeStained SDS-PAGE protein gel and confirmed using an autoradiogram (Figure 1e,f).

Enhancing Disulfide Bond Formation by Tuning the Oxidizing Environment. Our next goal was to synthesize full-length mAb using our optimized CFPS reaction conditions. This optimization required establishing an oxidizing reaction environment favoring disulfide bond formation, similar to the

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published effort toward making disulfide-bonded proteins in an *E. coli* CFPS system.[23] There, Yin and Swartz determined it was critical to both inactivate reductases in the cell extract using iodoacetamide (IAM), as well as tune the oxidizing environment through the addition of oxidized and reduced glutathione (GSSG and GSH, respectively). To identify the best strategy for disulfide bond formation of mAbs in a CHO-based cell-free environment, we first examined the stability of purified, intact mAbs in cell-free environments supplemented with combinations of 100 μM IAM extract-pretreatment and 4/1 mM GSSG/GSH buffer addition. CHO-based cell-free reactions were assembled without addition of plasmid template and, instead, reactions were spiked with purified mAb-A antibody at a final concentration of 100 μg/mL at the start of the reaction. mAb-A was chosen for experimental optimization because it is a standard antibody commonly used by MedImmune that expresses well *in vivo*. After 24 h of incubation, mAb-A from the cell-free reaction was compared to purified mAb-A using Western Blot analysis (Figure 2a). In the absence of additives, nearly all mAb-A incubated in the cell-free reaction was reduced to HC and LC (Figure 2a; lane 1) suggesting there were high levels of reducing agent present, creating an environment not conducive for mAb stability. Pretreating the extract with IAM improved mAb stability, suggesting that endogenous reductases in CHO extract may play a role in the poor disulfide bond stability. The largest benefit toward intact mAb preservation was from the addition of 4/1 mM GSSG/GSH, which helped to maintain an oxidizing cell-free environment and stabilize disulfide bonds. There was no additional benefit toward disulfide bond stability when a combination of IAM pretreatment and glutathione was used, making the glutathione buffer our primary target for optimization in our effort to synthesize and assemble mAbs using CFPS. Also of note, since the signal intensities obtained from performing Western Blots against LC using the same samples shown in Figure 2a exhibited a marked decrease in signal intensity (Supplementary Figure S1), future blots were performed against only the HC.

Next, we evaluated if the oxidizing environment generated with 4/1 mM GSSG/GSH was sufficient for the production of intact mAb. Specifically, mAb-A was synthesized by expressing both HC and LC (each on individual plasmids) in a cell-free reaction in the presence of the glutathione buffer (Figure 2b). In a control reaction (without glutathione additives), only HC was detectable (Figure 2b, lane 1). However, upon addition of 4/1 mM GSSG/GSH, species of HC at sizes larger than the monomer form were observed, likely as a result of disulfide bond formation and mAb assembly. Unfortunately, a decrease in overall HC production was observed. Together, our data suggested that a glutathione buffer aids our efforts toward mAb disulfide bond formation; however, optimization is required to reach an appropriate redox environment for disulfide bond formation and production of intact mAb.

To optimize the oxidizing cell-free environment, mAb-A CFPS reactions were performed over a range of GSH concentrations of 0.25 mM to 2 mM and GSSG concentrations of 0.5 mM to 8 mM (Figure 2c and Supplementary Figure S2). Overall, GSSG concentrations greater than 6 mM led to no detectable HC, indicating glutathione reached inhibitory levels. In addition, GSH levels greater than 1 mM made it difficult to generate mAb species greater in size than HC dimer, indicating that the environment was still reducing. GSSG levels less than 1 mM were insufficient for generating an oxidizing environment, where nearly all HC remained in its monomer form. The best condition for generating intact mAb obtained from these optimization studies was a ratio of 2/0.25 mM GSSG/GSH. This optimal ratio of GSSG/GSH was used for our remaining studies.

Lastly, we revisited the use of IAM pretreatment to identify any benefits toward mAb synthesis and assembly in CFPS. Interestingly, no intact mAb or HC-HC-LC species were observed in CFPS reactions supplemented with a range of glutathione additives upon pretreatment of the extract with IAM (Supplementary Figure S3). It may be speculated that enzymes involved in the formation and shuffling of disulfide bonds *in vivo*, such as ER-resident proteins (protein disulfide isomerase, PDI), are maintained in the extract and can be irreversibly negatively affected by IAM.[6] Additionally, IAM treatment is known to lower protein synthesis yields of reactions;[23] thus, the addition of glutathione without IAM pretreatment was chosen for further experiments. Results obtained for mAb CFPS using extract pretreated with IAM suggest that the addition of chaperones would be a crucial next step for improving mAb synthesis yields in our system.

Utilizing Protein Isomerases to Encourage Proper Disulfide Bond Formation. To test the importance of chaperone addition into our system for improving mAb production, commercially purified disulfide bond isomerase C (DsbC) chaperone was added into reactions with a 2/0.25 mM GSSG/GSH buffer (Supplementary Figure S4). A significant yield increase in mAb species in the presence of 5 μM DsbC was observed, suggesting that isomerization of disulfide bonds is important for synthesis of full-length mAb.

We then tested the effect of in-house purified isomerases on mAb synthesis. We chose to use purified yeast protein disulfide isomerase (yPDI) and *E. coli* DsbC (Supplementary Figure S5a,b), which have both been demonstrated to aid in disulfide bond formation in cell-free systems before.[14] The effect of individual additions of DsbC and yPDI are shown in Supplementary Figure S5c−e. Encouragingly, in-house purified DsbC yielded 1.9-fold more mAb chemiluminescence signal at 5 μM addition *versus* commercially available DsbC (Supplementary Figure S5e). Addition of 15 and 7.5 μM of in-house purified DsbC and yPDI individually yielded a 25 and 26-fold chemiluminescence signal increase, respectively, of intact mAb over control reactions with no chaperone addition. Furthermore, to test for an added benefit of dual chaperones addition on mAb assembly, a range of DsbC concentrations in combination with 7.5 μM yPDI were tested in CFPS reactions synthesizing mAb-A. Maximum intact mAb-A formation was identified upon addition of 7.5 μM yPDI and 10 μM DsbC (Figure 3a). All further studies used a 7.5 μM yPDI and 10 μM DsbC chaperone combination as optimal levels to encourage proper disulfide bond formation in cell-free synthesized mAbs.

Delayed Addition of HC Plasmid Reduces Aggregation Products. While we were able to see full-length mAb product, it was clear from the Western Blots that multiple mAb fragments and aggregates are also generated. Of note, the formation of HC alone, HC-HC-LC, and even aggregated products (as seen by the smear of species greater than 200 kDa) were observed (Figure 3; Supplementary Figure S6) and led to a decrease in intact mAb yields. Even when less plasmid encoding LC and HC is added at the start of the reaction, aggregated products are still observed despite the presence of high levels of disulfide bond isomerase chaperones (Supplementary Figure S7). Previously, Yin and colleagues showed in an *E. coli* based CFPS system that HC is unstable when

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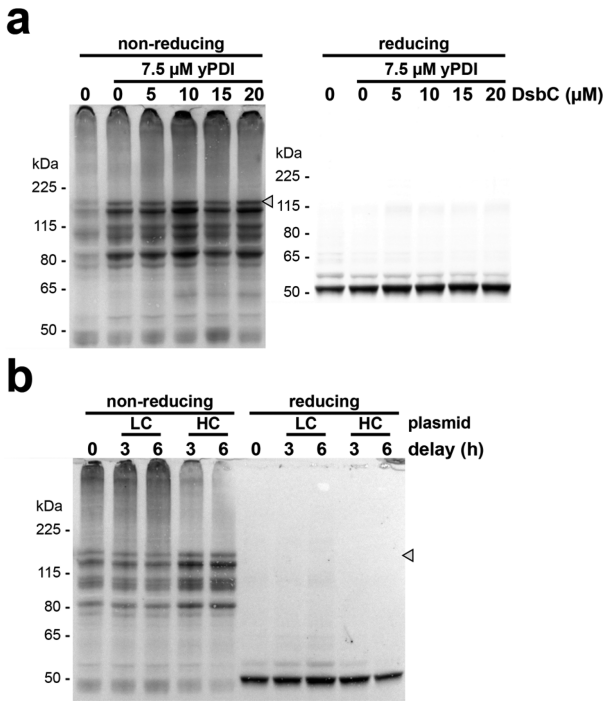

Figure 3. Improved mAb assembly resulting from dual chaperone addition and temporal delay in HC plasmid addition. (a) Western Blot probing for HC protein demonstrating the impact of yPDI and DsbC addition on mAb-A assembly in redox optimized CFPS reactions. (b) Western Blot probing for HC protein demonstrating the impact of delayed heavy chain (HC) and light chain (LC) plasmid addition on mAb-A assembly and aggregation products. Indicated lanes describe either LC or HC plasmid delayed by either 3 or 6 h. Arrow indicates intact mAb (~150 kDa) position.

expressed alone and mAb generation benefits greatly by delaying addition of HC plasmid to a batch cell-free reaction.[15] We sought a similar strategy to avoid aggregation issues and increase synthesis of intact mAb yields in our CHO-based platform. Semicontinuous reactions were performed with delayed addition of either LC or HC plasmid to the system (Figure 3b). Temporal addition of the second plasmid was delayed by either 3 or 6 h to align with the observed start of protein synthesis in our system (Figure 1c). Encouragingly, the appearance of aggregates was greatly minimized upon later addition of HC, and the converse occured with later addition of LC. Together, these results suggest that heavy chain synthesized in the absence of LC formed aggregates in cell-free reactions. Potentially, the LC serves as a scaffold for the HC to fold properly.[15] Using our CHO CFPS platform, we can now examine questions about protein stability (*e.g.*, identifying HC aggregates when expressed alone) and identify important factors that help improve solubility (*e.g.*, having LC present prior to expressing HC).

We next assessed whether we could improve mAb-A production by tuning the relative ratio of HC to LC plasmid added into the reaction (Supplementary Figure S8). This was accomplished by first adding LC plasmid at the specified concentration, followed by the addition of the complementary HC concentration 6 h after the start of the reaction, allowing for dual expression of HC and LC for the remaining 18 h. No clear benefit from plasmid tuning was observed, and the overall mAb species observed are consistent with previous results. Thus, we continued to use a 1 to 1 plasmid ratio of HC to LC along with a 6 h delay of HC for further experimentation. Overall, our redesigned CHO-based CFPS system shows the ability to make full-length intact mAb.

Determining Percent mAb Generated in Optimized Conditions. We next sought to better understand overall mAb synthesis improvements resulting from the development of conditions suitable for disulfide bond formation. We turned to radioactive amino acid incorporation for direct visualization and quantification of each mAb species using an autoradiogram. Specifically, CFPS reactions incubated with radioactive ^{14}C-leucine were performed using our newly designed CHO-based CFPS system with glutathione buffers and protein disulfide isomerases. Initially, samples were analyzed by both autoradiogram analysis and Western Blot analysis (Supplementary Figure S9). This allowed us to compare our two approaches and suggested two important high-level features of our data. First, we noticed some potential biases that likely arose from the transfer and probing steps in the Western Blots. Namely, we observed that (i) high molecular weight aggregates do not transfer well, (ii) the relative ratio of LC to HC intensity is biased by transfer efficiency, and (iii) the mAb band signal intensity is also affected by transfer efficiency. Second, general trends for full-length mAb synthesis were the same, suggesting our use of Western Blots for rapid screening is reasonable.

We next quantitatively analyzed the autoradiogram of the protein gel samples using densitometry analysis (Supplementary Figure S10 and Figure 4). Under nonreducing conditions, the emergence of mAb upon addition of an optimized glutathione buffer is clear and accounts for ~5% of total species synthesized, whereas the vast majority of synthesized HC forms aggregates (high molecular weight species). Upon optimizing yPDI and DsbC chaperones in the system, the percent of cell-free synthesized mAb increased to ~10%. Notably, by optimizing the delay of HC into the cell-free reaction by 6 h, a ~90% decrease in aggregates is observed and the percent mAb in the cell-free reaction surpasses 15%. Up to this stage, we were not able to clearly identify the population of LC monomer *via* Western Blot protein analysis. However, the results shown clearly indicate that LC is not limiting throughout the entire optimization process. A schematic highlighting the impact of each optimization step is illustrated in Figure 4e.

Ranking and Functional Analysis of Known High and Low *In Vivo* mAb Expressors. Given the ability to synthesize full-length mAb, we wanted to demonstrate the potential to use our CHO-based CFPS platform for rapid screening of mAbs. In this experiment, we assessed whether our CFPS system could differentiate between known high and low *in vivo* mAb constructs. To do so, we synthesized mAbs A, B, and C, which typically yield high, medium, and low mAb titers, respectively, using MedImmune's CHO-based *in vivo* platform (Table 1). Using autoradiogram analysis for ranking, our results suggest mAb-A ranks better than mAb-B, which ranks better than mAb-C (Figure 5 and Supplementary Figure S11). An estimated yield of 114 ± 8 and 58 ± 4 μg/mL was measured for intact mAb-A and mAb-B, respectively, whereas no observed full-length product was detected for mAb-C (Table 1). Supporting our radioactive ranking data, Western Blot results suggest mAbs A and B express well and rank better than mAb-C (Supplementary Figure S12). This experiment was repeated at least 8 times with our final optimized conditions and was shown to be reproducible (Supplementary Figure S13). The solubility of the mAbs was also assessed (Supplementary Figure

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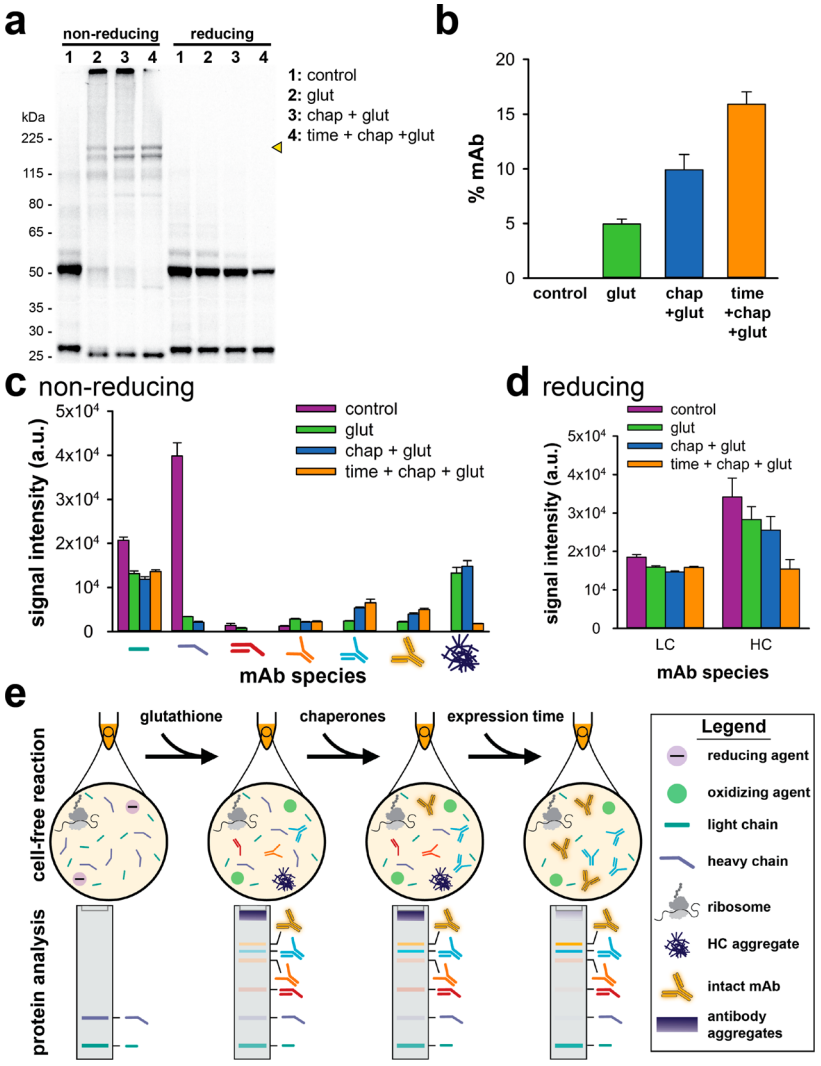

Figure 4. Holistic analysis of mAb production under optimized conditions show >15 mAb% relative to total products. (a) Autoradiogram analysis of mAb-A species under various optimization conditions. ^{14}C-Leucine incorporation into cell-free synthesized HC and LC proteins allowed for protein detection using phosphorimaging. Control reactions contain no additives. Glutathione optimized reactions (glut) contain 2/0.25 mM GSSG/GSH buffer. Chaperone optimized reactions (chap) contain 7.5 μM yPDI and 10 μM DsbC. Expression time optimized reactions (time) began expression of HC 6 h after the reaction start. (b) Percent mAb-A reported as quantified intact mAb-A observed under nonreducing conditions divided by total HC and LC quantified and observed under reduced conditions. mAb-A species were quantified using ImageJ software under (c) nonreducing and (d) reducing conditions. (e) Schematic detailing impact of optimization conditions on mAb-A production. Yellow arrow indicates intact mAb (~150 kDa) position. Error bars indicate standard deviation of three experiments.

Table 1. Yields of Intact mAbs Support Putative Ranking of Known High and Low *In Vivo* mAb Expressers[a]

		cell-free protein synthesis		
mAb	*in vivo* yields (mg/mL)	mAb %	relative expression	estimated yield (μg/mL)
A	6	20	1.00	114
B	2	11	0.51	58
C	1	ND	ND	ND

[a]Values used to analyze percent mAb, expression relative to mAb-A, and yield were obtained from Supplementary Figure S11. Data for *in vivo* expression were obtained using stable cell lines grown in fed-batch mode and standard MedImmune operating procedures.

S14). Western Blot analysis suggests the intact mAbs observed are soluble because the band intensity between nonreducing samples before and after centrifugation show no appreciable differences. While more constructs would be needed to demonstrate generality, our results suggest that CHO-based CFPS screening and ranking of mAb constructs could be achieved with future development.

To support downstream characterization activities and method development, it was important to test if the cell-free synthesized mAbs display bioactivity. To this end, we used a mAb binding assay to test the activity of mAb-A and mAb-B synthesized using CFPS in our optimized process (Figure 6). The antigen for both mAb-A and mAb-B is the hapten 4-hydroxy-3-iodo-5-nitrophenylacetyl (NIP). Negative controls, represented by CFPS samples with no plasmid addition or with GFP synthesis, show no hapten binding. A clear binding signal indicating activity toward NIP was observed for cell-free synthesized mAbs A and B as with purified mAb-A positive control from *in vivo* synthesis. Together, these results indicate that our optimized CHO-based CFPS platform is capable of properly assembling soluble, active mAbs. This is the first time,

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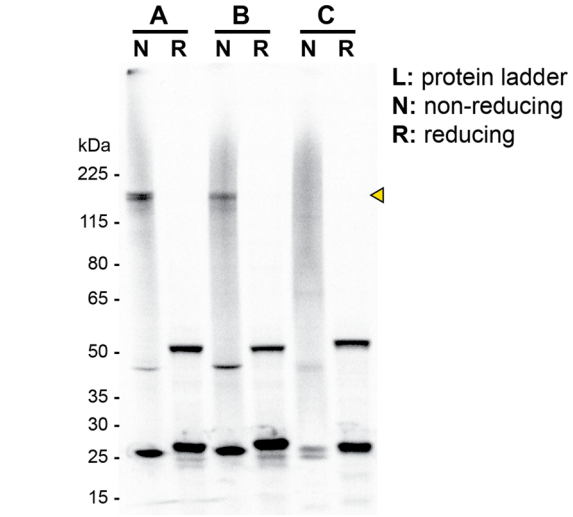

Figure 5. Ranking known high and low *in vivo* mAb expressers. Representative data of cell-free mAb-A, mAb-B, and mAb-C synthesis under final optimized conditions analyzed using autoradiography (Supplementary Figure S11). Yellow arrow indicates intact mAb (~150 kDa) position.

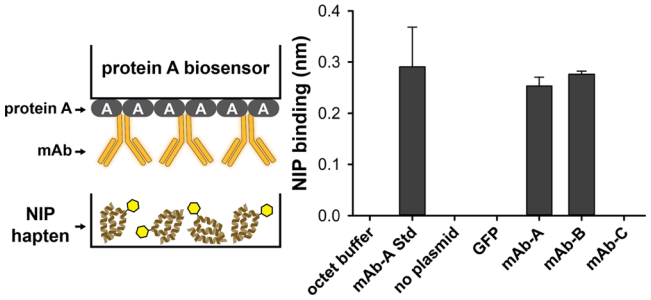

Figure 6. Cell-free synthesized mAbs demonstrate activity. The illustration on the left depicts the mAb binding assay used for activity testing. Unpurified mAb in a cell-free reaction is bound onto a protein A biosensor and then submerged into a solution of 0.1 M 4-hydroxy-3-iodo-5-nitrophenylacetyl hapten. Changes in wavelength of light passing through the mAb sample are measured and used to assess binding of mAb to antigen. Wavelength differences are shown on the right. Negative control CFPS reactions without plasmid and when expressing GFP are indicated. Three independent CFPS reactions were analyzed, and one standard deviation is shown.

to our knowledge, that an intact, active mAb has been synthesized in a eukaryotic-based CFPS system.

Summary. Here, we describe the development and optimization of a CHO-based CFPS system for the synthesis of soluble and active mAbs. We anticipate that a similar strategy may be employed to allow for mAb synthesis using the various *in vitro* translation kits commercially available (*e.g.*, Human Coupled IVT Kit); however, we focused on CHO cells lysates because of the use of CHO cells in an industrial setting. Protein titers in the system were increased by optimizing the cell-free reaction parameters using GFP as a reporter. To allow for disulfide bond stability and formation necessary for intact mAb synthesis, an optimized level of 2/0.25 mM GSSG/GSH buffer was added to the system, demonstrating initial success in formation of intact mAb. To enhance proper disulfide bond formation, chaperones such as DsbC and yPDI were added to the reaction. mAb titers were increased upon addition of either DsbC or yPDI and there was an additive benefit from supplementing with both isomerases. Additionally, we observed HC synthesis in the absence of LC led to aggregates. Applying this observation toward the synthesis of mAbs, improvements were observed from a 6 h delay in HC plasmid addition to the CFPS reaction as this increased the amount of mAb synthesized and minimized the amount of aggregated product we observed. The optimized system was also shown to rank known high and low producing mAb sequences in the same order as seen from the CHO cell-based platform in an active conformation.

As expected, creating an oxidizing environment and the use of disulfide bond isomerase chaperones were found to be crucial for producing high yields of mAbs in our CFPS system.[14,15] This is largely because formation of proper disulfide bonds is one of the rate-limiting steps toward intact mAb synthesis.[24] Another potentially limiting step for mAb folding *in vitro* is proline isomerization.[25,26] Accordingly, it stands to reason that addition of peptidyl prolyl isomerases (PPIases) would further benefit future mAb synthesis in CHO-based CFPS reactions. The need for proline isomerization could potentially be the main reasons for HC instability. In fact, PPIases have already been shown as a promising chaperone class for mAb formation in *E. coli* CFPS reactions.[14]

Looking forward, we believe this work sets the stage for future application of a CHO-based CFPS rapid prototyping platform for antibody screening. Such a platform could accelerate screening efforts aimed at relative productivity of the constructs or activity of mAbs to the order of days instead of weeks using conventional *in vivo* methods. While it is still early in the development of CHO-based CFPS systems, and this is the first demonstration of full-length, active aglycosylated mAb synthesis, our advances suggest promise for future development. However, due to the proprietary nature of the CHO-based commercial CFPS kit, additional manipulation of the reaction environment is difficult. Further optimization of the production of CHO extract, including complete control of the small molecules involved in the system, might dramatically help in efforts to generate an optimal cell-free environment for mAb formation. Improvements that increase the percentage of full-length mAb product and reductions in cost will open the way to even broader applications.[27]

METHODS

Cell-Free Protein Synthesis Reaction. All CHO cell extract-based reactions were performed using the 1-Step CHO High-Yield IVT Kit (Thermo Scientific, West Palm Beach, FL) following the manufacturer's instructions. Prior to the addition of the CFPS reagents, the cassette and dialysis buffer solution were incubated for 30 min at 30 °C. 100 μL CFPS semicontinuous reactions were performed using a 100 μL microdialysis cassette (Thermo Scientific, West Palm Beach, FL) in 2 mL microcentrifuge tubes with 1.4 mL dialysis buffer solution. Reactions were incubated in an Eppendorf Thermomixer C at 30 °C and 600 rpm for 24 h, unless otherwise indicated. Plasmid vectors for both heavy and light chain (HC and LC, respectively) were generated by DNA 2.0 (Newark, CA) using the backbone vector (pT7CFE1-NHis-GST-CHA) included with the 1-Step CHO High-Yield IVT Kit. A 2/0.25 mM GSSG/GSH buffer was added to both the dialysis buffer and the CFPS reactions to promote antibody assembly, unless otherwise indicated. For reactions testing the effects of iodoacetamide (IAM) addition, the CHO extract and accessory protein mixture was incubated for 30 min with 100 μM of IAM at room temperature. For HC expression time optimization

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experiments, reactions were prepared without the heavy chain plasmid, and the HC plasmid was added directly to the reaction 6 h from the start, unless otherwise indicated. The reactions then continued for 18 h more.

Isomerase Purification. Commercially purified DsbC was purchased from ENZO Technologies (Farmingdale, NY). In-house Strep-tag purification of yPDI and DsbC was performed as follows. *E. coli* BL21 (DE3) transformed with the pET28a plasmid encoding either yPDI or DsbC were grown in 1 L of 2 × YT broth to an OD_{600} of 0.6 at 220 rpm and 37 °C. Isomerase production was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma-Aldrich, St. Louis, MO) and cells were allowed to grow for an additional 3 h. Cells were harvested at 5000*g* for 15 min at 4 °C, washed with S30 buffer (2 mM DTT, 10 mM Tris-acetate pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate) and stored at −80 °C. Frozen cell pellets were thawed in 1 mL of Buffer W (IBA, Gottingen, Germany) per gram of wet cells, lysed using sonication, and centrifuged at 16 000*g* at 4 °C for 10 min. The chaperones were purified using 1 mL gravity flow Strep-Tactin Sepharose mini-columns (IBA, Gottingen, Germany). Purified sample was subsequently dialyzed against buffer composed of 50 mM Tris pH 7.5, 150 mM sodium chloride, 1 mM dithiothreitol (DTT), 1 mM EDTA, and 5% glycerol using a Slide-A-Lyzer G2 Dialysis Cassette (Life Technology, Grand Island, NY). The dialyzed sample was then concentrated using YM-10 Microcon centrifugal filter columns (Millipore, Billerica, MA). Purified chaperone samples were stored at −80 °C. Purified isomerase was quantified using the Quick-Start Bradford Protein Assay (Bio-Rad, Hercules, CA).

Quantification of Active GFP. Active full-length GFP protein yields were quantified by measuring fluorescence using a Synergy 2 plate reader (BioTek, Winooski, VT) with excitation at 485 nm, emission at 528 nm, and cutoff at 510 nm in 96-well half area black plates (Costar 3694; Corning, Corning, NY). Fluorescence units were converted into concentration by using a standard curve as previously described.[22,28]

Protein Gel and Western Blot Analysis. SDS-PAGE analysis was performed under both reducing and nonreducing conditions. For the reduced samples, DTT was added to a final concentration of 10 mM and heated to 95 °C for 10 min. Nonreducing samples were prepared at room temperature. Samples were loaded onto a 4−12% Bis-Tris protein gel (Thermo Scientific, West Palm Beach, FL). SimplyBlue SafeStained (Thermo Scientific, West Palm Beach, FL) protein gels were incubated in staining solution for 1.5 h, followed by a 3 h wash with water. Protein transfer was performed using the iBlot 2 Dry Blotting System onto nitrocellulose membranes (Promega, Madison, WI).

Membranes were blocked with 5% (w/v) milk in PBS with 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO) overnight. Membranes were then probed with antibody conjugated to HRP and diluted 10 000-fold in milk buffer for 1 h. For detection of the heavy chain, anti-Fc-γ-HRP antibody (Jackson ImmunoResearch, West Grove, PA) was used. For the detection of the LC, anti-Kappa LC-HRP (Rockland Immunochemicals, Limerick, PA) was used. Membranes were washed 3 times with 1XPBST for 10 min. Blots were processed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, West Palm Beach, FL) and visualized using the Chemidoc XRS (Bio-Rad, Hercules, CA) and the Odyssey Fc Imaging System (LI-COR, Lincoln, NE).

Autoradiogram Analysis. Radioactive ^{14}C-leucine was added into 1.4 mL of dialysis buffer at a final concentration of 10 μM prior to the reaction start. Protein yields were quantified by determining radioactive ^{14}C-Leu incorporation into trichloroacetic acid (TCA)-precipitated protein. Radioactivity of TCA-precipitated samples was measured using liquid scintillation counting (MicroBeta2, PerkinElmer, Waltham, MA). For autoradiogram analysis, 2 μL of each reaction was loaded on 10% Bis-Tris protein gel (Thermo Scientific, West Palm Beach, FL) after denaturing the sample. The gel was soaked in Gel Drying solution (Bio-Rad, Hercules, CA) for 30 min, fixed with cellophane films, dried overnight in GelAir Dryer (Bio-Rad, Hercules, CA), and exposed for 3 days on Storage Phosphor Screen (GE Healthcare Biosciences, Pittsburgh, PA). Autoradiograms were scanned using Storm Imager (GE Healthcare Biosciences, Pittsburgh, PA). Band intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD). Intact mAb yields were determined using a previously published formula.[29] Briefly, intact mAb yields were calculated using the following formula:

$$\mathrm{mAb}\left(\frac{\mathrm{mg}}{\mathrm{mL}}\right) = \frac{\text{washed} - \text{background}}{\text{unwashed}} \times \frac{\text{Leucine (mM)}}{\text{Leucine residues}} \times \mathrm{MW} \times \mathrm{mAb\%}$$

Washed and unwashed radioactive counts refers to the signal measured for reactions spotted on Whatman paper that were subsequently washed or not washed with TCA, respectively. Background corresponds to the counts obtained from a washed sample of a no plasmid control reaction. The leucine concentration was measured to be 0.7 mM in the reaction. The number of leucine residues present in assembled mAb-A, B, and C are 96, 100, and 86, respectively. The molecular weight for assembled mAb-A, B, and C are 144 854 g/mol, 145 440 g/mol, and 141 034 g/mol, respectively. Percent mAb in the reaction (mAb%) was calculated using densitometry as the mAb band signal under nonreducing conditions divided by the HC and LC signal observed under reducing conditions. This calculation allowed for estimating mAb yield in mg/mL.

Purified mAb Control. Purified mAb-A was generated using a proprietary CHO fed-batch cell culture process at MedImmune. The mAb secreted from the CHO cells was purified from the conditioned medium using a Protein A bead based capture method.

Activity Assay. Kinetic activity of cell-free synthesized mAb samples was measured using ForteBio Octet Bioanalyzer (Pall Life Sciences, Menlo Park, CA) which uses biolayer interferometry (BLI) to measure molecule binding to the biosensor. Protein A biosensors were preconditioned using pH 1 glycine buffer followed by neutralization in Octet buffer (1 g/L BSA and 0.02% Tween 20 in 1 × PBS) then used to measure binding with the following assay with a shaking speed of 400 rpm, sample temperature of 30 °C, and sample/reagent volumes of 100 μL per well. Binding of samples to Protein A was measured over 120 s, followed by 30 s measurement in Octet buffer to check for dissociation, followed by 120 s of binding of 0.1 M 4-hydroxy-3-iodo-5-nitrophenylacetyl hapten conjugated to BSA (Santa Cruz Biotechnology, Dallas, TX). Sensors were then regenerated using pH 1 glycine buffer followed by neutralization in Octet buffer before repeating measurements. Binding rate was measured using the initial slope binding rate equation over the 120 s acquisition time in ForteBio Data Analysis v 8.1.0.58 software.

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■ ASSOCIATED CONTENT

Supporting Information

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Figures S1−S14 (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: m-jewett@northwestern.edu. Tel: (+1) 847 467 5007. Fax (+1) 847 491 3728.

*E-mail: royv@medimmune.com. Tel: (+1) 301 467 0412. Fax (+1) 301 398 9665.

ORCID

Rey W. Martin: 0000-0002-9613-6951

Author Contributions

R. W. Martin, M. C. Jewett, and V. Roy designed the experiments. V. Roy constructed HC and LC plasmids. R. W. Martin, N. I. Majewska, and C. X. Chen performed cell-free protein synthesis reactions and Western Blot analysis. R. W. Martin performed autoradiogram analysis. T. Albanetti, R. Martin, and V. Roy performed activity analysis. R. W. Martin, M. C. Jewett, and V. Roy wrote the manuscript. A. E. Schmelzer provided input into experimental design and feedback on the manuscript. All authors discussed the experimental results and commented in writing the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CFPS, cell-free protein synthesis; CHO, Chinese Hamster Ovary; GFP, green fluorescent protein; HC, heavy chain; LC, light chain; HRP, horseradish peroxidase

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