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Research Article

A cell-free expression and purification process for rapid production of protein biologics

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Cell-free protein synthesis has emerged as a powerful technology for rapid and efficient protein production. Cell-free methods are also amenable to automation and such systems have been extensively used for high-throughput protein production and screening; however, current fluidic systems are not adequate for manufacturing protein biopharmaceuticals. In this work, we report on the initial development of a fluidic process for rapid end-to-end production of recombinant protein biologics. This process incorporates a bioreactor module that can be used with eukaryotic or prokaryotic lysates that are programmed for combined transcription/translation of an engineered DNA template encoding for specific protein targets. Purification of the cell-free expressed product occurs through a series of protein separation modules that are configurable for process-specific isolation of different proteins. Using this approach, we demonstrate production of two bioactive human protein therapeutics, erythropoietin and granulocyte-macrophage colony-stimulating factor, in yeast and bacterial extracts, respectively, each within 24 h. This process is flexible, scalable and amenable to automation for rapid production at the point-of-need of proteins with significant pharmaceutical, medical, or biotechnological value.

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Abbreviations: CECF, continuous exchange cell-free; **CFPS**, cell-free protein synthesis; **DEAE**, diethylaminoethyl; **EPO**, erythropoietin; **GM-CSF**, granulocyte-macrophage colony-stimulating factor; **GSH**, glutathione; **GSSG,** glutathione disulfide; **IAM**, iodoacetamide; **LC-MS/MS**, liquid chromatography-tandem mass spectrometry; **PDMS**, polydimethylsiloxane; **rhEPO**, recombinant human EPO; **rhGM-CSF**, recombinant human GM-CSF

1 Introduction

The safety and efficacy of protein biologics has led to an upswing in the number of such drugs being introduced into development pipelines toward clinical use. Additional benefits of protein drugs will be realized by way of their application for specialized needs that include both precision and personalized medicine, treatment of orphan diseases, and point-of-care delivery of medical products. Production of proteins for such applications will not be cost effective until there are techniques and systems available to enable production of single doses or

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small scale made-to-order products for individual needs that meet regulatory criteria for human use. Currently, the manufacture of protein biologics is primarily implemented with large scale heterologous expression in bacterial, yeast, and mammalian cell-based systems. These cell-based approaches are impractical for cost-effective and rapid manufacture of low doses of protein biologics because they require optimum and stable conditions for sustainable cell growth, long turnaround times between cell culture and protein isolation, laborious purification protocols, and dedicated facilities. In addition, protein production in living cells has other inherent limitations, including formation of insoluble protein aggregates, protein degradation by intracellular proteases, and inadequate target expression due to host cell toxicity of overexpressed protein [1–3]. Moreover, in some cases, protocol development is required for product export into the culture media as a means to improve production yields.

Cell-free protein synthesis (CFPS) systems have emerged as a powerful technology platform for rapid and efficient production of medicinal proteins [4–8]. Such systems have distinct advantages over in vivo methods for recombinant protein production [9–12]. Cell-free systems do not require ancillary processes for cell viability and growth, allowing optimization of manufacture for a single protein product. The absence of a cell membrane enables real-time monitoring, rapid sampling, and direct manipulation of the protein synthesis process. In addition, the cell-free concept avoids cell line generation, allowing for acceleration of the development process. Recent research advances have led to the production of robust cell-free systems for protein synthesis in high yields. These advances have been achieved by increasing reaction duration via continuous supply of substrates and removal of toxic reaction byproducts under a continuous exchange cell-free (CECF) format, activating metabolic networks in vitro for energy production, and improving extract preparation procedures [11, 13–19].

Automated systems for high-throughput cell-free production of proteins for structural and functional studies have been reported [e.g. 20, 21]. However, these robotic platforms are currently too large for point-of-care applications; additionally, they often use protein separation methods that result in products containing extra amino acids at their N-terminus not present in the natural protein sequences [22]. Development of microfluidic array devices for CECF protein synthesis using bacterial lysates has also been reported [3, 23]. In addition, microreactor array chips have previously demonstrated utility for cell-free synthesis of various proteins using bacterial cell-free extracts [24]. Although these microfluidic systems hold promise for high-throughput protein screening and analysis, they are not adequate for production and purification of proteins at pharmaceutical levels.

In this study we developed a fluidic process for rapid cell-free expression and purification of protein biologics at the µg-mg scale. We pursued both prokaryotic (*Escherichia coli*) and eukaryotic (*Saccharomyces cerevisiae*) organisms as source strains for producing lysates for CFPS to capitalize on the advantages of each system for manufacture of different protein biologics with varying expression, posttranslational modifications, folding kinetics and scale requirements. We evaluated the feasibility of this process as a protein biologics manufacturing platform by expressing and purifying two pharmaceutically relevant biologics, recombinant human erythropoietin (rhEPO) and recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), using methods that are amenable to a fully automated approach.

2 Materials and methods

2.1 DNA constructs

A plasmid encoding the protein sequence of rhEPO (Epoetin alfa; DrugBank Accession Number DB00016) was generated containing the 5'-UTR fragment of the Omega sequence from the tobacco mosaic virus [25] and used to produce polymerase chain reaction (PCR) DNA templates for combined transcription/translation in the yeast CFPS system. A plasmid encoding rhGM-CSF (Sargramostim; DrugBank Accession Number DB00020) was generated from the pY71 backbone [26] for use as a circular DNA template for the bacterial combined transcription/translation CFPS system. Both constructs contained a T7 promoter and signal peptide sequence located upstream of the mature rhEPO and rhGM-CSF coding regions; the rhEPO construct also contained three copies of a FLAG epitope located between the signal peptide and the protein coding region for use in an affinity chromatography-based approach for protein purification (see below). Signal peptide and gene sequences were codon modified based on a commercial proprietary method (RESCUE; Promosome, LLC, CA). The PCR templates for the yeast CFPS reactions were generated from the aforementioned rhEPO plasmid using a forward primer with the sequence: 5'-GTGATTC ATTCTGCTAACCAG-3', and a reverse primer with the sequence: 5'-T_{50}GTTAGCAGCCGGATCTCAGT-3'. PCR reactions were performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Inc., MA), 0.2 µM of each primer, and 0.1 ng/µL of template DNA. The PCR product was purified using QIAquick PCR Purification Kit (Qiagen, MD), quantified using a NanoDrop 1000 (Thermo Fisher Scientific, MA), and its quality assessed by agarose gel electrophoresis.

2.2 Yeast cell-free protein synthesis

Saccharomyces cerevisiae strain S288c [27] was used as the source strain for extract preparation that was per-

formed according to Hodgman and Jewett [19] with the exception of growing the yeast cells on synthetic complete media (6.7 g/L Yeast Nitrogen Base (YNB), 20 g/L glucose, 50 mM potassium phosphate buffer, pH 5.5, and 2.002 g/L Synthetic Complete Amino Acids Supplement (ForMedium, Norfolk, United Kingdom). The yeast CECF combined transcription/translation protein synthesis reaction had the following final composition: 22 mM HEPES, pH 7.4, 120 mM potassium glutamate, 5.5 mM magnesium glutamate, 1.5 mM of each ATP, GTP, CTP, and UTP, 0.08 mM of each of 20 amino acids, 25 mM creatinephosphate, 1.7 mM DTT, 2 mM putrescine, 0.5 mM spermidine, 0.4 mM cAMP, 0.27 mg/mL creatinephosphokinase, 1.3 U/μL T7 polymerase (Thermo Fisher Scientific, MA), 11% glycerol, 6.67 ng/μL PCR amplified DNA template, and 50% v/v yeast extract. The feeder solution had the same composition as the reaction mixture except for the absence of T7 Polymerase, DNA template, and extract and the presence of 115 mM mannitol. The reaction and the feeder solutions (1 and 10 mL, respectively) were loaded into the CECF reactor and incubated at room temperature for 6 h with continuous recirculation of the feeder solution at a rate of 1 mL/min. To assess the solubility of the expressed rhEPO in the CFPS system, translation mixtures were centrifuged at 16 000 ×g for 10 min at 4°C, the supernatant (soluble) fractions were isolated, the pelleted (insoluble) fractions were resuspended in the same volume of phosphate-buffered saline (PBS), and both samples were mixed with SDS loading buffer, heat denatured and subjected to SDS-PAGE followed by Western blot analysis [13]. Unless otherwise specified, reagents were obtained from Sigma-Aldrich Corporation (St. Louis, MO). Initial testing of product yields indicated that under these conditions the yeast CFPS system had a variability of approximately 6% between different lysate lots, and variability of less than 5% between replicate experiments using the same lysate lot on different days.

2.3 Bacterial cell-free protein synthesis

Bacterial S30 crude extracts were generated from a genomically recoded release factor 1 (RF1) deficient *E. coli* strain (*E. coli C321.ΔA.705*) [28] and described in detail in [29]. The bacterial combined transcription/translation protein synthesis reaction was performed in the PDMS reactor under a batch format and had the following final composition: 57 mM HEPES, pH 7.4, 12 mM magnesium glutamate, 10 mM ammonium glutamate, 130 mM potassium glutamate, 1.2 mM ATP, 0.85 mM CTP, 0.85 mM GTP, 0.85 mM UTP, 0.034 mg/mL folinic acid, 0.171 mg/mL tRNA, 2 mM of each of the 20 amino acids, 33.3 mM phosphoenol pyruvate, 0.33 mM NAD, 0.27 mM coenzyme A, 4 mM oxalic acid, 1 mM putrescine, 1.5 mM spermidine, 4 mM glutathione disulfide, 1 mM glutathione, 100 μg/mL disulfide bond isomerase DsbC (Enzo Life Sciences, Inc., NY), 0.1% Brij-35, 1.3 U/μL T7 Polymerase (Thermo Fisher Scientific, MA), 13.3 ng/μL plasmid, and 15% v/v *E. coli* extract. The necessity of addition of exogenous tRNA to the bacterial CFPS reactions was shown previously [30]. Prior to use, *E. coli* extracts were treated with 1 mM iodoacetamide (IAM) (Sigma-Aldrich Corporation, MO) at room temperature for 30 min. The reaction solution was loaded into the reactor and incubated at 30°C for 10 h. The solubility of the expressed rhGM-CSF protein was assessed as previously described. Initial assessment of protein yields showed that the bacterial CFPS system had variability of approximately 13% between different lysate lots, whereas variability between replicate experiments using the same lysate lot on different days was less than 5%.

Quantitative assessment of the cell-free rhEPO and rhGM-CSF products was performed using Western blot analysis in the presence of known amounts of protein standards and/or the WES system (ProteinSimple, CA). For rhEPO, Western blot analyses were performed using a rabbit anti-EPO antibody (H-162, Santa Cruz Biotechnology, Inc., TX) and a horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody (111-035-003, Jackson ImmunoResearch Laboratories, Inc., PA). For rhGM-CSF, Western blot analyses were performed using a rabbit anti-GM-CSF antibody (AbCam, PLC, MA) and an HRP conjugated goat anti-rabbit secondary antibody (111-035-003, Jackson ImmunoResearch Laboratories, Inc., PA). In both cases, proteins were analyzed using a Storm 840 PhosphorImager (GE Healthcare Bio-Sciences, PA).

2.4 Protein synthesis reactor

The bioreactor consisted of a 2 mL polydimethylsiloxane (PDMS) chamber divided into the reaction and feeding chambers (1 mL, each) by a 3.5 kDa molecular weight cut-off regenerated cellulose dialysis membrane (Spectra/Por 7 Dialysis Membrane, VWR, PA). Both halves of the reactor are implemented with 0.010–0.030 ID polyethylene (PE) tubing fluidic systems (Western Analytical Products, CA) to allow for recirculating flow, fluidic integration of the reactor module with the upstream feed and sample reagents modules, and for reaction material recovery.

2.5 Erythropoietin purification from a yeast cell free system

Purification of expressed FLAG-rhEPO protein from the yeast lysate was performed using Anti-FLAG M2 magnetic beads (Sigma-Aldrich Corporation, MO) according to the manufacturer's protocol with modifications. Magnetic beads were prepared as per the manufacturer's protocol and mixed with the CFPS sample at 1:10 v/v. The mixture was diluted three times with Tris-buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) in the presence of Tween-20 (0.1%). After incubation at room tempera-

ture for 4 h, affinity captured material was isolated by magnetic bead separation and the beads were washed three times with 10 times bead volume TBS. Elution of FLAG-tag protein was performed using five times the bead volume 0.1 M Glycine, pH 3.0. The supernatant containing the FLAG-protein was transferred to a vial containing Tris-HCl for re-equilibration to neutral pH according to the manufacturer's recommendations (Sigma-Aldrich Corporation, MO) followed by addition of NaCl and $CaCl_2$ to a final concentration of 50 mM and 2 mM, respectively. The sample was mixed with Enterokinase (Novagen, Inc., WI) (1 U of recombinant Enterokinase/100 ng FLAG-protein) at room temperature for 4 h. Enterokinase was removed via affinity-based capture using agarose matrix embedded with soybean trypsin inhibitor according to the manufacturer's protocol (Ekapture Agarose, Novagen, Inc., MI). The efficiency of the Enterokinase capture step was assessed using an Enterokinase-specific activity assay (SensoLyte Rh110, AnaSpec, Inc., CA). The de-tagged protein was purified using a 2 mL Sephacryl S200 HR (GE Healthcare Bio-Sciences, PA) size exclusion chromatography column (ratio 20:1, flow rate of 35 µL/min). Protein samples were analyzed in 4–12% Bis-Tris SDS-PAGE gels with SimplyBlue SafeStain (Life Technologies, Corporation, CA) according to the manufacturer's recommendations. Western blot analyses were performed as described above. Preliminary assessment of protein yields during the purification process was performed using yeast cell-free protein synthesis systems spiked in with known amounts of FLAG-EPO followed by protein purification using the resins and buffers described above, and indicated protein recovery at an estimated 50 ± 5%.

2.6 GM-CSF purification from a bacterial cell free system

A modular strategy was developed for purification of rhGM-CSF expressed in the bacterial CFPS system using a combination of ion exchange and size exclusion chromatography steps similar to the method described previously [12]. Bacterial CFPS samples were diluted 1:1 with 10 mM sodium phosphate, pH 6.5, during loading (flow rate of 250 µL/min) onto a 5 cm × 1.25 cm (ID) Diethylaminoethyl (DEAE)-Sepharose Fast Flow resin (GE Healthcare Bio-Sciences, PA) equilibrated at 10 mM sodium phosphate, pH 6.5. The column was washed and samples were eluted (flow rate of 500 µL/min) utilizing a step gradient with increasing concentrations of NaCl (0–1 M) in the same sodium phosphate buffer. GM-CSF eluted at 200 mM NaCl. The entire 200 mM salt fraction was collected, dialyzed and concentrated over a regenerated cellulose 3 kDa molecular weight cut-off membrane (Ultracel, Merk EMD Millipore, MA). The concentrated sample was then loaded on a 6 mL Sephacryl S200 HR (GE Healthcare Bio-Sciences, PA) size exclusion chromatography column (40:1 length:width) and developed at a flow rate of 20 µL/min. Total protein in fractionation samples was quantified using a Pierce™ BCA protein assay (Life Technologies Corporation, CA) and GM-CSF purity was assessed in 4–12% SDS-PAGE gels with SimplyBlue SafeStain (Life Technologies Corporation, CA) according to the manufacturer's recommendations. Preliminary assessment of rhGM-CSF yields upon purification was performed as described above and indicated protein recovery at an estimated 20 ± 3%.

2.7 Mass spectrometry

For protein mass spectrometric analysis, protein samples were analyzed in 4–12% SDS-PAGE gels and stained with SimplyBlue SafeStain (Life Technologies Corporation, CA) according to the manufacturer's recommendations. Gel bands migrating to the same molecular weight as the comparative control were excised and mass spectrometry was performed at the facilities of The Scripps Research Institute Center for Mass Spectrometry (TSRI, CA). The gel bands were destained, reduced (10 mM DTT), alkylated (55 mM idoacetamide), and digested with trypsin or Glu-C overnight before being analyzed by nano-LC-MS/MS.

2.8 Protein activity assays

The bioactivity of the purified recombinant human EPO and GM-CSF was assessed using the TF-1 cells proliferation assay; this cell line is derived from bone marrow cells and exhibits growth dependency on both EPO and GM-CSF proteins [31]. Briefly, TF-1 cells (ATCC #CRL 2003) were maintained in RPMI 1640 media (ATCC 30-2001) supplemented with Fetal Bovine Serum (10%), Penicillin/Streptomycin (100 U/mL) and rhGM-CSF (2 ng/mL), and grown at 37°C in the presence of 5% CO_2. For activity tests, cells were washed three times in growth medium without GM-CSF, plated in 96-well culture plates ($2 \times 10^4 - 1 \times 10^5$ cells/mL) in the presence of serial dilutions of the test sample or control protein (final volume 100 µL/well), and incubated at 37°C for 48–72 h. At the end of the incubation period, cells were removed from 37°C and allowed to equilibrate at room temperature for 30 min. An equal volume (100 µL) of room-temperature Cell Titer-Glo reagent (Promega Corporation, WI) was added to the cells followed by shaking of the plates at 900 rpm for two min. After incubation of the plates for 10 min at room temperature, luminescence was measured using a Mithras LB 940 multimode microplate reader (Berthold Technologies, USA LLC, TN). Human EPO (CYT-201, ProSpec-Tany TechnoGene, Ltd, NJ) and GM-CSF (C003, Novoprotein Scientific, Inc., NJ) proteins were used as controls. Data was analyzed and graphed using GraphPad Prism v6 (GraphPad Software, Inc., CA).

3 Results

3.1 Development of a fluidic process for rapid production of protein biologics

A schematic diagram for production of the two protein biologics is depicted in Fig. 1. The process incorporates a reactor system for CFPS of biologic target proteins using eukaryotic or prokaryotic lysates, protein purification modules (affinity or ion exchange chromatographies) for isolation of expressed protein from the lysate mixture, and a module for protein polishing and formulation (size exclusion chromatography). The rate of cell-free protein production decreases over time due to shortage of critical components, such as amino acids and nucleotides [13]. CECF protein synthesis systems allow replenishment of substrates and removal of low molecular-weight by-products by diffusional exchange across a membrane during protein synthesis [13]. Accordingly, a reactor was developed for supporting CECF protein synthesis (Fig. 2A

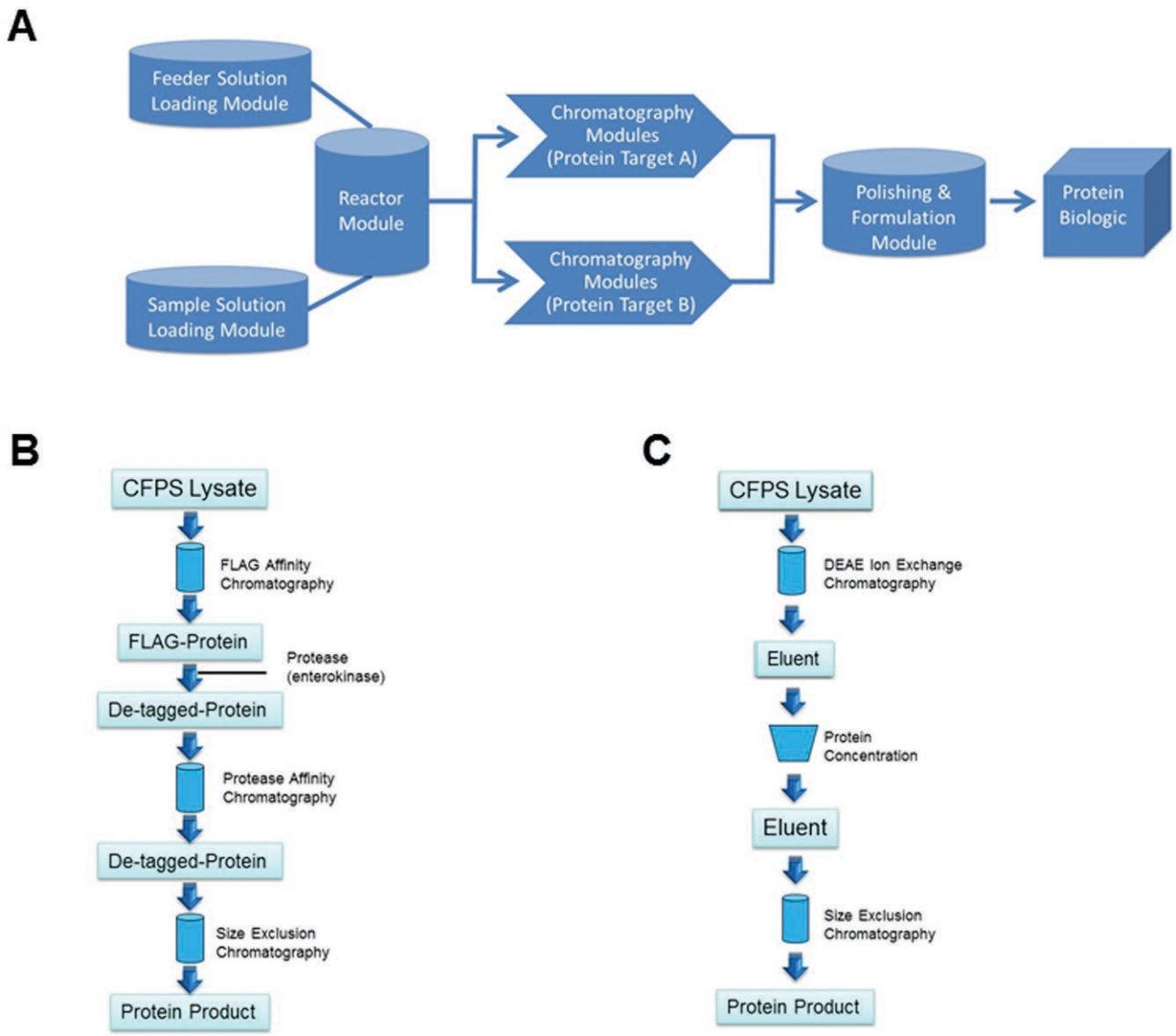

Figure 1. A schematic diagram of the fluidic process for cell-free production of protein biologics. (**A**). The fluidic process consists of: (i) Reagents (feeder and sample solutions) loading to the reactor system; (ii) Combined transcription/translation step taking place in a reactor module under continuous exchange or batch format; (iii) Protein purification steps for separation of the target proteins from the lysate mixtures using a variety of chromatography matrices depending on protein target modalities; and (iv) Protein polishing and formulation step, typically through the use of a size exclusion chromatography matrix. (**B**). Schematic representation of the purification process for recovery of rhEPO from a yeast cell-free protein synthesis system. This method incorporates FLAG-tag-based affinity chromatography for protein recovery from the cell-free system with subsequent removal of the affinity tag for recovery of the intended size protein target. (**C**). Process diagram for purification of rhGM-CSF from a bacterial cell-free protein synthesis system via the use of anion-exchange chromatography in combination with size exclusion chromatography.

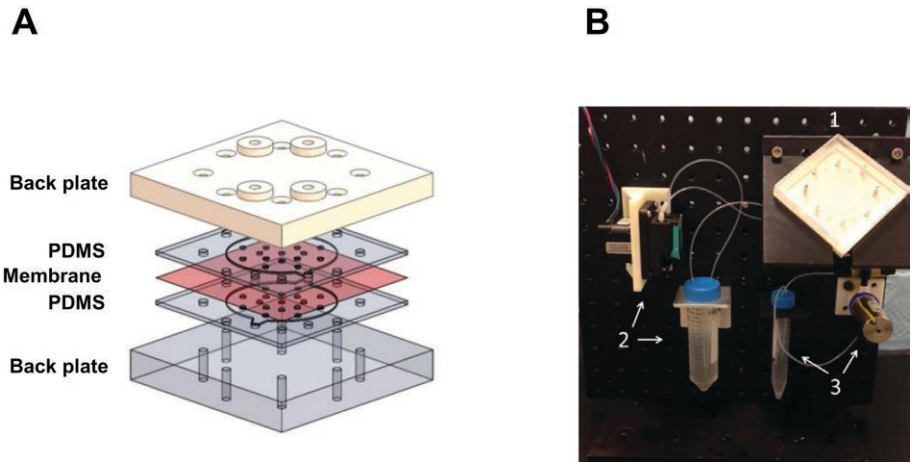

Figure 2. Protein synthesis reactor. (**A**) A computer-aided design model of the protein synthesis reactor for hosting cell free protein synthesis under continuous exchange format. Dotted features are "stand-off" supports to maintain channel volume by constricting dialysis membrane motion. (**B**) Protein synthesis reactor, feeder and sample loading modules. (1) Protein synthesis reactor. The reactor consists of a 2 mL polydimethylsiloxane (PDMS) chamber divided into the reaction and feeding chambers (1 mL, each) by a 3.5 kDa dialysis membrane. Molded PDMS chambers were produced from a stereo-lithographic printed mold. (2) Peristaltic pump and storage vial for feeder solution loading to the feeding chamber of the reactor and recirculation. (3) Diaphragm and vial for reagents loading to the reactor chamber and sample recovery.

and 2B). The reactor consists of a conventional regenerated cellulose dialysis membrane sandwiched between the reaction and feeding chamber, which are made of biocompatible materials (PDMS). The reactor is implemented with tubing fluidic systems to allow recirculating flow in the feeding chamber, fluidic integration with upstream reagent modules, and sample recovery for downstream processing. The design allows for use of various dialysis membrane types with different molecular weight cut-offs for optimum materials exchange as well as further volume scale up through parallel connection of reactor modules depending on yield requirements. The design also allows for direct connection of the two chambers in the absence of a dialysis membrane for supporting CFPS under a batch format.

A modular protein purification strategy was developed for purification of rhEPO from the yeast CFPS system (Fig. 1B). This strategy involves incorporation of three copies of a FLAG hydrophilic peptide at the N-terminus of the target protein (Fig. 3A); the FLAG-tagged protein target can then be purified using an immobilized monoclonal antibody matrix under non-denaturing conditions and eluted by lowering the pH or by adding competing amounts of free FLAG peptide. An important feature of the FLAG tag is the inherent Enterokinase cleavage site located at the C-terminus of the FLAG peptide sequence [22, 32]. Enterokinase cleaves the FLAG epitope without requiring a specific linker sequence and allows for the removal of the tag without leaving residual amino acids on the target protein [22, 32]. Subsequently, the enzyme can be removed using an Enterokinase-affinity chromatography and the target protein can be recovered and further purified through a size exclusion chromatography step (Fig. 1B). A modular strategy, similar to the method used previously [12], was developed for purification of GM-CSF from bacterial cell-free systems using a combination of ion exchange (DEAE Sepharose Fast Flow) and size exclusion (Sephacryl S200) chromatography matrices (Fig. 1C).

3.2 Rapid cell-free expression and purification of active rhEPO

3.2.1 Enhancement of rhEPO expression in a yeast cell free system through alleviation of substrate limitations and mRNA translation inhibitory factors

Our objective was to enhance product yields and functional activity of rhEPO expressed in a *Saccharomyces cerevisiae*-based CFPS system. We hypothesized that this would be achieved by a two-fold approach focusing on: (i) alleviating the exhaustion of essential substrates (e.g. nucleotide triphosphates and amino acids) that are consumed during protein synthesis and removal of toxic byproducts (e.g. inorganic phosphates) accumulated during protein synthesis [1, 18]; and (ii) eliminating translation initiation inhibitory features, such as canonical and non-canonical initiation codons contained within the coding sequence that cause ribosomal diversion and consequently reduction in the synthesis of the correct, full-length protein product [33, 34]. The former approach involved adaptation of a CECF protein synthesis format that has broadly been utilized for generation of highly productive CFPS systems [13]. The latter approach involved the use of synonymous mutations to either remove alternative

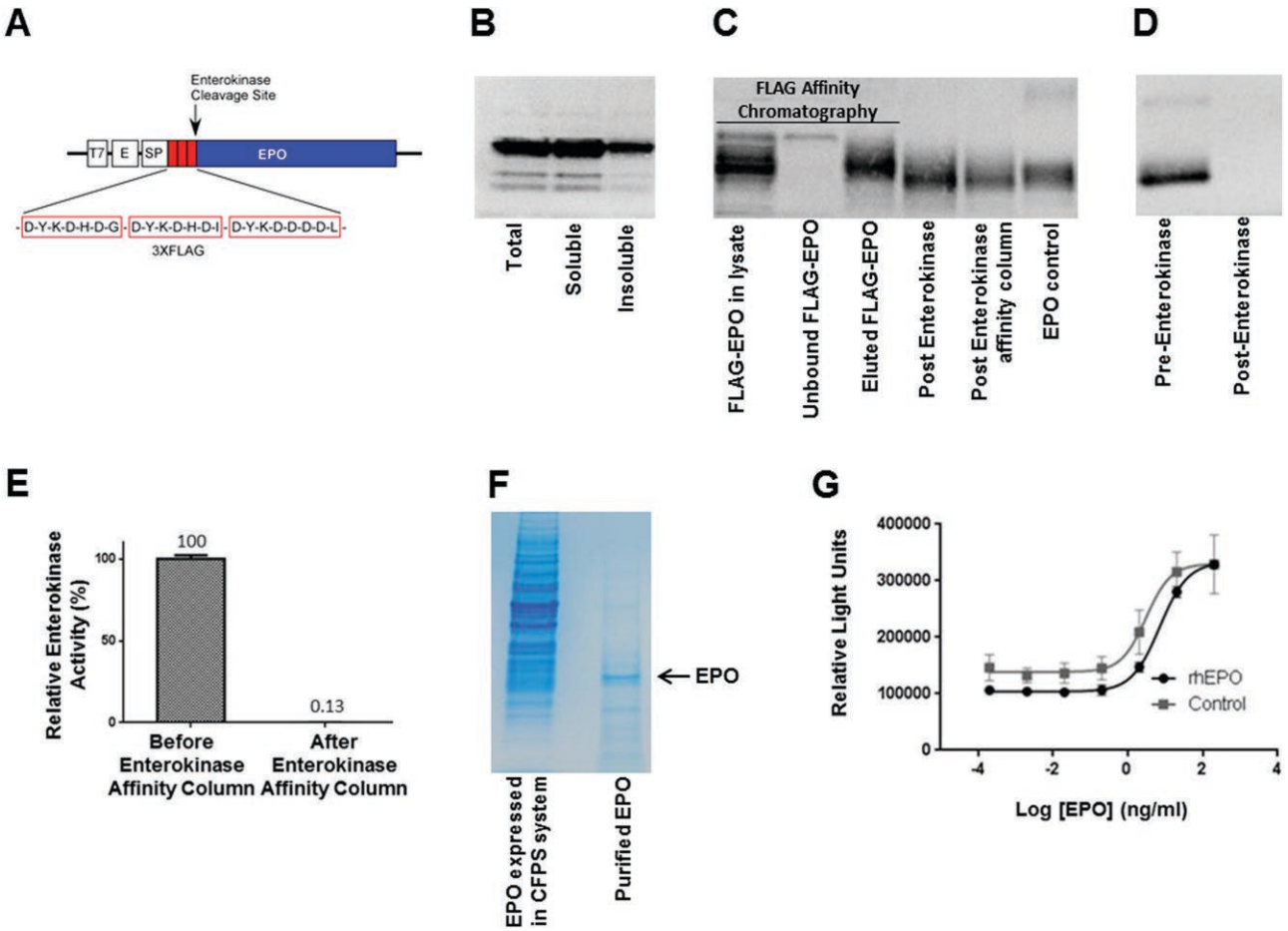

Figure 3. Expression and purification of recombinant human EPO using the fluidics system. (**A**) Schematic diagram of the construct used for cell-free expression of rhEPO in the yeast CECF protein synthesis system. The PCR template for rhEPO contains a T7 promoter (T7), translational enhancer (E), signal peptide (SP), and three FLAG epitopes, with an Enterokinase cleavage site upstream of the mature coding sequence. (**B**) Western blot analysis of rhEPO expression in a yeast cell-free protein synthesis system at 22°C. The majority of rhEPO expressed in the yeast CFPS system is present in the soluble fraction. (**C**).Western blot analysis (anti-EPO) of FLAG-rhEPO expressed in a yeast cell-free protein synthesis system during the various steps of protein purification. (**D**). Western blot analysis (anti-FLAG) of EPO sample before and after Enterokinase treatment for FLAG-tag removal. (**E**) The efficiency of the Enterokinase capture and removal step assessed using a fluorogenic substrate-based enzymatic assay; upon enzyme cleavage, this substrate generates a rhodamine fluorophore that is detected at excitation/emission = 490/520 nm. (**F**) Coomassie blue staining of protein samples before and after FLAG-affinity chromatography. (**G**) Biological activity of rhEPO synthesized in a PDMS-based reactor hosting a yeast CFPS system under continuous exchange format and purified as described in Fig. 1B. Protein biological activity was assessed using a standard human TF-1 cell-based proliferation assay. Shown are representative results from two biological replicates at multiple rhEPO protein concentrations. Data represent the mean ± SD of luminescence relative light units produced in the biological experiments.

initiation codons or decrease their utilization contained within the natural EPO signal peptide sequences. Signal peptides are short leader peptides found on the N-terminus of proteins destined for the secretory pathway. Signal peptides are portable, i.e. they can function on different genes, and are cleaved from the nascent polypeptide to generate the mature protein. To this end, EPO constructs carrying recoded signal peptide sequences upstream of the coding region as well as a FLAG sequence between the signal peptide and the coding region were generated and subjected to combined transcription/translation in the yeast CECF protein synthesis system (Fig. 3A). Using the yeast CECF protein synthesis system, rhEPO was produced at approximately 40 µg per mL of reaction with the majority of the expressed protein being present in the soluble fraction of the CFPS reaction mixture (Fig. 3B).

3.2.2 *A modular fluidic process for rapid purification of cell-free expressed rhEPO*

rhEPO expressed in the yeast CFPS system was purified according to the strategy described in Fig. 1B. The first step of the purification process involved separation of the expressed protein from the yeast lysate using anti-FLAG affinity chromatography. Typically this step requires overnight incubation of the protein materials with the beads; however, upon optimization of the binding conditions

(increasing the ratio of beads to protein) we were able to reduce this time to 4 h, during which time almost all available protein was bound to the beads (Fig. 3C). The next step included elution of the protein off the beads under acidic conditions (pH 3) (Fig. 3C). Treatment of the eluted protein with Enterokinase resulted in cleavage of the FLAG epitope from the tagged EPO protein as demonstrated by the reduction in the molecular weight of the protein product (Western blot using anti-EPO antibody, Fig. 3C) and the disappearance of the FLAG epitope (Western blot using anti-FLAG antibody, Fig. 3D). Enterokinase was then removed via the use of an agarose matrix embedded with soybean trypsin inhibitor that specifically binds to the enzyme. An Enterokinase-specific activity assay showed that this affinity-based enzyme capture step resulted in removal of the Enterokinase from the protein sample (Fig. 3E). The protein product was visualized using a Coomassie blue staining gel (Fig. 3F).

Mass spectrometry analysis showed that the nascent FLAG-rhEPO polypeptides synthesized in vitro were accurately processed enzymatically, generating mature rhEPO protein with a sequence that was identical to that of the pharmaceutical product (Epogen, Amgen, Inc., CA). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) confirmed the expected target sequences (86.7% coverage [144 of 166 amino acids] for rhEPO produced in the yeast system) as well as the presence of the correct N-terminal sequence in the rhEPO product as the result of enzymatic cleavage of the FLAG tag during the protein purification process (see also Supporting information, Mass Analysis: Section A; Table S1 and Fig. S1; Table S2 and Fig. S2).

3.2.3 *Production of bioactive rhEPO*

A standard EPO-dependent cell viability assay was employed to determine the bioactivity of the purified protein. This assay is based on quantitation of luminescent signal generated in the presence of ATP, which is directly proportional to the number of metabolically active cells present in the well. The results showed that produced EPO has activity that is comparable to that of a commercially available EPO protein (Fig. 3G).

3.3 Rapid cell free expression and purification of active rhGM-CSF

3.3.1 *Alleviation of mRNA translation inhibitory features and redox environment optimization increases expression of oxidized rhGM-CSF in a bacterial cell free system*

An assay optimization approach was used to maximize expression of functionally folded GM-CSF in the bacterial batch CFPS system. Similarly to the aforementioned yeast expression strategy, recoded signal peptides were generated and cloned upstream of the rhGM-CSF coding region to eliminate inhibitory features, such as secondary start sites, and consequently increase the productive ribosomal recruitment to the main mRNA initiation codon in the bacterial cell-free protein synthesis system (Fig. 4A). Additionally, cell-free reactions were performed in an oxidizing environment that favors disulfide bond formation (GM-CSF contains two intramolecular disulfide bonds in its native form) and supplemented with specific foldases and chaperone molecules, conditions that have been shown to allow for correct protein folding and synthesis of soluble and active target proteins [6]. This approach included: (i) IAM pre-treatment of cell extracts (IAM inactivates reducing activity in lysate by inactivating disulfide-reducing enzymes, such as thioredoxin reductase); (ii) adjustment of the redox potential by adding an oxidized glutathione buffer (GSH/GSSG); and (iii) addition of an *E. coli* periplasmic disulfide isomerase (DsbC) to facilitate formation of correct disulfide bonds (incorrect disulfide bonds may cause poor protein folding and lead to aggregation) [6]. In addition, we supplemented the CFPS system with the nonionic detergent Brij-35 that has been shown to prevent aggregation and improve solubility of expressed proteins [13]. Using these conditions, rhGM-CSF protein was expressed from the aforementioned constructs at approximately 2 mg per mL of reaction and found to be present almost exclusively in a soluble, oxidized form (Fig. 4B).

3.3.2 *A modular fluidic process for rapid purification of cell-free expressed rhGM-CSF*

rhGM-CSF expressed in the cell-free protein synthesis system was subjected to a protein purification scheme involving ion exchange and size exclusion chromatography (Fig. 1C), similar to the protocol described earlier [12]. Bacterial CFPS samples were loaded onto the ion exchange module and subjected to elution with increasing concentrations of NaCl. The 200 mM salt fractions containing rhGM-CSF were pooled, dialyzed, concentrated, and loaded on a size exclusion chromatography module. The Coomassie blue staining profiles of specific ion exchange and size exclusion samples are shown in Fig. 4C, indicating the isolation of a protein with the expected molecular weight of rhGM-CSF. LC-MS/MS analysis also confirmed the production of the expected target sequence (80.3% coverage [102 of 127 amino acids]) for the rhGM-CSF protein expressed in the bacterial system as well as established the presence of the correct N-terminal sequence in the protein product as the result of accurate cleavage of the signal peptide sequence from the nascent polypeptide in the bacterial lysate (see also Supporting information, Mass Analysis: Section B; Table S3 and Fig. S3; Table S4 and Fig. S4). The correct cleavage of the in vitro expressed rhGM-CSF protein is most likely due to signal peptidase activity present in the bacterial extract used in the CFPS system [35].

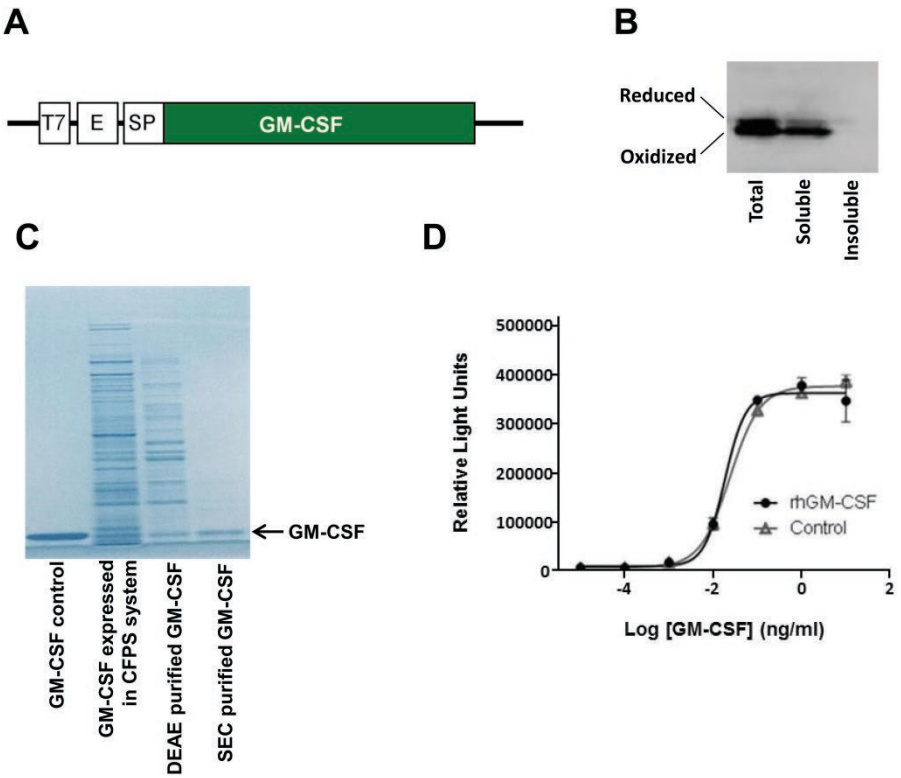

Figure 4. Expression and purification of recombinant human GM-CSF using the fluidics system. (**A**) Schematic diagram of the construct used for cell-free expression of rhGM-CSF in the bacterial cell-free protein synthesis system. The plasmid template for rhGM-CSF contains a T7 promoter (T7), translational enhancer (E), and signal peptide (SP) upstream of the mature coding sequence. (**B**) Western blot analysis of cell-free expressed rhGM-CSF in a bacterial lysate. CFPS reaction was performed in the presence of nonionic detergent Brij-35 (0.1%), IAM and DsbC at 30°C. (**C**) Purification of rhGM-CSF expressed in bacterial CFPS system. Coomassie blue staining of protein fractions during the various steps of the purification process. Commercial human GM-CSF was used as a control. (**D**) Biological activity of rhGM-CSF synthesized in a PDMS-based reactor hosting a bacterial CFPS system under batch format and purified as described in Fig. 1C. Protein biological activity was assessed using a standard human TF-1 cell-based proliferation assay. Shown are representative results from two biological replicates at multiple rhGM-CSF protein concentrations. Data represent the mean ± SD of luminescence relative light units produced in the biological experiments.

3.3.3 Production of bioactive rhGM-CSF

The biological activity of the expressed and purified rhGM-CSF product was evaluated using a standard cell-based assay that monitors the ability of the target protein to induce proliferation of a GM-CSF-dependent human cell line, TF-1. Purified GM-CSF was found to have similar activity to that of a commercially available control protein (Fig. 4D).

4 Discussion

4.1 Rapid cell-free expression and purification of protein biologics

In this work, we developed a process for rapid production of protein biologics at low doses. The process consists of combined transcription/translation of a DNA template encoding for the target protein under CECF or batch formats followed by a series of purification steps for separation of the protein biologic from the components of the cell-free system. The modularity of this process allows flexibility in using prokaryotic or eukaryotic lysates as well as combination of different protein purification workflows for production of various proteins depending on specific target modalities. Using this process, we demonstrated in vitro synthesis and purification of active rhEPO and rhGM-CSF, expressed in *S. cerevisiae* and *E. coli* lysates, respectively, in less than 24 h. These proteins contained the coding sequences for the FDA-approved protein products, Epoetin alfa, a recombinant version of endogenous hEPO, and Sargramostim, a recombinant version of endogenous hGM-CSF.

4.2 Development of a flexible process for production of protein biologics

The developed process exploits the unique properties of CFPS systems for rapid protein production from DNA templates that do not require cell cultures or insertion of

DNA sequences into cells. We employed both *S. cerevisiae* and *E. coli* CFPS systems to capitalize on the advantages of each platform for expression of different protein biologics with varying requirements. *E. coli* provides a suitable source organism since it is an FDA approved microorganism for protein production, it allows for inexpensive fermentation in large quantities using low-cost media, it has the lowest CFPS reaction cost among other lysates, and it generates the highest protein yields of all other lysates [9]. *S. cerevisiae* was used in this work as a source organism because, similarly to *E. coli*, it is an FDA-approved microorganism, it provides scalable methods for cell cultivation and lysate production, it is suitable for synthesis of complex proteins, its genome is well characterized, and there are advanced techniques for its genetic manipulation. The flexibility afforded by our developed process enables *S. cerevisiae* and *E. coli* CFPS systems to be used interchangeably for expression of different therapeutics with varying biochemical and pharmacological requirements. As described earlier, in this study, rhEPO was expressed in the yeast CFPS system whereas rhGM-CSF was produced in the bacterial CFPS system. Yet, parallel experiments showed production of rhEPO in the bacterial CFPS system at yields that were significantly higher than those obtained in the yeast system and similar to those of rhGM-CSF produced in the bacterial CFPS system, and with activities that were comparable to that of a commercially available control EPO protein (results to be described elsewhere). The flexibility of the described process also opens the possibility to use lysates from any organism for such purpose. In addition, reaction volumes in these systems can be scaled up through the use of larger reactors and/or parallelization to allow production of higher doses of protein biologics as needed.

The protein purification principles applied in the present work were focused on utilizing well-characterized resins and established approaches widely used for purification of protein biologics. The modularity of the purification processes, in combination with the flexibility of fluidics routing, can make possible the purification of different proteins on the same platform starting from DNA expressed in their respective CFPS system and continuing through the particular purification scheme optimized for each protein. In addition, complete automation is feasible by putting syringe drives and valves under computer control and the development of robust protocols that reduce/eliminate in-process decisional matrices.

4.3 Potential applications of a fluidic process for rapid production of protein biologics

Such a fluidic platform should have important applications for the production of protein biologics. Proteins that can be produced in the aforementioned platform may, for example, include FDA-approved protein therapeutics [36]. This system can also be used for rapid, cost-effective, production of limited dosages of biologics against rare medical conditions (orphan drugs), antibody vaccines, or protein-based medical countermeasures. Alternatively, it can be used to enable rapid synthesis and testing of proteins at small doses for drug screening or structure-function analysis during research and development efforts. Furthermore, it can be used for generating medications in areas where those are not available or in military medicine to increase medical capabilities of far forward providers and/or enable specific threat response.

Clearly more work is needed to enable production of pharmaceutical-grade proteins by a fully automated portable platform, a capability that could potentially allow reduction of pharmaceutical manufacture to an integrated fluidic process with a single dose lot size. Such a system would require production of analytical and statistical data demonstrating that the designed platform can reproducibly deliver a product to an established specification. In addition, it would require utilization of a Quality by Design approach that incorporates appropriate in-process check points and critical quality assurance assays in the final implementation of the manufacturing process to ensure that the limited lot size product meets those specifications. Nonetheless, this study provides a proof of concept that such a platform is feasible for rapid production of low doses of protein biologics at the point-of-need. In addition, the platform can be scalable and amenable to integration with fluidic analytical modules towards the generation of a fully automated system for production of proteins with significant pharmaceutical, medical, or biotechnological value.

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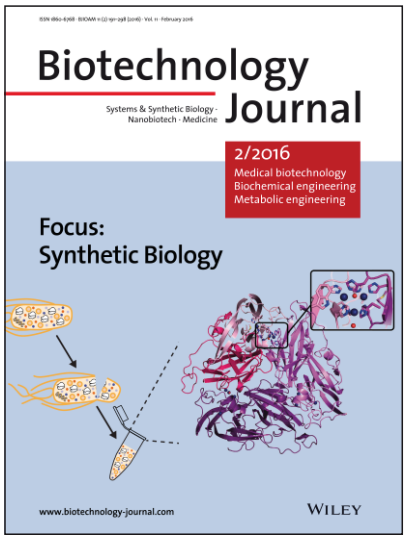

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Amin S. M. Salehi, Mark Thomas Smith, Anthony M. Bennett, Jacob B. Williams, William G. Pitt and Bradley C. Bundy

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