Integration of cell-free protein synthesis and purification in one microfluidic chip for on-demand production of recombinant protein

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(Received 31 May 2018; accepted 4 September 2018; published online 13 September 2018)

Recombinant proteins have shown several benefits compared with their non-recombinant counterparts in protein therapeutics. However, there are still some problems with the storage and distribution of recombinant proteins, owing to their temperature sensitivity. Microfluidic chips can integrate different functional modules into a single device because of the advantages of integration and miniaturization, which have the special potential to synthesize drugs when and where they are needed most. Here, we integrated cell-free protein synthesis and purification into a microfluidic chip for the production of recombinant protein. The chip consisted of a main channel and a branch channel. The main channel included two pinches, which were filled with template DNA-modified agarose microbeads and nickel ion-modified agarose beads as the cell-free protein synthesis unit and protein purification unit, respectively. The reaction mixture for protein synthesis was introduced into the main channel and first passed through the protein synthesis unit where the target protein was synthesized; next, the reaction mixture passed through the protein purification unit where the target protein was captured; and, finally, pure protein was collected at the outlet when washing buffer and eluting buffer were sequentially introduced into the branch channel. Enhanced green fluorescent protein (EGFP) was used as the model to investigate the performance of our chip. One chip could produce 70 μ l of EGFP solution (144.3 μ g/ml, 10.1 μ g) per batch, and another round of protein synthesis and purification could be performed after replacing or regenerating nickel ion-modified agarose beads. It should be possible to produce other recombinant proteins on demand with this chip by simply replacing the template DNA. *Published by AIP Publishing.*
<https://doi.org/10.1063/1.5042307>

I. INTRODUCTION

Recombinant proteins have attracted increasing attention in protein therapeutics^{1–3} and have been among the most rapidly developing trends in modern biotechnology^{4,5} because of their advantages in specific activity, produce, biosafety, and potential for modification. However, the storage and distribution of proteins in remote areas remains challenging, because they are temperature sensitive and require continuous cryopreservation. Even where suitable conditions for continuous

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cryopreservation can be provided, it is still a great challenge to preserve the biological function of the proteins during storage and distribution.^{6,7} In addition, traditional industrial production of biologics usually cannot meet the requirements of personalized medicine, resulting in a waste of resources. Producing a single-dose of recombinant protein drugs on demand provides a solution to the problem.⁸ However, since the traditional methods for preparing recombinant proteins often necessitate transfecting the recombinant DNA of the target protein into organisms, it is impractical to produce recombinant proteins on demand in this way.^{9–11}

In 1961, Nirenberg *et al.* developed cell-free protein synthesis (CFPS),¹² which brought hope for protein synthesis on demand. CFPS can synthesize target proteins on demand by mixing together the components required for protein synthesis: cell lysate, reaction mixture, amino acids, and template DNA.¹³ Shimizu *et al.* developed a protein-synthesizing system reconstituted from recombinant tagged protein factors purified to homogeneity, i.e., protein products without a His tag were purified easily by affinity chromatography to remove His-tagged translation factors.¹⁴ CFPS can be performed in remote areas without concern for the culture and activity of cells,^{3,15} minimizing the materials and facilities required. To date, many recombinant proteins have been synthesized by CFPS, including vaccines,¹⁶ antibodies,^{5,17–19} human growth hormone,²⁰ insulin,^{21–23} and erythropoietin.^{24,25} More importantly, CFPS possesses better flexibility than traditional methods and can be used to synthesize hard-to-express proteins *in vivo*, such as membrane proteins,^{26–29} toxic proteins,^{4,30} and some proteins with special functions, by introducing non-natural amino acids.^{31,32} However, protein expression yields were limited in the traditional batch reaction mode, owing to the accumulation of phosphate and the rapid consumption of raw materials, which resulted in high reagent consumption and costs. In order to improve protein yield, researchers developed the continuous-flow cell-free (CFCF)^{11,33,34} and continuous-exchange cell-free (CECF) methods.^{35–38} Both CFCF and CECF depend on a continuous supply of energy and substrate and the removal of reaction by-products to prolong reaction times and increase protein yield. However, it was still difficult to meet the needs of protein synthesis on demand with these devices, because of their large size and complex operation.

The emergence of microfluidics provides an alternative solution to the above problems. As protein synthesis is carried out in a micron-sized channel, the reagent consumption, reaction times, and reaction efficiency are improved³⁹ owing to the large surface area-to-volume,³³ short diffusion distance,³⁵ and ease of temperature control.⁴⁰ Both CFCF³³ and CECF³⁵ have been performed in microfluidic chips. Since proteins used at the point of care require a certain purity, there remains a need to integrate protein synthesis and protein purification on a microfluidic chip, utilizing the integral advantages of microfluidics,^{41,42} to obtain the desired recombinant proteins by a simple operation.

Previously, a microfluidic chip integrating positive and negative selection units for screening aptamers was developed by our group.^{43,44} The microfluidic chip was uncomplicated and could be used repeatedly. Here, we integrated two functional units, i.e., a protein synthesis unit and a protein purification unit, into the microfluidic chip, allowing pure proteins to be obtained without offline purification (Fig. 1). The chip consisted of a main channel and a branch channel. The main channel contained two pinches: the first pinch was filled with template DNA-modified agarose beads to form the protein synthesis unit, and the second pinch was filled with nickel ion-modified agarose beads [Ni-nitrilotriacetic acid (NTA) beads] as the protein purification unit.¹⁴ The components for protein expression, which included cell lysate, the reaction mixture, and amino acids, were introduced into the main channel (inlet 1) at a specified flow rate; after this solution passed through the protein synthesis unit, the target protein was produced. Subsequently, the mixed reaction solution passed through the protein purification unit, where the target protein was captured by Ni-NTA beads. To obtain pure protein, the washing buffer was first introduced from the branch channel (inlet 2) to remove non-specifically bound materials; next, the eluting buffer was introduced, again from inlet 2, after which the target protein could be collected at the outlet. Another round of protein synthesis and purification could be performed after replacing or regenerating the Ni-NTA beads. The chip platform was flexible, and we expected that it could be used to produce various recombinant proteins on demand, only by replacing the template DNA.

FIG. 1. Schematic illustration of the integration of cell-free protein synthesis and purification in one microfluidic chip. The reaction mixture for protein synthesis was introduced from inlet 1 and first passed through the protein synthesis unit, where the target protein was synthesized; next, the mixed solution passed through the protein purification unit, where the target protein was captured by Ni-NTA beads; finally, pure protein was collected at the outlet when washing buffer and eluting buffer were sequentially introduced from inlet 2. The 3D structure of His-tagged green fluorescent protein was reproduced with permission from Anal. Chem. 87, 6311 (2015). Copyright 2015 American Chemical Society.⁴⁴

II. EXPERIMENTAL

A. Materials, reagents, and instruments

RTS 100 *Escherichia coli* HY Kit was purchased from biotechrabbit (Germany). DNA Clean-up Kit was purchased from CWBiotech (Beijing). Bradford Protein Quantification Kit was purchased from Yeasen (Shanghai). 2× Power Taq PCR Master Mix was purchased from BioTeke Corporation (Beijing). DNA Marker and 6× Loading Buffer were purchased from Takara Bio (Dalian). SYBR Gold was purchased from Invitrogen (USA). Template dsDNA pET28a-EGFP was purchased from Miao Ling Biotechnology Co., Ltd. (Wuhan). HEPES was purchased from Solarbio Science & Technology (Beijing). KOH was purchased from Tianjin Fuchen Chemical Reagent Factory (Tianjin). Iminazole was purchased from Shanghai Second Pharmaceutical Factory (Shanghai). Streptavidin SepharoseTM High Performance (34 μm) and Ni SepharoseTM High Performance (34 μm) were purchased from GE Healthcare Life Sciences (USA). Polymerase chain reaction (PCR) primers, diethylpyrocarbonate-treated water, and microcentrifuge tubes

(DNase/RNase-free) were purchased from Sangon Biotechnology Co., Ltd. (Shanghai). Other chemical reagents were purchased from Sinoreagent (Shanghai). All the chemical reagents were of analytical grade or higher. Chrome plate and plate with holes were purchased from Shaoguang Microelectronics Co., Ltd. (Changsha). The film mask was purchased from Chengtian Culture Communication Co., Ltd (Hunan). The instruments used were as follows: Mastercycler[®] Pro 384 (Eppendorf) for PCR, fluorescence spectrophotometer (HITACHI F-7000) for fluorescence analysis of enhanced green fluorescent protein (EGFP), BioSpec-nano (SHIMADZU), centrifuge (Beckman Coulter, Microfuge 22R Centrifuge) for separating agarose beads, and inverted research microscope (Nikon, Ts2R-FL) for fluorescence microscopic images in the chip.

B. Design and fabrication of a microfluidic chip

The fabrication process for the chips was as described previously by our group,⁴³ using standard photolithographic and wet chemical etching techniques. The structure of the microchip, which was made of glass and packaged by thermal bonding, is shown in Fig. S1 in the [supplementary material](#). The chip consisted of a main channel (200 μm wide, 70 μm deep) and a branch channel (200 μm wide, 70 μm deep). The main channel contained two pinches (1 mm long, 30 μm wide, 15 μm deep), which were filled with template DNA-modified agarose beads and Ni-NTA beads to form the protein synthesis unit and protein purification unit, respectively.

C. The preparation of template DNA

For CFPS, the yield of the recombinant protein expressed by the template DNA prepared by PCR amplification was equivalent to that expressed by plasmid DNA.⁴⁵ Compared with plasmid DNA, the preparation of template DNA by PCR was not only an easier operation but also reduced time consumption.⁴⁶ In this work, the non-labeled template DNA EGFP sequence was obtained by PCR amplification of the plasmid pET28a-EGFP, performed by Wuhan Miao Ling Biotechnology Co., Ltd. To modify the template DNA on streptavidin-coated agarose beads, the template DNA was then amplified using a 5'-end biotin-labelled forward primer and a non-labeled reverse primer (see Table S1 in the [supplementary material](#)).⁴⁷ The PCR conditions were as follows: 94 °C for 5 min, 16 cycles of three-step PCR (94 °C for 30 s, 74.6 °C for 45 s, and 72 °C for 60 s), terminated by an extra extension at 72 °C for 5 min, followed by cooling at 4 °C. The PCR products were verified by 2% agarose electrophoresis and purified using a DNA Clean-up Kit according to the manufacturer's instructions.

D. Modification of template DNA on streptavidin-coated agarose beads

First, eight portions of 10 μl streptavidin-coated agarose beads were centrifuged at 10 000 $\times g$ and room temperature for 5 min, and the supernatant was removed. The streptavidin-coated agarose beads were washed three times with HEPES buffer (20 mM HEPES-KOH, pH 7.6, and 100 mM KCl); next, the pure biotinylated template DNA was mixed with streptavidin-coated agarose beads at different volume ratios and incubated at 1200 rpm and room temperature for 1 h. After centrifuging, the supernatant was retained to detect the amount of DNA. The template DNA-modified agarose beads were re-suspended in buffer and stored at 4 °C.

E. Template DNA-modified agarose beads for protein synthesis in microcentrifuge tubes

CFPS was performed in microcentrifuge tubes using an RTS 100 *E. coli* HY Kit according to the manufacturer's instructions. The 50 μl CFPS reaction volume comprised 12 μl of extract, 10 μl of reaction mixture, 12 μl of amino acids (excluding methionine), 1 μl of methionine, 5 μl of recombinant buffer, and 10 μl of template DNA-modified agarose beads. The reaction was performed at 1200 rpm and 30 °C for 6 h in a Thermomixer (Eppendorf), and the solution was collected and stored at 4 °C overnight. The fluorescence intensity of the EGFP synthesized in the microcentrifuge

tubes was measured using a Hitachi F-7000 fluorescence spectrometer. The excitation and emission wavelengths were 488 and 516 nm, respectively.

F. Purification of the protein with Ni-NTA beads in microcentrifuge tubes

A 10 μ l suspension of Ni-NTA beads was centrifuged at $10\,000\times g$ and room temperature for 5 min, after which the supernatant was removed and the Ni-NTA beads were washed three times with washing buffer (20 mM sodium phosphate, 0.5 M NaCl, 5 mM imidazole, pH 7.4). Then, the synthesized protein was added to the microcentrifuge tubes containing Ni-NTA beads and incubated at 1200 rpm and 30 °C for 1 h in a Thermomixer. After incubation, the mixture was centrifuged at $10\,000\times g$ and room temperature for 5 min, and the fluorescence intensity of the supernatant was measured. The protein-bound Ni-NTA beads were incubated with eluting buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4) at 1200 rpm and 30 °C for 30 min in a Thermomixer. After centrifuging at $10\,000\times g$ and room temperature for 5 min, the fluorescence intensity of the supernatant was measured.

G. Template DNA-modified agarose beads for protein synthesis in chip

First, inlet 2 was closed, and template DNA-modified agarose beads were aspirated from inlet 1 by a syringe (Hamilton) until the first pinch was filled; then, the reaction mixture for protein synthesis was introduced from inlet 1 at 10 μ l/min for 2.5 min; and, finally, the flow was adjusted to the desired rate, and the solution was collected at the outlet.

H. Ni-NTA beads for protein purification in chip

First, inlet 1 was closed, and Ni-NTA beads were aspirated from inlet 2 by a syringe until the second pinch was filled. Protein synthesized in the microcentrifuge tube was introduced, washing buffer and eluting buffer were sequentially injected from inlet 2, and the individual buffers were separately collected at the outlet. Finally, the fluorescence intensity of each buffer was measured.

I. Integration of protein synthesis and purification in one microchip

Before protein synthesis and purification, two kinds of beads were inhaled into the chip and blocked before the two pinches. First, inlet 2 was closed, and template DNA-modified agarose beads were aspirated from inlet 1 by a syringe and blocked at the first pinch; next, inlet 1 was closed, and Ni-NTA beads were aspirated from inlet 2 and blocked at the second pinch. For protein synthesis and purification, inlet 2 was closed, and the reaction mixture for protein synthesis was injected from inlet 1 and allowed to pass through the protein synthesis and purification units. For fluorescence analysis, the reaction mixture was collected at the outlet. Next, inlet 1 was closed, and the washing buffer and eluting buffer were sequentially injected from inlet 2 and allowed to pass through the purification unit. For fluorescence analysis, the washing buffer and the eluting buffer were collected at the outlet.

III. RESULTS AND DISCUSSION

In order to achieve the purpose of integrating protein synthesis and purification in one microfluidic chip, we first investigated the feasibility of each part: (1) template DNA-modified agarose beads for protein synthesis in microcentrifuge tubes; (2) Ni-NTA beads for protein purification in microcentrifuge tubes; (3) template DNA-modified agarose beads for protein synthesis on a chip; and (4) Ni-NTA beads for protein purification on a chip. After this step-by-step verification, we integrated protein synthesis and purification on a single chip.

A. Template DNA-modified agarose beads for protein synthesis in microcentrifuge tubes

In this section, first we determined whether the biotinylated template DNA has been successfully prepared; then, the feasibility of template DNA-modified agarose beads for protein synthesis in microcentrifuge tubes was investigated; finally, the density of the template DNA used to modify the agarose beads and the reaction temperature for CFPS were optimized.

To prepare the biotinylated template DNA, we optimized the annealing temperature and number of rounds of PCR amplification before performing batch PCR. In theory, the template DNA obtained by PCR was 1061 bp long. As shown in Fig. S2 in the [supplementary material](#), when the annealing temperature and the number of amplification rounds were 74.6 °C and 16, respectively, the electrophoresis bands were clear and bright and the PCR product was approximately 1000 bp according to the DNA marker, which met the theoretical values. Therefore, the subsequent experiments were carried out under these conditions.

The feasibility of biotinylated template DNA for protein synthesis in CFPS was verified. The reaction was performed using an RTS 100 *E. coli* HY Kit according to the manufacturer's instructions. The results were characterized by fluorescence (Fig. 2) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3). After addition of the template DNA-modified agarose beads to the CFPS system, the solution showed a clear emission peak at 516 nm, which was exactly the emission wavelength of EGFP [solid line in Fig. 2(a)]. However, after addition of non-modified agarose beads to CFPS, there was no emission peak at 516 nm [dotted line in Fig. 2(a)]. Figure 2(b) shows fluorescence photographs of the microcentrifuge tubes. These results showed that EGFP could be synthesized in a CFPS system with template DNA-modified agarose beads. In Fig. 3, there was a clear band at approximately 27 KD in lane 3 of the SDS-PAGE gel, indicating that the protein synthesized in the CFPS system was EGFP. These results demonstrated that the template DNA modified on agarose beads was able to successfully express protein in CFPS.

To investigate the relation between protein yield and the density of template DNA on agarose beads, pure PCR products were incubated with streptavidin-coated agarose beads at different volume ratios (Table S2 in the [supplementary material](#)). The density of template DNA on the agarose beads was characterized by ultraviolet-visible light (UV-vis) absorption spectra. UV-vis absorption spectra were obtained for the biotinylated template DNA before and after incubating with streptavidin-coated agarose beads. The total amount of DNA (N_{DNA}) modified on the agarose beads was calculated according to the following equation:

$$N_{\text{DNA}} = \frac{\Delta WV}{M} N_A, \quad (1)$$

FIG. 2. The feasibility of template DNA-modified agarose beads for protein synthesis. (a) The solid line is the fluorescence spectrum of reaction solutions after addition of template DNA-modified agarose beads. Non-modified agarose beads were used as control. (b) Fluorescence photographs of microcentrifuge tubes. Total reaction volume: 50 μ l; reaction conditions: 1200 rpm, 30 °C, 6 h.

FIG. 3. SDS-PAGE of target protein. Lane 1: marker; lane 2: adding non-modified agarose beads to CFPS; lane 3: adding template DNA-modified agarose beads to CFPS; lane 4: protein solution collected from the integration chip, i.e., purified protein from eluting buffer in Fig. 8(c).

where N_{DNA} is the amount of DNA; ΔW is the change in mass concentration of DNA before and after incubation with agarose beads; V is the volume of the DNA solution; M is the molecular weight of DNA; and N_A is Avogadro's constant. The number of streptavidin-coated agarose beads (N_{bead}) in the stock solution was calculated according to the following equation:

$$N_{\text{bead}} = \frac{V\varphi}{\frac{4}{3}\pi R^3}, \quad (2)$$

where N_{bead} is the number of agarose beads; V is the volume of the stock solution containing the agarose beads; φ is the volume fraction of agarose beads in the stock solution; π is the ratio of circumference to diameter; and R is the radius of agarose beads. The density of template DNA (D_{DNA}) on each bead was obtained using the following equation:

$$D_{\text{DNA}} = \frac{N_{\text{DNA}}}{N_{\text{bead}}}, \quad (3)$$

where D_{DNA} is the density of DNA on agarose beads. Non-specific interactions of the DNA with the beads were also investigated to correct the D_{DNA} by obtaining UV-vis absorption spectra of the unmodified template DNA before and after incubation with streptavidin-coated agarose beads.

The results are shown as Table S2 in the [supplementary material](#). As the ratio of DNA to agarose beads increased, the density of template DNA increased and reached a saturation point at 7:1. The changes in the density of template DNA were most obvious when the volume ratios were 1:1, 3:1, 5:1, and 7:1. We selected the four densities of template DNA corresponding to these ratios for protein synthesis, using 500 ng of DNA in each reaction. As shown in Fig. S3 in the [supplementary material](#), as the density of template DNA increased, the protein yield first increased and then decreased, since high densities increased steric hindrance between T7 RNA polymerase and the template DNA, which limited the transcription efficiency. Therefore, $1.53 \times 10^7/\text{bead}$ was chosen as the optimal density of template DNA for further experiments.

As temperature affected the activity of enzymes in CFPS, it was necessary to examine the effect of temperature on protein yield. As shown in Fig. S4 in the [supplementary material](#), the protein yield was higher at 30 °C than at 37 °C or 25 °C and was lowest at 37 °C. We selected 30 °C as the best reaction temperature for subsequent experiments.

B. Ni-NTA beads for protein purification in microcentrifuge tubes

Both the C-terminus and N-terminus of the template DNA were designed to express His6-tags so that the synthesized EGFP could be purified by Ni-NTA beads. The fluorescence intensity of the protein solution before incubating with Ni-NTA beads [Fig. 4(a)-i] was almost four times higher than that after incubating with Ni-NTA beads [Fig. 4(a)-ii], indicating that the protein was captured by the Ni-NTA beads. The protein-bound Ni-NTA beads were incubated with eluting buffer and then centrifuged. The fluorescence of the supernatant, as shown in Fig. 4(a)-iii, indicated that the protein bound to Ni-NTA beads was re-released in the eluting buffer. Figure 4(b) shows fluorescence photographs of microcentrifuge tubes at different steps. These results demonstrated that the protein was successfully captured and released by the Ni-NTA beads.

C. Template DNA-modified agarose beads for protein synthesis in-chip

In this section, which focuses on feasibility verification of template DNA-modified agarose beads for protein synthesis in-chip, optimizations of the filling length of template DNA-modified agarose beads and the flow rate of reaction mixture are discussed.

The feasibility of template DNA-modified agarose beads for protein synthesis in-chip was verified. As shown in Fig. 5(a), when the reaction mixture was introduced into the channel filled with the template DNA-modified agarose beads at $0.2\ \mu\text{l}/\text{min}$, the fluorescence spectrum showed an emission peak at 523 nm (solid line), indicating that the template DNA-modified agarose beads could successfully synthesize protein in the chip. The channel filled with non-modified agarose beads was used as the control, which resulted in no visible emission peak (dotted line). The fluorescence microscopic images of the channel shown in Fig. 5(b) confirmed these results. Compared with EGFP synthesized in a microcentrifuge tube, the emission peak was red shifted (from 516 to 523 nm); a possible reason for this might be the influence of the micron-scale channel on protein folding. In addition, the microfluidic platform improved the efficiency of protein synthesis. Based on the fluorescence intensities of EGFP as shown in Fig. 2(a) (about 1600) and Fig. 5(a) (about 2500), the yield of protein obtained in the $50\ \mu\text{l}$ reaction mixture in the chip was higher than that in the centrifuge tube. It should also be noted that the reaction time was shortened by more than 4.5-fold from 6 h to 1.3 h in the chip. More importantly, the device was easy to operate and required only two steps to prepare pure protein: introducing the reaction mixture for protein synthesis and introducing the wash buffer and elution buffer sequentially for protein purification. Compared with using centrifuge tubes, use of the device avoided repeated washing and centrifugation. Moreover, the device was easy to install and was reusable: agarose beads introduced from the chip inlet could be fixed at pinches, and the next round of protein synthesis and purification could be performed after replacing the Ni-NTA beads.

The amount of template DNA in the chip could be adjusted by changing the filling length of the template DNA-modified agarose beads. The fluorescence intensity slightly increased as the

FIG. 4. Feasibility verification of protein purification with Ni-NTA beads in microcentrifuge tubes. [(a)-i] protein solution that was not incubated with Ni-NTA beads; [(a)-ii] supernatant after incubation with Ni-NTA beads; [(a)-iii] supernatant of protein-bound Ni-NTA beads incubated with eluting buffer. (b) Photographs of microcentrifuge tubes. Incubation conditions: 1200 rpm, $30\ ^\circ\text{C}$, 1 h.

FIG. 5. The feasibility of template DNA-modified agarose beads for protein synthesis in-chip. (a) The solid line is the fluorescence spectrum of the reaction mixture after passing through the channel filled with template DNA-modified agarose beads. Non-modified agarose beads were used as control. (b) Fluorescence microscopic images of the channel filled with agarose beads. Filling length of agarose beads: 10 mm; flow rate of the reaction mixture: $0.2 \mu\text{L}/\text{min}$; reaction temperature: 30°C .

filling length increased (Fig. S5 in the [supplementary material](#)). Since longer bead filling length could induce higher column pressure in the chip, we selected 4 mm as the best filling length.

The flow rate of the reaction mixture was also optimized. As shown in Fig. 6, when the flow rate increased, the fluorescence intensity of the collected solution decreased, implying a decrease in protein yield. This phenomenon can be easily understood: when the flow rate was increased, the contact time between T7 RNA polymerase in the reaction mixture and template DNA on the agarose beads was reduced, and thus the amount of transcribed mRNA was reduced, resulting in a decrease in translated EGFP.

D. Ni-NTA beads for protein purification in-chip

In this section, which focuses on feasibility verification of protein purification by Ni-NTA beads in-chip, optimizations of the flow rates of the protein solution and eluting buffer and the filling length of the Ni-NTA beads are discussed.

FIG. 6. Fluorescence intensity of the different flow rates of the reaction mixture. Filling length of agarose beads: 4 mm; reaction temperature: 30°C . The error bars indicate the standard deviation of two experiments.

FIG. 7. Fluorescence microscopic images of the purification unit: (a) before the protein solution passed through the unit; (b) after the protein solution passed through the unit; (c) after the eluting solution passed through the unit. The flow rates of protein solution and eluting buffer were all $3 \mu\text{l}/\text{min}$. The filling length of Ni-NTA beads was 6–7 mm.

Fluorescence changes in the filling area of Ni-NTA beads (purification unit) were detected using an inverted fluorescence microscope (Fig. 7). The Ni-NTA beads showed very low fluorescence before the protein solution passed through the unit (A); however, the fluorescence intensity of the Ni-NTA beads significantly increased after the protein solution had passed through the unit (B) and disappeared (C) after the eluting buffer had passed through the unit. The results indicated that recombinant proteins were well captured and eluted by Ni-NTA beads on the chip. A video of protein purification in-chip is provided in the [supplementary material](#) (Protein purification video.mpg). In order to improve the contrast, 1 g/l methylene blue was introduced into the chip and the bright-field images were overlaid with fluorescence images.

To further confirm the above conclusions, the fluorescence intensities of the protein solution before and after passing through the purification unit were measured, as was that of the eluting buffer after passing through the unit. The results are shown in Fig. 8. Line a represents the protein solution before passing through the purification unit, with the highest fluorescence intensity; and line b represents the protein solution after passing through the unit, when the fluorescence intensity was significantly decreased, indicating that most of the proteins were captured by the Ni-NTA beads filling the channel. Line c represents the fluorescence intensity of the eluting buffer after passing through the protein-bound purification unit; the fluorescence intensity was obviously enhanced at this time, indicating that the protein bound to the purification unit was replaced by imidazole in the eluting buffer, while the protein was released into the eluting buffer. These results indicated that it was feasible to use Ni-NTA beads to purify proteins in-chip.

The flow rate of the protein solution was optimized by measuring the binding efficiency of protein to nickel beads at different flow rates, as shown in Fig. S6 in the [supplementary material](#).

FIG. 8. The feasibility of protein purification by Ni-NTA beads in-chip. [(a) and (b)] Protein solution before and after passing through the purification unit; (c) eluting buffer after passing through the protein-bound purification unit. The flow rates of protein solution and eluting buffer were all $3 \mu\text{l}/\text{min}$. The filling length of Ni-NTA beads was 6–7 mm.

The fluorescence intensity of the protein solution before being introduced into the purification unit was used as a control. Protein solution was introduced into the purification unit at 0.2, 0.5, 1, 2, and 3 $\mu\text{l}/\text{min}$. With increasing flow rate, the amount of protein in solution decreased, reaching a plateau at 1 $\mu\text{l}/\text{min}$, where the binding efficiency was as high as 93%. The increased protein binding efficiency accompanying an increased flow rate might have been due to the turbulence occurring at faster flow rates, which would have increased the effective collision efficiency between protein and Ni-NTA beads. To reduce the time required for purification, we selected 3 $\mu\text{l}/\text{min}$ as the optimal flow rate in the following experiment.

Next, the flow rate of eluting buffer was also optimized, as shown in Fig. S7 in the [supplementary material](#), using the fluorescence intensity of the original protein solution as a control. The eluting buffer was introduced into the protein-bound purification unit at different flow rates. When the flow rate increased, the protein content in the eluting buffer increased and reached a plateau at 3 $\mu\text{l}/\text{min}$. Therefore, we selected 3 $\mu\text{l}/\text{min}$ as the optimal flow rate for subsequent experiments.

Finally, the filling length for the Ni-NTA beads was also optimized. We investigated the fluorescence intensity of the protein solution after passing through the purification unit filled with different lengths of Ni-NTA beads, as shown in Fig. S8 in the [supplementary material](#). The control was the fluorescence intensity of the protein solution before being introduced into the purification unit. When the filling length of the Ni-NTA beads increased, the fluorescence intensity of the protein solution dramatically decreased. The protein in the solution was almost completely captured at a filling length of about 3 mm; thus, this was chosen as the optimal length in the following experiment.

E. Integration of protein synthesis and purification in one chip

According to the above results, in the protein synthesis unit, the slower the flow rate was, the higher the protein yield was; in the protein purification unit, however, the faster the flow rate was, the higher the binding efficiency and elution efficiency were. When integrating protein synthesis and purification on a chip, we only considered whether integration in the chip could be achieved, so the reaction mixture was introduced at 0.2 $\mu\text{l}/\text{min}$. The results are shown in Fig. 9. Line a in Fig. 9 represents the reaction mixture collected at the outlet after passing through the protein synthesis unit and purification unit from inlet 1 at 0.2 $\mu\text{l}/\text{min}$. The fluorescence intensity was significantly lower than that in Fig. 5 since the synthesized protein was captured by the purification unit.

To verify this deduction, washing buffer was introduced into the channel from inlet 2 at 3 $\mu\text{l}/\text{min}$. The washing buffer only passed through the purification unit and was collected at the outlet. The fluorescence intensity of the washing buffer (line b) was almost the same as that of the collected

FIG. 9. Integration of protein synthesis and purification in one chip. (a) Reaction mixture after passing through the synthesis unit and the purification unit in the chip; (b) washing buffer after passing through the purification unit; (c) eluting buffer after passing through the purification unit. Filling lengths of DNA-modified agarose beads: 4–5 mm; Ni-NTA beads: 6–7 mm; flow rate of eluting buffer: 3 $\mu\text{l}/\text{min}$; temperature: 30 $^{\circ}\text{C}$.

reaction mixture (line a) since the EGFP was captured by Ni-NTA beads. Next, we introduced eluting buffer into the channel from inlet 2 at 3 $\mu\text{l}/\text{min}$ and allowed it to pass through the purification unit. The fluorescence of the eluting buffer collected at the outlet was significantly increased (line c), proving that the captured protein was released. These results indicated that our microfluidic platform achieved the integration of protein synthesis and protein purification.

The eluting buffer collected at the outlet [Fig. 9(c)] was also verified by SDS-PAGE. The gel electrophoresis showed only the band at approximately 27 kD (Fig. 3, lane 4), indicating that pure protein can be obtained by a simple operation using our microfluidic chip.

Since the protein obtained in our microfluidic chip was pure, the Bradford Protein Quantification Kit was used to quantify the protein collected at the outlet. The results showed that 70 μl of 144.3 $\mu\text{g}/\text{ml}$ EGFP solution (i.e., 10.1 μg protein) was obtained in a single operation. Compared with other microfluidic platforms or commercial reactors (see Table S3 in the [supplementary material](#)), the main advantage of our microchip was the ability to harvest pure protein directly, which simplified operation and reduced time consumption. However, it would be desirable to improve the chip design to increase the protein yield.

IV. CONCLUSIONS

In this work, we integrated CFPS and purification into a microfluidic chip for on-demand production of recombinant protein. Only two steps were required in the process of protein preparation: (1) introduction of the reaction mixture into the channel and (2) sequential introduction of the washing buffer and eluting buffer into the channel to obtain pure protein. This platform was also flexible and could potentially be used to produce other recombinant proteins on demand, just by replacing the template DNA. Most importantly, our platform can produce proteins that bacterial protein expression systems cannot, such as anti-microbial peptides and proteins that are toxic to the producing cells. We will continue to develop this flow cell-based protein expression system in our future work.

SUPPLEMENTARY MATERIAL

See [supplementary material](#) for the primer sequences, density of template DNA on agarose beads, structure of the microfluidic chip, experimental condition optimization, and video of protein purification in-chip.

ACKNOWLEDGMENTS

This work was supported in part by the National Natural Science Foundation of China (Grant Nos. 21675047, 21735002, and 21521063) and the Key Point Research and Invention Program of Hunan Province (Grant No. 2017DK2011).

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