Solid-phase translation and RNA–protein fusion: a novel approach for folding quality control and direct immobilization of proteins using anchored mRNA

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ABSTRACT

A novel cell-free translation system is described in which template-mRNA molecules were captured onto solid surfaces to simultaneously synthesize and immobilize proteins in a more native-state form. This technology comprises a novel solid-phase approach to cell-free translation and RNA–protein fusion techniques. A newly constructed biotinylated linker-DNA which enables puromycin-assisted RNA–protein fusion is ligated to the 3′ ends of the mRNA molecules to attach the mRNA-template on a streptavidin-coated surface and further to enable the subsequent reactions of translation and RNA–protein fusion on surface. The protein products are therefore directly immobilized onto solid surfaces and furthermore were discovered to adopt a more native state with proper protein folding and superior biological activity compared with conventional liquid-phase approaches. We further validate this approach via the production of immobilized green fluorescent protein (GFP) on microbeads and by the production and assay of aldehyde reductase (ALR) enzyme with 4-fold or more activity. The approach developed in this study may enable to embrace the concept of the transformation of 'RNA chip-to-protein chip' using a solid-phase cell-free translation system and thus to the development of high-throughput microarray platform in the field of functional genomics and *in vitro* evolution.

INTRODUCTION

Cell-free systems have proved to have high utility at the genomic, transcriptomic and proteomic levels and to form a vital component of many aspects of recombinant gene expression, and of both structural and functional proteomics (1,2). The most recent advance using cell-free systems has been the development of protein display techniques using several genotype–phenotype linking methods. Among these, the coupling of translated polypeptides to the encoding mRNA by covalent linkage, i.e. RNA–protein fusion, is likely to be more generally applicable to *in vitro* protein selection and evolution, originally described in 1997 by Nemoto *et al.* (in-vitro-virus) and independently by Roberts and Szostak (mRNA display) [reviewed in (3)]. However, despite the encouraging results from advanced cell-free systems (4) there is still significant scope for improvement. First, exogenous mRNA is extremely labile in cell-free conditions over time, resulting in low yields. Second, there is still some uncertainty whether the correct protein conformations will be adopted in a crowded environment. Third, there is a need to develop simple and general post-translational techniques to immobilize functional proteins onto solid supports, a prerequisite for producing protein bio-chips. Many recent efforts have been successful in improving protein yields by controlling the stability of the cell-free system (4). However, improving the yields of stable and functionally active proteins in their proper native folding states has been less encouraging but is crucial for modern proteomic microarray methods.

Compared with DNA microarrays, protein bio-chips provide more challenges and have yet to be perfected due to the complexity and inherent difficulties with protein immobilization. First, proteins tend to adsorb non-specifically

to solid surfaces, leading not only to the inaccessibility of active sites but also to the possibility of denaturation and loss-of-function. Second, protein–ligand interactions are highly dependent upon the orientation and integrity of the protein conformation which is often reliant on post-translational modifications (5). Thus, direct improvements in translation efficiencies in cell-free systems and subsequent protein immobilization are a significant bottleneck in the advancement of protein microarray technology. For protein immobilization, the simplest method is physical adsorption to a solid surface (6). However, more stable and reliable methods using chemical bonding or linkages to solid supports have been more extensively utilized due to their ability to orient the immobilized molecule in a defined and precise fashion (7). A variety of affinity tags have been used in these linkage reactions, including whole proteins, antibody peptide tags and histidine tags (8–11) but do not necessarily provide the best general approach for protein immobilization. Nevertheless, it is crucial to select the right surface for immobilizing functional biomolecules that will retain their native conformational structure and optimal activity whilst forming a stable linkage.

To address these technical issues, we have developed a novel solid-phase approach to facilitate efficient cell-free protein translation. We herein describe a technology platform that facilitates not only the translation of more functionally active proteins using solid surface-bound mRNA-templates, but also the immobilization of native proteins on solid surfaces using puromycin-assisted mRNA–protein fusion. Hence, our novel methodology will enable high-throughput *in vitro* selection and functional proteomics.

MATERIALS AND METHODS

Construction of linker-DNA and mRNA-templates

The cDNAs for POU (POU-specific DNA-binding domain of Oct-1), GFP (green fluorescent protein) and ALR (aldehyde reductase) were amplified from linearized pUC19, pET-21a-d and pET-19b vectors harboring these inserts, respectively. Leader sequences including the T7 or SP6 promoters and a translational enhancer of TMV or Xenopus β-Globin UTR were inserted at the 5′-terminal region prior to initiation codon. A stretch of 3′UTR (GAATTCGAGCTCCGTCGAC) and a complementary sequence for linker-DNA (AGGAC-GGGGGGCGGCGGGGAAA) was inserted at the 3′-terminal ends. For templates used in genotype–phenotype linkage, the stop codon was removed by designing the PCR primers accordingly. PCR products were purified (Qiagen) and used as a template for cell-free transcription (RiboMAX, Promega). The capped mRNAs were purified (RNeasy, Qiagen) and assessed by spectrophotometry (Nanodrop) and PAGE.

Attachment of mRNA-template to solid surface via a linker-DNA bridge and synthesis of puromycin-linker DNA

The synthetic oligonucleotides, 'Puro-F-S': 5′-(S)-T-C-T(F)-(c18)$_4$-C-C-(Puro)-3′, where (S) is 5′-thiol-modifier-C6, T(F) is fluorescein-attached thymine, (Puro) is puromycin,

and (c18) is a phosphoramidite spacer; and 'Biotin-loop': 5′-CCCGGTGCAGCTGTTTCATC(T-B)CGGAAACAGC-TGCACCCCCCGCCGCCCCCCG(T)CCT-3′, where (T-B) is biotinylated thymine, (T) is amino-modifier-C6-dT, and the underlined sequence is a PvuII recognition site, were purchased from BEX Co. (Tokyo, Japan). To introduce a 5′-sulfhydryl (–SH) reactive group, 'Puro-F-S' (10–20 nmol) moieties were reduced by 4 mM TCEP (Pierce) in 100 μl of 50 mM phosphate buffer (pH 7) for 2–6 h at room temperature, and desalted on NAP-5 columns (Amersham) prior to use. Biotin loops (10 nmol) and the cross-linking agent, EMCS (Dojindo, Japan) (2 μmol), were added to 100 μl of 0.2 M phosphate buffer (pH 7). The mixture was incubated for 30 min at 37°C and ethanol precipitations were performed at 4°C to remove excess EMCS. The precipitate was then washed twice in 500 μl of pre-cooled 70% ethanol and dissolved in 10 μl of pre-cooled 0.2 M phosphate buffer (pH 7) and then mixed with the reduced Puro-F-S and stirred for 2 h at 37°C. The reaction was stopped by adding 5 mM DTT for 30 min at 37°C followed by ethanol precipitation to remove excess Puro-F-S. To remove excess Biotin-loop and uncrosslinked Biotin-loop-EMCS complexes, the precipitant was dissolved into phosphate buffer and purified with HPLC under the following conditions: column, Waters MA USA, AR-300, 5 × 250 mm; solvent A, 0.1 M TEAA; solvent B, acetonitrile/water (80:20, v/v); gradient, B/A (15–35%, 33 min); flow rate, 0.5 ml/min; detection, absorbance at 254 and 490 nm.

Ligation of mRNA to puromycin-linker DNA

The 3′-terminal ends of the mRNA molecules were hybridized to the complementary strands of the puro-linker-DNAs under annealing conditions (lowering the temperature linearly from 95 to −10°C) in ligation buffer (500 mM Tris–HCl, pH 7.5 containing 100 mM MgCl$_2$, 100 mM DTT, 10 mM ATP). Following the addition of T4 RNA ligase (40 U/μl, Takara), T4 Polynucleotide Kinase (10 U/μl, Takara) and SUPERase RNase inhibitor (Ambion), ligation reactions were performed in a dry incubator at 25°C for 90 min. Ligation optimization was carried out and the products were analyzed in denaturing polyacrylamide gels in 90 mM TBE (pH 8.5) buffer. The ligated products were visualized by FITC fluorescence, and non-ligated products were observed by subsequent staining with SYBR Gold (Molecular Probes) dye, using a fluoroimager (Typhoon, Amersham).

Immobilization of ligated mRNA-linker DNA onto beads

Streptavidin-coated super-paramagnetic hydrophobic (M280) and hydrophilic (M270) beads of 2.8 μm in size (Dynal) were used to immobilize mRNA-linker DNA products. Beads were initially washed for RNA manipulation as instructed by the manufacturer and resuspended in DEPC treated 0.1 M NaCl to a final concentration of 10 μg (6 × 10^5 beads)/μl. The binding capacity of these beads is dependent upon the size of the mRNA molecules and was determined to be ∼3 pmol of 0.3 kb mRNA or 1 pmol of 0.8–1.1 kb mRNA per 0.1 mg of beads. Immobilization was performed in binding buffer (10 mM Tris–HCl, pH 8.0 containing 1 M NaCl, 1 mM EDTA and 0.1% Triton X-100) at 25°C for

15 min in a dry thermo block rotator (NISSIN, Japan). The beads were subsequently washed twice with binding buffer to remove non-bound material, using a magnet and were stored until further use.

Solid-phase translation and IVV formation using bead-bound templates

Bead-bound mRNA templates were resuspended in the components of the cell-free translation system and incubated for 15 min under the conditions described by the manufacturer in dry thermo block rotator. For solid-phase translation, translated products were released from beads by PvuII restriction (Toyobo) and analyzed by either SDS–PAGE or a microplate reader. For solid-phase genotype–phenotype linkage, translated products were subjected to a fusion reaction (between mRNA and peptide chain) by the addition of a high-salt concentration ($MgCl_2$ and KCl to final concentration of 90 and 600 mM, respectively) followed by a second incubation for 90 min at 37°C. Fusion products were released from beads by PvuII restriction. The supernatant was then carefully removed and incubated with RNase-H (Toyobo) to release the mRNA component of the genotype–phenotype linked product. The remaining protein was further analyzed by SDS–PAGE.

Quantitative (production) and qualitative (folding) analysis of GFP formation in solid-versus liquid-phase

For comparative quantitative analysis of translated products generated in solid-phase versus liquid-phase, GFP was translated using fluorescently labeled lysine residues (FluoroTect, Promega) and analyzed by SDS–PAGE (10% T). Prior to electrophoresis, samples were heated at 70°C for 5 min for complete denaturation and removal of the inherent fluorescence of the folded GFP protein. After electrophoresis, the intensity of the FluoroTect labeled GFP bands was monitored by a fluoroimager which enabled accurate measurements of the differences in the relative intensity of the bands.

For comparative qualitative analysis of solid-phase versus liquid-phase produced GFP, the protein was translated without any fluorescence labeling and the intensities of the original inherent fluorescence, representing the amount of correctly folded GFP, was measured using a fluorescence microplate reader. Briefly, dilutions of translated GFP products were made with Tris buffer (10 mM Tris–HCl, pH 8.0), transferred to a 96-well microplate and assayed for fluorescence with a 485 nm excitation filter and a 535 nm emission filter. RFUs (relative fluorescence units) were recorded together with a negative control. Immobilized GFP was generated using the genotype–phenotype linkage method and observed using an inverted fluorescent microscope (Olympus IX71). These experiments were each conducted independently >3 times.

Enzymatic assay for ALR

ALR activity was determined microfluorometrically by measuring the rate of the enzyme-dependent decrease in NADPH fluorescence with an excitation wavelength of 360 nm and an emission wavelength of 465 nm. The reaction mixtures were prepared in a total volume of 100 µl containing 50 mM potassium phosphate buffer (pH 6.5), 10 mM glucuronate

as a substrate, and the ALR solution. The reactions were initiated by the addition of 0.2 mM NADPH and incubation at 30°C for 10 min, followed by measurement of the decrease in fluorescence at 30°C with a spectrofluorophotometer (FluPolo microplate reader, Takara).

RESULTS

General concepts for solid-phase translation and synthesis of immobilized proteins

A novel method to produce proteins in their native folded-state using solid-phase translation and in immobilized-state using solid-phase genotype–phenotype linkages is schematically outlined in Figure 1. The former requires the template (mRNA) in a stationary phase, which is achieved by immobilizing the mRNA molecules to a solid surface prior to translation. The latter is achieved by subsequent linkage between the mRNA molecules and their encoded protein products using genotype–phenotype linking methods such as in-vitro-virus (12).

In order to perform solid-phase translation, the immobilization of mRNA must satisfy several requirements: (i) mRNAs should be attached efficiently to the solid surface via a 3′UTR end linkage, (ii) the integrity of the mRNAs should not be affected by immobilization, (iii) the availability of the free 5′ end of the mRNA must be sufficient for translation and (iv) the properties of the solid surface must be compatible with translation. These are achieved by coupling the mRNA of interest to a solid surface via ligation to a synthetic biotinylated DNA oligomer (Figure 1c, step i) which is then immobilized to streptavidin-coated paramagnetic beads (Figure 1c, step ii). An efficient ligation is an essential part of solid-phase translation and for this purpose we have engineered a synthetic linker-DNA molecule (see Figure 1a, characteristics i–iii). To perform an efficient ligation between the mRNA and linker-DNA molecules, the 3′ ends of the mRNAs are first hybridized to the linker-DNA and then incubated with T4 RNA ligase (13,14). This reaction is efficient even at low concentrations of substrates as it is based on quasi-intramolecular ligation. Furthermore, the experimental conditions for ligation were optimized for various lengths of the mRNA and linker-DNA molecules (Figure 2). Ligation at 25°C for a period of 2 h using a 4-fold molar excess of linker-DNA results in a >90% yield, which was found to be independent of mRNA length (from 0.3 kb to 1.2 kb) (Figure 2a and a′). In the next step of solid-phase translation, the bead-bounded mRNA molecules are incubated in a cell-free translation system (Figure 1c, steps iii and iv).

To synthesize immobilized protein, solid-phase translation is followed by an mRNA–protein fusion method which requires the covalent joining of the mRNA to a short, synthetic oligonucleotide containing an adaptor molecule, typically puromycin, at its 3′ end. This is achieved by utilizing our recently engineered synthetic Puro-linker-DNA molecule which has four additional features (see Figure 1a, characteristics iv–vii). As shown in Figure 1d, an mRNA molecule with no termination codon is ligated to the puro-linker DNA and thus immobilized to the solid surface (steps i and ii). The resulting modified bead-bound mRNA is then used as template in the cell-free translation reaction (step iii).
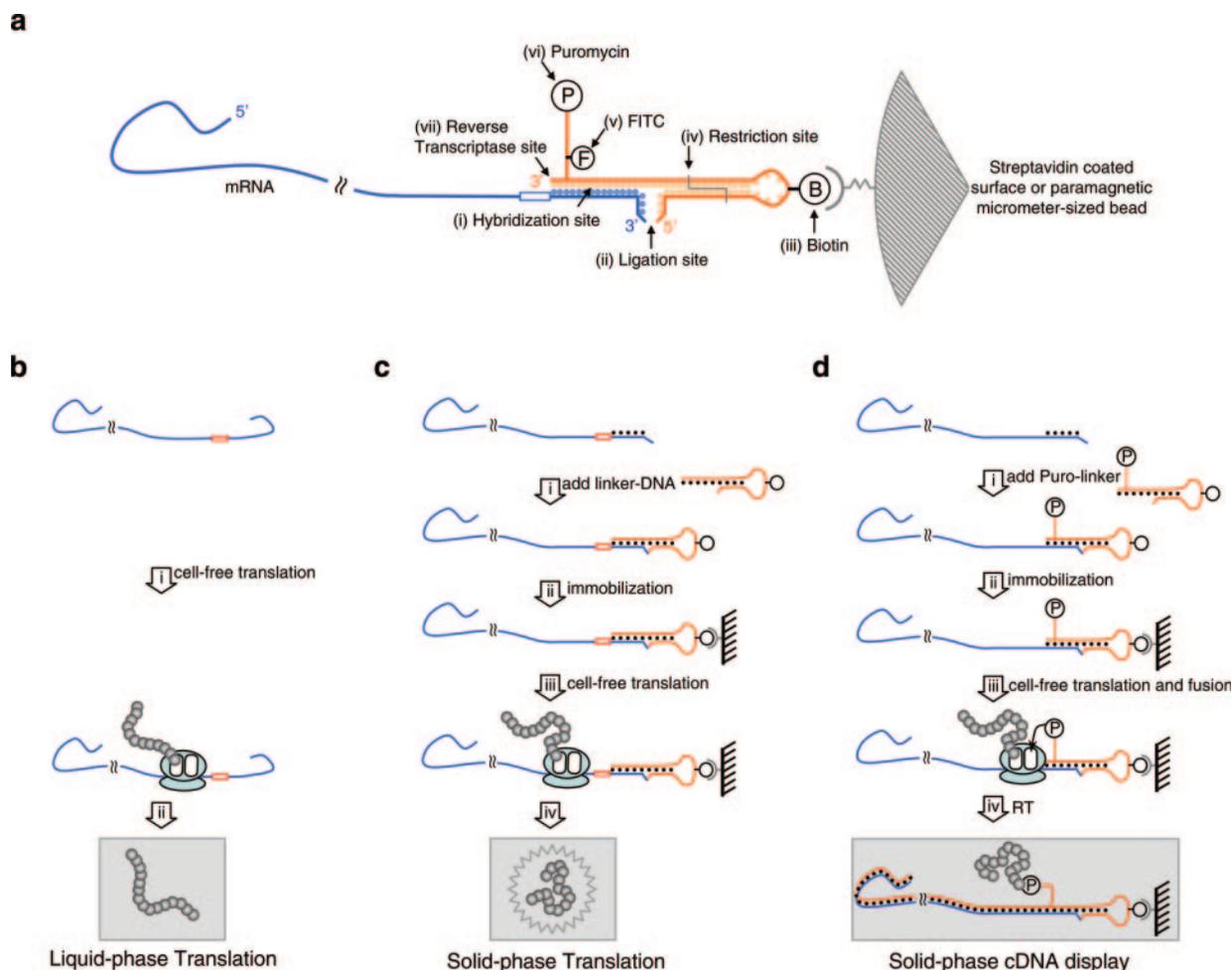
Figure 1. Schematic representation of a novel solid-phase cell-free translation system for synthesizing immobilized protein via two different covalent associations: between mRNA (ligated to biotinylated linker-DNA) and a solid surface (streptavidin-coated surface), and formed between the translated protein products and coded mRNA through puromycin-assisted genotype–phenotype linking. (**a**) Characteristics (i–vii) of Puro-linker-DNA molecule (red color) used for the immobilization of mRNA (blue color) to a solid support prior to cell-free translation. The Puro-linker-DNA comprises free terminal ends and one hairpin loop structure with seven principal silent features: (i) a hybridization site, a short sequence at the 3′-terminus which is complementary to the 5′-terminal end of the mRNA of interest and is used to hybridize the linker-DNA to mRNA molecule in such a way as to maintain a free 3′ end; (ii) a Y-ligation site, a free 5′-phosphoryl-terminated end which forms a phosphodiester bond with the free 3′-hydroxyl-terminated end of the mRNA (14,30); (iii) biotin, which will bind a streptavidin-coated solid surface and thus control the handling of whole template constructs during pre- and post-solid-phase translation reactions; (iv) a PvuII restriction endonucleases site; (v) FITC; (vi) puromycin, as a mimic of aminoacyl tRNA; and (vii) a reverse transcriptase site in which a free 3′-terminal end of the linker-DNA is used as the primer for reverse transcription. (**b**) Flow diagram of a conventional, liquid-phase translation approach. (**c**) Flow diagram of our current solid-phase translation approach. Following the hybridization and ligation of the 3′-terminal end of the mRNA to the complementary portion of the 5′ end of a biotinylated linker-DNA (situated after the termination codon, steps i and ii) the template is immobilized onto streptavidin-coated paramagnetic beads prior to translation (step iii). These templates are then incubated in a cell-free translation system which results in the generation of more native and correctly folded proteins (step iv). (**d**) Flow diagram of solid-phase genotype–phenotype linkage and the synthesis of immobilized protein. Following the same steps (i to ii) shown in (c) using mRNAs lacking a stop codon and using linker-DNA with a puromycin moiety (i.e. puro-linker-DNA), the ribosome stalls at the mRNA and linker–DNA junction during translation. This permits puromycin to enter the ribosomal A-site and to bind to the nascent polypeptide chain (step iii). This produces a covalent linkage between bead-bounded mRNA-template and coded peptide chain which is then followed by reverse-transcription to synthesize the cDNA form and finally a stably immobilized 'mRNA/cDNA–protein complex' (step iv) where the 'slash' represents hybridization between the RNA and DNA strands and the 'dash' indicates the covalent linkage between nucleic acids and proteins, and represents the transformation of the 'first' covalent linkage between mRNA and protein molecules into the 'second' covalent linkage between the cDNA and protein molecules.

After the translation machinery has reached at the end of the mRNA, i.e. the RNA–DNA junction, the ribosome stalls and allows the free puromycin moiety to enter into the peptidyl-transferase site and form a covalent bond to the C-terminal amino acid residue in the nascent peptide chain. This resulting linkage thus immobilizes the synthesized protein. In order to stabilize and amplify the resulting mRNA–protein complex, a cDNA chain complementary to the coded sequence can be generated using reverse transcription after

rapid purification from the crude lysate (step iv). The complex which is composed of mRNA, the coded polypeptide chain and a complementary cDNA sequence ('mRNA/cDNA–protein complex' which is designated as 'cDNA display' hereafter instead of 'in-vitro-virus' or mRNA-display) can be released from solid surface by PvuII restriction digestion.

Following ligation optimization, a liquid-phase genotype–phenotype fusion was performed to check the efficiency of
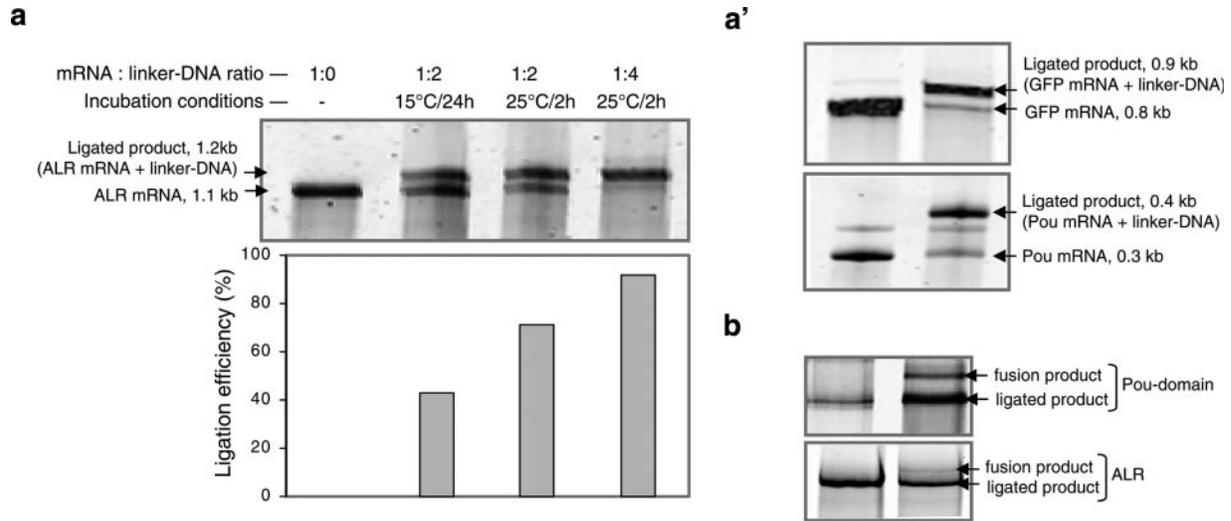
Figure 2. Optimization of ligation reactions. (**a**) Comparison of ligation efficiency between ALR-mRNA (1.1 kb) and linker-DNA molecules. Ligation products were evaluated by denatured PAGE (3.5% T gel consisting of 8 M urea and 90 mM TBE). With a 4-fold molar excess of linker DNA and 2 h incubation at 25°C, mRNA molecules could be ligated with a >90% efficiency. (**a′**) The same efficiency of ligation was observed with different-sized mRNA species (GFP, upper panel, 836 bases; POU, lower panel, 334 bases) to confirm the optimized conditions for ligation. (**b**) The integrity of the genotype–phenotype linkages was confirmed by production of an mRNA–protein fusion complex for shorter (upper panel, POU) and larger (lower panel, ALR) mRNA templates and analyzed by denaturing SDS–PAGE. Fusion products (ligated product, i.e. mRNA and puro-linker-DNA, attached to the encoded peptide chain) migrated with a reduced mobility compared with the ligated products. The nucleic acids and protein products which were covalently bound to the linker-DNA were visualized by the FITC fluorescence and non-ligated products were observed by staining with SYBR Gold dye.

the newly constructed puro-linker-DNA and generate mRNA–protein complexes. As shown in Figure 2b, the products of the translation and fusion steps can be observed by SDS–PAGE analysis and migrate with a decreased mobility, compared with the untranslated ligated products. This is consistent with the molecular weights of the ligated product and coded polypeptide chains. In addition, the successful fusion of larger proteins (ALR, 325 amino acids), with almost same efficiency (∼15–20% of the input template) of fusion formation of shorter protein (POU, 71 amino acids), is another important aspect of our newly designed puro-linker-DNA construct.

Yield of native (folded) proteins by solid-phase translation

To demonstrate the performance of our solid-phase translation system, GFP was chosen as the model protein. A T7 promoter-driven mRNA template encoding GFP with a stop codon and short stretch of complementary sequence of linker-DNA at the 3′ terminus was constructed (as shown partly in Figure 1a). This template was then ligated to linker-DNA and immobilized onto streptavidin-coated paramagnetic beads. Following cell-free translation in a wheat germ-based system, the beads were separated and the supernatant was analyzed quantitatively by SDS–PAGE and qualitatively (i.e. correct folding) by a fluorescence microplate reader. To compare the performances of solid-phase and liquid-phase systems, an identical quantity of free mRNA-template without ligation or immobilization was processed in parallel (as outlined in Figure 1b). To quantitatively compare the production between the solid- and liquid-phase methods, GFP was expressed using fluorescently labeled lysine residues. Translated products were heated at 70°C for 5 min for

complete denaturation and removal of the original fluorescence of the folded GFP protein, and resolved by SDS–PAGE. Heat-denatured (non-fluorescent) GFP migrates as a major band of ∼27 kDa (Figure 3a, right two lanes). The intensity of FluoroTect labeled GFP bands were monitored by a fluorescence imager. The average results obtained by four successive experiments clearly show that production of GFP using our solid-phase system was at ∼15% of the levels of the liquid-phase system (Figure 3b inset, black-border columns). However, the quality analysis, i.e. foldability, of the GFP, for these two systems obtained by measuring the intensity of original green fluorescence (Figure 3b inset, grey-border columns) showed similar results. The RFU values representing the foldability of GFP were directly measured using a fluorescence microplate reader, and for the solid-phase system was ∼80% of the liquid-phase system. This suggests that although the production of GFP using the solid-phase approach is considerably less compared with the liquid-phase method, the proteins produced in the solid phase are up to 4-fold more biologically active after normalization (Figure 3b). To confirm this finding, the solid-phase products were removed from the beads and then analyzed together with liquid-phase products by SDS–PAGE. The results showed a 37 kDa GFP product from the solid-phase reaction, which is shifted upwards from the denatured position predicted for its theoretical mass (27 kDa) due to its native folding (Figure 3a, left two lanes). This is consistent with previous reports (15).

The distance of the immobilized probes from their solid supports has been reported to be one of the most important properties of the solid-phase approach (16). We thus investigated whether the translation efficiency in our current system would be affected by varying the distance between the 3′-terminal end of the mRNA template from the surface of
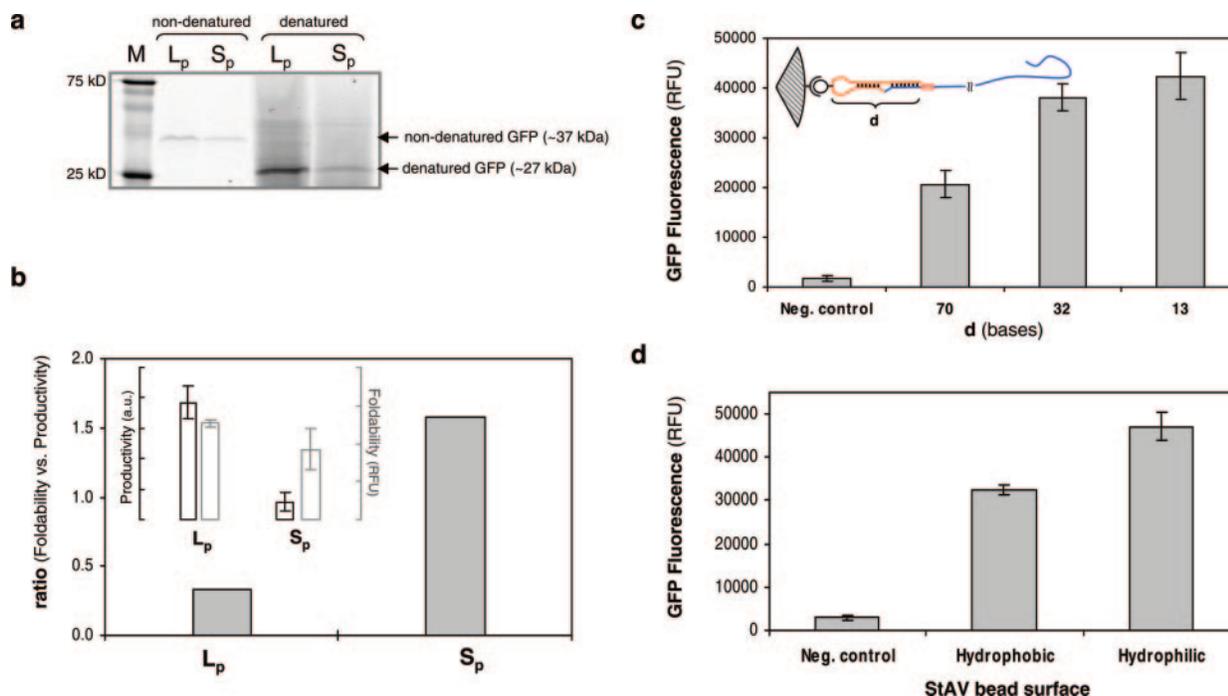
Figure 3. Comparison of the correct folding and productivity of GFP produced by solid-phase (Sp) and liquid-phase (Lp) cell-free translation systems. (**a**) SDS–PAGE of non-denatured (folded) GFP (leftmost two lanes) and denatured GFP (rightmost two lanes). (**b**) Quantitative measurements of the relative efficiency of Sp versus Lp in terms of ratio values were plotted after recombining the productivity (black-border column) and foldability (grey-border column) performance (shown in inset) from the liquid- or solid-phase systems. (**c**) RFU for GFP generated using various spacer lengths during the immobilization of the mRNA-template on solid-support for solid-phase cell-free translation. (**d**) RFU for GFP generated using either a hydrophobic (M280) or hydrophilic (M270) surface-bound mRNA-template. All plots and error bars represent average and standard coefficient values of more than four independent experiments. M, molecular weight markers.

solid support. For this purpose, biotinylated linker-DNAs ranging from 13 to 70 bases, a distance that approximates a spacer length of between 4.1 and 23.5 nm, were constructed and used to immobilize the mRNA-template on strepatavidin-coated beads. Following cell-free solid-phase translation, a pronounced effect of distance could be observed (Figure 3c and Supplementary Figure S1). For GFP translation, a significant increase in fluorescence of 2-fold or more was observed with the shorter 13-base spacer compared with the longer 70-base spacer. Hence, the activity of immobilized molecules that are more spatially separated from their solid support may approximate their liquid-phase counterparts. Nevertheless, this phenomenon may also be affected by the nature of the solid-support surface and we further investigated whether the GFP translation efficiency would be affected by using either a hydrophobic or hydrophilic surface. As shown in Figure 3d, GFP immobilized on hydrophilic beads shows greater levels of fluorescence in comparison with the immobilized GFP on hydrophobic beads. This is to be expected as biomolecules can unfold and expose their hydrophobic regions through adsorption.

Yield of immobilized native proteins by solid-phase genotype–phenotype linkage

To effectively immobilize functional proteins onto a solid support, we developed a combined approach comprising solid-phase translation and solid-phase genotype–phenotype linking. The former can attach mRNA-templates to a solid

surface and thus produce functional protein, whereas the latter can form a linkage between the mRNA-templates and the encoded growing peptide chains and thus serve as a technology platform for producing immobilized protein arrays. To demonstrate the feasibility of this proposed system, we used GFP as our model protein and constructed a template genome consisting of GFP mRNA without a stop codon, a requirement for puromycin-assisted genotype–phenotype linking. Following ligation using non-fluorescent labeled puro-linker DNA and immobilization as mentioned above, bead-bound mRNA was incubated in a wheat germ-based translation system to synthesize an amino acid chain, and then in high-salt buffer to convert the translation product into the fusion product. The beads were separated from the reaction solution, washed and directly observed by fluorescence microscopy (Figure 4). Few discernible beads were found to emit green fluorescence (Figure 4a and Supplementary Figure S2) which is probably due to the lower efficiency of solid-phase system and unexpected interference by the solid surfaces such as bead aggregation. This is partially supported by our observation of fluorescence switching that occurs when GFP molecules are observed individually and show fluorescence intensity fluctuations as a function of the excitation intensity over time (micro- to sub-second time range). We also observed that the excitation intensity for a couple of seconds has a dramatic effect on the nature of the 'on-off blinking effect' of GFP fluorescence and this has been reported previously (17). Optical images under white light were also generated to confirm any background fluorescence
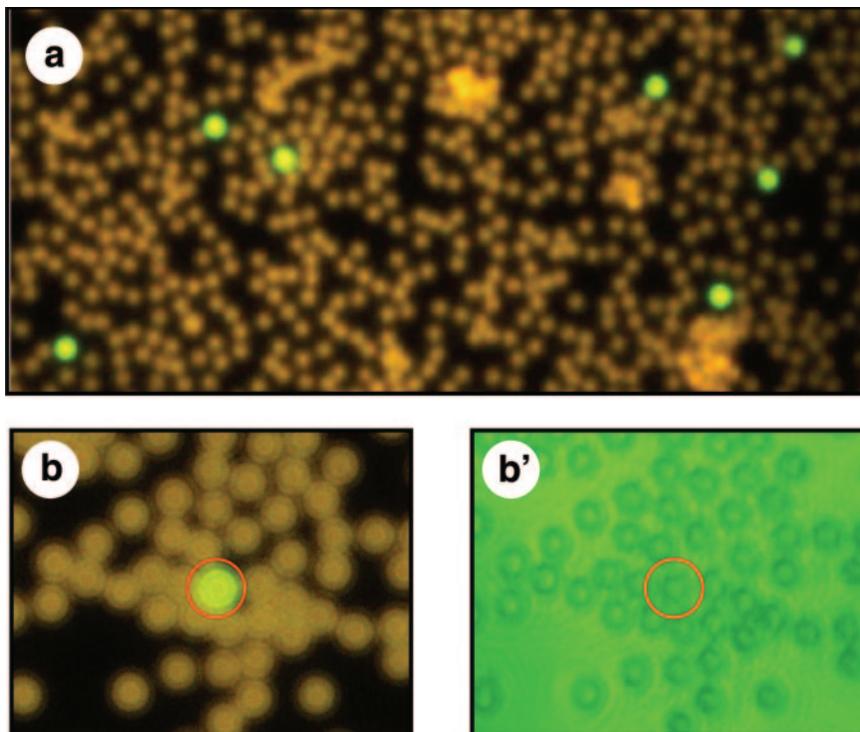
Figure 4. Detection of fluorescence of immobilized GFP. (**a**) Fluorescence microscopy image of bead-bound GFP molecules expressed using solid-phase genotype–phenotype linking. Fluorescence was observed using standard emission filters via inverted fluorescence microscopy. Analysis of fluorescence (**b**) and an optical image under white light (**b′**) from (a) at a higher magnification to assess for the presence of auto-fluorescence or artifacts. The polystyrene beads are 3 μm in dimension.

or artifacts (Figure 4b and b′). To further characterize the background or auto-fluorescence effects of the beads, if any, non-fluorescent protein was tested in this system and confirmed (data not shown). The results show that our method represents a universal approach to protein immobilization by the use of surface-bound mRNA and genotype–phenotype linking and that this technique has several advantages over traditional methods which are discussed later.

To further confirm the production of immobilized protein in a more active (native) form via solid-phase genotype–phenotype linkage, we utilized ALR enzymatic assay. An mRNA template for ALR was constructed and immobilized onto streptavidin-coated paramagnetic beads using puro-linker-DNA. Following the cell-free solid-phase translation, bead-bound fusion products for the ALR enzyme were generated and directly washed away from translation mixture and then separated from the beads. For comparison, the same quantity of ALR-mRNA was processed in liquid-phase, which requires post-translational immobilization for purification purposes. A portion of the ALR enzymes generated in both systems was directly analyzed by SDS–PAGE and showed a single band of ∼42 kDa (Figure 5a). A direct observation of the band intensity differences between liquid- versus solid-phase protein products showed about a 2-fold reduced level in solid phase (Figure 5a and inset in 5b, black-border columns). However, qualitative analysis via the ALR enzymatic assay (inset in Figure 5b, grey-border columns) showed that the solid-phase enzyme is more active when incubated with a reducing substrate (D-Glucuronate) in comparison with the activity of ALR produced in

liquid-phase. We calculated this to be about a 2-fold increase in RFU units of reacted NADPH using the solid-phase enzyme which amounted to an almost 4-fold increase following normalization (Figure 5b). This is another demonstration of the versatility of our solid-phase approach in generating more active immobilized proteins.

DISCUSSION

In our current study, we describe a novel solid-phase cell-free translation system that can generate more functionally active proteins using solid-surface bound mRNA which may then be efficiently immobilized via a covalent link between the gene and its encoded protein. This method offers a number of advantages over conventional approaches in liquid-phase, in addition to the generation of a stable genotype(mRNA)–phenotype(protein) linking complex. The solid-phase immobilization of biomolecules has been shown to have widespread applications for the analysis of both pre-translated (DNA/RNA) and post-translated (protein/enzymes) biomolecules (18–23). In addition, this technology can also be utilized in the development of microfabrication (24,25), high-throughput screenings and automation strategies in related areas including proteomics. In addition, our newly constructed linker-DNA reported herein is not only highly effective in facilitating the hybridization of mRNA, and thus the efficiency of ligation using T4 RNA ligase (in comparison with T4 DNA ligase or others), but is also advantageous for both the stability and handling of larger transcripts.
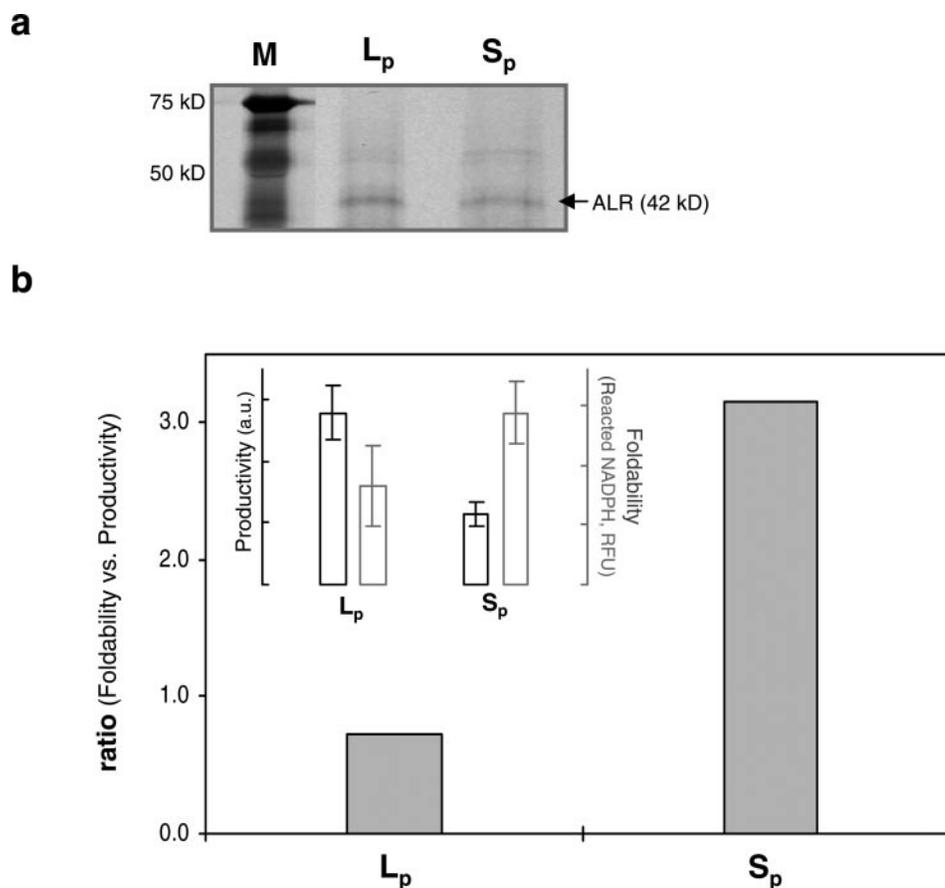
Figure 5. Comparison of the foldability and productivity of the ALR enzyme between solid-phase (S_p) and liquid-phase (L_p) cell-free translation systems. (**a**) SDS–PAGE of ALR produced by L_p, and by S_p genotype–phenotype linkage after subsequent cleavage of the encoding DNA strand. Arrow denotes the position of the expected band. (**b**) Quantitative measurement of the relative efficiency of S_p versus L_p in terms of ratio values that were plotted after recombining the productivity (black-border column) and foldability (grey-border column) performance (shown in inset) from the L_p and S_p systems. All plots and error bars represent average and standard coefficient values from three independent experiments.

Genotype–phenotype linkages for polypeptides of relatively short chain lengths (10–110 amino acids) have been successfully reported, but this is the first description of a high linkage efficiency under moderate conditions [without a light-induced psoralen crosslinking reaction (26)] for an mRNA of the size of the ALR enzyme (325 amino acids). Hence, we demonstrate the utility of our newly constructed linker-DNA that may prove to be a highly effective tool for generating arrays harboring larger proteins. Furthermore, the utility of our linker-DNA in immobilizing the end of an mRNA-template onto solid surfaces for solid-phase translation improves the half-life of the mRNA molecule, which is very short in cell-free systems, by protecting its 3′ terminus against contaminating nucleases (27). In this context, to confirm the stability of our immobilized mRNA, magnetic bead-bound GFP-mRNAs were used for multiple sequential solid-phase cell-free translation reactions. Our subsequent results show that bead-bound mRNA can be sufficiently stabilized by immobilization onto solid surfaces and be subjected to three or more rounds of cell-free translation without a significant loss in GFP production (data not shown). Although the immobilization of mRNA–protein fusion products to create protein chips has been described recently (28), in which mRNA–protein complexes self-assemble via hybridization to surface-bound DNA capture probes, this reported procedure is unlikely to maintain the stability and function of the mRNA–protein complex. One of our primary aims was therefore to develop a solid-phase system that maintains the integrity of mRNA during post-transcriptional reactions and can thus generate stable mRNA–protein complexes for assembling protein microarray chips.

To further validate our system, we focused on whether proteins could be synthesized with the correct folding and conformation in a cell-free system, where ribosomes are not bound to the endoplasmic reticulum that would promote maturation and translocation. The solid-phase approach however, controls cell-free protein synthesis reaction in a similar stationary mode using surface-bound mRNA, and this helps to direct protein folding. The misfolding of polypeptide chains is another drawback to the use of cell-free translation systems and is caused by collision and aggregation of growing polypeptide chains. Such aggregates of rapidly formed and partially folded intermediates would also be controlled by immobilization. In addition, using mRNA in a stationary phase will result in less interaction between growing peptides and reduce the incidence of other inappropriate interactions, and thus also prevent excessive protein misfolding, aggregation and degradation.

Finally, our genotype–phenotype fusion library (cDNA display library) appears to be more functional than those generated by liquid-phase mRNA display, or other methods where oligo-dT cellulose is required for purification and reverse transcription (29) and therefore are not necessarily suitable for larger proteins. The use of magnetic beads as a solid support, which circumvents the need for precipitation and centrifugation, is not only advantageous for the direct and efficient purification of expressed mRNA–protein fusion products, but also facilitates the control and quantification of the gene of interest in real time. Thus, our current technology could serve as the basis for an automated system of various cell-free methods and has enormous potential to contribute to the fields of evolutionary molecular engineering and functional genomics. The acquisition of an integrated microarray platform to bridge genomics and proteomics, and an effective solution for displaying fully functional proteins in a microarray format are the ultimate goals of future work.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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