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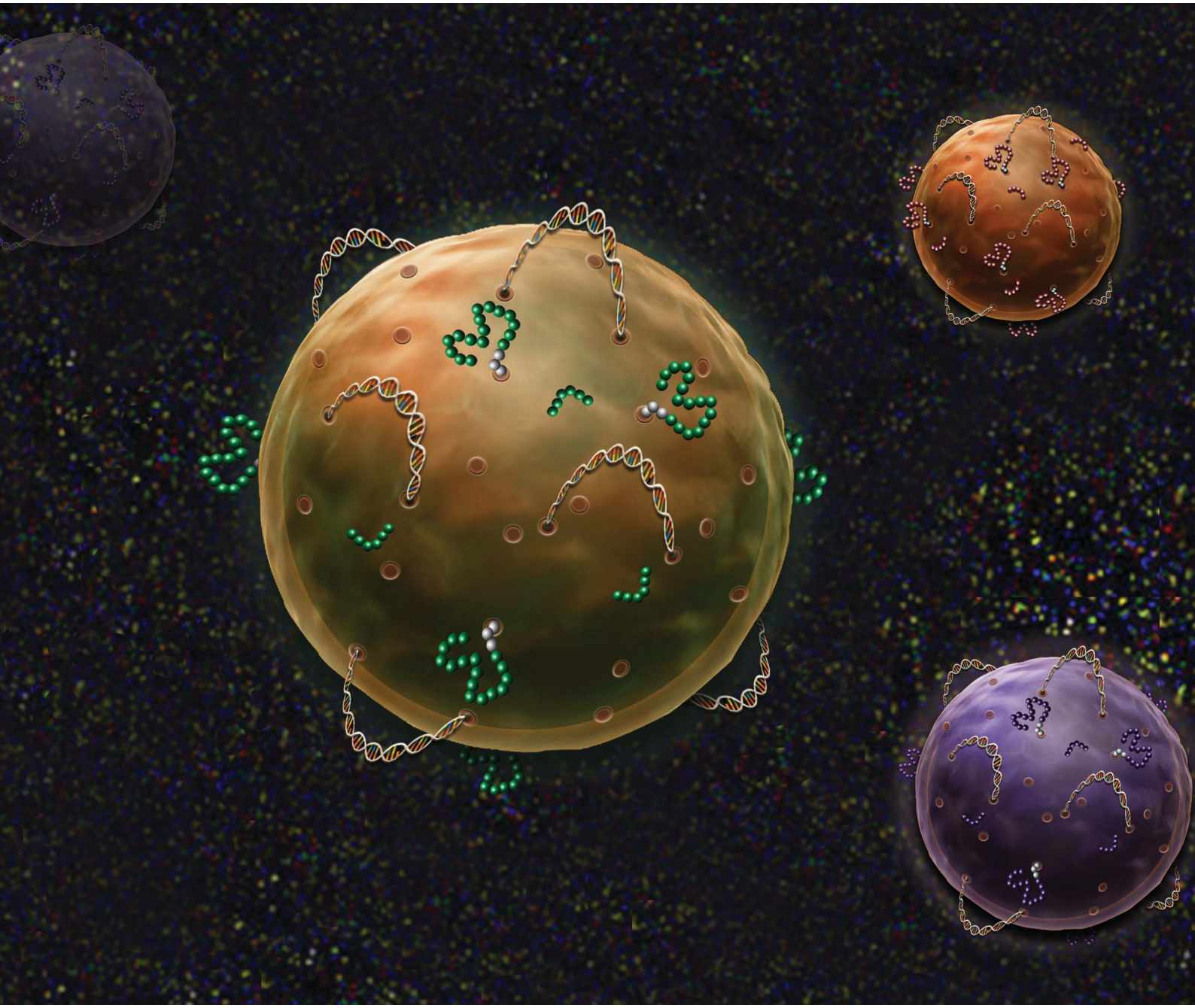

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PAPER

On-bead expression of recombinant proteins in an agarose gel matrix coated on a glass slide

Kyung-Ho Lee,[a] Ka-Young Lee,[b] Ju-Young Byun,[b] Byung-Gee Kim[c] and Dong-Myung Kim*[ab]

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A system for expression and *in situ* display of recombinant proteins on a microbead surface is described. Biotinylated PCR products were immobilized on microbead surfaces, which were then embedded in a gel matrix and supplied with translation machinery and substrates. Upon the incubation of the gel matrix, target proteins encoded on the bead-immobilized DNA were expressed and captured on the same bead, thus allowing bead-mediated linkage of DNA and encoded proteins. The new method combines the simplicity and convenience of solid-phase separation of genetic information with the benefits of cell-free protein synthesis, such as instant translation of genetic information, unrestricted substrate accessibility and flexible assay configuration design.

Introduction

While proteins are generally linked with encoding genes by their co-existence inside a cell, cellular containment of proteins often limits options that are available for subsequent screening and analysis. Assays of recombinant proteins often need to be preceded by lysis of host cells, for instance in studies of protein ligand binding activities.[1,2] Cell membranes also limit the types of substrates that can be used in enzymatic assays of the expressed proteins to those that can penetrate the lipid bilayer.[3] Thus, the development of autonomous processes, which expose expressed proteins to surrounding environments while maintaining their physical linkages to encoding genes, is of great value in the area of protein discovery and engineering.

In this regard, different versions of biological 'display' techniques have been devised to couple genes and expressed proteins and make them accessible to external substances.[4,5] Since the development of the phage display technology,[6] numerous methods have been devised for more efficient coupling of exposed proteins and encoding genes, including cell-surface,[7] ribosome[8,9] and mRNA displays.[10] Among these, phage and cell-surface displays rely on the containment of genes in biological compartments on which expressed proteins are covalently linked. As a consequence, expression and display of target proteins are limited by the growth and viability of cells. Display methods based on cells or viruses also employ the transformation procedure during which most genetic diversity is lost owing to its intrinsically low efficiency. Such limitations can be alleviated in ribosome or mRNA display where translation of genetic information takes place without the need of gene transformation and cell cultivation steps.[11] However, molecular displays of this type are conducted in homogeneous solution phases where mRNA species in the same reaction mixture should compete for limited amounts of ribosomes. As previously discussed by Ahn *et al.*, expression of mixtures of mRNAs in a homogeneous cell-free synthesis system can often lead to 'biased' expression of proteins and, consequently, misleading results.[12,13] Therefore, the availability of a display-expression platform that operates on individual species of mRNAs while retaining the relative advantages of cell-free synthesis would greatly benefit the expression screening of proteins.

In the study described below, we have developed a simple and universally applicable strategy, involving cell-free expression and instant immobilization of recombinant proteins on microbeads, for physical linkage of template DNA and encoded protein. In the procedure, template DNA is conjugated on the surface of a streptavidin (STV)-coated bead and expressed using the translation machinery prepared from *Escherichia coli* cells. The expressed protein was localized on the same bead by employing the following methods (schematic diagram shown in Fig. 1). First, after conjugation of biotinylated DNA, residual STV sites on the bead are modified with Ni–NTA to enable capture of histidine-tagged proteins expressed from the immobilized DNA. Second, the DNA-conjugated beads were incubated in hydrogel matrix that contains embedded translational machinery and substrates for protein synthesis. As a result, protein synthesis occurs on the surface of the DNA-bound bead being supported by the continuous supply of resources from the surrounding gel matrix. The expressed protein is then

[a]*Interdisciplinary Program for Nano-Technology, Chungnam National University, Daejeon 305-764, Korea. E-mail: dmkim@cnu.ac.kr; Fax: +82-42-823-7692; Tel: +82-42-821-5899*
[b]*Department of Fine Chemical Engineering and Applied Chemistry, Chungnam National University, Daejeon 305-764, Korea*
[c]*School of Chemical and Biological Engineering, Seoul National University, Seoul 151-742, Korea*

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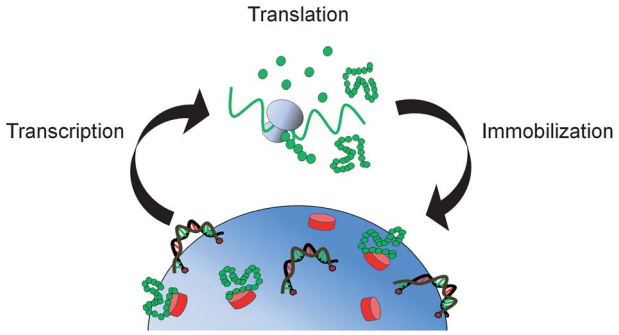

Fig. 1 Cell-free expression and instant immobilization of recombinant proteins on microbeads.

localized on the bead by the interaction of Ni–NTA groups on the bead with the histidine tag of the expressed proteins. Confocal microscopic and chemi-luminescence analyses clearly confirmed the expression and *in situ* immobilization of target proteins on the bead. Importantly, the presence of gel matrix surrounding the DNA-conjugated microbeads also restricts diffusional migration of protein, thus, preventing cross-contamination between the beads. These features of the presented method enable simplification and miniaturization of the protein expression/analysis platform without involving the many steps of cell-based expression and display.

Materials and methods

Materials

Nucleotide triphosphates, creatine phosphate (CP), and creatine kinase (CK) were purchased from Roche Applied Science (Indianapolis, IN, USA). Streptavidin agarose resin was obtained from Thermo Scientific (Rockford, IL, USA). Biotin-X-NTA was obtained from Anaspec, Inc (San Jose, CA, USA). All other chemical reagents were purchased from Sigma (St Louis, MO, USA) and used without further purification. The S12 extract was prepared using a minor modification of the previously described method.[14]

Preparation of template DNAs

Firefly luciferase sequence was PCR amplified from the plasmid pSP-luc + NF (Promega, Madison, WI) using the primers flanking NdeI and SalI sites. After digestion with NdeI and SalI, amplified genes were cloned into the pK7 plasmid between the T7 promoter and the T7 terminator sequences.[12] Similarly, the ORFs of enhanced green fluorescent protein (EGFP) and dihydrofolate reductase (DHFR) in the plasmid pIVEX 2.3d[13] were subcloned into the pK7 plasmid for subsequent experiments. For the conjugation of the template DNA to STV-coated beads, cloned target sequences were PCR-amplified using 5′-biotinylated T7 promoter specific (sense) primers. 5′-Cy3 labeled, 5′-biotinylated or unlabeled T7 terminator specific (anti-sense) primers were used depending on the experiment. All the PCR products were purified using a commercial PCR clean up kit (Promega, Madison, USA) prior to use.

Conjugation of STV beads with template DNA and Ni–NTA

A suspension (30 μL) of STV agarose resin was washed three times with DDW, and then mixed with 20 μg of biotin-labeled PCR products in 150 μL of conjugation buffer (5 mM Tris–HCl, pH 7.5 and 1 M NaCl). After being incubated at room temperature for 30 min, the microbeads were washed, and resuspended in 100 μL of PBS buffer (pH 7.4) containing 60 μg of biotin-X-NTA. The NTA groups conjugated to the microbeads were then charged with Ni^{2+} ion by adding 0.5 M $NiSO_4$ solution to the suspension. The microbeads were then washed and stored in PBS buffer prior to their use.

Cell-free protein synthesis

For conducting solution phase cell-free synthesis reactions, 1.53 nM of target genes (in the forms of plasmid, free PCR product or bead-conjugated PCR product) were added to 15 μL of the standard reaction mixture [57 mM Hepes–KOH (pH 7.5), 1.2 mM ATP, 0.85 mM each GTP, UTP, and CTP, 80 mM ammonium acetate, 34 μg mL^{-1} 1-5-formyl-5,6,7,8-tetrahydrofolic acid (folinic acid), 2.0 mM each of 20 amino acids, 0.3 U mL^{-1} creatine kinase, 67 mM creatine phosphate, and 4 μL of the S12 extract], and incubated at 30 °C with constant agitation.

In in-gel protein synthesis experiments, 5 μL suspension of the microbeads conjugated with PCR products were mixed with 65 μL of 3% low melting agarose (Bio-Rad Laboratories, Hercules, CA, USA) at 37 °C, and allowed to solidify in the Frame Seal Slide Chamber (Bio-Rad Laboratories, Hercules, CA, USA) at room temperature. 65 μL of standard cell-free synthesis mixture was added to the top of the solidified gel and allowed to absorb for 30 min at 4 °C. The protein synthesis reaction was initiated by placing the agarose gel cassette in a humidified incubating chamber set at 30 °C.

Analysis of proteins expressed from plasmid, free PCR product, and bead-immobilized PCR products

The fluorescence intensity of cell-free synthesized EGFP was measured by using a VICTORTM X2 multilabel plate reader (PerkinElmer, Waltham, MA, USA) after 10 fold dilution of the samples with PBS buffer. In-gel expressed proteins from the bead-bound PCR products were analyzed using a confocal laser scan microscope (LSM5, Carl Zeiss, Oberkochen, Germany) or GenPix 4200A laser microarray scanner (Axon Inc, Sunnyvale, CA, USA). Enzymatic activity of the bead-expressed luciferase was determined by using a chemiluminescence assay of the beads on the gel matrix. Luciferase assay buffer (200 μL) [0.5 mM D-luciferin, 0.2 mM ATP, 15 mM $MgSO_4$, 1 mM DTT, 4 mM EDTA and 15 mM KPO_4 (pH 7.8)] was overlaid onto the incubated agarose matrix after washing three times with PBS buffer. The chemiluminescence image was obtained using Molecular Imager ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). The same gel matrix was also stained with ethidium bromide to correlate the positions of the template DNA- and luciferase-immobilized beads.

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Results and discussion

Cell-free expression of bead-conjugated template DNAs

STV-coated microbeads were conjugated with biotinylated PCR products of the template genes. After the conjugation of DNA, residual STV sites on the beads were modified with Ni–NTA. It was estimated that approximately 0.3 nmol of PCR products were conjugated on 1 mL slurry of STV–agarose beads, which left most of the STV sites on the bead (more than 90%) available for subsequent NTA conjugation. As shown in Fig. 2a, microbeads conjugated with the PCR products of EGFP acquired fluorescence after the incubation of the microbeads in a standard solution phase cell-free synthesis reaction, confirming successful expression and immobilization of the expressed protein. The EGFP fluorescence was observed to be evenly distributed across the entire microbead while Cy3-labeled template DNA was shown to exist as a thin layer on the surface of the microbead. It appears that the protein molecules expressed on the bead surface diffuse into the matrix structure owing to their relatively small size compared to the DNA.

However, it was also found that proteins expressed from the template DNA were not solely localized on the same bead. For instance, when the protein synthesis reaction was conducted in the presence of a 5 : 1 mixture of the microbeads conjugated with EGFP or DHFR genes, fluorescence from the EGFP product was observed from the beads conjugated with DHFR gene as well as those conjugated with EGFP gene (Fig. 2b). This observation indicates that a substantial amount of bead-expressed proteins are not immobilized on the same bead, but instead are free to cross-contaminate other beads.

In-gel expression of bead-bound DNA

One distinct advantage of cell-free synthesis methods is that the physicochemical environment can be freely manipulated as long as the changes do not interfere with the protein synthesis reaction.[15] To restrict the diffusional migration of cell-free synthesized proteins, we attempted to express the microbead-conjugated DNA in a solid gel matrix. In the procedure, a low melting agarose solution (3% w/v) was mixed with DNA-conjugated microbeads and then solidified in a Frame-Seal Slide Chamber to form a thin agarose matrix layer. The 15 mm × 15 mm × 0.2 mm ($W \times L \times H$) dimensions of the agarose matrix were sufficient enough to cover a single layer of the dispersed beads that have an average diameter of 120 μm. After being treated with the reaction mixture used for cell-free synthesis (see Materials and Methods), the assembled cassette was incubated in a humidified chamber set at 30 °C for 1 h. The microbeads in the gel matrix were examined under a confocal laser microscope using unconjugated microbeads and microbeads conjugated with separately prepared EGFP as a negative and a positive control, respectively (Fig. 3a and b). While the cell-extract in the gel matrix gave significant background fluorescence (Fig. 3c), EGFP fluorescence could be observed with clear contrast after electrophoretic removal of extract-derived proteins in a horizontal electrophoresis tank (50 V, Tris–glycine buffer without sodium dodecyl sulfate, 15 min) as shown in Fig. 3d. In contrast to the DNA conjugated counterparts, unconjugated microbeads do not show detectable fluorescence in the electrophoretically washed gel matrix (Fig. 3a). DNA-conjugated microbeads also do not exhibit fluorescence if they are not subjected to prior treatment with Ni–NTA (Fig. 3e), indicating that the fluorescence on the bead was the result of *in situ* immobilization of the on-bead synthesized EGFP.

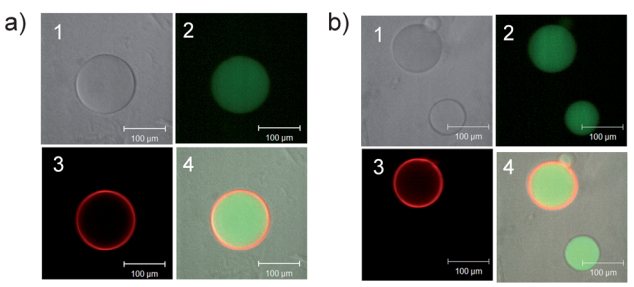

Fig. 2 Confocal laser microscopic images of the microbeads incubated in a solution phase cell-free synthesis system. (a) A microbead conjugated with Cy3-labeled EGFP DNA. (b) Cross-contamination between the microbeads. EGFP fluorescence is observed on the microbead conjugated with Cy3-labeled DHRF DNA as well as on the microbead conjugated with unlabeled EGFP DNA. 1, Optical images of the microbeads; 2, EGFP fluorescence on the microbeads; 3, Cy3 fluorescence on the microbeads; 4, merged images of 1, 2 and 3.

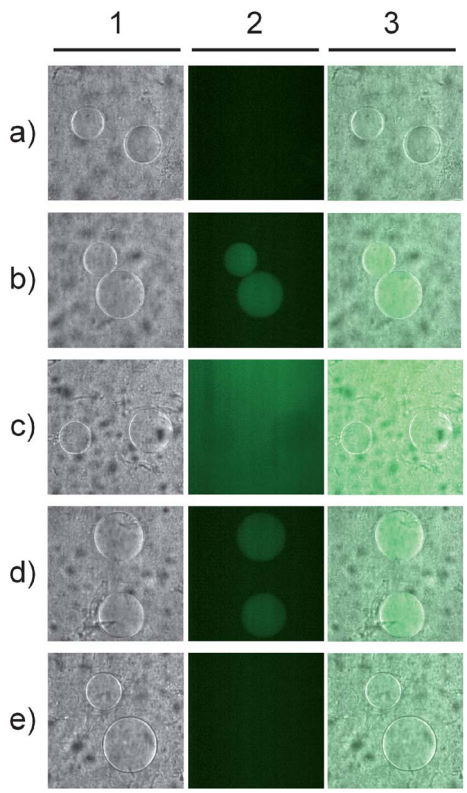

Fig. 3 Confocal laser microscopic images of the microbeads incubated in an agarose gel matrix containing embedded translation machinery. (a) Negative control microbeads without conjugated DNA. (b) Positive control microbeads that are conjugated with separately prepared EGFP. (c) Microbeads conjugated with EGFP DNA and NTA, before electrophoresis. (d) Microbeads conjugated with EGFP DNA and NTA, after electrophoresis. (e) Microbeads conjugated with EGFP DNA but not with NTA, after electrophoresis. 1, Optical images; 2, fluorescence images; 3, merged images.

In order to probe bead-to-bead transfer of cell-free synthesized EGFP, a mixture of EGFP DNA- and DHFR DNA-conjugated microbeads (5 : 1) was incubated in the agarose gel matrix. Unlike in the solution-phase expression system, only those beads that are conjugated with EGFP DNA gave rise to fluorescence and no detectable fluorescence emission is observed from nearly all of the control beads (Fig. 4). In addition to examining selected individual beads under a confocal microscope, the entire gel matrix was also analyzed using a laser microarray scanner. As the observations displayed in Fig. 5a show, green spots corresponding to EGFP-immobilized microbeads and red spots for Cy3-DHFR DNA-conjugated microbeads were clearly distinguishable in the scanned image. Yellow spots, which represent the presence of both EGFP and DHFR DNA on the same bead resulting from bead-to-bead transfer, are seen in less than 5% of the total number of beads counted.

Recovery of DNAs from the PCR product conjugated beads after the cell-free protein synthesis

Although linear PCR-amplified genes were used as the template for protein synthesis in crude cell extracts where the efficiency of protein synthesis can be severely impaired by the presence of

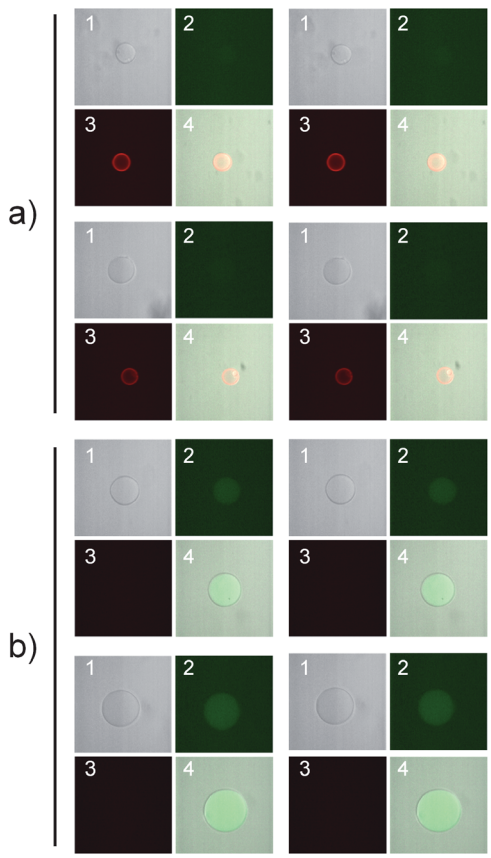

Fig. 4 Confocal laser microscopic images of the selected microbeads incubated in the same agarose gel matrix using a mixture of DHFR DNA conjugated beads and EGFP DNA conjugated beads. (a) Cy3 labeled DHFR–PCR conjugated beads. (b) Non-labeled EGFP-PCR conjugated beads. 1, Optical image; 2, Image detecting fluorescence from EGFP; 3, image detecting fluorescence of Cy3 dye; 4, merge of images of 1, 2 and 3.

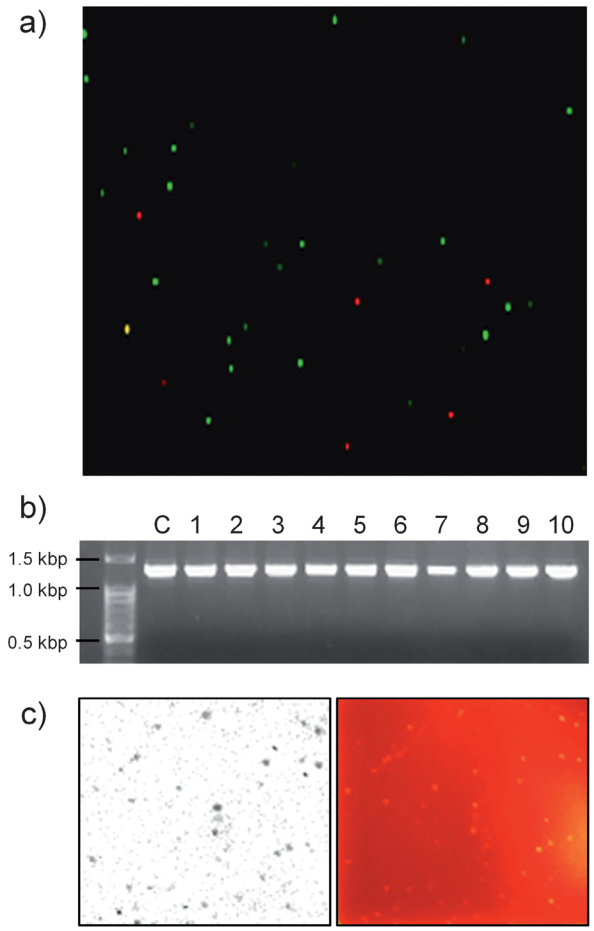

Fig. 5 Localized bead-immobilization of expressed proteins during in-gel synthesis. (a) The mixture of EGFP DNA and DHFR DNA-conjugated microbeads was incubated in the extract-embedded gel matrix. Only DHFR DNA was Cy-3 labelled, and unlabelled EGFP DNA was conjugated on the beads. Thus, green spots on the laser scanned image of the gel matrix represent EGFP immobilized microbeads, and red spots represent DHFR DNA conjugated microbeads. (b) DNA recovered from the EGFP fluorescent microbeads was PCR amplified and analyzed on a 1% agarose gel. Lane C is the own size (1300 bp) of PCR amplified EGFP gene. (c) Luminescence (left) and EtBr-stained (right) images after in-gel expression of microbead-conjugated luciferase DNA.

exonucleases,[16,17] to our surprise, bead-bound PCR products remained stable during incubation in the cell-extract. 10 separate microbeads that exhibit EGFP fluorescence were recovered from the gel matrix and the conjugated DNA was PCR-amplified using primers against the T7 promoter and the T7 terminator sequences. As shown in Fig. 5b, conjugated DNA from all of the 10 microbeads gave PCR products that have the same size of the EGFP gene, confirming the stable maintenance of the linear template DNA during its incubation in the extract-embedding gel matrix. It is proposed that the stability results from shielding of DNA ends by their conjugation on the microbeads. Supporting this presumption, bead-bound PCR products that were biotinylated at their both ends gave twice the yield of protein synthesis compared to those that had single biotinylated ends. On the other hand, immobilization of DNA also seems to restrict the access of translational machinery as

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well as the nucleases. The yield of protein synthesis from the DNA that was conjugated with single biotinylated end, and thus the other end of which remained exposed, was approximately 27% lower than that of free PCR products. It thus appears that bead-immobilized PCR products with dual biotin conjugation exhibit higher protein productivity than free PCR products as a compromised consequence of enhanced DNA stability and reduced ribosome accessibility (Fig. 6). The stability of intact template DNA not only improves the efficiency of on-bead expression, but it is also crucially important for extensions of the applications of the bead expression platform to the screening of protein libraries, where a discovered protein should be addressable to the encoding DNA.

In-gel bead expression of luciferase and analysis of its enzymatic activity

Owing to the fact that expressed proteins are localized on the microbeads that are exposed to the surrounding environment, the in-gel bead expression approach described above enables facile analysis of the biological activity of expressed proteins. In order to demonstrate this capability, the in-gel on-bead expression method was applied for expression and assay of the model enzyme firefly luciferase. Following in-gel expression of microbead-bound luciferase DNA utilizing the same procedure employed for EGFP expression, the agarose matrix was electrophoretically washed to remove extract-derived contaminating proteins and supplied with an assay solution containing D-luciferin and ATP. Addition of the assay solution resulted in the generation of luminescent spots that were detected using a CCD imaging device. The luminescent spots corresponding to the luciferase-bound microbeads overlapped well with DNA spots that were generated by staining the same matrix with ethidium bromide (Fig. 5c), providing an example of on-bead expression and analysis of enzymes from the bead-conjugated DNA.

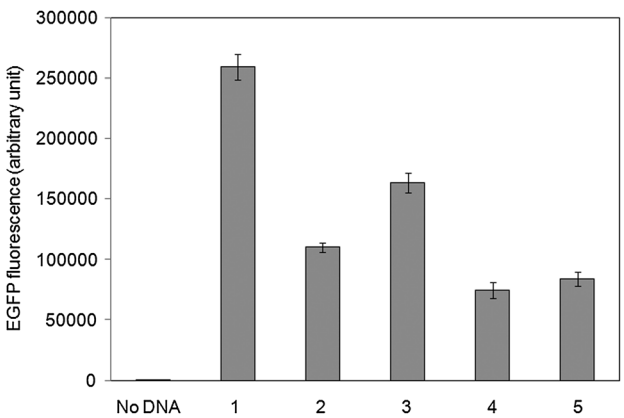

Fig. 6 Efficiency of protein synthesis from different types of template DNA. Standard cell-free protein synthesis reactions were conducted using plasmid (column 1), free PCR products (column 2), and microbead-conjugated PCR products of EGFP gene (columns 3–5) for the comparison of relative efficiency of protein synthesis. Column 3, PCR products biotinylated at both 5′-ends; column 4, PCR products biotinylated at the 5′-end of sense strand; column 5, PCR products biotinylated at the 5′-end of antisense strand.

Discussion

Microbead display technology has been developed for elucidating peptide–ligand interactions.[18] In addition to displaying specific ligand-binding peptides, the "split-and-mix" strategy of solid phase peptide synthesis has enabled the generation of bead-immobilized peptide libraries based on the one-bead-one-peptide principle.[19] Bead-displayed synthetic peptide libraries have been used for the identification of ligand-binding peptides.[20–22] While this display method based on solid-phase polypeptide synthesis enables the achievement of great diversity along with high degrees of freedom and flexibility in the discovery of novel binding affinity centered function, at present it only can be applied in the synthesis of peptides that have limited lengths and, thus far, the technology can not be employed for on-bead chemical synthesis of full-length functional proteins.

In the effort described above, we have developed a simple method for template-guided generation and *in situ* immobilization of functional proteins on the surfaces of microbeads. Through the co-conjugation of a template DNA sequence and a resulting protein on the same microbead, it is possible to address the protein function exhibited on the microbeads with the corresponding encoding gene. Although PCR-amplified genes were used as the template for protein synthesis in crude cell extracts that contain exonucleases,[16,17] bead-bound PCR products remained surprisingly stable during and after incubation in the cell-extract. It is proposed that the stability results from shielding of both ends of linear DNA by their conjugation with streptavidin coated on the microbeads. This finding should be generally applicable to cell-free synthesis reactions that produce proteins using PCR-amplified genes, where maintaining the stability of the genetic material is important for enhancing the efficiencies of protein production.

Although much of the expressed proteins bind with remote microbeads when solution phase reactions are used, successful local immobilization of the bead-expressed protein is achieved when the individual beads are encased in a gel matrix that retards diffusional migration of protein expressed on microbead surfaces. An additional advantage of the in-gel expression system is that extract components can be removed from the gel matrix by using a final electrophoretic step. We anticipate that the ability to obtain pure recombinant proteins after cell-free synthesis reactions will benefit the *in situ* generation of protein arrays, which is currently being explored in our laboratory. The method also substantially shortens the time needed to translate genetic information into functional proteins. In addition, unlike those used previously that are based on cell-free protein synthesis, in the newly developed procedure protein synthesis and analysis can be completed without employing complicated separation techniques and special laboratory equipment. In a manner that is similar to the conventional use of colony screening on agar plate, DNA-conjugated beads are spread on a gel matrix that contains translational components for expression and analysis of the encoded genes. The display of the expressed protein on the

solid surface also enables the analysis of the translation products against a virtually unlimited array of substrates.

Conclusions

The facile method described above, which enables direct linkage of DNA and encoded protein, should significantly benefit various applications that involve protein expression from addressable genetic templates, such as expression screening of ligand binding proteins and the discovery and engineering of novel enzymes, particularly in combination with the well-established single molecule gene amplification techniques.[23,24]

Acknowledgements

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