Cell-free protein synthesis for proteomics

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Date received (in revised form): 20th October, 2003

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Abstract

The use of cell-free expression systems as an alternative to cell-based methods for protein production is greatly facilitating studies of protein functions. Recent improvements to cell-free systems, and the development of cell-free protein display and microarray technologies, have led to cell-free protein synthesis becoming a powerful tool for large-scale analysis of proteins. This paper reviews the most commonly used cell-free systems and their applications in proteomics.

Keywords: *cell-free protein synthesis, proteomics, cell-free protein display and microarrays,* in vitro *transcription and translation*

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INTRODUCTION

With the completion of the genome sequences of many organisms, there is an increasing need for high throughput expression of the genome encoded proteins. Cell-based heterologous protein expression systems have been unable to meet this challenge due to time-consuming cloning procedures and the failure to generate functional molecules of many proteins in bacterial hosts.[1] The use of cell-free protein expression is now becoming the favoured alternative to cell-based methods as it offers a simple and flexible system for the rapid synthesis of folded proteins, drastically reducing the time taken to get from DNA sequence to functional protein. This paper reviews the most commonly used cell-free systems and the recent development of technologies derived from them. Their applications in proteomic studies will also be discussed.

CELL-FREE PROTEIN SYNTHESIS

General description

Cell-free expression systems exploit the cellular protein synthesis machinery to direct protein synthesis outside intact cells using exogenous messenger RNA (mRNA) or DNA as template. This has been achieved by combining a crude lysate from growing cells, which contains all the necessary enzymes and machinery for protein synthesis, with the exogenous supply of essential amino acids, nucleotides, salts and energy-generating factors.

Cell-free protein synthesis systems from several organisms have been developed.[2] The most commonly used systems are derived from *Escherichia coli* extracts, wheat germ extracts and rabbit reticulocyte lysates. Cell-free systems can be made to act either in a coupled manner, where DNA is used as template, or as an uncoupled system, which requires mRNA template produced from native sources or by *in vitro* transcription. The DNA template may be in the form of a plasmid or polymerase chain reaction (PCR) fragment but must contain a promoter (T7, SP6 or T3 are most commonly used) and a translation initiation signal such as a Shine–Dalgarno (prokaryotic) or Kozak (eukaryotic) sequence (Figure 1). A universal DNA sequence for protein initiation in both *E. coli* and eukaryotic systems has also been designed.[3] To increase the expression level, a transcription and translation termination region is also required (Figure 1). Cell-free systems can be used to express either a single gene or a DNA library.

In general, the level of protein expression is governed by two limiting factors: the efficiency of the energy supply and the accumulation of inhibitors. A

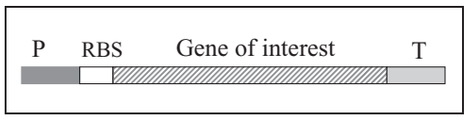

Figure 1: A general PCR construct for cell-free protein synthesis. P, promoter; RBS, ribosome-binding site, which is either Kozak sequence for eukaryotic systems or Shine–Dalgarno (S/D) sequence for *E. coli* S30 extracts; T, transcription and translation termination region

number of methods have been used in an attempt to overcome these limitations, including the use of concentrated batch systems,[4] continuous flow and continuous exchange methods,[4,5] the bilayer diffusion system[6] and systems employing hollow fibre membranes.[7] A combination of concentrated batch and continuous exchange methods has led to a reported yield of 6 mg/ml by an *E. coli* S30 extract.[8] Removal of a ribosome-inactivating protein from wheat germ extracts resulted in improved expression levels reaching 4 mg/ml.[9]

Improvement in protein yield

Cell-free systems are suitable for the expression of a wide range of protein families with molecular sizes up to 400 kilodaltons (see ref. 10, which is a comprehensive guide detailing the technologies and applications associated with *in vitro* expression and which includes valuable references and schematics). Some integral membrane proteins (receptors) have also been shown to insert into membranes with correct folding when expressed in the presence of canine microsomes.[11] An *E. coli* recombinant cell-free system (PURE) has been demonstrated in which purified recombinant protein components of the translation machinery are used.[12] This system reconstitutes the coupled transcription/translation process from 31 purified soluble protein factors with the addition of a mixture containing 46 transfer RNAs (tRNAs), the necessary substrates and their corresponding enzymes. The PURE system has been successfully used to express dihydrofolate reductase, λ-lysozyme, green fluorescent protein (GFP), glutathione S-transferase and the T7 gene 10 product, yielding around 100 μg protein/per ml after 1 hour.[12]

PURE system

Advantages of cell-free protein synthesis

Cell-free protein synthesis offers several advantages over conventional cell-based protein expression methods. First, cell-free systems can produce proteins directly from a PCR fragment or an mRNA template without the need for *E. coli* cloning, allowing it to be easily adapted for high throughput protein synthesis. In addition, cell-free systems can simultaneously express multiple templates, permitting the production of a protein population in a single reaction. Secondly, these systems often generate soluble and functional proteins, whereas cell-based methods yield insoluble aggregates for many proteins.[10] Cell-free systems therefore offer a rapid route to functional protein analysis. Thirdly, the protein synthesis conditions in a cell-free system can be adjusted and controlled by the addition of helper molecules, providing a defined environment(s) for the correct folding of individual proteins.[13] Furthermore, cell-free systems allow the efficient incorporation of non-natural or chemically-modified amino acids into the expressed protein at desired positions during translation, thereby generating novel molecules for proteomic applications. Finally, cell-free systems can produce proteins that are not physiologically tolerated by the living cell — eg toxic, proteolytically sensitive or unstable proteins.

Properties of cell-free synthesised proteins

Protein folding

Nascent proteins have been demonstrated to fold co-translationally on ribosomes in cell-free systems in a manner similar to protein folding *in vivo*, ie a growing peptide starts to fold as it emerges from the large ribosomal subunit or immediately at the end of translation prior

to release from the ribosome.[14,15] Molecular chaperones are also involved in the folding process for some proteins[16] and either ribosomes themselves or ribosomal RNA from either prokaryotic or eukaryotic sources have been demonstrated to contribute to the folding process.[17] The co-translational protein folding mechanism has also been supported by structural studies of the large ribosomal subunit.[18] Many proteins have been expressed in cell-free systems with correct folding and processing, yielding active molecules.[10] These proteins include various enzymes, growth factors, membrane proteins, protein complexes and viral capsids, demonstrating the feasibility of using cell-free systems to produce a variety of active, folded proteins.

Site-directed modification of proteins

Usually, cell-free systems contain dithiothreitol (DTT) to promote maximal protein synthesis and to preserve the cytoplasmic environment. This appears to have little effect on the folding of cytoplasmic proteins, but it may have different effects on the folding of those proteins that require the formation of disulphide bonds for activity.[13,19–22] For example, whereas some single-chain antibody fragments (scFv) showed no activity at all when produced in an *E. coli* S30 extract containing 1 mM DTT,[13] many scFvs including engineered mutants were actively generated in both *E. coli* S30 extracts and rabbit reticulocyte lysates containing 2 mM DTT.[20–22] Rabbit reticulocyte lysates containing DTT have been used to produce cysteine-containing proteins and receptors in their functional forms.[10] This system has also been used to re-fold proteins from a denatured state in an ATP-dependent manner.[23,24]

Cell-free protein folding

One of the advantages in cell-free expression is that the conditions for protein synthesis can be adjusted and/or controlled. For example, a defined folding environment can be generated for a particular protein by using glutathione redox buffers or by the addition of helper molecules such as protein disulphide isomerase.[13,19] Many proteins that fail to be functionally produced by cell-based methods can be actively expressed in a cell-free system with defined conditions, indicating that cell-free expression may be a more suitable system for producing folded proteins.[10,25]

Protein modifications

Cell-free systems allow modification of the protein during translation by including modified tRNAs to incorporate non-natural or chemically modified amino acids into the protein being expressed,[26] thus generating novel molecules.[27] This also permits the simple labelling of proteins with isotopic, fluorescent or biotinylated amino acids, or the incorporation of photo-reactive cross-linked groups for sensitive detection and functional analysis. The incorporation of such labels can be directed to various positions in the primary protein sequence. Utilising the fluorescently modified initiator, methionine tRNA (fmet-tRNA), proteins can be labelled at the N-terminus.[28] Labelling at internal sites can be achieved by using stop codon suppression methodology,[26,27] which introduces a stop codon at the desired position of the target gene and the cell-free extract is supplemented with a complementary suppressor tRNA carrying a labelled amino acid.[26,27] C-terminal labelling has been carried out using a puromycin analogue to attach a fluorescent group to the end residue of a protein.[8,29] Proteins generated by such cell-free modification methods have been used to study protein folding by nuclear magnetic resonance (NMR) spectroscopy[30] and the structural aspects of protein–nucleic acid[31] and protein–protein interactions.[29]

A variety of co- and post-translational modifications have been observed in proteins synthesised in eukaryotic cell-free systems (rabbit reticulocyte lysates and wheat germ extracts), both with and without the presence of canine pancreatic microsomal membranes.[10] These modifications include signal peptide cleavage, glycosylation, acetylation,

Comparison of *E. coli* S30, wheat germ and rabbit reticulocyte lysate

phosphorylation, isoprenylation, myristoylation, proteolytic processing and the action of degradation pathways.[10] Unlike in cell-based systems, cell-free modifications may vary depending on the nature of the expressed proteins and the type and conditions of expression system used.

Macromolecular protein complexes

The assembly of protein subunits into active complexes has been demonstrated in rabbit reticulocyte lysates for β-galactosidase, fibrinogen, connexins and steroid hormone receptors.[10] A number of viral coat proteins have also been successfully assembled into viral capsids in rabbit reticulocyte lysates. Cell-free synthesised human papillomavirus (HPV) L1 protein, after being assembled into virus-like particles, closely resembled HPV virions and retained various original conformational epitopes.[10]

Choice of cell-free system

The choice of a cell-free system for protein synthesis is generally based on the origin of the proteins to be expressed and their downstream applications. The commonly used rabbit reticulocyte lysates, wheat germ and *E. coli* S30 extracts are commercially available in both coupled and uncoupled formats. Coupled systems are generally simpler and more efficient; they also avoid problems of mRNA degradation and mRNA secondary structure.[32] Uncoupled systems control the amount of input mRNA and can express proteins in the absence of DTT.

The cell-free system chosen can affect the production of a particular protein. Parallel expression of five different coding sequences of bacterial and eukaryotic origin in either *E. coli* S30 extract, wheat germ extract or rabbit reticulocyte lysate systems has revealed that, while predominantly full-length products of all five sequences tested were generated in the two eukaryotic systems, many incomplete nascent polypeptides accompanied the full-length product in *E. coli* S30 extracts (Table 1).[33] It was suggested that the generation of these incomplete polypeptides was due to pausing of the *E. coli* ribosome.[33] In addition, co- and post-translational modifications can only be carried out in rabbit reticulocyte lysates and wheat germ extracts (Table 1).[10]

PROTEOMIC APPLICATIONS

Large-scale analysis of proteins

Cell-free expression has been widely used to determine the presence of a gene or open reading frame (ORF), especially when multiple samples or large genes are to be examined .[34] It can also be used to confirm an ORF predicted by DNA sequencing. In each case, the synthesised proteins are usually analysed for size, biophysical properties and function.[35] Cell-free protein synthesis has also been used as a routine screen for translation-terminating mutations in the diagnosis of genetic disease,[36] a rapid method referred to as a protein truncation test (PTT). The truncated mutant alleles can be easily distinguished from the normal full-length

Table 1: Choice of cell-free protein synthesis systems

	***E.coli* S30 extract**	**Rabbit reticulocyte lysate**	**Wheat germ extract**
Reported protein yield	6 mg/ml[8]	6 μg/ml*	4 mg/ml[9]
Post-translational modifications	No	Yes	Yes
Synthesised protein	Many incomplete polypeptides	Mainly full length	Mainly full length
Recommended template source*	Mainly bacteria	Animal, plant, bacteria, mammalian virus and plant virus	Animal, plant, bacteria and plant virus

protein product by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Screening of antibody mutants

PCR fragments can be directly applied as templates for coupled cell-free systems, thus allowing the rapid expression of large numbers of proteins for parallel activity screening. This is particularly useful for functional identification of candidates from a panel of engineered mutants. This idea has been used successfully to analyse an antibody combining site, where all possible amino acid substitutions were performed at six different positions within the ligand-binding domain.[20,21] A PCR-linked *in vitro* expression method has also been used for high throughput construction and screening of improved antibody mutants from a random mutagenesis library.[37] An improved wheat germ system has also been established for cell-free screening of enzyme activity.[38] It has been used for systematic screening of protein kinase genes from *Arabidopsis*, leading to the identification of autophosphorylation activity in at least 233 clones out of 530 genes.[38]

Protein synthesis for structural studies

IVEC technology

Cell-free systems have led to the establishment of a routine functional screening method known as *in vitro* expression cloning (IVEC).[39] In this method, a large complementary DNA (cDNA) library is broken down into pools of 50–100 clones of plasmid templates. The pools are expressed in a coupled cell-free system and screened for function. Plasmids from those pools giving a positive result are further deconvoluted and re-screened (Figure 2). The process is repeated until single clones are reached. IVEC has been used successfully to clone and identify enzymes,[40,41] protein substrates,[42,43] phospholipid-binding proteins[44] and a sister chromatid separation inhibitor.[45] One interesting application used IVEC to screen for mouse proteins that were degraded by the ubiquitin/proteasome-dependent N-end rule pathway.[46] This technique will only succeed if a sensitive assay is available for the protein function being tested, as each represented plasmid in the pool will give rise to only a small fraction of the expressed protein population. In addition, this technique cannot be used if the cell-free system itself contains the activity under examination.

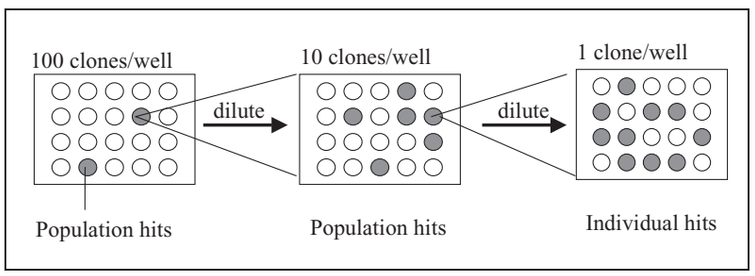

Figure 2: IVEC screening process

Recent improvements to cell-free systems have increased the expression level of functional proteins to milligram quantities.[8,9] This has led to the preparation of sufficient labelled proteins for structural determination by NMR and X-ray crystallography.[47,48]

Molecular interactions

Cell-free expression has been utilised for studies involving the interactions of various macromolecules including protein–protein, protein–DNA, protein–RNA, protein–ligand, DNA–RNA and even RNA–RNA complexes.[10]

Protein–protein interactions identified using the yeast two-hybrid system are commonly confirmed by cell-free expression techniques. In such a study, one protein partner is isotopically labelled during cell-free translation and incubated with the interacting protein which may be derived from expression in either *in vivo* or *in vitro* systems. The interaction complex is then immunoprecipitated with an antibody specific for the interacting protein followed by detection of the labelled protein using a variety of biochemical analysis methods such as SDS-PAGE or enzyme-linked immunosorbent assay.[49]

Protein–DNA and protein–RNA interactions are examined by mixing cell-

free synthesised protein with labelled oligonucleotides. The interaction is then detected by an electrophoretic mobility shift assay in which protein–oligonucleotide complexes are retarded by comparison with unbound oligonucleotide.[50] This approach has been used to investigate the DNA-binding properties of many proteins,[51] but cannot be used to examine any interacting protein that is endogenously present in the cell-free system chosen. So, for example, mammalian transcription factors are usually studied in wheat germ extracts rather than in rabbit reticulocyte lysates as the latter contain high levels of these effector molecules.

Use of cell-free expression systems in the study of DNA–RNA and RNA–RNA interactions is based on the ability of such interactions to inhibit cell-free transcription and translation.[52] Thus, the ability of oligonucleotides bearing a specifically designed sequence to hybridise with a target sequence may be assessed by determining the effect of that oligonucleotide on expression of the target template molecule.

Cell-free protein display

Cell-free systems have been crucial to the development of *in vitro* protein display technologies. By creating a physical link between genotype (gene) and phenotype (protein) (Figure 3), display technologies allow the selection of a gene through the function of its encoded protein. Repetition of the display process enriches selected molecules, enabling rare species to be isolated from a non-related population. Cell-free display technologies offer a number of advantages over cell-based display methods (see above). Perhaps most importantly, much larger DNA libraries can be rapidly generated by PCR and displayed. The library size in cell-free display systems can be up to 10^{12}–10^{14} members in comparison with 10^{7} –10^{10} members in cell-based methods. Larger library size increases the probability of discovering proteins with unique/desired properties.[53] At present, commonly used cell-free display methods include ribosome display,[54–56] mRNA display[57] and *in vitro* compartmentalisation.[58]

Ribosome display technology

Cell-free ribosome display couples mRNA to its encoded protein through the formation of a protein–ribosome–mRNA (PRM) complex (Figure 3A).[54,59] By fusing a gene encoding the protein of interest to a C-terminal spacer domain lacking a stop codon, a translating ribosome in the cell-free expression system is stalled at the end of the mRNA, thus preventing dissociation of the mRNA and nascent polypeptide from the ribosome.[54,55] A novel strategy has been described for producing stable PRM complexes by fusing the display protein with a C-terminal ricin A chain which inactivates and thus stalls the translating ribosome as it is synthesised. In this way, there is no need to remove the stop codon from ricin A.[60,61] Both prokaryotic and eukaryotic ribosome display systems have been developed[46–48] and successfully used for function-based selection of

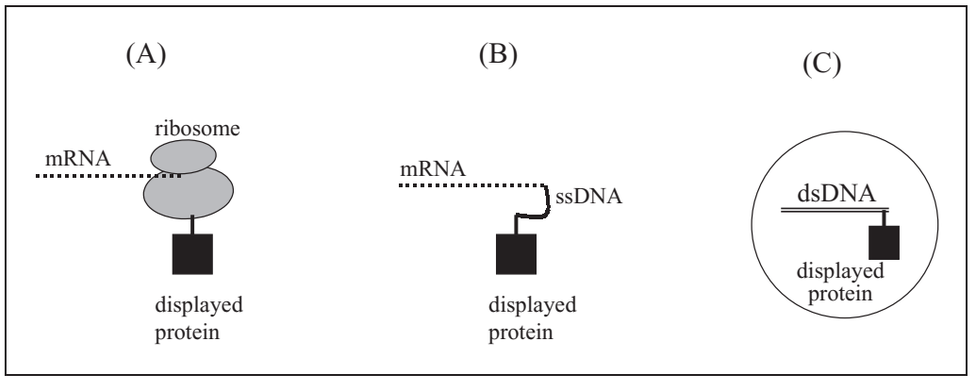

Figure 3: Linkage of phenotype and genotype. (A) Ribosome display; (B) mRNA display (ssDNA, single-stranded DNA); (C) *in vitro* compartmentalisation (dsDNA, double-stranded DNA)

various proteins including single-chain antibodies, peptides, enzymes and ligand-binding proteins.[62] Ribosome display has been demonstrated to be a very efficient and powerful tool for *in vitro* protein evolution.[62,63] This is largely due to the ease with which diversity/mutations can be continuously introduced into the template population either by PCR methods for DNA templates or including Qβ RNA-dependent RNA polymerase for mRNA templates[63] in subsequent display cycles without the need for cloning.[62,63]

Successful applications of ribosome display

Ribosome display has selected many high affinity antibodies (K_d as low as 80 pM) from different DNA libraries.[62] Antibodies whose combining sites demonstrate the recognition of conformation-specific epitopes or carry enzyme-like catalytic activities have also been isolated by ribosome display selecting on carefully designed antigens.[64,65] The screening of a very large synthetic DNA library (2×10^{13} members) has led to the isolation of a novel peptide which is bound specifically by streptavidin with a K_d of 4 nM, an affinity 1,000-fold greater than that previously reported for peptides selected by cell-based display methods.[66] One recent exciting application has been the comprehensive identification and recovery of immunologically relevant proteins of the human pathogen *Staphylococcus aureus* from genomic libraries,[67] showing for the first time the utility of ribosome display on a genomic scale. This study also revealed that a large fraction of the identified peptides could not be expressed and displayed by *E. coli*, demonstrating once again the advantage of the cell-free display method.[67]

mRNA display and *in vitro* compartmentalisation

mRNA display uses a different strategy to link the mRNA molecule to its encoded protein. It relies first on joining the mRNA covalently to a single-stranded DNA, which itself carries a puromycin moiety. When the ribosome reaches the RNA–DNA junction, the ribosome stalls and the puromycin moiety enters the peptidyl-transferase site of the ribosome to form a covalent linkage with the nascent polypeptide (Figure 3B).[57,59] As a result, an mRNA–protein fusion molecule without the ribosome is generated as the selection particle. Using this method, novel peptides binding to ATP with the best K_d of 100 nM were enriched from a library of 6×10^{12} members each containing 80 random residues after 18 rounds of display and selection.[68] This method has been used successfully to identify peptide aptamers with a K_d as low as 5 nM to a protein target.[69] A large number of Bcl-Xl binders have also been selected by display and screening of cDNA libraries.[70]

In vitro compartmentalisation[58] mimics cell compartmentalisation to maintain gene–protein linkage. In this method, each DNA molecule is separated into encapsulated aqueous 'compartments' formed in micro-droplets of an oil/water emulsion. Cell-free protein expression and formation of protein–DNA complexes takes place within the enclosed compartment (Figure 3C).[58] *In vitro* compartmentalisation has been used to screen libraries encoding methylase mutants. This method has been modified by coupling the gene to its synthesised protein through micro-beads in the 'compartments'. The resulting 'micro-bead libraries' — each member displaying a single gene and multiple copies of its encoded protein — can then be selected for catalysis or binding activity.[71] This approach has been demonstrated by the successful selection of a phosphotriesterase mutant with 63-fold improved turnover activity by comparison with the wild-type enzyme from a library of 3.4×10^7 mutated genes.[71]

Cell-free protein arrays

Cell-free systems have been exploited in the generation of protein arrays.[72–75] Protein arrays are useful tools in proteomics for high throughput analysis of molecular interactions, protein functions and expression patterns. The first cell-free method, described by He and Taussig,[72] was termed 'protein *in situ*

Protein *in situ* arrays

arrays' (PISAs) and generates protein arrays directly from PCR DNA in a single step. In this method, individual tagged proteins are synthesised in a cell-free system on a tag-binding surface, such that the tagged proteins are immobilised on the surface as they are produced (Figure 4A). The technology provides a rapid route for arraying proteins and protein domains that cannot be functionally produced by heterologous expression or for which the cloned DNA is not available.[72,76] PISAs have been successfully used to generate array elements to screen a panel of single-chain antibodies for binding to a specific ligand and also to demonstrate functional activity of an enzyme (luciferase) when bound to a solid surface.[72,76]

Self-assembling protein arrays

Cell-free protein arrays have also been generated by specifically capturing nucleotide-tagged proteins on high-density oligonucleotide arrays (Figure 4B).[73,75] A self-assembling protein array has been described[73] in which a cell-free system was used to express a protein containing biotinylated lysines. After purification, the biotinylated protein was tagged with an oligonucleotide of known sequence through streptavidin.[73] The resulting oligonucleotide-tagged protein complexes were then hybridised on a semiconductor oligonucleotide microarray and assembled at positions of the array designated by an oligonucleotide of the complementary sequence. The technology was exemplified by constructing an array of two model proteins, GFP and luciferase. The activities of both proteins were preserved on the array surface and, due to the semiconductor nature of the biochip, the arrayed proteins could also be detected by electrochemical methods.[73]

A more efficient procedure for the generation of protein arrays on high-density oligonucleotide arrays has also been reported.[75] In this approach, high-density oligonucleotide arrays were used to capture specifically mRNA–protein fusion molecules generated by mRNA display technology.[57,75] This technology has been demonstrated on a number of well-known peptide epitopes (FLAG, HA and MYC), showing specific detection of the mRNA–peptide fusion molecules at the expected sites of the array.[75] One useful application of this method would be to create protein microarrays by capturing libraries of mRNA–protein

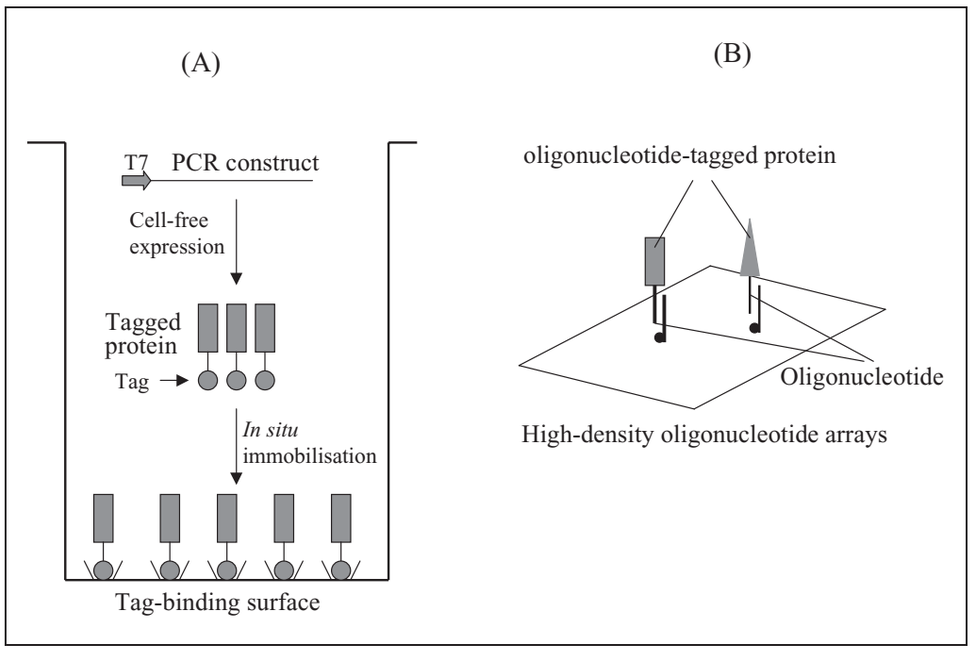

Figure 4: Cell-free protein arrays. (A) Protein *in situ* arrays (PISAs); (B) self-assembly of protein arrays

fusion molecules at specific sites of high-density oligonucleotide arrays, thus circumventing the need for the tedious purification or robotic printing of large numbers of proteins in the array.

The use of protein arrays to study protein–protein interactions has been carried out through cell-free expression of both arrayed and probing proteins. The arrayed proteins were produced as tagged molecules and captured on glass slides coated with tag-capturing reagents. The probing proteins were labelled by incorporation of a fluorophore (Cy3) during cell-free translation. Protein–protein interactions were then visualised using a microarray scanner .[74] This method has been demonstrated for three pairs of model proteins.[74]

CONCLUSIONS

Cell-free protein synthesis offers a number of advantages over cell-based methods in protein production. It provides a simple and flexible system to turn genes into folded proteins within hours and also allows protein production and modification under the type of defined condition(s) that living cells are incapable of reproducing. Recent improvements to cell-free systems and the development of cell-free display and protein array technologies have exploited cell-free expression to create a selection of very powerful tools for proteomic applications. Cell-free expression can rapidly provide proteins for screening protein function, for identifying molecular interactions and for structural studies. With cell-free display technologies, particularly ribosome display, it is possible to generate a large number of antibodies to study proteins and profile protein expression on a genomic scale. Furthermore, a cell-free display technology could be combined with a cell-free microarray method, giving high throughput identification of protein–protein interactions. In addition, it is possible to automate the cell-free expression process, allowing rapid generation of functional proteins from the whole genome. There is no doubt that cell-free protein synthesis and the technologies derived from it will become essential tools for bridging the gap between genomics and proteomics in the post-genome era.

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