Strategies for Immobilization of Biomolecules in a Microarray

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Abstract: Recent advances in the generation of peptide and protein microarrays are reviewed, with special focuses on different strategies available for site-specific immobilization of proteins and peptides.

Keywords: Proteomics, Microarray, Proteins, Peptides, Site-Specific Immobilization, Native Chemical Ligation, Intein.

1. INTRODUCTION

In the post-genomic era, the primary aim for researchers around the world is to fully characterize and understand all proteins encoded by the genome, or the so-called "proteome" [1]. Over the past few years, a variety of proteomic techniques have been developed, allowing many thousands of proteins to be studied based on either their relative abundance [1a], or their enzymatic activities [1b]. Most of these technologies, however, are based on the traditional protein separation technique, the 2-dimensional gel electrophoresis (2-D GE), which requires downstream instrumentations such as mass spectrometry in order to identify the proteins of interest individually. They are therefore time-consuming and not easily automatable. Newer technologies, especially those based on microarray platforms, have the potential to rapidly profile the entire proteome, thus are capable of revealing novel protein functions and mapping out comprehensive protein interaction networks of an organism [1c]. The miniaturization of high-throughput screening on a single microscope-sized glass slide has the undeniable advantage of needing only minute quantities of expensive reagents for most biological assays. Nevertheless, the challenges when dealing with proteins are numerous and complex, requiring intricate manipulation and care to ensure preservation of features such as spot uniformity, stable immobilization and preservation of desired protein activity in a microarray [1c].

A thorough literature search on PubMed reveals thousands of publications related to microarrays of biomolecules, most of which deal with DNA microarray and are therefore beyond the subject of this review. Herein, we focus on microarray technologies based on proteins and peptides that address the very important issue of immobilization of biomolecules onto a glass surface while maintaining their native properties. This is an area where researchers focus on generating different chemical surfaces on a plain glass, allowing efficient protein/peptide immobilization using appropriately chosen functional groups present on these biomolecules. For most biological assays to be successfully carried out in a microarray, it is crucial that immobilized proteins and peptides are oriented on the glass surface in an active state and with a high density. Traditional surfaces used for protein/peptide immobilization in a standard biochemical assay, including polystyrene, polyvinylidene fluoride (PVDF), agarose thin film and nitrocellulose membranes, could not be adopted easily in a microarray format, primarily because these surfaces use noncovalent forces (e.g. hydrophobic interactions) for immobilization, resulting in the generation of low-density arrays of biomolecules which are randomly oriented on the surface. As a consequence, these surfaces often give rise to relatively low signal-to-noise ratios in downstream protein/peptide screening assays. Glass slides, however, have the ideal surface for microarray applications because they are inexpensive and with low intrinsic fluorescence, at the same time also possessing a relatively homogeneous chemical surface, which, when used with appropriate bioconjugate chemistry, are capable of immobilizing biomolecules at very high densities. This directly translates into highly sensitive detection of proteins/peptides in most microarray assays.

2. HISTORY OF PEPTIDE/PROTEIN MICROARRAYS

Before we review some of the techniques currently used for efficient immobilization of biomolecules on a glass slide, it is necessary to introduce the history of microarray, so as to appreciate the significant role a carefully-chosen immobilization technique may play in the successful generation of a functional protein/peptide array.

The very first report on microarray technologies was credited to Fodor and coworkers in 1991 [2]. By exploring and adopting various attributes in solid-phase chemistry, photolabile protecting groups and photolithiography (a technique used routinely in the semiconductor industry for the fabrication of computer chips), the authors developed, for the first time, a new strategy for simultaneous generation of thousands of peptides on a small glass slide. By using a photolabile group NVOC as the N-terminal amino acid protecting group and the glass surface as the solid support, the authors were able to use solid-phase peptide synthesis to chemically synthesize thousands of peptides on the glass slide simultaneously. Each coupling cycle of the peptide synthesis was precisely controlled by a set of photomasks with predefined configurations, allowing for selective deprotection of the N-terminal amino group of the growing peptide chain, and thus leading to selective coupling of different amino acids onto different peptides. For the first time, this strategy demonstrated the feasibility of generating large numbers of μm-size spots of molecules within a small

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dimension, leading to potential miniaturization and high-throughput screenings of biological assays. Of course, the same technology was subsequently adopted by Affymetrix to generate the commercially available GeneChip™, the company version of DNA microarray [3].

Over the years, a host of new techniques have been refined and optimized for the generation and screening of peptide libraries including SPOT™ technology [4], one-bead-one-compound peptide libraries [5], and positional-scanning combinatorial libraries [6], etc. Few, however, have been applied to the microarray technology [7]. MacBeath and Schreiber were the first [8], in 1999, to generate a high-density microarray of proteins and peptides using an automatic robotic spotter, an instrument routinely used to fabricate DNA microarrays [9]. By taking advantage of μm-size features a spotter can generate on a standard microscopic slide, the authors cleverly and successfully immobilized thousands of well-defined protein and peptide spots in a 3" x 1" area, although only a handful of different proteins and peptides were used in their proof-of-concept experiment. Nevertheless, they further demonstrated that the strategy is well suited for simultaneous screenings of many biochemical assays in a miniaturized format. Their seminal work, although conceptually simple and primitive by today's standard, has inspired the rapid development of many other types of related technologies in the subsequent years [10-28]. Haab and others developed antibody arrays in 2001 and the following years [10-13]. Zhu and Snyder were the first to successfully spot 6000 yeast proteins onto a single glass slide to generate the so-called "proteome array" [14]. Their work was made possible by introducing site-specific immobilization of their proteins, which are all $(his)_6$-tagged, onto a glass slide functionalized with Ni-NTA. The first carbohydrate array was introduced by Wang *et al.* in 2002 [15]. Tissue arrays were first conceived in 1999 and subsequently developed extensively by various groups [16, 17]. Sabatini *et al.* first introduced the concept of cell array

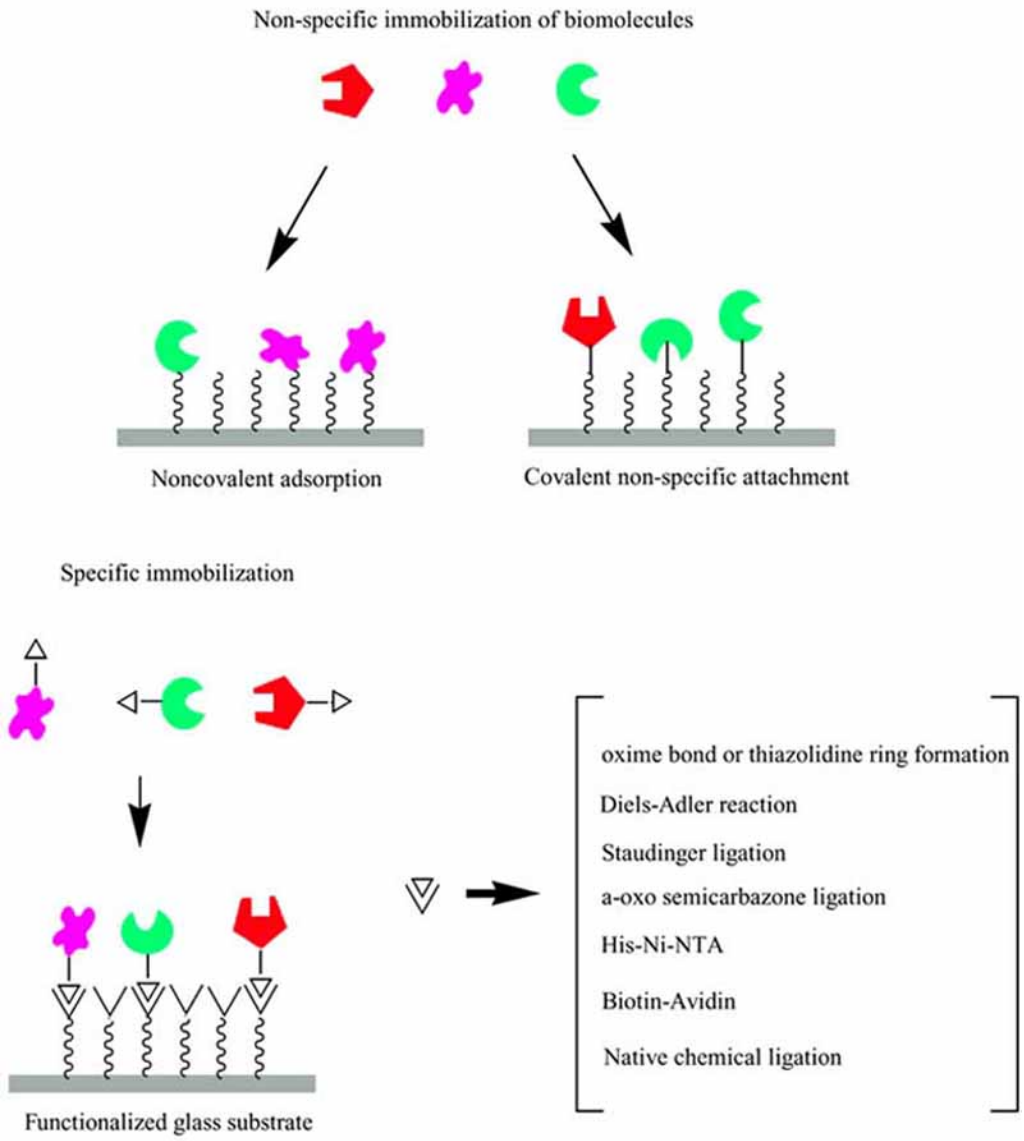

Fig. (1). Comparison of different strategies for immobilization of biomolecules. Non-specific immobilization results in biomolecules being orientated in a random fashion, while specific immobilization uniform orients the molecules, leading to full retention of their native biological activities criteria for downstream biochemical assays.

by cleverly combining the power of DNA and protein microarrays [18]. Schreiber and coworkers developed a number of platforms based on small molecule microarrays [19]. Lahiri *et al.* first successfully generated a protein array made of membrane proteins [20]. Since first conceived by MacBeath and Schreiber [8], a number of other research groups have further refined the concept of peptide microarray by introducing a variety of immobilization and screening methods [7, 21-23]. We and others recently introduced small molecule-based microarrays capable of high-throughput screening of enzymatic activities [24, 25]. We also developed a complementary approach to detect enzymatic activities in a protein array [26]. This strategy utilized mechanism-based inhibitors that are normally used for activity-based profiling of enzymes in a SDS-PAGE experiment [27]. It is advised that interested readers should be referred to a wealth of review articles which cover different areas of microarray technologies [28]. For the scope of this review, we will only review in details various immobilization strategies used for the generation of protein and peptide microarrays.

3. IMMOBILIZATION IN MICROARRAYS

The surface of the glass slide is usually derivatized with chemicals to generate different types of molecular layers. Immobilization of proteins/peptides is then subsequently carried out either by covalent linkage or non-covalent adsorption. Proteins in general, being polymers of amino acids and possessing immense chemical, physical and structural diversity, present additional problems when immobilized in a microarray. Despite these technical hurdles, several research groups including our own have successfully developed functional protein microarrays by using a variety of immobilization strategies (Fig. (**1**)). They will be presented in the following paragraphs.

3.1 Non-Specific Immobilization

This section summarizes different approaches, covalent and non-covalent, used to immobilize biomolecules onto a glass slide in a random fashion.

3.1.1 The Schreiber Approach

The first-ever protein (and peptide) microarray generated by MacBeath and Schreiber successfully and stably immobilized proteins (and peptides) using simple, nonspecific immobilization methods based on amine chemistry [8]. The authors covalently tethered proteins to aldehyde-derivatized glass surfaces through their lysine residues and α-amines at the N-terminus. By spotting their protein samples in a buffer containing PBS and glycerol, the authors were able to demonstrate, for the first time, that the microarray may be used to study protein-protein and protein-ligand interactions, as well as enzyme-substrate reactions. In order to minimize nonspecific protein absorptions to the surface and to maintain optimal spatial orientation, the authors used a molecular layer of BSA between the glass surface and the immobilized molecules (e.g. peptides). This was done by derivatization of the BSA layer with N-hydroxysuccimide (NHS). Upon spotting, nucleophilic groups in the peptide (-NH2, -SH, -OH, etc.) underwent covalent reactions with the reactive NHS group on the BSA surface, leading to subsequent immobilization. The biggest shortcoming of the strategy, however, was the non site-specific nature of the peptide immobilization on the glass surface. This disturbing issue may escalate if one were to generate a high-density microarray of peptides/proteins and, at the same time, retain their biological activities.

3.1.2 Antibody Arrays

Antibody arrays are used for high-throughput screening of antibody-antigen interactions, and they are generated by spotting many different antibodies, both monoclonal and polyclonal (and typically commercially available), onto glass slides using the Schreiber approach. In the first antibody-based protein array, Haab *et al.* used poly-L-lysine-coated glass slides to noncovalently capture more than 100 different monoclonal antibodies obtained by standard hybridoma technologies [10]. Other types of glass slides, including those coated with nitrocellulose membranes and polyacrylamide, have also been used to immobilize antibodies by noncovalent absorptions [11-13]. The effect of site-specific immobilization in an antibody array has not yet been studied, primarily because it was thought that the biggest problem facing the technology was not the immobilization, but rather the generation of a large collection of highly specific antibodies targeting different antigens. In spite of the many challenges remained in obtaining specific antibodies, many groups have successfully developed and employed antibody-based arrays for diagnostics of biomarkers, cancer, allergens, etc. [10-13].

3.1.3 Carbohydrate Arrays

By taking advantage of the strong noncovalent interaction between carbohydrates and nitrocellulose, Wang *et al.* directly spotted and immobilized about 50 different glycans from natural biological samples (including polysaccharides, glycosaminoglycans, glycoproteins and semi-synthetic glycoconjugates) onto a nitrocellulose-coated glass surface without any further treatments [15]. The resulting carbohydrate microarray was used to study biologically interesting protein-carbohydrate interactions. Although convenient and extremely simple to perform, this type of immobilization strategy may not be of general application to the immobilization of a variety of carbohydrates, as it was noted by the authors that the efficiency of immobilization differs greatly with the molecular mass of the carbohydrate: molecules with low-molecular weights were typically immobilized less efficiently. Nevertheless, the potential of this technology as a useful diagnostic tool was illustrated by the successful detection of a human serum from an individual infected with a pathogenic strain of *E. coli.*

3.1.4 Membrane Protein Array

Lahiri *et al.* created a membrane-like layer on a plain glass slide using a number of supported lipids anchored onto the glass surface modified with gamma-aminopropylsilane (GAPS). They subsequently spotted membrane proteins on top of these lipids to generate a “membrane protein array” [20]. Membrane proteins are extremely difficult to work with, requiring a membrane-like environment for retention of protein activity. Once again, the authors used simple noncovalent, hydrophobic interaction between the lipid and the membrane protein as their strategy of immobilization.

The generality of the strategy remains to be validated with other types of membrane proteins. However, the potential of this membrane protein array cannot be overlooked, as many membrane proteins are key drug targets in the pharmaceutical industry.

One of the most crucial disadvantages of non-specific immobilization is insufficient exposure of functional domains, largely due to a variety of unpredictable orientations the immobilized peptides/proteins can adopt upon binding to the glass surface. This often results in binding of an unnecessary fraction of biomolecules with improper orientation, thus impeding their binding with ligands and subsequent downstream biological assays. Another possible drawback is that noncovalent binding by hydrophobic interaction may cause protein denaturation and the loss of its functional activity. The molecules immobilized on the array may also be vulnerable to further manipulation, which may result in the graduate depletion of proteins adsorbed noncovalently.

3.2 Specific Immobilization

In order to ensure that all biomolecules are functionally active, it is imperative that they are aligned uniformly and optimally upon immobilization onto the glass surface. A variety of immobilization techniques have therefore been developed in the past few years, which allow site-specific immobilization of different molecules, including proteins, peptides and carbohydrates.

3.2.1 Oxime/thiazolidine Formation

In order to minimize the so called "orientation and effective interaction" problem faced in Schreiber's original peptide array, Falsey *et al.* developed a site-specific, covalent strategy to regiospecifically immobilize peptides on the glass slide at their N-termini [21]. The peptides were immobilized using the glyoxyl-derivatized glass slides via the formation of an oxime bond or a thiazolidine ring. Commercially available slides were derivatized with APETS to form amino slides which were converted to glyoxyl-derivatized glass slides via either (1) coupling of Fmoc-Ser followed by deprotection and oxidation, or (2) coupling with protected glycolic acid and followed by deprotection with acid treatments. Although this is the first report on site-specific immobilization of peptides using chemoselective ligation reactions, the relatively unstable oxime bond, and the unfavorably restricted orientation of the thiazolidine ring used in the strategy make it less amendable for large-scale production of many different peptides in a microarray.

3.2.2 Diels-Alder Reaction

Another method using chemoselective peptide immobilization was developed by first conjugating the peptide of interest to a cyclopentadiene linker (a diene) during the solid-phase peptide synthesis [22]. This peptide-cyclopentadiene conjugate was then spotted onto a glass surface pre-coated with a self-assembled monolayer (SAM) of alkanethiols containing benzoquinone groups (dienophiles). The Diels-Alder reaction between the diene on the peptide and the dienophile on the glass surface resulted in the rapid and site-specific immobilization of the peptides to generate the corresponding peptide microarray. This method has several advantages over existing methods as the self-assembled monolayers (SAM) are inert toward nonspecific absorption by proteins, eliminating the need of slide blocking procedures, and at the same time providing a regular, homogeneous environment for immobilized peptide ligands which make them well suitable quantitative assays. However, the requirement to generate peptides conjugated to an unnatural cyclopentadiene moiety is synthetically challenging and not easily accessible.

The same approach was subsequently employed to immobilize an array of chemically synthesized carbohydrates onto a hydroquinone-modified glass surface via covalent linkage [29], generating a site-specifically immobilized carbohydrate array. The carbohydrates were first synthesized by standard carbohydrate chemistry with appropriate modifications to allow the inclusion of a cyclopentadiene moiety. A hydroquinone-derivatized glass surface was then used to generate the carbohydrate array. Upon oxidation of the hydroquinone group to benzoquinone, which is a good dienophile, the carbohydrates were quantitatively immobilized onto the glass slide via a Diels-Alder reaction between the diene (e.g. cyclopentadiene) group on the carbohydrate and the benzoquinone on the glass. The density of immobilized carbohydrates could be precisely controlled by varying the concentration of the hydroquinone on the glass surface. In addition, since the linkage between the carbohydrates and glass surface occurs exclusively at the site of the Diels-Alder reaction, homogeneously oriented carbohydrate molecules could be generated on the surface, which should allow maximum binding between the carbohydrates and their interacting proteins to occur. Significantly, addition of a layer of ethylene glycol between the carbohydrate and the glass surface appeared to minimize the nonspecific absorption of interacting proteins to the glass slide, a problem often encountered in microarray experiments.

3.2.3 Staudinger Ligation

One of the most recent reports of site-specific immobilization of proteins/peptides onto a microarray involves a traceless version of the Staudinger ligation [30]. An azido group incorporated into either a side chain or the main chain in a protein (or peptide) can react with a phosphinothioester-derivatized surface to form an amide bond. This reaction is one of the most rapid and high-yielding coupling reaction at room temperature in aqueous or wet organic solvents. As a proof-of-concept experiment, a model protein ribonuclease S' (RNase S') consisting of 2 tightly associated fragments, S-peptide (containing azido group) and S-protein (binds tightly to S-peptide), were chosen for immobilization and activity assays. Presumably, this site-specific immobilization renders uniform orientation of the active sites towards the substrate, resulting in extremely high activities of up to 92% for the model protein. Immobilization was also extremely rapid with a $t_{1/2}$ < 1 min. As synthesis of azido-peptides [31] and azido-proteins [32] by semi-synthetic or even biosynthetic means is readily available, this new strategy appears to be promising.

3.2.4 α-oxo Semicarbazone Ligation

Oliver *et al.* delineated the preparation and characterization of semicarbazide glass slides for the fabrication of peptide and oligonucleotide arrays using the

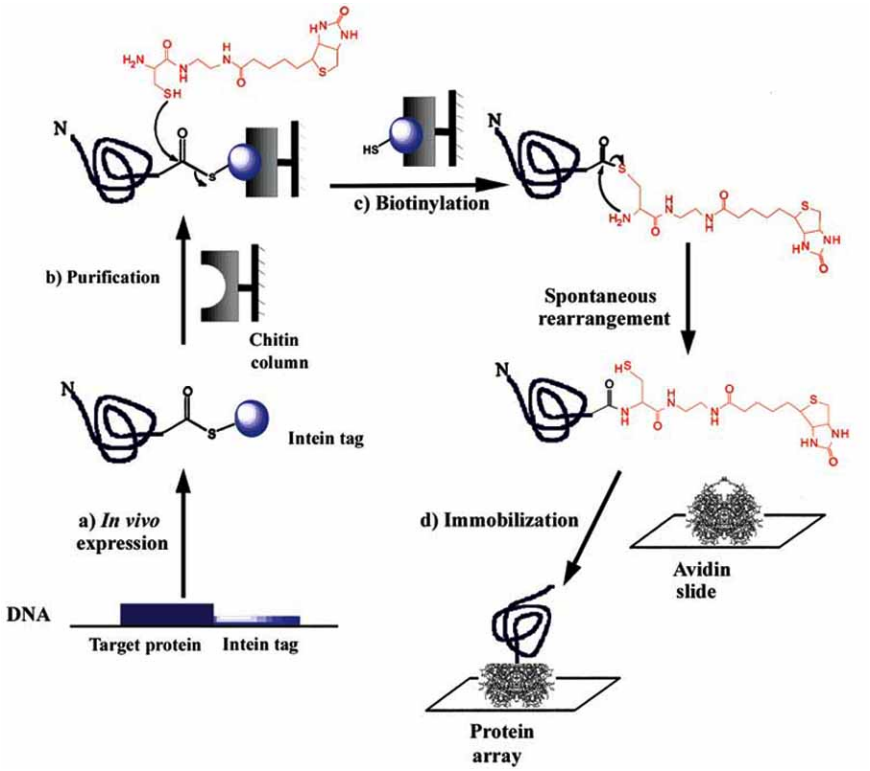

Fig. (2). Overview of the biotinylation strategy of proteins using intein-fusion and immobilization onto avidin glass slides [35a].

site-specific α-oxo semicarbazide ligation. The glass slides are silanized using either a multi-step procedure or a direct silanization with semicarbazide silane [33]. The functional density and homogeneity of the semicarbazide glass slides were optimized by analyzing the reactivity of the layer toward a synthetic glyoxylyl fluorescent probe. In summary, they presented a glyoxylyl peptide labeled with biotin and probed its immobilization on semicarbazide slides using streptavidin or an anti-biotin antibody. The hydrolytic stability of the α-oxo semicarbazone bond was also examined and found to be relatively stable.

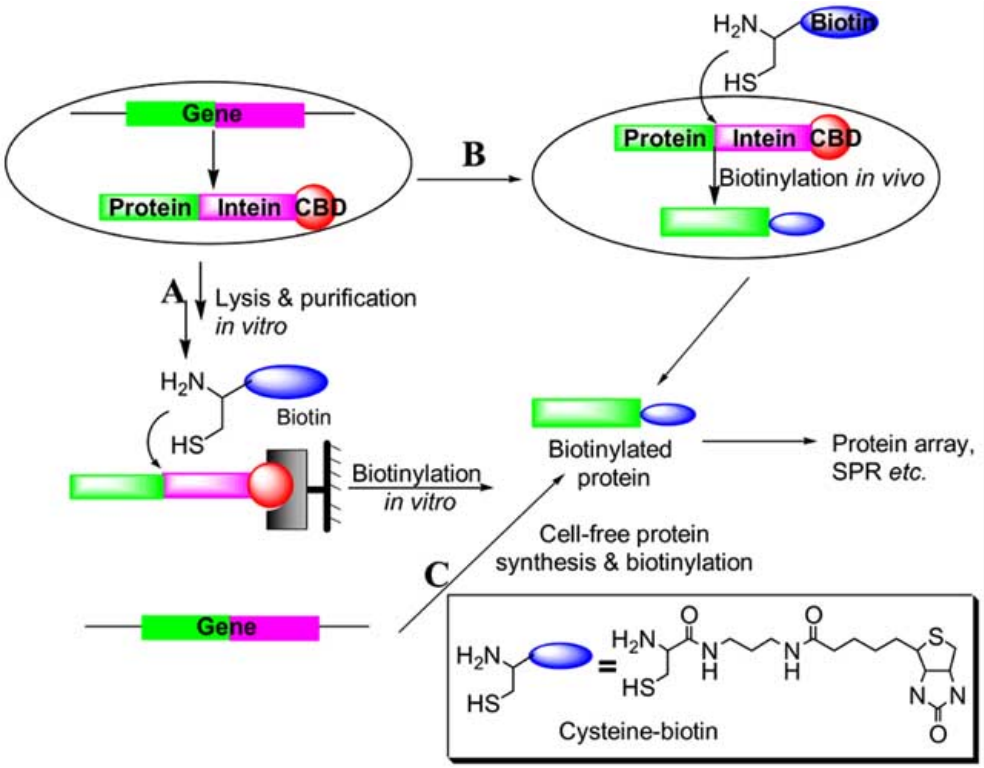

Fig. (3). Three intein-mediated protein biotinylation strategies: (A) *in vitro* biotinylation of column-bound proteins; (B) *in vivo* biotinylation in live cells; (C) cell-free biotinylation of proteins [35b].

3.2.5 Nitrilotriacetic Acid (NTA) Arrays

Recombinant proteins produced by genetic engineering have become an extremely important tool for biologists and biochemists alike. Proteins that are genetically modified to have specific affinity tags on the N- or C-terminus are easily generated in a standard biochemistry laboratory. One such affinity tag is polyhistidine ($(His)_6$-tag), which has a high affinity for the ligand NTA (i.e. Nitrilotriacetic acid) in the presence of nickel (Ni^{2+}). By taking advantage of this specific noncovalent interaction, Zhu and Snyder successfully arrayed about 6000 yeast open reading frames, producing for the first time the proteome microarray [14]. These gluthathione-S-transferase-polyhistidine (GST-$(His)_6$)-tagged proteins were immobilized either on aldehyde-functionalized slides, or Ni-NTA-coated slides, with the

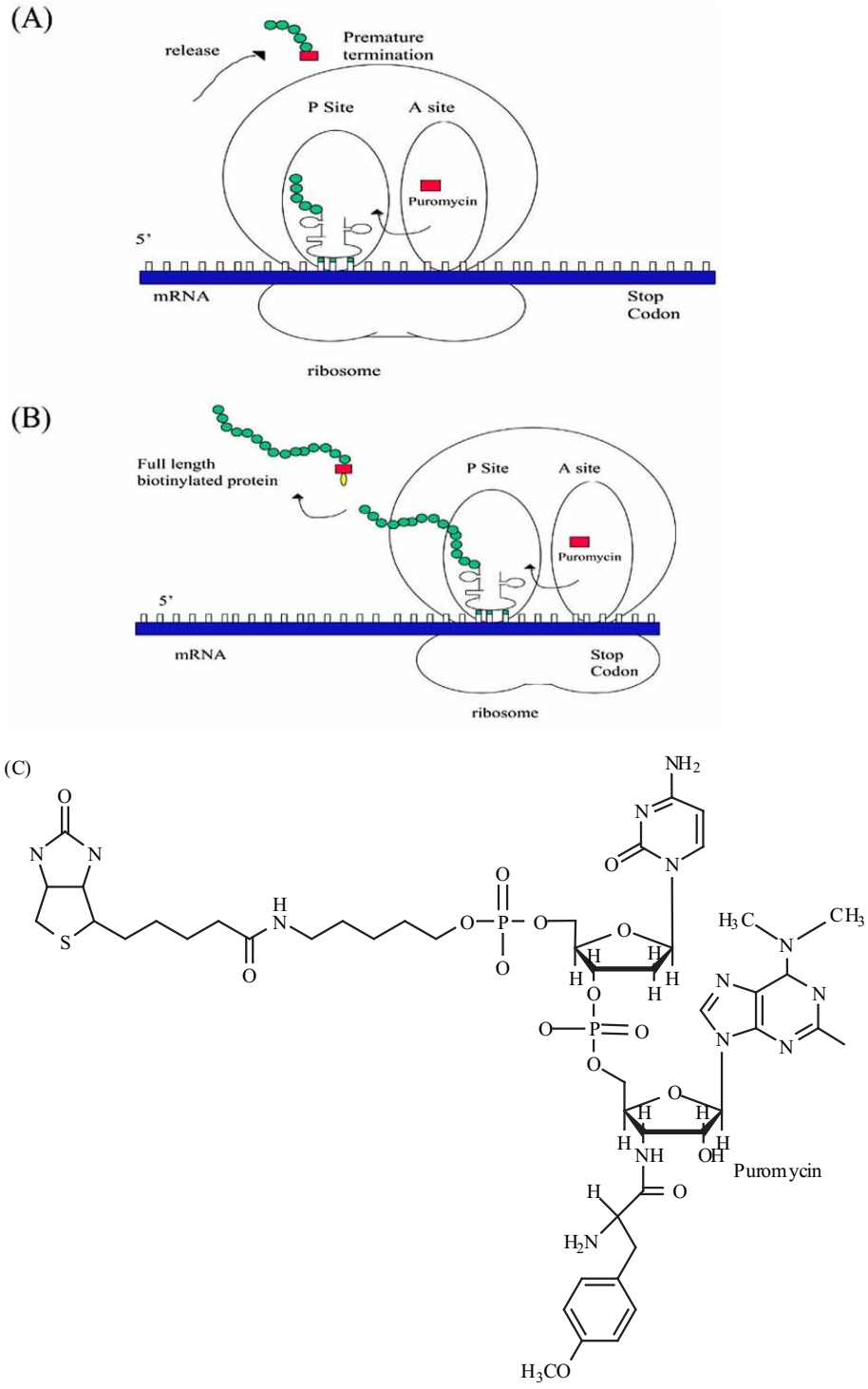

Fig. (4). Puromycin-assisted protein biotinylation [35c]. (A) At a high concentration, puromycin binds non-specifically to nascent protein, bringing about premature termination; (B) At a low concentration, puromycin binds to full length protein at the stop codon; (C) Structure of the 5'-biotin – dC – Puromycin used for protein biotinylation.

latter giving much better immobilization efficiency and conferring the further advantage of site-specific immobilization. The array was used to screen for phospholipid-interacting proteins, as well as calmodulin-binding proteins. The ability to detect post-translational modifications of proteins and screening for novel protein-ligand interactions was also established on the proteome array. This study demonstrated that, site-specific, well oriented immobilization strategies make it possible to generate high-density protein arrays on which the majority of the proteins may retain their full biological activities. Unfortunately, the binding between Ni-NTA and $(His)_6$-tag proteins is not very stable, often susceptible to interference by many commonly used chemicals and salts [34], making this immobilization method incompatible with a number of protein screening assays.

3.2.6 Biotin-avidin Arrays

To minimize the overwhelming shortcomings such as unstable peptide/protein attachment and "orientation and effective interaction" problem in peptide/protein microarrays, we first developed a new approach for site-specific immobilization of peptides on a glass plate using avidin-biotin interaction [23]. This strategy was recently extended to site-specific immobilization of proteins using recombinant protein engineering [35]. The crux of the new strategies relies on the well-known biotin-avidin interaction and the chemistry used in native chemical ligation. In our peptide microarray, N-terminally biotinylated peptides were conveniently synthesized by standard solid-phase peptide synthesis. The resulting peptides were then immobilized onto an avidin-coated glass slide using a conventional microarray spotter [23]. The approach exploits one of the strongest known non-covalent interactions, that of biotin and avidin [36], ($K_d = 10^{-15}$ M) to generate a peptide microarray on which all peptides are stably oriented in a uniform direction. The biotin-avidin interaction has been widely used in standard biochemical assays for labeling and immobilization purposes. Oriented monolayers using streptavidin have also been used in biosensors [37]. Avidin is an extremely stable protein, making it an excellent candidate for slide derivatization and immobilization. Each avidin/streptavidin molecule can bind rapidly and almost irreversibly up to four molecules of biotin, thus doing away with the long incubation time which alternative methods typically need for the critical immobilization step. Avidin also acts as a molecular layer that minimizes non-specific binding of proteins to the slide surface, thereby eliminating blocking procedures and minimizing background signals in downstream screenings.

Using a similar strategy, we spotted biotinylated proteins onto an avidin-coated slide to generate a protein microarray [35]. In order for the strategy to work, we developed a method to purify and site-specifically biotinylate recombinant proteins at their C-terminal end using an intein-mediated expression system (Fig. (**2**) and Figure (**3**), method A). Within a single column purification step, the expressed C-terminal fusion protein was purified and biotinylated in a single step on chitin beads [35a]. With this approach, we were able to generate site-specifically biotinylated proteins with high efficiency, which were then subsequently immobilized in a site-specific manner onto an avidin-functionalized glass slides (Fig. (**2**)). This highly robust novel protein array features uniformly oriented proteins, which ensures all immobilized proteins to retain their full biological activities. The use of biotin-avidin interaction for immobilization also allows the proteins to withstand even the most harsh conditions used for downstream screenings, thus making the protein array compatible with most biochemical assays. The use of intein fusion for cell-free and *in vivo* biotinylation of proteins had also been investigated (Fig. (**3**), methods B and C, respectively) [35b]. A cell-free system has many advantages over traditional recombinant methods used for protein expression. It eliminates problems of protein toxicity and inclusion bodies, which are typically encountered when live host cells are used. This is especially true when one attempts to express eukaryotic proteins in prokaryotic hosts. Other problems include potential proteolytic degradation of the protein by endogenous proteases, as well as expression of proteins toxic to the host cell. Cell-free protein synthesis provides an attractive alternative for protein expression which may potentially overcome many of these problems, and is well-suited for protein microarray applications because (1) minute quantities of proteins generated in cell-free system are sufficient for spotting in a protein array, and (2) the method could be easily adopted in 96- and 384-well formats with a conventional PCR machine for potential high-throughput protein synthesis. For *in vivo* biotinylation (Fig. (**3**), method C), a substantial level of the target protein was successfully biotinylated using the intein-mediated expression system. We further demonstrated that *in vivo* biotinylated proteins present in a crude cell lysate may be used directly for protein microarray applications [35c]. Overall, although the *in vivo* biotinylation of proteins using intein tag is less efficient as compared to the *in vitro* system, it nevertheless provides an alternative method for researchers to generate *in vivo*, without further processing (e.g. purification and elution, etc), a large array of biotinylated, ready-to-spot proteins in a truly high-throughput, high-content fashion.

We also developed a method for PCR-based, *in vitro* biotinylation of proteins using puromycin (Fig. (**4**)), which was subsequently used for site-specific immobilization of proteins on an avidin-coated glass slide [35c]. Puromycin is a naturally produced antibiotic, previously found to cause premature termination in protein synthesis in both and prokaryotes and eukaryotes [38]. As puromycin resembles the 3' end of the aminoacyl-tRNA, it competes with the ribosomal protein synthesis by blocking the action of the peptidyl transferase at high concentrations (Fig. (**4A**)). Interestingly, puromycin acts as a non-inhibitor of ribosomal protein synthesis when present in low concentrations, and gets incorporated at the C-terminus of the newly synthesized protein (Fig. (**4B**)) [39]. We have thus successfully exploited this phenomenon by extending it to the site-specific biotinylation of proteins [35c]. By use of a suitable amount of a puromycin-conjugated biotin (Fig. (**4C**)) in a cell-free protein expression reaction, the molecule can be efficiently incorporated to the carboxyl-terminal end of a mature protein. There are potentially numerous advantages of using proteins generated by cell-free systems over conventional *in vivo*-based recombinant expression, as mentioned above.

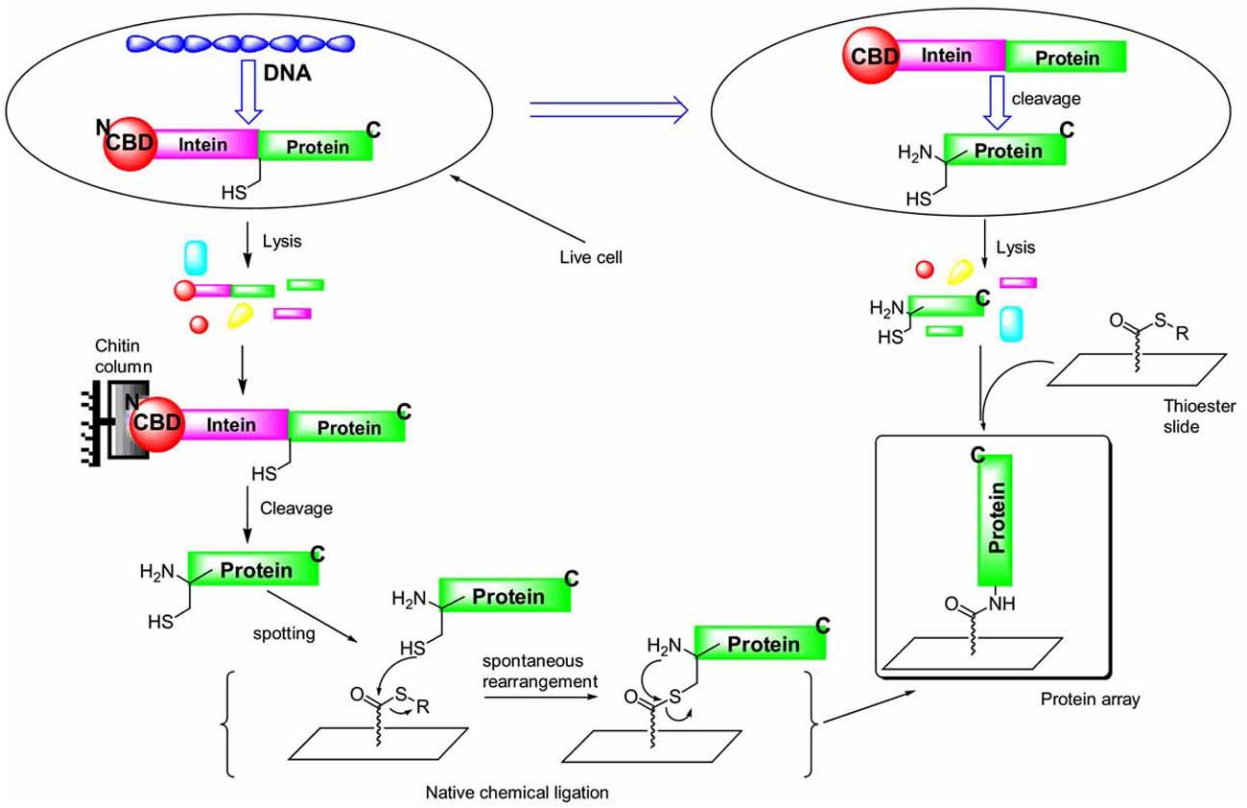

Fig. (5). Site-specific immobilization of N-terminal Cys proteins using thioester-derivatized glass slides [35c]. The N-terminal Cys proteins were expressed using intein-fused proteins.

This puromycin-based strategy should complement well with our intein-mediated methods.

3.2.7 Native Chemical Ligation

The chemoselective chemistry of native chemical ligation reaction is a very well-known one [40]. By employing glass slides that were modified to present thioester moieties, N-terminal cysteine-containing peptides [23] and proteins [41] were successfully immobilized to generate the corresponding peptide and protein microarrays [35c & 35d] (Fig. (**5**)). The N-terminal cysteine residue of a peptide/protein reacts chemoselectively with the thioester on the slide through the formation of a thioester intermediate followed by an N-C acyl shift to form a native peptide bond. The presence of other reactive amino acid side chains, including internal cysteines, is tolerated with this reaction [40]. This method is advantageous as peptides containing an N-terminal cysteine may be readily synthesized using established solid-phase peptide synthesis. As a proof-of-concept experiment, N-terminal-cysteine-containing kinase peptide substrates were immobilized onto a thioester-functionalized slide, and their activity was probed with the corresponding kinases, followed by successful detection with FITC-labeled anti-phosphotyrosine and antiphosphoserine [7, 23].

We recently extended the intein-mediated expression system (as mentioned in previous paragraphs) to the recombinant expression of proteins containing an N-terminal cysteine, which were subsequently immobilized onto thioester-functionalized glass slides to generate the corresponding protein microarray (Fig. (**5**)) [35c]. Once again, site-specific immobilization of proteins allowed the full retention of their biological activities [35c]. We also showed the strategy is extremely versatile, applicable to the immobilization of N-terminal cysteine proteins which are either purified prior to spotting, or present in crude cell lysates (e.g. unpurified).

4. CONCLUSION

In order to fully realize the tremendous potential of protein and peptide microarrays, there is an urgent need to develop strategies that allow site-specific and stable immobilization of proteins, peptides and other biomolecules onto a glass surface such that their native biological activities are fully retained. The past few years have witnessed an increasing number of reports that address this critical issue. A variety of chemical methods have been developed for the efficient immobilization of different molecules. However, the search for newer methodologies for improved immobilization of proteins in a microarray on a glass slides or any other platform will continue. Only when combining such immobilization strategies with improved arraying devices, labeling reagents, detection devices and data analysis tool, can the potential of microarrays to perform high-throughput protein studies be demonstrated to their fullest.

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