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DNA-directed assembly of protein microarrays

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1. ABSTRACT

Microarray technology has made it possible to simultaneously study the abundance, interactions, and functions of potentially tens of thousands of biological molecules. From its earliest use in DNA microarrays, where only nucleic acids were captured and detected on the arrays, applications of microarrays now extend to those involving biomolecules such as antibodies, proteins, peptides, and carbohydrates. In contrast to the relative robustness of DNA microarrays, the use of such chemically diverse biomolecules on microarray formats presents many challenges in their fabrication as well as application. Among the many methods that have been proposed to overcome these challenges, DNA-directed assembly (DDA) has emerged as a promising strategy for the high sensitivity and multiplexed capture and detection of various analytes. In this review, we explore the challenges faced during the design, fabrication, and utilization of protein microarrays and highlight how DDA strategies, together with other recent advances in the field, are accelerating the development of platforms available for protein microarray applications.

2. INTRODUCTION

The latest compilation of human gene annotations puts the number of protein-coding genes in the human genome at greater than 22, 000 (1). Having this relatively low number of genes responsible for the bewildering complexity of the human body requires the additional regulation of biological functions at the transcription, post-transcription, and post-translational levels (2). It has been estimated that the human proteome consists of several hundred thousand, possibly up to a million, different protein species generated by alternative splicing and post-translational events (3-5) with their concentrations covering a dynamic range as large as 10^6 (6, 7). In view of the complex and diverse nature of protein species, enabling technologies such as miniaturization, parallelization, and automation are being exploited to produce high throughput assays for proteomic analysis. These assays include tools for the global analyses of relative changes in protein concentrations, formation of protein complexes, interactions of proteins with other protein and non-protein biomolecules (e.g. carbohydrates, DNA), and the dynamic tracking of the resulting interaction networks that are functions of both time and cell state (8).

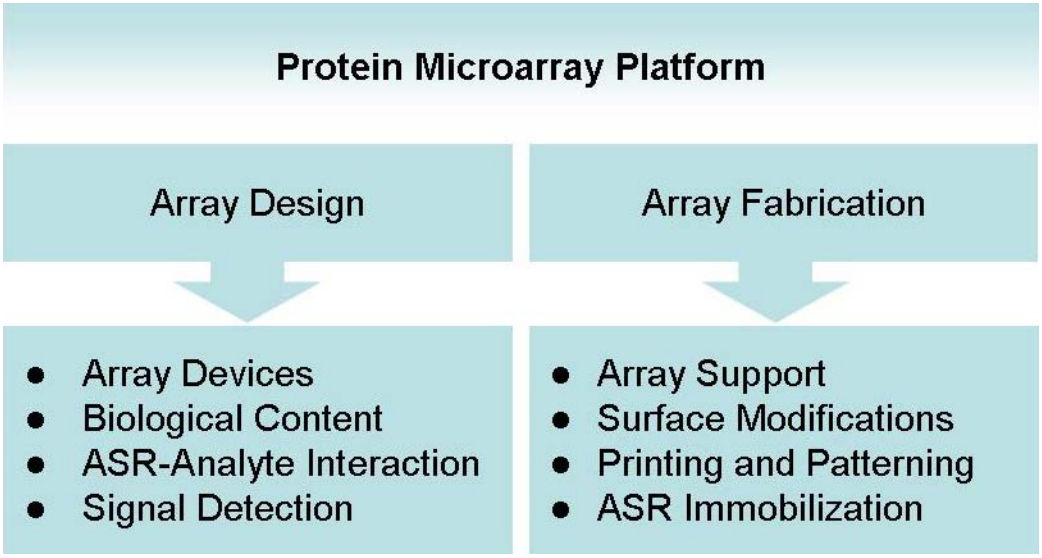

Figure 1. Overview of key considerations in the design and fabrication of protein microarray platforms.

The last decade has seen remarkable advancements, both conceptual and technical, in the development of DNA microarrays for high-throughput, large scale genomic analyses (9). Analogous to DNA microarrays, protein arrays are emerging as important tools for high throughput and multiplex protein profiling (10-17). Although protein microarrays, like DNA microarrays before them, are conceptually similar to macro-scale dot-blot techniques where DNA or protein molecules are directly immobilized onto membranes and probed (9), miniaturization of these assays results in a much lower consumption of reagents and samples while also increasing the sensitivity of detection. The rationale underlying the improved performance of microarrays over their macro-scaled counterparts has been theoretically examined and the reader is encouraged to explore the relevant literature (18-23) for a more detailed treatment of this subject.

Protein array platforms can take on any one of many formats that have been developed to date. Regardless of the array format used, there are several key considerations in the development and application of protein microarrays. These considerations can be broadly grouped into two inter-related aspects of protein array technology (Figure 1): the first aspect of array design encompasses the conceptualization of the overall experimental strategy while the second aspect of array fabrication deals with the technical issues involved in optimizing array conditions for proper execution of the array design. Initially, while considering the array design, it is necessary to choose a suitable device and the appropriate 'biological content' for capturing the target of interest (analyte) from samples. At this stage, the experimental strategy for capture and detection of the analyte is mapped out. The considerations of device to be used, 'content' to be arrayed, analyte to be captured, method of detection, and composition of sample mixtures will together point to how the array should be fabricated in order to allow high sensitivity analyte detection with a high signal-to-noise ratio. To achieve optimal capture and detection of the analyte while maintaining low background on the protein array platform, it is critical to select the appropriate array support, surface modifications, printing and patterning techniques, as well as 'content' immobilization methods.

Although a large body of knowledge concerning these issues is currently available, a more complete understanding of these factors affecting protein array performance is required for implementing rational approaches to the design, fabrication, and utilization of optimal protein arrays. A number of recent reviews on protein arrays have provided comprehensive discussions on probe immobilization strategies (24, 25), surface modifications (26-28), as well as various capture and detection methods (13, 29). Rather than attempt a comprehensive survey of this vast field, we will discuss each of the main factors briefly, seeking to highlight recent advances in protein array design and fabrication, while also laying out the challenges that have yet to be overcome effectively.

2.1. Advances in array design

Depending on their configuration, protein microarrays can be conceptually viewed as either analytical or functional microarrays (17). Analytical protein microarrays are useful in measuring the relative abundance of various proteins in samples collected from different subjects or under various conditions. Apart from analytical microarrays, functional protein microarrays have also been developed for the discovery and characterization of protein functions. These functions include protein-protein, protein-peptide, protein-DNA, protein-carbohydrate, and protein-small molecule interactions.

Detection of proteins or other biomolecules is usually achieved through the use of analyte-specific reagents (ASR) which can specifically capture their targets (analytes) from complex mixtures. The types of ASR

currently in use include antibodies, recombinant antibody fragments, protein scaffold, aptamers, peptides, organic small molecules, and other molecules that can serve as probes or binders of specific analytes. However, with miniaturization and the resulting increase in assay throughput and multiplexation comes the challenge of acquiring enough 'content' for screening large numbers of targets: there are simply not enough ASRs to detect such a large number and variety of analytes. Fortunately, this problem has since been recognized and the recent establishment of initiatives such as ProteomeBinders, a European consortium aiming to set up a comprehensive resource of well-characterized ASRs (30), will no doubt help to expand the current repertoire of 'content' available for future protein array applications.

There are several strategies commonly employed for capture and detection of analytes by ASRs. These can generally be classified into label-free methods and direct labeled methods (29). Label-free methods include those that make use of mass spectrometry, surface plasmon resonance, atomic force microscopy, micro-electromechanical system cantilevers, and quartz-crystal microbalance. More conventional direct labeled methods include detection through fluorescence, radioactivity, chemiluminescence, and electro-chemiluminescence. Although label-free methods are promising tools that can achieve high sensitivity detection of molecules on microarrays without the need to label proteins, they have yet to be commonly utilized mainly because of the requirement of highly sophisticated equipment that are not widely available (13). As a result, most protein arrays rely on fluorescence-based read-outs where either the analyte or ASR is labeled with fluorescent organic tags or otherwise detected using a secondary fluorescence-labeled probe (31).

Depending on the analyte capture and detection strategy utilized, arrays can be further divided into three types (Figure 2A): (i) forward-phase, (ii) reverse-phase, and (iii) sandwich arrays (29). In a forward-phase strategy, known ASRs can be immobilized and allowed to capture their unknown interaction analytes which are subsequently identified. Alternatively, in a reverse-phase strategy, various possible interaction analytes can be immobilized and probed with known ASRs. In sandwich arrays, a first immobilized ASR is used to capture its analyte which is then detected by a second labeled ASR.

Because target cellular proteins are generally present at low amounts, protein microarrays generally suffer from low signal-to-noise ratios because of low signal and high background in commonly used fluorescence methods where the signal is not amplified. Signal amplification methods like rolling circle amplification (RCA) have subsequently been developed to increase the sensitivity of assays (32). Recent developments in nanotechnology have also expanded the range of fluorescent tags available for a wider spectrum of analyses and this is likely to enable more extensive multiplexing in microarray applications (33). It is also interesting to note that surface modifications such as optical interference coating can result in significant signal enhancement (34), showing that optimization of individual processes in protein microarray fabrication can result in the overall enhancement of platform's performance.

To date, a number of devices have been successfully used for protein profiling studies. Apart from the most common planar format based on microarray glass slides, other devices that have been utilized include filtration based microarrays (35), microwells (36, 37), attovials (38), beads (39, 40), bead-microfluidics (41-43), microcantilevers (44, 45), fiber optic arrays (46), compact discs (47), nanowire sensor arrays (48), nanopore arrays (36), graphite electrode microarrays (49-51) and their variants. Except for the commercially available slide-based (e.g. Sigma's Panorama™ antibody microarray) and bead-based (e.g. Luminex's xMAP® technology) devices, most of the other devices are still under laboratory development.

To utilize the above-mentioned devices for protein microarrays, appropriate support surfaces have to be fabricated for the immobilization of biomolecules. The choice of support is dictated by several factors including, but not limited to: (i) biocompatibility; (ii) selectivity of ASR binding; (iii) ASR binding capacity; (iv) non-specific (background) binding; (v) accessibility and orientation of biomolecules; (vi) suitability of the support geometry (two-dimensional, three-dimensional, or quasi three-dimensional); and (vii) compatibility with downstream detection methods. The issues affecting array fabrication and the advances in this area are discussed in the following sub-section.

2.2. Advances in array fabrication

A critical element of any microarray platform is the surface or substrate on which capture and detection is carried out. It is necessary to have a surface that is chemically compatible with the probe immobilization strategy and yet optimize any ASR-analyte interactions that have to be carried out at the surface. This surface should ideally be resistant to non-specific binding of biomolecules so that background signal on the array is minimized during the detection step.

Many novel and diverse surfaces have been developed for both DNA and protein microarrays over the years (52, 53). However, it is evident that surfaces suitable for protein microarrays are vastly different from those required for DNA microarrays. Proteins tend to bind non-specifically to microarray surfaces because of a combination of interactions such as van der Waals forces, dipolar or hydrogen bonds, electrostatic forces, and hydrophobic effects (54), whereas polar and ionic interactions are the main concerns in fabricating DNA microarray surfaces (55). Because of this, finding appropriate array supports for DNA is relatively straight-forward as they are uniform, have stable structures, and share identical hydrophilic, negatively charged phosphate backbones. In contrast, the inherent diversity and unique physicochemical properties of proteins make it extremely challenging to choose suitable solid supports for protein arrays (11).

In DNA microarray fabrication, glass slides derivatized with organosilanes and other chemical functionalities are commonly used (52, 53). Similarly, silicon-based solid supports modified with various chemical functional groups are being used to generate optimal biocompatible surfaces suitable for ASR immobilization in protein microarrays (25). Although many different surfaces have been explored, they are far from ideal and there is certainly room for improvement (56).

Currently, methods for printing or patterning features in DNA microarrays are also being applied in protein microarrays to produce dense and uniform arrangements of features that possess good spot morphology. Many protein arrays are thus fabricated by depositing small volumes (in the pL range) of individual ASR or analytes onto solid surfaces at discrete positions in an ordered pattern. Depending on the functionalities on the solid support, immobilization of ASRs or analytes onto the substrate can be achieved through non-specific physical adsorption, covalent coupling, or interactions based on biological affinities (25-27, 57).

There are three fundamental ways of achieving efficient printing or patterning of a microarray: (i) contact printing, (ii) non-contact printing, and (iii) *in situ* synthesis (58). A number of nanolithographic methods commonly used in the semi-conductor industry have also been used to produce nano-analytical devices that can interrogate large number of samples in parallel (16, 59-64). Recently, there have been attempts to increase the content density of protein arrays through further miniaturization of these assays (16, 65-69).

Reducing the size of spots from micro to nano scale can result not only in the increase in number of spots that can fit onto an array, but also in the reduction of ASR molecules and samples required. Furthermore, it is estimated that the majority of analytes spread on a micro-scale array will never approach a relevant ASR domain within the incubation time while nano-scale arrays may overcome this problem through the reduction of array size, ensuring that every analyte molecule present samples the entire capture surface within a reasonable amount of time (67). However, there is also a concern that when spots fall below a certain size, there may not be enough biologically active or properly oriented ASR molecules in each spot to ensure accurate quantitation and a good dynamic range (67).

Although proteins are now routinely immobilized onto surfaces at high-density, it remains a challenge to retain the biological activities of protein ASRs after they are immobilized. This is because proteins tend to denature when immobilized onto two-dimensional solid supports. ASRs which require a proper orientation for analyte capture may also find their active or binding sites blocked when they are deposited onto array surfaces in a random and non-directed manner (Figure 2B). Analytes immobilized onto surfaces in reverse-phase arrays may also find their ASR-binding sites blocked.

Two approaches are commonly utilized to overcome this problem (Figure 2B). The first approach is to immobilize ASRs in a directed orientation using site-specific immobilization techniques like those using interaction of protein A, protein G, or protein L with the Fc region of antibodies (70), avidin-biotin interactions (71), and nitrilotriacetic acid (NTA)-histidine tag interactions (72) among many others. Although these strategies allow for directed orientation of ASR molecules, ASRs must be spotted onto separate sites on the array to enable capture and detection of different analytes on the same array. Also, there remains the problem of maintaining the correct conformation and activity of proteins on the array surface from the time of array fabrication through to the end of the experiment.

The other approach circumvents this problem by immobilizing proteins within porous structures formed by polymeric membranes or hydrogels. Although these three-dimensional supports show a high capacity for protein immobilization and provide homogeneous aqueous environments that can prevent protein denaturation, they suffer from mass transport effects and high background signals. As these effects can result in calculation of false kinetic rate constants during real-time protein-protein interaction measurements (21-23), attempts have been made to overcome this limitation through the use of methods such as micro-agitation to overcome mass transport effects (73-75).

A promising development in the generation of high-density bioactive microarray supports is the use of quasi-three-dimensional surfaces such as dendrimers. Glass surfaces derivatized with polyamidoamine (PAMAM) dendrimers was first reported by Benters *et al.* for fabricating high-density immobilization surfaces for nucleic acid and protein microarrays (76). This surface was modified to minimize non-specific binding by using carboxyl-terminated PAMAM (PAMAM-COOH) dendrimers (77). This resulted in negatively charged surfaces which substantially reduced non-specific binding (78). This dendrimeric carboxyl surface provided high-density immobilization of antibodies which retained high efficiency of antigen capture while maintaining low background even in the presence of a complex cell lysate mixture. PAMAM surfaces coupled with DNA-directed assembly (DDA) strategies for ASR-analyte immobilization were further shown to achieve high-sensitivity antigen detection in antibody microarray applications (77, 79, 80).

The DDA strategy makes use of the inherent robustness and unique recognition and binding properties of DNA as a spatial address tag on proteins and biomolecules. Different ASRs can be tagged with unique address sequences which will direct them to specific sites on the microarray to be immobilized either before or after ASR-analyte binding. The ability to immobilize ASR-analyte complexes after binding eliminates the problems of protein stability during storage, protein orientation, and ASR activity retention. These unique features make DDA strategies extremely promising and versatile approaches for

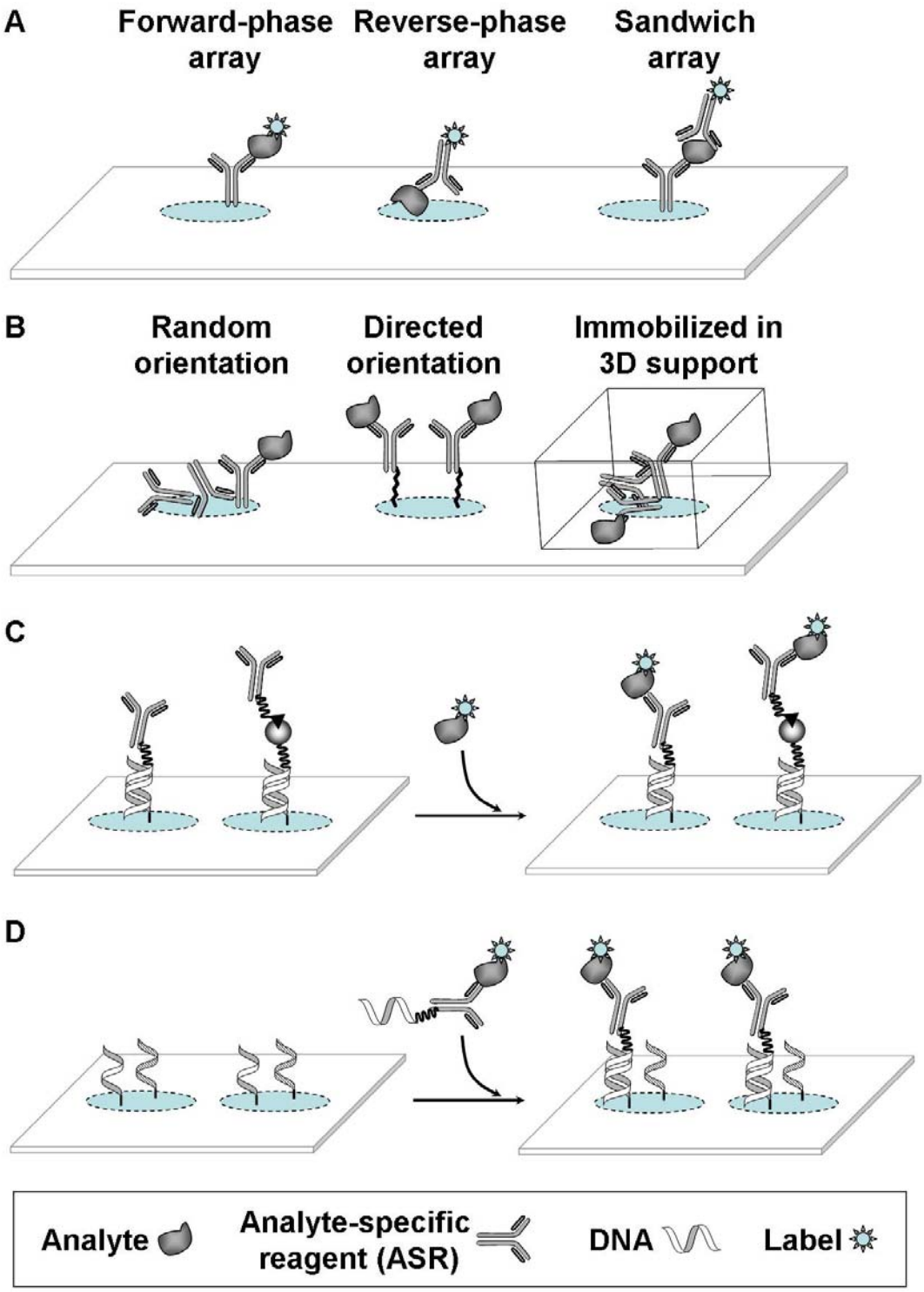

Figure 2. Schematic representation of various protein microarray formats. A, Conventional capture and detection strategies in forward-phase, reverse-phase, and sandwich protein microarrays. B, Orientation of analyte-specific reagents (ASRs) on arrays generated by random immobilization, directed immobilization, and 3D support immobilization. C, DNA-directed pre-assembly strategy. The DNA-ASR conjugate is first immobilized onto the array surface before analyte capture and detection. D, DNA-directed self-assembly strategy. ASR-analyte binding is allowed to take place in solution phase before the ASR-analyte complex is immobilized onto the array surface using the DNA address tag.

protein array platforms. The rest of this review will first focus on issues pertinent to the fabrication of protein arrays using the DDA strategy and thereafter highlight recent work applying this strategy in various protein microarray applications.

3. DNA-DIRECTED ASSEMBLY OF PROTEIN ARRAYS

It has long been recognized that the highly specific molecular recognition properties of nucleic acids make them convenient building blocks for the self-assembly of supramolecular structures (81). DDA has been used to organize a wide variety of nano- and micro-scale particles and nanowires (82, 83), quantum dots (84), and dendrimers (85). Generation of DNA-ASR conjugates for use in protein microarrays combines the unique molecular recognition properties of DNA with the wide and diverse range of functionalities of ASRs like antibodies, peptides, and other biologically relevant molecules (86).

Pioneering work in antibody immobilization using DNA was demonstrated using a streptavidin-biotin system to produce polyadenylic acid-conjugated antibodies which could be immobilized onto magnetic beads for cell sorting applications (87). This was quickly extended to the assembly of biotinylated immunoglobulin G onto macroscopic arrays (88). This DNA-directed immobilization of antibodies onto surfaces was found to be not only reversible but also more efficient than conventional immobilization techniques such as the direct binding of biotinylated proteins to streptavidin-coated surfaces (89). A detailed comparison of antibody microarrays fabricated by DNA-directed immobilization, direct spotting or streptavidin-biotin attachment showed that although all three strategies had similar detection sensitivity, DNA-directed immobilization resulted in microarrays with better spot homology and less variability while using at least 100-fold less antibody material than is required for direct spotting microarrays (79).

3.1. DNA-directed self-assembly *versus* pre-assembly

There are principally two approaches in the use of the DNA-directed strategy. The pre-assembly approach requires the ASR to be immobilized before analyte capture (Figure 2C) while the self-assembly approach allows ASR-analyte interactions to take place in a homogenous phase before the ASR-analyte complex is assembled onto DNA microarrays (Figure 2D). Since the term immobilization is commonly used to describe the placement of ASR onto arrays prior to analyte capture in protein microarray applications, we use the term assembly in DDA to include DNA-directed immobilization strategies previously described as well as incorporate various strategies that do not make use of pre-immobilized ASRs. Although the difference may appear trivial, the possibility of allowing ASR-analyte interactions to take place in an optimized and homogeneous solution phase is a key advantage of the DNA-directed self-assembly strategy.

A long-held belief is that biological interactions that take place in solution phase inside a physiological environment may not be recapitulated on an artificial solid-liquid interface. Although studies have reported that antibody-antigen capture and other biological interactions can take place on microarray surfaces, there has so far been no comprehensive study on the effect of interfacial conditions on biological interactions. Given the tendency of proteins to denature and bind onto array surfaces (90), such probe-target interactions are clearly better carried out in solution before the immobilization and detection step.

3.2. Generation of DNA-conjugated analyte-specific reagents

An important step in the fabrication of DNA-assembled protein microarrays is the attachment of single stranded DNA (ssDNA) to the ASR molecule which can be a protein, antibody, peptide, double stranded DNA (dsDNA), carbohydrate, or small molecule. As many ASRs currently in use are proteins, we focus on methods for generating DNA-protein conjugates. However, these methods can also be applied to other non-protein ASRs.

The simplest way to generate a DNA-protein chimeric molecule is to directly attach the ssDNA to the protein through covalent coupling. This can be achieved through the use of oligonucleotides containing a thiopyridyl disulfide which, when activated, will couple to proteins containing reactive cysteines or recombinant proteins that have been tagged with a terminal cysteine residue (91). While this method potentially allows for the attachment of ssDNA at specific positions on the protein, the disulfide bond is unstable under reducing conditions. An alternative method is to create a hydrazone bond between the protein and the oligonucleotide by modifying the protein with a hydrazide group and coupling that to an aldehyde group on oligonucleotides (92, 93).

Other than direct coupling, which may be limited as it requires the modification of proteins that may not contain the required functional groups, homo- or hetero-bifunctional linkers have also been extensively used to produce DNA-protein conjugates. Homobifunctional linkers like bis(sulfosuccinimidyl)suberate (BS) and heterobifunctional crosslinkers like succinimidyl 4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylate (SMCC), *N*-[γ-maleimidobutyryloxy]succinimide ester (GMBS), succinimidyl 4-hydrazinonicotinate acetone hydrazone (SANH), succinimidyl 4[p-maleimidophenyl]-butyrate (SMPB), and their derivatives like sulfo-SMCC and sulfo-SMPB have been used for DNA-protein conjugation (25, 94). These crosslinking agents take advantage of functional amino or thiol groups either on the oligonucleotide or protein to form a covalent link.

Although the use of crosslinkers increases the options available for the selection of attachment site on the protein, it is often not possible to control the precise site of attachment as proteins may have more than one amino or thiol group present on the protein exterior available for coupling. As proteins require unhindered access to active and binding sites for retention of their functions, the random attachment of the oligonucleotide to one of these sites may result in reduced functionality of proteins and

antibodies. This was demonstrated through the observation that higher DNA-linker to antibody ratios used in the conjugation reaction resulted in lower antibody-antigen capture (94). Therefore, the conjugation reactions between DNA, crosslinking agents, and various proteins have to be carefully optimized to ensure maximal retention of protein function while also controlling the number of oligonucleotides coupled to each protein molecule. In contrast to protein-DNA conjugation, the attachment of ssDNA to peptides, dsDNA, carbohydrates, and small molecules are generally more straightforward. These can be directly synthesized or coupled using the chemistry described above without affecting the function of the biological molecules.

To overcome possible reduction in protein function after ssDNA attachment, chemoselective ligation of oligonucleotides to recombinant proteins through expressed protein ligation (EPL) has been proposed (95, 96). This involves the use of oligonucleotides containing terminal cysteine residues which will be reacted with recombinant intein fusion proteins each containing a C-terminal α-thioester to form DNA-protein attachments away from the functional sites. Recent work had shown that the thiol group formed through EPL can be further labeled with maleimide-containing small molecule tags to enable site-specific probe labeling (97). A related method used site-specific labeling of recombinant human O^6-alkylguanine-DNA-alkyltransferase fusion proteins with O^6-benzylguanine-PEG-maleimide-oligonucleotides during the purification of the recombinant protein (98).

While various proteins and antibodies have been coupled directly to oligonucleotides using the methods described above, the attachment of oligonucleotides to large proteins is still a poorly developed, yet crucial step in the fabrication of nucleic acid-assembled protein microarrays. Since proteins are a highly diverse group of biomolecules with different physicochemical properties, it is unlikely that a single conjugation method or condition will be optimal for all the proteins that will be used on a multiplexed protein array.

One way to overcome the diversity of proteins in protein-DNA conjugation is to couple ssDNA to intermediate adapter molecules which remain stable and functional after conjugation. Specific proteins to be used in the protein microarrays can then be bound to various adapters with their respective ssDNA addresses through affinity-based interactions. A common adapter protein used in this way is streptavidin which takes advantage of its exceptionally high binding affinity with biotin (10^{-12} M) and high stability in adverse conditions (e.g. in urea) (88). The streptavidin moiety can also be used to bind ssDNA directly (99). Although functional ssDNA-streptavidin conjugates can generated with ease, this method still requires the biotinylation of various protein ASRs which may suffer from similar problems associated with the retention of protein functions. Other than proteins, antibodies, and enzymes, the use of streptavidin-DNA conjugates also makes it possible to attach other biological molecules like peptides, carbohydrates, and small molecules if they can be tagged with biotin, although the size of streptavidin may reduce optimal ASR-analyte interactions due to steric hindrance (100). Another recently reported strategy using adapter proteins to produce DNA-assembled and self-oriented antibody microarrays is the use of *Streptococcus* protein G-DNA conjugates for the assembly of antibodies onto surfaces (101).

Recently, the use of locked nucleic acids (LNA) and peptide nucleic acids (PNA), analogues of RNA and DNA which do not occur naturally, have been described for programmable assembly applications (102). These oligomers retain the molecular recognition characteristics of DNA and RNA, but possess greater affinity for their complementary DNA strand or with each other compared to the natural complementary nucleic acids, making them attractive materials for directing probe assembly in microarray applications. Another artificial oligonucleotide that has attracted interest for such applications is L-DNA which is the perfect mirror-image form of the naturally occurring D-form DNA (103). Because of the difference in chirality, L-DNA does not bind to its D-DNA counterpart, thus removing the possibility of hybridizing with any naturally occurring complementary sequence present in cell lysates. L-DNA oligonucleotides are also less sensitive to nucleases and may be good raw materials for generating protein-binding aptamers (104).

4. APPLICATIONS OF DDA PROTEIN MICROARRAYS

The development of DNA-directed assembly strategies for protein microarray platforms is still in its infancy. However, a steadily increasing number of studies have shown that this strategy is suitable for application in both analytical and functional protein microarrays. Some of these studies have shown that DDA protein microarrays perform favorably when compared with microarrays fabricated using other conventional methods. We will highlight some applications of nucleic acid-directed assembly in protein microarrays which show that this is a viable and promising fabrication strategy.

4.1. Antibody microarrays

Antibody microarrays are one of the most common protein microarrays in use and fabrication of spatially addressable antibody arrays using antibody-DNA conjugates have been reported (79). Since it is much simpler to tag antibodies with oligonucleotides than to do the same to all the proteins present in a complex biological mixture like cell lysate or serum, DNA-assembled antibody microarrays are largely used in the forward-phase mode.

Wacker *et al.* developed a chip-based microarray-fluorescence immunoassay formed by DNA-directed immobilization (DDI-microFIA) for the multiplex detection of the tumor marker human carcinoembryonic antigen (CEA), recombinant mistletoe lectin rViscumin, ceruloplasmin, and complement-1-inactivator in human blood samples (80). In this system using DNA-directed immobilization of antibodies in a sandwich immunoassay, detection limits down to 400 pg/ml were reached. Two

detection schemes using DDI-microFIA were evaluated in this study: a three-step scheme where the antibody was first immobilized onto the array before capturing the antigen and then finally detection of the antigen with a second fluorophore-conjugated antibody; the second one-step scheme combined all three steps in the previous scheme to reduce handling time. It was found that both schemes resulted in comparable detection limits of about 10 ng/ml for detecting CEA in serum.

Biosensors fabricated by the DNA-directed immobilization of antibodies onto a self-assembled ssDNA/oligo(ethylene glycol) thiol terminated monolayer were reported by Boozer *et al.* (105, 106). Antibody-DNA conjugates were used for detecting three fertility hormones: human chorionic gonadotrophin (hCG), human luteinizing hormone, and follicle stimulating hormone using surface plasmon resonance on a multichannel biosensor surface. Although a detection limit of 100 pg/ml was reported for hCG, this was carried out in a model system of detecting hCG in a buffer solution and is unproven in a complex biological mixture like serum or cell lysate.

Recent work by Bailey *et al.* (94) and our group (107) using antibody-DNA conjugates have resulted in DNA-directed self-assembly antibody microarrays where the antibody-antigen interaction was allowed to take place in a homogeneous solution before the DNA-probe-target complex is spatially separated onto a spotted DNA array for detection. Our spatially addressable protein array (SAPA) was used for the detection of fluorophore-labeled rabbit immunoglobulin G and green fluorescent protein (GFP) from both buffer solution as well as fluorophore-labeled cell lysate with a detection limit of as low as 1 pM (about 150 pg/ml) without amplification (107). The DNA-encoded antibody library (DEAL) technique reported by Bailey *et al.* uses a sandwich immunoassay approach for the detection of the human cytokines interferon-gamma, tumor necrosis factor-alpha, and interleukin-2 (IL-2). Using a fluorescently labeled secondary antibody for detection of IL-2 in a microfluidic-based assay resulted in a detection limit of 10 pM while the use of an amplification strategy employing secondary antibodies labeled with gold nanoparticles detected by electroless metal deposition further increased the sensitivity of this assay 1000-fold to 10 fM (94). This shows the potential of the DNA-directed assembly strategy when the various parameters affecting antigen detection is optimized in an antibody microarray.

4.2. Aptamer microarrays

Aptamers are DNA or RNA molecules that can bind to other molecules such as nucleic acids, proteins, and organic compounds. Aptamers that can recognize and bind a wide range of proteins have been identified, making them potential probe molecules for protein microarrays. As they are single strand oligonucleotides, aptamers can be hybridized to DNA microarrays through a short stretch of complementary DNA. Hao and coworkers have reported the use of aptamers as spatially addressable protein-binding molecules that can be used to detect proteins like thrombin in solution before self-assembly onto DNA microarrays (108). They further produced signaling aptamers for thrombin detection by substituting a fluorescent nucleotide analogue into the aptamer at a position near to the binding site (109). This signaling aptamer generated a significant increase in fluorescence when bound to thrombin and the fluorescence detected after the signaling aptamer is self-assembled onto DNA arrays. A further development of this platform involves the simultaneous detection of the aptamer-binding molecules thrombin and ATP together with DNA targets on a DNA tile array using a novel strand displacement detection strategy (63).

Aptamers are useful protein-binding molecules especially for DDA protein microarrays, but one potential limitation is the availability of aptamers that can bind a wide variety of proteins or other biomolecules. Although the range of proteins that can be bound by aptamers is currently limited, improved methods for the evolution of aptamers are being developed with the number of reported aptamer-binding molecules steadily increasing (110). Coupled with the fact that aptamers are oligonucleotides and can easily be conjugated to a ssDNA address, the use of protein-binding aptamers would be ideal in a DNA-directed assembly protein microarray format. Apart from their use as ASRs in analytical microarrays, aptamers may also be used as adapters to immobilize specific proteins onto DNA microarrays for functional protein microarray applications. However, the challenge is to ensure that these proteins captured by aptamers and displayed on microarray surfaces retain their functionality or protein-binding ability.

4.3. Peptide microarrays

Peptide microarrays are useful tools for characterizing protein functions as it has been shown that short peptide sequences that make up part of the much larger protein molecule can recapitulate much of the biological activities of that protein (111). This has resulted in microarrays fabricated through the immobilization of protein binding domains for discovering interaction partners (112) and cleavage or phosphorylation sites on protease (113) and kinase (114) substrates for measuring the activities of these enzymes.

Since peptide probes are much smaller than their protein targets, it is important that the peptide remain recognizable and accessible to its protein interaction partner. Williams *et al.* showed that a DNA-tagged myc-peptide epitope can be immobilized onto a DNA array and subsequently detected by an anti-myc mouse antibody (115). Recently, DNA-directed immobilization has also been used for the generation of live-cell microarrays through the capture of specific cell surface ligands like integrin using RGD peptide ligands (116).

Screening of libraries of peptide substrates is necessary for the determination of protease activity and identification of its substrates. It is therefore crucial that such screening steps be carried out in high-throughput and preferably multiplexed assays. Two groups have reported the use of PNA-encoded protease substrate libraries for screening of protease activity (117-119). The PNA tags are used to address individual protease substrates to DNA microarrays for detection after protease cleavage of its

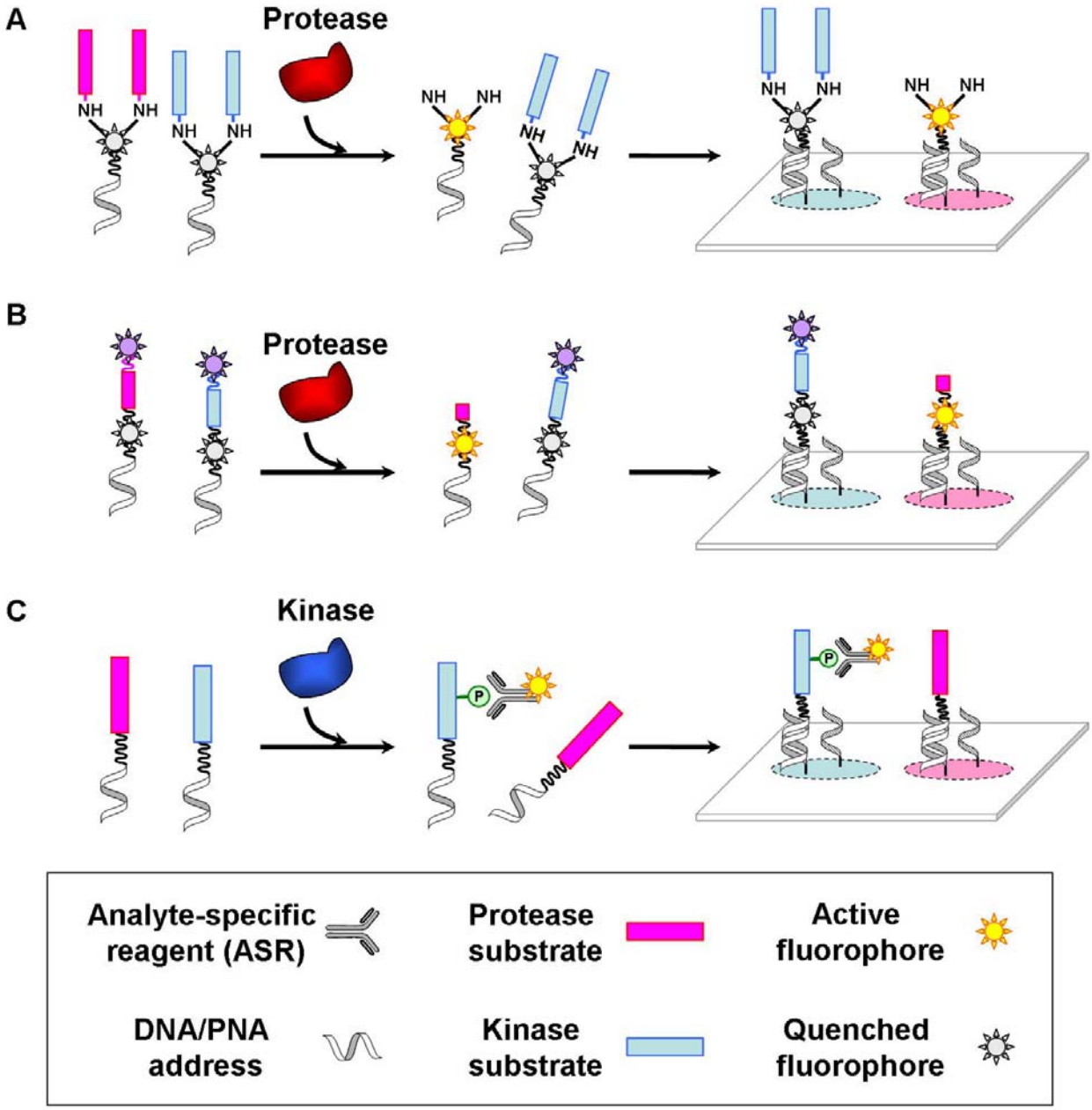

Figure 3. DNA-directed self-assembly strategies for screening protease and kinase substrates. A, latent fluorogenic substrates and B, FRET-based peptides were used to screen for protease substrates. The cleavage of the PNA-encoded substrates results in activation of the quenched or latent fluorophores which are then detected on DNA microarrays. C, DNA-addressed kinase substrates are phosphorylated in solution-phase reactions before being detected with ASRs against phospho-specific residues on DNA microarrays.

substrates in solution (Figures 3A and 3B). Winssinger *et al.* reported the use of latent fluorogenic substrates linked to PNA for the screening of 192 protease substrates prepared by split and mix combinatorial synthesis (117) (Figure 3A) while Diaz-Mochon *et al.* synthesized 10, 000 PNA-encoded FRET-based peptides for high-throughput analysis of protease cleavage specificity (118) (Figure 3B).

Another peptide microarray fabricated using DNA-direct self-assembly is a multiplexed protein kinase assay reported by Shults *et al.* (120) (Figure 3C). DNA-tagged peptide substrates were first phosphorylated in a solution-phase reaction. The phosphorylated serine, threonine, and tyrosine residues were then chemically labeled and subsequently hybridized onto a DNA microarray for detection and characterization of kinase activity.

An alternative method of preparing self-assembling protein microarrays and avoiding direct spotting of proteins onto surfaces utilizes *in vitro* transcription and/or translation to achieve on-chip synthesis of peptides and proteins using their cDNA or mRNA coding sequences (121). Most applications using such methods anchor the protein-encoding DNA (122), mRNA (123), or PCR products (124) directly to the microarray surface before the *in vitro* protein expression step. However, Weng *et al.* have described a modification of the method using covalent mRNA-protein fusion (PROfusion) technology where the mRNA segment of the mRNA-protein fusion is used not only as a coding sequence for *in vitro* translation but also for site-specific immobilization of the translated protein onto a DNA microarray (125). Each mRNA-protein fusion possesses a unique epitope which can then be detected by autoradiography or through the use of specific antibodies. Multiplex detection of the epitopes for three proteins (myc, FLAG, and HA11) was demonstrated with sub-attomole quantities of displayed protein being detected without any signal amplification methods employed.

4.4. Protein-DNA microarrays

DNA-protein interactions are central to many cellular functions such as the gene transcription, DNA repair, enzymatic restriction, and genomic replication. Protein-DNA binding microarrays have been reported for the study of transcription factor binding (126) and restriction endonuclease specificity (127). These are usually modified DNA microarrays consisting of ssDNA immobilized onto the microarray substrate and subsequently hybridized to form dsDNA containing specific protein binding or cleavage sites. However, many DNA-protein complexes consist of large, multimeric components which are unlikely to assemble efficiently with DNA directly immobilized onto microarrays.

Hauser *et al.* have shown that it is possible to generate dsDNA probes linked to a single strand L-DNA tag for the study of protein-DNA binding (103). These probes were used to capture fluorescently-labeled NF-kappaB transcription factors. The probe and target were subsequently crosslinked and hybridized onto a L-DNA ZIP-code microarray for detection.

We have developed a surface addressable DNA array (SADA) for the analysis of RNA polymerase (RNAP) binding to DNA using DNA-directed self-assembly (128) (Figure 4). In this platform, a probe containing a ssDNA address linked to a dsDNA binding sequence was generated using PCR. The probe was then allowed to bind RNA polymerase in a solution under optimal binding conditions. After binding, we separated the bound probes from unbound probes using polyacrylamide gel electrophoresis and recovered the RNAP-probe complex which is then hybridized onto specific sites on a DNA microarray surface through its ssDNA address tag. The detection of hybridized DNA-probe complex is achieved through a fluorophore tag on the DNA probe molecule. We have also extended this platform to screen for inhibitors against DNA-modifying enzymes that suppress viral replication.

4.5. Small molecule microarrays

DDA of chemical compounds for enhancement of reactions has been reported (129), demonstrating the usefulness of DNA attachments on organic or inorganic molecules through various means. In addition to their use as reactants in chemical synthesis reactions, small molecules are also routinely used as inhibitors of biologically active proteins. Small molecule inhibitors of enzymes such as kinases and proteases play important roles in delineating the functions of these enzymes in regulating cellular functions through post-translational modifications that can change protein activities (130).

Parallel to studies using PNA-encoded peptides for the study of enzyme activities, Winssinger and coworkers have reported the use of PNA addressable small molecule probes for the analysis of enzyme activities (Figure 5). Winssinger *et al.* used PNA-tagged libraries of mechanism-based inhibitors to screen for molecules that bind to the cysteine proteases caspase-3 and cathepsin K (131). PNA-inhibitor probes were added to crude cell lysate or protein libraries for solution-phase binding. Unbound PNA-probe molecules were separated from those that were bound to the proteases by size exclusion filtration and subsequently hybridized to DNA microarrays. Detection was achieved through a fluorophore attached to the PNA segment of the probe. An extension of this work by Urbina *et al.* showed that this platform can be used to profile two cysteine proteases, cathepsin K and cathepsin F, for activity against 625 PNA-encoded tetra-peptide acrylates with dectection limits of down to 10 pM (132). A systematic evaluation of various detection methods like direct fluorescence labeling, antibody-mediated fluorescence amplification, and biotin-gold nanoparticle detection was also reported in this study.

4.6. Carbohydrate microarrays

Oligosaccharides on gycoproteins, glycolipids, and glycosaminoglycans are known to play important roles in cellular processes like cell adhesion, differentiation, immune response, and trafficking through their interactions with carbohydrate-binding proteins (133). Several applications employing oligosaccharide microarrays have been reported for the study of carbohydrate-protein binding (134, 135) or for screening enzyme activities (136) but these glycan microarrays consist predominantly of oligosaccharides immobilized directly onto microarray surfaces. Although there has so far been no reports of DNA-directed assembly being used to fabricate carbohydrate-protein microarrays, synthetic carbohydrate-DNA conjugates have been generated through chemical coupling of modified glycopolymers to thiolated DNA (137), or through direct incorporation of glycol-modified deoxyuridine phosphoramidite to DNA (138) for the construction of self-assembled glyco-clusters. With the widespread adoption of oligosaccharide microarrays in recent years (139, 140), it is likely that DDA of carbohydrates for protein microarray applications will be a subject of future microarray studies.

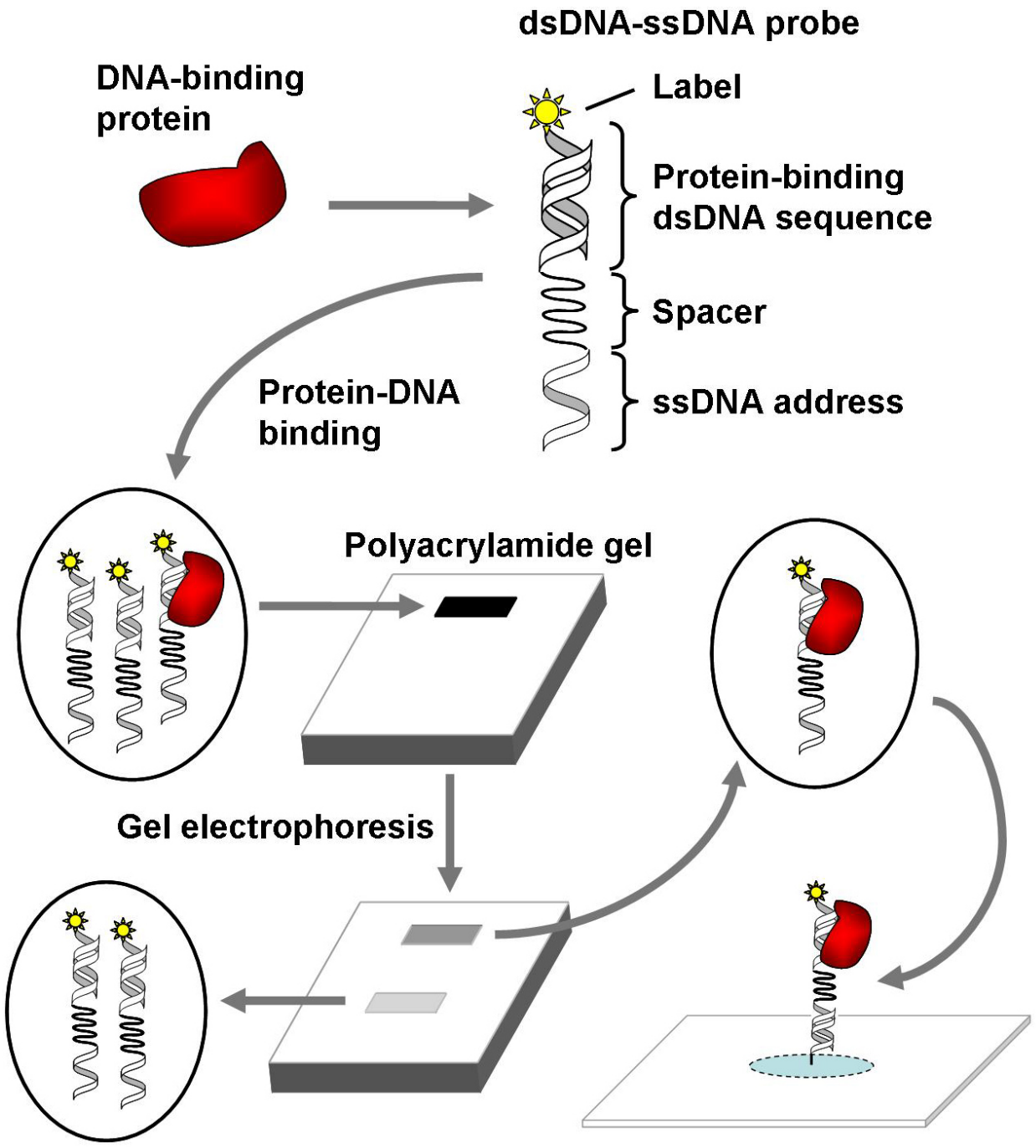

Figure 4. A surface addressable DNA array (SADA) for the analysis of protein binding to DNA using DNA-directed self-assembly.

5. PERSPECTIVE

Protein microarrays hold great potential as tools that can be used to study the composition and functions of large numbers of proteins. In this review, we have highlighted many exciting advances which are driving this rapidly developing field. Though these advances have brought protein arrays closer to the realization of their full potential, many formidable challenges remain. We have focused on the use of DDA as a particularly promising strategy for protein arrays as it can provide high-sensitivity analyte detection while circumventing many of the issues relating to protein stability and ASR-analyte capture efficiency that are faced by almost all microarray platforms. By incorporating various state-of-the-art technologies in array design, fabrication, and detection strategies, we are confident that the DDA protein array platforms will find widespread application as robust, high

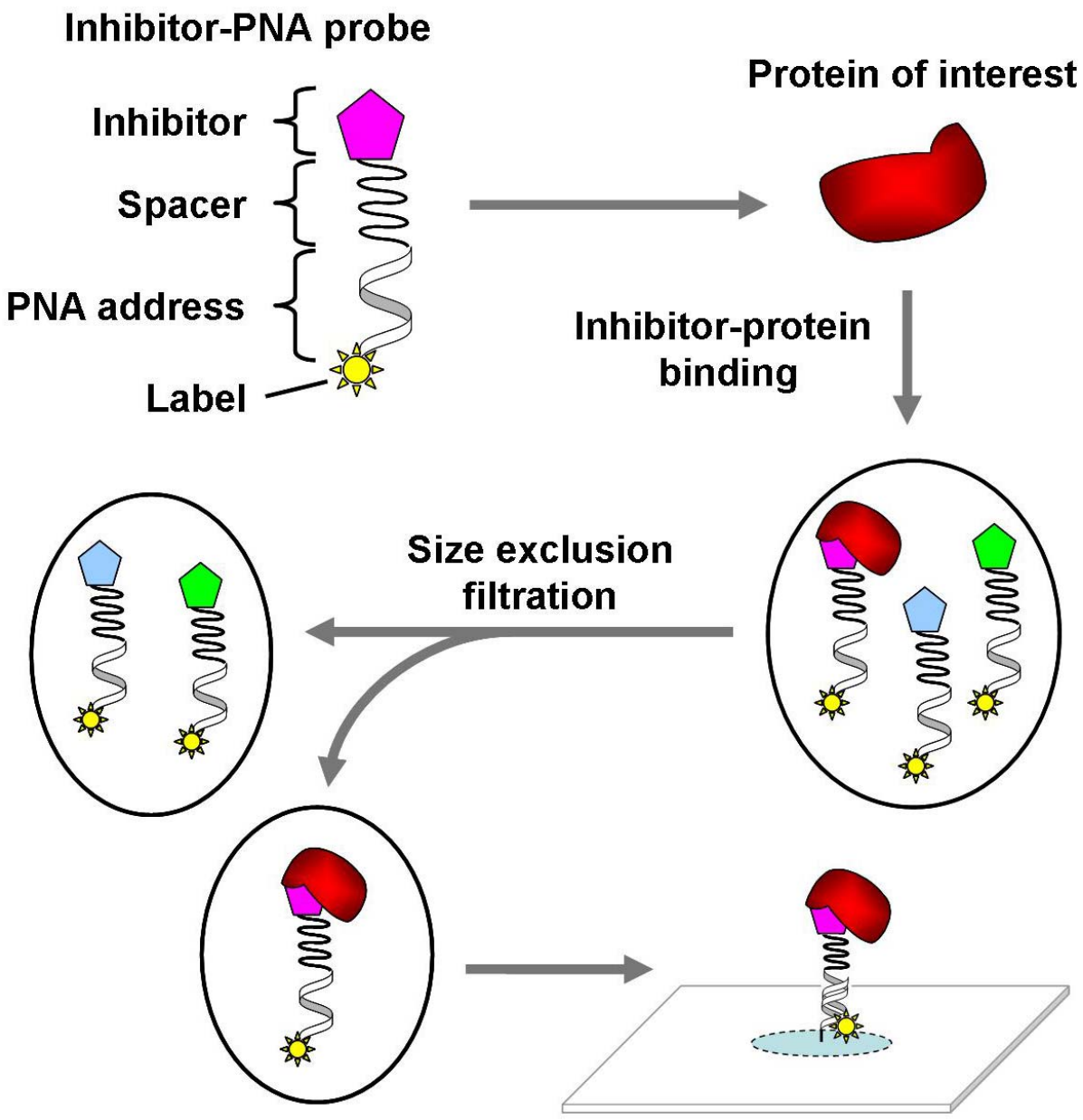

Figure 5. DNA-directed self-assembly strategy for screening libraries of PNA-encoded small-molecule inhibitors for activity against enzymes.

throughput, and high sensitivity assays capable of detecting a wide range of diverse analytes in a multiplexed manner.

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Abbreviations: DDA: DNA-directed self-assembly, ASR: analyte-specific reagent, RCA: rolling circle amplification, NTA: nitrilotriacetic acid, PAMAM: poly(propyleneimine), PAMAM-COOH: carboxyl-terminated PAMAM, ssDNA: single stranded DNA, dsDNA: double stranded DNA, BS: bis(sulfosuccinimidyl)suberate, SMCC: succinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate, GMBS: N-[γ-maleimidobutyryloxy]succinimide ester, SANH: Succinimidyl 4-hydrazinonicotinamide acetone hydrazone, SMPB: succinimidyl 4[p-maleimidophenyl]-butyrate, EPL: expressed protein ligation, LNA: locked nucleic acid, PNA: peptide nucleic acid, DDI-microFIA: microarray-fluorescence immunoassay formed by DNA-directed immobilization, CEA: carcinoembryonic antigen, hCG: human chorionic gonadotrophin, SAPA: spatially addressable protein array, GFP: green fluorescent protein, DEAL: DNA-encoded antibody library, IL-2: interleukin-2, SADA: surface addressable DNA array, RNAP: RNA polymerase.

Key Words: Protein array, Microarray, DNA-directed assembly, Self-assembly, Analyte-specific reagents, DNA-protein conjugates, Review

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