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Review

Current peptide and protein arrays and their applications in biomedical research

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ABSTRACT

In the past decade, peptide and protein arrays have become a substantial tool for systematic, large scale and high-throughput analysis in basic research, diagnostics, drug discovery, and functional genomics. A great variety of array formats and variants has been developed, accompanied by substantial progress in array production. Compared to mass-spectrometry-based proteome analyses, peptide and protein arrays might have less comprehensiveness and quantitative accuracy. However, they may allow for rapid and parallel screening of thousands of samples in a single experiment and represent a convenient tool for a broad spectrum of biomedical applications. Accordingly, peptide and protein arrays have been applied to analyse e.g. protein-protein, antigen-antibody or protein-small molecule interactions, while whole cell or tissue lysate (reverse phase) arrays have proven useful for diagnostic biomarker discovery and analysis of signaling pathways. This article reviews the most common peptide and protein arrays presently available for biomedical research, providing information on synthesis techniques, applications, and future perspectives within this field.

KEYWORDS: peptide array, protein array, antibody array, reverse phase array, biochips

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INTRODUCTION

The first blueprint of the human genome has given rise to a broad spectrum of novel possibilities but also to new questions. Based on genome information, DNA microarrays have been set up which allow for genome-wide profiling of the expression levels of genes as well as for detecting 0.5 up to 1.0 million naturally occurring genetic alterations via so-called SNP-chips in a single experiment [1-6]. Undoubtedly, these arrays have significantly been pushing molecular analyses, drug target and drug discovery forward in major human diseases. Furthermore, DNA arrays are meanwhile at the point of transition towards the use as diagnostic tools for personalized medicine [7, 8].

Simultaneously, decoding the human genome raised the demand for handling the next level of complexity, i.e. deciphering the functions of the encoded proteins and their complex relationships to signaling pathways and diseases. With the advent of novel high-throughput DNA sequencing methods [9, 10], information about genetic variation can be expected to grow exponentially, adding the need to analyze functional differences between protein variants with subtle variations caused by genetic alterations. Further, alternative splicing, cellular miRNAs, and post-translational modifications (of which up to date around 300 have been recorded) modulate the functions and active quantities of the proteins, giving rise to a large and complex network of permutations.

There is general consent that the knowledge of protein functions and proteome changes is critical for understanding the emergence of common diseases and to define new starting points for diagnosis and therapy. Accordingly, huge efforts are in progress to profile the proteomes at maximal depth or to analyze a portion of the proteome down to single proteins in large numbers of samples.

For addressing these issues, mass-spectrometry and peptide and protein microarrays have become essential tools within two major fields of application that are defined by mode of detection. State of the art mass-spectrometry approaches provide quantitative data for several thousands of proteins, including post-transcriptional and -translational modifications in a systematic, non-hypothesis-driven manner [11]. Using quantitative metabolic labeling strategies, 4400 proteins have been profiled in yeast [12] and a set of 5100 proteins has been measured in mouse embryonic stem cells [13]. It is, however, assumed that this still represents only a fraction of the mammalian proteome. Peptide and protein microarrays allow for studying selected sets of proteins and peptides in up to several thousands of samples in a single experiment in miniaturized format.

In principle peptide and protein microarrays represent assay systems, which display a library of ligands bound to or immobilized on a solid-phase. Libraries, in which peptides represent the ligands bound to polymer beads, ribosome-display or phage-display libraries, and libraries synthesized via so-called multipin strategies have proven to be useful tools [14-20]. This review will focus on microarrays generated on planar two-dimensional solid surfaces. Such microarrays can be divided into subclasses according to the synthesis technology, the kind of solid support used for immobilization, their field of application, or the nature of immobilized ligands. We will use the latter criterion for classification into peptide arrays, protein/antibody arrays, and reverse phase arrays, ordering the arrays in correspondence to the increased complexity of the immobilized polypeptides. The different array formats are associated with individual demands with regard to array production and are suitable for different biomedical applications. We will provide a survey of the present state of the art in this field.

General immobilization and detection strategies for peptide and protein arrays

Common to peptide, protein/antibody and reverse phase arrays is the requirement of a matrix for polypeptide immobilization. The mode of immobilization is critical for peptide and protein microarrays. It must be firm enough to withstand post-arraying experimental procedures, but has to be compatible with peptide/protein chemistry and should have minimal effects on conformation and activity. Further, the ligands need to be immobilized in sufficient high densities without interfering with accessibility. This often includes that an oriented immobilization is the most favorable goal.

For immobilization physical, covalent and bioaffinity-based strategies can be employed [21]. Physical immobilization is commonly used, when pre-synthesized peptides or proteins are immobilized on cellulose or nitrocellulose membranes, which is one of the simplest methods. Van der Waals forces and/or ionic interactions cause the stable but non-coordinated adsorption to the surface. Covalent coupling can be achieved via a chemical ligation step, resulting in an oriented attachment to the surface or in a non-oriented immobilization via general cross-linking approaches. Finally, bioaffinity-based arrays, also referred to as capture arrays, possess tags for an oriented immobilization. The tags can consist of amino acids (e.g. His-tags) or of other non-amino acid groups, such as biotin. In this case, the immobilization matrix displays the respective interaction partner of the tags (e.g. Ni-NTA or streptavidin). As the basic support, functionalized glass or gold slides, membranes and polymer substrates are most commonly used [21]. The choice of the matrix and immobilization strategy strongly depends on the kind of microarray, the downstream biomedical application, and the detection method of choice. For example, for the detection of a specific antibody in a complex sample in many cases a correct protein configuration is not necessary, while a good accessibility of the small linear epitope is critical.

The development of detection methods for peptide and protein arrays is a highly dynamic field. In general, labeled antibodies, recombinant fusion proteins and radioactive enzyme substrates are most commonly employed (Figure 1a-e, g, h). Besides that also luminescent bacteria to identify antimicrobial peptides, colorimetric assays (Figure 1f), and label-free detection methods, such as atomic force microscopy (Figure 1i), have been used [22, 23, 24].

Construction of peptide arrays

The foundation for peptide array synthesis had been laid by Merrifield in 1963. Merrifield developed a novel method for the combinatorial synthesis of peptides on solid supports [25, 26], which was acknowledged with the Nobel Prize in chemistry in 1984. An amino acid is coupled to a solid support (Merrifield resin) and afterwards the target peptide is synthesized from the C- to the N-terminus by coupling further amino acids in stepwise cycles. The side chains of the amino acids and the N-terminus of the newly added amino acid are protected by chemical groups to avoid undesired side reactions. Accordingly, each synthesis cycle starts with de-protection of the N-terminal end of the already synthesized peptide. By repeating the coupling and N-terminal group de-protection process, the peptide is stepwise synthesized on the supporting matrix. In a last step the protective groups of the peptide side chains are cleaved off, resulting in the mature peptide.

After 1963 the method has stepwise been improved. For example, novel protection groups (e.g. 9-fluorenylmethoxycarbonyl-, tert-butoxycarbonyl-, t-butyle-, tert-trityl-groups) and activation methods have been developed (e.g. via 1-hydroxybenzo-triazole, pentafluorophenyl ester) [27, 28]. Furthermore, improved solid supports have been implemented, such as modified polystyrene and polymer supports [29]. Because maximal steric accessibility of the peptides is critical for efficient detection with antibodies and for interaction studies, additionally linkers and spacers have been developed for peptide immobilization [30, 31]. Due to the sequential construction of the peptide, Merrifield's synthesis method has rapidly been advanced to automation, employing columns as solid phase. The resulting peptide synthesizers are nowadays well established and broadly used equipment [32].

The first two-dimensional, i.e. planar peptide arrays were synthesized by Fodor *et al.* in 1991 [33] and by Frank in 1992 [34]. Fodor used a photolithographic synthesis principle and achieved the parallel synthesis of 1024 peptides on a functionalized glass surface, employing light-sensitive instead of chemically cleavable protection groups [33]. Via positioning of a mask the amino acids are de-protected at the desired locations by light irradiation. Afterwards the array is exposed to a coupling solution containing the next defined amino acid, so that a local coupling of the next monomer in the peptide sequence can take place (Figure 2a). Gao and co-workers improved the basic photolithographic method by using light-sensitive acids as terminal protection groups to increase de-protection efficacy [35, 36]. But because in each single layer every amino acid of the peptide has to be coupled separately, the technique requires a huge number of coupling steps. For example, for the synthesis of 8-mer peptides with all possible permutations of the 20 amino acids it would require 160 coupling steps. Although this method has not been finding broad application for peptide array synthesis, the principle is still the most recent state-of-the-art to synthesize DNA/RNA arrays. The technique is better applicable for these arrays, because the number of coupling cycles is reduced to 32 for randomized 8-mer synthesis due to the fact that only four building blocks, i.e. the four nucleotides, are necessary (dATP, dGTP, dCTP, dTTP).

Frank used classical Merrifield peptide synthesis chemistry in his so-called SPOT-synthesis (Figure 2b). Here, pre-activated amino acids in solution are drop-wise positioned onto a functionalized cellulose membrane. The dissolved amino acids are coupled immediately to the surface in the spatially separated reaction volume defined by the droplet. Afterwards the terminal groups of all immobilized amino acids on the membrane are cleaved in one single step and the local spotting process is repeated with the next amino acid. Repetitive spotting and cleavage steps result in the parallel synthesis of the desired peptides at defined positions (spots) on the array [34].

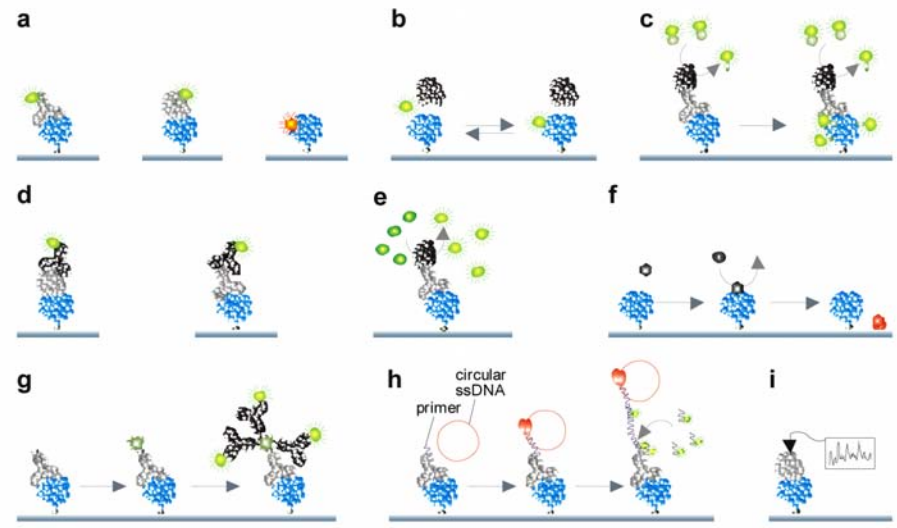

Figure 1

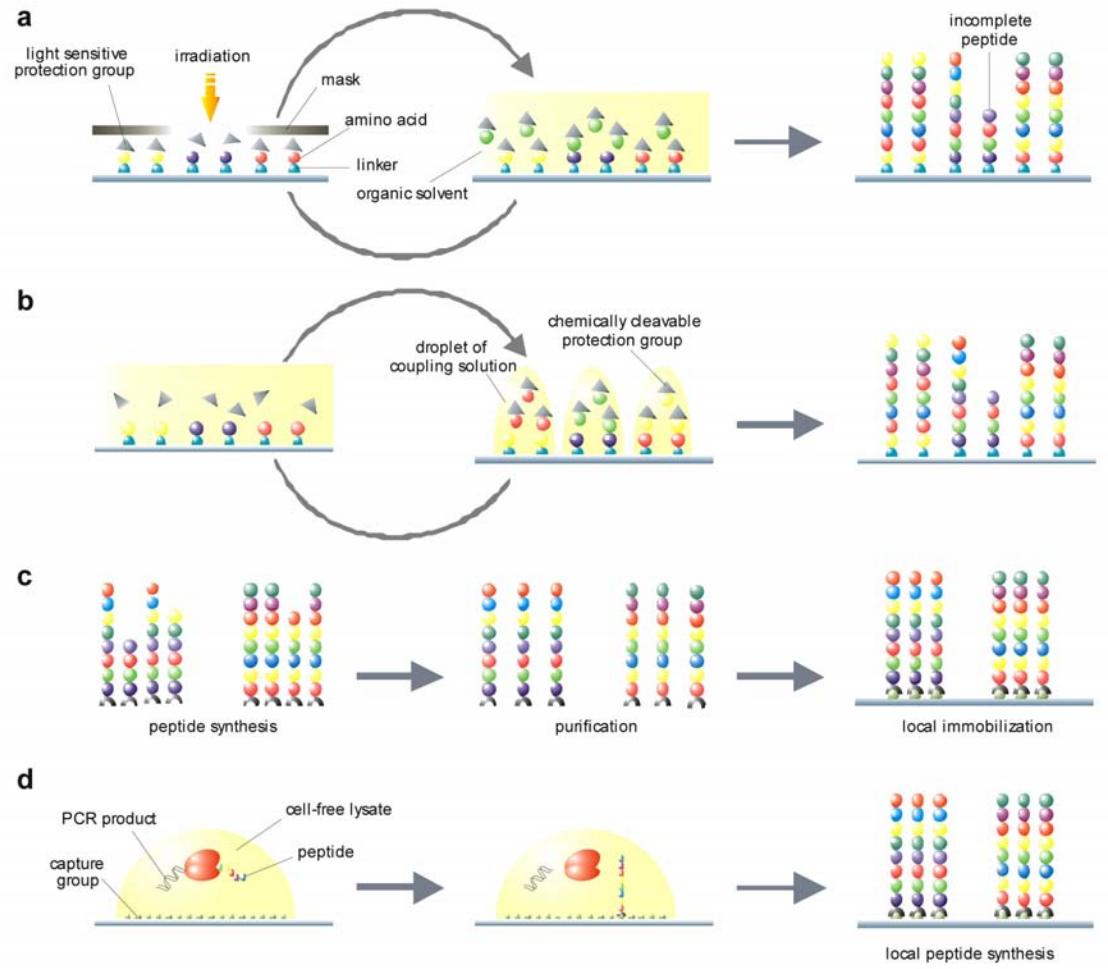

Figure 2

Both methods, i.e. photolithographic and SPOT-synthesis have some drawbacks in common. First, incompletely synthesized peptides are still present on the array and actually reduce the amount of the target peptide per location or could lead to undesired false positive reactions in consecutive applications. Second, the concentration of peptide per synthesis location might vary due to different coupling efficiencies of the different amino acids and the lack of a suitable method for concentration measurement, which might produce systematic errors. The production of array copies with homogeneous quality is thus made difficult and normalization after array application is almost impossible due to the lack of suitable methods for local target peptide concentration measurements.

After 1992 several procedures have been developed to face these challenges and to further improve peptide arrays with respect to spot density and conformity. Today's peptide array comprise three further groups in addition to the peptide arrays that are generated by the above-mentioned chemical *in situ* synthesis directly on the solid support. First, this includes peptide arrays, on which pre-synthesized and purified

Legend to Figure 1. Common detection strategies for peptide and protein arrays. The detection methods can be classified into three major categories. In direct labeling methods the signal for readout is directly attached to the immobilized polypeptide (peptide, protein or antibody; blue) or directly attached to its binder (a-c). In indirect labeling methods the signal group is indirectly attached via secondary binders or stays in the local proximity of the immobilized polypeptide without attachment (d-h). In label-free methods (i), the readout is performed by measuring alterations of the physico-chemical properties upon binding. a, the immobilized polypeptide is detected via the specific binding of a labeled antibody or protein (grey) or by a radioactive metal compound (orange). b, enzymatic attachment or removal of a signaling agent provides the readout, which is commonly used for analyzing enzyme-substrate interactions. c, the immobilized polypeptide is directly labeled via an enzyme (black; e.g. horse radish peroxidase) that is coupled to an antibody (grey). d, so-called sandwich assays represent one of the most commonly used indirect detection methods. The first antibody or protein ligand (grey) interacting with the immobilized polypeptide is detected by a secondary antibody (black) with the signal group. e, in enhanced chemiluminescence detection a specific primary antibody (grey) is coupled to an enzyme (black; e.g. horse radish peroxidase). The enzyme causes a chemical compound (dark green; e.g. luminol) to emit a chemiluminescent signal. f, colorimetric assay, in which a bound metal compound (black hexagon, e.g. nickel ion) reacts with a reagent (black oval; e.g. dimethylglyoxime), so that a colored metal-complex (red) is formed in the local proximity. g, a primary biotinylated antibody (grey) is used. Biotin-streptavidin (light green) interaction consecutively allows for binding of multiple biotinylated secondary antibodies (black), which results in signal amplification. h, the primary detection antibody (grey) is coupled to a primer. A circular single stranded DNA molecule interacts with the primer via complementary base-pairing, so that a polymerase (red) can be used for rolling circle amplification. Small labeled oligomers hybridize to the resulting single-stranded DNA product, providing an amplified signal for readout. i, label-free methods, in which the binding event is, for example, detected by atomic force microscopy or plasmon surface resonance.

Legend to Figure 2. Strategies for the synthesis of peptide microarrays. a, photolithographic peptide array synthesis. The first layer of immobilized and protected amino acids (colored cycles) is locally irradiated by light via application of a mask. The irradiation causes local cleavage of the protection group (grey triangle) from the amino acids. Afterwards, the second protected amino acid is coupled and remaining non-protected amino acids are blocked. Repetition results in the local synthesis of different peptides on the array, which may contain incompletely synthesized peptides. b, spot synthesis. The protection groups of the complete first layer of immobilized amino acids are chemically cleaved off. The second amino acid with protection group is then locally deposited by positioning droplets to the respective localization on the array. Remaining free amino acids are blocked and the synthesis cycle is repeated. c, arrays consisting of pre-synthesized peptides utilize tags and capture groups for immobilization. Incompletely synthesized peptides can be excluded by purification prior to immobilization. d, peptide *in situ* synthesis. Products from polymerase chain reaction (PCR) or other DNA sources are used for a combined *in vitro* transcription/translation via cell-free lysates in a locally restricted droplet. The array matrix is functionalized by a capture group and the first amino acid is coupled to a corresponding tag. This results in the local production of immobilized peptides.

peptides are immobilized on a planar surface (Figure 2c). Second, this comprises bio-synthesized arrays employing cell-free lysates (Figure 2d), which will be discussed in more detail in the protein and antibody array section below [37]. Third, this encompasses peptide arrays, which employ mixed or downstream optimized approaches, as, for example, so-called CelluSpots [38, 39] or solid particle-based peptide synthesis [40, 41].

The immobilization of pre-synthesized peptides bears the advantage that the peptide concentration per spot can be controlled and that target peptides can be separated from undesired incomplete synthesis products prior to arraying. Further, once a peptide or peptide library has been synthesized via automated processes, several identical array copies can be generated. This strategy is, however, disadvantageous with regards to the laborious and cost-intensive peptide purification steps.

The CelluSpot method is based on the original SPOT-technology, but it prints pre-synthesized peptides still bound to cellulose (peptide-cellulose conjugates) in multiple copies. This reduces production costs, allows for adjustment of peptide concentration and increases reproducibility. Furthermore, the CelluSpots build 3-dimensional spheres for increased accessibility and higher peptide loading per area.

Fluid spotted solutions tend to spread on the solid support depending on the physical and chemical properties of the surfaces, so that an increase of spot diameters has to be accepted, which in turn counteracts miniaturization. Furthermore, a minimal volume per spot is needed to avoid evaporation of the solvent and consecutively decreased amino acid coupling efficacy. The available spotting systems allow for adjustment of air humidity during the spotting process [42], but still major limitations exist with regard to reducing the reaction volumes. Solid, so-called amino acid particles may contribute to overcome these hurdles. Here, the pre-activated and side-chain protected amino acids are embedded in a solid matrix of about 7 µm in size, which is equivalent to a droplet volume of 0.5 pL. The particles are deposited on a functionalized glass surface via a modified laser printer, which is equipped with 20 amino acid particle cartridges. Alternatively, particle deposition can be performed via an addressable microchip device, which represents a modified central processing unit as commonly used in computers. After deposition the particles are melted at increased temperatures, so that the solid particle matrix liquefies and the coupling reaction can take place. The method results in a notable 16- to 1,600-fold increase of spots per area from about 25 spots/cm^2 achieved by the SPOT-technology to 400 spots/cm^2 by using the laser printer, and up to 40,000 spots/cm^2 by employing the microchip technology. It can be expected that this results in a substantial decrease of the array production costs while simultaneously providing increased information content per array [40, 41]. While nowadays the SPOT technology and immobilization of pre-synthesized peptides are the leading peptide microarray production technologies, laser printer- and microchip-based strategies represent promising future options.

A general advantage common to peptide arrays is that most of the formats can be produced synthetically in a guided manner, so that any desirable permutation of amino acids - including peptide chains that do not occur naturally - can easily be generated. This facilitates construction of “theme”-chips. However, except for bio-synthesized peptide arrays, there are limitations regarding the maximal length of the polypeptides, which are defined by the coupling efficacy. Under optimal conditions automated synthesizers are suitable to generate peptides of up to 70 amino acids in length, which is still well below the size of most human proteins. In addition, the lack of post-translational processing normally taking place within the living cells means that the peptides only resemble to a limited extent the binding and other functional properties of proteins. On the other hand, however, the fact that peptide arrays can be synthetically produced by combining appropriate chemical and physical methods results in a great potential for generating high-diversity miniaturized arrays.

Biomedical applications of peptide arrays

Due to the relative ease, with which peptide arrays can be constructed compared to protein and

antibody arrays with a corresponding complexity, they have been utilized for numerous applications in biomedical research.

The identification of protein epitopes which are recognized by antibodies is of general importance for biomedical research. Such analyses allow for characterizing antibody specificity and affinity as well as for identifying potentially cross-reacting non-target structures. Peptide arrays offer the possibility to perform such an epitope mapping in a reliable manner. In most cases the protein will be presented in portions of small overlapping peptides with 6-15 amino acids in length. For example, Daskalow *et al.* used a small library of hexapeptides with an overlap of 2 amino acids synthesized on cellulose to map the epitopes of antibodies directed against S-tagged fusion proteins and molecular weight markers [43]. Alberts *et al.* used a peptide array with 781 13-mers with a 10 amino acid overlap of the clotting factor VIII. They identified the B-cell epitope of the monoclonal anti-factor VIII antibody ESH8 [44]. Reineke *et al.* used a peptide array of 5520 randomly generated sequences and identified distinct antibody epitopes and mimotopes of an anti-p24 antibody recognizing human immunodeficiency virus (HIV-I)-1, an anti-Interleukin-10, and an anti-transforming growth factor α (TGFα) monoclonal antibody [45].

The identification of epitopes via systematic mapping can provide a reasonable basis for the development of improved diagnostic assays or for vaccine and drug design. Soutullo *et al.* systematically analyzed the major core protein of the equine infectious anemia virus (p26) and identified two promising epitopes via peptide arrays [46]. Lottersberger *et al.* uncovered two potentially suitable epitopes within LipL32, which is an antigenic outer membrane protein from the pathogenic *leptospires* [47]. Peptide arrays were also employed to gain structural information about epitopes of the pregnancy-associated Malaria protein VAR2CSA [48] and for the prediction of fibrinogen-binding motifs within a major fibrinogen receptor of group B *streptococci* [49].

Peptide arrays have successfully been used for the identification of enzyme substrates and inhibitors as well as for the determination of enzyme substrate specificity. Especially kinases, phosphatases and proteases as main regulators in protein signaling cascades have been in the focus of peptide microarray-based approaches, but also chaperones have been investigated. Houseman *et al.* immobilized 24 pre-selected and pre-synthesized peptides on gold-surfaces and employed the resulting arrays for the quantitative measurement of protein kinase activity [50]. The activity of the c-Src kinase, the catalytic subunit of the protein kinase A and the cAbl tyrosine kinase were monitored by using radioactive labeled [γ-^{32}P]ATP. Panse *et al.* used the same radioactive assay to profile casein kinase 2, employing a peptide library of more than 13000 peptides deposited on glass slides [51]. They additionally used fluorescein-labelled antibodies for the profiling of generic anti-phosphopeptides applying a smaller phosphopeptide array that displayed 2923 peptides. Schutkowski *et al.* used analogous strategies for systematically deciphering kinase specificity [52]. Here, arrays were used containing 6912 13-mer peptide sequences extracted from annotated human phosphorylation sites as well as comprising phosphopeptides to study kinase autophosphorylation events. Knoblauch *et al.* investigated the substrate specificity and the principles of action of the bacterial SecB chaperone via peptide arrays with 2688 peptides covering sequences of 23 selected proteins [53]. Salisbury *et al.* employed a peptidyl coumarin substrate to investigate protease substrate specificity [54]. In this assay, the proteolytic cleavage of the peptide by the protease released the fluorescent coumarin group, allowing the determination of cleavage rates.

Peptide arrays were used for functional characterization of key proteins playing roles in cancer or microbial infections. In addition, the arrays served to identify peptidic lead structures for drug development. Gail *et al.* used GFP (green fluorescence) fusion proteins for the systematic delineation of the differential β-catenin interactions with Tcf4 (T-cell factor 4), E-cadherin and *Adenomatous Polyposis coli* (APC) on a peptide array [55]. The arrays consisted of the β-catenin binding sites of Tcf4, E-cadherin and APC and various amino acid permutations of

the natural binding sites. By modifying the classical SPOT technology, they achieved synthesis of peptides with a remarkable length of up to 53 amino acids. The study led to the identification of known and novel β-catenin binding peptide motifs. Furthermore a hydrophobic pocket interacting with Tcf4, but not with E-cadherin was identified, representing an interesting site for targeted drug design. Recently, Zhang *et al.* employed peptide arrays displaying the primary structure of the mature death receptor 5 via overlapping 12-mer peptides [56]. A novel epitope in the N-terminal region was identified. Antibodies raised against this epitope showed high affinities for death receptor 5 and triggered caspase activation. Via peptide arrays, Butterfield *et al.* achieved the identification of peptides binding chondroitin sulfate, which is an extracellular matrix component involved in the regulation of growth factor activity [57].

Rationalized drug design itself became an area, in which peptide arrays were used for various approaches. Malin *et al.* identified ^{99m}technetium-binding 6-mer peptides, which can be fused to antibodies for tumor imaging purposes [58]. Peptide arrays with non-natural amino acids (e.g. D-amino acids) or artificial amino acids became a crucial tool as well, because such modifications increase the half-life of peptides *in vivo*. Scheerer *et al.* used peptide arrays to stepwise convert natural L-epitopes for the anti-p24 (HIV-1) antibody CB4-1 and the anti-cholera toxin antibody TE33, respectively, into D-epitopes and analyzed their binding affinities [59]. In a different approach Kramer *et al.* employed not only the non-natural D-amino acids but also cyclic peptide structures to identify structure-based binding differences of the monoclonal antibody Tab2, which is directed against TGF-β [23]. Microarrays presenting peptidic nucleic acids (PNAs) or peptidomimetics such as peptomers or peptoids represent further options for such approaches [60, 61].

In conclusion, peptide arrays have experienced broad applications in biomedical research. Due to the linearity of the peptides bound to the array, their application is limited with regards to resembling the original conformations of proteins. Furthermore there are synthesis-inherent limitations with regards to the lengths of the polypeptides. Nonetheless, they offer as a convenient screening tool for the characterization of biomolecules and drug development.

Construction of protein and antibody arrays

While peptide arrays bear the advantage of synthetically creating a high diversity, they are associated with obvious limitations regarding the polypeptide length, post-translational modifications and the degree, by which they reflect the natural conformations of proteins. Thus, there is a persisting demand for protein arrays.

Historically, immunoassays represented the basis for today's protein and antibody microarrays. In 1959 Yarlow and Berson introduced radio-immunoassays (RIA) [62] followed by the first publication of the nowadays well-established enzyme-linked immunosorbant assay (ELISA) by Engvall and Perlmann in 1971 [63]. The basic principle of a protein array was first suggested by Ekins in the late 1980s [64]. However, it required another decade until the feasibility of microarray-based immunoassays was demonstrated. Silzel and co-workers spotted monoclonal antibodies directed against the four human immunoglobulin-γ (IgG) subclasses onto a thin sheet of polystyrene [65]. For this purpose, they employed a standard ink-jet printer, which gave rise to spots with a diameter of 200 µm. In a sandwich assay, subclass specific recognition of human myeloma proteins with minimal cross-reactivity was shown, and dose-dependent signals for each subclass were obtained. In 2000 MacBeath and Schreiber published one of the first comprehensive studies, which employed high density protein arrays (1600 spots per cm^2) to screen for protein-protein interactions, for the identification of protein kinase substrates and for recovering protein targets of small molecules [66]. A further step towards higher complexity was achieved by Zhu co-workers, who implemented so-called proteome chips [67, 68, 69]. They cloned 5800 yeast open reading frames and consecutively overexpressed, purified and printed the resulting proteins onto slides. The array was screened for interactions with proteins and phospholipids and revealed novel calmodulin- and phospholipid interacting proteins [67].

Because a complete synthesis of proteins via matrix-based chemical synthesis methods is not feasible at the present state of the art, the individual proteins or antibodies need to be either purified from natural sources or first expressed *in vivo* in cells or *in vitro* via cell-free lysates, followed by a purification step. Protein expression is often performed in *E. coli* for practical and cost reasons (Figure 3a).

Alternatively, eukaryotic expression systems or cell-free protein synthesis can be performed, using, for example, PCR products or plasmids as template. Cell-free lysates can be prepared from different prokaryotic or eukaryotic cell types. This method is particularly suitable for the production of proteins, which are difficult to express with *in vivo* systems. Using the cell-free protein biosynthesis method, protein (and also peptide) arrays can be generated *in situ*, i.e. the individual spots of the array are directly produced on a solid surface. This principle is applied in Protein *In Situ* Arrays (PISAs; Figure 3b) [70] or PISA variants [71], Nucleic Acid Programmable Protein Arrays (NAPPAs; Figure 3c) [72], and DNA Array to Protein Arrays (DAPAs; Figure 3d) [73] as well as for systems that utilize RNA as templates [74, 75]. This strategy largely facilitates array production, because it eliminates time-consuming steps for protein expression and purification prior to assembly on the array. In the DNA-based approaches the open reading frames (ORFs) of the respective genes are amplified from a library by PCR and the resulting PCR product is then used to locally synthesize the target protein by incubation with the cell-free lysate of the prokaryotic or eukaryotic species of choice (Figure 3b-d). Depending on the protein collection, which shall be displayed on the microarray, the one or the other of the afore-mentioned methods may be best suited. One has to acknowledge that if protein arrays are intended to take advantage of what discerns them from peptide arrays, i.e. displaying proteins in their natural conformation and with natural post-translational modifications, an expression of human proteins in mammalian cells would be required, which represents the most labor-intensive procedure.

Using *in vivo* expression strategies, the recombinant proteins have to be purified prior to spotting, printing, or immobilization on the solid support. The purification is usually performed by employing well-established affinity tags (e.g. His-tag, Gluthathione-S-transferase/GST-tag) attached to the C- or N-terminal of the protein and consecutive chromatography. In a technique developed by Wong *et al*. non-purified tagged proteins from a cell lysate mixture are bound directly to the microarray surface in a site-specific and covalent manner by using a phospho-pantetheinyl transferase-catalyzed surface ligation [76]. This allows for performing purification and immobilization in a single step. For a site-specific and oriented immobilization of the antibodies and the proteins capture arrays can be employed. There is the possibility to subsequently introduce certain post-translational modifications, such as phosphorylation, methylation or glycosylation via enzymatic or chemical approaches.

For the construction of antibody arrays essentially the same immobilization strategies are used (Figure 3e). Instead of producing antibodies in animals or via hybridoma cell lines there are a number of further options. Recombinant single chain fragments (scFc) expressed in *E. coli* and obtained from phage or ribosome display libraries as well as single V-domains from engineered human equivalents can serve as alternative starting material. Immobilization is mainly performed via capture arrays to guarantee the correct orientation.

The localized deposition of the proteins or antibodies is commonly performed with an ink-jet printer or with different arrayer devices, including non-contact printers, pipetting systems, or devices with solid pin printer heads. Arraying devices employing pipette tips, pipes or nozzles may bear the drawback that they tend to clog, depending on the viscosity of the protein solutions, which can cause problems in array production. On the other hand, they offer the advantage of more precise volume adjustment. Solid pin printer technology can be considered as more robust and has experienced broader application and acceptance within the past years.

Classical planar supports for protein spotting are glass surfaces, membranes (PVDF) and microtiter plates, which additionally can be functionalized with a poly-L-lysine layer, silicon, HydroGel,

polymer or nitrocellulose coatings. Balboni *et al.* published a comprehensive study of commonly used surfaces and their suitability for antibody array applications [77]. The choice of the support and the microarray design in general is influencing the detection systems that can be used [78, 21]. For example, while nitrocellulose-covered microscope slides allow for loading of higher protein amounts, the surface cover interferes with the signal detection in the green-fluorescent range. The choice of an appropriate detection system is particularly critical, if low abundance proteins have to be detected in a complex samples. For this purpose signal amplification systems based on enzymatic reactions (Figure 1c, e) or rolling circle amplification (Figure 1h) [79] are beneficial, but also fluorescein or biotin labels can be used (Figure 1a, g).

In summary, the construction of protein and antibody arrays is associated with considerable larger efforts compared to peptide arrays with the same complexity, but these array formats have the advantage to explore proteins more closely to their natural context.

Biomedical applications of protein and antibody arrays

With regards to their applications, protein and antibody arrays can be divided into two major subtypes. Analytical (capture) arrays primarily contain antibodies for the detection and quantification of analytes in complex mixtures, which includes, for example, blood plasma or serum, tissue extracts, or cell lysates. By contrast, functional arrays contain functional full-length proteins or protein domains for analyzing biochemical processes.

Analytical arrays have been employed in various approaches to recover diagnostic markers or signatures. For example, Ingvarsson *et al.* used a recombinant antibody microarray for monitoring the presence of pancreatic cancer by profiling patient sera [80]. This revealed 19 non-redundant proteins discriminating between healthy and cancer patients. Bartling *et al.* employed an antibody array to identify 29 proteins, which discerned human squamous cell lung cancer carcinoma from normal tissue [81]. Further comprehensive studies to profile cancer cells via antibody arrays have been performed by Sreekumar *et al.* [82], Madoz-Gurpide *et al.* [83] and Knezevic *et al.* [84].

In their studies, Gnjatic *et al.* used a commercially available protein microarray displaying >8000 human antigens to profile the sera of ovarian and pancreatic cancer patients versus sera of age-matched healthy donors [85]. Sets of 202 and 29 proteins were identified, against which antibodies were present in the sera of ovarian and pancreatic cancer patients, respectively.

Likewise, protein arrays have been used for studying infectious disease. Felgner *et al.* employed a protein microarray containing 1,205 *Burkholderia pseudomallei* proteins for screening patient sera, which identified 170 reactive antigens [86]. Arraying this set of reactive antigens, 747 sera from 10 different patient groups were consecutively assayed. This identified 49 reactive antigens, which selectively occur in sera of patients with melioidosis but not in sera of healthy individuals or of patients with other types of bacterial infections.

Functional arrays are commonly utilized whenever interaction partners, substrate specificity or a general mapping of the role of a protein in signaling pathways is aimed at. O'Connell *et al.* employed a pre-manufactured protein array of the human brain library (37,200 proteins, hEx1) for the identification of potential calmodulin binding proteins [87]. This finally yielded a set of 76 high-affinity interaction partners, of which 72 were novel, not previously identified binders. Four known and 8 novel calmodulin-binding proteins represented candidates with functions in the organization of postsynaptic density.

Salamat-Miller *et al.* screened five polyanionic biomolecules (actin, tubulin, heparin, heparan sulphate, and DNA) with a protein array containing 4985 proteins of the human UltimateTM ORF clone collection [88]. This identified 397 different human proteins as polyanion-binding proteins, of which 25 proteins interacted with all five polyanions.

Wang *et al.* used 43 cytokine- and chemokine-specific monoclonal antibodies (mAbs) printed on a protein array to screen and quantify cytokines and chemokines in conditioned culture media, cell lysates, and human plasma [89]. They identified cytokines associated with breast cancer and chemokines associated with cervical cancer. Uetz *et al.* used a protein array constructed of about 6000 yeast transformants, each expressing one of the open reading frames of *Saccharomyces cerevisiae* as a fusion to an activation domain [90]. They used the microarray to screen for interactions with a set of 192 yeast proteins. In conjunction with yeast two-hybrid-screening this allowed mapping of 957 putative protein-protein interactions in yeast.

DNA-protein interactions, which are involved in the regulation transcription, have also been studied using protein microarrays. Via a functional protein microarray for the analysis of the DNA-binding activity of p53 mutants Malcikova *et al.* uncovered substantial differences in DNA-binding activity compared to the wild type p53 [91]. However, further downstream studies revealed, that the potential to bind DNA does not correlate with the transcriptional activation in living cells. These findings also point to a critical drawback of protein microarrays. Despite their advantages compared to peptide arrays, the data may not necessarily reflect the processes in the living cells and may not provide conclusive information with regard to cell- or disease-related phenotypes.

Construction of reverse phase protein arrays

The so-called reverse phase protein arrays (RPAs) represent the array format with the highest level of complexity. Here, the biological samples under investigation are immobilized and afterwards assayed for proteins of interest. RPAs are related to tissue microarrays (TMAs), for which cylinders are punched out of tissues, as for example surgically removed tumor samples, and subsequently fixed in a paraffin block. The TMA technique allows for arranging up to several hundreds of tissue samples on a single microscope slide. Consecutive immunohistochemical analysis enables to study this huge number of samples in a single experiment [92]. Kononen *et al.* employed one of the first TMAs. In this study, six gene amplifications, p53 and estrogen receptor expression were monitored simultaneously in a set of 645 breast cancer tissue samples [93].

RPAs use cell or tissue lysates instead, which are arrayed on to the slide surface (Figure 4). The source of the lysates can either be archival paraffin-embedded tissues, cells isolated by laser-capture microdissection [94], cells from frozen tissues [95], plasma or serum samples [96], or cultivated cells [97, 98]. Most commonly, the detection of the target proteins is carried out via antibodies, which need to be confirmed for their specificity by Western blotting beforehand. Typically, only every third antibody is suitable for RPA applications [99].

To cover a sufficient dynamic range for relative protein quantification serial dilutions of the lysates are spotted or printed in multiple copies onto a solid support, such as nitrocellulose covered glass slides or modified silicon substrates [100]. Because of the viscosity of the lysates, this is commonly performed with solid pin devices. After production, the lysates can be stored at -80°C at least for one year without substantial loss of performance [98].

RPAs have been shown to possess high sensitivity. Detection of target proteins is possible in lysate spots containing as few as the protein amount equivalent of only 10 down to 0.4 cells [99, 101]. Reference peptides or proteins can be mounted as standards onto the arrays or the lysates themselves can be spiked with such standards as positive controls for the target protein [101]. Spotting of recombinant expressed proteins of known concentrations can further be used for absolute quantification of the target protein in the arrayed samples. A whole protein content quantification is performed by e.g. Sypro ruby staining, which serves as normalization [98]. Via this approach a quantification of proteins down to the pico- to femtomolar range (10^{-12} to 10^{-15} M) is possible. However, the detection limit may strongly depend on the choice of the antibody, the optimal blocking, and the detection methods [98, 102].

An obvious advantage of RPAs is that they may come closest to the situation in the living cells.

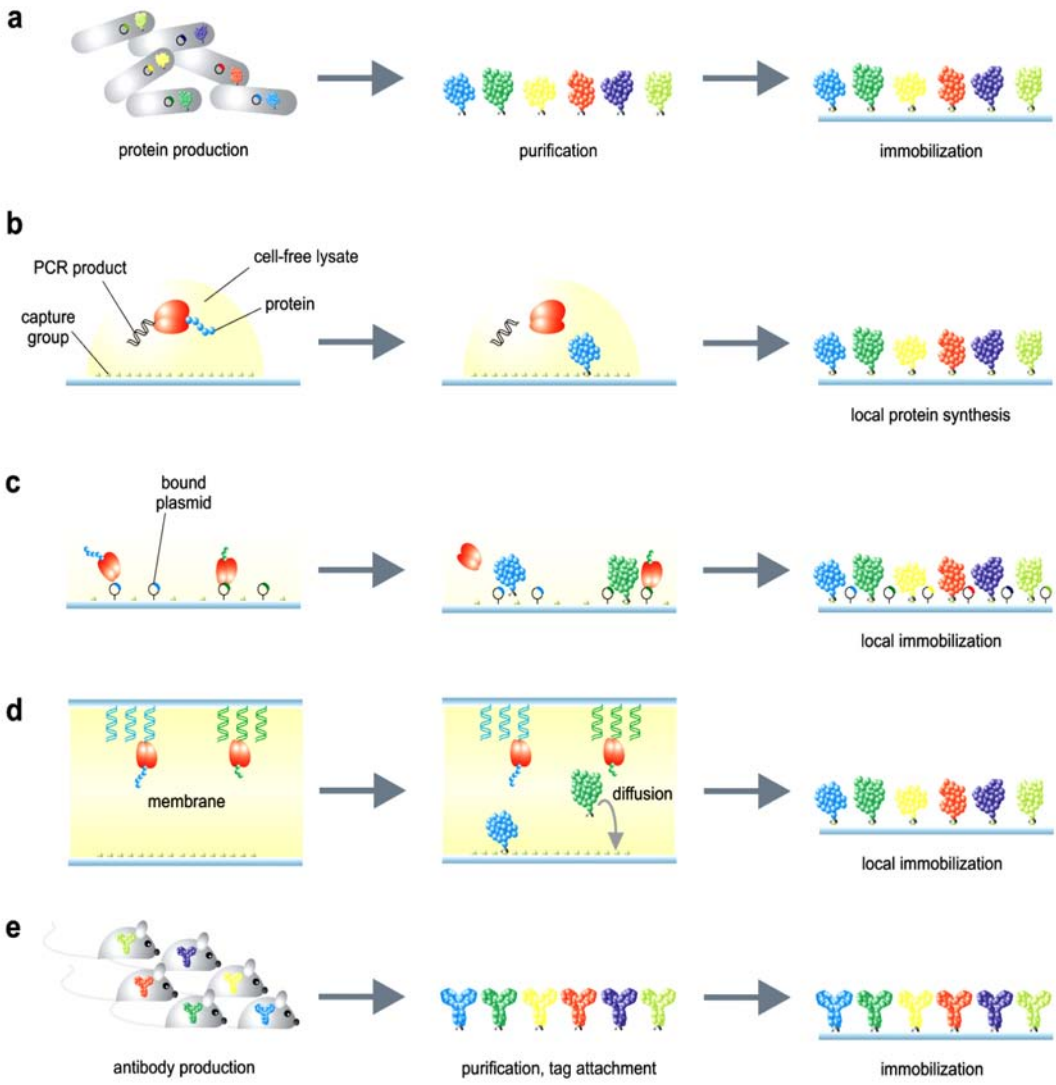

Figure 3

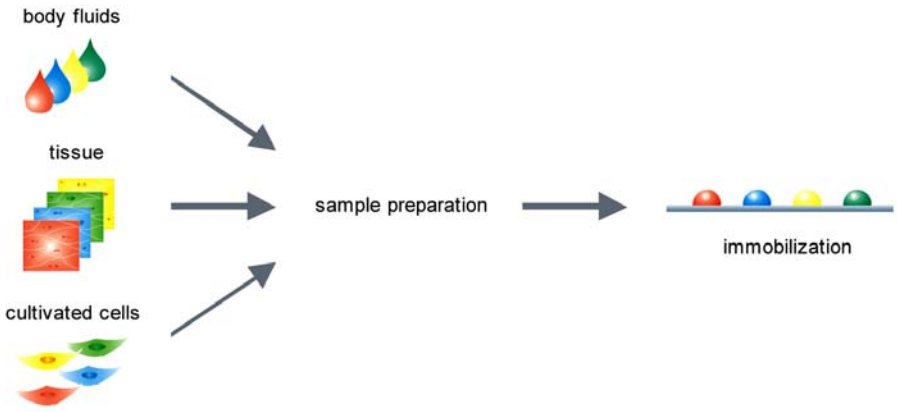

Figure 4

Because the proteins are produced in their natural environments, they *a priori* have the corresponding post-translational modifications and the correct folding. However, to which extend these properties and also the protein quantities are preserved is influenced by the lysate source and the procedures upstream to the arraying process. Espina and co-workers demonstrated that the differential stability of 53 phosphoproteins depends on addition of phosphatase and kinase inhibitors, time, and tissue type [103]. Winters and co-workers showed that the composition of the lysis buffer and the buffer volume can exert critical influence on RPA results [104].

Furthermore, because the cellular compartments are disrupted by the necessary lysis, the detected protein amount may not necessarily be identical to the active protein amount. This applies to proteins that require a translocation, for example, to the nucleus prior to fulfilling their functions. Moreover, it cannot necessarily be discerned between the amounts of protein that are presently active within the cells and that are inactive, because they are in the process of being synthesized or scheduled for degradation. Being aware of these limitations, RPAs nonetheless represent the array format that allows for studying protein occurrence and quantitative changes closest to the situation within the living cells.

Biomedical applications of reverse phase arrays

The biomedical applications of RPAs are defined by their strengths and weaknesses, which result from the design of this array format. Due to the fact that the sample of interest is immobilized on RPAs, these arrays are commonly not applied to assay huge numbers of proteins within one or few biological samples. In this case, protein or antibody arrays would represent the format of choice. In general, RPAs are used, when a limited number of proteins have to be analyzed in a large number of biological samples. A realistic range for the number of pre-selected target proteins is one up to several hundreds. The amount of the biological sample is rate-limiting in this case. It may not be possible to produce a sufficiently high number of array copies from limited resources such as archival tissue specimen for assaying large numbers of individual proteins, because a protein amount equivalent to about 10 cells per spot on the array is required when using such sources [101, 105]. It is, however, well conceivable to produce larger sets of array copies from cultivated cells, which are commonly used as model systems in disease research. Here, a protein amount equivalent to as few as 0.4 cells per spot seems to be sufficient [99, 106].

Legend to Figure 3. Strategies for the synthesis of protein microarrays. a, arrays with pre-synthesized proteins. Recombinant proteins are expressed *in vitro* or *in vivo* (e.g. in *E. coli*) and are tagged. After purification, they are immobilized on a capture array via the tags. b, Protein *In Situ* Synthesis (PISA). PCR products are locally transcribed and translated via cell free lysate into tagged proteins. The tagged proteins are locally immobilized via capture groups. c, Nucleic Acid Programmable Protein Arrays (NAPPA). Plasmid DNA containing the open reading frames for the proteins are immobilized on the array and incubated with cell-free lysates for combined *in vitro* transcription and translation. The tagged proteins are immobilized via capture groups in the vicinity of the bound plasmids. d, DNA Array to Protein Array (DAPA). The protein encoding PCR products are displayed on a separate array. The cell-free protein synthesis is performed in a membrane located between the DNA array and the target matrix for the protein array. Individual tagged proteins are translated within the membrane, which is soaked with cell-free lysate. After diffusion, the proteins are immobilized via capture groups on the target array. e, antibody array. Antibodies are conventionally produced in mice, purified, provided with a tag, and locally immobilized via capture groups on the array.

Legend to Figure 4. Generation of reverse phase arrays (RPAs). RPAs can be generated from different biological samples, such as body fluids (blood, plasma, serum, urine), tissue samples (fresh, paraffin-embedded or frozen tissue), or cultivated cells. The latter can be pre-treated in various fashions, for example with drugs, chemical compounds, antibodies, or siRNAs. The sample preparation depends on the nature of the actual starting material. Immobilization is commonly achieved by local spotting of the prepared samples onto a protein-binding matrix, for example onto nitrocellulose-covered glass slides.

Using whole tissue and cell lysates also sets natural limits regarding the minimal volume to be immobilized, because of increasing viscosity with decreasing volume. This is associated with limitations with respect to the minimal spot size, which is about 85 µm in diameter. Accordingly, with the present techniques densities of about 1600 spots/samples per cm^2 are feasible [99]. Immobilization of the undiluted sample plus four serial dilutions thus means that about 3200 biological samples can be mounted on one slide.

RPAs have successfully been applied for exploring a variety of signaling pathways and biomarkers in particular within the field of cancer research. Grubb III and co-workers screened lysates of 36 microdissected prostate cancer tissue samples with 39 different antibodies and identified potential metastatic and prognostic signatures [107]. In a study published by Kornblau *et al.* leukemia-enriched cells from 256 newly diagnosed acute myeloid leukemia patients were analyzed for a set of 51 proteins [108]. This recovered seven protein signature groups, which correlated with cancer remission, relapse, and overall survival of cancer patients. RPAs were used for profiling of signaling pathways in ovarian cancer [101], phosphoprotein levels in non-small cell lung cancer [109] as well as for the recovery of villin and moesin as potential biomarkers for distinguishing between ovarian and colon cancer [110]. Moore *et al.* included RPAs in their approach, which resulted in the identification of IGFBP2 as a candidate biomarker and potential therapeutic target for high-grade gliomas [111]. Grote *et al.* printed 71 sera and 78 plasma samples from patients with diagnosed pancreatic cancer or chronic pancreatitis as well as from healthy controls and used the RPAs for determining the level of the pancreatic cancer marker CA19-9. The data indicated that RPAs might operate with higher sensitivity compared to standard ELISA techniques [96].

Applied to *in vitro* cultivated cells, RPAs have proven as a valuable tool for drug and drug target discovery as well as for systematic functional genomics approaches. Sevecka and MacBeath used immortalized A431 cells, which were treated with 84 small molecule kinase and phosphatase inhibitors at different concentrations. Consecutively, the effect on 12 different components of the ErbB signaling pathway was examined via RPAs, which allowed for mapping of small molecule actions [99].

Boyd and co-workers examined via RPAs the phosphorylation status of 100 proteins in 30 different breast cancer cell lines, resulting in breast cancer subtype-specific signatures [94]. Further, they used RPAs to monitor effects of siRNA-mediated PTEN knockdown, and of chemical inhibition of MEK1/2, PI3K/mTor and EGFR on 24 selected signaling pathway proteins. This revealed novel pathway connections of the PI3K/Akt signaling [94].

In a recent study, Leivonen *et al.* employed RPA analysis for the identification of estrogen receptor (ER) signaling regulating miRNAs in MCF7 and BT-474 breast cancer cell lines [106]. The cells were transfected with 319 pre-miRNAs, lysed, arrayed and screened for the ERα level, resulting in a total of 1280 data points. This recovered 21 miRNAs, which down regulated ERα and provided potential novel starting points for breast cancer therapy.

Taken together, these examples demonstrate that RPAs offer a convenient tool, when it is aimed at analyzing a limited number of selected target proteins in a larger number of biological samples.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The methods for the construction of peptide arrays have experienced substantial advances in the recent past, suggesting that miniaturized high-diversity chips can be manufactured at affordable costs within the near future. Such peptide chips may substitute protein arrays in certain applications, e.g. for pre-screens of ligands or enzyme substrates, where the construction of protein or antibody arrays might be associated with over-dimensioned efforts for the individual biomedical research laboratory. Protein and antibody arrays are an appropriate tool, when the natural context in terms of conformation and/or post-translational modifications is of importance or a spectrum of analytes has to be detected from a complex biological sample with high sensitivity. These applications define an overlap with reverse phase arrays, which allow detection

of proteins in whole tissue or cell lysates. Depending on the source of the tissue or cells, the reverse phase arrays may be superior to protein arrays, because analyzing the proteins from and within their authentic source grants a higher likelihood that the natural conformation and modifications are preserved. Protein and antibody arrays allow to analyze a higher number of target molecules if the amount of the biological sample is rate-limiting. On the other hand, reverse phase arrays may rather be suitable for analyzing a defined number of target molecules in a large number of biological samples. The specific advantages and limitations of each of the three types of arrays suggest that all of them will find broader future applications in basic biomedical research and diagnostic approaches. Future developments in mass spectrometric quantitative proteomics will probably exert substantial influence on the use of these array formats. There are, however, scenarios visible, where the complementary approaches may converge in the future and initial attempts were already published to merge both techniques [112, 113].

Reverse phase arrays had a remarkable “reverse” development. In first instance primarily designed for diagnostic approaches, they recently have also been applied for systematic functional genomics approaches [110]. Such manipulated cell arrays represent an attractive tool for systematic studies of signaling pathways, miRNA functions, drug actions, and general cellular processes related to human diseases. They further enable drug target and drug discovery approaches that intend to readout protein levels and/or activities within the cellular context without using reporter systems. In conjunction with high-throughput compound screens this may particularly be a powerful technique in cancer and stem cell research.

ABBREVIATIONS

deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), deoxyribonucleic acid (DNA), deoxy-thimidine triphosphate (dTTP), DNA Array to Protein Arrays (DAPA), enzyme-linked immuno-sorbant assay (ELISA), horse radish peroxidase (HRP), nickel-nitrilotriacetic acid(Ni-NTA), Nucleic Acid Programmable Protein Array (NAPPA), open reading frame (ORF), polymerase chain reaction (PCR), polyvinylidene fluoride (PVDF), Protein *In Situ* Arrays (PISA), radioimmunoassays (RIA) reverse phase array (RPA), ribonucleic acid (RNA) Single-nucleotide polymorphism (SNP), tissue microarray (TMA)

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