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The role of peptide microarrays in biomedical research

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Peptide microarrays, comprising hundreds to thousands of different peptides on solid supports in a spatially discrete pattern, are increasingly being used as high throughput screening tools with high sensitivity in biomedical science. The application of this technology to profile the antibodies, enzymes and ligands of target samples including body fluids, tissues and cells results in the discovery of new biomarkers for the precise diagnosis of diseases. Additionally, the identification of inhibitors of enzymes using peptide microarrays might offer an opportunity for the further development of enzyme-targeted drugs in a fast and efficient manner. This review provides an overview of the main peptide microarray formats currently used with their applications in the biomedical field and discusses the current challenges of this technology.

Introduction

Microarray development started at the early 1990s due to the imperative need for global and high throughput techniques for the systematic deciphering of genomic and postgenomic information.[1–3] Peptide microarrays are planar solid substrates (*e.g.*, glass slides) with a collection of microscopic peptide spots and are high throughput devices for various applications in many fields of biochemistry and medicine ranging from basic research to clinical diagnosis.[4–13] For example, a peptide microarray is considered as an ideal platform for studying the functionalities of enzymes and screening their inhibitors and enhancers since the microarray allows for undertaking hundreds to thousands of enzymatic reactions simultaneously.[6,7] Peptide microarray-based immunoassays can provide accurate information for decision-making in clinical situations, and require only a low volume (at the μL level) of patient samples to simultaneously test large numbers of antigen epitopes.[4,8]

In comparison with proteins, peptides are relatively easy to produce, chemically stable, and compatible with various immobilization chemistries. In addition, peptides preserve partial aspects of protein function. Therefore, peptide microarrays enable and support proteomic studies in a reductionist way. As a result, the scope of application of peptide microarrays has been constantly expanding since Frank's group and Fodor's group published the first two papers on the fabrication of peptide arrays in 1991.[1,2] Some of these studies have been reviewed elsewhere with a focus on the fabrication and applications of peptide microarrays or as subclassifications in more generalized overviews of the biological applications of microarrays.[4–14] In this review, we will focus on the applications of peptide microarrays in biomedical areas, including disease-related enzyme function and inhibition, disease biomarker (*e.g.*, antibodies and affinity peptides of viruses) discovery and drug development, highlighting some of their technical challenges and the new trends by means of a set of selected recent publications.

A brief overview of peptide microarray fabrication and detection methods

Peptide microarrays are composed of large numbers of peptides with known specificities, which are either *in situ* synthesized step by step or printed/spotted on suitable solid substrates, such as microscopic glass slides, silicon wafers, and other functionalized substrates.[1–14] Flexible porous substrates, such as cellulose, membranes and cotton are preferentially used for *in situ* synthesis.[15] In the printed/spotted case, peptides are normally synthesized by the Merrifield solid-phase peptide synthesis (SPPS) method[16] and loaded onto the solid substrate by a robotic arrayer. The detailed advantages and drawbacks of these two microarray fabrication procedures have been summarized in a recent review by Mrksich and his colleagues.[14] The surface chemistry plays a critical role in peptide microarray fabrication since the immobilization efficiency, surface density and reaction activity of peptides are defined by the specific chemical groups on the solid substrate and compatibility of the substrate with samples.[17]

Currently, peptide microarrays are mainly fabricated using presynthesized peptides since the quality of presynthesized peptides is higher than that of *in situ* synthesized peptides.[9] Fig. 1 shows the basic fabrication and detection principle of peptide microarrays which are prepared using presynthesized peptides. The presynthesized peptides are first transferred onto

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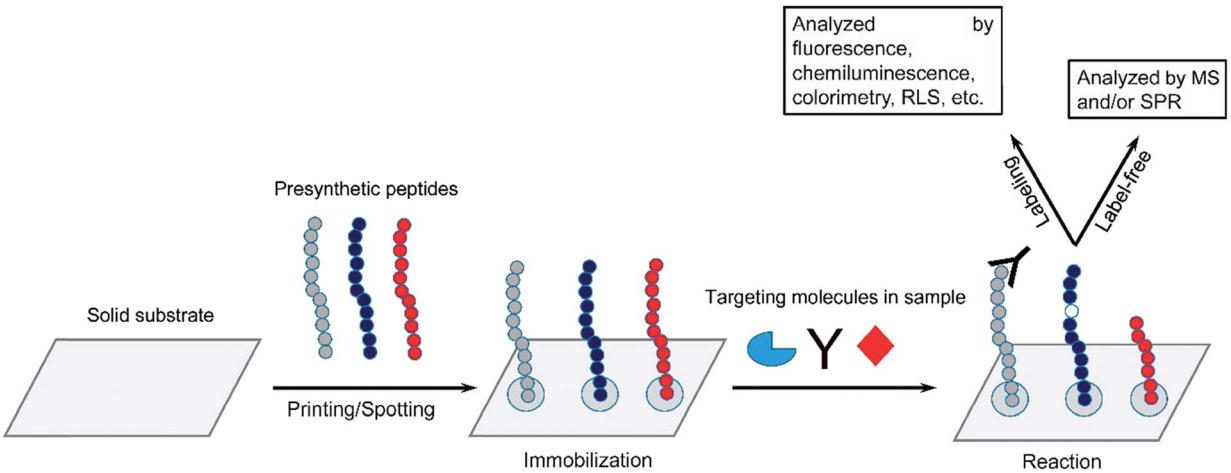

Fig. 1 Schematic representation of the fabrication and detection principle of peptide microarrays which are prepared using presynthesized peptides.

the solid substrate using a robotic arrayer in contact printing mode or non-contact printing mode. The presynthesized peptides can be immobilized on the solid substrate through three general strategies including physical adsorption, covalent reaction and specific biorecognition (biotin–avidin reaction, DNA hybridization, *etc.*). Physical adsorption is the simplest method for immobilizing peptides on a solid substrate. However, physical adsorption is not strong enough to yield stably immobilized peptides capable of withstanding the necessary washing steps and incubation conditions used in biological studies in subsequent reactions. In addition, it is difficult to control the surface densities and orientations of peptides immobilized on the microarray spots through physical adsorption. These phenomena result in a decrease in the reproducibility and sensitivity of peptide microarray-based assays. Covalent reaction is the most common strategy for immobilizing peptides on a solid substrate since the covalent immobilization of peptides on a solid substrate offers high stability and is demonstrated to be quite robust.[4–14] In comparison with the physical adsorption immobilization strategy, covalent binding is normally more complex, sometimes requiring intensive work on modification of the surface of the solid substrate. The covalent immobilization of peptides on solid substrate surfaces is normally based on the reaction of the nucleophilic α-amino group of peptides with the relevant surface functional groups of the solid substrate, such as carboxylate groups, aldehyde groups, epoxy groups, *etc.* In general, the covalent reaction should have a high reaction efficiency and does not affect the structure and properties of peptides while the reaction is employed to immobilize large numbers of peptides in a microarray. Solid substrates functionalized with groups that provide affinity sites for the binding of bio tags have also been used for the specific attachment of peptides.[18–20] For instance, the specific interaction of biotin and avidin/streptavidin has been used for the attachment of biotinylated peptides on avidin/streptavidin-functionalized solid substrates.[18,19] A peptide microarray can also be generated by the hybridization of a collection of peptide–oligonucleotide conjugates to an oligonucleotide microarray.[20] The biorecognition immobilization procedure may be advantageous because of a high binding constant between these molecules. In addition, the surface orientations of immobilized peptides can be finely defined by the biorecognition reaction. Due to the steric hindrance of the heterogeneous reaction, only some of the surface functional groups react with peptides. On subsequent assaying, nonspecific interactions between analytes and excess functional groups on the solid substrate reduce the detection sensitivity and specificity. This is particularly serious in the case of protein targets because they have a large number of amino acid residues. This phenomenon can be eliminated by suitable blocking steps following peptide immobilization.

After the specific reaction with target molecules (*e.g.*, enzymes, antibodies, proteins, *etc.*) in samples, the peptide microarray-based assays are analyzed by both label-dependent methods (*e.g.*, fluorescence,[21,22] chemiluminescence,[21,23] colorimetry,[24] resonance light scattering (RLS),[25] *etc.*) and "label-free" methods (*e.g.*, surface plasmon resonance (SPR)[21,22] and mass spectrometry[26,27]). The vast majority of peptide microarrays are analyzed by fluorescence-based methods because of well-developed laser confocal fluorescence microarray scanners. The peptide microarray-based RLS assays exhibit a relatively higher sensitivity and lower cost than peptide microarray-based fluorescence assays. The SPR readout format can only incorporate the peptide microarrays which are prepared on gold or silver films. In addition, the "label-free" methods circumvent the requirement for labels, which may perturb enzyme activity and enzyme substrate specificity.

Studying the functionality and inhibition of enzymes

It is believed that enzymes (*e.g.*, kinases, proteases, phosphatases, *etc.*) are involved in every cellular process and play a critical role in numerous pathological and physiological processes. In particular, the dysfunction of enzymes is closely related to

numerous diseases such as inflammation, cancer, cardiovascular disease, and diabetes, making enzymes important biomarkers for early disease diagnosis and targets for therapeutic drug development. As important tools for high-throughput screening and multiple analyses, peptide microarrays have gained great success in studying the functionalities and inhibitors of enzymes.[9,28–35] Here, we provide an overview of recent examples of peptide microarray-based assays involved in (i) the determination of enzyme substrate specificity,[36–44] (ii) the detection of enzyme activities in complex biological samples[45–58] and (iii) the screening of enzyme inhibitors.[59–65]

Determination of enzyme substrate specificity

Identification of the substrate specificity of an enzyme can be used to speculate its natural substrate, which is essential for defining its biological function and designing its selective inhibitors.[9] Peptide microarrays have been widely used to determine the specificities of enzyme substrates.[36–44] As early as 2002, Ellman and colleagues fabricated microarrays of peptidyl coumarin substrates for the determination of protease substrate specificity.[36] Using a mass spectrometer as a detector, Mrksich and colleagues developed a label-free peptide microarray for profiling the substrate specificities of several histone deacetylases (HDACs).[38] Schmidt and colleagues employed a peptide microarray-based fluorescence polarization assay to identify the peptide substrates of kinase Myt1, a member of the Wee-kinase family involved in G2/M checkpoint regulation of the cell cycle.[42] They found that several cellular protein-derived peptides can be used as Myt1 kinase substrates. This result may help to clarify potential downstream cellular targets of Myt1 and further define the biological role of Myt1 and other proteins in cellular processes. There is a strong demand for highly efficient techniques that can be used to identify O-GlcNAc transferase (OGT) substrates and study their function because altered O-GlcNAcylation has been demonstrated in cancer, diabetes and neurodegenerative diseases. Recently, Pieters and colleagues developed a peptide microarray of 144 peptides for discovering novel OGT substrates and studying their specificity (shown in Fig. 2).[44] They found that the protein RBL-2 (retinoblastoma-like protein 2), a key regulator of the entry into cell division as well as a tumor suppressor, can be O-GlcNAcylated by three isoforms of OGT. This finding demonstrated that the peptide microarray is a useful tool for both the discovery of novel OGT substrates and for studying OGT specificity.

Detection of enzyme activities in complex biological samples

The systematic study of aberrant enzyme activities leading to distinctive corresponding protein post-translational modification (PTM) patterns would be important for understanding the molecular mechanism of particular disease states. This knowledge is essential for determining disease progression and developing enzyme-targeted therapeutics. The enzymatic activities of different members of the same enzyme family could be studied, both qualitatively and quantitatively, though the microarrays of peptide substrates with enzymatic specificities. In the early 2000s, Mrksich and colleagues first fabricated the

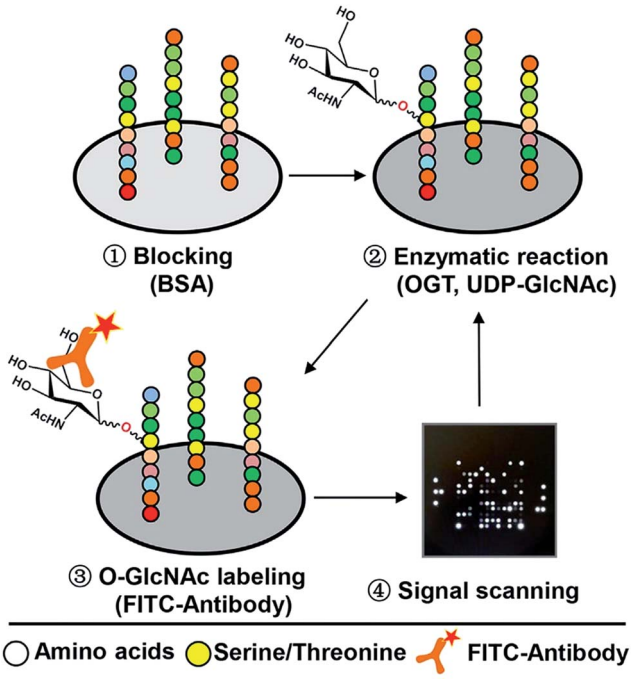

Fig. 2 The schematic depiction of the peptide microarray process for discovering OGT substrates. The microarray is blocked with bovine serum albumin (BSA), followed by the addition of OGT (s-OGT, m-OGT, and nc-OGT), uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), and a fluorescein isothiocyanate (FITC) conjugated antibody. The enzymatic reaction is run by pumping the mixture up and down through a porous Al_2O_3 chip material. Images of the fluorescent signals were generated every 10 min for 4 h (kinetic readout) (reprinted from ref. 44 with permission).

peptide microarray on a gold film and used it for the quantitative evaluation of tyrosine kinase c-Src activity.[45] Yao and colleagues first developed a peptide aldehyde small-molecule microarray (SMM) for profiling the activities of cysteine proteases in fluorescently labeled biological samples, including pure proteins, cellular lysates, and infected samples.[48] Clear signatures were obtained that were readily attributable to the activity of the constituent proteases within the lysates of *Plasmodium falciparum*-infected apoptotic red blood cells and HeLa cells. Using gold nanoparticles as detection probes, we have developed a peptide microarray-based RLS assay for profiling the activity of protein kinase A (PKA) in five cancerous cell lysates including two nerve cell (SHG-44 and PC-12) lysates and three somatic cell (HeLa, MCF-7, and SW-620) lysates and studying chemical-mediated PKA activity fluctuation in living cells.[50] The peptide microarray-based RLS assay has a relatively high sensitivity, and can detect PKA activity in 0.1 μg total cell proteins of the SHG-44 cell lysate (corresponding to 200 cells). Adopted from immunodetection methods, Denu and colleagues developed a peptide microarray-based dual-channel fluorescence assay for identifying altered histone acetyltransferase (HAT) activity in prostate cancer through printing cellulose-conjugated peptides on nitrocellulose-coated microscopic slides.[54] The experimental result revealed upregulated HAT activity against specific histone H3 sites in a castration-resistant

prostate cancer cell line compared to its hormone-sensitive isogenic counterpart. This finding uncovered a distinct molecular pathway in the progression of castration-resistant prostate cancer, which may help to develop personalized medicine for its treatment by inhibiting the HAT activity. Very recently, we proposed a fluorescence resonance energy transfer (FRET)-peptide microarray-based metal enhanced fluorescence (MEF) assay for the sensitive profiling of the activities of five matrix metalloproteinases (MMPs) on a novel Au/Ag@SiO_2 substrate *via* fluorescence recovery by the MMP cleavage of quenched peptide motifs, being further enhanced by MEF (as shown in Fig. 3).[55] Using papillary thyroid carcinoma (PTC) patients as a typical example, the relationship between the MMP activity pattern and progression of PTC is evaluated by the FRET-peptide microarray-based MEF assay, and the obtained result is comparable with the result of the pathological analysis. In the light of its throughput, robustness and sensitivity, the FRET-peptide microarray-based MEF assay can further be used to determine the clinical utilities of aberrant MMPs activities for the early diagnosis of aggressive cancers. Using a commercial protein tyrosine kinase (PTK) peptide substrate microarray (PTK PamChip® 4 microarray[56,57]), Arni and colleagues successfully analyzed PTK activities in different tissue lysates of lung adenocarcinoma (LuAdCa) resection specimens.[58] This result demonstrated that peptide microarrays could be used for the discovery of molecular prognostic signatures for LuAdCa as well as the identification of potential novel targets for future anti-lung cancer therapies.

Screening of enzyme inhibitors

The high throughput nature of the peptide microarray makes it an ideal platform for screening the inhibitors of enzymes.[59–65] We developed a peptide microarray-based spectroscopic assay with two readout principles, fluorescence and RLS, for screening kinase inhibitors from a commercial inhibitor library, a collection of 80 kinase inhibitors.[61] 11 inhibitors (2 for PKA (protein kinase A) and 9 for LCK (leukocyte-specific protein tyrosine kinase)) were defined. On combining the self-assembled monolayer desorption ionization (SAMDI) technology and the peptide microarray method, Scholle and colleagues discovered a novel, specific inhibitor of sirtuin 3 (SIRT3) from a 100 000-compound library.[62] Very recently, we developed a biotinylated peptide microarray-based fluorescence assay for evaluating MMP inhibitors on poly(glycidyl methacrylate-*co*-2-hydroxyethyl methacrylate) (P(GMA–HEMA)) brush modified glass slides through the recognition of biotin with Cy3 modified avidin (Cy3-avidin) (as shown in Fig. 4).[65] In the proof-of-principle experiment, 9 known MMP inhibitors against two MMPs (MMP-2 and MMP-9) were selected to demonstrate the practicability of the assay. The inhibition results of MMP activities both *in vitro* and *in vivo* by selected inhibitors demonstrate that the proposed assay can be used to not only screen the inhibitors of MMPs, but also quantitatively analyze the inhibitory potencies (IC50) of the inhibitors. These results suggest that peptide microarrays have great potential in the discovery of enzyme-targeted drugs.

The accessibility of the immobilized peptide to the enzyme plays a critical role in the enzyme function and inhibition study because there is an intrinsic steric hindrance between the active site of the immobilized peptide and the catalytic pocket of the enzyme. This potential problem can be circumvented through the insertion of a spacer (*e.g.* inert linear molecules) between the active motif of the peptide substrate and the surface and/or the immobilization of the peptides on a solid support with a hierarchical nanostructure (also known as three dimensional (3D) microarray substrates).

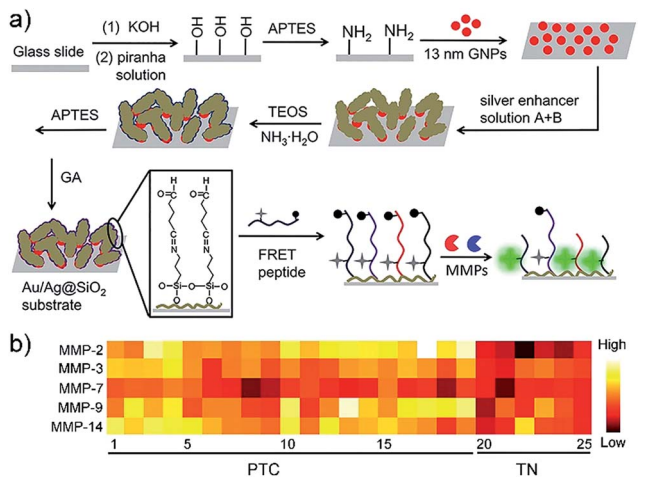

Fig. 3 (a) Preparation of the Au/Ag@SiO_2 substrate and the principle of the FRET-peptide microarray-based MEF assay for multiple profiling of MMP activities. (b) MMP activity profiling of crude thyroid tissue samples. 19 thyroid tissues of papillary thyroid carcinoma (PTC) patients and 6 thyroid tissues of thyroid nodule (TN) patients were collected using standard surgical procedures (reprinted from ref. 55 with permission).

Peptide microarray-based immunoassays

Autoantibodies are a hallmark of autoimmune diseases such as lupus and have the potential to be used as biomarkers for diverse diseases, including immunodeficiency, infectious diseases, and cancer. More precise detection of antibodies with specific targets is needed to improve the diagnosis of such diseases. Peptide microarrays have been utilized for the mapping of antibody binding sites at the beginning of their development. Some early research studies were summarized in previous reviews.[66,67] During the past 15 years, peptide microarray-based immunoassays have been further adopted for profiling antibodies in humoral samples, such as cerebrospinal fluid (CSF) and serum, studying antibody responses to host proteins after immunotherapy and detecting allergen-specific immunoglobulins in food.[67–87] For instance, Glocker and colleagues fabricated a series of peptide microarrays for displaying the epitope diversities of antibodies.[68–70] They demonstrated that peptide microarrays display epitopes with secondary structure conformations. Intrathecal immunoglobulin G (IgG) synthesis and oligoclonal IgG bands in CSF are

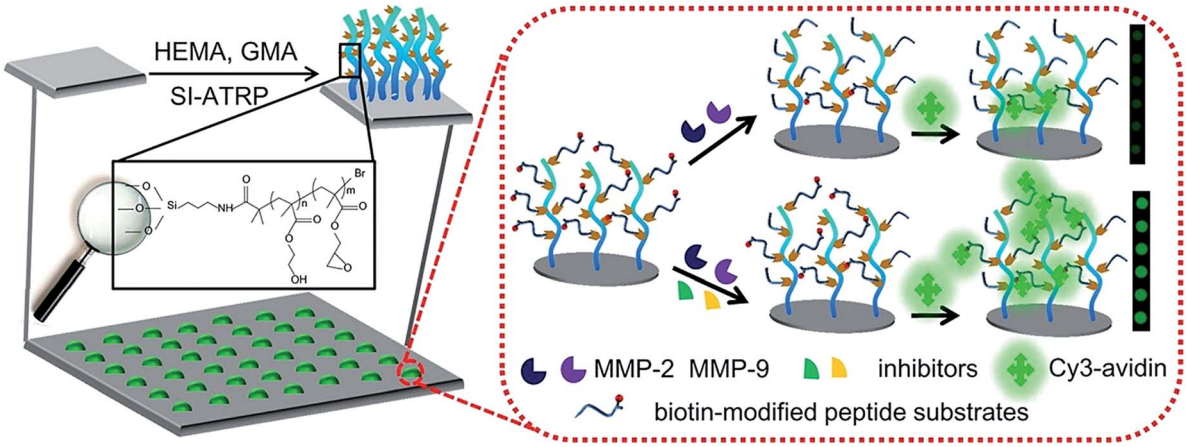

Fig. 4 Schematic representation of the peptide microarray-based fluorescence assay for MMP activity and inhibition analysis on a polymer brush substrate (reprinted from ref. 65 with permission).

hallmarks of multiple sclerosis (MS), but the antigen specificities remain enigmatic. Hecker and colleagues first investigated the autoantibody repertoire in paired serum and CSF samples from patients with relapsing-remitting MS (RRMS), primary progressive MS (PPMS), and other neurological diseases with the use of a high-density microarray with 3991 peptides including selected viral epitopes.[72] They found 54 peptides to be recognized significantly more often by serum or CSF antibodies from MS patients compared with controls (p values < 0.05). The results for RRMS and PPMS clearly overlapped. However, PPMS patients presented a broader peptide-antibody signature. The reported data confirmed several known MS-associated antigens and epitopes, and they delivered additional potential linear epitopes. Recently, Ayoglu and colleagues employed different peptide microarray formats to characterize autoantibody repertoires and identify new autoantigens of autoimmune diseases (as shown in Fig. 5).[74] Using an unbiased and untargeted strategy, the plasma and serum samples from multiple sclerosis patients, narcolepsy patients and healthy persons were profiled on three different peptide microarray formats: a high-density peptide microarray of 2.2 million overlapping 12-mer peptides representing the protein products of all human protein-coding genes, a targeted peptide microarray with 174 000 12-mer peptides of single amino acid lateral shift, and a bead-based peptide microarray. The result revealed different IgG reactivity patterns between and within the narcolepsy and multiple sclerosis sample groups. They also demonstrated two potentially new autoantigens: MAP3K7 in multiple sclerosis patients and NRXN1 in narcolepsy patients. Interestingly, Wang and colleagues reported a reusable giant magnetoresistive (GMR) nanosensor microarray with *in situ* synthesized peptides for the sensitive detection of antibodies binding to linear peptides with a resolution of a single post-translationally modified amino acid.[87] The GMR nanosensors added functionality to the microarrays, including kinetics monitoring and quantitative measurement. Regeneration of microarrays combined with the portability of the GMR nanosensor could further facilitate the measurement of antibodies in point-of-care (POC) settings.

Screening affinity agents for viruses

Viruses, such as influenza, Zika virus and Ebola virus (EBOV) are major threats to public health. Peptide microarrays are a potential tool for the complete characterization of antibody specificities associated with virus or bacterial infection.[88–97] Barouch and colleagues developed a global HIV-1 peptide

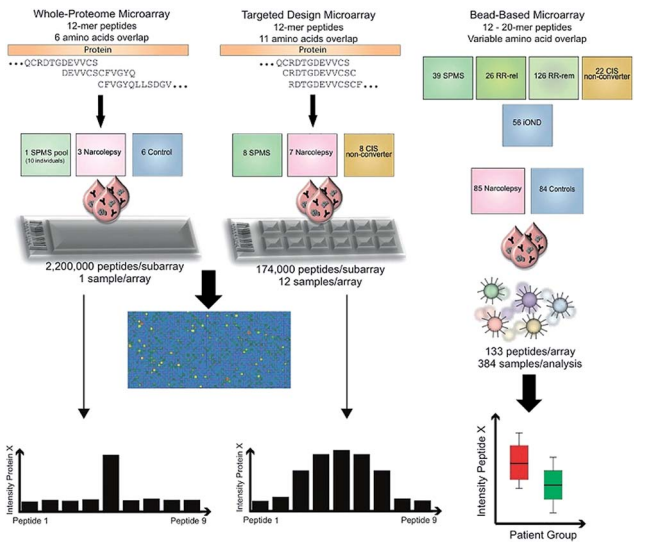

Fig. 5 Overview of the study design with three different peptide microarray platforms. The whole-proteome peptide microarrays were utilized as an initial screening platform and consisted of 2.2 million peptides representing the whole human proteome in 12-mer peptides with a six amino acid overlap. Peptides showing differential plasma/serum reactivity between the SPMS plasma pool, the narcolepsy samples, and the control samples were then selected to be analyzed on the arrays with a targeted design. Here the selected 12-mer peptides had an overlap of 11 amino acids. Because of the smaller number of peptides on the arrays (174 000 peptides), more samples could be analyzed, including samples from SPMS, narcolepsy, and CIS patients with no conversion to MS. Peptides that once again revealed differential reactivity among the groups were selected for further analysis on a bead-based array format, where a higher sample throughput could be achieved (reprinted from ref. 74 with permission).

microarray containing 6564 peptides from across the HIV-1 proteome and covers the majority of HIV-1 sequences in the Los Alamos National Laboratory global HIV-1 sequence database, which was used to quantify the magnitude, breadth, and depth of IgG binding to linear HIV-1 sequences in HIV-1-infected humans and HIV-1-vaccinated humans, rhesus monkeys and guinea pigs.[93] The results suggest that the global HIV-1 peptide microarray may be a useful tool for both preclinical and clinical HIV-1 research. With the aid of the computationally predicted Zika virus NS1 protein putative antigenic region, Cretich and colleagues developed a microarray by combining a limited set of readily accessible linear peptides belonging to the Zika virus NS1 protein putative antigenic region.[97] The method is able to differentiate Zika infected individuals from healthy controls. This may circumvent the requirement to build high-throughput screening platforms of large-scale linear and mixed peptide libraries, a severe limitation of current strategies. It paves a new path for the further development of simple and low-cost peptide microarray-based diagnostic immunoassays. Although these antibodies are extensively used for virus detection, the yield and stability of the antibody are relatively low. It is highly desirable to produce alternative affinity ligands for research, diagnostics, and therapeutics of virus-related epidemics. Synthetic biomolecules such as aptamers and/or peptides are ideal alternative viral-binding agents. Diehnelt and colleagues developed a peptide microarray with 10 000 peptides for identifying influenza-binding peptides (as shown in Fig. 6).[98] In this method, candidate peptides were obtained by whole virus screening against the peptide microarray. Subsequently, the candidate peptides were conjugated with bis-maleimide peptide scaffolds to yield a high-affinity synbody to influenza. A specific synbody to the 1934 H1N1 virus was found, which bound H1N1 with a dissociation constant (K_D) of <1 nM, comparable to that of a monoclonal antibody for neuraminidase (NA). This approach is simple and rapid and offers an efficient tool for developing viral-binding agents. The Diehnelt group further employed this approach to screen the peptide binders of the Ebola Zaire (EBOV) virus glycoprotein.[99] They discovered two synbodies with K_D < 200 nM for the large viral glycoprotein.

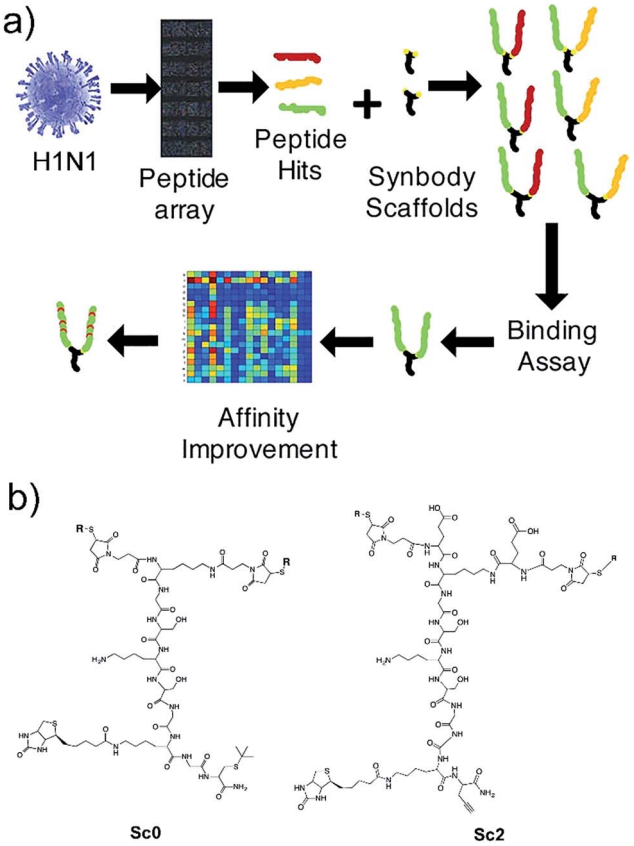

Fig. 6 The schematic representation of the synbody development process (a) and the structure of scaffolds (b) used to construct influenza synbodies (reprinted from ref. 98 with permission).

Studying and identifying ligands for cell behavior

Cell behavior *in vivo* is normally guided by the extracellular microenvironment which contains many different biomolecules, such as extracellular matrix (ECM) proteins, growth factors, and proteoglycans. For example, the cancer cell transformation and metastasis are strongly dependent on the tumor microenvironment. Controlling the interactions of cells with various components of the microenvironment will be invaluable in developing new diagnostic and therapeutic strategies for various diseases including cancer and tissue damage.[100–102] However, it is difficult to decipher specific interactions of ligands with cellular receptors because there is a dynamic and multivariate presentation of many signalling molecules on the cellular surface. As early as 2001, Lam and colleagues first tried to use peptide microarrays to study cell adhesion.[100] Recently, Zhang and Kilian developed a peptide microarray strategy using copper catalyzed azide–alkyne cycloaddition chemistry to immobilize mixtures of different peptide ligands on a gold surface.[101] Using matrix assisted laser desorption ionization (MALDI) mass spectrometry as the detection method, the peptide microarray was employed for investigating the combinatorial effects of three peptides including a cell adhesion peptide (YIGSR), a bone morphogenetic protein (BMP) growth factor derived peptide (KPSSAPTQLN) and a heparin binding peptide (KRSR) on the adhesion characteristics of two cells, mouse embryonic fibroblasts (MEFs) and adipose derived stem cells (ADSCs). They demonstrated that BMP-7 derived peptides alone or in combination with ECM adhesion peptides can enhance the expression of the osteogenic markers Runx2 and osteopontin of ADSCs. Subsequently, the Kilian group developed a versatile peptide microarray with 78 peptide combinations derived from proteins present in the melanoma microenvironment for screening cancer cell phenotypic changes in response to ligand–receptor interactions (as shown in Fig. 7).[102] They identified a proteoglycan binding and bone morphogenic protein 7 (BMP7) derived sequence that selectively promotes the expression of several putative melanoma initiating cell markers, suggesting that proteoglycan binding

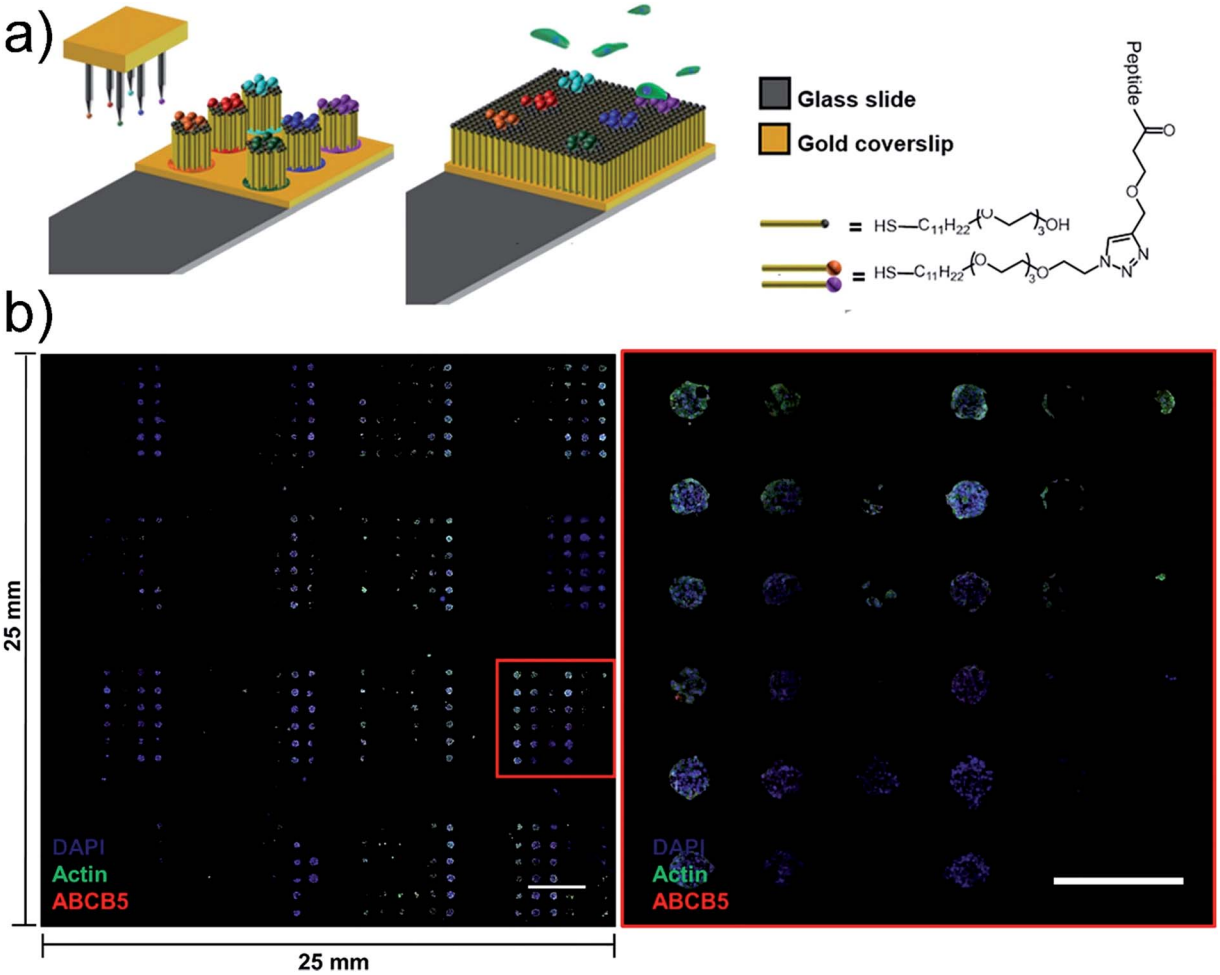

Fig. 7 Schematic for generating peptide microarrays. An OmniGrid microarray spotter deposits nanoliters of a spotting solution containing EG3-terminating alkanethiols, and peptide-terminating alkanethiols onto a gold surface. A background EG3-terminating alkanethiol passivates the nonspotted regions, and seeded cells adhere only to peptide-terminated regions of the self-assembled monolayer (a). Representative image showing B16F0 melanoma cells adhering to the array (b) and a representative subarray (inset). Scale bar is 1500 and 700 μm for inset (reprinted from ref. 102 with permission).

can modulate cell adhesion through the Smad2/3 signaling pathway. These results demonstrate that the peptide microarray-based approach can be used to explore relationships between matrix signals and cell metastatic behavior.

Screening therapeutic peptides

Peptide microarrays have also been used for screening therapeutic peptides for different diseases.[103–110] Svarovsky and Gonzalez-Moa fabricated a high density peptide microarray with 10 410 presynthesized 20-mer random sequence peptides for screening bacterial binding peptides.[103,104] They demonstrated that bacterial lipopolysaccharides (LPS) are the major targets of the bacterial binding peptides. Importantly, the bacterial binding peptide–nanoparticle conjugates show potent antibacterial agglutination activity. This result suggests that the as-prepared peptide microarray is general enough to potentially create antimicrobial agents to virtually any pathogen.[103,104] More specifically, peptide microarrays have been used with success to identify ligands for short RNA hairpins. In 2012, Shin and colleagues utilized a peptide microarray-based approach to profile the binding affinities of six RNA hairpins including IRES, RRE, TAR, 16S-rRNA, IRE and TS-mRNA with 111 amphiphilic peptides.[107] The peptides adopt a helical structure and have been shown to bind tightly to hairpin RNAs.[108] After modification of the hydrazide group at their C-terminals, the peptides were immobilized on an epoxide-derivatized glass slide through the covalent reaction of hydrazide with epoxide. In this study, the authors designed the peptide library based on a 16-mer peptide (peptide sequence of LKKLLKLLKKLLKLKG), which was *de novo* designed to bind to calmodulin and later shown to bind to hairpin RNAs with high affinity. They found that the peptide (peptide sequence of LKKLLKLLKKLLKLKG-NH_2) has high binding affinity for TAR RNA, efficient cell permeability and strong inhibition of TAR-Tat protein interaction. In a follow-up study, Shin and colleagues constructed a peptide microarray with 185 peptides for evaluating the peptide binding properties of pre-miRNA-155, one of the most potent oncogenic miRNAs

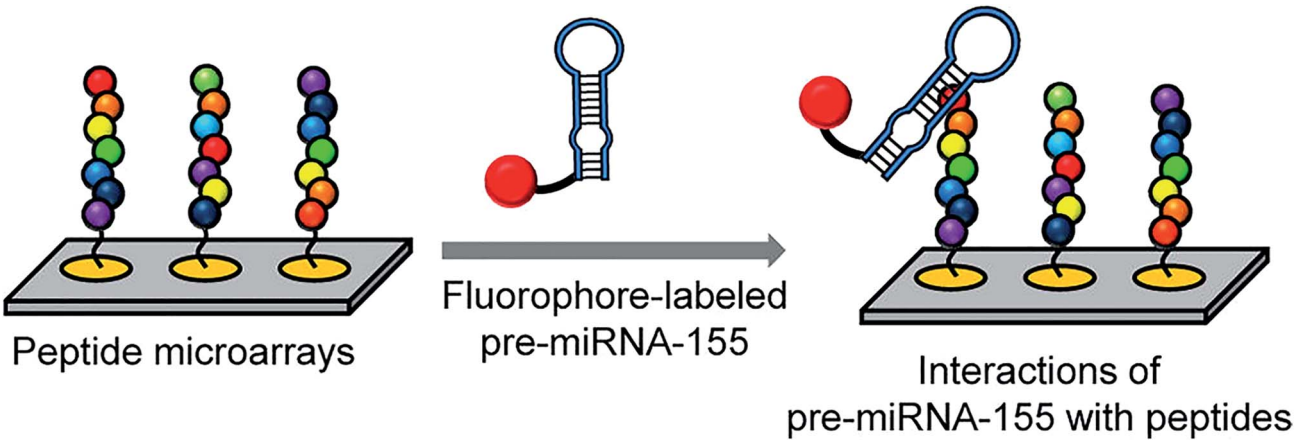

Fig. 8 Peptide microarrays for studies of interactions of stem-loop pre-miRNA-155 with peptides. Peptide microarrays immobilized by various peptides are probed with fluorophore-labeled pre-miRNA-155 (reprinted from ref. 110 with permission).

(as shown in Fig. 8).[110] Two miRNA-155 binding peptides were identified by the peptide microarray. The cell experiment further demonstrated that the miRNA-155 binding peptides promoted apoptotic cell death *via* a caspase-dependent pathway. These results indicate that peptide microarrays can be used to identify miRNA-targeted ligands/inhibitors in a rapid manner.

Conclusions and outlook

After development for nearly thirty years, peptide microarrays have been extensively used in various areas of biochemistry and medicine for identifying substrate specificities of enzymes, simultaneously determining multiple enzyme activities in complex biological samples, screening enzyme inhibitors, mapping epitopes, analyzing the antibody profiles in human serology and studying the interactions of cells with various components of their microenvironment. The study of enzyme functionality and inhibition provides valuable information for the design of enzyme-targeted drugs and development of new therapeutic strategies. In particular, peptide microarrays can break down a polyclonal immune response into a singular response, *i.e.* monoclonal antibody specificities, and discriminate even subtle differences in antibody abundance and specificity. Therefore, peptide microarrays may be one of the most promising tools for discovering diagnostic antibodies which can be used as biomarkers to identify patient subgroups in autoimmunity, allergy, infection and even cancer. Furthermore, serological biomarkers could be used to evaluate the severity of diseases and assist in medical decisions.

In comparison to commercially available and widely used oligonucleotide microarrays, peptide microarrays are primarily used as research tools. Currently, although a few peptide microarrays are commercially available, they still face many challenges in practical applications. For instance, it is impossible to optimize solid substrates and detection methods of peptide microarrays for their broad range of applications. It is necessary to prepare specific 'inert' surfaces and develop efficient blocking strategies for each of these diverse applications, because nonspecific reactions/interactions of peptide microarrays with non-target components in the sample often lead to false positive and negative results. Peptide library construction is the most basic strategy for the selection of biomolecule recognitions (*e.g.*, screening specific peptide substrates of an enzyme, studying the interactions of peptides with other biomolecules) on the microarray. Peptide libraries normally comprise either peptides derived from natural proteins (*e.g.*, protein substrates of enzymes and antigens) or combinatorial and randomly generated peptides. Therefore, designed peptides may represent a key point in the advancement of the construction of peptide libraries. The reasonable use of computational and bioinformatics tools (*e.g.*, statistical analyses and theoretical simulations) can not only reduce the costs of peptide libraries but also improve the screening efficiency. In addition, short peptide sequences usually have a linear rather than a 3D structure and are rarely capable of intramolecular interactions. This phenomenon results in the fact that the interactions of immobilized peptides with other biomacromolecules (*e.g.*, antibodies and cell-surface receptors) are strongly dependent on the location of key epitope residues in peptide sequences and the peptide surface orientation on the solid support. Therefore, simple physisorption is an unfavourable peptide immobilization method for developing peptide microarray-based immunoassays. High apparent affinities of antibodies and/or ligands can be achieved through the covalent immobilization of peptide probes along with a free amino or carboxy terminus and unrestrained key epitope residues, resulting in high sensitivity and selectivity of the immunoassay. Traditional microarrays are normally analyzed by fluorescence imaging, which involves the fluorescence labeling of peptides or antibodies. The labeling procedure is manual and tedious, resulting in the greatly increased cost of the peptide microarray-based assay. Recently, label-free imaging techniques including SPR imaging and MS imaging have matured. Integration of label-free peptide microarrays with microfluidic devices has become a current trend in developing lab-on-chip devices for hands-free, ultra-high throughput studies of enzyme functionalities and drug discovery. In addition, the detection sensitivity

of peptide microarrays can be improved significantly when nanoscale materials are used for modifying solid substrates and/or labeling biorecognition/bioreaction events. With the increasing interest in high throughput, automation and miniaturisation in both basic research in life science and clinical analysis, peptide microarrays should become one of the most important tools in bioanalysis and biomedicine. This will eventually lead us to a new era of "personalized medicine."

Conflicts of interest

There are no conflicts to declare.

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