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Progress in miniaturization of protein arrays—a step closer to high-density nanoarrays

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Protein microarrays is a technology with great promise for high-throughput proteomics. Designing high-performance protein microarrays for global proteome analysis has, however, turned out to be challenging. To this end, major efforts are under way to design novel array formats capable of harboring the tremendous range of probes required to target complex proteomes composed of more than 10 000 analytes. By adopting nanotechnology, the first generation of miniaturized nanoarrays has recently emerged, which opens up new avenues for global proteome analysis and disease proteomics. This review describes the progress and key issues in designing miniaturized protein arrays.

Introduction

Protein microarrays is a new technology with great promise within proteomics [1–6], where the need for multiplexed protein measurement technologies is significant [1,7,8]. Monitoring numerous proteins simultaneously is desirable for biological discovery with proteins since they constitutively function within pathways and networks, and so on [7,9–11]. Detecting protein expression signatures, or protein atlases, instead of single proteins could provide unique information about intricate events occurring at the molecular level in both health and disease, providing novel opportunities for protein network modeling, disease diagnostics, and patient stratification [1,7–11].

Miniaturization of protein arrays

The current concept of designing and fabricating protein arrays is based on the arraying of small amounts (pL scale) of individual proteins, for example antibodies or antigens, carrying the desired specificity/biological function in discrete positions (<300 μm sized spots) in an ordered pattern, a microarray, onto a solid support where they will act as specific probes for the target analytes [3–5,8] (Table 1). Such microarrays, often less then 1 cm^2 in size, have been fabricated harboring anything from a few up to about 1000 proteins, at a density <2000 probes/cm^2. In some cases, larger arrays that composed of about 10 000 proteins have been manufactured. The microarrays are then incubated with small amounts of sample (μL scale), for example labeled serum, whereafter specifically bound analytes are detected and quantified, using mainly fluorescence as the detection method. Assay sensitivities in the pM to fM range have been observed. Finally, the microarray signals are transformed into protein expression profiles, or protein atlases, revealing the composition of the analysed proteome.

To date, protein microarrays, and in particular antibody microarrays, have been successfully used for a variety of applications, such as autoantibody profiling, antibody response profiling, identification and detection of bacterial and protein analytes, as well as disease proteomics with a clear focus towards oncoproteomics [12–16], for review see [1–4,8,17]. In these efforts, low-to-medium density antibody microarrays (<1000 probes/array) and low-to-high-density protein arrays (<10 000 proteins/array) have been used [1,3–5,8]. Albeit successful, these examples illustrate one of the key issues in designing protein microarrays for global proteome analysis, namely that of designing protein array format(s) harboring sufficient number of probes [3–5,8,18]. A complex proteome, for example a cell or tissue lysate, is anticipated to be composed of >100 000 proteins, highlighting the need for mega-dense arrays to be fabricated (>10 000 probes/array) [5,7,8,18]. To accommodate such array densities, nanotechnology-based approaches [18–21] must be adopted to generate nanosized features (nm range) in miniaturized arrays (mm^2 scale) at a high density (>50 000 features/mm^2) (Table 1). The main reasons for

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TABLE 1

Comparison of parameters for protein microarrays vs. protein nanoarrays

Parameter	Microarray	Nanoarray
Spot (feature) size	100–300 μm	3–1000 nm
Array density (probes/mm^2)	<2000/cm^2	<1 × 10^6/mm^2
Total no. of unique probes	<1000 (normal) <10 000 (rare)	<10 (normal)[a]
Array size	cm^2 range	mm^2 range

[a] As mainly proof-of-concept studies have been generated, the total number of unique probes is still low, and thus not representative for the inherent capacity of the nanoarray design. The anticipated value is >10 000.

the currently observed limitation in array density include the lack of large enough numbers of high-performance probes, lack of substrates enabling non-purified probes to be directly applied, speed of the dispenser, spot size, as well as lack of robust nanotechnology approaches enabling us to extend the current microarray format into nanoarrays [5,8,18–21]. In the following, we will discuss the current status of the protein array technology focusing on recent technological advances and key issues in the process of evolving high-density miniaturized nanoarrays.

Fabrication and functionalization of nanoarrays

The process of fabricating and functionalizing high-density protein nanoarrays in an efficient manner will be a key challenge. A variety of tentative techniques are available for generating nanopatterns with biological functions (for review see [21,22]), such as soft-lithographic techniques, microcontact printing (muCP), nanoimprint lithography (NIL) [23], nanosphere lithography (NSL), electron beam lithography (EBL) [24–26], focused ion-beam lithography (FIBL), conductive atomic force microscopy (c-AFM) [27], dip-pen nanolithography (DPN) [28–31], native protein nanolithography [32] as well as nanodispensing [33–35] (Table 2). While these techniques can be applied to generate patterns of nanosized features, for example vials, spots or wires, the issue of functionalizing the individual features with different proteins is a key issue that remains to be resolved. Initially, in many cases, this critical step has been circumvented by functionalizing all features with the same protein probe. However, designing multiplexed setups will require each feature to be individually addressed/functionalized with different probes, a task that has so far been accomplished in only a few cases. Further, additional key issues, such as printing time, scaling-up, and compatibility with printing reagents (e.g. PBS, to avoid the impairing effects associated with clogging due to buffer evaporation) are also crucial features that have not yet been addressed.

Nanopatterning techniques—proof-of-concept

DPN has been used to generate protein arrays with features often smaller than 100 nm [28–31] (Table 2). Interestingly, DPN [31] and electrochemical DPN [28] have been used to immobilize his-tagged proteins on nickel surfaces, paving the way for affinity-based immobilization of the probes, a highly attractive feature when designing protein arrays [5,8,28]. While DPN could possibly be used to generate arrays containing several different probes, the issues of speed, reagent compatibility and scaling-up are still limiting factors that need to be considered. In comparison, Gu *et al.* have presented another novel approach to fabricate nanoarrays, on the basis of avidin-binding to templates generated by c-AFM on oligo(ethylene glycol) (OEG)-terminated monolayers on silicon substrates [27]. In parallel, an alternative AFM-based approach, native protein nanolithography, has recently been developed, which may be used for writing, reading, and erasing of protein arrays down to 50 nm resolution [32].

Ultra-small features <3 nm in diameter, composed of atomic clusters, for example gold clusters, on surfaces which would enable the binding of single proteins, has been suggested as one viable approach to generate nanoarrays (Table 2) [36]. In comparison, Hoff *et al.* have produced nano-sized features (10 nm) by adopting a NIL approach [23]. A different methodology to fabricate nanoarrays was proposed based on adopting plant viral capsides as nanobuilding blocks [37]. In this study, the authors used the virions of Cowpea mosaic virus (CPMV), which have a diameter of about 28 nm, as programmable nanobuilding blocks for functional devices at the nanoscale. Going from more conventional planar array designs to alternative layouts, a 3D protein nanopatterning method has been devised, based on local activation of porous silicon by electron beam lithography [24]. The increased surface area provided by sponge-like substrates opens up new possibilities to fabricate nanosized features with increased amounts of immobilized probes, a crucial feature for improving the sensitivity of any array setup [4,5,8].

Nanodispensing—a viable approach?

In some cases, a nanodispenser has been designed and used to fabricate nanoarrays (Table 2) [33–35]. Bruckbauer *et al.* have used an in-house designed nanopipette to dispense probes into individual vials (about 300 nm in diameter) on nanostructured surfaces [33]. Another nanodispenser, the Nano eNabler system, is a surface-patterning tool, based on microcantilever-based microfluidic devices. It is capable of printing ≥250 nm sized spot features [34,35] and now commercially available (http://www.bioforcenano.com). Although these setups are promising and readily allow individual features to be functionalized with different probes, it could be argued whether such direct spotting is compatible with the logistics of fabricating high-density arrays, irrespective of working in the micro or nano format [5,8].

Novel approaches-fluidics, self-addressing and self-assembly

Another way of functionalizing nanoarrays could be to adopt nanofluidic approaches (Table 2) [18,38–42]. Recent work has shown that protein microarrays can be designed to be compatible with self-regulating microfluidic networks [38,39,42], which open up new avenues for probe and sample loading. Two other approaches (Table 2), which have been validated in the microarray format, could be adapted for the use in nanoarray format: self-addressable [43,44] or self-assembling protein arrays [45–47]. For the former, the probes, are equipped with unique zip-code tags. They are then simply added to the chip in bulk solution, and are guided to their spot(s) on the chip by the tag. In the latter case, the proteins are expressed directly on the chip in the desired spot, using cell-free expression. Although promising, additional work will be required to adapt these methodologies to the nanoarray format.

TABLE 2

Overview of tentative techniques for generating nanopatterns with biological functions

Technique	Comments/Refs
Nanopatterning	For review see [21,22]
Soft-lithographic techniques	
Microcontact printing (muCP)	
Nanoimprint lithography (NIL)	10 nm sized dots [23]
Nanosphere lithography (NSL)	
Electron beam lithography (EBL)	100 nm sized features [26]. 200 nm–5000 μm sized vials [25] 3D nanopatterning, based on local activation of porous silicon [24]
Focused ion-beam lithography (FIBL)	300 nm sized features [33]
Conductive atomic force microscopy (c-AFM)	[27]
Atomic clusters	<3 nm sized features [36]
Nanopatterning and/or probe deposition	
Dip-pen nanolithography (DPN)	<100 nm sized features [28–31]
Programmable nanobuilding blocks	Based on 28 nm sized features generated by nanobuilding blocks [37]
Native protein nanolithography	Can be used for reading, writing and erasing protein arrays [32]
Probe deposition	
Nanodispensing	Direct dispensing
Nanopipett	Nanopipett capable of dispensing into ∅ 300 nm sized vials [33]
Nano eNabler	Printer, based on microcantilevers, generating >250 nm sized spots [34,35]
Nanofluidic	[38–42]
Self-addressable probes	The probes can be added in bulk, and they will find the way to their unique spot(s) on the chip on their own via a zipcode–anti-zipcode system [43,44]
Self-assembling protein arrays	The probes can be expressed in the desired spot directly on the chip by using cell-free expression [45–47]

TABLE 3

Overview of first generations protein nanoarray designs

Design	Features (features size/array densities/proof-of-concept applications)	Refs
Planar arrays	From 10 to 350 nm features, up to ∅ 2 μm sized dots 1×10^4 dots/mm^2 to 1×10^6 spots/mm^2 Detection of antigen (antibody arrays). Analysis of protein interactions (protein arrays)	[23,26,28–31]
Well-based arrays	6 to 8 nL reaction volumes 25-well array Enzymatic assays	[48]
Nanovial arrays	100 nL reaction volumes 12 × 8 nanovial array Enzymatic assays	[45]
Attovial arrays	6 (∅ 200 nm) to 4000 aL (∅ 5 μm) sized vials 225 vials/mm^2. Tentative densities of 90 000 to 225 000 vials/mm^2 Detection of antigen (antibody arrays)	[25,33]
Nanowire arrays	nm scale Single biosensors to 200 individually addressable devices/array Antigen (e.g. cancer biomarker or pathogen) detection (antibody arrays)	[49–53]
Random arrays	nm scale – Antigen detection (antibody arrays)	[54]
Bead arrays	900 nm sized features (using 1 μm beads) (dependent on the size of the bead applied) Enzymatic assay	[55]
Nanoparticle arrays	100 nm sized features 300 spots/sensing surface Antigen detection (antibody arrays)	[56]
Cantilever arrays	500 μm long and 100 μm wide (common size of a single cantilever) Mainly 8 cantilever silicon arrays Antigen detection (e.g. proteins or pathogens) (antibody microarrays). Detection of specific protein conformations, protein DNA interactions, and protein ligand interactions (protein arrays)	[57–66]

Designs and applications of nanoarrays

The nanoarray design must allow an efficient fabrication of high-density nanoscale arrays compatible with sensitive read-out systems. The precise choice of design will be dependent on an intricate combination of several factors, such as choice nanopatterning technique, properties of the substrate and the probes, as well as compatibility with the detection method. To date, proof-of-concept studies have been performed for a wide range of first generation nanoarray designs (Table 3), including planar arrays [23,26,28–31], well-based arrays [48], nanovial arrays [45], attovial arrays [25,33], nanowire arrays [49–53], random arrays [54], bead arrays [55], nanoparticle arrays [56], and cantilever arrays [57–66]. A range of protein probes have been used in those studies, for example enzymes, polyclonal and monoclonal antibodies, recombinant single-chain Fv (scFv) antibody fragments as well as various model proteins (e.g. biotin–streptavidin and integrin $\alpha_v\beta_3$–vitronectin).

Planar nanoarrays

For planar arrays, the feasibility of a variety of substrates has been demonstrated in several proof-of-concept studies. Substrates tested include gold-coated silicon wafers [30], nickel oxide surface [31], metallic nickel surface [28], gold surface [29] as well as biotin/streptavidin functionalized aminosilane surface [23]. In general, these nanosized spots ranged in size from 10 nm up to 350 nm [23,26,28–31], and although the array densities were not optimized, tentative array densities of about 1×10^4 spots/mm^2 (∅ 2 μm) [23] to 1×10^6 spots/mm^2 [29] were achieved. In comparison, array densities of about 1×10^7 spots/mm^2 have been shown for DPN fabricated oligonucleotide arrays [67]. These proof-of-concept applications were mainly low-density arrays, where all features were functionalized with the same probe. For example, antibody nanoarrays were designed and used for antigen detection [23,26,29] and protein nanoarrays were used for analysis of protein interactions [30].

Well-and vial-based nanoarrays

Using well-based [48] and vial-based nanoarrays [45], enzymatic assays for, for example alcohol dehydrogenase and pyruvate kinase, have been successfully performed. For this purpose, 25-well nanoarrays or 12×8 nanovial arrays with a sufficient reaction volume of 6–8 nL [48] or 100 nL [45] have been devised, respectively. Recently, the vial format has been developed into atto-vial based arrays by fabricating EBL [25] or gallium focussed ion beam microscope [33] nanostructured surfaces. In the former case, vials ranging in volume from 6 (∅ 200 nm) to 4000 aL (∅ 5 μm), were fabricated at an array density of 225 vials/mm^2 [25]. In theory, the EBL step allows array densities of up to about 225 000 vials/mm^2 to be produced. The arrays were used to generate recombinant scFv nanoarrays, that were successfully used to detect high-abundant as well as low-abundant (pg/mL range) analytes in complex proteomes, such as serum. In the latter study, Bruckbauer *et al.* generated 7×7 arrays with a 20×20 μm footprint, displaying a tentative array density of 90 000 vials/mm^2 [33]. In this case, the vials were individually functionalized with polyclonal antibodies using a nanopipette and subsequently employed for antigen detection.

Nanowire nanoarrays

Proof-of-concept studies for nanowire-based biosensors have been performed on polyaniline nanowires [52], gold nanowires (gold posts) [49,51] and boron-doped silicon nanowires [50]. These biosensors, functionalized with streptavidin or antibodies have been used for, for example the detection of Bacillus species [52] and *Escherichia coli* O157:H7 [49], displaying a sensitivity in the picomolar range [50,51]. The nanowire format has recently been extended into true silicon-based nanowire arrays, containing about 200 individually addressable devices [53]. These nanowires were successfully functionalized with antibody in bulk or via conventional spotting, and the arrays were used to screen crude serum samples for cancer markers, such as prostate specific antigen (PSA). While assay sensitivities in the sub pg/mL range were observed, the robustness and reproducibility of the setup remains to be fully validated.

Random nanoarrays

In contrast to orderly arranged nanowire arrays, Tamiya *et al.* have designed randomly self-assembled nanoarrays [54]. The array design was based on barcoded metallica particles, carrying two functional sides, one face for biomolecular attachment and one for mediating random self-assembly in spatially discrete microwells on the chip surface. The applicability of the setup was demonstrated by simultaneous multianalyte immunoassays of human IgA, IgG and IgM.

Bead and particle-based nanoarrays

Pammer *et al.* have designed a novel, simplified bead-based approach to generate nanopatterns of biomolecules (DNA and proteins) [55]. In the case of protein, his-tagged probes were first bound to Ni-NTA modified beads (∅ 1 μm), and then dispensed onto aldehyde-functionalized solid supports allowing covalent immobilization of the probes (while still non-covalently attached to the beads). After blocking of non-reacted surfaces, only the beads, determining the spot-size, were eluted, exposing the about 900 nm sized featured probe functionalized spots. In comparison, Endo *et al.* have recently generated an array by constructing a core-shell-structured nanoparticle (∅ 100 nm) layer, which provided 300 nanospots on the sensing surface [56]. The spots were functionalized with Protein A, and an antibody array was generated by dispensing of the probes in the nL scale; the array was then successfully used for antigen detection.

Cantilever nanoarrays

Finally, a range of different cantilever-based arrays have been designed [57–66]. These arrays are mainly based on eight cantilever silicon arrays functionalized by, for example inkjet printing [59] or by insertion into aligned microcapillaries [57,58], the latter enabling the cantilevers to be individually addressed with different probes. Proof-of-concept studies have demonstrated the capability of these arrays to analyse (i) specific protein conformations [59,63], (ii) membrane protein-based receptor–ligand interactions [59], (iii) pathogens [65], and (iv) protein DNA interactions [61]. In addition, recent efforts have shown that antibody-based cantilever arrays could be generated and used for detection of antigens [57,58,60,66]. In most cases, assay sensitivities in the μM to nM

TABLE 4

Overview of detection technologies for protein nanoarrays

Technology	Assay sensitivity	Refs
Label-dependent		
Fluorescence	pg/mL range (non-fractionated complex proteome)	[25,26]
Label-free		
Electrical read-out	pg/mL range (arrays). Picomolar range (biosensors)	[49–53,71]
AFM		[26,32–34,72]
Localized surface plasmon resonance (LSPR)	pg/mL range	[56,73]
Scanning Kelvin nanoprobe		[74]
Nanoscale interferometry		[75]
Cantilever	μM to nM range	[57–66]

range have been reported [57,58,60,61,63], while a recent report claim fg/mL sensitivities [66].

Clearly, a wide variety of nanoarray designs, based on various surface chemistries and materials, have so far been generated. From this, it is clear that additional work will be needed before any leading array design(s) can be identified. In addition, it is also evident that several technological features must be evolved further, in a process similar to what conventional protein microarrays have already passed through [5,8,68,69], before the potential of the technology will become transparent. In particular, key issues concerning scaling up, reproducibility, sensitivity, applicability, sample loading and compatibility with complex samples and reagents/buffers in relation to the various array designs remain to be explored. In the next five years, the first medium-density to high-density nanoarray designs (>100 probes) will start to emerge and be used for discovery projects. Based on these results, tentative biomarker signatures can be identified that will be validated and exploited for, for example disease diagnostics, using re-focussed arrays, based on a much smaller number of probes (15–20) and fabricated in the microscale format.

Detection methods for nanoarrays

The detection methods for current nanoarray designs are either based on label-dependent (fluorescence) [25,26] or label-free detection [70] (Table 4). For label-free detection technologies, proof-of-concept has been generated for methodologies, such as electrical read-out [49–53,71], AFM [26,33,34,72] localized surface plasmon resonance (LSPR) [56,73] scanning Kelvin nanoprobe [74], nanoscale interferometry [75], and cantilevers [57–66]. In general, label-dependent read-out systems will provide a higher sensitivity than label-free detection methods.

Assay sensitivities in the pg/mL range have been observed for non-fractionated directly labelled proteomes using fluorescence as read-out system [25]. Further, by adopting dye-functionalized nanoparticles, a system for reaching ultra-sensitive detection or for reagent tagging enabling multiplexing may be devised [76–78]. Obviously, the application of the label-dependent approach for nanoarrays is dependent on having a scanner or microscope with a sufficient resolution to discriminate between the individual nano-sized features. Notably, a total internal reflection fluorescence (TIRF) microscope is capable of observing features less then 200 nm, opening routes even for single molecule detection.

By adopting label-free detection methods, nanoarrays will, like microarrays, benefit from the key issue of not having to label complex proteomes, provided that sufficient sensitivities can still be obtained for these miniaturized nanodevices [5,8,18]. Using an electrical read-out, assay sensitivities in the picomolar range have been shown for nanowire-based biosensors [50,51] and in the pg/mL range for nanowire arrays [53]. Although looking very promising, the development of protein nanowire arrays (linked to electrical read-out) is still in its early stages, and more work will be required to establish the assay sensitivities that could be obtained on a regular basis for such platforms.

It has been shown that nanowires [79] and gold nanoparticle arrays [80] can also be interfaced with LSPR-based detection, although this was not applied to proteins. Recently, Endo *et al.* have used the latter format to include protein analytes and devised a core-shell-structured nanoparticle layout compatible with LSPR read-out with a detection limit in the pg/mL range [56]. In comparison, Dahlin *et al.* have designed a system for LSPR-based sensing of lipid-membrane-mediated biorecognition events using nanometric holes on a gold surface [73].

Moreover, cantilever-based sensing has also been used, providing initial limits of detections in the μM to nM range [57,58,60,61,63]. By embedding a metal-oxide semiconductor field-effect transistor (MOSFET) into the base of the cantilever, a fg/mL sensitivity may be achieved [66]. As for most of the setups described above, these studies have involved mainly low-density proof-of-concept designs, which in the end will require additional work to demonstrate sufficient sensitivity and compatibility with required reagents (buffers etc.), sample complexity and multiplexity (scaling-up).

Content rules

The properties of the probes, that is the content, are a key issue to consider whether microarrays or nanoarrays are to be fabricated [3–5,8]. Probes validated for microarray applications, are likely perform well also in the corresponding nanoarray applications. However, when increasing the probe density, logistical issues linked to the efforts of generating a sufficient number of relevant probes in an efficient manner will add yet another level of complexity [5,8,18]. Hence, to realize true megadense nanoarray layouts, automated systems for probe selection, production and down-stream processing, for example purification, must be implemented. Novel substrate designs compatible with affinity-based immobilization of the probes could eliminate the need for pre-purifying the probes could be eliminated. Further, large probe sources, providing access to an almost limitless

number of high-quality probes, which are adapted to microarray use by molecular design, should also be adopted [5,8,18]. To this end, large human recombinant scFv antibody libraries composed of $>10^{10}$ members, have been proposed as a leading probe format of the future, at least for array applications within affinity proteomics [5,8,18,81]. Microarray engineered scFv antibodies have been found to display high functionality and stability, both in solution and on-chip, making them compatible with long-time storage [4,5,8]. Other affinity-based reagents available in a library format, such as aptamers and affibodies, may provide an alternative approach in respect of on-chip synthesis strategies [4,5]. The perspective, advantages and disadvantages of using antibodies or other affinity-based reagents for array applications have been discussed in more details in recent reviews [4,5]. When designing functional protein arrays, probe sources based on for, example cDNA expression libraries [82–84] have been used to generate arrays ranging in density from about 100 through 6200–37 200. Recently, commercial functional protein arrays based on plus 8000 human recombinant proteins have been released by both Protagen (http://protagen.de) and Invitrogen (http://invitrogen.com). Although the need for additional probe sources will be significant, if and when the use of nanoarrays becomes more general, the current availability of (a few) probe sources based on numerous (high-performing) probes should be more than sufficient to fabricate (at least) the first generation of truly high-density protein nanoarrays.

Conclusions

Current efforts have generated compelling evidence for the first generation of miniaturized protein nanoarrays consuming only minute amounts of reagents and samples. The work has outlined a variety of technologies that can be used to fabricate the arrays, adopting one of several tentative array designs. Sensitive systems for both label-dependent and label-free detection have been shown. While mainly low-density arrays based on a single or a few different probes have been manufactured, large numbers of probe formats are now readily available that could serve as (initial) probe sources for future high-density nanoarrays. Despite the success, major technological efforts remains to be resolved in order to transform these promising setups into robust, sensitive and truly high-density layouts for multiplex profiling of complex samples, which will be required to become an established proteomic technology. The anticipated technological developments taking place during the coming years will be crucial for setting the future stage for miniaturized nanoarrays within high-throughput proteomic applications.

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