Biocompatibility of surfaces for antibody microarrays: design of macroporous silicon substrates

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Abstract

Major efforts to develop antibody microarray technology to enable global proteome analysis to be performed in a facile manner are under way. In this process, the design and the properties of the substrate will play crucial roles. In the present study, we have developed novel, highly biocompatible solid supports for microarrays, using adsorbed recombinant human single-framework antibody fragments as probes. Several silicon-based supports, including planar silicon, micro- and macroporous silicon, and nitrocellulose-coated variants thereof, were designed and evaluated in a stepwise procedure. The surfaces were scored based on biocompatibility and probe binding capacity as judged by spot morphology, signal intensities, signal to noise ratios, dynamic range, sensitivity, and reproducibility. A set of five commercially available substrates, selected to represent a set of supports providing different surface and coupling chemistries, was used as reference surfaces. The results showed that several well-performing silicon-based supports could be designed; in particular, a nitrocellulose-coated macroporous variant, MAP3-NC7, received the highest scores. In comparison, MAP3-NC7 displayed properties equal to or better than those of the reference substrates. Taken together, designed surfaces based on silicon can undoubtedly meet the requirements of the next generation of solid supports for antibody microarrays.

Keywords: Antibody microarrays; Protein microarrays; Substrates; Solid supports; Macroporous silicon

During recent years, DNA microarray technology has played a central role in global gene expression profiling of complex biological systems, providing transcriptional fingerprints of human diseases, such as cancer, allergy, and autoimmunity [1–3]. In analogy to DNA arrays, protein microarrays offer a new distinct possibility to develop a high-throughput global proteome analysis [4–6]. By comparing proteomic maps of healthy vs diseased samples, protein microarrays will play a vital role within disease stratification, biomarker discovery, and drug target identification [7–9]. Further, protein microarrays are anticipated to finally close the existing information gap between genomics and proteomics [4–6].

Major efforts to develop the protein microarray technology are currently under way and the first generations of mainly low-density microarrays have already been successfully designed [4–6,10,11]. In this process, it has become evident that the design of both the probes and the solid support will be essential [5,6,12,13]. In contrast to DNA, proteins are a large group of heterogeneous molecules displaying a wide range of properties, such as molecular stability, affinity, and specificity, making them differently suited to array applications [5,6,13–16]. In addition, the deposition of protein-based probes onto
solid supports often results in partial or complete denaturation and consequently loss of biological activity [5,13,15,16]. This clearly merits the use of uniform probes based on the same molecular scaffold directly designed for microarray applications and displaying similar biophysical properties. We have recently shown that a human recombinant single-chain Fv (scFv) antibody library, the n-CoDeR library genetically constructed around one molecular framework [17], was an excellent source for microarray probes [4,13,14,18–21]. Microarrays based on these single-framework antibody fragments (SinFabs) were shown to be highly uniform and functional, providing assay sensitivity down to 300 zmol of analyte [13,14,18–21]. In addition, the SinFabs were extremely stable and arrayed; dehydrated probes retained their activity in room temperature for more than 200 days [13] (C. Steinhauer et al., unpublished observations).

The surface chemistry of the solid support will have a major impact on the quality and performance of the microarrays [6,12,13,16]. Early work showed that the standard surfaces used for DNA microarrays or for enzyme-linked immunosorbent assay and radioimmunoassay could not be directly applied [12]. It is essential that the support displays high biocompatibility and probe binding capacity, while nonspecific binding (background) is minimized. In recent work, more sophisticated supports designed for protein microarrays have been developed and evaluated (e.g., [12,16,21–31]). To date, the substrates have mainly been based on glass, gold, plastics, membranes/substrates have mainly been based on glass, silicon also may be used as a preferred substrate for protein microarrays [13,18,32]. Based on the coupling chemistry, the supports are often classified into three major groups: (i) covalent chemistry (e.g., [22,24,30]), (ii) affinity binding (e.g., [19,21,25,26,31]), and (iii) adsorption (e.g., [13,18,19,27–29,32]). Despite the increased selection of surfaces, providing a wide range of properties, the performances of the current supports vary and no substrate or group of substrates can still meet all of the demands required of a high-performance substrate. Taken together, there is a clear need for novel supports directly designed for antibody microarrays with superior characteristics.

The aim of this study was to develop new biocompatible silicon-based supports for protein microarrays based on adsorbed SinFabs, selected from the n-CoDeR library. To this end, iterative cycles of surface design, array production, and array evaluation were performed. The results showed that novel, highly functional, and biocompatible macroscopic silicon-based supports for SinFab microarrays could be designed. The significance of these supports for the design of antibody microarrays is demonstrated.

**Materials and methods**

**Antibodies and antigen**

The single-chain Fv antibody fragments directed against cholera toxin subunit B (CT) (clone CT-17) and fluorescein 5(6)-isothiocyanate (FITC) (clone FITC-8) were selected from the human recombinant scFv antibody phage-display library, n-CoDeR [17], and kindly provided by Bioinvent International AB (Lund, Sweden). The scFvs were produced in *Escherichia coli*. Soluble scFv molecules, all carrying a C-terminal His6 tag, were purified from the periplasmic space and the expression supernatant by affinity chromatography on Ni2+-NTA (Qiagen, Hilden, Germany). Bound molecules were eluted with 250 mM imidazole (in 50 mM sodium phosphate, 300 mM sodium chloride, pH 8), dialedyzed against phosphate-buffered saline, pH 7.4 (PBS), 0.05% sodium azide, and stored at 4°C until further use.

The activity and purity of the purified scFv were evaluated by the means of antigen-specific dot blot analysis [13], SDS–PAGE, and Western blot. The antibody concentration was determined using a MicroBCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA).

CT (Sigma, St. Louis, MO, USA) was labeled with Cy5 monoreactive dye following the recommendations of the manufacturer (Amersham Biosciences, Uppsala, Sweden). Excess dye was removed by extensive dialysis against PBS, 0.05% sodium azide, whereafter CT-Cy5 was stored in the dark at 4°C until further use.

**Production of in-house designed substrates**

Several silicon-based supports were produced, including (i) planar silicon, unmodified (electropolished) and derivatized with silane or nitrocellulose, (ii) microporous (MIP) silicon of different pore size and depth (MIP1-7), (iii) macroporous (MAP) silicon of different pore size and depth (MAP1-3), and (iv) macroporous silicon coated with different amounts of nitrocellulose (MAP3-NC1/4/7). The production details are given only for the key intermediates presented in Fig. 1.

Except for silanized silicon, all silicon substrates were prepared from p-type silicon wafers with ⟨100⟩ crystal orientation purchased from Addison Engineering (San Jose, CA, USA) and Topsil Semiconductor Material A/S (Frederikssund, Denmark) (MAP3 only).

In the case of silanized silicon, planar n-type silicon dice with ⟨100⟩ orientation were derivatized with 3-aminopropyltrimethoxysilane (APTES) from vapor phase for 2 h. They were then baked at 150°C overnight.

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1 **Abbreviations used:** CT, cholera toxin subunit B; FITC, fluorescein 5(6)-isothiocyanate; MAP, macroporous; MIP, microporous; nc, nitrocellulose; scFv, single-chain fragment variable; SinFab, single-framework antibody fragment; PBS, phosphate-buffered saline; HF, hydrofluoric acid; DMF, dimethyl formamide; SEM, scanning electron microscopy.
Fig. 1. Comparison of the key intermediates in the development of a silicon-based support for sinFab microarrays with respect to (A) highest signal intensity at 80 nM analyte, (B) detectable amount of analyte [80 nM (white), 8 nM (gray), and 0.8 nM (black)], (C) relative dynamic range, (D) signal to noise ratio for 11 fmol probe at an analyte concentration of 80 nM (white) and 8 nM (gray), and (E) mean coefficient of variation (CV). The CV values shown represent the mean CV value observed for three probe concentrations (2, 5.5, and 11 fmol probe/spot) at one analyte concentration (8 nM). On each surface, three identical 8 x 8 arrays, composed of an anticholeratoxin sinFab molecule (clone CT-17) arrayed in seven serial dilutions ranging from 11 fmol/spot to 111 amol/spot, were incubated with Cy5-labeled analyte, choleratoxin subunit B (CT-Cy5), at a concentration of 80, 8, or 0.8 nM. A nonspecific scFv (clone FITC-8) was used as negative control. To allow a direct comparison, one standard protocol for producing the microarrays and for performing the subsequent assays and array quantification was adopted for all surfaces. Saturated signals (due to the selected scanner settings) are marked by an asterisk.
Planar silicon coated with nitrocellulose was produced by airbrushing a 2-mg/ml (0.2%) nc solution onto the dice. The nc solution was prepared by dissolving 0.45 μm nitrocellulose trans-blot transfer medium (Bio-Rad Laboratories, Richmond, CA) in acetone.

Porous silicon substrates were fabricated by anodic dissolution of the silicon wafers in hydrofluoric acid (HF). This process was performed in a two-compartment electrochemical cell providing an electrochemical (HF) interface to the silicon surfaces. Current was passed through the wafers to initiate and continue the porous silicon layer formation. Different pore sizes and depths were accomplished by varying the current and process time. MIP-7 was produced by anodization of silicon wafers of 1–20Ωcm resistivity in a 20% HF/48% ethanol electrolyte solution for 10 min with a current density of 30 mA/cm². MAP2 was generated by anodization of silicon wafers of 1–20Ωcm resistivity in a 4% HF/88% dimethyl formamide (DMF) electrolyte solution. A current density of 2 mA/cm² was applied for 2 h. MAP3 was produced from silicon wafers with 10–15Ωcm resistivity. The substrate was anodized in 4% HF/88% DMF for 1 h with a current density of 2 mA/cm². The backside of the substrate was illuminated during the process. MAP3 was coated with nc by dipping the dice into 0.1-mg/ml (0.01%) (MAP3-NC1), 0.4-mg/ml (0.04%) (MAP3-NC4), or 0.7-mg/ml (0.07%) (MAP3-NC7) nc solutions in acetone.

The morphology of MAP2, MAP3, and MAP3-NC7 was examined by scanning electron microscopy (SEM) analysis (Philips SEM 515; Philips/FEI, Eindhoven, NL) (Fig. 2).

Reference substrates

Five commercially available glass substrates, providing different surface chemistry and coupling chemistry, were selected and used as reference surfaces. SpotOn slides (undisclosed surface chemistry/covalent binding via ε-amino groups) were kindly provided by Scandinavien Micro Biodevices A/S (Copenhagen, Denmark). Silane slides (aminoalkylsilane derivatized/adsorption) (Sigma), Xenoslde N (Ni³⁺-chelate derivatized/affinity binding) (Xenopore, Hawthorne, NJ, USA), FAST slides (nitrocellulose coated/adsorption) (Schleicher & Schuell, Dassel, Germany), and HydroGel slides (modified polyacrylamide gel/adsorption) (Perkin–Elmer Life and Analytical Sciences, Boston, MA, USA) were purchased from the suppliers.

Production and evaluation of SinFab microarrays

To allow a direct comparison, one standard predetermined protocol for producing the microarrays and for performing the subsequent assays and array quantification was adopted for all surfaces. Consequently, the performances of the various surface designs could be directly compared, allowing a relevant ranking of the substrates to be performed in a facile manner. On each surface three identical 8 × 8 arrays were generated. A CT-specific scFv (clone CT-17) was arrayed in seven serial dilutions ranging from 1 mg/ml (11 fmol/spot) to 10 μg/ml (111 amol/spot) in PBS. A 0.5-mg/ml nonspecific scFv (clone FITC-8) was used as negative control.

The scFv microarrays were generated using the noncontact Biochip Arrayer (Perkin–Elmer Life and Analytical Sciences). The scFvs were deposited as single droplets (333 pl) at a spot to spot distance of 200 μm. Except for HydroGel and SpotOn slides (incubated overnight in a humidity chamber at 4 °C), the arrays were directly blocked with 5% (w/v) fat-free milk powder in PBST for 1 h. Subsequently, the arrays were washed four times with 0.5% Tween 20 in PBS (PBST) and incubated with 0.8, 8, or 80 nM CT-Cy5 in 1% (w/v) fat-free milk powder in PBST for 1 h. All incubations were made in a humidity chamber at room temperature. Finally, the
arrays were washed, as described above, and allowed to dry.

The arrays were scanned, using the confocal ScanArray Express microarray scanner (Perkin–Elmer Life and Analytical Sciences) with fixed settings of 100% laser intensity and a PMT gain of 75%. The ScanArray Express software V2.0 (Perkin–Elmer Life and Analytical Sciences) was used to quantitate the intensity of each spot, using the fixed circle method. Each data point represents the mean value of eight replicates after subtracting local background and negative control (nonspecific scFv) values. The relative dynamic range is defined as the relative range of linear signal intensities observed for a given analyte concentration at the range of probe concentrations applied (see above). The signal to noise ratios represents the mean signal intensity divided by the mean local background. The coefficient of variation (CV) is defined as the standard deviation divided by the mean signal intensity. The CV values represents the mean CV value obtained for medium to high probe concentrations (2, 5.5, and 11 fmol probe/spot) at medium analyte concentration (8 nM). A detectable signal was defined as being at least twice as high as that of the negative control.

**Results**

**Design of silicon-based substrates**

Several silicon-based substrates, ranging from planar silicon to nitrocellulose-coated macroporous silicon, were produced and evaluated as support for antibody microarrays. In Fig. 1, the key intermediate surface variants generated in this process are shown. The substrate designs were scored based on biocompatibility and probe binding capacity as judged by spot morphology, signal intensity, sensitivity, dynamic range, signal to noise ratio, and reproducibility.

Planar, nonmodified silicon provided a biocompatible substrate yielding high signal intensities (Fig. 1A). However, only a high concentration of analyte (80 nM) could be detected (Fig. 1B), indicating a poor assay sensitivity. Furthermore, the substrate displayed a high background, as illustrated by a low and variable signal to noise ratio (Fig. 1D). In addition, a high CV value (almost 40%) was observed, indicating a large spot to spot variation (Fig. 1E).

In a first attempt to improve the performance, in particular the surface uniformity of planar silicon, the substrate was modified by either electropolishing (eliminating any impurities) or silanization (making it more hydrophobic). The results showed that electropolished silicon displayed properties similar to those of nonmodified silicon (data not shown). In contrast, silanization of silicon significantly reduced both the CV value (from 40 to 15%) (Fig. 1E) and the amount of analyte required (from 80 to 8 nM) (Fig. 1B). Despite these improvements, the signal to noise ratio was still low and variable (Fig. 1D), and the dynamic range was considerably impaired (Fig. 1C). The latter observation would imply that the biocompatibility and/or probe binding properties of silanized silicon were reduced.

To enhance in particular the biocompatibility and probe binding capacity of silicon, the substrate was then coated with a thin layer of 0.2% nitrocellulose. This resulted in a biocompatible substrate providing high signal intensities (Fig. 1A) and a high signal to noise ratio (>30), i.e., low background (Fig. 1D). In turn, this enabled the lowest amount of Cy5-labeled analyte (0.8 nM) to be readily detectable (Fig. 1B). Further, the spot to spot variation as illustrated by the CV value (Fig. 1E) and the spot morphology (data not shown) were improved. Although the relative dynamic range of nc-coated silicon was still limited (Fig. 1C), the results showed the potential of using nc-coated silicon as a substrate.

**Design of silicon-based substrates with maximized surface area**

To specifically target the probe binding capacity and the dynamic range, we increased the available surface area per spot by introducing pores into the silicon surface. To start with, several microporous structures with pore structures of different sizes, depths, and densities were designed and evaluated (MIP1 to MIP7). Except for the amount of analyte required (8 vs 80 nM), the best-performing MIP substrate, MIP7, displayed properties of inferior quality compared with those of plain nonmodified silicon (Fig. 1). In particular, MIP7 displayed a low dynamic range and a low signal to noise ratio (<5), the latter indicating a high background (Fig. 1D). In fact, SEM analysis revealed that the pores of MIP7 were small (<10 nm) and flat (Fig. 2A), indicating the need for larger, more accessible pore structures.

To generate a substrate with increased pore size, a set of macroporous substrates were subsequently designed and evaluated (MAP1 to MAP3). Compared to MIP7, SEM analysis of MAP2 showed that it was made up of larger (0.5–1 μm) and deeper (about 10 μm) pores (cf. Figs. 2A and B). Of note, MAP2 was found to display properties equal to or better than those of planar nonmodified silicon (Fig. 1). Although the maximum signal intensity was lower (Fig. 1A), the amount of analyte required (Fig. 1B), the relative dynamic range (Fig. 1C), and the signal to noise ratio (Fig. 1D) were better or equal, indicating an improved probe binding property and/or biocompatibility. In addition, the CV value was significantly improved (<10%) (Fig. 1E). Thus, the results showed the potential of using MAP constructs as substrates for SinFab arrays.
Next, MAP2 was further improved with respect to its pore structure to be able to combine its features with the beneficial properties of nc coating without restricting the access to the pores. SEM analysis of MAP3 showed that both the pore openings and the pore density were increased compared to those of MAP2 (Figs. 2B and C). Except for an increased maximum signal intensity and a slightly higher CV value, MAP3 displayed properties similar to those of MAP2 (Fig. 1). MAP3 was subsequently coated with an increasing amount of nc, using a 0.01, 0.04, or 0.07% nc coating solution. All three nc-coated variants of MAP3, denoted MAP3-NC1, MAP3-NC4, and MAP3-NC7, displayed excellent properties, with MAP3-NC7 setting a new standard (Fig. 1). SEM analysis of MAP3-NC7 showed that the pores were still accessible, even after the introduction of the nc layer (Figs. 2C and D). In fact, MAP3-NC7 was found to be the best-performing substrate of all the in-house-developed variants. High signal intensities (Fig. 1A), low amount of analyte required (Fig. 1B), high relative dynamic range (Fig. 1C), high signal to noise ratio (Fig. 1D), and a low CV value (5%) (Fig. 1E) were observed for MAP3-NC7. Thus, a novel, highly biocompatible, well-performing substrate for SinFab microarrays had been developed.

Comparison of MAP3-NC7 with commercially available solid substrates

To evaluate the performance of our in-house-developed substrates the properties of the best-performing variant, MAP3-NC7, were compared with those of five commercially available substrates. The supports were selected to represent a set of well-performing substrates providing different surface and coupling chemistries and consisted of Silane slides (aminoalkylsilane derivatized/adsorption), FAST slides (nitrocellulose coated/adsorption), Xenoslide N (Ni²⁺-chelate derivatized/affinity binding), SpotOn slides (proprietary information/covalent binding via ε-aminogroups), and HydroGel slides (modified polyacrylamide gel/adsorption). The substrates were evaluated and compared based on biocompatibility and probe binding capacity as judged by spot morphology, dynamic range, signal intensity, sensitivity, signal to noise ratio, and reproducibility (CV value).

The spot morphology on MAP3-NC7 was found to be distinct, uniform, and of similar size (240–270 μm) independent of the probe concentration used (Fig. 3A). In the case of Silane slides (Fig. 3B) and Xenoslide N (Fig. 3C), the spots were less distinct and contained hot spots. Furthermore, the spots on FAST slides were slightly larger (250–310 μm) and less distinct (Fig. 3D). The spot morphology and in particular the spot size were highly dependent on the amount of probe deposited on SpotOn slides (Fig. 3E) and HydroGel slides (Fig. 3F). The spot sizes were found to vary between 280–370 μm and 160–370 μm. Thus, MAP3-NC7 displayed optimal spot morphology despite the fact that it, like the FAST and HydroGel slides, has a three-dimensional surface structure.

The three supports carrying a three-dimensional surface coating, MAP3-NC7 (Fig. 3A), FAST slides (Fig. 3D), and HydroGel (Fig. 3F), also displayed the best dynamic ranges, indicating a superior probe binding capacity and/or biocompatibility. While MAP3-NC7 and HydroGel performed best at higher concentrations of analyte (8–80 nM), the FAST slides displayed best performance at lower analyte concentrations (0.8–8 nM). In contrast, the dynamic ranges of Silane slides (one-dimensional coating) (Fig. 3B) and Xenoslide N (two-dimensional coating) (Fig. 3C) were limited, indicating a lower probe binding capacity. Noteworthy, the SpotOn slides (one-dimensional coating) displayed adequate dynamic range at low concentrations of analyte (Fig. 3E).

The highest signal intensities were also obtained on MAP3-NC7 (Fig. 3A), FAST slides (Fig. 3D), SpotOn slides (Fig. 3E), and HydroGel slides (Fig. 3F). Using the current scanner settings, the lowest amount of analyte (0.8 nM) applied was sufficient for detection on MAP3-NC7, FAST slides, and in particular SpotOn slides, while higher concentrations of analyte were required on Xeno-slide N (8 nM), HydroGel slides (8 nM), and Silane slides (80 nM). Hence, MAP3-NC7 provided an assay sensitivity comparable to that obtained on the two best-performing commercial substrates.

In Fig. 4, the signal to noise ratios observed at different probe and analyte concentrations are shown. In the case of MAP3-NC7 (cfs. Figs. 3A and 4A), SpotOn slides (cfs. Figs. 3E and 4E), and the HydroGel slides (cfs. Figs. 3F and 4F), the signal to noise ratios correlated well with the observed high signal intensities, indicating low background. While the SpotOn slides performed well at low probe and/or analyte concentrations (<20) (Fig. 4E), the highest signal to noise ratios (<30) were observed on MAP3-NC7 (Fig. 4A). Despite the high signal intensities observed on the FAST slides (Fig. 3D), the signal to noise ratios were low (Fig. 4D), indicating high background. In contrast, Xenoslide N displayed high signal to noise ratios (Fig. 4C), but low signal intensities (Fig. 3C), indicating low background.

The coefficients of variation values displayed by the different substrates are shown in Table 1. The results showed that the CV value for all surfaces tested was below 20%. However, a superior reproducibility could be observed on SpotOn slides and MAP3-NC7 (<5%).

Taken together, the results clearly demonstrated an enhanced performance of MAP3-NC7 as compared with that of the other substrates, including the five reference substrates. Ranking of the five reference substrates revealed a top trio, composed of FAST, HydroGel, and SpotOn slides, followed by Xenoslide N and then Silane slides. In the top trio, the individual ranking (choice) will
be dependent on the actual assay requirements at hand (sensitivity, sample complexity, and coupling chemistry, etc.).

**Discussion**

Despite recent efforts [12,16,21–31], there is a growing need for novel high-performance substrates for protein microarrays. In this study, we have developed macroporous silicon substrates for antibody microarrays displaying improved properties (Figs. 1–3 and Table 1). Our data indicate that silicon-based substrates undoubtedly will provide a platform for designing the next generations of solid supports for antibody microarrays and protein arrays in general.

The process of developing new substrates is, however, still a major challenge and the choice of material is one of the key issues. We have focused our efforts on using silicon as the base for our arrays [13,18,32]. Over the years, silicon has emerged as a promising support for protein immobilization in general (e.g., [34–36]), and silicon displays several features making it suitable for protein microarray applications. The material is (i) biocompatible (Fig. 1), (ii) readily redesigned with respect to its physical properties (e.g., surface structure)
(Fig. 1), and (iii) compatible with the major modes of detection, such as fluorescence [13,32] and mass spectrometry [18], that are used within protein array technology. In addition, silicon displays a high protein adsorption capacity (Figs. 1 and 3A) [13,18], allowing the simplest and most applicable of the three major probe coupling chemistries to be directly used. Commercially available supports, compatible with physical adsorption, includes both one-dimensional coatings, e.g., Silane slides (silanized glass slides) (Fig. 3B) [37], and three-dimensional surfaces, e.g., FAST slides (nitrocellulose-coated glass slides) (Fig. 3D) [27,28] and HydroGel slides (polyacrylamide-coated glass slides) (Fig. 3F) [29].

Attaching probes via physical adsorption is, however, a process that is difficult to control, since it is nonselective and reversible [12,16]. These issues are commonly addressed by employing derivatized glass and gold slides to which the probes are covalently (irreversible) anchored at high density [15,22,24]. However, this process and choice of materials could impair the biological function of the probes, require the reaction conditions to be optimized for each individual probe, and/or cause nonuniform spots [15,16,22,24]. Of note, SpotOn slides (Fig. 3E) are among the few substrates that provide covalent chemistry that have been designed to allow one coupling protocol to be used for all probes [30].

However, in accordance with previous results [18], the high signal intensities observed on our silicon substrates despite noncovalently coupled probes (Figs. 1 and 3A) indicated that dissociation due to washing and incuba-
tion was not a problem. Thus, the data demonstrated that our SinFabs were strongly adsorbed to silicon-based supports, further meritng our choice of material.

Another way of addressing the issues discussed above is to use affinity anchoring [6,10,16]. The approach could allow crude probe preparations to be selectively purified, coupled, and oriented in a one-step procedure directly on the chip. However, the process will require access to affinity-tagged probes. Further, as for covalent coupling, a humid environment is often recommended during the binding process [31], which may be a limiting factor in the microarray format, as the minute volumes arrayed tend to evaporate within a few seconds. Although glass slides are commonly used, e.g., Xenoslides N (Ni2+-coated glass slides) (Fig. 3C) [31], the approach is not limited to any particular material as long as the surface can be functionalized with the corresponding affinity ligand, e.g., Ni2+ ions [21,31,33] or streptavidin (e.g., 25).

Currently, we are investigating whether silicon substrates can be made compatible with affinity binding.

To further improve, in particular, the probe coupling (adsorption) capacity of silicon (Fig. 1), we redesigned the surface properties by modifying the surface structure and adding a layer of nitrocellulose coating (Figs. 1C–3A). By introducing pores into the surface structure of silicon (Figs. 1 and 2), the substrate design was converted from a one-dimensional to a three-dimensional substrate. The initial microporous designs (MIP1-7) were directed toward more favorable macroporous designs (MAP1-3) by increasing the size, depth, and density of the pores (Fig. 1). In this process, the background signals were significantly reduced (Figs. 1A and D) and the binding capacity was increased (as reflected by the increased dynamic range) (Fig. 1C). Reflection of background signals may be explained by the larger and deeper pores in MAP that reflected the incoming laser signal to a lower extent than in the case of MIP, while the increased binding capacity was due to a larger and more accessible surface area [12]. In accordance, the highest binding capacity was observed for the three-dimensional substrates evaluated, MAP1-3 and variants thereof (Figs. 1C and 3A), FAST slides (Fig. 3D), and HydroGel slides (Fig. 3F).

Using three-dimensional supports may, however, lead to larger and less distinct spots as the arrayed probes could diffuse into the substrate [12]. This was also the case for HydroGel slides and FAST slides (Fig. 3). In contrast, MAP3-NC7 was found to display among the best spot morphology of all substrates evaluated (Fig. 3). The spot morphology is highly dependent on the wetting ability of the substrate. A low wetting ability of the surface will result in more confined spots (of smaller size). Accordingly, the wetting properties of MAP were recently shown to be significantly lower than those of both silanized glass and ordinary glass slides (i.e., one-dimensional substrates) [32]. Noteworthy, this feature of MAP will also provide possibilities to create microarrays with higher spot densities.

Furthermore, the addition of a nitrocellulose layer significantly increased the binding capacity and/or biocompatibility of silicon (Fig. 1). Nitrocellulose is also a proven material well known for its high protein adsorption capacity and for providing a biocompatible environment. However, the intrinsic fluorescent properties of nitrocellulose and the issue of blocking nonspecific adsorption must be considered to minimize background signals [27,28]. For example, high background signals were observed on the FAST slides (Figs. 3D and 4D). In fact, high background signals are among the major problems in running microarray analysis using the currently available substrates. This will reduce the assay sensitivity and complicate the analysis of complex samples, such as cell lysates containing thousands of different proteins. However, the results showed that a thin surface layer of nitrocellulose could be added to our MAP substrates to improve the signal intensities and sensitivity without increasing the background (Fig. 1), further supporting our choice of substrate design. Taken together, the results showed that MAP3-NC7 displayed properties (biocompatibility and probe binding capacity as judged by spot morphology, signal intensity, sensitivity, dynamic range, signal to noise ratio, and reproducibility) equal to or better than those of most substrates evaluated, including the reference substrates. However, in accordance with previous studies of various substrates [12,22,23], our data do not suggest one support to be the optimal substrate for any given protein microarray application. The precise choice will be dependent on several factors, such as the probes source, the coupling chemistry needed, the sample complexity, the sensitivity required, etc. In conclusion, we have successfully designed new, highly biocompatible, well-performing silicon-based supports that have the capacity to play significant roles within antibody and protein microarray applications.
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