REGULAR ARTICLE

Versatile protein microarray based on carbohydrate-binding modules

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Non-DNA microarrays, such as protein, peptide and small molecule microarrays, can potentially revolutionize the high-throughput screening tools currently used in basic and pharmaceutical research. However, fundamental obstacles remain that limit their rapid and widespread implementation as an alternative bioanalytical approach. These include the prerequisite for numerous proteins in active and purified form, ineffectual immobilization strategies and inadequate means for quality control of the considerable numbers of multiple reagents. This study describes a simple yet efficient strategy for the production of non-DNA microarrays, based on the tenacious affinity of a carbohydrate-binding module (CBM) for its three-dimensional substrate, *i.e.*, cellulose. Various microarray formats are described, *e.g.*, conventional and single-chain antibody microarrays and peptide microarrays for serodiagnosis of human immunodeficiency virus patients. CBM-based microarray technology overcomes many of the previous obstacles that have hindered fabrication of non-DNA microarrays and provides a technically simple but effective alternative to conventional microarray technology.

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1 Introduction

Protein and non-DNA microarray technology has become one of the most promising approaches for large-scale analysis of the whole proteome [1–3]. The complexity of printing non-DNA microarrays, *e.g.*, protein and peptide microarrays, is one of the major drawbacks of this approach. In this context, the use of affinity immobilization strategies may provide a general solution for fabrication of such microarrays. Immobilization of ligands based on their affinity to specific substrates has been widely established in a variety of biotechnological applications [4]. However, affinity-immobilization strategies have not been extensively exploited for fabrication of protein microarrays [5, 6]. An exception is the recent study of Zhu *et al.* [7], where the authors utilized a double-tagging system to express ~ 5800 *Saccharomyces cerevisiae* genes in the form of fusion proteins that contain both glutathione *S*-transferase (GST) and polyhistidine (HisX6) tags. The expression step was followed by a laborious affinity-purification step, which involved the purification of the expressed proteins on a glutathione-Sepharose column and subsequent fabrication of the purified recombinant proteins onto a nickel-coated glass slide.

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Abbreviations: BNHS, biotin *N*-hydroxysuccinimide ester; **CBM**, carbohydrate-binding module; **GST**, glutathione *S*-transferase; **HIV**, human immunodeficiency virus; **HRP**, horseradish peroxidase; **NHS**, *N*-hydroxysuccinimide; **QC,** quality control; **scFv**, single-chain antibodies; **ZZ domain**, synthetic IgG-binding domain derived from staphylococcal protein A

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In order to overcome some of the major obstacles faced with protein microarray construction and application, we explored the combined use of the carbohydrate-binding module (CBM) and its three-dimensional substrate, *i.e.*, cellulose, for efficient and versatile production of peptide, protein and small molecule microarrays. CBMs are key components of cellulolytic microorganisms and are involved in the targeting and binding of polysaccharide-degrading enzymes onto cellulose and related polysaccharide substrates. More than 200 different CBM sequences from hundreds of different species have been identified, and have been grouped into at least 37 distinctive families on the basis of their amino acid sequences similarities, their binding specificity and three-dimensional structure. Thus far, CBM molecules have been successfully exploited as affinity tags for expression and purification of a variety of peptides and proteins [8–11]. In this work, we employed the exceptionally stable, family-3a CBM from the cellulosome, a multicomponent multifunctional cellulase complex from the bacterium *Clostridium thermocellum* [12–14].

This paper describes the use of CBM for effective fabrication and application of non-DNA microarrays. The CBM-based microarray technology described herein offers fundamental advantages over current non-DNA microarray technology, such as retention of protein functionality after immobilization, ease of fabrication, high S/N, extended stability of the printed microarray, integrated test for quality control (QC) and the capacity to print test proteins devoid of a purification step. Together, these features allow efficient production of protein and peptide microarrays, which can be used in a variety of potential applications that are technically impractical *via* conventional microarray technologies.

2 Materials and methods

2.1 Materials

All fine biochemicals and solvents were obtained from Sigma (St. Louis, MO, USA). Cy5 and Cy3 reactive dye were purchased from Amersham Biosciences (Uppsala, Sweden). Biotin *N*-hydroxysuccinimide (BNHS) was a product of Pierce (Rockford, IL, USA). Streptavidin, Alexa Fluor 546 conjugate was obtained from Molecular Probes (Eugene, OR, USA), Cy3-labeled goat anti-human IgG, (horseradish peroxidase) HRP-conjugated rabbit anti-human IgG, HRP-conjugated rabbit anti-human IgA, HRP-conjugated rabbit anti-human IgM, rabbit anti-chicken IgY and rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA); rabbit anti-P2X1, rabbit anti-P2X2 and rabbit anti-HCN2 were from Alomone Labs (Jerusalem, Israel); and rabbit anti-GST was a product of Zymed Laboratories (San Francisco, CA, USA). Recombinant gp41 was from Maine Biotechnology Services (Portland, Maine, USA). Rabbit reticulocyte lysate was obtained from Promega (Madison, WI, USA), specific ion-channel peptides were from Alomone Labs, and GST was from Sigma. Activated slides. (SpotOn Protein MicroArray) were from SMB (Farum, Denmark), NC-coated FAST slides were from Schleicher & Schuell (Keene, NH, USA); MaxiSorp slides were from Nunc (Roskilde, Denmark); regenerated-cellulose, cellulose acetate, and cellulose ester slides were from Zephyr ProteomiX (Kiryat-Shemona, Israel). Xenoslide N/nickel-chelate slides were from Xenopore (Hawthorne, NJ, USA), and microcrystalline cellulose TLC plates were from Merck (Darmstadt, Germany). Human immunodeficiency virus (HIV)-1 infected and healthy sera were obtained from Kaplan Hospital (Rehovot, Israel).

2.2 Methods

2.2.1 Antibody production and preparation

Polyclonal rabbit anti-biotin and rabbit anti-CBM production was carried out as described previously [12]. Chicken anti-CBM IgY was obtained from Yamit Biotechnology (Kiryat-Shemona, Israel).

2.2.2 Construction and bacterial expression of CBM and CBM derivatives

CBM was overexpressed and purified as described previously [12]. Preparation of CBM-fused single-chain antibodies (scFv) and selection of specific antibody were carried out as reported [15, 16]. Immunogenic HIV-related peptides were genetically fused at either the *C*- or *N*-terminus of the CBM molecule. *Escherichia coli* XL1-Blue bacteria were used in all of the cloning procedures and *E. coli* BL21 (DE3) bacteria were used for expression using the vector pET3d (Novagen, Madison, WI, USA). CBM-fused peptides were purified by affinity chromatography on cellulose [12]. Two sequential synthetic IgG-binding domains (ZZ) derived from *staphylococcal* protein A [17] were fused to the *N*-terminus of the CBM by replacing the scFv with the ZZ domain, cloned as a CBM-fused protein in the pET-derived CBM-scFv expression vector as previously described [18]. The resultant CBM-ZZ construct contained a His tag at the *N*-terminus. The His-tagged CBM-ZZ was purified by metal-chelate affinity chromatography on a Ni-NTA resin according to the manufacturer's instructions (Qiagen, Hilden, Germany).

2.2.3 ELISA

ELISAs were carried out essentially as described [19]. Briefly, wells of microtiter plates were coated with 0.1 mL antigen solutions, blocked with BSA and incubated with diluted tested sera. Detection was with a mixture of (HRP)-conjugated rabbit anti-human antibodies and development was carried out with tetramethylbenzidine (TMB) as the chromogenic substrate (DAKO, Carpinteria, CA, USA).

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2.2.4 Conjugation of antibodies and proteins

NHS-ester-activated Cy-3 and Cy-5 dyes were resuspended in 0.1 M sodium hydrogen carbonate buffer, pH 8.1, mixed with the desired purified proteins and quenched with a 10% volume of 1 M Tris-HCl, pH 8.1, according to the manufacturer's instructions. Free dye was removed by dialysis against PBS. Biotinylation was carried out using BNHS as described [20].

2.2.5 Printing CBM-based microarrays

A manual spotter MicroCASTer (Schleicher & Schuell) was utilized to print proteins onto the cellulose-coated glass slides. Protein samples were dissolved in PBS, pH 7.4, or diluted with appropriate solutions to a final concentration as specified in the figure legends. The proteins were printed in spot diameters of ~450 μm and at ~1.5 mm intervals, center to center. The printed microarrays were air-dried with a hair dryer and kept at room temperature in the absence of desiccant prior to application.

2.2.6 Microarray probing

The printed microarrays were blocked by incubating the slides in 0.5% w/v BSA in PBS at room temperature for 30 min. The slides were then incubated at room temperature with the desired fluorescence-labeled proteins or unlabeled serum, serially diluted with BSA solution for different time periods, and incubated with secondary fluorescence-labeled antibody as specified in the figure legends. The probed slides were washed five times with PBS with 0.1% v/v Tween 20, air-dried and scanned for fluorescence signals using a ScanArray Lite scanner (Packard BioChip Technologies, Billerica, MA, USA).

3 Results and discussion

3.1 Examining support matrix and conditions for protein fabrication

To explore the overall concept of microarray formats, based on CBMs, standard glass slides were coated with various cellulose derivatives, *i.e.*, regenerated cellulose, cellulose acetate, and cellulose ester membranes. In addition, commercially available TLC plates, precoated with microcrystalline cellulose, were evaluated. In preliminary experiments, the feasibility of the concept was examined using the avidin-biotin system. Briefly, cellulose-coated glass slides were printed with purified biotinylated-CBM in various concentrations (0.01–100 μg/mL) and with CBM (100 μg/mL). The latter slides were immediately blocked with a solution of BSA followed by incubation with fluorescence-labeled streptavidin. The results (not shown) demonstrated that glass slides coated with either regenerated cellulose or cellulose-acetate membranes provide enhanced binding of CBM and low nonspecific binding to the membrane, compared to the other tested cellulose derivatives. In particular, the regenerated cellulose membrane provided the best overall performance in terms of binding capacity, spot morphology and sensitivity, and was subsequently used in this work as the support of choice.

3.2 Characteristics of CBM-based protein microarrays

The lowest detection limit and the S/N are fundamental features of protein microarray technology. To investigate the former parameter, a protein-ligand (avidin and biotin) and protein-protein interaction (*staphylococcal* protein A-derived IgG-binding domain and antibody) were selected as model cases. In the first example, purified biotinylated-CBM was printed on cellulose-coated slides. The printed microarray was blocked for 2 min with BSA and the response to various concentrations of fluorescence-labeled streptavidin was determined after a short (10 min) incubation. Figure 1A shows a representative image of responses obtained for direct interaction between biotinylated-CBM and streptavidin. Even with very short incubation periods, significant signal levels could be measured for concentrations as low as 1 ng/mL of fluorescence-labeled streptavidin. In a separate

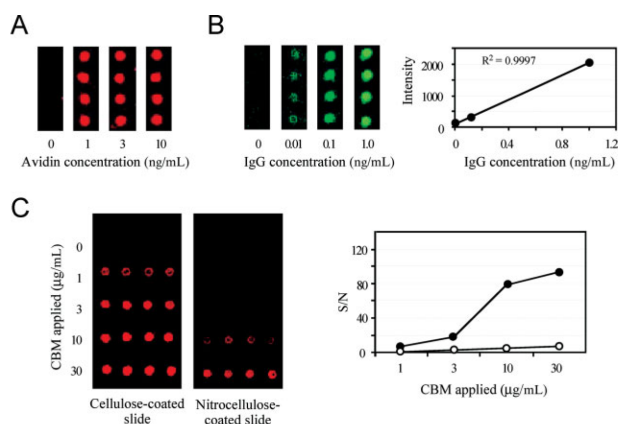

Figure 1. Efficacy of CBM-based microarray technology. (A) Detection for rapid incubation-time assay. Purified biotinylated-CBM was serially diluted and fabricated onto cellulose-coated glass slides. The slides were blocked for 2 min followed by incubation for 10 min with 1, 3 and 10 ng/mL of AlexaFluor streptavidin, and the microarray was scanned. (B) Representative images and dose-response curve for analysis of lowest detection limits. A purified form of the CBM-based Ig-binding fusion protein, CBM-ZZ, was serially diluted and printed onto cellulose-coated slides. The slides were incubated for 2 h with 10, 100 and 1000 pg/mL of Cy5-labeled rabbit IgG. (C) Representative images and comparative dose-response curve for S/N on polymer-coated glass slides. Glass slides, coated with either NC or cellulose, were fabricated using various concentrations of CBM-ZZ. The slide was blocked and allowed to incubate for 1 h with 1 mg/mL Cy5-labeled rabbit IgG. Data from six samples (four shown in Fig.) for cellulose-coated (closed circles) *versus* NC-coated (open circles) slides were analysed statistically.

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experiment, CBM, fused to two sequential synthetic IgG-binding domains derived from *staphylococcal* protein A [17] (CBM-ZZ), was printed on cellulose-coated slides, blocked as above and incubated for 2 h with various concentrations of Cy-5-labeled rabbit IgG. Figure 1B shows the fluorescence images of the resultant microarray and the corresponding response curve. As expected, the fluorescence signals increased with increasing concentrations of the labeled rabbit IgG, thus providing a lower detection limit of 50 attomoles/mL. Similar or lower limits of detection have been described previously, using special detection instruments, such as planer waveguide technology [21], or amplification strategies, *e.g.*, rolling circle amplification technology [22].

Microarray technologies, based on fabrication to three-dimensional polymers, such as NC membranes, usually generate high signal intensities. However, this is often accompanied by high background, thus rendering the apparent advantage of strong signals rather useless [23]. To examine this issue, a capture immunoassay format was employed. For this purpose, serial dilutions of CBM-ZZ were printed on glass slides coated with NC and cellulose. The slides were allowed to interact with rabbit IgG followed by incubation with fluorescence-labeled chicken anti-rabbit antibody. The signal was measured using different laser intensities and variable levels of detection. Signal intensities using the cellulose-coated slides were about 25 times higher than those obtained using the NC-coated slides (Fig. 1C). More notably, the S/N for the highest concentration of applied protein reached a level of ~100. Low S/N values have been reported earlier for commercial NC-coated slides [23]. Raising the laser intensity or the level of detection was accompanied by a significant increase in background intensity for the NC-coated slide, as opposed to the relatively low background for cellulose-coated slides, observed under the same conditions (data not shown). The significant advantage of the CBM-based microarray can mainly be attributed to the relatively low nonspecific binding of the secondary antibodies to the cellulose membrane compared to that obtained for NC membranes. Moreover, correct orientation of the captured rabbit antibody onto the CBM-based microarray may also contribute to the observed enhancement in signal intensity [24].

3.3 Fabrication of purified *versus* unpurified protein

In the past, the fabrication of proteins and peptides in microarray format, whether on chemically activated slides or on three-dimensional coated matrices such as NC-based slides, has been restricted to purified samples [2, 3]. This has been a major limiting factor influencing the design, production and implementation of non-DNA microarrays. In fact, the prerequisite for purified material is also a critical and relevant issue for tag-based microarrays, whereby large numbers of proteins are cloned expressed and purified using a specific tag (such as a His-tag) [7]. Thus far, the CBM molecule has been successfully exploited as an affinity tag for expression and purification of a variety of peptides and proteins [10, 13, 25, 26].

The high affinity of the CBM towards cellulose should enable fabrication of various proteins directly from crude protein mixtures, without further purification and without reduction in immobilization efficiency. To examine this premise, we compared the performance of the CBM-based microarray to the affinity-immobilization approach based on His-tagged proteins. For this purpose, we took advantage of the His tag, located at the *N*-terminus of our CBM-ZZ protein, that enables immobilization of the protein on Ni-chelate-coated surfaces. The proteins were affinity purified to homogeneity as revealed by SDS-PAGE (data not shown). Subsequently, the CBM-ZZ purified samples were spiked and serially diluted into PBS on the one hand and into rabbit reticulocyte lysate (RRL) on the other, in order to obtain purified and crude protein samples, respectively. The latter crude preparation was designed to simulate proteins expressed by coupled *in vitro* transcription and translation. The samples were printed onto cellulose- and Ni-chelate-coated slides, blocked and incubated with Cy3-labeled rabbit IgG. For the CBM-ZZ printed on cellulose surfaces, the fluorescence signals resulting from purified and unpurified samples were almost identical for all the tested concentrations (Figs. 2A and B). In contrast, printing of unpurified His-tagged samples on Ni-chelate-coated slides failed to produce detectable signals, indicating that the RRL completely inhibits the binding of this molecule to the Ni-chelate surfaces (Figs. 2A and B). Moreover, the fluorescence signals generated by the purified His-tagged CBM-ZZ were significantly lower than the corresponding signals generated by CBM-ZZ

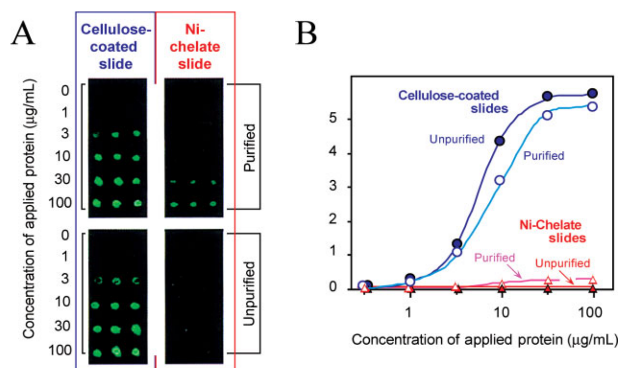

Figure 2. CBM-based affinity immobilization of crude protein samples. (A) Images of purified and unpurified samples fabricated onto cellulose *versus* Ni-chelate slides. (B) Quantitative evaluation of fluorescence signal intensity *versus* concentration of the printed CBM-based microarray for purified and unpurified samples. His-tagged CBM fused to the *staphylococcal* protein A IgG-binding Z domain-dyad (CBM-ZZ), was expressed and affinity-purified using Ni-NTA chromatography. The purified CBM-ZZ was spiked and serially diluted in PBS or in rabbit retriculocyte lysate RRL and printed on cellulose-coated or nickel-chelate coated glass slides. The slides were blocked and allowed to react with 1 µg/mL Cy5-labeled rabbit IgG for 1 h.

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immobilized onto the cellulose-coated slides. Similar results were obtained by spiking the CBM-ZZ into *E. coli* cell extracts, designed to simulate bacterial expression systems (data not shown).

These findings are in accordance with those of Zhu *et al.* [7], who fabricated ~5800 *S. cerevisiae* His-tagged proteins on nickel-chelate glass slides but emphasized the need for purification prior to application. More recently, an intein-mediated protein biotinylation strategy has also been employed for production of biotinylated proteins for avidin-coated slides [27]. This approach, however, is tedious and requires multiple steps of manipulation, such as cleavage, biotinylation and affinity purification.

In view of the above, the CBM-based technology is highly advantageous compared to conventional microarray technologies. Unlike the use of activated slides, the cellulose-coated slides can be stored indefinitely and essentially have no expiration date. In addition, the high level of expression of the CBM-containing hybrid proteins and the fact that their purification is usually unnecessary further attest to their favourable qualities. Even if purification is required in some cases, the purification step is simple and inexpensive due to the use of the cellulose resin. In addition to its other attributes, the CBM-based microarray is thus cost-effective compared to standard methodologies.

3.4 Binary detection as a tool for end-user quality control

QC is an indispensable step during the production process of biological products. This is even more crucial when massive numbers of proteins or peptides are implemented in a single biological device, such as for microarrays. Currently, nondestructive QC tests are available only for the DNA microarray sector. The tests are based on either inherent self-fluorescence properties of DNA or hybridization with a randomly selected fluorescent-probe [28]. Unfortunately, these methods cannot be adapted for non-DNA microarrays, due to the high complexity and low-intensity of auto-fluorescence in proteins. In this context, we have devised two different nondestructive QC tests that are based on direct and indirect detection (Fig. 3). In the direct approach, CBM was sequentially labeled with fluorescent dye and with biotin, whereby the latter serves as a model for small ligand-protein interaction. The double-labeled molecule was serially diluted and printed on a cellulose-coated slide, which was allowed to interact subsequently with fluorescence-labeled streptavidin (Fig. 3A). It is clearly evident that this approach does not interfere with either the binding of the CBM to its cellulosic matrix or the streptavidin-biotin interaction. The lower limit of detection observed with this method is about 6 pg of CBM which is equivalent to 0.3 fmoles of protein. In the indirect approach, a test was designed using a model protein-protein interaction (Fig. 3B). To evaluate this approach, CBM-ZZ was serially diluted and printed on a cellulose slide. The blocked slide was allowed to incubate with Cy3-labeled rabbit IgG.

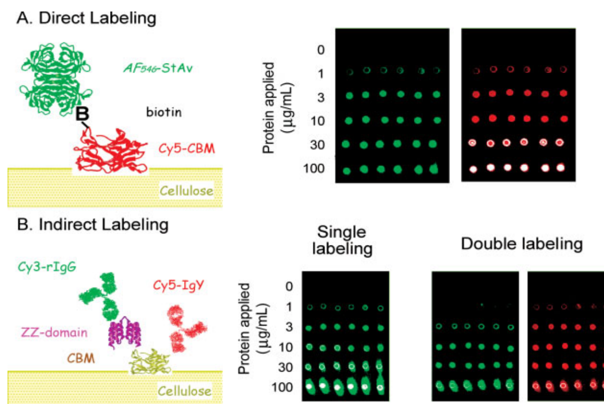

Figure 3. Integrated QC approaches for CBM-based microarrays. (A) Direct quality QC. CBM, double labeled with biotin and Cy5, was serially diluted and printed at the indicated concentrations onto cellulose-coated slides. The slides were blocked with BSA, treated for 1 h with 0.5 µg/mL Alexa Fluor 546-labeled streptavidin (AF546-StAv), and viewed using two separate filters. (B) Indirect QC strategy. CBM-ZZ fusion protein was serially diluted and printed onto cellulose-coated slides. The BSA-blocked slides were treated with 0.1 µg/mL Cy3-labeled rabbit IgG for 60 min and scanned. Double-labeling involved initial treatment of the printed slides with Cy3-labeled rabbit IgG for 40 min and supplemental addition (without washing) of 1000-fold diluted Cy5-labeled chicken anti-CBM (IgY). Double-labeled slides were viewed using two separate filters. Note the reduction in signal intensity of the primary antibody in the double-labeled slides.

The latter slides were either scanned immediately or treated additionally with Cy5-labeled chicken anti-CBM and then viewed. The results indicate that the interaction of the ZZ-domain with its antibody was somewhat impaired by parallel immunochemical interaction of the CBM molecule. Analysis of the median fluorescence intensity achieved by the two strategies indicated that values for double labeling were on average 50.7% those obtained by single labeling. Nevertheless, this approach enables similar levels of detection as observed for the direct labeling strategy. From the practical point of view, the proposed indirect QC test may to a great extent be preferred as a general QC test. Thus, the fluorescence-labeled antibody may provide a simple, nondestructive and general QC test that can be performed simultaneously with the direct measurement of the target protein. In fact, this approach, designed for CBM-based microarrays, is essentially unique and unavailable with existing technology, since common antigenic epitopes are virtually absent in the various immobilized proteins.

3.5 Peptide microarray for HIV serodiagnosis

As an example of the feasibility of CBM-based microarray technology, we examined its application for detection and assay of HIV-related antibodies in the serum of HIV-positive patients. Indeed, assays for simultaneous detection of antibodies with different specificities are potentially applicable in the diagnosis of a broad range of disorders, including aller-

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gies [29–31] autoimmune [32] and infectious diseases [33, 34]. The diagnosis of infectious diseases in serum samples of HIV patients is currently carried out using recombinant proteins or synthetic immunogenic peptides [35, 36]. To achieve the highest sensitivity of immunodiagnosis with peptide microarrays, a sufficient amount of peptide in the correct orientation should be immobilized on the microarray surface. In view of the difficulties arising during printing of small peptides in active form in microarray formats, we considered using the CBM as a scaffold molecule for displaying linear peptides for serodiagnosis of HIV-1. For this purpose, a repertoire of immunogenic HIV-related peptides were genetically fused at either the *C*- or *N*-terminus of the CBM molecule, expressed and purified to homogeneity (Table 1). Initially, the specificity and sensitivity of the CBM, fused to a 12-mer transmembrane protein of gp41 (gp41a peptide), was evaluated and compared with the performance of a conventional ELISA format, consisting of the full-length gp41 (positive control) and the CBM molecule alone (negative control; Fig. 4A). For this purpose, sera from either HIV-

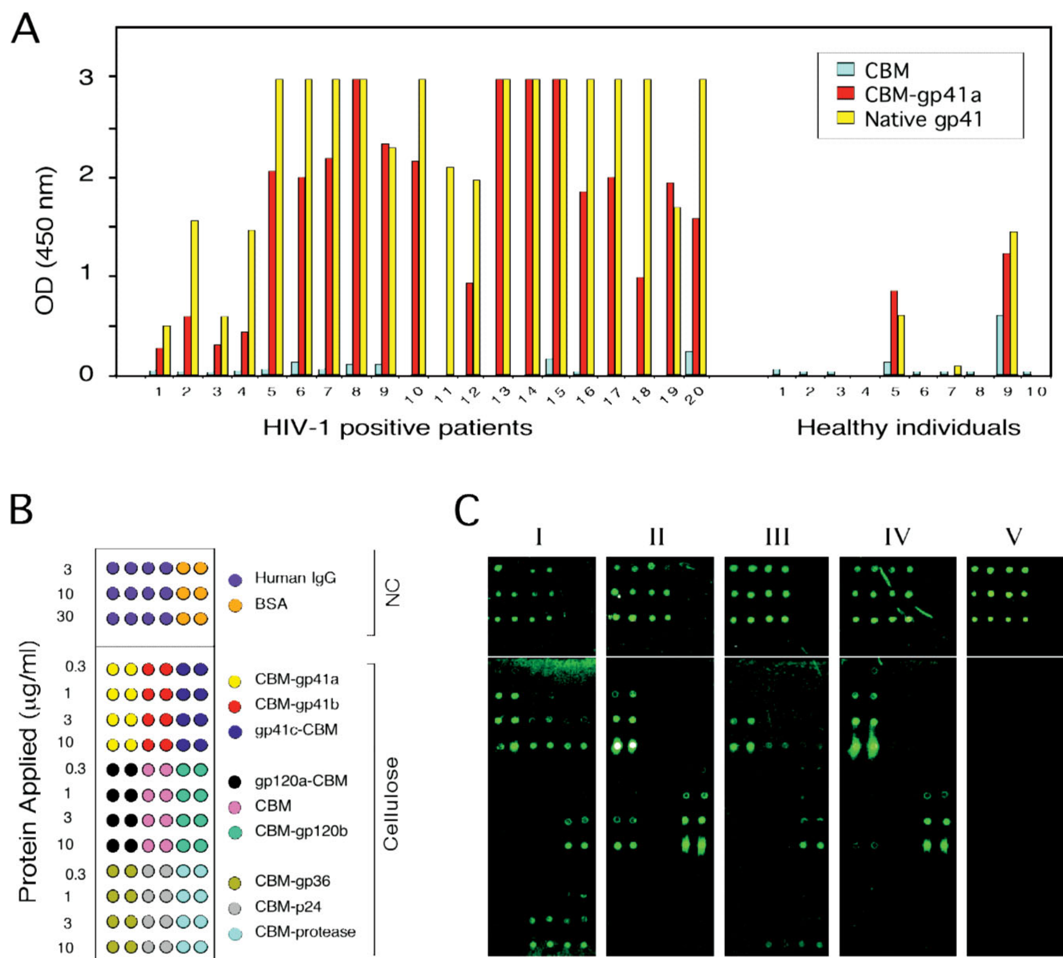

Figure 4. Immunogenic peptide microarray for serodiagnosis of HIV-1. (A) Immunoreactivity of recombinant gp41, CBM-fused gp41a peptide and native CBM in a conventional ELISA assay. Serum samples from 20 HIV-1-infected patients and 10 healthy individuals as controls were examined. Microtiter plates were coated with solutions (0.1 mL) containing 2 μg/mL of the test protein antigens, blocked with BSA and incubated for 1 h at 23°C with the test sera diluted 2×10^5-fold. The plates were washed and incubated with a 5000-fold diluted mixture of HRP-conjugated rabbit anti-human antibodies (anti-IgG, anti-IgA and anti-IgM). After 1 h incubation, the plates were washed extensively, and chromogenic substrate (tetramethylbenzidine) was added. The reaction was terminated by acidification and read at 450 nm. (B) Schematic representation of CBM-based peptide microarray used in serodiagnosis of HIV-1 in human sera. (C) Glass slides, coated with NC (top) and cellulose (bottom), were printed with human IgG and the various CBM-fused immunogenic peptides as shown in (B). The peptide numbers correspond to those shown in Table 1. The slides were blocked with BSA and allowed to react with 10^4-fold dilutions of HIV-infected sera (arrays I-IV) or with pooled sera from healthy individuals (array V). The CBM-fused peptides were fabricated in duplicate in three different concentrations. HIV-1 immunogenic peptide microarray was generated by fabricating seven distinct recognized peptide antigens on cellulose-coated glass slides. Following 1 h incubation, the slides were washed and allowed to interact with fluorescence-labeled rabbit anti-human IgG.

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Table 1. Immunogenic peptides used in this study for serodiagnosis of HIV

Protein	Abbreviation	Sequence	HXB2 Location	Position
HIV-1 Glycoprotein gp120 (V3 loop)	gp120a-CBM	TRKSIHIGPGRAFYTTG	303–321	*N*-linked
HIV-1 Glycoprotein gp120 (V3 loop)	CBM-gp120b	TRKSIHIGPGRAFYTTG	303–321	*C*-linked
HIV-1 Transmembrane protein gp41	CBM-gp41a	LGIWGCSGKLIC	598–609	*C*-linked
HIV-1 Transmembrane protein gp41	CBM-gp41b	KNEQELLELDKWAS	656–669	*C*-linked
HIV-1 Transmembrane protein gp41	gp41c-CBM	KNEQELLELDKWAS	656–669	*N*-linked
HIV-1 Protease	CBM-protease	PKMIGGIGGFIKVRQ	44–58	*C*-linked
HIV-1 Capsid protein p-24	CBM-p24	GATPQDLNTMLNTVG	178–192	*C*-linked
HIV-2 Transmembrane protein gp36	CBM-gp36	LNSWGCAFRQVC	592–603	*C*-linked

infected patients or healthy donors were diluted, allowed to interact with different test antigens, and then detected using HRP-conjugated to rabbit anti-human antibodies. A similar specificity profile was obtained for full-length gp41 and CBM-fused to the gp41a peptide for both HIV-infected and healthy patients (Fig. 4A). The negative control failed to react with any of the HIV-infected patients and cross-reacted with one of the false positive control sera.

In the second phase, the immune response of the patients towards selected HIV-related peptides was estimated. In order to do so, CBM-fused synthetic immunogenic peptides (Table 1) were printed onto cellulose-coated glass slides (Fig. 4B). For semiquantitative analysis, part of the same slide was coated with a NC membrane, which was printed with an internal standard calibration curve for human IgG. Additional negative samples (BSA and free CBM) were incorporated onto the slide, in order to assess the level of nonspecific interference in the assay. The printed slides were incubated with either diluted sera obtained from individual HIV patients or with pooled healthy donor sera diluted in the same manner. The detection in this experiment was based solely on fluorescence-labeled rabbit anti-human IgG.

Figure 4C shows representative microarray images obtained for four HIV patients (arrays I-IV) and for the pooled sera of healthy donors (array V). The pooled sera exhibited no detectable reactivity against any of the tested HIV-related peptides. In contrast, serum derived from HIV-1 patients generally interacted with at least one of the specific test antigens. All four of the HIV-infected patients reacted with gp41a and gp120b, which were the most reactive peptides of the panel used in this microarray. As expected, no reactivity was detected with gp36, a peptide similar in sequence to gp41a but associated with HIV-2. The reactivity with the other CBM-fused HIV-related peptides was variable, either in preference for a specific peptide or in the intensity of the signal. Although the concept of immunoassay in microarray format has been previously demonstrated for various infectious diseases, such as rubella virus, cytomegalovirus and herpes simplex virus [33], this is, to our knowledge, the first report demonstrating the application of microarray technology using immunogenic peptides for immunodiagnosis of HIV-1. The findings of this study clearly demonstrate the specificity and sensitivity of the peptide microarray format for serodiagnosis purposes. Furthermore, this approach serves to validate results based on Western blotting, thus precluding additional confirmation tests (*e.g.*, ELISA) used routinely in the diagnosis of seropositive patients.

3.6 CBM-based *versus* conventional antibody microarrays

The most common and commercially promising form of microarrays is the antibody microarray, in which antibodies that bind specific antigens are printed at high density on various solid surfaces [23, 24, 37]. The antibody microarray opens up new possibilities for screening more samples in a shorter time period and at greater sensitivity. However, the generation of antibody microarrays is complicated by several common biophysical and chemical properties of the antibodies themselves and of the supports used for their immobilization [38, 39]. Owing to our above-described experience with the CBM-ZZ, we decided to evaluate the employment of this molecule for construction of antibody microarrays and to compare its application to several conventional approaches for antibody immobilization. In preliminary experiments, the optimal molar ratio between the CBM-ZZ and specific IgG was determined to be about 1:8. In addition, excess fluorescence-labeled rabbit IgG failed to compete with the resultant complex, thus demonstrating the stable nature of this interaction (data not shown).

In order to assess the possible advantages of CBM-based antibody microarrays over conventional antibody microarray technologies, six different rabbit antibodies were printed on different types of commercial glass slides, either *via* chemical interaction with or *via* passive adsorption to the surface (Fig. 5). In all cases, antibodies at a final concentration of 1 mg/mL were printed either directly on commercial slides or bridged *via* the CBM-ZZ on cellulose-coated slides. The slides were blocked and incubated with a mixture of fluorescence-labeled antigens. The performance of the fabricated antibodies was assessed by the criteria of signal intensity, background level and spot morphology (quality). In general, the overall best performance was observed with the CBM-based technology, followed by the NC-coated glass slide

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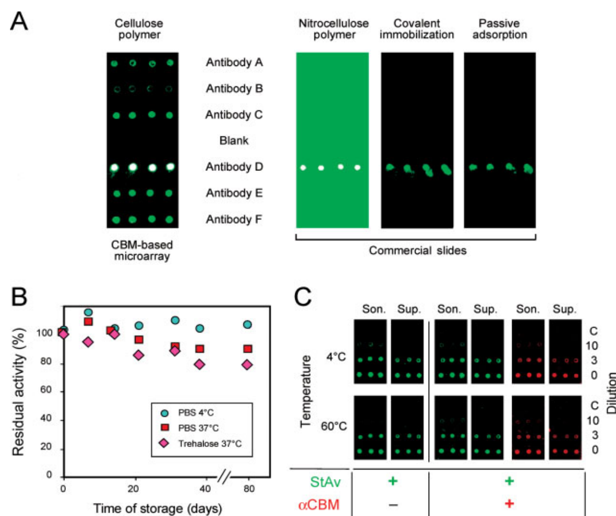

Figure 5. CBM-based *versus* conventional antibody microarrays. (A) Antibodies A-F (rabbit anti-chicken IgY, rabbit anti-ion-channel antibodies anti-P2X1, anti-P2X2 and anti-HCN2, rabbit anti-biotin and rabbit anti-GST, respectively) and BSA (blank) were diluted in PBS, each to a final concentration of 1 mg/mL, and applied directly to the commercial slides. For application to cellulose-coated slides, the latter antibodies, at the same concentration, were preincubated with 0.1 mg/mL CBM-ZZ and then printed. Slides were blocked with BSA and treated, according to the manufacturer's instructions, with a mixture of fluorescence labeled antigens at a final concentration of 1 μg/mL. Commercial slides used in this experiment were, from left to right, Fast slide (NC), SpotOn (covalent immobilization), and MaxiSorp slides (passive immobilization). (B) Rabbit anti-chicken IgY (1 mg/mL) was preincubated with CBM-ZZ in PBS or trehalose solution (10% w/v) and printed on cellulose-coated slides. The slides were sealed in nylon bags and maintained at either 4°C or 37°C. At the specified time intervals, the slides were removed from the bags, blocked with BSA and incubated for 1 h with 1 μg/mL fluorescence-labeled chicken IgY. (C) *E. coli* BL21 cell lysate (Son.) and growth medium (Sup.) after expression of anti-streptavidin CBM-scFv were incubated for 30 min at 4°C and 30°C and centrifuged for 10 min at 20 800 *g*. The supernatant fluids were diluted three and ten times with PBS and then fabricated on cellulose-coated slides. The BSA-blocked slides were treated for 60 min with 0.5 μg/mL Alexa Fluor 546-labeled streptavidin and scanned. Double-labeled slides were first incubated for 40 min with Alexa Fluor 546-labeled streptavidin followed by direct addition of 1000-fold diluted Cy5-labeled chicken anti-CBM. After 20 min, the slides were viewed using two separate filters.

(Fig. 5A). The performance of the other slides was comparatively poor for five out of the six antibodies tested. Comparison of the sole active antibody (antibody D) common to all of the slides revealed superior spot morphology and signal intensity generated by the cellulose-coated slide. Evaluation of the three-dimensional polymer-coated slides, *i.e.*, NC and cellulose-coated slides, indicated high performance for the NC membrane in terms of signal intensity. However, the background level was also unacceptably high, rendering the advantage of strong absolute signals inadequate. In contrast, the background generated by the cellulose-coated slide and the other commercially available slides was relatively low. These results clearly demonstrate the advantages of CBM-based antibody microarrays over conventional technologies.

The long-term stability of the CBM-based antibody microarray was also assessed. For this purpose, antibody D was diluted with either PBS or trehalose solution and printed on a cellulose-coated slide. The fabricated slides were maintained at either 4°C or 37°C and tested for their capacity to recognize the target antigen. The fabricated antibody stored at 4°C maintained its activity without any apparent decline over a period of at least 2 months (Fig. 5B). Under accelerated storage conditions at 37°C, a 20-30% decrease in activity was detected over the same period. These results support the overall performance and stability of the CBM-based antibody microarray. It should be noted that the presence of trehalose, that has been widely used as a preservative agent [40], failed to confer added stability to the fabricated antibody.

Normally, native full-size antibodies are preferred for use in antibody microarrays. However, the question remains as to whether such antibodies are sufficiently specific for this purpose [41–43]. One means of addressing this problem is to design a recombinant antibody library on the basis of a single framework, such as the scFv [44]. To evaluate the implementation of scFvs in our CBM chip technology, we employed the Ronit 1 library [15], a library of CBM-fused single-chain antibodies. A clone encoding for CBM-fused anti-streptavidin scFv, was isolated from this library [15], and the fusion protein was expressed in *E. coli*. The cell lysate and the growth medium were treated at 60°C for 30 min, which resulted in a partially purified protein due to precipitation of most contaminating *E. coli* proteins (data not shown). The heat-treated and untreated samples were then fabricated onto cellulose-coated glass slides, followed by incubation with fluorescence-labeled streptavidin. Specific and near-identical fluorescence signals were generated by the CBM-fused scFv that was isolated from the lysate and from the growth medium with and without the 60°C treatment (Fig. 5C). The expression level of CBM-fused single-chain antibodies was also monitored independent of the specific measurement, by using the indirect methods described above (Section 3.4). Employment of fluorescence-labeled anti-CBM reveals that the expression level is correlated with the specific activity.

The results indicate that neither immobilization nor activity of the scFv fused to CBM was affected either by the presence of impurities or by the heat treatment. It should be noted, however, that the high stability characteristics of this particular single-chain antibody does not necessarily reflect the stability of other CBM-fused scFvs, derived from this library. Nevertheless, we have shown earlier that denatured CBM-fused scFvs can be refolded into an active state while immobilized onto cellulose [18]. Our results clearly demonstrate the potential use of CBM-based microarrays for production of recombinant antibody fragments in a simple yet efficient manner, without the need for purification steps prior to fabrication. In addition, the level of the expressed molecule

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can be evaluated semiquantitatively in a simple non-destructive manner. The future challenge for CBM-fused scFvs will involve extension of the antibody microarray to enable global analysis of tens of thousands of proteins with the ultimate goal of fabricating an entire library on a single chip.

4 Concluding remarks

Here we describe a simple, efficient and general technology for fabrication of non-DNA microarrays, based on the combination of a CBM and cellulose-coated glass slides. Recombinant CBM-containing fusion proteins were designed that bind inherently to the cellulose surface. The CBM-based technology has thus been employed for production of peptide and antibody microarrays, and its superiority over existing commercial technologies has been demonstrated. To our knowledge, this is the first technology that enables production of non-DNA microarrays without the need of prior purification of the arraying protein. This feature should prove invaluable for the efficient fabrication of affordable microarray libraries.

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