



Research paper

Surface immobilized hydrogels as versatile reagent reservoirs for microarrays

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ABSTRACT

The accuracy of antibody-based microarrays depends on eliminating or at least minimizing the effect of cross-reactive components. In this work, a method to create a versatile, compartmentalized storage system for antibodies and soluble microarray reagents is described. These containers are made of hydrogel plugs immobilized on a slide facing the corresponding sub-array on a printed surface.

The hydrogels were polymerized using a 4% w/v *N,N*-dimethylacrylamide and 3% w/v bisacrylamide solution, thus generating large pore sizes to allow the facile transfer of intact, functional biomolecules. The hydrogel microenvironments can be desiccated and rehydrated with a desired solution, and they can store reagents in a dry form.

These hydrogels were shown to provide limits of detection similar to those obtained by conventional incubation conditions in an assay for HIV p24 antigen. Moreover, they prevent cross-reactivity issues in the detection of *Staphylococcus aureus* enterotoxins A and B and promote the accurate quantification of interleukin 10 by a microarray-integrated calibration curve.

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1. Introduction

In recent years, the use of DNA microarrays has become quite widespread due to their superior utility in molecular profiling of gene expression patterns for prognosis and diagnosis (Ewis et al., 2005). Accordingly, protein microarrays have been adopted to investigate protein expression patterns and the function of entire proteomes (Kung et al., 2009), to identify biomarkers in serum (Bohm et al., 2011) and to predict evolution of cancer disease (Carlsson et al., 2011). Despite their tremendous potential, protein and antibody microarrays still have not drastically changed human diagnostics because technical and operational challenges hinder

their implementation in clinical settings (Ellington et al., 2010). Most of the issues in the development of protein microarrays stem from the complexity of protein-based interactions. Unlike the predictable sequence-specific hybridization chemistry of DNA arrays, proteins exhibit extraordinary diversity in their structures and affinities (Gaster et al., 2011). The selectivity of microarray-type immunoassays results from the specificity of the antigen–antibody reaction. In many cases, however, the specificity compromised the ability of other molecules with structurally similar or identical epitopes to bind to the same antibody. This competition is commonly referred to as cross-reactivity. The use of monoclonal antibodies has greatly increased the specificity of immunoassays; similarly, the use of two-site (or sandwich) assays that require two distinct epitopes on the antigen to be recognized greatly decreases cross-reactivity but does not fully eliminate this interference (Diamandis and Christopoulos, 1996). The accuracy of microarray-based immunoassays depends on the elimination or at least the minimization of antibody cross-reactivity.

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The ELISA format is the gold standard for multiplex protein profiling. In this type of assay, the dual binding of the target analytes to the pairs of capture and detection antibodies yields greater specificity than using singular analyte detection. However, when the detection antibodies are used in mixture, as in microarray based multiplex assays, cross-reactivity between non-matched pairs of antibodies is often observed. This effect produces background noise, which affects sensitivity and causes false positive results (Ellington et al., 2010). As a consequence, the development of multiplex immunoassays on a planar microarray format usually requires extensive validation and a time-consuming development process (Gonzalez et al., 2008).

Due to the vulnerability of multiplex sandwich assays to cross-reactivity (Pla-Roca et al., 2012), it was found that the number of required antibody pairs increases with the number of analytes, making the large-scale application of multiplex sandwich assays unfeasible.

To overcome this problem, assay components are physically separated in multiple bead-based assays (Schneiderhan-Marra et al., 2010) or by antibody colocalization microarrays that deliver detection antibodies specifically to the locations of the matched capture antibodies (Pla-Roca et al., 2012). However, multiplex sandwich assays so far have only been used simultaneously for tens of targets (Pla-Roca et al., 2012; Djoba Siawaya et al., 2008).

Recently, the SnapChip method was demonstrated (Li et al., 2012) to solve the problem of cross-reactivity in multiplex immunoassay. In this approach detection antibodies, spotted on a “transfer slide” are transferred at once by snapping on an “assay slide” with capture antibodies and antigens. Though interesting, this approach is limited by the spot volume in the amount of material to be transferred.

In this work, we present the use of hydrogels as a versatile method to create compartments for parallel analysis in microarrays. Hydrogel compartments of different formats can be polymerized on slides, desiccated and rehydrated with solutions containing microarray reagents. During the incubation step, hydrogel plugs are placed on the corresponding sub-array printed on the microarray slide to allow entrapped reagents to be exposed to the printed surface. Hydrogel compartments are versatile reservoirs that can be used to avoid cross-reactivity by separating single detection antibodies, to incorporate samples when low incubation volumes are needed or to contain calibration standards used for accurate quantification. Hydrogel compartments can also be used for long-term reagent storage in dry conditions. Examples of the applicability of hydrogel plugs in microarray technology are shown in several relevant applications. In particular, immunoassay for HIV p24 antigen has been chosen to demonstrate the equivalence in sensitivity when using either hydrogel plugs or standard incubation conditions due to the low limits of detection required by clinical assays for infectious disease diagnostics; interleukin 10 detection has been selected to demonstrate accuracy in quantification when using an integrated calibration curve since evaluation of cytokine expression levels is a critical step in biomarker discovery and development process; detection of *Staphylococcus aureus* enterotoxins A and B is used to show how hydrogel plugs prevent cross-reactivity issues since the detection antibodies selected in this work are good examples of cross-reacting reagents.

2. Materials and methods

2.1. Materials

Phosphate saline buffer (PBS), Trizma base (Tris), HCl, ethanolamine, NaCl, Tween 20, ammonium sulfate, *N,N*-dimethylacrylamide (DMA), 3-(trimethoxysilyl)propyl methacrylate (MAPS), ammonium persulfate (APS), tetramethylethylenediamine (TEMED) and *N,N'*-methylenebisacrylamide (Bis) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cyanine 3 labeled goat anti-rabbit secondary IgG (anti-Rabbit IgG Cy3) and rabbit IgG were obtained from Jackson ImmunoResearch (West Grove, PA, USA). Silicon oxide slides were bought from Silicon Valley Microelectronics (Santa Clara, CA, USA). Multiwell cell culture slides were purchased from Grace Bio-labs (Redmond, OR).

2.2. Coating of microarray slides with poly(DMA-co-NAS-co-MAPS)

Poly(DMA-co-NAS-co-MAPS)-coated silicon microarrays were fabricated as previously described (Cretich et al., 2004). Briefly, silicon slides were immersed for 30 min in a poly(DMA-co-NAS-co-MAPS) solution (1% w/v in 0.9 M (NH₄)₂SO₄). The chips were then rinsed with DI water, dried with nitrogen and finally cured under vacuum at 80 °C. A picture of the silicon slides used in this work is shown in the Supplementary information.

2.3. Fabrication of hydrogel plugs

Multiwell cell culture system slides (see Fig. 1) used to compartmentalize eight hydrogel plugs onto the same slide were pre-treated with oxygen plasma for 10 min. The silanization solution was prepared by dissolving MAPS in toluene at a final concentration of 10% v/v. Aliquots (30 µL) of this solution were added to each well. Slides were stored in a chamber saturated with toluene vapor for 30 min, washed with acetone, dried with nitrogen and cured in a vacuum oven at 80 °C for 30 min. A DMA-Bis solution (4% T, 3% C) was prepared by diluting a stock solution (20% T, 3% C) in deionized (DI) water (where % T is [grams(acrylamide + methylenebisacrylamide) / total volume] × 100 and % C is [grams(methylenebisacrylamide) / g(acrylamide + bis-acrylamide)] × 100). APS (40% w/v) and TEMED were added to give a final concentration of 5 µL/mL for both components. The monomer solution (30 µL) was immediately loaded into each well and polymerized for 30 min at room temperature. After polymerization the slides were washed twice with DI water (10 min each) and with a solution of 1% v/v glycerol in DI water (10 min) on a shaker. The excess water was removed by evaporation with nitrogen, and the hydrogel plugs were finally dried in a vacuum oven at room temperature. The hydrogels at the bottom of the wells were rehydrated simply by filling the wells with a volume of solution containing the protein of interest.

2.4. Microarray experiments: compartmentalization of secondary antibodies

Rabbit polyclonal antibodies against *Staphylococcus* enterotoxin A (SEA) and *Staphylococcus* enterotoxin B (SEB)



Fig. 1. Picture of the multiwell system used to compartmentalize eight hydrogel plugs. The hydrogels are made of a DMA-Bis solution (4% T, 3% C) that can be desiccated and rehydrated in 60 min by adding 30 μ L of solution.

(from Sigma-Aldrich, St. Louis, MO) as well as streptavidin labeled with Cy3 as a reference were patterned on each silicon chip using a SciFlexArrayer S5 spotter from Scienion (Berlin, Germany) according to the scheme reported in Fig. 3. The printed chips were placed in a humidity chamber and incubated at room temperature overnight. The chips were then blocked by 50 mM ethanolamine in 1 M Tris/HCl (pH 9) for 1 h, washed with DI water and dried with nitrogen. Then, the chips were incubated for 2 h with a protein solution made of a mixture of 100 ng/mL SEA and 10 ng/mL SEB (Sigma-Aldrich, St. Louis, MO). The chips were washed with the washing buffer (0.05 M Tris/HCl (pH 9), 0.25 M NaCl, 0.05% v/v Tween 20) for 10 min while stirring and next rinsed with DI water. For the solution incubation methods, the chips were incubated with a mixture of labeled secondary monoclonal antibodies at 1 μ g/mL in the incubation buffer (50 mM Tris–HCl, 150 mM NaCl and 0.02% Tween 20) for 1 h. The mouse monoclonal antibody against SEA (HyTest, Turku, Finland) was labeled with Cy5 (GE Healthcare, Buckinghamshire, UK), whereas the mouse monoclonal antibody against SEB (HyTest, Turku, Finland) was labeled with Cy3 (GE Healthcare, Buckinghamshire, UK). For the hydrogel plug-mediated incubation method, the chips were placed in contact for 1 h with the hydrogel plugs, previously rehydrated for 1 h with 20 μ L of labeled antibody at 1 μ g/mL in the incubation buffer. The

antibodies were compartmentalized in separate plugs as shown in Fig. 2. Each plug was placed in contact with the corresponding sub-array spotted on the chip. After removal of the plugs, the chips were washed with PBS (10 min) and DI water (10 min). Fluorescence evaluation visualization was performed by a ProScanArray scanner from Perkin Elmer (Boston, MA); the silicon slides were analyzed using 90% photomultiplier (PMT) gain and laser power. The fluorescence intensities of 12 replicated spots were averaged.

2.5. Microarray experiments: HIV p24 assay

Mouse monoclonal antibodies against HIV p24 and the corresponding antigen were gifts from DiaPro srl (Milan, Italy).

Four arrays of the capture antibody against HIV-p24 and streptavidin labeled with Cy3 as a reference were patterned on each chip. Each antibody was spotted in PBS. The printed chips were placed in a humidity chamber and incubated at room temperature overnight. The chips were then blocked by 50 mM ethanolamine in 1 M Tris/HCl (pH 9) for 1 h, washed with DI water and dried with nitrogen.

DMA hydrogel plugs were rehydrated for 1 h with 20 or 10 μ L of the HIV-p24 antigen solutions at 0, 1, 2, and 5 ng/mL in the incubation buffer. The chips were incubated for 2 h on the corresponding hydrogel plugs. Simultaneously, the control chips were incubated in the conventional manner, with 20 μ L of the HIV-p24 antigen solutions at 0, 1, 2, and 5 ng/mL in the incubation buffer for 2 h. The chips were then washed with the washing buffer for 10 min while being stirred, rinsed with DI water, and incubated with 100 μ L of 1 μ g/mL biotinylated secondary antibody against HIV-p24 in incubation buffer for 1 h in static conditions. The chips were washed with PBS (10 min) and DI water (10 min) and incubated with 100 μ L of 1 μ g/mL streptavidin labeled with Cy3 (Jackson ImmunoResearch, West Grove, PA) in PBS for 1 h. The chips were washed again with PBS (10 min) and DI water (10 min). A calibration curve was created using the fluorescent intensities obtained upon incubation with several concentrations of HIV p24 antigen in the ng/mL range. The limit of detection (LOD) was extrapolated from the fluorescent value of blank samples plus three standard deviations (3σ). Three replicates of each experiment were performed.

2.6. Microarray experiments: calibration curve

Eight arrays of the capture antibody against IL-10 (BioLegend, San Diego, CA) and streptavidin labeled with Cy3 as a reference (Jackson ImmunoResearch, West Grove, PA) were patterned on a silicon slide using a SciFlexArrayer S5 spotter according to the scheme reported in Fig. 4. The printed slide was placed in a humidity chamber and incubated at room temperature overnight. The slide was then blocked by 50 mM ethanolamine in 1 M Tris/HCl (pH 9) for 1 h, washed with DI water and dried with nitrogen.

Six of the eight hydrogel plugs were hydrated with 20 μ L of the incubation buffer containing 1 μ g/mL of the biotinylated detection antibodies against IL-10 and 0, 2, 5, 10, 20 and 40 ng/mL of IL-10 (BioLegend, San Diego, CA) to obtain the calibration curve. The dried plugs were stored at 4 °C. Upon use, the two remaining plugs were rehydrated with

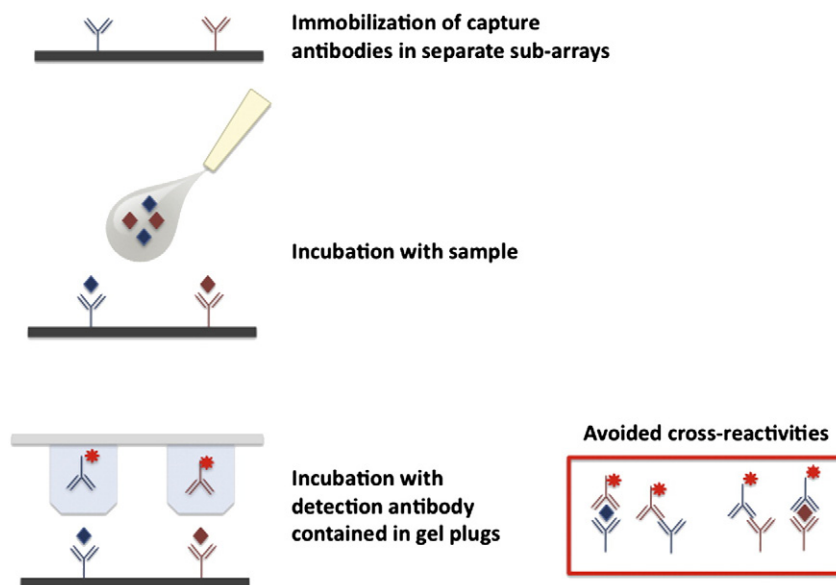


Fig. 2. Scheme of the *Staphylococcus aureus* enterotoxins A and B (SEA and SEB) detection assay. The sample was incubated on a microarray spotted with two polyclonal capture antibodies. The toxins were then detected with a mixture of the two monoclonal detection antibodies. To overcome cross-reactivities, the capture antibodies for SEA and SEB were spotted in two separate sub-arrays. The entire area of the microarray slide was then incubated with the sample, and toxins were revealed using the two detection antibodies confined in different hydrogel plugs.

sample solutions of 3 and 10 ng/mL of IL-10 (BioLegend, San Diego, CA).

The silicon-arrayed slide was incubated for 2 h at room temperature on the corresponding rehydrated hydrogel plugs. The slide was then washed with the washing buffer for 10 min while being stirred, rinsed with DI water and incubated with 1 µg/mL Cy3-streptavidin in the incubation buffer for 1 h. The slide was washed with PBS (10 min) and DI water (10 min). Fluorescence visualization was performed by a ProScanArray scanner; the slide was analyzed using 90% photomultiplier (PMT) gain and laser power. The fluorescence intensities of nine replicated spots were averaged.

Multiwell cell culture system slides (Fig. 6, panel a) with fifty wells were used for the fifty-plug format. The assay was performed as described above using IL-6 ELISA kit (BioLegend, San Diego, CA). Calibration mixtures were composed of 0, 2, 5, 10, 50 ng/mL IL-6 and the biotinylated detection antibody at 0.01 mg/mL.

3. Results and discussion

3.1. Compartments for secondary antibodies

The cross-reactivity between capture and detection antibodies is one of the most critical issues when developing multiplex sandwich immunoassays. To show how hydrogels can prevent antibody cross-reactivity, the detection of *S. aureus* enterotoxins A and B (SEA and SEB) by microarrays was investigated. In a normal microarray, the sample contacts a glass slide spotted with two polyclonal capture antibodies, and the toxins are then detected with a mixture of the two fluorescently labeled monoclonal antibodies.

The specificities of the detection antibodies selected in this work are poor. In fact, the SEA detection antibody cross-reacts with SEB and its capture antibody, whereas the SEB detection

antibody slightly interacts with SEA and its capture probe (Fig. 2). To overcome this issue, hydrogel plugs to confine the detection antibodies were developed. The scheme of the assay using hydrogels is depicted in Fig. 2. The capture antibodies for SEA and SEB were spotted on the same slide in two separate sub-arrays. The entire slide was then incubated with the sample, and the toxins were revealed using the two detection antibodies confined in the different hydrogels. The spotted capture antibody sub-arrays and hydrogel plugs, which were rehydrated either with the anti-SEA antibody labeled with Cy5 or with the anti-SEB antibody labeled with Cy3 in the incubation buffer, were aligned. The chip bearing the hydrogels was placed in contact with the microarray slide and incubated for 60 min. The spotting scheme and the fluorescence results are reported in Fig. 3. In panel a, the fluorescence of the assay using a mixture of the SEA and SEB detection antibodies is shown. The array was first incubated with a solution containing a mixture of SEA (100 ng/mL) and SEB (10 ng/mL) as described in the [Materials and methods](#) section and then with a mixture of the two monoclonal detection antibodies in the incubation buffer at 1 µg/mL. The Anti-SEA antibody was labeled with Cy5, and the anti-SEB antibody was labeled with Cy3. The fluorescence signals found for both fluorophores are shown in panel a. The specific signals originating from the capture and detection antibody sandwiches are clearly visible on the left sub-array of the Cy5 channel for SEA and on the right sub-array of the Cy3 channel for SEB. The cross-reacting antibody signals, Cy3 anti-SEA on anti-SEB and Cy5 anti-SEB on anti-SEA, are also visible on the left sub-array of the Cy3 channel and right sub-array of the Cy5 channel, respectively (highlighted). When the antibodies are labeled with the same fluorophore (as in most assays), it is not possible to distinguish this type of nonspecific binding from a real toxin detection event, leading to false positive results.

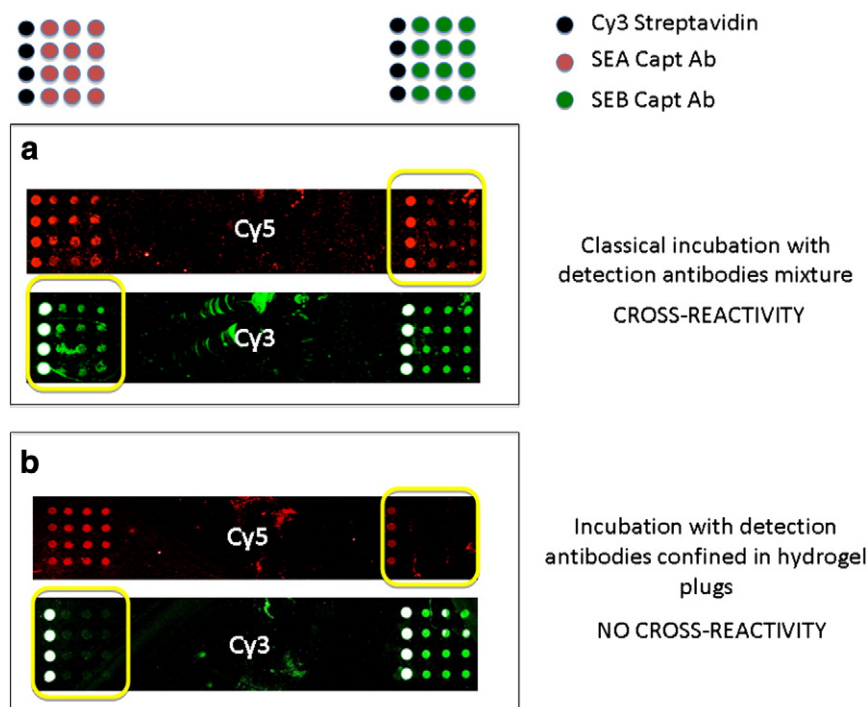


Fig. 3. Spotting scheme and dual channel detection of SEA and SEB. Panel a: Fluorescence detection of the assay performed using a mixture of the SEA and SEB detection antibodies. The specific signals originating from the perfect match between capture and detection antibodies mediated by the toxin are clearly visible on the left sub-array of the Cy5 channel for SEA and on the right sub-array of the Cy3 channel for SEB. However, the cross-reacting antibody signals, are visible as well for each sub-array on the same chip, as highlighted in the rectangle. Panel b: Fluorescence detection of the assay performed using hydrogel-plugs to confine the detection antibodies: in this case, the labeled detection antibodies were entrapped in hydrogel plugs facing the corresponding sub-arrays on the slide. Specific toxin signals are clearly visible on the left sub-array of the Cy5 channel for SEA and on the right sub-array of the Cy3 channel for SEB, whereas nonspecific fluorescence, originating from antibody cross-reactivities, is not detectable (as highlighted in the rectangle).

The fluorescence detection results using the hydrogels to confine the detection antibodies are shown in panel b. The toxins were analyzed as described above except that the labeled detection antibodies were entrapped in hydrogel plugs facing the corresponding sub-arrays on the slide. Because the toxins on the surface were contacted only by their specific detection antibodies, only the signals from those detection antibodies were observed, and the nonspecific, cross-reactivity fluorescence was not present (see highlighted areas on the left sub-array of the Cy3 channel and right sub-array of the Cy5 channel, respectively).

3.2. Compartments for samples

Hydrogel plugs can also help avoid liquid spreading on the microarray surfaces. Confining the target within a hydrogel of a defined shape provides a way to increase its local concentration, resulting in improved signal intensities. In a standard microarray experiment, the target solution covers the entire surface, including unspotted areas. Concentrating the target in a specific zone of the slide reduces the cost per experiment. Moreover, compartments are often used to incubate calibration standards and the unknown sample at the same time to increase the accuracy of quantitation. Multi-well formats are commercially available for parallel analysis of multiple samples; however, they rely on the use of hydrophobic patterning, adhesive silicon chambers or microplates to be

used in combination with multiple wells. A way to use hydrogel compartments for multiplexing analysis is reported below.

It was demonstrated that a sandwich immunoassay for the HIV p24 antigen using a conventional incubation method with the target in solution provided results that were identical to those obtained with the desiccated hydrogel-plaques rehydrated with the sample solution.

In this assay, poly(DMA-co-NAS-co-MAPS)-coated silicon slides were spotted with capture anti-p24 monoclonal antibodies (1 mg/mL) in PBS (pH 7.2). After an overnight binding step followed by a blocking step as described in the [Materials and methods](#) section, the slides were incubated for 2 h either with 20 μ L of HIV p24 antigen dissolved in the incubation buffer or with hydrogel-plaques rehydrated with the same antigen solution. To detect the captured antigen, a conventional incubation method with the detection antibody was performed in both cases. Several antigen concentrations were assayed from 0 to 5 ng/mL, and calibration curves were built to extrapolate the limits of detection (LODs) as described in the [Materials and methods](#) section. The conventional incubation method provided a LOD value of 37 pg/mL and the incubation method with the hydrogel plugs gave a value of 45 pg/mL. Calibration curves are reported in the Supplementary information. The sensitivity obtained for the model test demonstrates that the use of the hydrogel is as effective as the conventional method. Moreover, we noticed that when

using 6 mm diameter hydrogel plugs, a reduction of rehydration volume from 30 to 10 μL does not affect the sensitivity of the assay, allowing us the use of a reduced sample volume.

3.3. Integrated calibration curves

Dry-form reagent storage is particularly useful for its ability to preserve reagents without using refrigeration, to simplify assay automation, which can improve assay repeatability, to reduce training requirements and to facilitate integration into lab-on-a-chip devices (Stevens et al., 2008). Hydrogels easily store reagents such as secondary antibodies or calibration curve standards. To prepare the storage hydrogels, solutions of the reagents at the required concentrations are used to rehydrate dry hydrogels, which are then desiccated and stored. Immediately before usage, the hydrogels are rehydrated with DI water and used in the assay. To demonstrate the feasibility of the method, a sandwich immunoassay for interleukin 10 (IL-10) detection with an integrated calibration curve was carried out. A picture of the hydrogel compartments and the spotting scheme is shown in Fig. 4. In this assay, an eight-plug format was used. Six of the eight hydrogels (a to f) contained the standard mix for the calibration curve (0, 2, 5, 10, 20 or 40 ng/mL IL-10 and the biotinylated detection antibody at 0.001 mg/mL), and two of the hydrogels (g and h) were used to assay unknown samples. The chip bearing the hydrogels was stored in its dry form. Before use, the calibration hydrogels were rehydrated with the incubation buffer, and the sample hydrogels were rehydrated with the sample dissolved in the

incubation buffer. The IL-10 capture antibody was spotted in 8 replicate sub-arrays that were placed in contact with the hydrogel compartments for 1 h. Washing steps and incubation with Cy3-labeled streptavidin were carried out as described in the Materials and methods section. Fig. 4 reports the fluorescence results obtained with a slide in contact with the calibration hydrogels described above and two samples in which IL-10 was spiked at concentrations of 10 ng/mL (array g) and 3 ng/mL (array h). Using the calibration curve (Fig. 5) generated by incubating with the calibrating hydrogel plugs (blue dots), samples in arrays g and h provided mean fluorescence signals equal to 8524 (SD = 1773) and 3849 (SD = 682) (red dashes). According to the calibration curve generated using the concentrations from 0 to 20 ng/mL, the signals were quantified as 11.14 ng/mL and 2.27 ng/mL, respectively.

We have tested the scalability of this approach using a 50 hydrogel format to demonstrate its usefulness in high-density microarray technology. Fig. 6, panel a) shows a picture of a 50 multiwell cell culture system where the 50 gel plugs were re-hydrated with a Bromophenol Blue solution. A sandwich immunoassay for interleukin 6 (IL-6) detection was carried out. Panel b) shows the fluorescence results of a slide where each of the 50 gel plugs was re-hydrated with 7 μL of IL-6 at the concentration of 50 ng/mL resulting in an average fluorescence value (at 70% PMT and laser power) of 29582 ± 3589 . Eight spots per each replicate were measured.

In a parallel experiment, 10 replicated calibration curves were analyzed: the 50 hydrogel plugs contained 10 replicates of each of the standard mixtures for the calibration curve (0, 2,

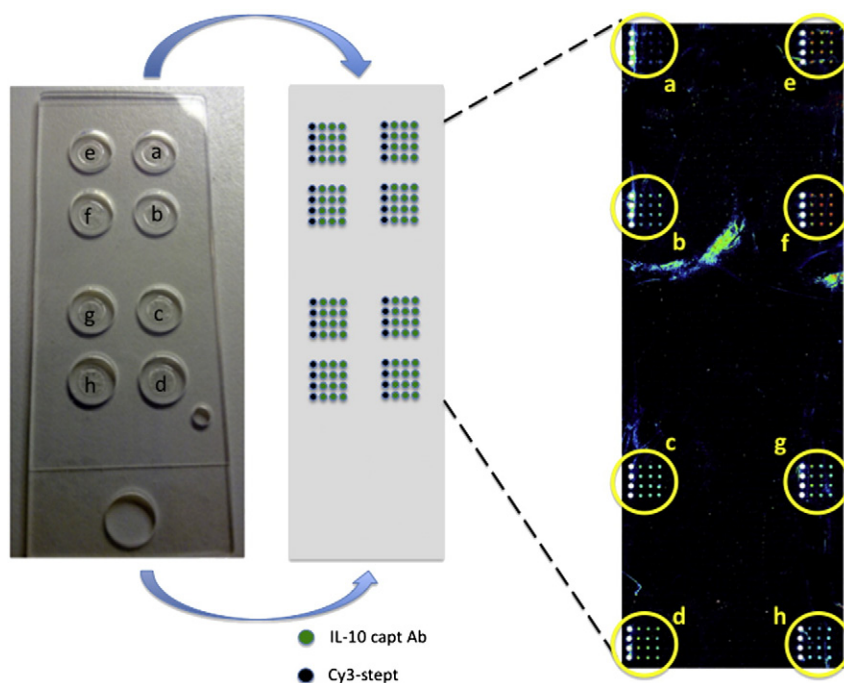


Fig. 4. Picture of the hydrogel plugs and spotting scheme. Six of the eight hydrogel plugs (a to f) contained a standard mix for a calibration curve (0, 2, 5, 10, 20 or 40 ng/mL IL-10 and the biotinylated detection antibody at 0.01 mg/mL), and two of the hydrogel plugs (g and h) were used to assay unknown samples. The IL-10 capture antibody was spotted in eight replicated sub-arrays that were placed in contact with the hydrogel plugs for 1 h. The right panel reports the fluorescence results obtained with a slide in contact with the calibration plugs described above and two samples in which IL-10 was spiked at concentrations of 10 ng/mL (array g) and 3 ng/mL (array h).

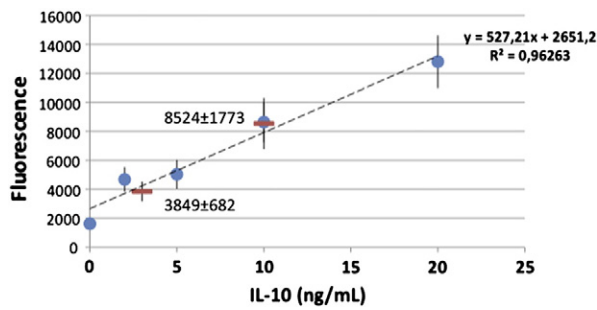


Fig. 5. Calibration curve based on fluorescent signals obtained on arrays a–e (blue dots). The fluorescence of array f was not included because it was out of the linear range. Samples in arrays g and h (red dashes) provided fluorescence signals equal to 8524 ± 1773 and 3849 ± 682 . According to the calibration curve, these signals were quantified as 11.14 ng/mL and 2.27 ng/mL, respectively.

5, 10, 50 ng/mL IL-6 and the biotinylated detection antibody at 0.001 mg/mL). The resulting calibration curve is shown in Fig. 6, panel c).

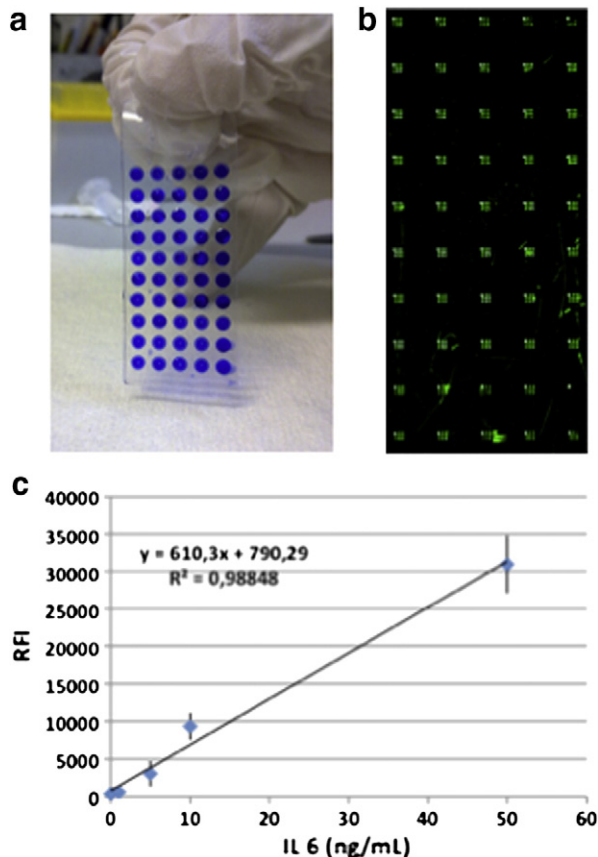


Fig. 6. Multiplexing of the system and calibration curve. Panel a) Picture of the multiwell system used to compartmentalize fifty hydrogel plugs re-hydrated with a Bromophenol Blue solution. Panel b): Fluorescence results obtained with a slide in contact with the calibration plugs in which IL-6 was spiked at concentrations of 50 ng/mL. Panel c): Calibration curve based on fluorescent signals obtained from 10 replicates of each calibration mixtures (0, 2, 5, 10, 50 ng/mL IL-6 and the biotinylated detection antibody at 0.01 mg/mL).

Using hydrogels to create controlled microenvironments has been proposed to immobilize proteins (Rubina et al., 2003) and DNA (Rubina et al., 2004) to study protein interactions with the goal of mimicking the complexity of the crowded cellular environment (Moorthy et al., 2007) and to characterize antigenicity and antibody functionality for biosensor applications (Charles et al., 2004).

This work aimed to develop hydrogels in which proteins can be stored and transported but not irreversibly trapped. Hydrogels define a highly aqueous environment because they are ~97% water, which causes the physical localization of a protein while maintaining its integrity and functionality.

According to Holmes and Stellwagen (Holmes and Stellwagen, 1991), the pore size of a polyacrylamide hydrogel strongly depends on the concentration of the acrylamide monomer (% T) and of the bisacrylamide cross-linker (% C). At a fixed concentration of cross-linker, the apparent pore radius decreases from 140 to 20 nm while % T is increased from 3 to 10%. In this work, the hydrogels were made of DMA (4% T) cross-linked with Bis (3% C). The low monomer concentration was used to facilitate protein diffusion and increase transfer efficiency. DMA was used due to its greater stability compared to polyacrylamide to create round hydrogels with a diameter of 6 mm covalently attached to a glass slide to facilitate their transport.

To the best of our knowledge there are no other examples in the literature on the use of hydrogels as reagent's reservoirs.

4. Conclusions

Due to the highly complex structure of each protein, antibodies used in high-density arrays often bind aberrantly to epitopes with similar structures, resulting in nonspecific cross-reactive signals. The accuracy of antibody-based microarrays thus depends on eliminating or at least minimizing this cross-reactivity. In this work, a way to create compartments for detection antibodies and soluble microarray reagents was described. The containers are made of hydrogels immobilized on a slide facing the corresponding sub-array on the printed surface. The porosity of the hydrogels made of the DMA-Bis solution (4% T, 3% C) allowed the easy transfer of biomolecules while preserving their integrity.

The use of hydrogels prevented cross-reactivity issues in *S. aureus* enterotoxins A and B detection assay and provided an accurate quantification of IL-10 by a microarray-integrated calibration curve. Surface-attached hydrogels can therefore be used as a versatile compartmentalization tool for the storage and the transport of soluble microarray reagents.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jim.2013.02.013>.

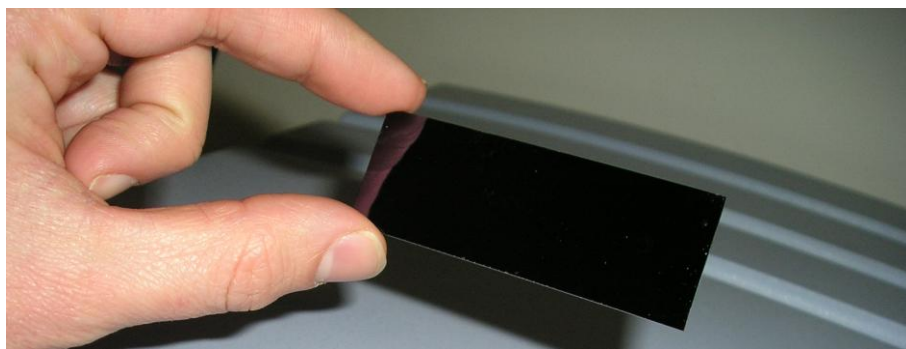
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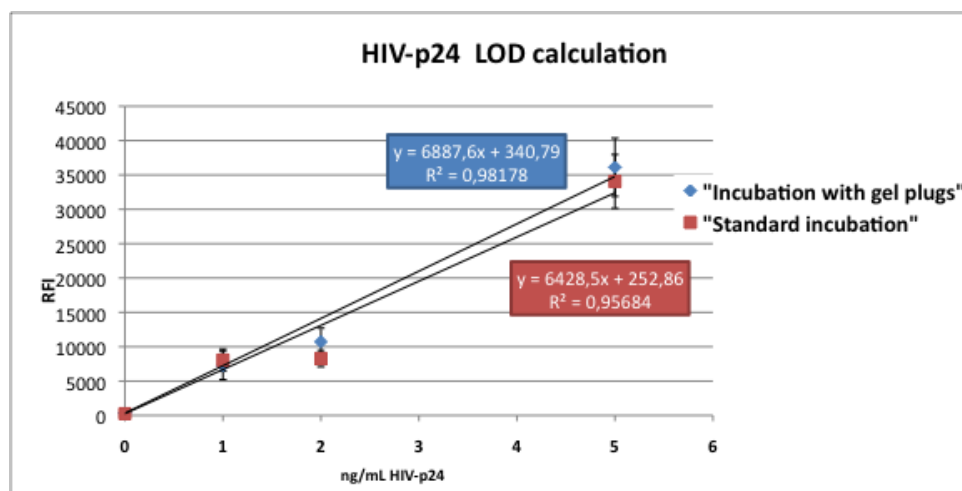
Surface immobilized hydrogels as versatile reagent reservoirs for microarrays

Laura Sola, Paola Gagni, Marina Cretich and Marcella Chiari*

SUPPLEMENTARY INFORMATION



Picture of the silicon slides used in this work



Calibration curves used to calculate the limits of detection (LODs) for HIV p 24 antigen when using either standard incubation (red dots) or hydrogel plugs.

Curves led to LOD of 37 pg/mL for standard incubation and 45 pg/mL for incubation with gel plugs.