



Notes & Tips

Overcoming mass transport limitations to achieve femtomolar detection limits on silicon protein microarrays

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ARTICLE INFO

Article history:

Received 19 May 2011

Received in revised form 4 July 2011

Accepted 5 July 2011

Available online 14 July 2011

Keywords:

Protein microarrays

Polymeric coating

Fluorescence

Limit of detection

Infectious disease

ABSTRACT

Here we combine the use of fluorescence-enhancing silicon substrates coated by copoly(DMA–NAS–MAPS), a ter-copolymer based on *N,N*-dimethylacrylamide (DMA), *N*-acryloyloxysuccinimide (NAS), and 3-(trimethoxysilyl)propyl-methacrylate (MAPS), with an efficient dynamic incubation to overcome mass transport limitations and obtain femtomolar limits of detection. The high sensitivity was obtained with a conventional microarray scanner without the use of any sophisticated detection strategy or protocol. When the method was applied, an improvement of the analytical sensitivity of approximately three orders of magnitude was achieved for antibody detection when compared with the same assay performed on regular glass slides and static conditions. Moreover, limits of detection of 45 and 54 pg/ml were obtained for hepatitis B superficial antigen and HIV p24 antigen, respectively.

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Protein microarrays have become well-established research tools; their enormous potential for in vitro diagnostics already generated the first products on the market [1]. High sensitivity is the key factor that drives the process so that new tests are more likely to reach the clinic and the market. Multiple factors affect detection sensitivity in fluorescent microarrays, including (i) surface chemistry that promotes binding of proteins in native conformation and suppresses nonspecific analyte adsorption [2], (ii) new labels or detection strategies to amplify the signals [3], (iii) optimal assay design to compensate for kinetic limitations [4], and (iv) use of antibody pairs with optimal dissociation constants (K_D) [5] and with low or zero cross-reactivity. Moreover, in fluorescent microarrays, the specificity of labeled secondary antibodies directly influences analytical sensitivity by affecting the fluorescent signal detected for blank samples.

We recently reported on the use of silicon with a silicon dioxide top layer of appropriate thickness to enhance fluorescence on the surface of a microarray substrate [6]. On these slides, fluorescence is intensified due to optical constructive interference between the incident and reflected light of the fluorescent radiation. The silicon oxide surface was functionalized by adsorption of copoly(DMA–NAS–MAPS), a ter-copolymer based on *N,N*-dimethylacrylamide (DMA),¹ *N*-acryloyloxysuccinimide (NAS), and

3-(trimethoxysilyl)propyl-methacrylate (MAPS) [7] that does not alter the optical properties of the optimized SiO₂ layers and allows us to produce microarray supports characterized by a very low nonspecific background [8].

In this article, we demonstrate how critical mass transport limitations are for the outcome of microarray assay sensitivity. By replacing regular glass slides with silicon slides and by applying an efficient mixing of the sample to avoid kinetic limitations, we demonstrate a strong sensitivity improvement for selected antibodies with a reduction of two orders of magnitude in the lowest detectable concentration.

Ovalbumin, β -lactoglobulin B, and α -lactalbumin were immobilized in three different pH conditions on copoly(DMA–NAS–MAPS)-coated microscope glass slides (Sigma, St. Louis, MO, USA) and silicon slides (SVM, Santa Clara, CA, USA). The chips were incubated with several concentrations of anti-ovalbumin, anti- β -lactoglobulin B, and anti- α -lactalbumin polyclonal antibodies in static (using a cover slip) and dynamic (in a Petri dish on a horizontal shaker at 50 rpm) conditions, followed by incubation with secondary antibodies. A calibration curve reporting the fluorescent intensities obtained on incubation with the specific antibodies in the nanogram/milliliter (ng/ml) range was built for each protein specific antibody. Each specific antibody limit of detection (LOD) was extrapolated from the fluorescent value of blank samples plus 3 standard deviations (3σ) [9]. In Fig. 1, the antibody LOD values (in g/L) are reported. For each specific antibody (anti-ovalbumin, anti- β -lactoglobulin B, or anti- α -lactalbumin), three immobilization conditions for the target protein are compared: 20 mM 6- ϵ -aminocaproic acid (6-EACA)/acetate (pH 4.4), phosphate-buffered saline (PBS, pH 7.2), and

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¹ Abbreviations used: DMA, *N,N*-dimethylacrylamide; NAS, *N*-acryloyloxysuccinimide; MAPS, 3-(trimethoxysilyl)propyl-methacrylate; LOD, limit of detection; 6-EACA, 6- ϵ -aminocaproic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Hbs Ag, hepatitis B superficial antigen.

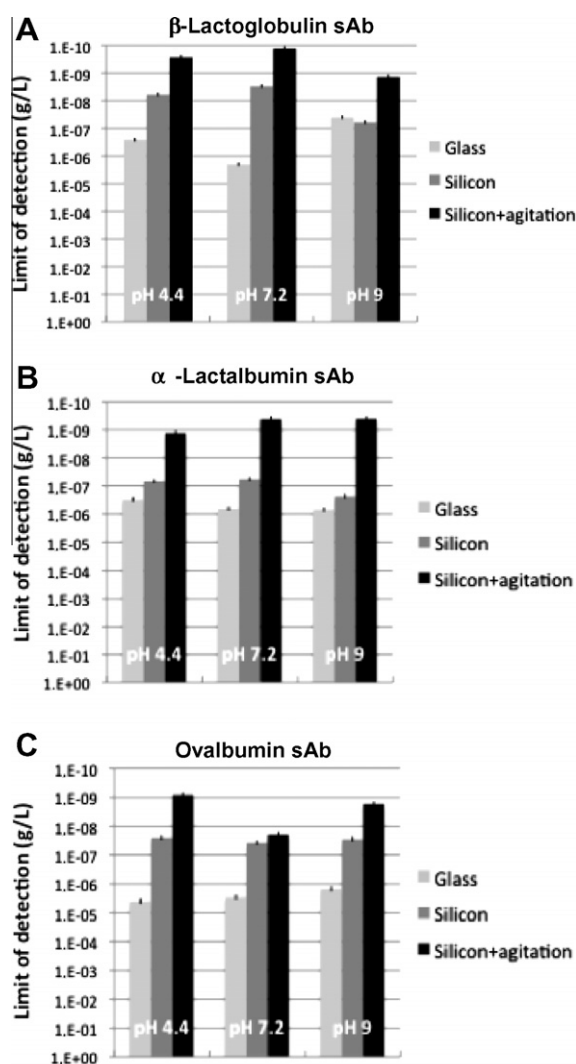


Fig. 1. LODs on glass and silicon incubated in static conditions and on silicon incubated under mixing for anti- β -lactoglobulin specific antibody (A), anti- α -lactalbumin specific antibody (B), and anti-ovalbumin specific antibody (C). Glass and silicon were coated by copoly(DMA–NAS–MAPS). LODs are reported in grams/liter (g/L, reverse order values, logarithmic scale). Except for anti- β -lactoglobulin B and anti- α -lactalbumin, when the proteins were immobilized at pH 9.0, the shift from glass to silicon provided an improvement of the LOD of one or even two orders of magnitude. Similarly, incubation of silicon chips under mixing, except for anti-ovalbumin antibody when the protein is immobilized at pH 7.0, provided an improvement of the LOD up to two more orders of magnitude. sAb, specific antibody.

50 mM borate/NaOH (pH 9.0). Printed slides were placed in a humid chamber and incubated at room temperature overnight. The slides were then blocked by 50 mM ethanolamine in 1 M Tris/HCl (pH 9.0) for 1 h, washed with water, and dried by a stream of nitrogen.

Table 1

LODs expressed as molar concentrations for β -lactoglobulin B, α -lactalbumin, and ovalbumin specific antibodies when different protein immobilization conditions are used on glass and silicon under static or dynamic incubation.

Immobilization condition	Static incubation on glass			Static incubation on silicon			Dynamic incubation on silicon		
	pH 4.4	pH 7.2	pH 9.0	pH 4.4	pH 7.2	pH 9.0	pH 4.4	pH 7.2	pH 9.0
LOD (M) β -Lactoglobulin B sAb	1.73E–12	1.33E–11	2.67E–13	4.00E–14	2.00E–14	4.00E–13	1.80E–15	8.67E–16	9.20E–15
LOD (M) α -Lactalbumin sAb	2.00E–12	4.27E–12	4.67E–12	4.47E–13	3.80E–13	1.55E–12	8.73E–15	2.73E–15	2.67E–15
LOD (M) Ovalbumin sAb	2.67E–11	1.91E–11	1.00E–11	1.67E–13	2.47E–13	1.87E–13	5.47E–15	1.30E–13	1.13E–14

Note. sAb, specific antibody.

Arrayed slides were then incubated with the solutions of antibody in incubation buffer (0.05 M Tris/HCl [pH 7.6], 0.15 M NaCl, and 0.02% Tween 20) with 1% (w/v) bovine serum albumin (BSA) for 2 h. Slides were then washed with washing buffer (0.05 M Tris/HCl [pH 9.0], 0.25 M NaCl, and 0.05% Tween 20) for 10 min, rinsed with water, and incubated with a labeled secondary antibody (0.001 mg/ml in incubation buffer) for 1 h. Slides were then washed with PBS (10 min), rinsed with water, and dried with a nitrogen stream. Scanning for fluorescence evaluation was performed by a ProScanArray scanner from PerkinElmer (Boston, MA, USA). Fluorescence intensities of five replicated spots were averaged. For each condition, the LOD was determined on copoly(DMA–NAS–MAPS)-coated glass slides and silicon slides (both incubated with the sample in static conditions) and on copoly(DMA–NAS–MAPS) silicon slides on which sample was incubated under mixing.

Except for anti- β -lactoglobulin B and anti- α -lactalbumin, when the proteins were immobilized at pH 9.0, the shift from glass to silicon provided an improvement of the LOD of one or even two orders of magnitude. This is due to the fluorescence-enhancing effect of the 100-nm SiO₂ optical interference layer coupled to the favorable nonfouling properties of the copoly(DMA–NAS–MAPS) polymeric coating.

Similarly, incubation of biofunctionalized silicon chips under mixing, except for anti-ovalbumin antibody when the protein is immobilized at pH 7.0, provided an improvement of the LOD up to two more orders of magnitude, leading to the outstanding LODs reported in picograms/milliliter (pg/ml) in Fig. 1 and antibody molar concentrations in Table 1. Indeed, efficient mixing of the sample on the microarray surface compensates for mass transport limitations, which are a key physicochemical limitation of the current generation of microarray immunoassays [4].

The optimized protocol described above was applied to detection of infectious diseases by the assay of viral antigens with diagnostic and prognostic value: hepatitis B superficial antigen (Hbs Ag) and HIV p24 antigen. Briefly, copoly(DMA–NAS–MAPS)-coated silicon slides were spotted with capture anti-Hbs Ag and anti-p24 monoclonal antibodies (1 mg/ml) in three spotting buffers: 20 mM 6-EACA/acetate (pH 4.4), PBS (pH 7.2), and 50 mM borate/NaOH (pH 9.0). After overnight binding and blocking as described above, the slides were incubated with Hbs Ag or HIV p24 in incubation buffer for 2 h under mixing. The antigens were then revealed using monoclonal specific secondary antibodies in-house labeled with Cy3. Slides were scanned at 80% laser power, and PMT gain and LODs were extrapolated by calibration curves as described above. Antigens and antibodies were a kind gift from DIA PRO (Milano, Italy). Fig. 2 shows the images of the arrays incubated with 0, 0.01, 0.1, and 1 ng/ml antigens. The detection of Hbs Ag, when the primary antibody was spotted in PBS, provided an LOD of 45 pg/ml, whereas for HIV p24 an LOD of 54 pg/ml was obtained immobilizing the capture antibody in borate buffer. Given molecular weights of 24 kDa for HIV p24 antigen and 98 kDa for Hbs Ag, we obtained LODs equal to 2.25 and 0.46 fmol, respectively.

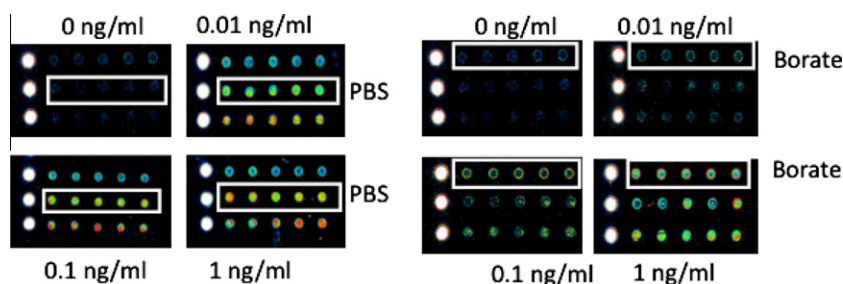


Fig.2. Microarray images (80% laser power and PMT gain) of the detection of 0, 0.01, 0.1, and 1 ng/ml Hbs Ag (left panel) and HIV p24 (right panel). Assays were performed using dynamic incubation on silicon substrates coated by copoly(DMA–NAS–MAPS). The detection of Hbs Ag, when the primary antibody was spotted in PBS, provided an LOD of 45 pg/ml, whereas for HIV p24 an LOD of 54 pg/ml was obtained immobilizing the capture antibody in borate buffer.

In conclusion, the use of fluorescence-enhancing silicon substrates coated by copoly(DMA–NAS–MAPS) and efficient dynamic incubation to overcome mass transport limitations provided sub-femtomolar LODs for specific antibodies and infectious disease-related antigens. High sensitivity was achieved with the use of a conventional microarray scanner without applying target amplification strategy or cumbersome protocols.

Acknowledgments

This article was published with the support of Fondazione Cariplo, project Spintronic Biosensors for Medicine (Grant 2008-2330), and Regione Lombardia, projects FREE-IMAGER (Rif. SAL-39) and SEPSIS (ID 13269241).

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