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Characterization of a new fluorescence-enhancing substrate for microarrays with femtomolar sensitivity

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ABSTRACT

The demand for high sensitivity in microarray technology has stimulated the research of new labeling strategies, new substrates with increased loading capacity and new approaches to amplify the fluorescence signals. Although these methods improve the sensitivity, they are based on non-routine procedures which limit their widespread usage. A simple way to achieve fluorescence enhancement is the optical interference (OI) coating technology based on the use of substrates with films of well-defined thickness that maximize photoabsorption of the dye molecules in the vicinity of the surface and reflect the emitted light toward the detector. Here we introduce a new substrate for fluorescence enhancement composed of a thin aluminum mirror and a single quarter wave silicon oxide as a dielectric layer. The use of such material allows the development of a substrate suited for parallel production in conventional IC (Integrated Circuit's) technology, which produces a 20-fold enhancement of fluorescence compared to glass, which is the most common material used in microarrays. The new substrate was coated with a copolymer of *N*,*N*-dimethylacrylamide, *N*-acryloyloxysuccinimide, and 3-(trimethoxysilyl)propyl methacrylate, copoly(DMA-NAS-MAPS), which forms, by a simple and robust procedure, a functional nanometric film that covalently binds bio-probes on the surface and efficiently suppresses non-specific adsorption. The performance of the new fluorescence-enhancing substrate in microarray technology was demonstrated for the detection of a panel of inflammation biomarkers pushing detection limits into the femtomolar range.

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1. Introduction

The identification of disease-specific protein biomarkers in serum is a challenging task due to the presence of proteins in a wide dynamic range of concentration and the low abundance of some of them [1]. Successful detection of low abundance biomarkers will depend upon development of novel methods to separate the low- from the high-abundance proteins. Detection will also depend on robust detection techniques with high sensitivity, specificity and multiplexing capability [2]. Protein microarray-based biosensing is an active area of research with strong potential for the development of novel multiplexed diagnostic assays. In this technology, fluorescent signals are often used to estimate analyte concentration; the signals are provided by fluorophore-labeled molecules on microarray supports. Optical fluorescence detection of microarray spots is now an established laboratory technique thanks to its high sensitivity, good resolution, and acceptable dynamic range. The demand for even higher sensitivity in microarray technology has stimulated the research of new labeling strategies, for example the use of quantum dots [3], of new substrates with increased loading capacity [4], as well as new approaches to amplify the fluorescence signals [5]. Although these methods improve the sensitivity of traditional microarray technologies, they are either based on non-routine procedures which modify the experimental protocols and introduce further steps in the procedure, or made use of expensive reagents with limited shelf-lives. From the perspective of the end-user, the employment of an optimized microarray substrate which enhances fluorescence is the simplest way to achieve a sensitivity improvement since it would not require changing the experimental parameters, would not add any assay steps, and would use a conventional fluorescence scanner for detection. One such simple enhancement would be optical interference (OI) coating technology, using substrates with films of a well-defined thickness that maximize photoabsorption of the dye molecules in the vicinity of

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the surface and reflect the emitted light toward the detector [6]. This strategy has been introduced by Drexhage et al. [7] and applied in the microarray field using glass slides coated with multiple layers of metallic or dielectric films. For example, Choumane et al. [8] designed a multilayer dielectric mirror coating for bicolor microarrays leading to improvements in both fluorescence excitation and collection, each about four fold with an overall improvement 10–15 fold as compared to a standard glass slide. Similarly, Fouquè et al. [9] introduced a multilayer mirror (MOTL) with alternative thin layers of SiO_2 and HfO_2. MOTL slides prevent fluorescence absorption, allowing the simultaneous enhancement of the fluorescence signals of both Cy3 and Cy5 intensities, enhanced by 5–8-fold.

We have recently introduced a (SiO_2/Si) crystalline silicon substrate coated with thermal silicon oxide with a thickness of 100 nm, optimized for fluorescent enhancement [10]. The substrate was functionalized with a polymeric coating [11] to improve microarray sensitivity. The combination of the fluorescence-enhancing properties of silicon with the optimal binding and non-fouling properties of the polymeric coating was applied to challenging diagnostic applications such as allergy [12], viral antigens detection [13] and Alzheimer's disease early diagnosis [14].

Here we introduce a new substrate for fluorescence enhancement, based on the aforementioned OI coating technology. The OI substrate is composed of a thin aluminum mirror and a single quarter-wave silicon oxide as the dielectric layer leading to a multilayered (Si/Al/SiO_2). The proposed configuration allows the development of a substrate with high fluorescence-collection efficiency, suited for parallel production in conventional IC technology and even more efficient than the SiO_2/Si slide.

The substrate was coated with a copolymer of *N*,*N*-dimethylacrylamide, *N*-acryloyloxysuccinimide, and 3-(trimethoxysilyl) propyl methacrylate named copoly(DMA-NAS-MAPS). This coating forms, by a simple and robust procedure, a functional nanometric film able to covalently bind bio-probes and to suppress non-specific adsorption thus allowing the running of bioassays on the modified surface. X-ray photoelectron spectroscopy (XPS) was used to study the polymer-coated surface material composition. Surface roughness was investigated by Atomic Force Microscopy (AFM).

To demonstrate the performance of the new Si/Al/SiO_2 fluorescence-enhancing substrate coated with copoly(DMA-NAS-MAPS), a microarray for the detection of a panel of inflammation biomarkers was developed leading to femtomolar detection limits for C reactive protein (CRP), cytokines Interleukin 6 and 10 (IL-6 and IL-10) and tumor necrosis factor alpha (TNFα).

2. Materials and methods

2.1. Materials

Tris, bovine serum albumin (BSA), Triton X-100, Tween 20, phosphate-buffered saline (PBS) tablets, *N*,*N*-dimethylacrylamide (DMA), and [3-(methacryloyloxy)propyl]trimethoxysilane (MAPS), Cy3-streptavidin were from Sigma (St. Louis, MO). *N*-Acryloyloxysuccinimide (NAS) was from Polysciences (Warrington, PA). Human CRP antibodies and calibrators were from R&D Systems (Minneapolis, MN), human TNF-α, Il-6 and IL-10 antibodies and calibrators were from BioLegend (San Diego, CA).

2.2. Si/Al/SiO_2 slides

Substrate for high-efficient fluorescent detection were constructed on a cleaned, flat, Czocralsky (1 0 0) silicon substrate. A 50 nm Al layer was deposited on the substrate by Ar sputtering of an aluminum target, and successively a 78 nm silicon oxide layer was deposited by oxygen sputtering of a silicon target.

2.3. Slide coating

Copoly(DMA-NAS-MAPS) was synthesized and characterized as described in [11]. After 10 min of oxygen plasma pretreatment using a Plasma Cleaner from Harrick Plasma (Ithaca, NY, USA) with the oxygen pressure set to 1.2 bar and the power at 29.6 W, the Si/Al/SiO_2 slides were immersed for 30 min in a copoly(DMA-NAS-MAPS) solution (1% w/v in a water solution of ammonium sulfate at a 20% saturation level (0.8 M)). The slides were rinsed with water and dried under vacuum at 80 °C. The same coating procedure was applied to glass microscope slides (Sigma, St. Louis, MO) and SiO_2/Si slides (SVM, Santa Clara, CA).

2.4. Characterization

Si/Al/SiO_2 substrates, before and after coating, were analyzed by X-ray Photoelectron Spectroscopy (XPSon a PHI ESCA/SAM 5600 Multitechnique spectrometer. The analyses were carried out at a photoelectron take-off angle of 80°; and the X-ray beam was obtained by exciting the K line of an aluminum target. Operated with monochromatized beam (photon energy = 1486.6 eV) and a pass energy of 11.75 eV for all elements except Ar (pass energy 58.7 eV), the spectrometer had an energy resolution which we estimate in the interval 0.21–0.23 eV [15].

For the samples coated with copoly(DMA-NAS-MAPS), a tentative decomposition of the C 1s peak in its putative components (carbon in amide configuration, carbon in alkane configuration, and carbon bonded with a double bond to oxygen) is also proposed.

AFM measurements were carried out in the tapping mode with either a PSIA XE-150 apparatus or an NT-MTD SOLVER P47 instrument adopting a silicon probe with a nominal 10 nm tip curvature radius and a typical force constant of 5 N/m.

2.5. Microarray experiments

Cy3-labeled streptavidin at 100 μg/mL, 10 μg/mL, 1 μg/mL, was spotted in 20 replicates for each concentration on glass, SiO_2/Si and Si/Al/SiO_2 substrates each coated with copoly(DMA-NAS-MAPS), and Mirror Epoxy slides (Arrayit, Sunnyvale, CA). The slides were analyzed immediately after spotting at 70% laser power and PMT without washing.

For the inflammation microarray, capture antibodies were dissolved in PBS at a concentration of 1 mg/mL and patterned using a SciFlexArray spotter from Scienion (Berlin, Germany) in 15 replicates each. Cy3-labeled streptavidin was used as position reference for each array. The printed Si/Al/SiO_2 supports were placed in a humid chamber and incubated at room temperature overnight. The slides were then blocked with 50 mM ethanolamine in Tris/HCl 1 M pH 9 for 1 h, washed with water and dried under a stream of nitrogen. Microarrays were then incubated with the calibration solutions of CRP, IL-6, IL-10 and TNF-α (from 0 to 1 ng/mL) in incubation buffer (Tris/HCl 0.05 M pH 7.6, NaCl 0.15 M, Tween 20 0.02%) with 1% w/v BSA, for 2 h under mixing. The slides were then washed with the washing buffer (Tris/HCl 0.05 M pH 9, NaCl 0.25 M, Tween 20 0.05%) for 10 min, rinsed with water and then incubated with the mixture of biotinilated secondary antibodies (0.001 mg/mL) in incubation buffer for 1 h following by an incubation of 1 h with Cy3-labeled streptavidin at 0.001 mg/mL. The slides were then washed with PBS (10 min), rinsed with water and dried with a nitrogen stream. Fluorescence measurements were performed with a PerkinElmer Scanarray Express laser scanner apparatus based on a confocal architecture. For the detection of Cy3 fluorophores, a 543 nm laser beam for the

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