

REVIEW

Interferometric silicon biochips for label and label-free DNA and protein microarrays

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Protein and DNA microarrays hold the promise to revolutionize the field of molecular diagnostics. Traditional microarray applications employ labeled detection strategies based on the use of fluorescent and chemiluminescent secondary antibodies. However, the development of high throughput, sensitive, label-free detection techniques is attracting attention as they do not require labeled reactants and provide quantitative information on binding kinetics. In this article, we will provide an overview of the recent author's work in label and label-free sensing platforms employing silicon/silicon oxide (Si/SiO₂) substrates for interferometric and/or fluorescence detection of microarrays. The review will focus on applications of Si/SiO₂ with controlled oxide layers to (i) enhance the fluorescence intensity by optical interferences, (ii) quantify with sub-nanometer accuracy the axial locations of fluorophore-labeled probes tethered to the surface, and (iii) detect protein–protein interactions label free. Different methods of biofunctionalization of the sensing surface will be discussed. In particular, organosilanization reactions for monodimensional coatings and polymeric coatings will be extensively reviewed. Finally, the importance of calibration of protein microarrays through the dual use of labeled and label-free detection schemes on the same chip will be illustrated.

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1 Introduction

The progressive demand for high-throughput detection of biomarkers has sped up the development of a large variety of biosensors [1]. In the last decade, microarray tests providing multiple, parallel, protein and nucleic acid measurements on the same specimen have grown in popularity.

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Abbreviations: APTES, 3-aminopropyl(triethoxysilane); ATRP, atom transfer radical polymerization; CaFE, calibrated fluorescence enhancement; DMA, *N,N*-dimethylacrylamide; NAS, *N*-acryloyloxysuccinimide; POEGMA, PEG-like polyoligo(ethylene glycol)methacrylate; RAFT, reversible addition-fragmentation chain transfer polymerization; SSFM, spectral self-interference fluorescent microscopy; WL, white light

One of the key issues for the successful protein microarray translation into diagnostics is the ability to control the quantity and the integrity of surface-immobilized proteins. The challenge of specific analytical and quality control requires the development of tests to confirm the attachment density and proper folding for all proteins arrayed on the surface [2]. This article reviews recent publications detailing the development of microarray sensing platforms employing silicon oxide/silicon (Si/SiO₂) substrates of variable thickness and surface chemistries for interferometric and/or fluorescence detection. Si/SiO₂ substrates enable (i) the enhancement of surface fluorescence intensity by optical interference, (ii) the quantification with sub-nanometer accuracy of the axial location of fluorophore-labeled probes tethered to the surface, and (iii) the label-free detection of biomolecular-binding interactions by optical interferometry. In addition, a section of

Colour Online: See the article online to view Figs. 1–8 and Scheme 2 in colour.

the article will review different approaches of biofunctionalization of the SiO₂. Finally, the calibration of protein microarrays through the merger of labeled and label-free modalities will be reported.

2 Modulation of fluorescence intensity by construction of optical interferences on oxidized silicon substrates

In the field of molecular biology, fluorescence measurement of labeled secondary probes bound to targets is the prime mode of detection for microarrays [3–6]. For highly sensitive detection, this technique relies upon the difference in the absorption and emission spectrum of fluorophores called the Stokes shift. After a photon is absorbed by a fluorophore, some energy is lost before emission, lowering the photon's energy. Spectral isolation of the emission spectrum leads to a low background. With a minimized background, further advancement in the LOD must be pursued through maximizing the collected signal.

The strength of the collected signal is controlled by two factors: the emitted intensity and the collection efficiency. To improve the emitted intensity, a more powerful excitation source can be implemented to drive the fluorophores through more absorption and emission cycles. However, turning up the source intensity will offer limited improvement due to more rapid photobleaching effects. The collection efficiency is limited by the collection optics, which only collects a fraction of the omnidirectional intensity emitted by fluorophores.

The implementation of a reflective substrate to redirect the emitted photons typically lost in the glass substrate toward the collection optics has demonstrated an enhancement of the fluorescence signal [7,8]. A reflective substrate often includes a transparent spacer layer on the surface of the substrate to generate constructive interference of the excitation wavelength for enhanced emission [9,10]. Reflective substrates can also be crafted into a complex structure, such as a photonic crystal, to confine and direct the excitation and emission wavelengths for more than 100-fold enhancement of the fluorescence signal over than glass substrate [11–13]. These configurations prove that glass substrates for fluorescence microarrays are not the optimized platform for sensitive detection. The selection of an alternative scheme, which is compatible with existing chemistries and processes, can provide immediate improvement to existing systems.

A particularly promising replacement for glass slides is Si/SiO₂ substrates. This layered substrate configuration is widely available, inexpensive, compatible with established glass surface chemistries, and provides a tuneable wavelength enhancement [14]. The optically transparent SiO₂ acts as a spacer to bring the fluorophore into the plane of constructive interference. By varying the thickness of the SiO₂ layer, the emission wavelength of any fluorophore of choice can be enhanced by constructive interference. Using the dipole emission model [9,15,16] to simulate fluorophore emitters

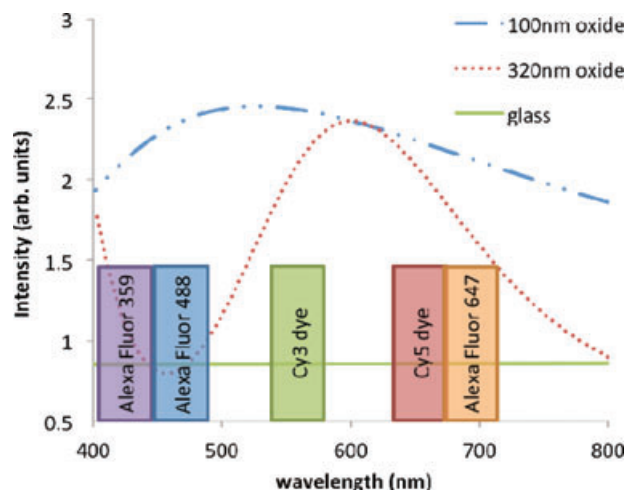


Figure 1. Simulated fluorescence enhancement of 100-nm SiO₂, 320 nm SiO₂, and glass for commonly used fluorophores. Reproduced with permission from [17].

near a dielectric interface on a layered substrate, the structure can be designed and optimized for a range of wavelengths (Fig. 1).

This concept has been proven with the development of a 100-nm SiO₂ layer for broadband enhancement and a 320-nm SiO₂ layer for the selective enhancement of Cy3 and Cy5 fluorophores [17].

3 Application of resonance to fluorescence microscopy: SSFM

While fluorescent enhancement chips are developed to improve the detection sensitivity of biomolecules by measuring fluorescence intensity, spectral self-interference fluorescence microscopy (SSFM) uses Si substrates with a thick SiO₂ layer to measure biomolecule conformational change upon binding in microarrayed format and provides additional information on specific biomolecule interactions. SSFM is an interferometric technique that maps the spectral oscillations emitted by an ensemble of fluorophores located above a reflecting interface (Si/SiO₂ interface) into a precise (sub-nanometer (nm)) height determination of the fluorophores in a broad range from a few nanometers to more than 100 nm [16,18–20]. In contrast to earlier fluorescence interference microscopy techniques [21,22], that only rely on intensity variation of total fluorescent emission, SSFM utilizes spectral information to provide higher precision in a single measurement. As a consequence, the results of SSFM are immune from factors that can affect the fluorescence intensity, such as local fluorophore density, photobleaching, and/or change of buffer environment. Thus, SSFM allows quantification of the axial locations and conformations of fluorophore-labeled

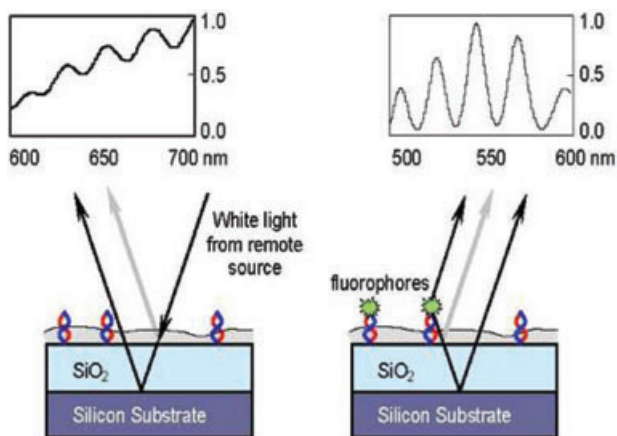


Figure 2. Schematic representation of the interferometric experimental configurations with examples of measured spectra for each technique. The spectra show normalized interference intensity plotted against wavelengths. The thickness of SiO₂ in both cases is 5 μm with a roughness of 2 nm. In white light (WL) spectroscopy interference of light from the top surface and a buried reference surface results in periodic oscillations (left panel). SSFM maps the spectral oscillations emitted by a fluorophore located above a reflecting surface into a precise position determination with sub-nanometer accuracy (right panel). Reproduced from [19] Copyright (2006) National Academy of Sciences, USA.

probes tethered to the surface with sub-nanometer accuracy (Fig. 2).

Early proof-of-concept results of SSFM have resolved the 5 nm-step between fluorophores atop the protein streptavidin from those on a bare surface and the different height of center sources of emission between quantum dots and fluorescein bound to the surface with sub-nanometer precision [16]. In combination with white light (WL) reflectance spectrometry, which detects additional mass on a surface by measuring optical thickness, the SSFM platform can perform assay of surface-bound DNA hybridization and study its conformational change [19]. The following example illustrates one application of SSFM combined with WL reflectance spectrometry (Fig. 3). Immobilization of unlabeled ssDNA leads to an observable increase in the optical thickness of a layered surface. At this point, the actual conformation of oligonucleotides on the surface is unknown and the WL measurement shows that monolayers of ssDNA of 21 or 50 nucleotides are 1.0–1.5 and 2.0–2.5 nm thick, respectively. Adding complementary second strands to 50-mers results in an increase in the film thickness by approximately 1.0 nm corresponding to a hybridization efficiency of approximately 50%. These second strands are fluorescently labeled at either surface distal or proximal end as shown in Fig. 3(B) and (C), in which incomplete hybridization is schematically illustrated. In both cases, the nonspecific binding of the second strand is very low, so virtually all of the labels are attached to double helices. The results demonstrate that whereas ssDNA can go downcoiled

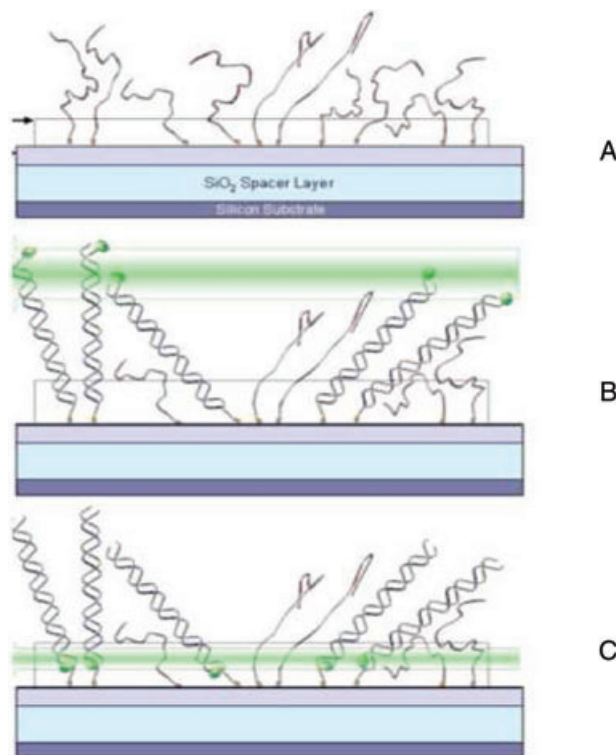


Figure 3. Schematic representation of surface-bound ssDNA and dsDNA. (A) Nonlabeled ssDNA covalently bound on the surface (B) after hybridization with complementary strand labeled at the 5' (distal)-end and (C) 3' (proximal)-end. The measured white light (WL) and fluorescence spectra similar to those in Fig. 2 were fitted to an algorithm to deduce biomass heights and fluorophore heights. Reproduced from [19] Copyright (2006) National Academy of Sciences, USA.

on the surface, dsDNA molecule is rigid and standing up (by ~ 10 nm for 50-mers) from the surface.

As illustrated in the previous example, SSFM platform allows precise quantification of intrinsic conformations and conformational changes of DNA on substrate in the native environment. Other potential applications include quantification of protein-induced conformational change of DNA with SSFM. One example is the detection of binding of integration host factor to surface-bound dsDNA tagged with a fluorophore at the distal end to the surface. Integration host factor induces a bend of 160° at the binding location of DNA, resulting in a measurable fluorophore height change. This measured fluorophore height change allows precise quantification of protein-induced conformational change of dsDNA [23]. Therefore, SSFM platform can potentially detect specific DNA-protein binding and protein-induced conformational change of DNA on thousands of spots in one assay, similar to protein-binding microarrays [24]. Such a high-throughput approach can increase efficiency and decrease the costs (time and money) required to investigate specific molecular interactions.

4 Label-free microarray imaging techniques

Although techniques using a label have produced highly sensitive and specific measurements, labeling procedures can be cumbersome and interfere with biofunctionality. For decades, numerous efforts have pursued label-free technologies to provide direct measurements. A successful method of label-free technology has been through the use of common path interferometry on layered substrates [25–29]. These optical interrogation techniques utilize a broadband or narrow-band illumination source to probe the substrate's interference signature. Binding of minute amounts of proteins or DNA to the substrate induce an alteration to the interference signature. These shifts are then characterized to the amount of bound mass in order to provide a sensitive, quantified method of direct detection [30].

One particular method of label-free detection is the interferometric reflectance imaging sensor (IRIS). This platform is a microarray sensing technique that utilizes an Si/SiO₂ biochip for high throughput, multiplexed detection. IRIS utilizes optical interferometry with a buried reference plane to detect binding of biomolecular interactions on the surface. Binding interactions are recorded through sampling the reflectivity signature of the surface with multiple wavelength sources and a camera. After a stack of images is acquired, the reflectivity curves for each area mapped by a pixel are found through fitting to the Fresnel reflectivity equation of the silicon chip structure [29]. This technique has been demonstrated to offer picogram per square millimeter sensitivity, real-time kinetics monitoring, and the binding of multiple analytes to immobilized probe molecules—all in a simple platform amenable to high-throughput screening [29, 30] (Fig. 4).

5 Optical biosensors for nanoparticle detection

Silicon biochips are not limited to ensemble measurements of binding interactions, but can also be engineered to enable the imaging of single nanoparticle attachment. Moving away from ensemble measurements and toward single event measurements offers more information about the surface to address issues of nonspecific binding. Daaboul et al. have shown that by thermally growing a thin layer of SiO₂ on Si, the signal from individual nanoparticles can be visualized using a CCD camera [31]. Similar to fluorescence enhancement, the SiO₂ layer thickness is designed to enhance the signal from a nanoparticle. Typically, for a nanoparticle in free space or on a noninterferometric surface, the detected intensity is only composed by the scattered electric field, which is proportional to the radius of the particle to the sixth power. However, by placing the nanoparticle on an interferometric surface, the detected intensity relies upon three components: the scattered

field from the particle, the reference field from the buried interface, and the interference of these two fields. By optimizing the interference term, the detected intensity is enriched, leading to a higher nanoparticle contrast in images. Unlike other interferometric imaging techniques, the phase between the reference and scattered field is known from the thickness of the SiO₂ layer; therefore, the optical response can be calculated for different nanoparticles radii. If the refractive index of the nanoparticle is known, the measured optical response can be correlated to the size of the nanoparticle bound to the sensor's surface [31]. Recently, this technique was applied to detect individual viruses captured on the surface using an antibody array. The authors also show preliminary data on how virus size and shape can be calculated from the measurement, which will improve specificity of the sensor by discriminating against nonspecific binding [32].

6 Surface functionalization of silicon slides

The surface of silicon biosensing devices, independently from their sensing principles or surface geometry must be modified in order to provide a suitable interface between silicon-based transducer and immobilized biomolecules. A frequently employed strategy for the preparation of functional surface layers is by the self-assembly of organic monolayers on solid substrates. Self-assembly of organic monolayers, produced by organosilanization reactions, introduce chemical moieties reactive toward biomolecules on silicon oxide surfaces. An alternative approach involves formation of functional polymeric coatings with a high concentration of reactive groups.

It is generally recognized that the three-dimensional conformation, and consequently the activity of a protein on a surface, is greatly influenced by the strategy adopted for immobilization. Compared to other classes of biologically relevant compounds, proteins tend to bind to surfaces in a disordered orientation and as a result, may lose their biological activity [33–35]. The extent of the conformational change of adsorbed proteins depends on the nature of the surface including hydrophobicity/hydrophilicity and on the environmental conditions, such as temperature, pH, and ionic strength of the solution. For this reason, protein microarrays are generally more difficult to fabricate than DNA microarrays. Furthermore, to obtain high S/N ratio, proteins must be immobilized in high densities in very small areas in moisturized environments [36]. Silicon, characterized by its chemical homogeneity and stability, can be modified with a wide range of chemical functionalities. However, this material is characterized by a lower content of surface silanol (Si-OH) groups when compared to glass substrates. This lack of anchoring groups may render the functionalization process more challenging.

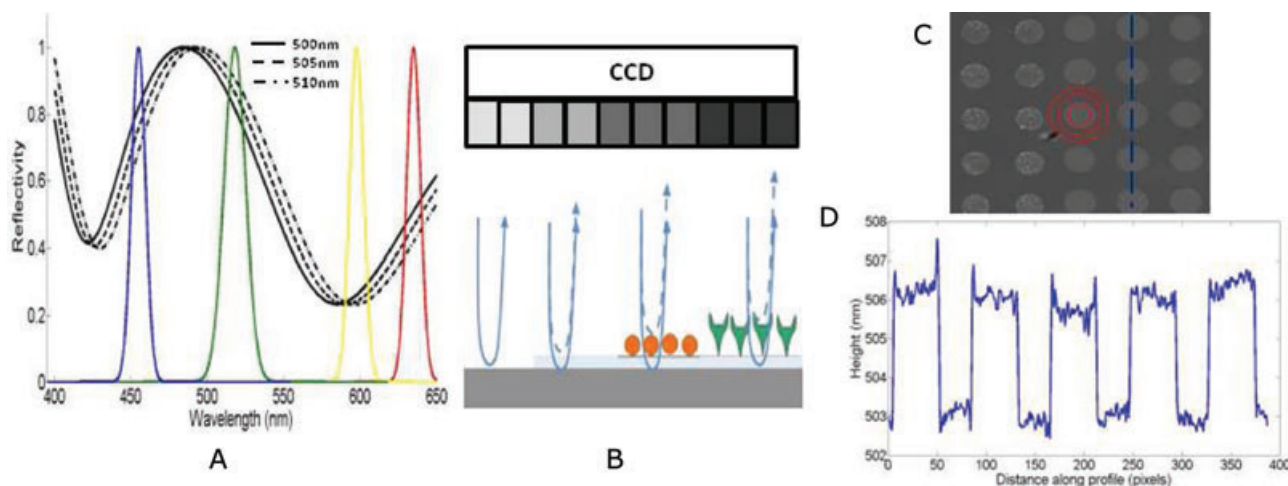


Figure 4. Interferometric reflectance imaging sensor (IRIS) method of detection: (A) theoretical reflectivity for 5-nm shifts in the thickness on the surface. The colored Gaussians represent the four LEDs used to sample the curve. (B) Sensor's imaging path illustrating the grayscale intensity change due to biomass accumulation. (C) Image of the sensor's surface with an array of protein spots. (D) Height profile across dashed line in (C).

6.1 Organosilane coatings

Covalent immobilization, which establishes a strong and stable linkage from biomolecule to surface, is the preferred method for attaching proteins to Si/SiO₂ substrates. The most widely used method functionalizes Si/SiO₂ surfaces via silane-coupling reactions. Despite reports describing organosilanization as the simplest approach to build up biocompatible layers for the attachment of biomolecules to solid surfaces, successful and reproducible deposition of a monolayer on Si/SiO₂ substrates is not a simple task. Furthermore, the mechanism of silanization is still not thoroughly understood. In particular, it is difficult to control the density of silanol groups (Si–OH) and the presence of surface water layer, which ultimately controls the properties of the deposited layer. If excess surface water was present, an ordered two-dimensional polymerization of the organosilane with no strong bonds to the surface would occur. Alternatively, the predominance of surface hydroxyls would establish a stable anchor for the molecules onto the surface by chemical bonds [37, 38]. The prevalence of one condition over the other is the result of different substrate pretreatments.

Depending on the application, SiO₂ layers on Si may be added via thermal oxidation, chemical vapor deposition, or sputter deposition. In many sensor applications, the oxide layer presents levels of defects that diminish the optical or electrical signals needed for detection [39]. Thermal growth of oxide on silicon provides the best oxide quality. The ability to tightly control the growth rate results in highly reproducible oxide thickness, an important feature when the thickness of the oxide layer is critical for the sensing principle. However, thermal oxide films produced by dry oxidation on silicon have a lower surface content of hydroxyl groups

and are more difficult to modify [40, 41]. To facilitate subsequent functionalization, activation steps, which do not alter the topology or thickness of the oxide layer, are needed. A number of different protocols for wet cleaning and activation of thin silicon oxides have been investigated. Han et al. [42] discuss the efficiency of several cleaning methods studied by contact angle measurements, atomic force microscopy, and Fourier-transform infrared spectroscopy. Furthermore, x-ray photoelectron spectroscopy was used to characterize the oxides after the cleaning and silanization procedures. Reliability and repeatability of silanization conditions has also been extensively studied in reference [43].

Following the proper pre-activation step, functional trialkoxysilanes of general structure (RO)₃Si(CH₂)_nX or trichlorosilanes are then usually used to introduce functional groups on the surface [44–47]. A large variety of silane reagents are commercially available, bearing amine, thiol, carboxy, epoxide, and other functionalities for subsequent modification steps. Various protocols of silanization can be found in the literature, implying deposition of silanes from aqueous or organic solutions, from the gas phase, or by chemical vapor deposition [48]. Silane-coupling reactions are capable of providing high surface loading (10¹² molecules/cm²), and the oxide interface typically shows good thermal stability [49]. Compared to adsorption, covalent immobilization of protein provides higher irreversible surface loading and improved stability to operational conditions [50]. However, it must be pointed out that the attachment of proteins to the surface is always driven by a combination of covalent and adsorption mechanisms. There are evidences in the literature that the dominant “loss” mechanism of covalently immobilized protein is the continuous desorption of noncovalently bound protein left from incomplete washing after the immobilization procedure [51].

In some cases, such as using amino-terminated organosilanes, the introduction of chemical moieties that are useful in biological applications is hindered by the fact that they introduce more complexity into the silanization process. In a recent publication, an extensive investigation on the characteristics of a silicon surface modified with 3-aminopropyl(triethoxysilane) (APTES) was reported [52]. The surface silanization was performed mainly via APTES hydrolysis followed by surface condensation reaction with the hydroxyl groups of the hydrophilized silicon surfaces. As clearly shown by x-ray photoelectron spectroscopy, a film with short APTES hydrocarbon chains terminated with NH_2 groups that point away from the surface siloxane network is formed. However, an alternative, although less effective, coupling mechanism was also identified with protonated amino groups pointing toward (charged) silicon. The interaction between amino and surface hydroxyl groups makes it difficult to form ordered layers as NH_2 groups act like build-in catalyst for the reaction of alkoxy [53] and chlorosilane [54]. When the functionalities to be introduced conflict with the silanization process, an alternative approach is to modify a less problematic silane monolayer using chemical surface reactions. This method offers the possibility to introduce a wide range of terminal end groups on well-defined base monolayers. A recent review highlights a selection of chemical surface reactions, i.e. nucleophilic substitution, click chemistry, and supramolecular modification, which have been used for the functionalization of solid substrates [55].

6.2 Polymeric coatings

Silane coatings are normally classified as monodimensional coatings [56]. In this type of coating, steric hindrance may significantly reduce the reaction between surface functional groups and probe molecules leading to poor grafting density. In addition, the direct attachment of a protein to a surface without a spacer can cause steric constraint of the protein's reactivity or interaction capability compared to the protein in solution [57]. Moreover, multiple direct contacts with the surface can induce denaturation or partial denaturation. By introducing a spacer between the protein and the reactive group on the surface, these effects can be minimized. An effective way to space a protein from the surface is by the introduction of a polymeric coating [58], which, besides reducing steric hindrance, increases the density of protein per unit area by providing a three-dimensional binding scaffold.

Polymers can be immobilized on a surface by physisorption, chemisorption, or covalent grafting. Many methods to immobilize PEG on surfaces have been developed based on each of these different approaches [59–66]. However, PEG brushes usually bear only one reactive group per chain. To increase the density of reactive groups, other types of radical homo- and block-copolymers brushes bearing chemical functionality for the covalent attachment of biomolecules obtained by “grafting to” or “grafting from” methods were intro-

duced [67]. Most approaches that aim at attaching polymers to a surface use a system where the polymer carries an “anchor” group either as an end group or in a side chain. This anchor group can react with appropriate sites at the substrate surface, thus yielding surface-attached monolayers of polymer molecules (termed “grafting to”). Another technique is to carry out a polymerization reaction in the presence of a substrate onto which monomers had been attached leading to the so called “grafting from” approach. The “grafting from” approach is particularly advantageous as it allows to obtain high graft density of polymer chains.

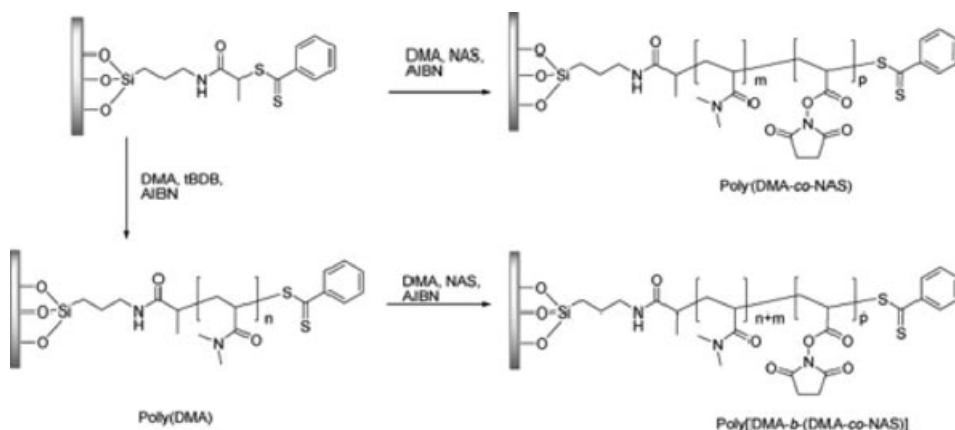
Silicon oxide was functionalized with brushes obtained by surface initiated, controlled radical polymerization [68]. Controlled radical polymerization techniques, including nitroxide-mediated radical polymerization [69], atom transfer radical polymerization (ATRP) [70], and reversible addition-fragmentation chain transfer polymerization (RAFT) [71], are excellent methods for the preparation of well-defined polymer structures such as block copolymers, star shape polymers, and interpenetrating polymer networks [72].

Using ATRP, a technique that affords good control of polymerization kinetics, compatible with a range of solvents, PEG-like poly oligo(ethylene glycol)methacrylate (POEGMA) functionalized polymer brushes were produced [73]. There are a number of advantages in this procedure: (i) ATRP initiator can easily be immobilized onto surfaces by various methods, such as covalent coupling, Langmuir–Blodgett deposition, plasma grafting, and chemical self-assembly [74–76], (ii) the POEGMA chains are larger than the initiator and this allows to form a dense brush coating from a low initiator surface density, (iii) the polymer graft density and thickness are easy to control. POEGMA-brush surfaces can be easily functionalized as they present reactive hydroxyl groups that can be easily derivatized with complementary reactive moieties.

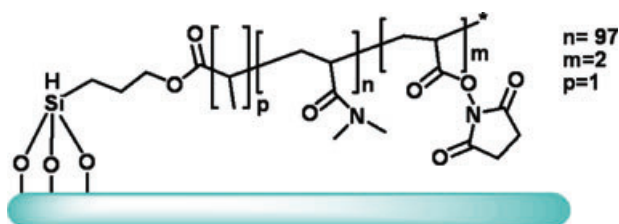
RAFT polymerization was used to functionalize microarray silicon oxide slides with block polymer brushes of *N,N*-dimethylacrylamide (DMA) and *N*-acryloyloxysuccinimide (NAS) (graft-poly[DMA-*b*-(DMA-co-NAS)]) [77]. A polymer layer with di-block architecture was prepared from a novel RAFT chain transfer agent bearing a silanating moiety directly anchored onto the glass surfaces. A schematic representation of the reactions involved in the mono-/di-block coating preparation are shown in Scheme 1. The coating conformation was characterized by SSFM. The di-block polymer has chemical reactive moieties located on the external block as demonstrated by SSFM which shows different heights for DNA probes immobilized in the mono block coating (about 10 nm) compared to those in the di-block coating (about 35 nm).

Although surface-initiated polymerization techniques enable the synthesis of polymer brushes with a high surface density and thickness, the synthesis requires several steps including an initial step of organosilanization to graft a silane initiator.

In an effort to simplify the coating process, a ter-copolymer that is the result of an optimized composition of DMA,



Scheme 1. Schematic synthesis of the RAFT-mediated brush polymers: mono-block, and di-block architectures.



Scheme 2. Sketch of copoly(DMA-NAS-MAPS) coating.

3-(trimethoxysilyl)propyl methacrylate (MAPS), and NAS [78] (Scheme 2) was used to form a thin film on silicon oxide by a combination of physisorption and chemisorption. The coating was prepared by “dip and rinse” approach immersing a silicon oxide substrate in an aqueous solution of the copolymer (10 mg/mL) at ambient temperature followed by washing with water. The substrates were then cured at 80°C for 30 min. When Si/SiO₂ wafers were immersed in the copolymer solution for 30 min, ultrathin films of the polymer were generated with an average thickness of approximately 15 nm in a wet environment [79]. The formation of the layer was not dependent on polymer concentration within a large interval of values (unpublished data) indicating that the adsorbed poly-

mer layers can limit further attachment of additional polymer chains to the surface. The surface topography of the polymer layer on Si/SiO₂ wafers was examined by atomic force microscopy. Figure 5 clearly shows the surface of the coating on Si/SiO₂ wafers was very smooth with average roughness of 2 nm in dry conditions [14]. The procedure provides a fast and robust method for producing hydrophilic functional surfaces bearing active esters, able to react with amino groups in proteins and peptides or in modified DNA. Most of the applications reported in this review employ this coating.

7 Applications of silicon biochips

The majority of the microarray community utilizes glass substrates as the “gold standard” for biosensing. Two specific limitations exist with this choice of substrate. First, fluorescence assays conducted on simple glass slides (or other high-index substrates) reduce fluorescence yield. Numerous layered structure designs for enhanced fluorescence measurement of DNA and protein microarrays have been investigated to replace glass [7, 80–83]. As an attractive layered structure

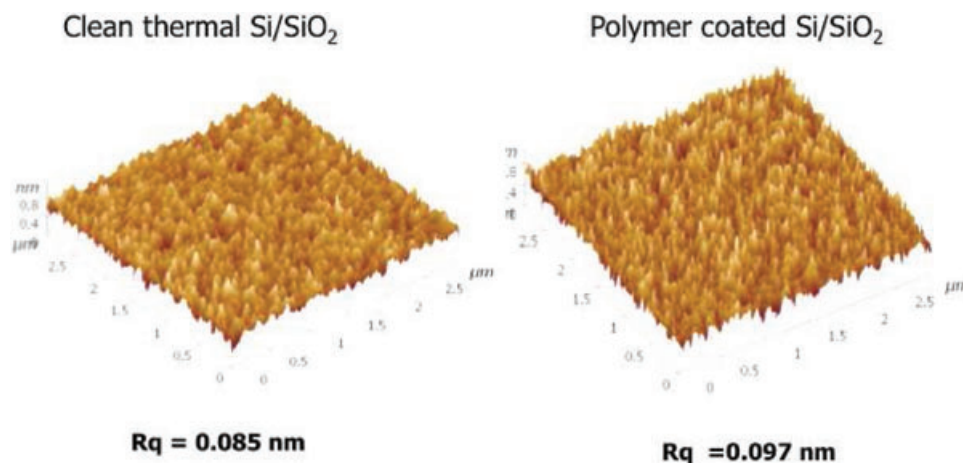


Figure 5. Atomic force microscopy scanning showing roughness of bare silicon oxide (left panel) and copoly(DMA-NAS-MAPS) coated silicon (right panel). Rq, the roughness parameter is the standard deviation of the Z values within a given area. The low Rq value indicates the smoothness of the surface before and after the coating.

for fluorescence enhancement, silicon chips are low cost and possess well-established and controllable characteristics.

A second important drawback of glass substrates is connected to the challenge of collecting quantitative results in the standard fluorescence assay. This difficulty arises in the differences in the amount of immobilized probe on the substrate surface, which has shown to affect target capture and quantification [84–86]. This variation is primarily due to the technical issues (array printing, sample processing, etc.) but also due to the characteristics of the proteins, which exhibit different reactive groups on their surface [2, 87, 88]. This variability is common to both glass and silicon substrates. However, Si/SiO₂ substrates allow to calibrate fluorescence for the amount of probe actually immobilized on the surface as it will be illustrated in Section 7.3. In the next sections, we will highlight applications in the fields of protein and DNA microarray technology in which the use of silicon oxide, flat substrates has been critical to reach detection performance and accuracy when using label, label-free, and dual detection schemes. In particular, Section 7.1 will report on enhanced labeled detection whereas Section 7.2 exhibits examples of IRIS, the technology extensively described in Section 3. Finally, in Section 7.3, the combination of label and label-free detection schemes will be presented and the advantages provided by the dual detection scheme will be illustrated.

7.1 Enhanced label based detection

Modulation of the fluorescence intensity by the construction of optical interferences on oxidized silicon substrates was first demonstrated with immobilized green fluorescent protein [89] and Cy3 labeled oligonucleotides [90]. This simple configuration provides fluorescence enhancement close to that obtained by the sophisticated multilayer substrates reviewed in [91].

An optimized layer of thermally grown silicon oxide of a highly reproducible thickness, low roughness, and fluorescence background can be produced to exploit the fluorescence intensification due to the constructive interference of the fluorescence radiation as mentioned in Section 2. When a 100-nm thick silicon oxide surface is coated by copoly-(DMA-NAS-MAPS), the polymeric coating presents a thickness that does not alter the optical properties of the silicon oxide, optimizes the binding efficiency of the slides, and maintains a low background due to its antifouling properties, leading to a high S/N ratio. This approach was demonstrated first using protein and peptide arrays [14] obtaining a 5–10-fold enhancement of the fluorescence signals with respect to glass slides for specific antibody detection. A superior performance of copoly-(DMA-NAS-MAPS) silicon slides in comparison to glass slides and commercially available substrates was demonstrated for allergy diagnosis in real clinical samples plotting receiver-operating characteristic curves [92]. Figure 6 reports the results of the assay for allergen specific IgE detection in a pool of sera on microarrays. In this comparative ex-

periment, silicon substrates provide a fluorescence intensity that is higher than each signal detected on the other supports and is the only surface able to correctly diagnose, in the pool of sera, the presence of IgE specific to the six allergens Alt a 1, Bet v 1, Der p 1, Del p 2, Fel d 1, and Phl p 1. Even if sensitivity and precision of this diagnosis would be simply improved by using higher laser power and PMT gain, the authors underline that there are conditions in which the highest laser power of commercial scanners is not sufficient to detect low abundance biomarkers. Moreover, the use of slides capable of enhancing the fluorescence signals allows the use of less sophisticated and powerful lasers making possible the development of cheaper, simpler, and smaller detection setups. In this work, analytical robustness on the silicon substrate was consistently better than on glass as a result of an overall increase in signal intensity. When background noise is kept low, enhanced fluorescence produces higher S/N ratios even for low abundance analytes resulting in a wider concentration ranges for stable analytical imprecision (CV%). The analytical performance of the allergen microarray was thus significantly enhanced, as compared to glass, in accurately identifying allergic patients spanning a wide range of specific IgE titres to the considered allergens.

In the field of DNA microarrays, an important clinical application in which high sensitivity is a key issue is the identification of minority mutated alleles due to its implications in early diagnosis, prognosis, and new noninvasive diagnostics. Minority mutations (i.e. a few mutants within a high excess of wild-type alleles) are important in early tumor detection and molecular genetics. Fetal DNA released into maternal plasma is an example of minority mutation that allows not invasive, prenatal diagnosis of genetic diseases. The discrimination between fetal and maternal sequences differing by as little as one base pair is challenging. Despite the scarcity of fetal DNA in maternal plasma, due to optimal extraction protocols and new detection schemes, the identification of genomic sequences, which belong to the fetus, such as those inherited from the father, can be performed with an accuracy close to 100%. One way to overcome the problem of the unbalanced concentration of the two alleles is to use an enrichment strategy based on the inhibition of the amplification of the predominant sequence. To this aim, peptide nucleic acid, a DNA analogue, is used to bind maternal wild-type DNA that represents over 95% of total plasma DNA thus interfering with its amplification while still allowing the mutant DNA amplification. Fluorescence enhancement on Si/SiO₂ coated by copoly-(DMA-NAS-MAPS) was exploited in the noninvasive prenatal diagnosis of beta-thalassemia [93]. In this work, the sensitivity in detecting low concentrations of mutated sequences by hybridization was assessed and compared to that provided by phyrosequencing, a technique [94] that monitors the incorporation of nucleotides in a primer-directed polymerase extension reaction. Both approaches, microarray and phyrosequencing, were able to monitor the variation of wild-type-to-mutated allele fluorescence ratio in the presence of different amounts of wild-type peptide nucleic acids.

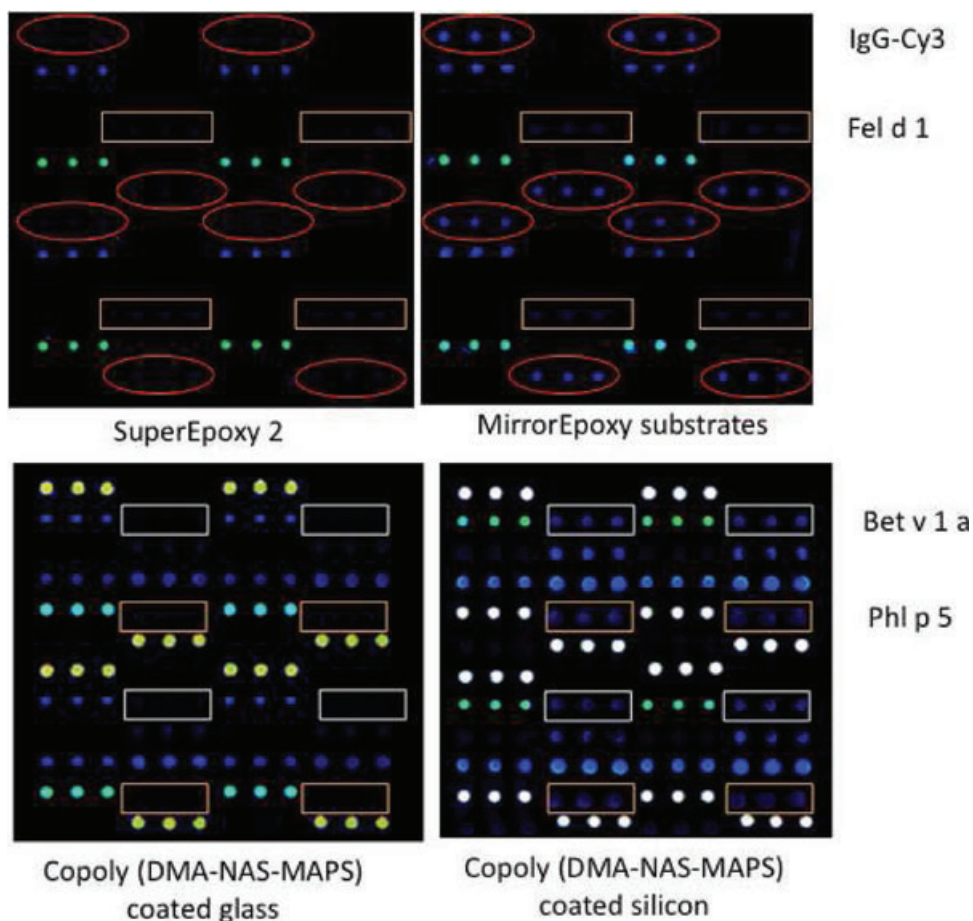


Figure 6. Results of the assay for allergen-specific IgE detection in the pool of sera on microarrays analyzed at 60% laser power and PMT gain. The upper panel reports the results of the analysis of the pool of sera on SuperEpoxy 2 and MirrorEpoxy substrates (Arrayit, Sunnyvale, CA, USA) the lower panel the results on copoly(DMA-NAS-MAPS) coated glass and silicon. In the detection conditions used, MirrorEpoxy substrates reveal the presence of IgE specific to the allergen Fel d1 that was not detected on SuperEpoxy 2. The superior performance of MirrorEpoxy substrates is clearly visible from the enhanced fluorescence of the reference Cy3 labeled IgG (red circles) and is due to the fluorescence enhancement produced by its reflective backside mirror coating. Similarly, signals of IgE specific for Bet v 1 and Phlp 5 are detectable on silicon substrates but not visible on glass slides and the intensity of fluorescence of the reference Cy3-labeled IgG (red circles) is higher on silicon than on glass. Reproduced with permission from [92].

The lowest detectable amount of mutated DNA was assessed by adding decreasing amounts (from 100 to 6.25 pg) of genomic DNA from a homozygous subject carrying the mutation to 1 ng of DNA from a wild-type homozygous subject. The system based on Si/SiO₂ microarrays coated by copoly-(DMA-NAS-MAPS) was able to detect the presence of 12.5 pg of mutated DNA in 1 ng of wild-type DNA. A similar result was also obtained with commercial slides (ready-to-coat dual-color AmpliSlides 570/670 Genewave, Paris, France) [95] that were also proposed as substrates with optimal optical properties. However, in this case, a more complex strategy was adopted to achieve fluorescence enhancement based on the use of multiple layers of materials with different refractive index.

The high intensity of the fluorescence detected on Si/SiO₂ slides was also exploited in protein microarray experiments. Silicon slides with an oxide layer coated by copoly-(DMA-NAS-MAPS) provided antibodies detection with femtomolar sensitivity. The fluorescence was measured with a conventional laser scanner without the use of any sophisticated detection strategy. This astonishing sensitivity was achieved only overcoming mass transport limitations [96]. LODs of 45 and 54 pg/mL (lower than that obtained by ELISA using the same

antibodies) were achieved for hepatitis B superficial antigen and Human Immunodeficiency Virus (HIV) p24 antigen, respectively. In this work, once calibration curves were built, LODs were extrapolated from the fluorescent value of blank samples plus three standard deviations (3σ).

7.2 Label-free detection and IRIS

Development of label-free biosensors that can simultaneously monitor multiple molecular interactions is of great interest to biomedical research and clinical applications. Detection of DNA–DNA, DNA–protein, and antibody–antigen interactions has been established using fluorescent microarrays. Label-free technologies can be advantageous because they monitor the interactions directly and alleviate the need for labeled detection probes, which are costly and may interfere with biomolecule functionality [97].

Technologies have been developed based on silicon chips that use a thin silicon dioxide film to form a common path on-chip interferometer [25, 98]. The advantage of silicon chip interferometric techniques is that the glass surface chemistries

developed for the well-established fluorescent microarrays may be used.

Si/SiO₂ substrates are applied to label-free detection of protein–protein interactions by spectroscopic ellipsometry [99] and by interferometric detection schemes for monitoring biomolecular interactions [29]. Ozkumur et al. have shown single nuclear polymorphism detection by monitoring with IRIS the melting of DNA complexes [100]. Recently, advancement has shown that IRIS platform can be further simplified by replacing the tuneable laser source with discrete LED sources making it translatable to a point of care device [101]. IRIS has been continuously verified through the detection of viruses [102], protein binding [101], and surface effects due to buffer interactions [103].

7.3 Dual detection schemes

Silicon technology and tuned modulation of the oxide layer facilitate dual detection of label-based and label-free detection schemes on a single chip. This combination chip is of high practical utility during the assay development process to image arrays of proteins, check consistency and quality of the spotted array, quantify the amount of immobilized probes, and detect fluorescence of bioassays. Awareness of real immobilization yields for different proteins in distinct immobilization conditions can help in developing consistent protein chips. The absolute amount of immobilized material, the activity of the immobilized proteins, and the specificity of the secondary antibodies will ultimately determine the sensitivity and reproducibility of protein microarrays.

This dual detection scheme is particularly effective for allergen chips, which suffer from large immobilization variability that may result in underestimation or failure of detection of captured targets [84, 86]. To address protein microarrays reproducibility issues, the calibrated fluorescence enhancement (CaFE) method, which integrates quantitative IRIS label-free sensing and enhanced fluorescence measurement, has been developed. Two CaFE platforms have been designed and optimized for dual label-free and fluorescence detection based upon the dipole emission model and label-free modeling [17, 104]. The first platform consists of two oxide islands, one optimized for fluorescence detection for all fluorophore emission wavelengths and one optimized for IRIS (label free) sensing. In this particular silicon biochip, a spot located on the label-free oxide island is used to calibrate a spot of the same protein located on the optimized fluorescence island and was shown to be useful in the assay development process [104]. In Fig. 7, the silicon chip and the concept of dual detection is shown. The chip is a 15 × 15 mm square silicon slide divided into four quarters (panel a), three regions of the chip exhibit a silicon oxide layer of 100 nm, whereas one region displays an oxide thickness of 500 nm. Each quarter of the chip is spotted with the same protein array within the same spotting session. After binding of proteins and blocking of unreacted sites, the quarter of the chip

exhibiting the 500-nm oxide layer is imaged using the LED-based IRIS and the amount of immobilized probe for each condition is quantified; the morphology and consistency of spots are also verified by visual inspection of the images generated by the IRIS setup. After the interferometric imaging, that quantifies the amount of surface immobilized probe, the chip undergoes a conventional fluorescence assay. The silicon biochip for dual label-free and fluorescence detection proposed can be of high practical utility during the microarray assay development process. Without altering the standard microarray protocol, the new tool can be implemented in a routine use as an internal quality control for protein array production.

However, this platform relies on repeatability within a chip. The second platform [17] consists of a single oxide thickness designed to operate in both label-free and enhanced fluorescence modalities. In this platform, each spot is measured with both fluorescence and label-free measurements, and therefore calibrated, effectively accounting for any spot-to-spot variation frequently reported in microarrays. In this particular case, this platform was optimized for Cy3 and Cy5 fluorescence enhancement due to these fluorophores extensive use in the literature. Both platforms are realizations of the CaFE method; one platform uses optimized oxide islands for enhanced fluorescence measurement that is calibrated by the label-free IRIS measurement, while the second platform calibrates the enhanced fluorescence measurement of a specific fluorophore to the label-free IRIS measurement on the same spot.

By combining sensitive fluorescence measurement with the quantified probe measurement, the CaFE method addresses variation of microarrays by (i) quantifying probe with IRIS, (ii) measuring fluorescence enhancement of secondary detection antibodies, and (iii) self-calibrating fluorescence signal to quantitative assessment of probes with IRIS. This method is applicable to any microarray assay that utilizes a ligand-analyte detection scheme. Improving upon quality and quantity control in microarrays, the CaFE method demonstrates (i) a linear correlation between immobilized probe density and fluorescence signal despite chip-to-chip variation and (ii) the importance of quantifying probes (i.e. major allergens) to ensure reproducibility of fluorescence measurement due to the possibility to calibrate fluorescence resulting from the bioassay by the amount of immobilized probe.

Figure 8 illustrates how the dual detection platform improves assay precision in the diagnosis of allergy. In panel (a), the fluorescence of the anti-IgE detection antibody is plotted against allergen spotting concentration (Ara h1 peanut allergen on the left and Phl p1 timothy grass allergen on the right). In panel (b), the fluorescence of anti-IgE antibody is divided by the effective amount of probe immobilized detected by IRIS. The fluorescence is corrected for the actual amount of surface probe. The CaFE platform, panel (b), provides calibrated linear responses for allergy testing analysis compared to traditional semiquantifiable analysis in panel (a). In addition to the CaFE method, a separate dual detection

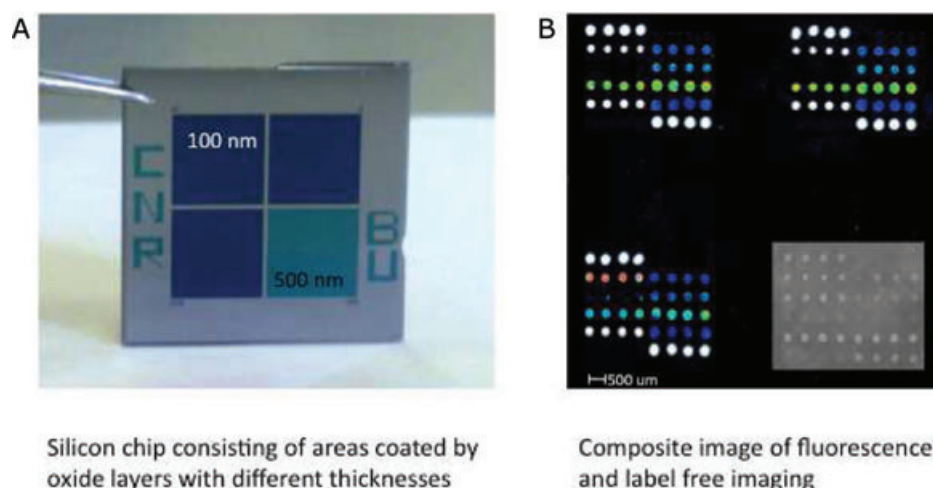


Figure 7. The silicon chip is a 15 × 15 mm square silicon slide divided into four quarters (panel A), three regions of the chip exhibit a silicon oxide layer of 100 nm whereas one region displays an oxide thickness of 500 nm. Each quarter of the chip is spotted with the same protein array within the same spotting session. The quarter of the chip exhibiting the 500-nm oxide layer is imaged by the LED-based interferometric reflectance imaging sensor (IRIS), the other three quarters are analyzed by a fluorescence scanner. Panel B reports a composite image obtained, for the same allergen microarray, before the serum incubation (label-free detection of immobilized allergens) and after incubations with an allergic patient's serum and fluorescent secondary antibody. Reproduced with permission from [105]

platform for combined chemiluminescence and label-free detection was developed to quantify hepatitis B superficial antigen [105]. The dual detection system presented in this work enables high density arrays to be developed using label-free

detection as an analytical tool on a platform that is amenable to becoming a clinical diagnostic platform using labeled detection without necessitating a change in surface, chemistry, or format.

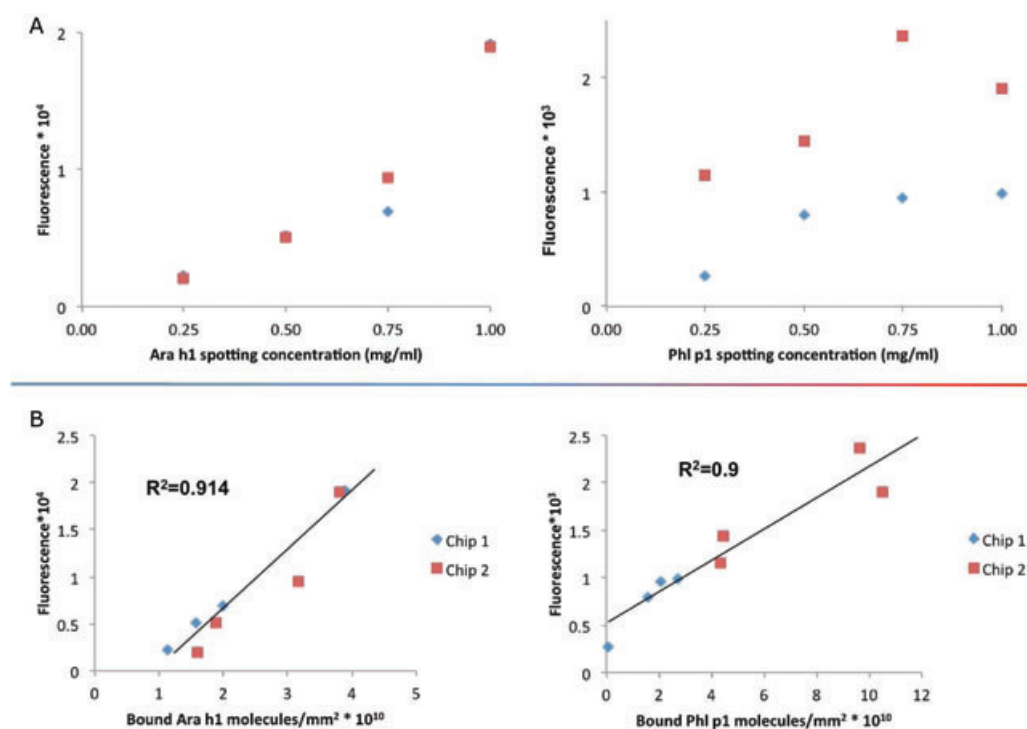


Figure 8. (A) The standard calibration of secondary antibody for diagnosis of allergy is measured as the degree of fluorescence as a function of spotting concentration is shown for Ara h1 peanut allergen (left) and Phl p1 timothy grass allergen (right) for two chips. Using the self-calibration method provided by CaFE (B), results show calibrated, linear responses for allergy testing analysis compared to traditional semi-quantifiable analysis in (A). Reproduced with permission from [17]

8 Conclusion

The article reviews the recent work of the authors in label and label-free sensing platforms employing Si/SiO₂ substrates for interferometric and/or fluorescence detection of microarrays. This widely available, inexpensive material, compatible with established glass surface chemistries is suitable as microarray substrate in the interferometric detection techniques. Si/SiO₂ layered substrates find application in label and label-free biosensing platforms. By varying the thickness of SiO₂, it is possible to enhance the emission of any fluorophore of choice by constructive interference with significant improvements in detection sensitivity.

The material finds application in SSFM, an interferometric technique that allows to measure the height of an ensemble of fluorophores located above a reflecting interface with sub-nanometer precision. This technique supports conformational study of DNA and proteins immobilized on surfaces.

The article discusses working principles and applications of IRIS, a platform amenable to high-throughput screening for label-free multiplexed detection. This technique provides picogram per square millimeter sensitivity, real-time kinetics monitoring of multiple analytes binding to immobilized probe molecules.

Finally, modulation of the oxide layer enables the development of dual label-based and label-free detection schemes on a single chip. This combination chip is of high practical utility during the assay development process to quantify the amount of probe immobilized on a surface. This information, generally not available in glass microarray technology, is crucial to determine consistency and quality of the spotted array.

Whatever the modulation of the oxide layer thickness is, surface chemistry of silicon substrates plays a fundamental role in bioprobe immobilization and in suppression of non-specific interactions. Hence, the paper presents an extensive review of organosilanization reactions as well as polymeric coatings useful for biofunctionalization in microarray technology.

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9 References

- [1] Ray, S., Reddy, P. J., Jain, R., Gollapalli, K. et al., Proteomic technologies for the identification of disease biomarkers in serum: advances and challenges ahead. *Proteomics* 2011, 11, 2139–2161.
- [2] Kricka, L. J., Master, S. R., Quality control and protein microarrays. *Clin. Chem.* 2009, 55, 1053–1055.
- [3] Pepperkok, R., Ellenberg, J., High-throughput fluorescence microscopy for systems biology. *Nat. Rev. Mol. Cell. Biol.* 2006, 7, 690–696.
- [4] Schena, M., Shalon, D., Davis, R. W., Brown, P. O., Quantitative monitoring of gene-expression patterns with a complementary-DNA microarray. *Science* 1995, 270, 467–470.
- [5] Neumann, B., Held, M., Liebel, U., Erfle, H. et al., High-throughput RNAi screening by time-lapse imaging of live human cells. *Nat. Methods* 2006, 3, 385–390.
- [6] Noyce, R. S., Bondred, D. G., Ha, M. N., Lin, L. T. et al., Tumor cell marker PVRL4 (Nectin 4) is an epithelial cell receptor for measles virus. *PLOS Pathog.* 2011, 7, e1002240.
- [7] Domnanich, P., Pena, D. B., Preininger, C., Xanthan/chitosan gold chip for metal enhanced protein biomarker detection. *Biosens. Bioelectron.* 2011, 26, 2559–2565.
- [8] Touahir, L., Allongue, P., Aureau, D., Boukherroub, R. et al., Molecular monolayers on silicon as substrates for biosensors. *Bioelectrochemistry* 2010, 80, 17–25.
- [9] Choumane, H., Ha, N., Nelep, C., Chardon, A. et al., Double interference fluorescence enhancement from reflective slides: application to bicolor microarrays. *Appl. Phys. Lett.* 2005, 87, 031102.
- [10] Mangeat, T., Berthier, A., Elie-Caille, C., Perrin, M. et al., Gold/silica biochips: applications to surface plasmon resonance and fluorescence quenching. *Laser Phys.* 2009, 19, 252–258.
- [11] Huang, C. S., Chaundhery, V., Pokhriyal, A., George, S. et al., Multiplexed cancer biomarker detection using quartz-based photonic crystal surface. *Anal. Chem.* 2012, 84, 1126–1133.
- [12] Ganesh, N., Block, I. D., Mathias, P. C., Zhang, W. et al., Leaky-mode assisted fluorescence extraction: application to fluorescence enhancement biosensors. *Opt. Express* 2008, 16, 21626–21640.
- [13] Cui, X. Q., Tawa, K., Hori, H., Nishii, J., Duty ratio-dependent fluorescence enhancement through surface plasmon resonance in Ag-coated grating. *Appl. Phys. Lett.* 2009, 95, 133117.
- [14] Cretich, M., di Carlo, G., Longhi, R., Gotti, C. et al., High sensitivity protein assays on microarray silicon slides. *Anal. Chem.* 2009, 81, 5197–5203.
- [15] Hellen, E. H., Axelrod, D., Fluorescence emission at dielectric and metal-film interfaces. *J. Opt. Soc. Am. B* 1987, 4, 337–350.
- [16] Moiseev, L., Cantor, C. R., Aksun, M. I., Dogan, M. et al., Spectral self-interference fluorescence microscopy. *J. Appl. Phys.* 2004, 96, 5311–5315.
- [17] Monroe, M. R., Reddington, A. P., Collin, A. D., LaBoda, C. et al., Multiplexed method to calibrate and quantitate fluorescence signal for allergen-specific IgE. *Anal. Chem.* 2011, 83, 9485–9491.
- [18] Swan, A. K., Moiseev, L., Cantor, C. R., Davis, B. J. et al., Towards nanoscale optical resolution in fluorescence microscopy. *IEEE JSTQE* 2003, 9, 294–300.

- [19] Moiseev, L., Ünlü, M. S., Swan, A. K., Goldberg, B. B. et al., DNA conformation on surfaces measured by fluorescence self-interference. *PNAS* 2006, **103**, 2623–2628.
- [20] Dogan, M., Yalcin, A., Jain, S., Goldberg, M. B. et al., Spectral self-interference fluorescence microscopy for subcellular imaging. *IEEE JSTQE* 2008, **14**, 217–225.
- [21] Braun, D., Fromherz, P., Fluorescence interferometry of neuronal cell adhesion on microstructured silicon. *Phys. Rev. Lett.* 1998, **23**, 5241–5244.
- [22] Parthasarathy, R., Groves, J. T., Optical techniques for imaging membrane topography. *Cell. Biochem. Biophys.* 2004, **41**, 391–414.
- [23] Spuhler, P. S., Knezevic, J., Yalcin, A., Bao, Q. Y. et al., Platform for in situ real-time measurement of protein-induced conformational changes of DNA. *PNAS* 2010, **107**, 1397–1401.
- [24] Newburger, D. E., Bulky, M. L., Universal protein-binding microarrays for the comprehensive characterization of the DNA-binding specificities of transcription factors. *Nat. Protocols* 2009, **4**, 393–411.
- [25] Zhao, M., Wang, X. F., Nolte, D. D., Molecular interferometric imaging. *Opt. Express* 2008, **16**, 7102–7118.
- [26] Piehler, J., Brecht, A., Gauglitz, G., Affinity detection of low molecular weight analytes. *Anal. Chem.* 1996, **68**, 139–143.
- [27] Brecht, A., Gauglitz, G., Recent developments in optical transducers for chemical or biochemical applications. *Sensor. Actuator. B: Chem.* 1997, **38**, 1–7.
- [28] Nikitin, P. I., Gorshkov, B. G., Nikitin, E. P., Ksenevich, T. I., Picoscope, a new label-free biosensor. *Sensor. Actuator. B: Chem.* 2005, **111**, 500–504.
- [29] Ozkumur, E., Needham, J. W., Bergstein, D. A., Gonzalez, R. et al., Label-free and dynamic detection of biomolecular interactions for high-throughput microarray applications. *PNAS* 2008, **105**, 7988–7992.
- [30] Ozkumur, E., Yalcin, A., Cretich, M., Lopez, C. A. et al., Quantification of DNA and protein adsorption by optical phase shift. *Biosens. Bioelectron.* 2009, **25**, 167–172.
- [31] Daaboul, G. G., Yurt, A., Zhang, X., Hwang, G. M., et al., High-throughput detection and sizing of individual low-index nanoparticles and viruses for pathogen identification. *Nano Lett.* 2010, **10**, 4727–4731.
- [32] Daaboul, G. G., Yurt, A., Goldberg, B. B., Connor, J. H. et al., Optical biosensors for virus detection and characterization. *IEEE J. Select. Topic. Quant. Electron.* 2011.
- [33] Bhatia, S.K., Shriver-Lake, L. C., Prior, K. J., Georger, J. H. et al., Use of thiol-terminal silanes and heterobifunctional crosslinkers for immobilization of antibodies on silica surfaces. *Anal. Biochem.* 1989, **178**, 408–413.
- [34] Shriver-Lake, L. C., Donner, B., Edelstein, R., Breslin, K. et al., Antibody immobilization using heterobifunctional crosslinkers. *Biosens. Bioelectron.* 1997, **12**, 1101–1106.
- [35] Leea, S. C., Keener, M. T., Tokachichu, D. R., Bhushan, B. et al., Protein binding on thermally grown silicon dioxide. *J. Vac. Sci. Technol. B* 2005, **23**, 1856–1865.
- [36] Zhu, H., Snyder, M., Protein chip technology. *Curr. Opin. Chem. Biol.* 2003, **7**, 55–63.
- [37] Wituck, G. L., A silane primer: chemistry and applications of alkoxysilanes. *J. Coating Technol.* 1993, **65**, 57–60.
- [38] Rye, R. R., Nelson, G. C., Dugger, M. T., Mechanistic aspects of alkylchlorosilane coupling reactions. *Langmuir* 1997, **13**, 2965–2972.
- [39] Bunimovich, Y. L., Ge, G., Beverly, K. C., Ries, R. S. et al., Electrochemically programmed, spatially selective biofunctionalization of silicon wires. *Langmuir* 2004, **20**, 10630–10638.
- [40] Verwey, F., Amerasekera, E. A., Bisschop, J., The physics of SiO₂ layers. *Rep. Prog. Phys.* 1990, **53**, 1297–1331.
- [41] Revesz, G., Hughes, H. L., The structural aspects of non-crystalline SiO₂ films on silicon: a review. *J. Non-Cryst. Solids* 2003, **328**, 48–63.
- [42] Han, Y., Mayer, D., Offenhäusser, A., Ingebrandt, S., Surface activation of thin silicon oxides by wet cleaning and silanization. *Thin Solid Films* 2006, **510**, 175–180.
- [43] Halliwell, C. M., Cass, A. E. G., A factorial analysis of silanization conditions for the immobilization of oligonucleotides on glass surfaces. *Anal. Chem.* 2001, **73**, 2476–2483.
- [44] Cui, Y., Wei, Q., Park, H., Lieber, C. M., Nanowire nanosensors for highly sensitive and selective detection of biological and chemical species. *Science* 2001, **293**, 1289–1292.
- [45] Tan, W., Wang, K., He, X., Zhao, X. J. et al., Bionanotechnology based on silica nanoparticle. *Med. Res. Rev.* 2004, **24**, 621–638.
- [46] De Vos, K., Bartolozzi, I., Schacht, E., Bienstman, P. et al., Silicon-on-Insulator microring resonator for sensitive and label-free biosensing. *Opt. Express* 2007, **15**, 7610–7615.
- [47] Raiteri, R., Grattarola, M., Butt, H. J., Skladal, P., Micromechanical cantilever-based biosensors. *Sens. Actuator B Chem.* 2001, **79**, 115–126.
- [48] Kitsara, M., Misiakos, K., Raptis, I., Makarona, E., Integrated optical frequency-resolved Mach-Zehnder interferometers for label-free affinity sensing. *Opt. Express* 2010, **18**, 8193–8206.
- [49] Jonkheijm, P., Weinrich, D., Schröder, H., Niemeyer, C. M. et al., Chemical strategies for generating protein biochips. *Angew. Chem. Int. Edit.* 2008, **47**, 9618–9647.
- [50] Williams, R. A., Blanch, H. W., Covalent immobilization of protein monolayers for biosensor applications. *Biosens. Bioelectron.* 1994, **9**, 159–167.
- [51] Golbik, U. R., Schellenberger, A., Protein adsorption and leakage in carrier-enzyme systems. *Biotech. Bioeng.* 1991, **37**, 280–287.
- [52] Leckband, D., Langer, R., An approach for the stable immobilization of proteins. *Biotech. Bioeng.* 1991, **37**, 227–237.
- [53] Awsiuka, K., Bernasik, A., Kitsarac, M., Budkowskia, A. et al., Spectroscopic and microscopic characterization of biosensor surfaces with protein/amino-organosilane/silicon structure. *Colloid. Surface. B* 2012, **90**, 159–168.
- [54] Blitz, J. P., Murthy, R. S. S., Leyden, D. E., Ammonia-catalyzed silylation reactions of Cab-O-Sil with

- methoxymethylsilanes. *J. Am. Chem. Soc.* 1987, 109, 7141–7145.
- [55] Tripp, C. P., Hair, M. L., Chemical attachment of chlorosilanes to silica: a two-step amine-promoted reaction. *J. Phys. Chem.* 1993, 97, 5693–5698.
- [56] Haensch, C., Hoepfner, S., Schubert, U. S., Chemical modification of self-assembled silane based monolayers by surface reactions. *Chem. Soc. Rev.* 2010, 39, 2323–2334.
- [57] Kusnezow, W., Hoheisel, J. D., Solid supports for microarray immunoassays. *J. Mol. Rec.* 2003, 16, 165–176.
- [58] Milum, J., Sadana, A., Influence of different parameters on a dual-fractal analysis for antigen–antibody binding kinetics for biosensor applications. *J. Colloid Interface Sci.* 1997, 187, 128–138.
- [59] Nakanishi, K., Sakiyama, T., Kumada, Y., Imamura, K. et al., Recent advances in controlled immobilization of proteins onto the surface of the solid substrate and its possible application to proteomics. *Curr. Proteome.* 2008, 5, 161–175.
- [60] Lee, J. H., Kopecek, J., Andrade, J. D., Protein-resistant surfaces prepared by PEO-containing block copolymer surfactants. *J. Biomed. Mater. Res.* 1989, 23, 351–368.
- [61] Elbert, D. L., Hubbell, J. A., Reduction of fibrous adhesion formation by a copolymer possessing an affinity for anionic surfaces. *J. Biomed. Mater. Res.* 1998, 42, 55–65.
- [62] Liu, V. A., Jastromb, W. E., Bhatia, S. N., Engineering protein and cell adhesivity using PEO-terminated triblock polymers. *J. Biomed. Mater. Res.* 2002, 60, 126–134.
- [63] Prime, K. L., Whitesides, G. M., Adsorption of proteins onto surfaces containing end-attached oligo(ethylene oxide) – A model system using self-assembled monolayers. *J. Am. Chem. Soc.* 1993, 115, 10714–10721.
- [64] Xia, N., Hu, Y. H., Grainger, D. W., Castner, D. G., Functionalized poly(ethylene glycol)-grafted polysiloxane monolayers for control of protein binding. *Langmuir* 2002, 18, 3255–3262.
- [65] Bearinger, J. P., Terrettaz, S., Michel, R., Tirelli, N. et al., Chemisorbed poly(propylene sulphide)-based copolymers resist biomolecular interactions. *Nat. Mater.* 2003, 2, 259–264.
- [66] Amiji, M., Park, K., Surface modification of polymeric biomaterials with poly(ethylene oxide), albumin, and heparin for reduced thrombogenicity. *J. Biomater. Sci. Polym.* 1993, 4, 217–234.
- [67] Llanos, G. R., Sefton, M. V., Does polyethylene oxide possess a low thrombogenicity. *J. Biomater. Sci. Polym.* 1993, 4, 381–400.
- [68] Jain, P., Baker, G. L., Bruening, M. L., Applications of polymer brushes in protein analysis and purification. *Annu. Rev. Anal. Chem.* 2009, 2, 387–408.
- [69] Yu, Q., Zhou, M., Ding, Y. H., Jiang, B. B. et al., Development of networks in atom transfer radical polymerization of dimethacrylates. *Polymer* 2007, 48, 7058–7064.
- [70] Rowe-Konopacki, M. D., Boyes, S. G., Synthesis of surface initiated diblock copolymer brushes from flat silicon substrates utilizing the RAFT polymerization technique. *Macromolecules* 2007, 40, 879–888.
- [71] Tsujii, Y., Ejaz, M., Sato, K., Goto, A. et al., Mechanism and kinetics of RAFT-mediated graft polymerization of styrene on a solid surface. 1. Experimental evidence of surface radical migration. *Macromolecules* 2001, 34, 8872–8878.
- [72] Li, C., Benicewicz, B. C., Synthesis of well-defined polymer brushes grafted onto silica nanoparticles via surface reversible addition-fragmentation chain transfer polymerization. *Macromolecules* 2005, 38, 5929–5936.
- [73] Mammen, M., Dahmann, G., Whitesides, G. M., Effective inhibitors of hemagglutination by influenza-virus synthesized from polymers having active ester groups – insight into mechanism of inhibition. *J. Med. Chem.* 1995, 38, 4179–4190.
- [74] Hucknall, A., Rangarajan, S., Chilkoti, A., In pursuit of zero: polymer brushes that resist the adsorption of proteins. *Adv. Mater.* 2009, 21, 2441–2446.
- [75] Prucker, O. R., Ruhe, J., Mechanism of radical chain polymerizations initiated by azo compounds covalently bound to the surface of spherical particles. *Macromolecules* 1998, 31, 602–613.
- [76] Ejaz, M. Y., Yamamoto, S., Ohno, K., Tsujii, Y. et al., Controlled graft polymerization of methyl methacrylate on silicon substrate by the combined use of the Langmuir-Blodgett and atom transfer radical polymerization techniques. *Macromolecules* 1998, 31, 5934–5936.
- [77] Di Carlo, G., Damin, F., Armelao, L., Maccato, C. et al., Synthesis and conformational characterization of functional diblock copolymer brushes for microarray technology. *Appl. Surf. Sci.* 2012, 258, 3750–3756.
- [78] Pirri, G., Damin, F., Chiari, M., Bontempi, E. et al., Characterization of a polymeric adsorbed coating for DNA microarray glass slides. *Anal. Chem.* 2004, 76, 1352–1358.
- [79] Yalcin, A., Damin, F., Ozkumur, E., Di Carlo, G. et al., Direct observation of conformation of a polymeric coating with implications in microarray applications. *Anal. Chem.* 2009, 81, 625–630.
- [80] Moal, E., Fort, E., Leveque-Fort, S., Janin, A. et al., Mirror slides for high-sensitivity cell and tissue fluorescence imaging. *J. Biomed. Opt.* 2007, 12, 024030.
- [81] Akimoto, T., Yasuda, M., Fluorescence enhancement and reflection of the excitation light observed with a multilayered substrate. *Appl. Optics* 2010, 49, 80–85.
- [82] Sullivan, K., Hall, D. G., Enhancement and inhibition of electromagnetic radiation in plane-layered media. I. Plane-wave spectrum approach to modeling classical effects. *J. Opt. Soc. Am. B* 1997, 14, 1149–1159.
- [83] Rajendra, R., Niko, S., Volker, S., Luis, B. et al., Signal and sensitivity enhancement through optical interference coating for DNA and protein microarray applications. *J. Biomol. Tech.* 2006, 17, 122–130.
- [84] Shreffler, W. G., Microarrayed recombinant allergens for diagnostic testing. *J. Allergy Clin. Immunol.* 2011, 127, 843–849.
- [85] Balboni, I., Limb, C., Tenenbaum, J. D., Utz, P. J., Evaluation of microarray surfaces and arraying parameters for autoantibody profiling. *Proteomics* 2008, 8, 3443–3449.

- [86] Klin, J. B. L., Shreffler, W. G., Microarrayed allergen molecules for diagnostics of allergy. *Methods Mol. Biol.* 2009, 524, 259–272.
- [87] Chuaqui, R. F., Bonner, R. F., Best, C. J. M., Gillespie, J. W. et al., Post-analysis follow-up and validation of microarray experiments. *Nat. Genet.* 2002, 32, 509–514.
- [88] Ellington, A. A., Kullo, I. J., Bailey, K. R., Klee, G. G., Measurement and quality control issues in multiplex protein assays: a case study. *Clin. Chem.* 2009, 55, 1092–1099.
- [89] Volle, J. N., Chambon, G., Sayah, A., Reymond, C. et al. Enhanced sensitivity detection of protein immobilization by fluorescent interference on oxidized silicon. *Biosens. Bioelectron.* 2003, 19, 457–464.
- [90] Bras, M., Dugas, V., Bessueille, F., Cloarec, J. P. et al. Optimisation of a silicon/silicon dioxide substrate for a fluorescence DNA microarray. *Biosens. Bioelectron.* 2004, 20, 797–806.
- [91] Bally, M., Halter, M., Voeroes, J., Grandin, H. M., Optical microarray biosensing techniques. *Surf. Interface Anal.* 2006, 38, 1442–1458.
- [92] Cretich, M., Breda, D., Damin, F., Borghi et al. Allergen microarrays on high-sensitivity silicon slides. *Anal. Bioanal. Chem.* 2010, 398, 1723–1733.
- [93] Galbiati, S., Restagno, G., Foglieni, B., Bonalumi, S. et al. Different approaches for noninvasive prenatal diagnosis of genetic diseases based on PNA-mediated enriched PCR. *Ann. NY Acad. Sci.* 2006, 1075, 137–143.
- [94] Fakhrai-Rad, H., Pourmand, N., Ronaghi, M., Pyrosequencing: an accurate detection platform for single nucleotide polymorphisms. *Hum. Mutat.* 2002, 19, 479–485.
- [95] Bruno, F., Damin, F., Causarano, V., Galbiati, S. et al., High-sensitive microarray substrates specifically designed to improve sensitivity for the identification of fetal paternally inherited sequences in maternal plasma. *Clin. Chem. Lab Med.* 2009, 47, 818–823.
- [96] Cretich, M., Bagnati, M., Damin, F., Sola, L. et al., Overcoming mass transport limitations to achieve femtomolar detection limits on silicon protein microarrays. *Anal. Biochem.* 2011, 418, 164–166.
- [97] Bergese, P., Cretich, M., Oldani, C., Oliviero, G. et al. Advances in parallel screening of drug candidates. *Curr. Med. Chem.* 2008, 15, 1706–1719.
- [98] Lu, J. H., Strohsahl, C. M., Miller, B. L., Rothberg, L. J., Reflective interferometric detection of label-free oligonucleotides. *Anal. Chem.* 2004, 76, 4416–4420.
- [99] Kodera, S., Okajima, T., Iwabuki, H., Kitaguchi, D. et al. Detection of protein-protein interactions on SiO₂/Si surfaces by spectroscopic ellipsometry. *Anal. Biochem.* 2003, 321, 65–70.
- [100] Ozkumur, E., Ahn, S., Yalcin, A., Lopez, C. A. et al., Label-free microarray imaging for direct detection of DNA hybridization and single-nucleotide mismatches. *Biosens. Bioelectron.* 2010, 25, 1789–1795.
- [101] Daaboul, G. G., Vedula, R. S., Ahn, S., Lopez, C. A. et al., LED-based interferometric reflectance imaging sensor for quantitative dynamic monitoring of biomolecular interactions. *Biosens. Bioelectron.* 2011, 26, 2221–2227.
- [102] Lopez, C. A., Daaboul, G. G., Vedula, R. S., Ozkumur, E. et al., Label-free, multiplexed virus detection using spectral reflectance imaging. *Biosens. Bioelectron.* 2011, 26, 3432–3437.
- [103] Ahn, S., Spuhler, P., Chiari, M., Cabodi, M. et al., Quantification of surface etching by common buffers and implications on the accuracy of label-free biological assays. *Biosens. Bioelectron.* 2012, 36, 222–229.
- [104] Cretich, M., Reddington, A., Monroe, M., Bagnati, M. et al., Silicon biochips for dual label-free and fluorescence detection: application to protein microarray development. *Biosens. Bioelectron.* 2011, 26, 3938–3943.
- [105] Pereira, C., Yalcin, A., Cretich, M., Chiari, M. et al. Synergistic chemiluminescence and label-free dual detection for developing a hepatitis protein array. *J. Immunol. Methods* 2011, 371, 159–164.