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Biosensors @ CSEM

Hans Sigrist* and Hui Gao

Abstract. Biosensor systems are man-made operational mimics of nature's design and efficiency. They are hybrid constructs consisting of biomolecules, transducers and instrument components, designed to specifically detect and identify substances that are relevant for diagnosis or process surveillance. The Swiss Center for Electronics and Microtechnology (CSEM) fosters biosensor-system applications for the detection of constituents and additives in food, and for prognostic or diagnostic identification of drugs and metabolites. Instruments and biosensor platforms are developed on the basis of advanced detection principles. Optical and electrochemical platforms are improved in efficiency and value through assay-specific surface bioengineering. Device miniaturization and large-scale manufacturability, combined with instrumental simplicity are adopted properties of CSEM's application-specific biosensor systems. Major current biosensor developments include the detection of infectious prion proteins in livestock and antibiotics in milk.

1. Introduction

Medical diagnosis and food technology are increasingly confronted with issues that are essential to personal welfare and public health. Individuals, private institutions and governmental agencies demand bold and substantial analytical systems that satisfy personal needs, that help to control processes, and ascertain the observance of legal regulations. These requests call for advanced analytical systems with improved detection sensitivity, short analysis time and biotechnology-empowered analytical selectivity.

Biosensors combine the selectivity of biological recognition with the transfer features of transducer materials. In nature, biomolecule-based sensing is a fundamental principle of communication. Responsive recognition is part of the communication between molecules, subcellular components, and cells; even among organs of an organism. In biological systems, arrays of enzymes and receptors, combinations of hormones, neurotransmitters and/or antigens are capable of individually receiving and propagating signals at the molecular level. Biosensor technology and biomaterial engineering request communicative molecular interactions between bioconstituents and material surfaces, enabling molecular signal-transfer and biorecognition. The successful combination of biological functions with transducer surfaces often demands mediating interfaces. Surface bioengineering is the art of furnishing molecular environments on transducer surfaces in a way that biomolecules are able to operate as in native biosystems.

*Correspondence: PD Dr. H. Sigrist
Centre Suisse d'Electronique et de Microtechnique
Jaquet-Droz 1
CH-2007 Neuchâtel
Tel.: +41 32 720 52 92
Fax: +41 32 720 57 20
E-Mail: hans.sigrist@csem.ch

2. Biosensors @ CSEM

The CSEM is currently developing biosensor systems for commercial applications. Activities of the biosensor team include the development of instruments for optical signal detection. These efforts are accompanied by the development of optical, electrochemical and surface-acoustic wave-transducer platforms. Major activities include bioengineering of either type of transducer. Surface bioengineering covers all aspects of biomolecule immobilzation on material surfaces. Specific attention is paid to the manufacturability of referenced multiplex-analysis systems and the mass production of bioengineered transducer platforms.

2.1 *Instruments for Optical Signal Transduction*

Optical detection principles used are based on the behavior of light at boundaries of different refractive index. Beyond the critical angle, light will be totally internally reflected at the interface of the higher-refractive-index medium (*e.g.*, the sensor platform) and a lower-refractive-index medium (*e.g.*, a sample solution that contains biomolecules). When total internal reflection occurs, an electromagnetic field – the evanescent wave – passes from the interface into the lower-refractive-index medium. It decays exponentially over a short distance of a few hundred nanometers. In resonant structures (waveguide grating, waveguide-prism or plasmon-prism couplers), the evanescent wave is greatly enhanced at the resonance angle. The resonance angle is extremely sensitive to the refractive index of adlayers at the sensor surface. The interaction of medium- and high-molecular-weight analytes with surface-immobilized sensing molecules is detected by recording the changes of the effective refractive index. Molecules that form biospecific interactions, *e.g.*, antibodies, oligonucleotides, cell-binding oligosaccharides, and receptors may serve to specifically detect and identify analyte constituents such as antigens, complementary-DNA fragments, and specific cell types. Physico-chemical or covalent attachment of ligate molecules followed by ligand binding, renders quantifiable information on the kinetic properties and the binding specificity of biomolecular interactions. Refractive-index-based detection does not require labeled components and is thus advantageous for field testing of biotic fluids.

One instrument that is currently developed is a small compact sensor unit com-

prising a diode-laser light source (785 nm diode laser), an optical detection module, and an integrated optical transducer. Distinct diffraction functionalities are implemented in a recently designed optical-sensing scheme (*Fig. 1a*) [1]. The integrated optical transducer consists of one input and one output pad with chirped gratings. By illuminating the input grating-pad at a fixed angle, the coupling occurs at one position only (due to the chirp) and defines a fine line. Macromolecule-binding to bioengineered surfaces causes a refractive-index change. This effect in turn shifts the position of the coupled line. The shift distance is dependent on the analyte mass deposited on the grating surface. Medium- and high-molecular-weight biomolecules are detectable in nano- to picomolar quantities with this biosensor system.

A second biosensor system that is currently developed for the detection of low-molecular-weight analytes is depicted in *Fig. 1b*. This detection system makes use of fluorescent probes, *e.g.*, fluorescein or cyanine dyes, that flag analyte analogs. By applying competitive analysis-schemes, fluorescence-labeled analytes are detected in sub-nanomolar concentrations. As the integrated optical sensor system described above, this second system also uses the evanescent field of guided light. In this approach, however, the evanescent light serves to excite the fluorescent molecules [2]. Technically, the system relies on a laser light source (488-nm laser light), single-mode waveguides and parallel submicron gratings. The detection of molecular interactions is based on the following principle: the light of a laser is coupled in the waveguide layer with a first grating and propagates in the waveguide (*Fig. 1b*). The evanescent light excites the fluorescent labels that are within reach of the evanescent field. A part of the emitted fluorescent light enters into, and propagates along the waveguide. The guided fluorescent light is out-coupled, optionally at a second grating, and directed to a detector. An optical filter quenches the excitation component of the activating light. This second optical detection system thus allows time-dependent fluorescence monitoring and the setting of system-specific coupling angles. The amount of fluorescence detected on the sensor surface reports on the biochemical reaction, *e.g.*, the competition between an analyte and its fluorophore-labeled analogue.

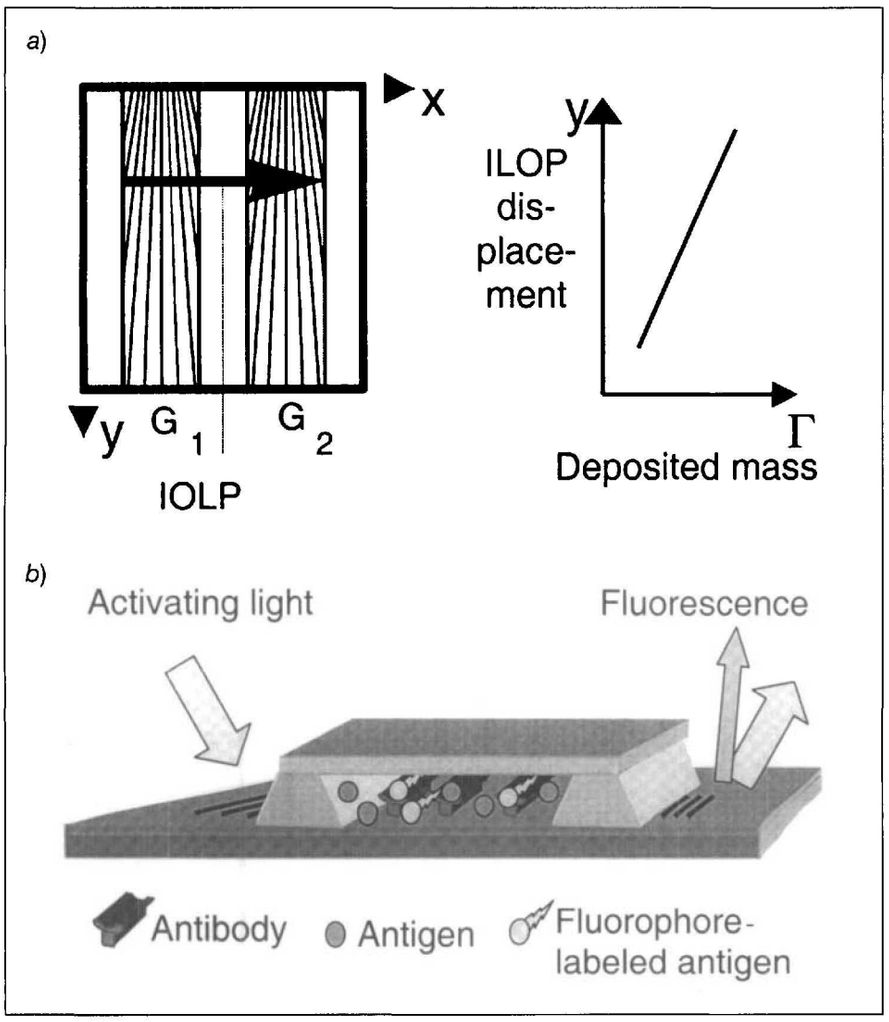

Fig. 1. *a) Optical detection scheme for quantifying the mass deposited on replicated optical devices.* Laser light impinging on a chirped grating G_1 generates an optically detectable light path (integrated optical light pointer, IOLP) in the waveguide layer between the incoupling grating G_1 and the outcoupling grating G_2. The line spacing defines the position of the light pointer in a given medium. The light pointer changes its position along with a change in refractive index and thickness. Index changes are effected by the addition of mass (*e.g.*, by selective binding of biomolecules) to the waveguide layer. The integrated optical platform thus allows the detection of bi-molecular interactions occurring in the evanescent field. *b) Detection of fluorescence-labeled analytes on a microstructured silicon/silicon-nitride platform.* Activating light is incoupled in the waveguiding layer, fluorescent probes residing in the evanescent field are activated, and generated fluorescence is detected at a distinct outcoupling angle. The scheme details a competitive fluorescence assay.

In both optical detection systems, highly refractive materials such as silicon nitride or metal oxides serve as waveguides. Thin films of either material are deposited on silicon wafers or on polycarbonate substrates by low-pressure chemical vapor-plasma deposition or magneton sputtering, respectively. Diffraction gratings are generated either by microstructuring of silicon wafers, by hot embossing, or polymer moulding [3]. The substrate materials mentioned have been selected in view of transducer manufacturing in high numbers. The mass production of high-quality optical microstructures in silicon and replicated polycarbonate is an essential contribution to the commercialization of both optical biosensor systems.

2.2. *Electrochemical Transducers*

Thin-film carbon deposition is a process mastered by CSEM and applied to the manufacturing of various electrochemical platforms. Microelectrodes are manufactured with adapted microelectronic processes based on photolithography, plasma deposition, and plasma etching. Common to interdigitated and disk microelectrodes is the combination of conducting (α-carbon) and non-conducting (diamond-like) thin-film carbon [4]. Thin-film technology led to the manufacturing of all-carbon electrodes. Carbon microelectrodes are particularly well-suited to contious monitoring where high stability of the carbon is combined with the flow-independent be-

haviour of the microelectrodes. A tested transducer device used for enzyme-mediated amperometric biosensing is shown in *Fig. 2a*. The transducer chip consists of six α-carbon coated, electrically conductive pads. Non-conductive surfaces are passivated with diamond-like carbon. The dimensions of the electrically transducing areas are 0.375 and 0.75 mm^2. The array of electrodes allows quantitative analysis of enzyme-catalyzed biochemical reactions and enables appropriate electrochemical referencing with a reference electrode and a counter electrode [5]. *Fig. 2b* shows the results obtained for referenced amperometric detection of D-glucose and L-glutamate on an electrochemical all-carbon electrode furnished with photobonded glucose oxidase and glutamate oxidase. The configuration is advantageous for biosensing in that the interaction of analyte components (*e.g.*, proteins) occurs with one type of material only. Adsorption of components of biotic fluids with carbon-based materials is factual [6]. As a consequence, differentiating (material-specific) adsorption is prone to hamper the performance of multi-material electrodes (*e.g.*, platinum or Ag/AgCl as reference). All-carbon electrodes, however, enable unifying control of ubiquitous biomolecule adsorption. The detection limits of enzyme electrodes depend on both the characteristics of the enzyme, and the electrochemical properties of the detected product. Concentration limits for the glutamate-oxidase and glucose-oxidase sensor systems were 0.05 mM L-glutamate and 0.2 mM D-glucose. The shelf-life time at 4 °C of glucose oxidase photobonded to conductive carbon is more than 3 months.

2.3. *Surface-Acoustic Wave Sensors*

Surface-acoustic wave sensors (SAWs) are increasingly used for biosensing and the detection of bi-molecular interactions [7]. In this system, molecular interactions are registered by determining the changes in surface-wave velocity, predominantly caused by mass adsorption or viscosity changes. For biosensing purposes, transducer systems are selected that generate horizontally-polarized shear waves on piezoelectric substrates (*e.g.*, $LiTaO_3$). Adsorption or binding of biomolecules to the SAW surface alters the wave propagation. Observed frequency changes thus report on analyte binding. However, the exposure of commercial SAW devices to physiological electrolyte solutions is detrimental to the metal parts and electrical connections. For immunosensing in physiological media, pretreatment of SAW devices

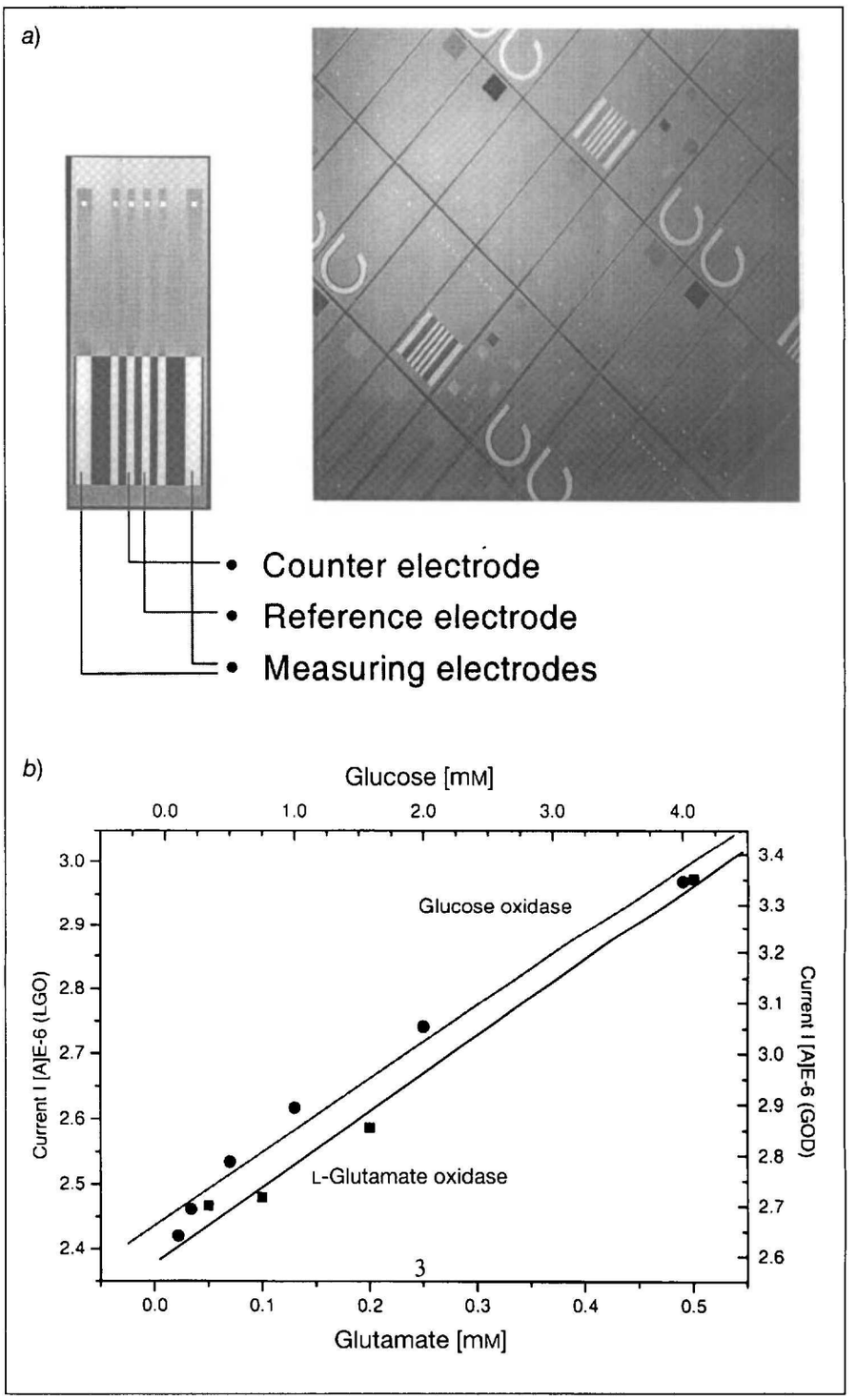

Fig. 2. *a) All-carbon electrodes.* An individual all-carbon electrode chip (7 x 2.8 mm) with six conductive electrode pads is depicted (left) next to a section of a wafer with various electrode designs. *b) Enzyme-mediated amperometric detection of* D-*glucose and* L-*glutamate on an all-carbon electrode.* Thin-film α-carbon conductive electrodes were microstructured on silicon. After packaging in measuring devices, the measuring-electrode pads were modified with the enzymes glucose oxidase (GOD) and L-glutamate oxidase (LGO). Enzyme immobilization was achieved by photobonding: Mixtures of the photolinker polymer T-BSA and enzyme were deposited on selected conductive electrode pads. For photobonding, the chip surfaces were dried and exposed to activating light (365 nm, 20 min, 0.7 mW/cm^2). Referenced amperometric measurements were carried out sequentially with the enzyme electrode using a third pad as reference and a fourth pad as counter electrode. Incremental amounts of D-glucose or L-glutamate were added to the reaction medium (5 ml, r.t.) and enzyme-mediated H_2O_2 production was detected with an applied working potential of 1.1 V (Potentiostat, *EG&G Instruments*). Registered current intensities (saturation values) were correlated with the (enzyme) substrate concentration in the analyte solution.

with passivating layers is essential. Polyimide and parylene were found to provide exceptional passivation properties. Polymer coating enables serial production and subsequent surface bioengineering [8]. At the CSEM, SAW immunosensors are developed in collaboration with the Research Center, Karlsruhe, Germany. Established detection systems are applied to detect antigens in the low-nanomolar range [9].

3. Surface Bioengineering for Improved Biomolecule Detection

Surface bioengineering in sensor technology covers all aspects that lead to optimal efficiency of the sensing biomolecule. The first and ultimate goal in surface bioengineering is the expression of unlimited biological activity of the sensing biomolecules, *e.g.*, enzymes, antibodies, or oligonucleotide probes. Good biosensors further request properties like high biomolecule density at the surface, low-level non-specific interaction, and biolayer stability. Advanced biosensor devices additionally call for signal amplification, optionally at the level of the biolayer, and array deposition of biomolecules for multiplex analysis.

With increasing complexity and demands for future biosensor generations, there is an emerging trend towards the design and manufacturing of efficacious biosensor constructs; constructs that optimally interface biological activity with the transducer material: The analytical system must be capable of acquiring minute signals. It needs to process multidomain information and generate unique sensor responses. Such complex bioinformation processing requires both competence in surface bioengineering at the chemical and biochemical level, and advanced microstructuring (biopatterning) technologies. Biointerfacing of transducer surfaces is not trivial. The connection of biomolecules with transducers demands the retention of physical properties of the signal transducing material in the presence of biomolecules. Moreover, signal transduction must remain functional in media wherein biomolecules can express and retain their biological functions. Such considerations apply to all transducer systems including optical, electrochemical and surface-acoustic wave devices.

3.1. *Surface Modification and Photobonding of Biomolecules*

A first step in numerous sensor bioengineering schemes is chemical treatment of the material surface. Preferred modes of transducer-surface functionalization initiate with surface silylation. The extent of surface silylation critically depends on the number and structural availability of surface hydroxy functions. The latter may vary depending on history and age of the device surfaces. Surface hydroxylation by oxygen-plasma treatment is a recommended approach to timely unify the chemical properties of transducer surfaces. Such a treatment makes, for example, TiO_2-surface silylation reproducible and increases the wetability of silicon nitride [10].

Immobilization of biomolecules on transducer surfaces can be effected by various means; the route chosen may depend on the characteristics of the sensing biomolecule and the analytical assay conditions. Adsorptive biomolecule binding followed by (non-covalent) blocking of the surface with serum albumin or casein is a routine protocol in enzyme-linked immunosorbent assays (ELISAs). Such procedures are easily adaptable to biosensor systems.

Complex analyte mixtures containing a multitude of proteins – each of them expressing characteristic surface interactions – may call for covalent immobilization of the sensing biomolecules [11]. Covalent biomolecule binding adds molecular stability to biosensor surfaces throughout the analytical measurement and may allow for biosurface regeneration [12]. An elegant mode of biomolecule immobilization is effected by the use of photoactivatable reagents. The interaction of light with photoreagents such as aryldiazirines, arylazides or benzophenones leads to the generation of reactive intermediates: carbenes, arylnitrenes and ketyl radicals, respectively. Whereas carbenes and nitrenes undergo insertion reactions with covalent chemical bonds of adjacent surface materials or biomolecules, photoactive benzophenones establish covalent bonds by radical-mediated processes. Either of the reagents mentioned can be chemically grafted to polymers. The products, for example multiply aryldiazirine-substituted bovine serum albumin (T-BSA) or amino-dextran, are used to covalently bind biomolecules (antigens, allergens, enzymes, antibodies, oligonucleotides) to material surfaces in a single-step photoreaction. Photolinker polymer and sensing molecules are mixed, spread on the target surface and irradiated with light. The product of such light-addressable reagent immobilization is a surface with covalently attached 'sensing' molecules. Such photoreagent-mediated immobilization opens new routes to fast and effective surface engineering: the photoreaction is independent on reaction conditions, multicomponent immobilization is applicable, the extent of biomolecule immobilization can be controlled by the irradiation time, and photolithographic procedures or direct fiber guided immobilization are feasible. Light-dependent immobilization of biomolecules on material surfaces implies widespread applications due to the broad reactivity of photogenerated intermediates.

Several surface-modification studies have provided direct and indirect evidence that photogenerated carbenes bind to covalently bonded materials ranging from carbon-based organic polymers to metal oxides [13]. Carbene insertion into surface-bonded oxygen was recently evidenced by TOF-SIMS [14]. To date, aryldiazirines are approved reagents for biomolecule immobilization acting either as bifunctional crosslinkers [13][15] or as light-sensitive units of photolinker polymers [12][13]. Moreover, photolinker-polymer-mediated immobilization allows for the simultaneous immobilization of mixtures of components. This property is unique to the photobonding process and enables complex surface bioengineering in a single step. Substances that stabilize the sensing biomolecule, that suppress non-specific binding, that improve biomolecule function, and/or that simulate the native molecular environment of the sensing biomolecule can be co-immobilized on the sensor surface. An example for multicomponent photobonding is described in *Fig. 3*. Selective immunodetection of recombinant prion protein has been achieved by co-immobilization of the prion protein with casein, the photolinker polymer T-BSA serving as light-activatable linking agent.

3.2. *Biopatterning*

The availability of biosensor arrays is essential for efficient biosensing and multiplex analyte detection in high-throughput assay formats. Biosensor arrays are prepared by depositing biomolecules into spatially defined sensing domains. For calibration purposes, individual domains may contain individual composition and defined molecular complexity.

Biosensor arrays on transducer surfaces are manufactured either by microcontact printing, ink-jet bioprinting, mask-assisted patterning, or by laser writing [16]. The latter two methods can be accomplished by light-dependent processes. Mask-assisted biopatterning with light confers with standard photolithography commonly used in microtechnology. Experimentally, a surface is coated with a

thin film of a biomolecule/photolinker polymer mixture and dried. The film is then exposed to activating light. A structured mask serves as stencil that is used in contact or near-contact mode. Non-bonded biomolecules are removed from protected regions by rinsing with aqueous media.

Mask-assisted printing of biomolecules can be realized on various substrates. To date, surface patterning and retention of biological functions are documented on silicon and silica-based materials by applying photolinker-polymer-mediated immobilization procedures. In view of sequential patterning of different biomolecules on a single substrate surface, overlay-patterning processes have been established. *Fig. 4a* shows a criss-cross overlay pattern consisting of fluorescein-labeled bovine serum albumin (vertical bars) and rhodamine-labeled bovine serum albumin (perpendicular bars), the second layer being deposited after completion of the first bioprint. The absence of fluorescence from areas hidden in both successive printing steps qualifies the method for multi-step mask-assisted array printing. Bioprinting in conjunction with photobonding technologies offers the advantages of fast and spatially selective deposition and immobilization of biomolecules. Mask-assisted biopatterning is the method of choice for biomolecule deposition for array systems with neglegible cross-contamination, *e.g.*, few sequential deposition steps and low-level surface interaction in the absence of activating light.

3.3. *Ink-jet Bioprinting*

The possibility of adapting ink-jet printing technologies to the requirements of biomolecule dispensing is very attractive. Ink-jet printing eliminates the need for contact or near contact with the target substrate. The dispensing speed and throughput can be increased while getting rid of the potential problems of cross-contamination. The most notable attempts to adapt ink-jet technology are those that use drop-on-demand dispensing. In a drop-on-demand system, the user programs the action of a valve that controls the dispensing function in such a way that each actuation of the valve produces a single drop of reagent. We have applied a drop-on-demand ink-jet system based on piezoelectric valves to dispense nanoliter quantities of solutions containing biomolecules. Bioprinted enzymes and antibodies remain biologically active. Ink-jet bioprinting procedures in conjunction with photobonding are routinely used for local deposition of 'sensing' proteins, protein mix-

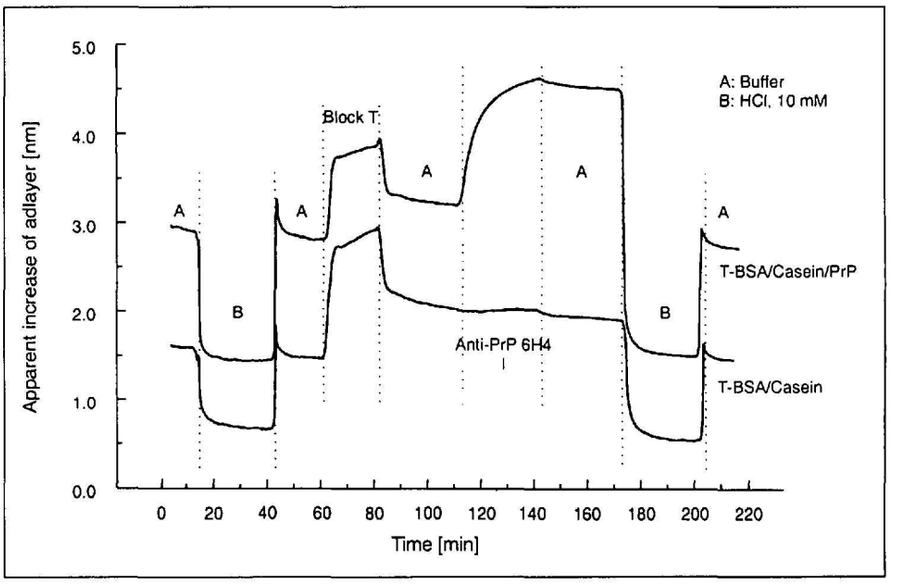

Fig. 3. *Application-specific surface engineering for optical detection of recombinant prion protein.* In order to suppress non-specific interaction, the optical sensor platform (grated SiO_2/TiO_2 waveguide on glass) was coated with a 1:1:2 mixture of recombinant prion protein (PrP), casein and the photolinker polymer T-BSA (control without recombinant prion protein). For photobonding, dried surfaces were activated with light (365 nm, 20 min, 0.7 mW/cm^2, *Stratalinker* light source). Modified surfaces were then washed with phosphate-buffered saline containing detergent (PBS/0.02% *Tween 20*). Immunocomplexion of photobonded prion protein was analyzed with the monoclonal anti-prion antibody 6H4. Additional treatment of the surface with casein (Block T) or BSA was essential for suppression of non-specific binding. Binding of the probing antibody was detected by recording the change of effective refractive index using the integrated optical sensor instrument *BIOS 1* (upper line). Immunocomplexation reverted to an active biosensor platform after low-pH treatment (10 mM HCl). The generated signal was found to be specific for the 6H4 antibody. An identically treated sample without photobonded PrP served as control (lower line). Non-specific binding was not observed under analysis conditions. Recombinant prion protein and the monoclonal anti-PrP antibody were provided by *Prionics*, Zürich, Switzerland.

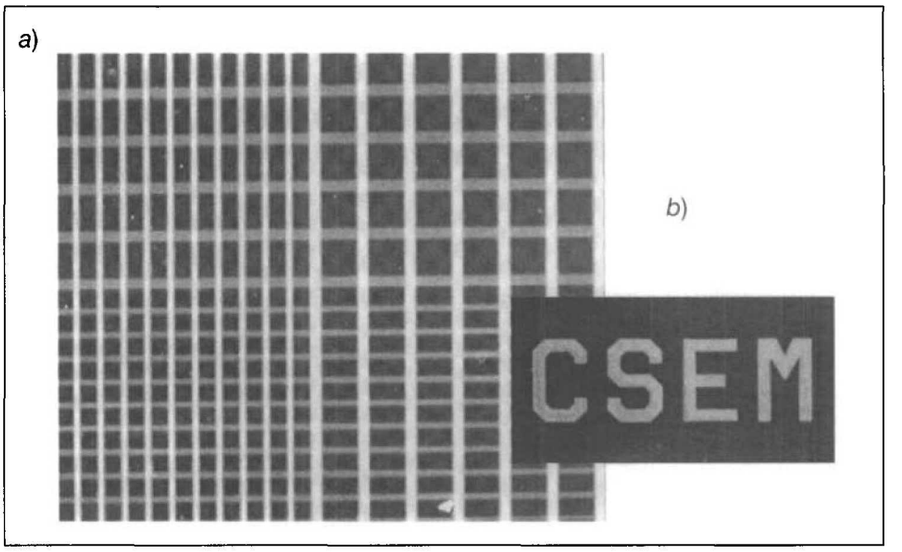

Fig. 4. *Mask-assisted biopatterning.* *a*) Overlay-photopatterned lines of fluorescein-labeled serum albumin and rhodamine-labeled serum albumin on glass. Fluorophore-labeled serum albumin was mixed with the photolinker polymer T-BSA, dried on acid-treated AF glass and exposed to the collimated radiation of a mercury lamp (*Oriel* Xe/HgXe Arc lamp, irradiance 11 mW/cm^2). After photobonding of the first fluorophore-labeled protein, the process was repeated with a rhodamine-labeled serum albumin, setting the mask perpendicular to the first pattern. In both lithographic processes, the stencil lines (10 μm and 5 μm) were separated by 40-μm and 20-μm bridges, respectively. *b*) Photolithographic bioprinting of 40-μm letters on glass. The CSEM logo was 'written' with a fluorescein-labeled BSA/T-BSA 1:1 mixture as detailed in *a*) and recorded by fluorescence microscopy.

tures, or interphasing dextran on electrochemical and optical sensor platforms.

4. Future Tools to Satisfy User Needs

The beneficial effects of technological advancement and globalization are associated with the increased exposure of living organisms to multitudes of agents that may interfere with the user's welfare and health. A plurality of convenience products and processed food for daily use and consumption, increasingly stimulate the intrinsic defense mechanisms in man and animals. The user and service institutions thus appreciate the availability of cost-effective tools that provide fast and reliable prognostic information on health status, food quality and composition. Current biosensor systems are able to detect and quantitate bi-molecular binding events. It is most probable that in the near future, biosensors will be essential tools of daily life. This prospect demands biosensor systems with improved sensitivity and long-term stability, decreased cost per mass-produced item, and simple device manipulation.

Molecular characteristics of biological interactions that regulate biosystems will be increasingly integrated in 'smart' biosensor systems: A 'smart' biosensor system features structural and functional control of vectoriality of the sensing biomolecule. It further includes mastering the complexity of biosystems and bio-amplification (signal amplification by the appropriate choice of bioconstituents). On the instrument level, a major driving force is the development of compact sensor systems that are small and easy to use. Public services and end-users (*e.g.*, medical and veterinarian offices) demand accurate and cost-effective systems. Test results provided by successful biosensor systems must be available within a time frame that meets the timing of the process in question. Moreover, systems designed for medical diagnosis and approved food analysis demand sophisticated system engineering, intrinsic referencing and quantitation in order to exclude false-positive results. Finally, the result of biomolecule-based detection is optionally reported to the user in a format that is easy to interpret and answers the relevant questions. Such biosensor systems represent parts of novel health-control concepts. They are applicable for the detection of additives or (infectious) contamination of food products, and for prognostic health care.

In a competitive market, the end-user prefers consumer goods that are controlled with respect to harmful components. Information on the geographical origin of the product or the processes applied to make the product is certainly of minor importance. Current biosensor activities at CSEM focus on the topics of food analysis and the manufacturing and improvement of prognostic and diagnostic tools. Application-specific biosensor systems are developed to properly answer and meet the end-user's questions and requests.

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