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The ligand-receptor interactions based on silicon technology

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ABSTRACT

Characterization of the extraordinary complexity of ligand-receptor interactions, represent one of the prominent goals in today biological research. To approach this theme, we explored the use of porous silicon (pSi) technology for the construction of a biotechnological device, in which the ligand-receptor interactions are revealed by means of laser optical measurements. Here we report the settling of chemical procedures for the functionalization of the silicon wafers and for the subsequent anchoring of biological molecules such as a purified murine monoclonal antibody (UN1 mAb), an antibody anti-P8 protein of M13 phage and an antibody anti-A20 murine lymphoma cell line. The optical analysis of the interaction on the biochips between the bound biomolecules and their corresponding ligands indicated that the pSi is suitable for this application.

KEYWORDS: silicon biochip, chemical functionalization, fluorescence labelling, M13 phage, monoclonal antibody, protein-protein interaction

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ABBREVIATIONS

APTES, Aminopropyltriethoxysilane; a.u., absorbance units; cSI, crystal silicon; FITC, fluorescein isothiocyanate; FT-IR, Fourier transformed infrared spectroscopy; GA, glutaraldheyde; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mAb, monoclonal antibody; ON, overnight; OSA, optical spectra analysis; PA, protein A; PBS, phosphate buffer saline; pSI, Porous silicon; RT, room temperature; SIO, silicon oxide

INTRODUCTION

The extraordinary complexity of ligand-receptor interaction limits our knowledge of the molecular mechanisms of tumorigenesis and is a major obstacle in developing cancer-specific therapeutic agents. Pioneer large-scale analysis of protein complexes has revealed that a given protein can physically associate to tens (>200) of different proteins [1]. In the case of eukaryotic cells, 20,000 proteins or more may establish an extraordinary large number of functional interactions with cell receptors, making unrealistic a systematic approach where each ligand-receptor interaction is studied as a single network. To overcome these hurdles, we dedicated to a comprehensive analysis of ligand-receptor interactions in mammalian cells [2, 3], developing a novel biochip based on the chemical and optical properties of porous silicon [4]. In this device, the

ligand-receptor interaction is transduced, at high levels of specificity and sensitivity, in an optical signal generated by change of pSI refractive index [5-7]. The strategy includes the chemical functionalization of the silicon surface by using bifunctional linker molecules; the anchoring to the functionalized surface of specific antibodies; the biochip activity assay by evaluating its ability to reveal the corresponding antibody-ligand interaction.

Silicon wafer chemical functionalization

The binding of an antibody to the biochip was achieved through the specific molecular interaction between the antibody and protein A (PA). We adopted the following procedure in order to bind protein A to the wafer. pSI or crystal silicon (cSI) surface was functionalized by a first step oxidation process, performed at 900°C for 15 minutes, that produced a layer of about 80 nm of silicon oxide (SIO). In the second step, the oxidized wafer was treated at room temperature (RT) for 30 minutes with a water solution containing 5% of APTES (aminopropyl-triethoxysilane) in 50% methanol, followed by several washes in 50% methanol and drying under a stream of nitrogen. The wafer was then heated on a heating plate at 100°C for 10 minutes. Figure 1A shows the corresponding schematic chemical reaction occurring in the first two steps.

In the third step, the chip was treated at RT for 30 minutes with a solution of 2.5% Glutaraldehyde (GA) in 20 mM HEPES, pH 7.4. The chemical bond APTES-GA was then stabilized by incubating the supports at RT for 2 hours in 50 mM $NaCNBH_3$ in 10 mM NaOH (Figure 1B). The bifunctional GA linked to the surface was then available for the subsequent linkage of PA. 2 mg/ml of commercial recombinant PA from *Staphylococcus aurens* in 0.1 M sodium carbonate, pH 9 were incubated overnight (ON) at 4°C with the functionalized wafers [8]. After extensive washes of the biochips surface with water, specific antibody was bound to the PA ON at 4°C at a concentration of 2 mg/ml in 1x PBS (Figure 1C).

Interaction between a monoclonal antibody and its ligand peptide

The progressive functionalization steps of the porous silicon biochip were analyzed by ellipsometry, optical measurements and FT-IR spectroscopy (not shown). As reported in Table 1, each chemical treatment induced the formation of

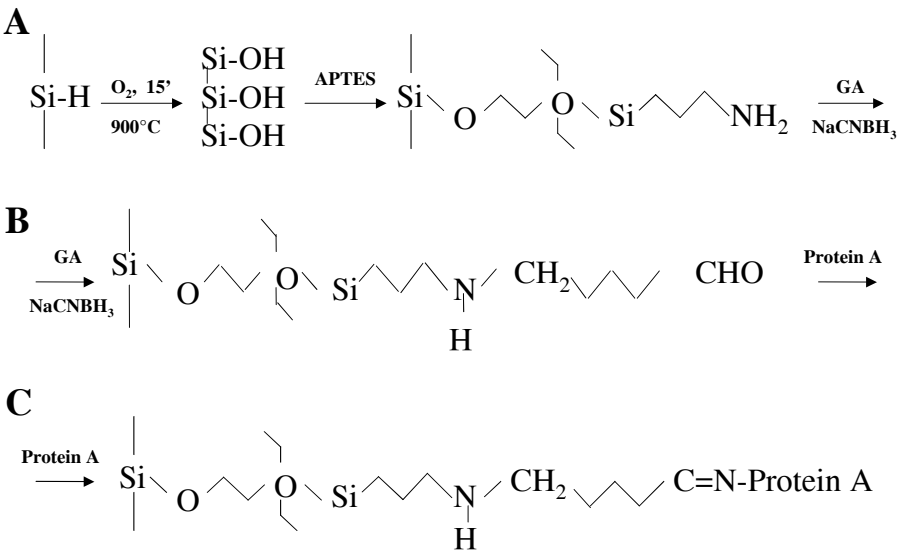

Figure 1. Reaction scheme of the porous silicon chemical functionalization process.

Table 1. Thickness of the biochip chemical layer after functionalization steps.

Layer	Thickness (nm)
IgG	6.18
PA	0.48
GA	2.31
APTES	2.95
SIO	74.91

a chemical layer with a total increase of the surface thickness of about 85 nm.

The fabrication process of the biochip was also monitored by fluorescence intensity measurements starting from PA treatment step using a fluorescence macroscope (Z16 APO) equipped with a high precision 100 W Hg lamp. Leica QWIN V3 software allowed image computation. Each reaction step was carried out using fluorescent biomolecules labelled with fluorescein or rhodamine (fluorescein green, FITC, λ_{ext}, 492 nm, λ_{em}, 520 nm; rhodamine red, λ_{ext}, 570 nm, λ_{em}, 590 nm), thus allowing determining their chemical anchoring to the biochip surface. The antibody used was UN1, a murine monoclonal antibody (UN1 mAb) previously selected for the specific reactivity with human thymocytes as compared to peripheral blood cells [9, 10]. The antigen recognized by UN1 mAb is a 100-120 kDa transmembrane glycoprotein showing biochemical features of cell membrane-associated mucin-like glycoproteins, a class of macromolecules that are involved in cell-to-cell interactions and cancer progression [11, 12]. UN1 mAb interacts at high affinity with a specific 23mer peptide, G23 (SFAATPHTCKLLDECVPLWPAEG), with a k_d = 0.155 ± 0.003 μM. To determine the optimum concentration of UN1 mAb binding to the biochip, an antibody titration uptake was performed. As reported in Figure 2, the uptake of UN1 mAb on the biochip, evaluated by OSA (Figure 2A) and fluorescence measurements (Figure 2B), increased at increasing concentration of antibody and reached a plateau at a concentration of about 1.0 mg/ml corresponding to 6.8 μM. In addition, reported in Figure 2C, the amount of fluorescence intensity of labeled G23 bound to the biochip increased

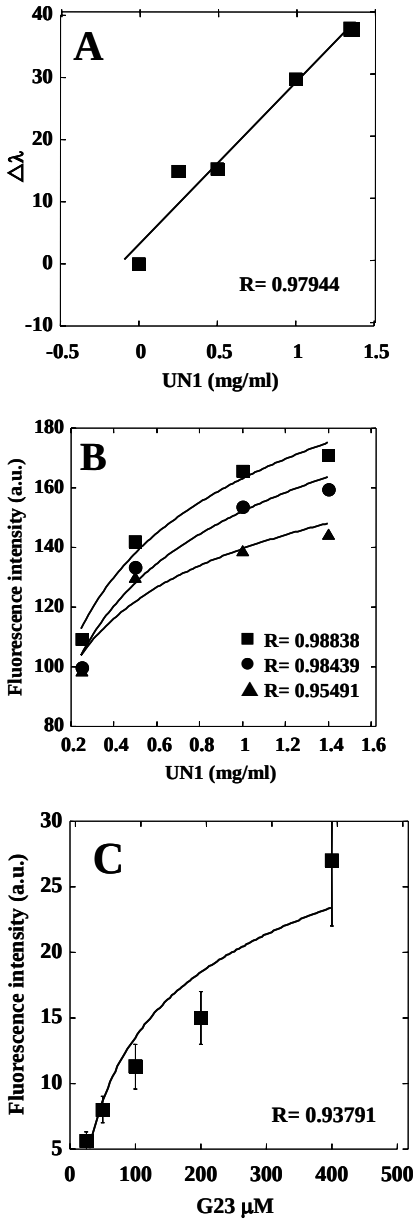

Figure 2. Titration of the binding of UN1 mAb to the PA-functionalized biochip. A) Average difference of the refractive index (△λ) of pSI at increasing concentration of UN1 mAb. B) Fluorescence intensity of the biochip at increasing concentration of UN1 mAb, (■) no-wash, (●) wash, (▲) dialysis. C) Fluorescence intensity of the biochip at increasing concentration of labelled G23. 150-200 μg of antibody (or peptide) in 0.1 M $NaHCO_3$ were mixed with 5-10 μl of rhodamine (0.5-1 μg in 20 μl DMSO) in a final volume of 100 μl and incubated for 1 hour at RT. The labelled molecule was then purified by Sephadex G50 gel filtration (0.5 x 5.0 cm spin-down column at 3000 rpm for 30 seconds).

proportionally to the increase of the peptide concentration up to about 400 μM.

The UN1 mAb concentration determined for the saturation of biochip was then used to test the ability of the biochip to bind its ligand peptide, G23. In Figure 3 are reported the fluorescence images of the biochips during its fabrication and monitored by using for each step the corresponding labelled biomolecules (Figure 3A-3C). The fluorescence intensity observed appeared to decrease with the progression of the biochip construction steps. This behaviour was probably due to a concomitant reduction of the binding efficiency of the biochip in terms of yield. Nevertheless, the results obtained strongly indicated that the biochip is able to reveal the specific interaction antibody-ligand (UN1 mAb-G23).

Interaction between antibody anti-P8 M13 protein and M13 phage on silicon wafer

The strategy is directed toward the fabrication of a new system-array for the screening of random peptide libraries (RPL) displayed on phages to identify novel peptide ligands of highly polymorphic (>10E11) receptor such as B cell (BCR) and T cell receptor (TCR) complexes of B and T cells, respectively. To fulfill this task, we started to build a system able to detect the molecular interaction between the antibody anti-P8 M13 phage protein and the M13 phage. Having settled the condition to construct a PA-based pSI-biochip, we applied this fabrication procedure to crystalline silicon. Therefore, the antibody anti-M13 phage was bound to PA-functionalized cSI biochips at a concentration 6.8 μM, ON at 4°C. In order to eliminate nonspecific binding of the phage to the silicon support, before the incubation with the phage solution, the biochips were passivated by incubation with a solution of 5% nonfat dried milk in 1x PBS at RT for 1 hour. As negative control, we used UN1 mAb that does not bind to the M13 phage. As reported in Figure 4, the M13 phage, labelled with FITC, did bind to the biochip containing the anti-P8 phage antibody (AbAnti-P8) whereas it did not bind to the biochip containing UN1 mAb and to the non-functionalized biochip treated with anti-P8 phage antibody. Evaluation of fluorescence intensity (a.u.) at macroscope showed a high value only in the case of biochip A (chip A, after washes: 32.81, after dialysis: 23.53; chip B, after washes: 25.85, after dialysis: 3.0; chip C, after washes: 15.6, after dialysis: 3.0). Also the ellipsometric analysis of the samples reported in Table 2 indicated a higher increase of the biochip thickness only in the case of wafer A thus indicating the effective anchoring of the M13 phage to the biochip throughout its interaction with antibody anti-P8.

Immobilization on the silicon wafer of a murine lymphoma cells

We next explored the application of the functionalized biochip for its capability to bind a cell line. As model, it was chosen a murine lymphoma cell line (A20) expressing high levels of membrane IgG. For this purpose, an antibody anti-membrane immunoglobulins was bound to the functionalized biochip as previously described. The biochip was then incubated in the presence of A20 cells at a concentration of 5Exp6 cells/ml in 1 x PBS at RT for 2 hours. After several washes in 1 x PBS, the wafers were analyzed under a light microscope (Leika DM6000M). As experimental control it was used a biochip that was incubated with a cell line that

Table 2. Thickness of biochips during fabrication steps.

Chip	SIO (nm)	APTES+GA (nm)	PA+Ab+M13 phage (nm)	$\chi 2$
A	72.06±0.09	1.33±0.07	5.35±0.06	0.5
B	72.56±0.09	2.10±0.09	2.35±0.05	0.4
C	75.11±0.06	No treatment	0.47±0.03	0.4

$\chi 2$ is a measure of the deviation of sample from expectation.

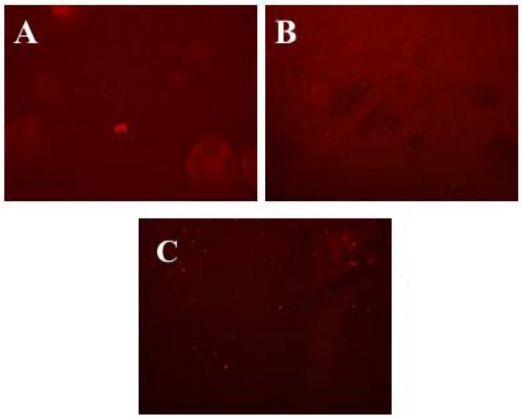

Figure 3. Fluorescence images of biochips after binding of labelled biomolecules. A) biochip after treatment with rhodamine-labelled protein A. B) biochip after incubation ON at 4 °C with rhodamine-labelled UN1 mAb (1mg/ml in 1 x PBS, pH 7.5). C) biochip after incubation for 2 hours at RT with rhodamine-labelled peptide G23 (200 μM in 1 x PBS, pH 7.5).

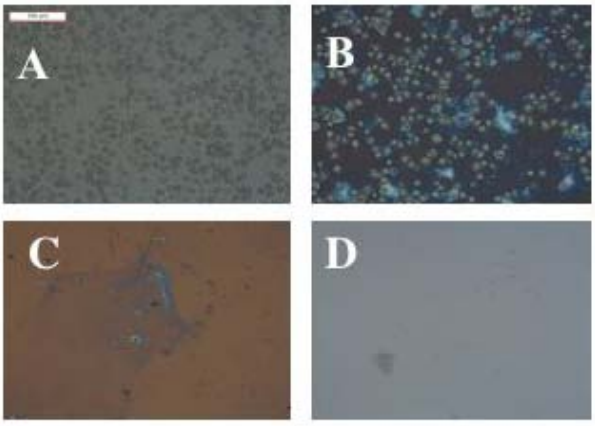

Figure 5. Binding of murine lymphoma cells (A20) to the silicon biochip. Optical images of the functionalized biochips treated with antibody anti-IgG (6,8 μM) and then incubated with A20 cells. (A) Before washes and (B) after washes (20 x enlargement). (C) Optical image after washes of the biochip functionalized with anti-IgG and incubated with CB1 cells. (D) Optical image after washes of the biochip lacking anti-IgG and incubated with A20 cells.

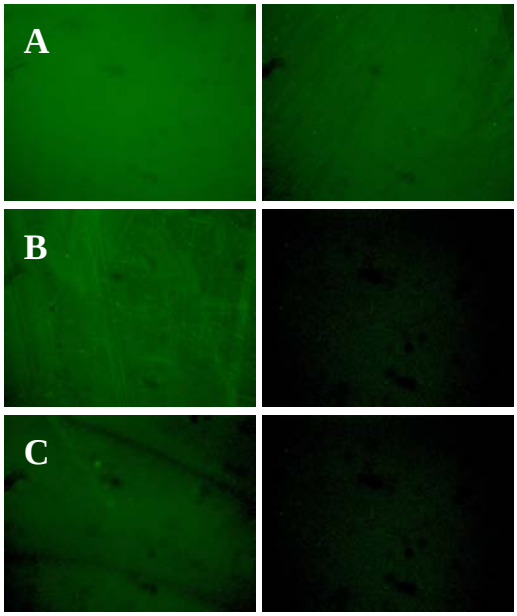

Figure 4. Fluorescence images of biochips after the interaction with labelled M13 phage. FITC labelled M13 phage was incubated with differently functionalized biochips according to the following schemes: A) SIO + APTES + GA + PA + AbAnti-P8; B) SIO + APTES + GA + PA + UN1 mAb; C) SIO + PA + AbAnti-P8. Left panels, biochips after washes with 1 x PBS; right panels, biochip after extensive dialysis against 1 x PBS.

does not express membrane IgG (CB1) and a biochip lacking the antibody anti-IgG. As reported in Figure 5, the IgG-functionalized biochip was able to bind specifically the A20 cells (Panels A and B, Figure 5) whereas it did not recognize different cells (Panel C, Figure 5). In addition, A20 cells did not show non-specific binding to the biochip lacking antibody anti-IgG (Panel D, Figure 5) thus suggesting the versatility of the engineered biochip.

CONCLUDING REMARKS

In this work, we describe the use of the silicon technology in order to construct a biochip that allows the identification of specific ligand-receptor interactions. The micro-fabricated biochips we tested appear to be suitable to reveal several specific bindings such as that between a) an antibody and its ligand peptide, b) an antibody and a recombinat phage expressing an antibody ligand epitope and c) cell surface proteins and corresponding specific antibody. On the bases of the results obtained, we further plan to use this specific technology for anchoring phage virions to silicon surface and to select for the recognition of the phage virions by specific

T hybridoma cells. Once the system has been validated, the application of this technology will be expanded studying crude T cell populations obtained from different sources (i.e. tumor infiltrating lymphocytes and tissue target of autoimmune diseases).

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