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Surface functionalization of germanium ATR devices for use in FTIR-biosensors

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ABSTRACT

Biosensors based on intrinsic detection methods have attracted growing interest. The use of Fourier transform infra-red (FTIR) spectroscopy with the attenuated internal total reflection (ATR) mode, in the biodetection context, requires appropriate surface functionalization of the ATR optical element. Here, we report the direct grafting of a thin organic layer (about 20 Å depth) on the surface of a germanium crystal. This covering, constructed with novel amphiphilic molecules **2b** (namely, 2,5,8,11,14,17,20-heptaoxadocosan-22-yl-3-(triethoxysilyl) propylcarbamate), is stable for several hours under phosphate buffered saline (PBS) flux and features protein-repulsive properties. Photografting of molecule **5** (namely, O-succinimidyl 4-(*p*-azidophenyl)butanoate) affords the activated ATR element, ready for the covalent fixation of receptors, penicillin recognizing proteins BlaR-CTD for instance. The different steps of the previous construction have been monitored by water contact angle (θ_w) measurements, spectroscopic ellipsometry (covering depth), X-ray photoelectron spectroscopy (XPS) by using a fluorinated tag for the control of surface reactivity, and FTIR-ATR spectroscopy for the structural analysis of grafted molecules. Indeed, contrarily to silicon device, germanium device offers a broad spectral window (1000–4000 cm^{-1}) and thus amide I and II absorption bands can be recorded. This work lays the foundations for the construction of novel FTIR biosensors.

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1. Introduction

Label free detection of "ligand–receptors" interaction at the surface of a device has become the subject of growing interest, in connection with the development of biosensors [1,2]. Amongst the reliable detection methods [3–5], surface infrared spectroscopy (FTIR-ATR, Fourier transform infrared using the attenuated total internal reflection mode) offers the advantage of coupling the detection with the collect of structural information about interacting partners [6,7]. We have already described a new type of biosensor, called BIA-ATR [8], based on a functionalized ATR germanium crystal and the detection of "ligand–receptor" interactions in aqueous media, at the water–device interface, by FTIR spectroscopy. For instance, the ATR element was hydrophobized by reaction with octadecyltrichlorosilane (OTS) under adapted wet-chemistry conditions and then coated with phospholipid membranes fragments containing a few percents of phosphatidylserine as receptors. This device allowed the specific detection of coagulation factor VIII in the presence of a large excess of other proteins [9].

We are now interested in the covalent grafting of receptors (mainly proteins) on the surface of ATR elements in view to detect low molecular weight ligands. The mostly used ATR crystals are made of silicon; the material is quite inexpensive and the surface chemistry of silicon has been widely described [10]. However, a problem of silicon, not encountered with germanium, is its opacity below 1550 cm^{-1} [11]. Yet, the fingerprint region of organic molecules below 1550 cm^{-1} is essential for univocal structure assignment. Therefore, BIA-ATR biosensor includes preferably a germanium optical element. Due to the cost of germanium and its enhanced sensitiveness comparatively to silicon *versus* surface degradation [12–14], the crystal preparation for the covalent anchorage of a biomolecule of interest has been carefully examined.

The requirements of this organic covering are as follows: (i) easiness and reproducibility of the surface chemistry; (ii) receptor (biomolecule) fixation *via* a tunable functionalization method; (iii) selectivity *versus* the (non-covalent) adsorption of biomolecules; (iv) stability *versus* chemical hydrolysis under physiological condi-

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tions (phosphate buffered saline (PBS) for instance); (v) simple conditions of storage and multi-use.

In this paper, we describe the design, the synthesis, and the validation of an organic layer which meets all the above conditions. Our strategy has been first established on silicon ATR elements, and then successfully applied to germanium. The different steps of the construction were surface-analyzed by X-ray photoelectron spectroscopy (XPS), while the stability and selectivity were assayed by FTIR-ATR analysis.

2. Materials and methods

2.1. Materials

ATR crystals were silicon or germanium internal reflexion elements (IRE) ($50 \times 20 \times 2$ mm^3) with an internal incidence angle of 45° (ACM, Villiers St Frédéric, FR).

The molecular clip **5** (O-succinimidyl 4-(*p*-azidophenyl)butanoate) was prepared according to reference [15]. PEG molecules **1a** and **1b** (2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane and trichlorosilane, 90%) were purchased from ABCR (Germany). Complete procedure for the preparation of PEG molecule **2b** and its spectroscopic characterization are provided as supplementary material.

Phosphate-buffered saline (PBS) solution at pH 7.4 was prepared from NaH_2PO_4 (0.26 g), and Na_2HPO_4 (1.146 g) in MilliQ water (100 mL) containing 0.9% NaCl.

BlaR-CTD protein, generously furnished by Prof. Bernard Joris (Centre d'Ingénierie des protéines, Université de Liège, Belgium), was used in a concentration of 300 µg/mL in PBS at pH 8 (20 mM NaH_2PO_4 and 250 mM NaCl).

2.2. Methods

Advancing contact angles of MilliQ water drops (θ_w) were measured using a custom-built gionometer with an analogue camera as described in reference [8].

The thickness of the grafted layers was determined by spectroscopic ellipsometry. The measurements were carried out with a GESP5 rotating polarizer instrument operated in scanning mode, with a spectral resolution of 5 nm. The ellipsometer was operated in parallel beam configuration at three angles of incidence: 65°, 70° and 75°. The polarizer rotated at 9 Hz and the analyzer angle was adjusted at each wavelength, according to a tracking procedure.

XPS spectra were recorded on a Kratos Axis Ultra spectrometer (Kratos Analytical, Manchester, UK) equipped with a monochromatized aluminium X-ray source (powered at 10 mA et 15 kV). The pressure in the analysis chamber was around 10^{-6} Pa. The angle between the normal to the sample surface and the lens axis was 0°. The hybrid lens magnification was used with the slot aperture and the iris drive position set at 0.5 resulting in an analysed area of 700 µm × 300 µm. The constant pass energy of the hemispherical analyser was set at 40 eV. In these conditions, the energy resolution gives a full width at half maximum (FWHM) of the $Ag_{3d_{5/2}}$ peak of about 1.0 eV. Charge stabilization was achieved by using the Kratos Axis device. The binding energies were calculated with respect to the C–(C, H) component of the C_{1s} peak fixed at 284.8 eV. Data treatments were done with the CasaXPS program (Casa Software Ltd, UK) with a Gaussian/Lorentzian (70/30) product function and after subtraction of a linear baseline.

FTIR-ATR spectra were obtained on a Bruker IFS 55 FTIR spectrophotometer (Ettlingen, Germany) equipped with a MCT detector (broad band 12000–420 cm^{-1}, liquid N_2 cooled, 24 h hold time) at a resolution of 2 cm^{-1} with an aperture of 3.5 mm and acquired in the double-sided, forward-backward mode. The experimental details were described in reference [8]. The software used for data processing was written in MatLab 7.1 (Mathworks Inc, Natik, MA).

2.3. Surface chemistry

Silicon crystals were washed with chloroform (2×5 min) under sonication, and then submitted to UV-ozone treatment (2×10 min, on each face). Oxidation was performed with 95% H_2SO_4/30% H_2O_2 (70/30, v/v) during 10 min at 110–130 °C. The devices were abundantly rinsed with MilliQ water and dried under N_2 flux (17 h).

Germanium crystals were washed with 38% HNO_3 (1 min) and rinsed with MilliQ water. Oxidation was performed with 99+% oxalic acid/35% H_2O_2 (10/90, v/v) during 5 min at 20 °C, and washing with MilliQ water. This was repeated three times. The devices were dried under N_2 flux (17 h).

The grafting protocol of silanes (**1a**, **1b**, **2b**) was the same for Si–OH and Ge–OH surfaces. The crystals were immersed into 2% PEG solutions in CCl_4, under N_2 atmosphere, during 3 h at reflux. The crystals were washed with methanol (2 h) and with tetrahydrofuran (2 h) in a soxhlet apparatus. The resulting surfaces are named Si-PEG **1a**, Si-PEG **1b**, Si-PEG **2b** and Ge-PEG **1a**, Ge-PEG **1b**, Ge-PEG **2b**, respectively.

The photografting of azide **5** on Si-PEGs and Ge-PEGs was performed similarly. A solution of molecular clip **5** in benzene (5 mg/1.5 mL) was sprayed on the device and the solvent was evaporated under air flux in the dark (deposition of 0.1 to 0.2 mg **5**/cm^2). The device was submitted to UV irradiation during 2 h at room temperature (3 lamps of 8 W and λ_m 254 nm, placed at a distance of 10 cm). The device was rinsed with THF (10 min) and $CHCl_3$ (5 min) under shaking at 20 °C (Edmund Bühler stirrer, model KL-2, 150 rpm). The resulting surfaces are named Si-PEG-NHS and Ge-PEG-NHS. The blank sample was similarly prepared, but with omitting the irradiation.

The covalent coupling of the amine **6** (fluorinated tag = 3,5-*bis*(trifluoromethyl)benzylamine) was performed similarly on Si-PEG-NHS and Ge-PEG-NHS. The device was immersed into a solution of **6** in CH_2Cl_2 (0.2 g/250 mL), under argon atmosphere for 2 h, at reflux. The device was rinsed with CH_3CN (2×10 min) at 20 °C under shaking (150 rpm). The resulting surfaces are named Si-PEG-F6 and Ge-PEG-F6.

2.4. Fixation/adsorption of proteins

A solution containing the receptor (BlaR-CTD protein) was passed over the ATR crystal for 3 h with a discontinue flow of 20 µl/min speed. A total volume of 200 µL was needed for the covalent binding of BlaR-CTD on Ge-PEG-NHS. Then the covalent binding was stabilized by passing the PBS solution for 30 min with a continue flow of 20 µl/min speed. A spectrum was recorded every 30 min during the binding, then every 10 min for the stabilisation. In this work, the spectra of biological samples were registered under medium flow, thus with an excess of water. This contribution of the medium was eliminated by subtracting of the medium spectra by a coefficient 1 (100%) for all the registered spectra.

3. Results and discussion

3.1. Design and synthesis of the organic reagent

Polyethylene glycols (PEGs) are widely used to reduce non-specific adsorption of biomolecules (proteins) on surfaces [16,17]. When chemical grafting is concerned instead of coating, oligomers of ethylene oxide (chains containing 3 to 9 EO units) are also efficient [18–20]. Surface treatment of glass and metal oxides is

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