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# Optimization and characterization of a homogeneous carboxylic surface functionalization for silicon-based biosensing

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ABSTRACT

A well-organized immobilization of bio-receptors is a crucial goal in biosensing, especially to achieve high reproducibility, sensitivity and specificity. These requirements are usually attained with a controlled chemical/biochemical functionalization that creates a stable layer on a sensor surface. In this work, a chemical modification protocol for silicon-based surfaces to be applied in biosensing devices is presented. An anhydrous silanization step through 3-aminopropylsilane (APTES), followed by a further derivatization with succinic anhydride (SA), is optimized to generate an ordered flat layer of carboxylic groups. The properties of APTES/SA modified surface were compared with a functionalization in which glutaraldehyde (GA) is used as crosslinker instead of SA, in order to have a comparison with an established and largely applied procedure. Moreover, a functionalization based on the controlled deposition of a plasma polymerized acrylic acid (PPAA) thin film was used as a reference for carboxylic reactivity. Advantages and drawbacks of the considered methods are highlighted, through physico-chemical characterizations (OCA, XPS, and AFM) and by means of a functional Protein G/Antibody immunoassay. These analyses reveal that the most homogeneous, reproducible and active surface is achieved by using the optimized APTES/SA coupling.



## 1. Introduction

Several biological applications, especially those related to biosensors and bioassays, depend on the immobilization of a particular biosensing element, such as an enzyme, a receptor, or an oligonucleotide probe, able to detect a particular analyte with high specificity and sensitivity [1]. Many efforts have been spent in the last decades to lower the detection limits, to increase the signal to noise ratio (S/N), challenging tasks related to biomolecules immobilization, and finally to reach the single molecule detection [2]. In order to match these requirements, the immobilization of biorecognition elements should be the most efficient, with a high and stable surface coverage, preserving the chemical and biological activity of the components [3].

The most commonly employed methods of immobilization of proteins and oligonucleotide probes are physical adsorption [4], covalent bonding [5], cross-linking [6] and entrapment [7]. Physical adsorption is usually a cheap and fast method, but it does not ensure the retention of the proper folding and/or activity of the sensing element, even if this process is regarded as a mild immobilization method [3]. Furthermore, adsorption can be reversible, resulting in a small surface loading, which can provide bad sensitivity and reproducibility [8]. Another technique is based on the entrapment of biospecies by means of different kinds of hydrogels or simply membranes [7,9]. Usually these kinds of immobilizations preserve the appropriate structure and activity of proteins and probes, but suffer from mass transfer limitations and long preparation protocols [10,11]. Covalent bonding and cross-linking are usually applied to reach a stable and effective immobilization of biological species, especially of enzymes and antibodies [3,6]. Generally, the surface of the sensor is coated with a functional layer that exposes a particular functional group (carboxylic acid, amine, etc.): the biomolecules can be directly coupled to the substrate [12], or can be grafted through cross-linking agents, such as glutaraldehyde (GA) [6,13–15], or a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxy-succinimide (NHS) [16–19]. This is particularly relevant for silicon substrates, a very attractive

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material for biosensing because of its ease of coupling biological systems to microelectronics [20–22].

The silicon surface is typically oxidized, modified by silanization with organosilanes such as 3-aminopropyltriethoxysilane (APTES), and eventually further derivatized [23–25]. This kind of surface modification is extremely versatile and the nucleophilicity of the amine group ($-NH_2$) is exploited in the production of different composites and not only for biomolecules surface grafting [26,27]. Unfortunately, this kind of aminosilane often creates a disordered layer on the surface, because of the many possible ways for it to interact with surface silanol/silanolate groups and because of its tendency to polymerize in the presence of water. As a matter of fact, this polymerization gives rise to multilayers, due to horizontal and vertical polymerization, and to an uncontrolled clustering of organosilane molecules on the surface [24]. This effect can be further amplified by using two-step protocols involving homo-bifunctional cross-linking agents such as GA. This reactant frequently produces polymeric structures due to intra/inter-molecular reactions easily occurring in aqueous solution. The resulting unstable 3D matrix usually increases the variability of the biomolecule immobilization and functionality [28,29].

A different approach to obtain a stable and reproducible functionalization is based on the controlled deposition of polymeric conformal coatings obtained by means of spin-coating [30] or by plasma-polymerization [31]. These functionalization methods, well-known in the domain of biomedical applications, show advantages such as the room temperature operation and the low amount of wet chemicals used during the surface modification treatments, but they require a long and tedious optimization of the controlling parameters: for the former, the spinning centrifugal force (rpm) and time, while for the latter, the input power and wave modulation, the precursor/gas carrier ratio, working pressure, flow rate, etc. Nevertheless, after the surface functionalization, the grafting capability depends on many different aspects, such as time-dependent degradation (e.g. oxidation), pH and ionic strengths of working solutions; so that an activation/re-activation of the functional groups, for instance by means of EDC/NHS is often required [17,19].

Recently, a new functionalization, rarely applied to the immobilization of biomolecules and based on succinic anhydride (SA), was reported. The chemical reaction of the SA with the amine termination of APTES, already grafted to a silicon surface, can be exploited to introduce carboxylic functionalities that can be successfully used to immobilize biological species. Very few applications of this surface modification are found in literature and most of them are based on protocols starting from a non-optimized APTES functionalization of silica surface and followed by SA derivatization in organic solvents. The whole procedure is highly time consuming, given that it lasts at least 24 h [16,32–34]. Furthermore, some steps after APTES reaction, such as additional washing, heating or sonication, are reported.

In this work, an optimized 3 h long APTES/SA protocol is presented and compared to the well-known surface chemistry functionalizations based on APTES/GA and PPAA, in terms of homogeneity, reproducibility and chemical stability. The physico–chemical properties of APTES/SA functionalization were investigated by Optical Contact Angle (OCA), Atomic Force Microscopy (AFM) and X-ray Photoelectron Spectroscopy (XPS) and the related results are compared to those obtained for the other modified surfaces. In order to attain high quality results, both the functionalization and the EDC/NHS activation were optimized, paying a particular attention to the maximization of the S/N. Moreover, the surface density of immobilized molecules was estimated by means of a Protein G/Antibody immunoassay, highlighting the improvements in terms of molecular biorecognition given by the optimized APTES/SA protocol.

## 2. Experimental

### 2.1. Materials and chemicals

See Supplementary Data.

### 2.2. Silicon samples preparation and silanization

Squared silicon samples ($5 \times 5\,mm^2$) were functionalized through an APTES silanization [25] (APTES samples), as reported in Supplementary Data (Fig. S1). Briefly, freshly cleaned substrates were incubated with 1% (v/v) solution of APTES in anhydrous toluene at 70 °C for 10 min, rinsed several times with toluene and dried in $N_2$ stream.

### 2.3. APTES samples modification through GA and SA

In order to introduce an aldehydic functionality (Fig. S1c), APTES samples were modified following a previously reported protocol [25], as described in the Supplementary Data. Shortly, samples were incubated at room temperature in a 0.5% (v/v) GA solution in 100 mM Borate Buffer Saline (pH 8.5) for 1 h. $NaBH_3(CN)$ solution was added to a final concentration of 50 mM after 15 min of incubation. These substrates are referred hereafter as APTES/GA samples.

In order to introduce a carboxylic functionality (Fig. S1d), two different methods were tested. Starting from the one reported by Lue et al. [34], APTES samples were incubated at room temperature in a solution made of 18 mg/mL SA dissolved in DMF for 30 min. After the reaction, the substrates were deeply rinsed with DMF, cured for 2 min in boiling water, washed with ethanol and finally Milli-Q™ water. The functionalized samples were dried under a $N_2$ stream and stored in a sealed desiccator until needed. These substrates are referred hereafter as APTES/SA1 samples. At the same time, by modifying Kim et al. [16], APTES samples were incubated at room temperature in a solution made of 5 mg/mL SA and 5% (v/v) TEA dissolved in THF for 2 h, unless differently stated. After the reaction, the substrates were thoroughly rinsed with THF, ethanol and finally Milli-Q™ water. The functionalized samples were dried under $N_2$ stream, and stored in a sealed desiccator until needed. These substrates are referred hereafter as APTES/SA2 samples.

### 2.4. Silicon PPAA functionalization

The thin film deposition of PPAA on squared silicon samples was performed in a Low Pressure Plasma Enhanced CVD reactor (ION-VAC PROCESS S.r.l., Pomezia, Italy) (Chamber Base Pressure = 28 mTorr; RF = 13.56 MHz). The system is equipped with a delivery frame, allowing the injection of organic vapors coming from liquid reactants stored in quartz reservoirs intercepting the carrier gas lines. Acrylic acid vapors (vapor pressure = 3.1 Torr at 20 °C) are produced by bubbling argon (flow = 20 sccm) in the liquid acrylic acid. Argon acts as gas carrier and is needed to sustain the plasma discharge. The mixture of acrylic acid vapors and argon is uniformly admitted into the reactor by the upper showerhead electrode. Polymerization was performed by a pulsed plasma discharge and obtained by applying a RF power of 200 W with a duty cycle of 10% ($t_{on}$ = 10 ms, $t_{off}$ = 90 ms); a deposition time ($t_{dep}$) of 10 min that corresponds to a film thickness of ~80 nm, as measured by AFM on a flat silicon substrate. After deposition, the samples were soaked (30 min) in Milli-Q™ water to remove unstable surface oligomers formed at the end of the plasma process [31].