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“Versatile toolset” for DNA or protein immobilization: Toward a single-step chemistry

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ABSTRACT

Covalent immobilization of non-modified biological materials as proteins or nucleic acids has been performed through a single and soft method. Based on diazonium salt chemistry, this protocol leads to an ultrathin grafted film, on metallic or polymer materials, which can eventually be used as a self-adhesive primer for immobilizing biological materials from aqueous solutions through a simple dipping step. Moreover, this self-adhesive primer may be patterned by cheap and easy methods as ink or UV masking. Biological models as low molecular weight DNA from salmon sperm and glucose oxidase (GOD) were covalently immobilized by this soft procedure. In order to evaluate the consequences of this non-specific covalent immobilization method on biological activity, enzymatic activity of GOD was monitored by electrochemical detection of hydrogen peroxide (H_2O_2). We thus demonstrate that such a self-adhesive primer represents a new and alternative process offering a versatile toolset for immobilizing biological material for biosensor development on conductive and non-conductive materials.

1. Introduction

Biology as the electronic is following a path of miniaturization and large-scale integration process that will revolutionize biotechnology [1]. These ongoing processes are compiled in devices known as biosensors and/or biochips, including microarrays and lab-on-a-chip microfluidic devices [2–4]. In biosensors the recognition species are either proteins (enzymes, receptors, antibodies, peptides, etc.) [1,5] or nucleic acids (DNA, RNA, etc.) [6,7]. Two key parameters appear to govern the overall performance of biosensors are both the surface chemistry and the method used for biomolecule immobilization.

Nowadays, the microarray material and the type of biomolecules often determine the method used for immobilization. Moreover, the detection method to visualize biological event is conditioned itself by the nature of microarray material [1,5–7]. In order to overcome all these conventional drawbacks, a versatile surface chemistry capable of selective functionalization of raw conductive, semiconductive and insulating materials, with surface density control, and compatible with chemical and biological element immobilization is highly desirable. An obvious need exists to develop a flexible immobilization process or “versatile toolset” to provide strong, stable, and accessible binding of the sensing element (DNA, proteins and peptides) with the same chemistry, thus leading to sensitive and reproducible biosensor performances.

Methods used for biomolecule immobilization are classified into two categories: (i) non covalent attachment which includes sol–gel encapsulation, polymer entrapment and physical adsorption (electrostatic and hydrophobic adhesion) and (ii) covalent attachment [8,9]. Concerning the first category it is noteworthy that three-dimensional supports are generally plagued by problems relating to mass transport effects and high background signals, resulting in false kinetic rate constants. The question of which strategy can be considered as the best one is still open [1,5–7]. Concerning the second category many chemical routes have been explored, the first one consists to only prepare the surface to react with biological materials the second one consists to modify randomly the biological materials in order to react with the surface. The formation of irreversible covalent bonds between biomolecules and reactive groups on the support is one of the most widely investigated methods [8,10]. However, the disadvantages of most covalent attachment schemes are: (i) the surface activation methods may be incompatible with biological

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medium, (ii) the coupling procedures may require harsh conditions or release byproducts which can produce artefacts and (iii) the covalent attachment may be detrimental for the biological activity. The formation of surface functional groups (carboxyl, amino, etc) through various chemistries such as silane on glass or silicon [11,12], alkanethiol monolayers on Au [13] and conducting polymers [14,15] such as modified polypyrrole and polyaniline have been employed to improve surface functionalization and to immobilize biomolecules onto surfaces. Recently a method proposed by Bailey's group use benzophenone-modified substrates to photochemically control covalent coupling of solution-phase biomolecules [16,17].

In this context, using aryldiazonium salts, which substituent may be selected at will, appears attractive because the grafted polyaryl layers obtained from aryldiazonium salts exhibit a very high stability, in particular at high temperature, in harsh solvent conditions, under sonication, limitation of non-specific protein adsorption and may be stored without any damage over several months in air [18,19]. Indeed, diazonium chemistry was already used to attach a variety of biomolecules including DNA, proteins and peptides on conductive or semiconductive materials [8,18,20–24].

There are actually two generic methods used to attach biomolecules with diazonium chemistry. The first one is the chemical modification of the biomolecule with a diazonium group. The modified biomolecule is then used to functionalize surfaces by electrografting a conductive material. This process has two drawbacks: (i) the chemical modification of biomolecules can damage their biological activity and is time-consuming (purification, etc.) and (ii) this process is only limited to conductive materials. The second one is a multistep process which consists to electrograft onto surfaces a diazonium layer bearing a functional group (for example an amino group). Afterwards, the chemical ligation between the biomolecules and this layer can be performed with generic coupling agents such as carbodiimide or aldehyde for example. This process also presents some drawbacks: (i) this process is also limited to conductive materials and (ii) some side products can be generated which can affect the activity of the biomolecule or create artefacts.

Recently, we have shown that redox grafting of diazonium salt provide a very simple way to modify any surface (metallic, semiconductor or insulating) of any complex geometry with robust polyphenylene-like films [25,26]. Chemical reactivity of these layers is used to graft polymers, metallic nanoparticles and MWCNT in one step immobilization procedure [27,28]. Concurrently, an electrochemical process has been reported for the immobilization of Horseradish peroxidase on gold electrode surface [29]. Nevertheless, this protocol presents some drawbacks: (i) the electrochemical reduction of diazonium salt for layer synthesis and (ii) the electrochemical conversion of the nitro to amine groups reduce the support used for biosensor synthesis to conductive or semiconductive materials.

Herein, we present, for our knowledge, the first "versatile toolset" for biosensor synthesis. Based on a purely chemical reduction of diazonium salts, it can be applied for the surface modification of both conductive and insulating materials with a simple, robust, "green" and soft procedure. Afterwards, aryldiazonium layers can react with a multitude of biomolecule functions which lead to covalent bonds between the surface and proteins or nucleic acids. Results based on the use of these self-adhesive surfaces and the grafting procedure concerning different biomolecules as protein (glucose oxidase) and DNA strands are reported, together with preliminary results on the formation of patterned self-adhesive surfaces. In order to confirm the potential of that immobilizing method for biosensing, the biological activity of the immobilized enzyme was also checked.

2. Experimental

2.1. Chemicals

All standard chemicals and solvent were of research grade, purchased from Sigma–Aldrich and used as received. Iron particles were purchased from VWR (98%, mean particle size 45–100 mm). Glucose oxidase from *Aspergillus niger* and DNA from salmon sperm were purchased from Sigma–Aldrich and used as received. Glucose solution BioUltra grade, 20% in H_2O for molecular biology and PBS were purchased from Sigma–Aldrich and used as received. Gold substrates were obtained by successive metallic evaporation of 5 nm of chromium underlayer and 200 nm of gold as top coat on microscopic glass slides. PP (polypropylene) films were purchased from Goodfellow and cleaned before use with water and ethanol under ultrasonic treatment for 10 min.

2.2. Self-adhesive surfaces preparation

The synthesis of self-adhesive surfaces was performed in open air in a simple flask. The grafting of polyaminophenylene (PAP) onto gold and PP surfaces was realized according to the process summarized here and detailed in a previous paper [25]. After addition of sodium nitrite (Eq. (1)) to an acidic solution (HCl 0.25 N, pH 0.3) of *p*-phenylenediamine, iron powder was added to reduce the aryldiazonium salt in solution and start the overall grafting and growing chemical process. Then gold or polymeric substrates were introduced and left in the solution for 15 min to 2 h at 38 °C. Note that the film thickness increased with the reaction time whereas the film properties are the same whatever the film thickness [25,27]. The film thickness was appreciated with the highest IR band intensity as presented in Section 2.5. Afterwards, samples were rinsed with deionized water and acetone in an ultrasonic bath for 5 min. The resulting PAP film on gold was analyzed by FTIR-ATR (Fig. 1b). The diazotisation of the grafted PAP layer on substrates could be performed either in aqueous or in organic medium. In aqueous medium, the PAP layer was soaked 60 s with acidic nitrite sodium solution in the same way that the first diazotisation, rinsed with dry acetone or acetonitrile then finally dried with a flow of nitrogen. In organic medium, the PAP layer was soaked 60 s in acetonitrile with $NOBF_4$.

2.3. Patterning strategies

2.3.1. Ink patterning

Small squares representing the future spots of grafting were drawn by using a simple indelible commercial marker (Stabilo OHPen universal). After the PAP grafting step, ink was eliminated by a rinsing in acetone. Then, after the surface drying, the diazotisation step was carried out.

2.3.2. UV patterning

After the conversion of PAP layer into PDP reactive thin layer, samples were exposed to UV (Spectra Physics, 200 W mercury–xenon lamp with a 380 nm cut off filter) through a mask for 10 min. Afterwards, UV protected areas were used to graft biomolecules.

2.4. Spontaneous grafting and rinsing

The spontaneous grafting of organic and bio-organic molecules onto self-adhesive (PDP) surfaces was performed by a simple droplets deposition. After 10 min, the substrates were sonicated for 10 min in an appropriate solvent. This rinsing treatment allowed discarding all the physisorbed matter without preserving the biological activity. Glucose oxidase (1 mg) was dissolved in 3 mL of