Regular Article

Designing a high performance, stable spectroscopic biosensor for the binding of large and small molecules

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GRAPHICAL ABSTRACT

ABSTRACT

In the context of FTIR ATR-based sensors, the organic layer covering the ATR element has to be as stable as possible for optimal spectroscopic measurements. Previously, this self-assembled covering was considered stable after several hours under a PBS flux, probably due to a hydrophobic barrier, which prevents water penetration into the grafted network. Stability and reactivity, measured simultaneously using FTIR ATR, identify the limits of the previously used molecular construction. For the first time, surface etching of the previous functionalised Ge devices (Ge-PEG-NHS), a few minutes after BSA injection, was observed. It was concluded that the molecular chain deformation of Ge-PEG-NHS likely occurred when large molecules were bound. BSA loaded onto a Ge-PEG-NHS surface led to network deprotection, with the probable disruption of hydrogen bonds for single barrier-based networks. This, in turn, was presumably influenced by the random deposition of the NHS moiety on the PEG chain. A new functionalised germanium device, using a rapid three-step in situ procedure, provides an efficient robust network composed of two protective barriers, ideal for the binding of various sized molecules. The Ge-APS-PEG-NHS device has shown exceptional sensitivity with regards to BSA and ethanolamine target molecules while offering homogeneous NHS distribution.

Abbreviations: APS, aminopropylsiloxane; APTES, 3-aminopropytriethoxysilane; ATR, Attenuated total reflection; BIA, Biospecific interaction analysis; BSA, Bovine serum albumin; DPCI, N,N'-Diisopropylcarbodiimide; DMAP, 4-diméthylaminopyridine; dPEG, Di-acid polyethylene glycol; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EthNH2, Ethanolamine; FTIR, Fourier transform infrared; Ge, germanium; IR, infrared; IRE, infrared element; NHS, N-Hydroxysuccinimide; OTS, Octadecyltrichlorosilane; PEG, Polyethylene glycol; PBS, Phosphate buffered saline; QCM, Quartz crystal microbalance; SAMs, self-assembled monolayers; SCFH, standard cubic feet per hour; SD, standard deviation; SPR, Surface plasmon resonance; SSNR, spectroscopic signal to noise ratio.

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1. Introduction

Biodetection is a very active field of research in very diverse areas such as medicine, pharmacy, defence, food safety... The main challenge in this field is to detect tiny amount of ligands in very complex media without false positive. One of the route to solve this problem is to graft specific bioreceptors to a surface to design an affinity biosensor. Many examples have been developed in the literature ([11] and references therein). The quality of the grafting, a typical problem for interface science, is of course key in the biodetection process especially in the presence of complex media such as body fluids. Let us here stress that the stability of the grafting is of paramount importance in this problem. This is precisely what we are willing to explore for a given technique, the BIA-ATR (Biospecific interaction analysis- Attenuated total reflection) technology ([12] and references therein). This is a spectroscopic technique which allows to detect tiny amount of ligands in complex media. It uses an organic layer directly grafted onto the chemically activated surface of a silicon or germanium ATR crystal with an internal incidence angle of 45°. The functionalised layer of these ATR crystals is usually built by wet chemistry, in order to detect ligand in solution, as previously explained [3]. The organic anchoring layer was initially obtained by the grafting of small molecules like Octadeyltrichlorosilane (OTS) or Polyethylene Glycol (PEG) onto the ATR element surface [2]. In the past, infrared elements (IRE) grafted using home-made amphiphilic silane have shown exceptional stability in conditions devoid of extraneous amines, thanks to the presence of a shielding barrier, composed of hydrogen bonds, inside the molecules’ network [3].

Scheme 1. Simplified representation of the Ge-PEG-NHS surface using the previously reported functionalization method [3]. [A] NH₂-terminated biomolecules react with NHS ester ends of the old organic layer, [B] Then, after this covalent reaction, NHS molecules are released from the layer.

Furthermore, the ultimate stability of the functionalised surfaces is crucial to reduce the noise in the infrared measurements while offering the best ultimate sensitivity. A general claim that a more reactive surface gives better sensitivity cannot be made. The more realistic claim is that a more reactive and stable surface will give better spectroscopic sensitivity. The reactivity obtained by the sensor used here often varied along the functionalised crystal due to the numerous limitations related to the photochemistry processes. Until now the results of detection have varied according to the lane used [9]. Poor reproducibility upon the binding of the molecule of interest was initially observed on the various lanes of the sensor. Even though a method has been established to resolve this drawback, by normalising the detection results as a function of the receptor quantities, the need to fully understand and control all the mechanisms involved regarding these reproducibility problems has given rise to an original method of functionalisation for designing robust spectroscopic biosensors. Despite many studies dealing with the chemical functionalisation of germanium oxide surfaces, a lack of precise characterisation of the SAMs on oxide interfaces has made it difficult to fully understand and, therefore, control the self-assembly processes. Some researchers have already studied the physisorption or chemisorption of diverse SAMs while monitoring the in situ processes [10–14]. Until now, most techniques used to monitor these functionalisations in real-time have been limited to physical measurements using, for example, Quartz Crystal Microbalance (QCM) [10,11,12], Surface Plas-
mon Resonance (SPR) [13] or electrochemical devices [14], based on weight, refractive index or electrical variations respectively. IR spectroscopy has played a key role in the optimisation of SAMs on IRE [15]. Fourier Transform Infrared (FTIR) spectroscopy is an ideal quantitative tool for studying molecular monolayers at solid-liquid interfaces while providing an actual Infrared (IR) fingerprint of each assembled molecule [16]. Monitoring organic layer formation by FTIR in real-time can provide very useful information with regards to grafting quality and layer integrity. In this article a new in situ method to characterise the organic anchoring of layers onto germanium surfaces while using FTIR spectroscopy in ATR mode for real-time monitoring is proposed. The functional organic layer on the surface of the germanium ATR element is built according to a rapid three-step procedure, thus reducing the time needed to functionalise IRE, the cost of use, and, therefore, the time to detect an analyte of interest in solution. Another advantage of the original method described here over the previous method [3] is that it provides a more stable surface which is composed of many well distributed and accessible individual tethers of NHS-terminated azide on the organic layer made of PEG-silane. The stable coating proposed here, based on double protective barriers, also allows a significant reduction of the noise in the spectroscopic measurements. This new technique of functionalisation provides a useful additional protective barrier over the previous method. Different behaviours of the silane-PEG layer stability were observed according to the size of the studied analyte molecules and the considered molecular construction. Besides the decrease in chemical consumption, and thus the cost of use, this new method offers much more stability and accessibility for targeting molecules.

2. Material and methods

2.1. Reagents

Unless otherwise stated, the chemicals purchased were of analytical grade. 3-aminopropyltriethoxysilane (APTES), N,N’-diisopropylcarbodiimide (DIPCI), 4-dimethylaminopyridine (DMAP), H2SO4, H2O2, N,N-bis[2-[(3-carboxy-1-oxopropyl)amino]ethyl] polyethylene glycol (di-acid PEG, dPEG, Mr. = 2000), NHS, 1-ethyl-3-[(3-dimethylaminopropyl) carbodiimide (EDC), Bovine serum albumin (BSA), Ethanolamine (EthNH2), other chemicals, the buffers, and organic solvents were purchased from Sigma Aldrich (Belgium). Water was purified using a Millipore Milli-Q water purification system.

2.2. Cleaning and activation of germanium prisms

Germanium crystals were cleaned and activated by first submerging the surface in HNO3 and then in oxalic acid/H2O2 mixture solutions, as previously described [3]. This was done three consecutive times. Each of these steps was separated by intermediate washing using MilliQ water. After drying using nitrogen flux, the hydroxylated germanium surface was then immediately incubated with fresh EDC at 0.4 M (1:1; v:v) for 10 min. This mixture was then injected into the flow cell and was allowed to react for 1 h at room temperature. Dry acetonitrile is an ideal media for the NHS reagent because it provides an anhydrous environment which minimises the partial hydrolysis of NHS molecules.

2.3. APTES in situ grafting

A solution of APTES was diluted in pure acetonitrile at a concentration of 1% (v:v) and then injected into the flow cell to generate the chemically reactive amino groups on the hydroxylated germanium substrate. An aminopropylsiloxane (APS) surface was obtained, with the APTES molecules being covalently bound to the germanium oxide via Si–O bonds.

2.4. In situ functionalisation of dPEG

Surface modification was continued by the injection of a solution containing dPEG, at a concentration of 0.1% (w:v) dissolved, in ethanol with DIPCI at 0.2% (w:v) and DMAP at 0.05% (w:v) into the flow cell. DIPCI was used here to activate the carboxylic acid end groups of the dPEG chains, and DMAP helped to catalyse this reaction. This modified APS surface is ideal because the PEG chain is connected via two carbamate links on each side. Their key functions are to provide two steric barriers by generating hydrogen bonds between vicinal chains, avoiding both penetration into the network and hydrolysis of the lower silanized layer.

2.5. In situ grafting of NHS using EDC

EDC was associated with NHS to perform a reactive succinimide surface while improving the efficiency of the reaction with an intermediate O-acylisourea-terminated pegylated surface. The NHS was dissolved in acetonitrile at a concentration of 0.1 M and immediately incubated with fresh EDC at 0.4 M (1:1; v:v) for 10 min. This mixture was then injected into the flow cell and was allowed to react for 1 h at room temperature. Dry acetonitrile is an ideal media for the NHS reagent because it provides an anhydrous environment which minimises the partial hydrolysis of NHS molecules.

2.6. Surface stability experiments under PBS flux

Stability experiments were monitored in real-time by FTIR ATR spectroscopy. They consisted of estimating the spectral deviation observed in the spectrum baseline after 1 h of buffer flowing (PBS flux at 50 µl/min) onto different germanium surfaces. This IR signal deviation, called mean noise under PBS flux, was measured in triplicate. Noise in the spectrum baseline was calculated by the peak-to-peak value observed in the final spectrum between 4000 and 650 cm−1. Stability under PBS flux was firstly estimated from a cleaned germanium prism, and then along the entire molecular construction for each differently grafted molecule (Table 1). In the same manner, stability assessments of grafted-germanium devices, using the new or old functionalisation protocol, were performed. The obtained surfaces were denoted Ge-APS-PEG-NHS and Ge-PEG-NHS. This set of stability measurements, with respect to the different functionalised-IRE, are also reported in Table 1.

2.7. Surface stability and reactivity measurements after EthNH2 and BSA binding

A second set of stability experiments, also done in triplicate, was determined, this time after molecule binding using an appropriate spectral range for stability determination. Indeed, mean noise, corresponding to the peak-to-peak measurement after 1 h

<table>
<thead>
<tr>
<th>Surfaces</th>
<th>Mean noise under PBS flux (Absorbance × 10−4)</th>
<th>Peak-to-peak</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ge</td>
<td>25.0</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Ge-APS</td>
<td>33.9</td>
<td>0.3</td>
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<tr>
<td>Ge-APS-PEG</td>
<td>29.4</td>
<td>0.4</td>
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<tr>
<td>Ge-PEG-NHS</td>
<td>37.9</td>
<td>0.2</td>
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<tr>
<td>Ge-APS-PEG-NHS</td>
<td>39.5</td>
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of target flowing (BSA or EthNH₂) was processed between 3000 cm⁻¹ and 2800 cm⁻¹. This selected infrared range is highly appropriate for studying hydrolysis phenomena, which can produce spectral variation around CHₓ stretching bands. Reactivity quantification (called mean signal) was also estimated during the same experiment by measuring the peak area of the amide I band.

Fig. 1. Simultaneous monitoring of molecule binding and coating stability using Ge-PEG-NHS devices. Each curve represents the mean of three identical experiments including the SD. The filled circles represent IR variation of the peak area of amide I bands over time, and are representative of surface sensor reactivity estimation (bold axes). The open squares correspond to the peak area evolution around CH₂ stretching bands over time. A preliminary buffer solution (PBS) was injected into the flow cell before target injection (step A). Step B is related to the injection of the studied analyte of interest. Finally, a rinsing step was also performed after target binding (step C). Each step is separated by vertical lines. [A]: FTIR ATR results obtained before, during and after BSA sample injection. [B]: FTIR ATR results obtained before, during and after EthNH₂ binding.
after washing. Stability and reactivity assessments of grafted IRE were simultaneously analysed by monitoring the spectral evolution of properly selected specific IR ranges in real time. The sensitivity of different functionalised germanium prisms was investigated (Ge-PEG-NHS and Ge-APS-PEG-NHS) regarding EthNH₂ or BSA targets. Fig. 1A and B, respectively, represent spectral evolutions over time around the amide I and CH₂ bands when BSA or EthNH₂ was injected into the functionalised-germanium device using the previous protocol and a home-made compound. The same experiments were performed on the functionalised-germanium prism using commercial products and following the new in situ grafting protocol described here. Fig. 2A and B concern...

Fig. 2. Same IR dynamics curves as in Fig. 1 but using Ge-APS-PEG-NHS substrates. The symbols used are the same as in Fig. 1. [A]: FTIR ATR results related to BSA binding study. [B]: FTIR ATR results using EthNH₂ as target.
BSA and EthNH₂ chemisorption respectively. Each target was mixed with Phosphate Buffered Saline (PBS) solution at pH 7.3 in order to minimise the succinimidyl reaction. Preliminary experiments were performed to select the ideal concentration of each ligand to ensure the saturation of the NHS reactive ends. EthNH₂ (500 μl at 10 mM) or BSA (500 μl at 5 mg/ml) samples were finally injected into the flow cell at a flow rate of 10 μL/min (semi-continuous), in order to react with the free sites of succinimide (step marked B in Figs. 1 and 2). Before and after each sample, a buffer solution (250 μl at 50 μl/min) was injected into the flow cell. The first PBS injection allowed the background spectrum to be acquired while observing the baseline evolution over time (step A in Figs. 1 and 2). The second injection was useful for removing the unreacted excess of EthNH₂ or protein molecules (step C in Figs. 1 and 2). Detecting small molecules like EthNH₂ is challenging for biosensors because the response in classical label-free methods is usually proportional to the mass of the molecule of interest. Detecting larger molecules, such as BSA, is also important because proteins are one of the most commonly used biological receptors [19].

2.8. Reference spectra of pure films

To determine the IR zones of interest related to each grafting step, and to prove the success of the graftings realised directly in the flow cell, ATR crystals were dip-coated for comparative purposes using the same in situ experimental conditions described previously. FTIR ATR spectra after solvent evaporation were acquired between 4000 cm⁻¹ and 650 cm⁻¹ (spectra not shown). APTES pure film showed interesting main adsorption bands in the C=H stretching region (3000–2800 cm⁻¹) due to aliphatic hydrocarbons. More precisely, this coated surface showed two peaks at 2931 cm⁻¹ and 2875 cm⁻¹, related to CH₂ and CH₃ in stretching vibration mode respectively. Furthermore, the APTES pure layer contained two major vibrational modes at 1575 cm⁻¹ and 1485 cm⁻¹ according to amino groups, which is consistent with other works [20]. The di-acid PEG layer showed similar features in the 2800–3000 cm⁻¹ region due to CH₂ stretching vibration. The C=O and C=O in stretching modes were visible at 1100 cm⁻¹ and 1729 cm⁻¹ respectively. A broad peak around 3500 cm⁻¹, and two main peaks at 1655 cm⁻¹ and 1550 cm⁻¹, are appropriate for the amino and hydroxyl groups according to the chemical structure of the molecule. The germanium sample coated with pure NHS gave two characteristic bands appearing around 1700 and 1216 cm⁻¹, corresponding to C=O and C=O stretching, useful for quantifying the implanted NHS during in situ functionalisation.

2.9. Grafting procedure

The functional organic layer at the surface of the IRE can be built easily following a one-step procedure ex-situ and a three-step pro-

![Scheme 2](image-url)
procedure inside the flow cell. In this experiment, immediately after
an oxidation process to increase the density of Si-OH groups at
the surface, the hydroxylated germanium samples were placed in
an ATR flow cell (Specac, UK) connected to a Watson-Marlow
403 U/VM2 peristaltic pump (Farmount, UK) to allow organic
molecular construction by wet chemistry. These three steps, con-
sisting of silanization, then pegylation and finally NHS grafting,
were monitored in real-time by an IR spectrophotometer, enabling
better control of the germanium surface functionalisation.
Scheme 2 outlines the simplified chemistry of these reactions. IR
spectra were recorded on an FTIR Iz10 spectrophotometer
(ThermoFischer) equipped with a Mercury-cadmium-telluride
(MCT) detector at a resolution of 4 cm$^{-1}$, with a mirror speed of
0.6329 cm/s, and continuously purged using an air dryer (Parker-
Zander, Germany) at a flow rate of 25 standard cubic feet per hour
(SCFH). Each experiment was performed on a single lane under a
semi-continuous flow. Firstly, 1200 µl of the APTES was flowed
into the ATR flow cell to obtain the APS surface and was held there
for 1 h to allow the reaction. The flow cell was then rinsed using
APTES-free solvents for 10 min (500 µl at 50 µl/min) to remove
the loosely physisorbed APTES. Then, 1200 µl of the mixture con-
taining the dPEG, DMAP and DIPCI was injected at a flow rate of
12 µl/min for 1 h. The lane was then washed with pure ethanol
(500 µl) at a flow rate of 50 µl/min to remove the activator and
catalyser agents and the unbound dPEG reagents. After the pegyla-
tion of the APS surface, 1200 µl of the NHS/EDC mixture was then

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Fig. 3. Spectral evolution during the three different grafting steps leading to the Ge-APS-PEG-NHS surface. The dotted spectrum represents the IR baseline before any grafting. The dashed, thin and thicker spectra represents the IR signature obtained after 20, 40 and 60 min of in situ grafting respectively. [A] Firstly, APS anchored layer formation on germanium oxide surfaces can easily be observed between 3600 cm$^{-1}$ and 2700 cm$^{-1}$. [B] Secondly, the chemisorption of APS on hydroxylated germanium crystal can also readily be observed between 3600 cm$^{-1}$ and 2700 cm$^{-1}$. [C] IR spectra related to the in situ pegylation of the Ge-APS surface as function of time was clearly visible by the increase of two positive peaks between 3100 cm$^{-1}$ and 2700 cm$^{-1}$. [D] FTIR ATR spectra obtained at various times during NHS in situ implantation onto the Ge-APS-PEG surface were readily observable around 1645 cm$^{-1}$. To improve spectra quality, “automatic smoothing” function from the OMNIC 7.3 software (Thermo Electron Corporation, USA) was applied.
injected at a flow rate of 12 μl/min for 1 h. The washing was done using acetonitrile for 10 min at 50 μl/min. This led to a highly reactive succinimide surface called Ge-APS-PEG-NHS, able to interact with primary amine groups by covalent reaction.

3. Results and discussion

3.1. APS formation

Each grafting step was investigated via FTIR ATR spectroscopy to prove the successful immobiliation of each molecule on the germanium prism (Fig. 3). APS modification on oxide germanium surfaces was first performed in the flow cell and monitored in real-time by infrared spectroscopy. The reaction efficiency was easily quantifiable (Fig. 3A) by the decrease in absorption peaks related to CH2 asymmetric at 2975 cm\(^{-1}\) and to Ge-OH stretching vibrations around 3500 cm\(^{-1}\) (broad band). This means that the APTES molecules were covalently bonded onto the surface. In addition, an increase in several infrared areas was observed, such as the CH2 asymmetric and symmetric stretching bands observed at 2915 cm\(^{-1}\) and 2848 cm\(^{-1}\) respectively, as shown in Fig. 3A. Other positive bands appeared (Fig. 3B) revealing that the chemisorbed APTES molecules were increasingly present on the surface during the grafting process. Among those, the asymmetric and symmetric stretching bands for chemisorbed APS at 1070 cm\(^{-1}\) and 1022 cm\(^{-1}\), and the symmetric and asymmetric vibration modes of the hydrogen bonded amine group at 1576 cm\(^{-1}\) and 1491 cm\(^{-1}\), respectively.

3.2. Instability of APS surface in PBS media

The prerequisite for using infrared elements in biosensor construction is the stability of the organic covering under the conditions usually applied for measuring “receptor–ligand” interactions. In this experiment this was monitored by FTIR ATR spectroscopy under PBS flux (3000 μl at 50 μl/min) at room temperature for 60 min. The spectra of the APS surface, recorded in negative mode as a function of time, showed the progressive etching of the surface by the disappearance of the CH2 stretching bands in the 2800–3000 cm\(^{-1}\) region, indicating a loss of material (see Fig. 4). After about 40 min, the organic layer created from commercial APTES was almost completely removed, in accordance with previous research [15,21]. APS instability could also be observed by the high peak-to-peak value, reported in Table 1. Therefore, both to avoid damage to the APS film, and to prevent nonspecific interactions, a shielding layer able to form double steric barriers was then deposited.

3.3. Functionalisation using dPEG, antifouling behaviour

When investigating interactions of biomolecules in complex media, nonspecific binding to the sensor surface must be limited. A commercial mixture solution containing dPEG, DMAP and DIPCI was therefore injected directly into the flow cell, onto the APS germanium oxide surface, while monitoring using FTIR ATR. Fig. 3C shows the IR spectrum measured during dPEG grafting with two main peaks attributed to the ethylene glycol CH2 asymmetric and symmetric stretching vibrations at 2917 cm\(^{-1}\) and 2848 cm\(^{-1}\) respectively, with an increase during grafting. To provide more information on the extent of the antifouling capabilities of these silane PEG germanium surfaces, FTIR ATR analysis was performed on the pegylated surface after exposure to the protein solutions. The silane PEG germanium surfaces were tested in this way for the adsorption of bovine serum albumin (BSA). IR spectra were recorded during the injection of 1000 μl of BSA at 5 mg/ml (flow rate at 20 μl/min) and after washing. As expected, BSA was not adsorbed onto the silane PEG layer, proving that the surface was completely antifouling to proteins (results not illustrated). The washing, using 1000 μl of PBS at 50 μl/min, was enough to desorb protein from the silane-PEG germanium oxide surface. Furthermore, during BSA flowing, no negative bands were initially observed around the CH2 deformation bands. As expected the APS coating was protected from any hydrolysis by the upper PEG layer containing two steric barriers.

3.4. NHS binding

To create a sensor surface, a further step, related to the addition of NHS, was needed. The grafting of the NHS-terminated pegylated surface, and its stability, could be quantified and monitored on-line.
from the FTIR intensity of the stretching vibration of C=O bands around 1645 cm\(^{-1}\) (Fig. 3D). The quantification of the anchored NHS (mean of three sets of experiments with standard deviations) of various functionalised germanium surfaces is reported in Table 2A, using the peak area value of the C=O band after washing. The implanted NHS could also be quantified from another IR band centred at 1210 cm\(^{-1}\), due to the C–O stretching deformation (not shown).

### 3.5. Contact angle measurement

The grafting quality of the previously used PEG-terminated Ge devices has already been reported [3]. In the same manner, the quality of the new grafted organic layer was investigated (Ge-APS-PEG) by static advancing contact angle measurements using milli-Q water drops. The wettability of five grafted surfaces was characterised by successive depositions of about 20 droplets on each. Advancing contact angles (θ \(a\)) ranging between 39° and 44° were obtained. These values are consistent with the reported wettability of previous Ge-PEG substrates, revealing that the surface grafting quality is similar whatever the method used. In addition, receding contact angles (θ \(r\)) were measured on Ge-APS-PEG surfaces to determine the contact angle hysteresis. Hysteresis (θ \(a\)–θ \(r\)) below 5° was obtained suggesting a compact and well-ordered organic layer.

### 3.6. Stability under PBS flux

Usually, sensor layer stability is simply concluded when signal intensity reaches a plateau during various layer formations. In the past, the stability experiments of the used organic covering were limited to studying spectral variation under simple media and in ideal conditions. Indeed, the stability of functionalised ATR substrates was initially checked by flowing PBS into the flow cell onto the thin organic layer made of PEG-silanes. The FTIR ATR spectrum of this functionalised IRE was found to be identical to that of the starting material, even after several hours under PBS flux [3]. Stability of proteins anchored onto functionalised ATR elements was also proved using PBS, by monitoring the amide band intensity over time. As no spectral intensity changes were observed regarding PBS flowing, a conclusion was able to be reached regarding protein layer stability [9,22]. In the same manner, the stability of various functionalised Ge surfaces was investigated by estimating the noise level variation under PBS flux (Table 1). The APS surface gave, as expected, a high peak-to-peak value due to its instability in aqueous media. Table 1 also clearly demonstrates the exceptional stability of the Ge-APS-PEG, and, as such, the pegylation benefits of APS surfaces. The peak-to-peak value on the Ge-APS-PEG was very close to the measured value on cleaned germanium (Ge). In fact, the noise after pegylation was very near to the shot noise. As expected, higher peak-to-peak values were obtained using NHS-terminated surfaces due to the partial hydrolysis of the NHS ends. However, such stability characterisation (using PBS) did not allow the different surface sensors (Ge-PEG-NHS and Ge-APS-PEG-NHS) to be differentiated. At first sight, and after 1 h of PBS flushing, no significant difference was observed in the mean noise estimation between the various molecular constructions studied.

### 3.7. Stability after EthNH\(_2\) or BSA binding

Although many studies have already focused on layer stability [23], the lack of a precise characterisation method for surface sensor stability has made it difficult to completely understand, and so control, the involved processes. Even if the problem of long-term stability of SAMs in biological fluids [24] can be partially resolved by using amphiphilic silane derivatives for reactions on oxidised surfaces, there is an urgent need to properly characterise SAMs’ stability to improve sensor layer quality. In addition, most of the reported studies have only focused on controlling a variety of external environmental parameters which cause layers to degrade [23]. Even though studying surface stability under the conditions usually applied (like PBS) is an essential prerequisite for biosensor efficiency, it does not always provide complete information about layer stability. Indeed, studying the long-time stability of biosensors, particularly during biomolecular recognition events, is crucial to get a clear understanding of the possible mechanisms. Various molecules injected during the binding of any bioreceptors or ligands can dramatically affect the primarily observed stability of the layer. In the literature, very few studies have investigated the
stability of functionalised surfaces during such binding processes. This is because only IR characterisation allows the recognition of two independent simultaneous events thanks to molecular signature differentiation. IR spectroscopy has proven to be essential for oxidised surface characterisation, also providing precise and useful information about both the synthesis and stability of thin organic layers [25]. Here, for the first time, the stability of the functionalised Ge surface, and the binding of various biomolecules, have been investigated simultaneously. Coating stability is a function of time which classically depends on different parameters such as pH, ionic strength, chain length or the nature of the involved biomolecules [23,26]. Furthermore, biomolecules of various size can react in different manners with an organic layer. For this reason, BSA and EthNH2 interactions with functionalised germanium devices were chosen for the study. Figs. 1 and 2 show simultaneous IR monitoring, with differences in binding dynamics and stability behaviour, according to the biomolecules and the used functionalised ATR surfaces. This set of experiments was analysed to characterise the sensitivity of each reactive Ge surface (Table 2). This sensitivity was calculated by measuring the ultimate noise and the reactivity of each prepared surface when EthNH2 or BSA solution flowed. Table 2 presents a comparison, in terms of sensitivity, between the method of functionalisation previously used (Ge-PEG-NHS) [2,3,9,22] and the new method (Ge-APS-PEG-NHS) proposed in this article. The SSNR for each function- alised surface was determined, with respect to the EthNH2 and BSA molecules. The normalised mean signal, corresponding to the target studied and the lane used along the Ge-PEG-NHS prism. The high values observed in the SD of implanted NHS molecules. Nevertheless the sensitivity, with regards to the target molecules, was better using Ge-APS-PEG-NHS. The higher the SSNR, the more sensitive the biosensing method. This is due to the mean noise decrease observed using the newly proposed method. This benefit could be attributed to the additional hydrophobic barriers resulting from the hydrogen bond network, which offers more layer stability. Interestingly, when a larger molecule, such as BSA protein, is flowed onto the Ge-PEG-NHS surface, the mean noise increases considerably (about six times more than injecting EthNH2). This is due to negative peaks that sometimes appear as a function of time around CH2 stretching absorption bands. While it is sometimes difficult to determine the causes of such spectral observations, or even what their physical meaning is, negative peaks around CH2 stretching vibrations clearly indicate the hydrolysis of monolayers after NHS moiety grafting. More precisely, base-surface etching probably appears when water penetration occurs inside the grafted hydrogen bond network, involving nucleophilic substitutions by Cl- and phosphate anions, and/or OH- attacks on the Ge—O—Si—O ether bond. The mean noise calculated during EthNH2 injection was lower than during BSA flowing for the Ge-PEG-NHS device. The single hydrophobic barrier seems to be more sensitive to high molecular weight molecules that can break the weaker hydrogen bonds, as illustrated in Scheme 3A. The surface device becomes vulnerable and surface etching rapidly occurs, featuring highly sensitive Ge—O—Si bonds during large molecule binding. Moreover, Table 2A clearly shows that the Ge-PEG-NHS surface is affected by heterogeneities problems in the NHS deposition, due to the photochemistry process. Indeed, the amount of implanted NHS varies according to the target studied and the lane used along the Ge-PEG-NHS prism. The high values observed in the SD of implanted NHS, with respect to the Ge-PEG-NHS surface, were also in agreement with the heterogeneities evidence. On the other hand, the

![Scheme 3](image-url)

Scheme 3. Schematic representation of mechanisms involved regarding stability behaviour of various NHS-terminated Ge surfaces when large (R1) and small (R2) target molecules were fixed to these. [A] Instability of Ge-PEG-NHS device when large molecules, such as BSA, were bound. [B] Stability of Ge-PEG-NHS device when small molecules, such as EthNH2, were fixed. [C] Stability of the new molecular construction (Ge-APS-PEG-NHS) with respect to the large and small sized molecule binding.
quantity of anchored NHS, regarding the Ge-APS-PEG-NHS surface, varied very little with lower SD values. The in situ method improved the NHS deposition by providing reproducible amounts of NHS molecules on the pegylated ATR device. Contrary to the photochemistry process, which randomly inserts the NHS moiety into the C–H bonds of the pegylated chains, in situ grafting offers well distributed NHS ends with high quality deposition. Furthermore, Table 2A highlights that the Ge-PEG-NHS surface is more sensitive to small molecules, like EthNH₂, than large BSA molecules. The previous method of functionalisation, based on a short spacer arm, probably causes steric hindrance for larger molecules. On the other hand, Ge-APS-PEG-NHS, based on longer spacer arms, reduces steric hindrance while enhancing interactions with EthNH₂ and BSA. This is clearly evidenced by the high SSNR values calculated for the Ge-APS-PEG-NHS surfaces. BSA contains fifty-nine primary amines which are susceptible to react with NHS moiety. EthNH₂ only possesses one available reactive site per molecule. This difference could explain why the Ge-APS-PEG-NHS device offers higher sensitivity to BSA molecules with a higher SSNR value. Compared to EthNH₂, BSA binding seems to provide a higher IR signal response whatever the functionalised surface used (Table 2A). However, after signal normalisation the opposite was observed, as shown in Table 2B. Indeed, the peak area becomes higher after EthNH₂ than after BSA binding. This is probably due to the small sized EthNH₂ molecules which can reach free and reactive NHS sites more easily than large molecules. The normalisation process allowed better identification of the different behaviours of the Ge-PEG-NHS surface when the BSA was injected. Effectively, the observed noise per reactive molecule was relatively constant, except for BSA detection using the previous functionalisation method (Table 2B). Normalisation and Fig. 2 clearly reveal that surface hydrolysis is never observed when using Ge-APS-PEG-NHS surfaces, whatever the size of the bound molecule. Additionally, surface etching does not appear when small molecules, like EthNH₂, are injected into the flow cell containing the Ge-PEG-NHS surface. This is clearly evidenced by the high SSNR values calculated for the Ge-APS-PEG-NHS surfaces. BSA contains fifty-nine primary amines which are susceptible to react with NHS moiety. EthNH₂ only possesses one available reactive site per molecule. This difference could explain why the Ge-APS-PEG-NHS device offers higher sensitivity to BSA molecules with a higher SSNR value. Compared to EthNH₂, BSA binding seems to provide a higher IR signal response whatever the functionalised surface used (Table 2A). 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A network containing two hydrophobic barrier sheets certainly provides a more rigid molecular structure than a structure based on a single barrier. This rigidity allows the disruption of hydrogen bonds to be avoided (Scheme 3C), otherwise this can occur when large molecules are fixed onto spacer arms interconnected by a single barrier. Networks based on a single barrier are sensitive to large molecule binding (Scheme 3A). Their excessively high flexibility can probably induce a sort of “creep deformation” of the spacer arm after large molecule fixing. This “biological cold flow” was observed, even though an amphiphilic silanization reagent composed of short alkyl and PEG chains was selected. Other possible scenarios could be the SAMs deformation by compression, or BSA molecule penetration inside the network involving a “domino effect”. After about 10 min under BSA flow, the spacer arm moved slowly, and permanently disrupted the weaker hydrogen bonds under the influence of the mechanical stress involved by the applied BSA load. This could be readily observed by FTIR ATR spectroscopy, as the negative peaks around CH₂ stretching vibrations appeared as a function of time about 10 min after BSA flowing (Fig. 1A). This time delay did not vary a lot according to the BSA concentration or flow rate variations. “Cold flow” takes about 10 min (±1 min) when varying the BSA concentration from 1 to 10 mg/ml or the flow rate from 10 to 50 μl/min (results not presented).

4. Conclusion

This work has revealed the limits of the previously reported functionalised germanium surface using the grafting method described by Devouflage et al. [3]. The sensitivity of the silane-PEG-NHS layer grafted onto the germanium surface, and its removal when introducing BSA molecules, have been demonstrated. Nevertheless, surface etching was not observed when small molecules, such as EthNH₂, were attached to these old functionalised germanium surfaces. Hence, “molecular cold flow”, when binding large molecules, had to be invoked, even though this home-made amphiphilic silanization reagent was composed of short alkyl and PEG chains. The spacer arm movement was suggested up to the point that the weaker hydrogen bonds were permanently disrupted under the influence of the mechanical stress involved by the applied BSA load. Therefore, a new procedure using a dPEG was used to improve both the surface stability and the NHS deposition of a grafted germanium device. The use of this rapid three-step procedure, performed directly in an ATR flow cell, has provided complete control throughout the molecular construction. This work has shown that replacing the previously used short organic layer by a longer counterpart could considerably improve the quality of the grafting. FTIR ATR spectroscopy has allowed the simultaneous monitoring of multiple events. Layer stability investigations during the binding of various sized biomolecules (EthNH₂ or BSA) have proven that a robust and stable sensor surface (Ge-APS-PEG-NHS) made of germanium could be produced from this new in situ grafting protocol. Even though larger molecules were expected to be more sensitive to the “molecular cold flow”, the addition of an upper sterical barrier to the PEG chains has probably reduced the flexibility of the long chain molecules, thus avoiding hydrogen bond disruption. This work has demonstrated that additional hydrogen bonding played a key role in the greater stability observed, particularly after the binding of high molecular weight molecules onto the Ge-APS-PEG-NHS surfaces. In addition, this work has proven that longer organic layers, containing an upper shielding barrier on the PEG units, can be used in the functionalisation of an ATR element for detecting an analyte of interest. Even though, in ATR mode, the intensity of the evanescent wave exponentially decreased with the distance from the interface, the use of longer organic molecules reduced steric hindrance while improving receptor-ligand interactions. The use of this new Ge-APS-PEG-NHS surface in target detection has shown exceptional surface reactivity, probably due to the homogeneous distribution of NHS moiety on the new functionalised germanium device. Furthermore, the noise measured in the biosensing measurements was considerably reduced, thus improving the performance of spectroscopic biosensors. More specific results related to biodetection will be published elsewhere. Further studies are under consideration to determine the influence of the PEG chain length on spectroscopic performance and biosensor efficiency. It is anticipated that the new organic layer proposed here will offer a useful and robust basis for biosensor construction, and will help many researchers working on surface treatment.

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Author contributions

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