Ready-to-Use Germanium Surfaces for the Development of FTIR-Based Biosensors for Proteins

Pascale Blond, Robin Bevernaegie, Ludovic Troian-Gautier, Corinne Lagrost, Julie Hubert, François Reniers, Vincent Raussens, Ivan Jabin

Ready-to-Use Germanium Surfaces for the Development of FTIR-Based Biosensors for Proteins

Article *in* Langmuir · October 2020

DOI: 10.1021/acs.langmuir.0c02681

8 authors, including:

Pascale Blond
Université Libre de Bruxelles
4 PUBLICATIONS **41** CITATIONS
SEE PROFILE

Robin Bevernaegie
Université Catholique de Louvain - UCLouvain
13 PUBLICATIONS **103** CITATIONS
SEE PROFILE

Ludovic Troian-Gautier
Université Libre de Bruxelles
66 PUBLICATIONS **867** CITATIONS
SEE PROFILE

Corinne Lagrost
CNRS-Université de Rennes 1
97 PUBLICATIONS **3,015** CITATIONS
SEE PROFILE

Ready-to-use germanium surfaces for the development of FTIR-based biosensors for proteins

Pascale Blond,[‡,¥] *Robin Bevernaegie,*[‡] *Ludovic Troian-Gautier,*[‡] *Corinne Lagrost,*[⊥] *Julie Hubert,*[‖] *François Reniers,*[‖] *Vincent Raussens,*[*,¥] *Ivan Jabin**[,‡]

[‡] Laboratoire de Chimie Organique, Université libre de Bruxelles (ULB), avenue F. D. Roosevelt 50, CP160/06, B-1050 Brussels, Belgium.

[¥] Laboratory for the Structure and Function of Biological Membranes, Centre for Structural Biology and Bioinformatics, Université libre de Bruxelles (ULB), boulevard du Triomphe, CP206/02, B-1050 Brussels, Belgium.

[⊥] Univ Rennes, CNRS, ISCR – UMR 6226, 35000 Rennes, France.

[‖] Chemistry of Surfaces, Interfaces and Nanomaterials, Université libre de Bruxelles (ULB), boulevard du Triomphe, CP 255, B-1050 Brussels, Belgium

KEYWORDS. Biosensor, Germanium, Calixarenes, FTIR spectroscopy, Fluorescence microscopy

ABSTRACT. Germanium is particularly suitable for the design of FTIR-based biosensors for proteins. The grafting of stable and thin organic layers on germanium surfaces remains however challenging. To tackle this problem, we developed a calix[4]arene-tetradiazonium salt decorated with four oligoethylene glycol chains and a terminal reactive carboxylic group. This versatile

molecular platform was covalently grafted on germanium surfaces to yield robust ready-to-use surfaces for biosensing applications. The grafted calixarene monolayer prevents nonspecific adsorption of proteins while allowing bioconjugation with biomolecules such as Bovine Serum Albumin (BSA) or biotin. It is shown that the native form of the investigated proteins was maintained upon immobilization. As a proof of concept, the resulting calix[4]arene-based germanium biosensors were used through a combination of ATR-FTIR spectroscopy and fluorescence microscopy for the selective detection of streptavidin from a complex medium. This study opens real possibilities for the development of sensitive and selective FTIR-based biosensors devoted to the detection of proteins.

INTRODUCTION

Easy-to-use (bio)sensors are highly sought-after systems in a large number of fields that include medicine, food safety and environment.[1-3] They enable the detection and sometimes the quantification of an analyte in a complex medium, based on its affinity for receptors immobilized on the sensor. Detection is performed through classical analytical methods including various spectroscopic techniques. In the specific context of proteins detection, Fourier transform infrared (FTIR) spectroscopy is particularly well adapted, notably for identifying the secondary and tertiary structures of the target protein.[4-8] In addition, a multitude of biophysical and chemical information can be obtained from the spectral signature of proteins (*e.g.* post-translational modifications). Attenuated total reflectance (ATR) FTIR-based biosensors make use of an organic layer, directly grafted onto the internal reflection element (IRE), to which a biological receptor is bound.[8-14] Common infrared-transparent materials for the IRE include semiconductors such as silicon and germanium. Germanium exhibits a high refractive index and a broader spectral window,[10] which makes it particularly suitable for ATR analysis of proteins. However, due to the unstable nature of

their surface oxydes,[15] only a few methods involving silanes or thiols have been developed for the grafting of organic layers on germanium substrates.[10-12,16-17] Unfortunately, these methods require laborious preliminary treatments of the germanium surfaces (*i.e.* oxidation and passivation) and lead usually to a weak reproducibility. Moreover, once modified, the surfaces present a poor stability, limiting their use in the field of biosensing. Another strategy relies on the reductive grafting of aryldiazonium salts, which leads to a strong and durable attachment of an organic layer onto the germanium surface.[18-19] However, due to the high reactivity of the radicals produced upon the reduction of diazonium cations, disordered multilayers are generally obtained through this method. The preparation of well-ordered monolayers with diazonium chemistry remains thus highly challenging,[20-24] though this is a key point when immobilizing receptors in order to warrant an efficient and sensitive sensing.[25-26]

In this context, we have recently developed a general strategy for surface modification, which consists in the covalent grafting of molecular platforms based on calix[4]arene-tetradiazonium salts.[27-31] As demonstrated on various materials or nanomaterials, the unique macrocyclic structure of calix[4]arenes enables the formation of robust, dense and compact monolayers of controlled composition.[32-33] Based on this expertise, we have recently reported that a thin (ca. 3 nm) and robust coating of calix[4]arenes bearing oligo(ethylene glycol) (oEG) chains can be obtained on germanium IRE (Figure 1a).[31] It was shown that such oEGylated calix[4]arene-based coatings reduce nonspecific adsorption of Bovine Serum Albumin (BSA) by more than 85%. To the best of our knowledge, this was the first study to show such a significant reduction of nonspecific adsorption through the use of a stable and extremely thin coating of organic molecules. The thickness parameter is of crucial importance to ensure optimal IR signal, as sensitivity decreases exponentially with distance from the IRE. Hence, the calixarene-based methodology appears very

promising for the preparation of ready-to-use germanium surfaces that can serve to the design of ATR-FTIR-based biosensors.

Herein, we describe the synthesis and incorporation in Ge-based biosensors of a novel calix[4]arene-tetradiazonium salt bearing four oEG chains. Importantly, one of the oEG chains is equipped with a terminal reactive carboxylic acid group for further post-functionalization. After grafting on germanium surfaces, this polyfunctional platform displayed antifouling properties while allowing the bioconjugation of biomolecules under classical conditions such as peptide type coupling (Figure 1b). Besides, with these versatile calixarenes, the ratio of functionalized/non functionalized oEG chains is controlled within the molecular platform itself, avoiding the formation of binary mixed layers, which is particularly challenging.[26,34] The usefulness of this approach for the design of FTIR-based ready-to-use germanium biosensors is illustrated by the specific detection of streptavidin (SA) in a medium containing other proteins.

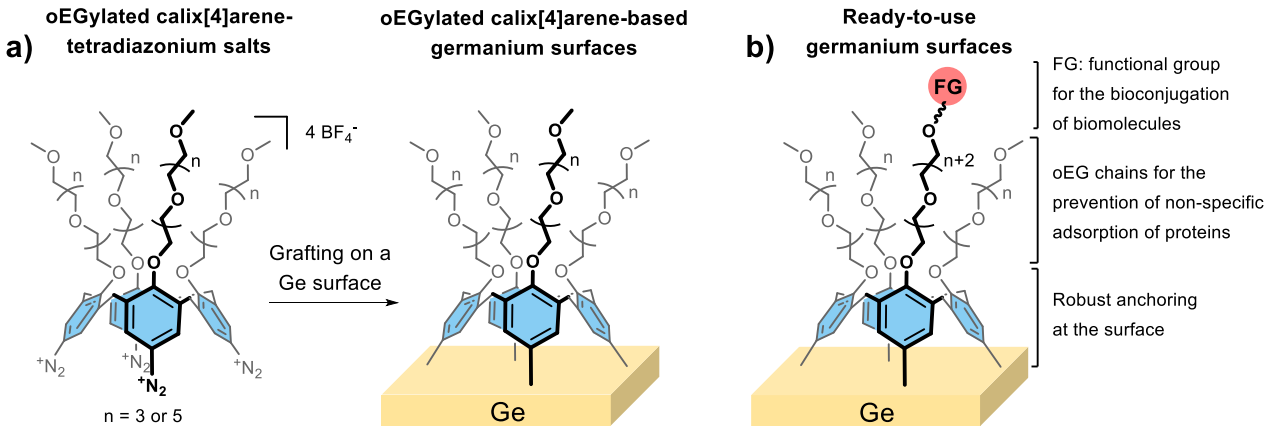
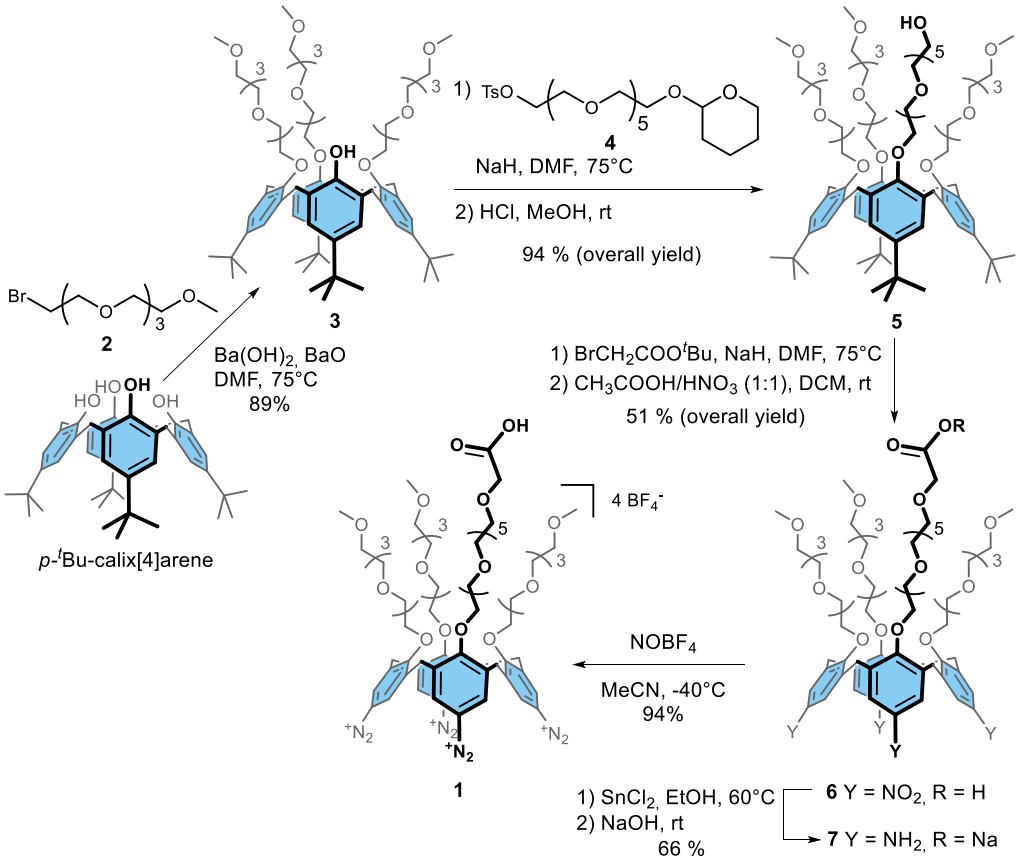


Figure 1. a) previous work on oEGylated calix[4]arene-based germanium surfaces displaying antifouling properties;[31] b) this work on ready-to-use germanium surfaces for the bioconjugation of biomolecules.[35]

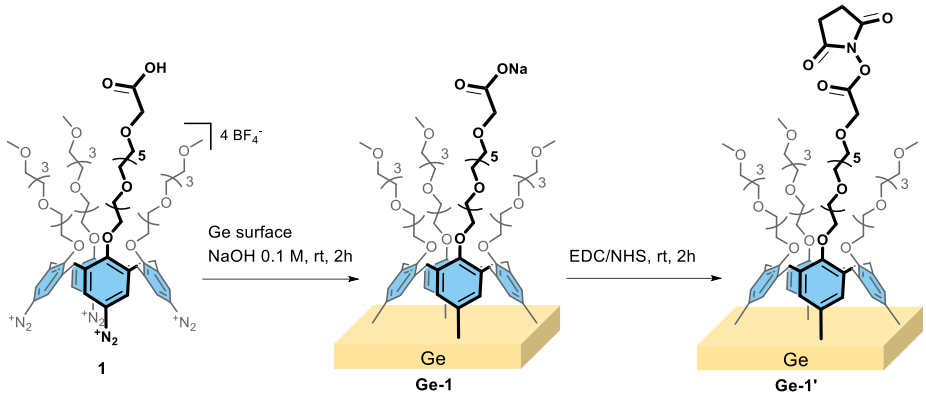
RESULTS AND DISCUSSION

Synthesis and characterization of calix[4]arene-tetradiazonium salt 1. Synthesis of calix[4]arene-tetradiazonium salt **1** was achieved for the development of the targeted ready-to-use germanium surfaces (Scheme 1). The oEG chain bearing a carboxylic acid moiety is longer than the three others in order to facilitate bioconjugation of biomolecules after grafting on a surface. Firstly, *p*-^{t}Bu-calix[4]arene was alkylated with bromo-tetra(ethylene glycol) monomethyl ether[36] **2** in the presence of $Ba(OH)_2$/BaO. Under these conditions, which are known to lead to tris-O-alkylated derivatives,[37] calix[4]arene **3** was isolated in 89% yield. Introduction of the fourth arm was then considered through alkylation of the remaining phenolic group by a tosyl-hexa(ethylene glycol) derivative terminated by a protected carboxylic group. However, this synthetic route led to non-reproducible results due to the deprotection of the carboxylic group under the harsh basic reaction conditions, which hindered complete alkylation of calix[4]arene **3** (see the Supporting Information). Another strategy consisting in the use of the tosyl-hexa(ethylene glycol) derivative terminated by an acetal protecting group **4** was thus developed. Alkylation of calixarene **3** by **4** led to the corresponding tetraalkylated compound whose hydroxyl group was directly deprotected under acidic conditions (94 % overall yield from **3**). Further alkylation of the hydroxyl group of **5** by ^{t}Bu-bromoacetate followed by *ipso*-nitration of the aromatic units led to the tetranitro derivative **6** in 51 % overall yield**.** Reduction of the corresponding nitro groups by tin(II) chloride afforded the corresponding tetraaniline **7** in 66 % yield. Finally, diazotation using $NOBF_4$ in acetonitrile led to the desired tetradiazonium salt **1** in 94% yield. Compound **1** was unambiguously characterized by IR ($\nu_{(N_2^+)}$ = 2268 cm^{-1}) and NMR spectroscopy (see the Supporting Information).



Scheme 1. Synthesis of calix[4]arene-tris-oEG$_4$-mono-oEG$_6$-acid-tetradiazonium salt **1**.

Covalent grafting of an oEGylated calix[4]arene-based layer on germanium. To achieve the grafting of the calixarene-based layer, we used a previously reported procedure that consists in generating the desired aryl radicals from the aryldiazonium groups under alkaline conditions.[29,31] All the grafting experiments were repeated at least three times to assess reproducibility (see the Supporting Information). In practice, the germanium substrates were immersed for 2 hours in an aqueous 0.1 M NaOH solution containing calixarene **1** (5 mM) (Scheme 2). The resulting different batches of germanium surfaces **Ge-1** were then thoroughly washed with water and acetonitrile under sonication.



Scheme 2. Preparation of ready-to-use calixarene-based germanium surfaces.[35]

The different batches of **Ge-1** were analyzed by ATR-FTIR spectroscopy and by ellipsometry to assess the grafting and to estimate the thickness of the organic layer. The IR absorbance spectrum of **Ge-1** exhibit bands that are typical of the calixarene core and of the oEG chains, as shown by the comparison with the spectrum of calix[4]arene **7** (Figure 2) (see the Supporting Information for complete assignment of the spectrum). More specifically, the asymmetric COC stretching from the oEG chains around 1100 cm^{-1} is visible in both spectra. Also, other bands such as the symmetric COC_{Ar} stretching around 1050 cm^{-1} and the aromatic ring stretching around 1460 cm^{-1} unambiguously confirm the presence of calix[4]arene moieties on the Ge surface.[11,31,33,38] It is noteworthy that the typical vibrational bands for carboxylate groups at ~1600 cm^{-1} and ~1420 cm^{-1} are too weak to be significantly distinguished, in accordance with the low number of carboxylate groups.

A thickness value of 2.85 ± 0.80 nm was found by ellipsometric measurements. This value corresponds well to the estimated height of calixarene **1** (ca. 3.0 nm estimated from MM2 energy minimizations with ChemBio3D software). This is in agreement with previous results showing that the calixarene-based methodology leads to the formation of monolayers, *i.e.* through AFM scratching experiments or ellipsometry measurements on various substrates.[27,29,31]

The grafting of calix[4]arene-tetradiazonium salt on germanium surfaces was further assessed through X-ray photoelectron spectroscopy (XPS) analyses. For that purpose, a CF_3-containing calix[4]arene was used. as described in previous articles,[27,32,29] Indeed, the CF_3 groups provide a characteristic chemical tag for XPS studies. The survey scan exhibits photoelectron peaks and Auger peaks due to C, O, Ge and F elements (see the Supporting Information). The absence of N1s peak at 403.8 eV indicates the absence of weakly bound diazonium at the surface. The high-resolution core level spectrum of C1s could be decomposed into several components, namely components at 286.5 ±0.5 eV and at 292.8 ±0.5 eV that correspond to carbons involved in C-O-C linkage and CF_3 groups. The atomic ratio of these two component C-O-C / CF_3 is equal to 1.07. These observations are in full agreement with the calixarene backbone and unambiguously confirm the grafting of calix[4]arene tetradiazonium salts.

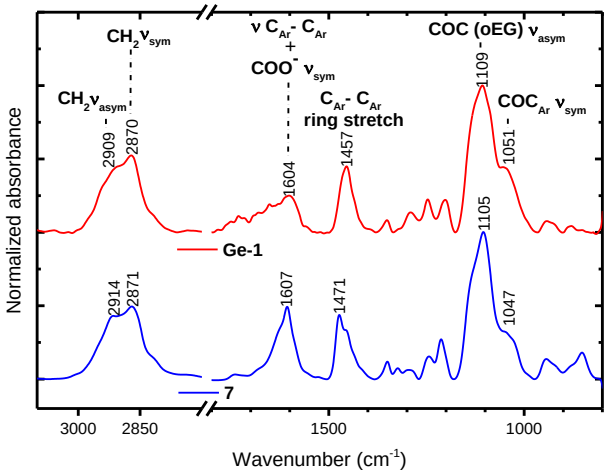
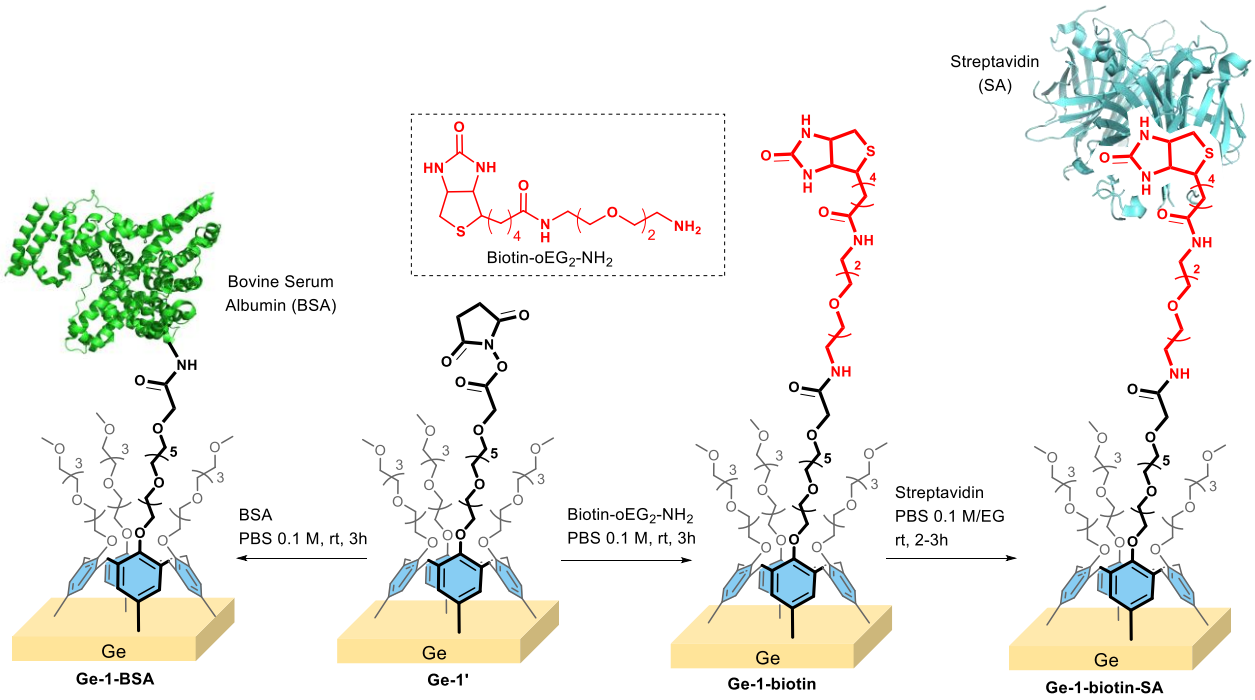


Figure 2. ATR-FTIR absorption spectra (3100-2700 cm^{-1} and 1800-800 cm^{-1}) of modified surface **Ge-1** (red) and calix[4]arene **7** (blue). All spectra were normalized with respect to the intensity at 1459 cm^{-1}.

We can conclude from the combination of these IR and ellipsometric measurements that thin films of oEGylated calix[4]arenes decorated with a carboxylate group are grafted on germanium surfaces. The great robustness of the calixarene-coated surfaces is also demonstrated by their resistance to the aggressive washing treatments (*i.e.* organic solvent under sonication) (see the Supporting Information).

Post-functionalization of the ready-to-use calixarene-based germanium surfaces. Bovine Serum Albumin (BSA) was used as a model protein to investigate the post-functionalization of the grafted calix[4]arene layer through peptide-like coupling reactions. First, the terminal carboxylate groups present at the surface of **Ge-1** were converted to the corresponding activated esters using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) (Scheme 2). The possibility to bioconjugate BSA to the resulting activated surface **Ge-1'** was then evaluated by ATR-FTIR spectroscopy and fluorescence microscopy.



Scheme 3. Post-functionalization of the calixarene-based layer with BSA (left) or biotin-oEG$_2$-NH$_2$ (right) and specific recognition of streptavidin (SA).

Regarding the IR experiments, different batches of surfaces **Ge-1'** were incubated for 3 hours in a 0.1 mg/mL BSA solution [0.1 M phosphate-buffered saline (PBS) buffer at pH 7.4)] (Scheme 3). The resulting surfaces **Ge-1-BSA** were then thoroughly washed with ultrapure water under sonication. The bioconjugated BSA is characterized by an amide-I band at 1657 cm^{-1}, which is mainly associated with the C=O(NH) stretching vibrations, and an amide-II band at 1547 cm^{-1}, which results mainly from in-plane NH bending vibrations (Figure 3, green spectrum). It is noteworthy that an absorption maximum at 1657 cm^{-1} for the amide-I band corresponds predominantly to proteins whose major secondary structure is an α helix,[5] which is the native structure of BSA (Figure 3, black spectrum). Therefore, BSA maintains its native structure after grafting, indicating that the modified germanium support has a protein-friendly character. In a control experiment, a non-activated surface **Ge-1** was exposed to the same experimental bioconjugation and washing conditions. No characteristic bands associated with BSA were observed when analyzing the resulting surface **Ge-1-control** (Figure 3, blue spectrum). When subtracting the spectrum of **Ge-1-control** from that of **Ge-1-BSA**, only the signals of the bioconjugated proteins were clearly observed (Figure 3, magenta spectrum), confirming the covalent immobilization of BSA on the surfaces **Ge-1-BSA**. The non-detection of BSA on the surface **Ge-1-control** also highlights the remarkable antifouling properties of the oEGylated calixarene-based layers.

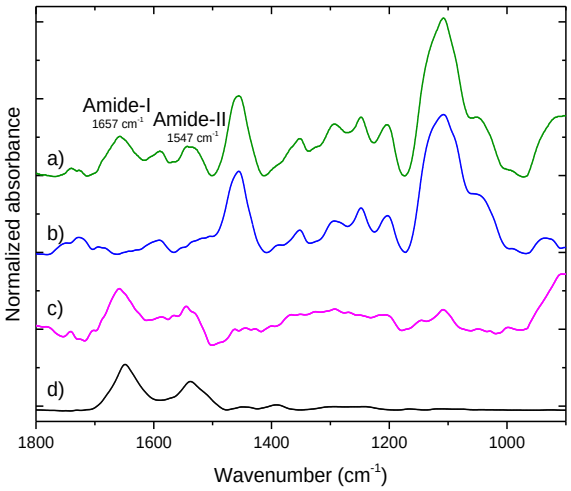


Figure 3. ATR-FTIR absorption spectra (1800-900 cm^{-1}) of a) **Ge-1-BSA** (green) and b) **Ge-1-control** (blue). Spectrum c) (magenta) is a subtraction of spectrum b to a. Spectrum d) corresponds to native free BSA (black). Spectra a) and b) were normalized with respect to the intensity at 1459 cm^{-1} and spectra c) and d) were normalized with respect to the intensity of the amide-I band.

Regarding the fluorescence microscopy studies, fluorescein isothiocyanate-BSA conjugate (FITC-BSA) was used in place of BSA for the grafting experiments. Microarrays of FITC-BSA were printed on germanium surfaces **Ge-1'** with an Arrayjet Marathon non-contact inkjet Microarrayer (ArrayJet, Roslin, UK). Drops of *ca.* 100 pL of FITC-BSA were deposited to form regular arrays. After 3 hours of incubation, the slides were rinsed with ultrapure water and analyzed. From top to bottom in Figure 4, the concentration of FITC-BSA gradually decreases from 10 to 0.5 mg/mL, allowing a qualitative comparison. In all cases, protein spots are clearly visible, indicating that the bioconjugation of FITC-BSA is effective in the whole concentration range. Fluorescence intensity increased from 0.5 mg/mL to 5 mg/mL but no further improvement was observed by the use of 10 mg/mL solutions. Hence, a concentration of 5 mg/mL leads to saturation of the surface. In a control experiment, a non-activated surface **Ge-1** was exposed to the

same experimental bioconjugation and washing conditions (see the Supporting Information for the fluorescence image). In this case, the fluorescence is very weak for the high concentration conditions and undetectable for the lowest concentration. This result is not quantitative but gives a good indication of the remarkable antifouling character of the modified germanium surface.

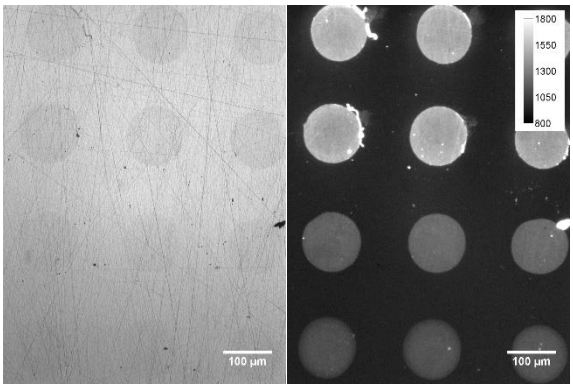


Figure 4. Bright-field (left) and fluorescence images (right) acquired on germanium surface **Ge-1'** that was locally post-functionalized by "printing" (see text) a fluorescent FITC-BSA solution of 10, 5, 1, and 0.5 mg/mL (from top to bottom) (three replicates presented).

Development of a germanium-based biosensor for proteins. The streptavidin-biotin recognition system was used to validate protein detection by the calixarene-coated germanium surfaces. First, fluorescence microscopy analysis was performed to investigate the interaction behavior of fluorescent streptavidin ATTO-655 with surfaces **Ge-1-biotin spotted**. These surfaces were obtained by printing microarrays of a solution containing a biotin derivative bearing a terminal amino group, *i.e.* biotin-oEG$_2$-NH$_2$ (Scheme 3, inset), on germanium slides **Ge-1'** (see the Experimental Section for the details). The surfaces **Ge-1-biotin spotted** were then incubated with a 0.5 µL drop of a solution containing 100 µg/mL of fluorescent streptavidin ATTO-655 and 100 µg/mL of fluorescent FITC-BSA in 0.1 M PBS buffer at pH 7.4/ethylene glycol 1/1 v/v. After 2 hours of incubation, the slides were rinsed with ultrapure water. A microarray that was generated

by the specific interaction of streptavidin ATTO-655 with the immobilized biotin was detected by fluorescence microscopy (Figure 5b). The border of the incubation drop was visible on the left of Figure 5b. The level of fluorescence in the area outside of the incubation drop was close to that observed in the interstices of the microarray, where incubation was carried out, but biotin was not immobilized. Hence, these interstices serve as a control area to evaluate the streptavidin nonspecific adsorption which is extremely limited according to the very weak fluorescence intensity in these regions. Moreover, the absence of fluorescence associated with FITC-BSA (Figure 5c) shows the absence of BSA nonspecific adsorption. These fluorescence microscopy results highlight the remarkable selectivity of our biosensing system as the presence of other proteins in the incubation media does not interfere with the recognition process.

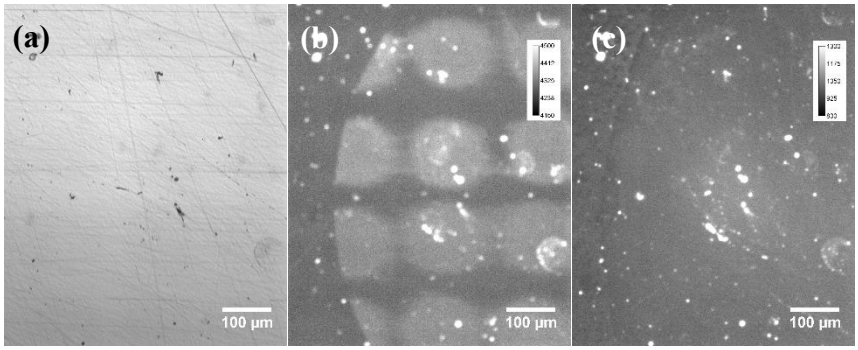


Figure 5. Bright-field (a) and fluorescence images (b and c) acquired on surface **Ge-1-biotin spotted** that was locally incubated with fluorescent streptavidin ATTO-655 and fluorescent FITC-BSA. The images were acquired with specific parameters (see experimental section) to only see the fluorescence of ATTO-655 (b) or FITC dyes (c).

Finally, protein detection was investigated by IR spectroscopy. For this, different batches of surfaces **Ge-1'** were incubated for 3 hours in presence of biotin-oEG$_2$-NH$_2$ (in 0.1 M PBS buffer at pH 7.4) (Scheme 3). The resulting surfaces **Ge-1-biotin** were then thoroughly washed with ultrapure water under sonication and dried under argon flow. Surfaces **Ge-1-biotin** were then

incubated with drops of 1 µL solution of 100 µg/mL streptavidin in 0.1 M PBS buffer at pH 7.4/ethylene glycol 1/1 v/v. Other incubation experiments were conducted under the same conditions except that BSA (100 µg/mL) was also added. After 3 hours of incubation, all the surfaces were rinsed with ultrapure water. In all cases, streptavidin was efficiently detected by the characteristic amide-I and amide-II bands at 1637 cm^{-1} and 1535 cm^{-1}, respectively (Figure 6). The location of the absorption bands indicates that the streptavidin has a β-sheet structure,[5] which corresponds to its native conformation. Very interestingly, no contribution from BSA at 1657 cm^{-1} can be detected in the spectra obtained from incubation experiments in presence of this protein. This demonstrates the remarkable antifouling properties of the calixarene-coated germanium surfaces.

Altogether, these IR and fluorescence microscopy results indicate that the surface modification strategy based on the calix[4]arene-based platform allows on the one hand to immobilize receptors that retain their recognition properties and on the other hand to selectively detect a protein in a medium containing other proteins.

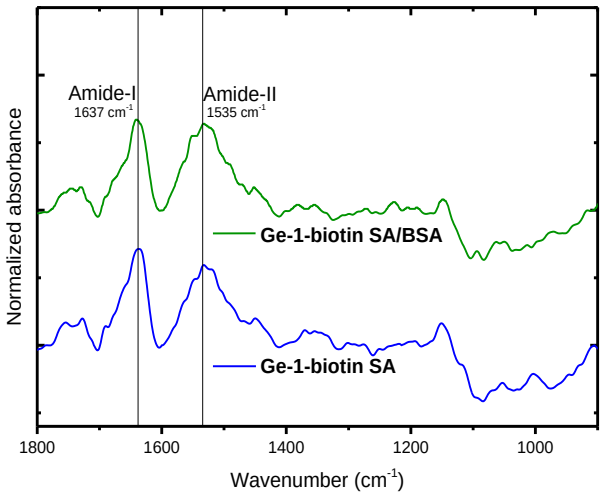


Figure 6. ATR-FTIR absorption spectra (1800-900 cm^{-1}) obtained after incubation of surfaces **Ge-1-biotin** in either solutions of streptavidin (blue) or streptavidin/BSA (green). All spectra were normalized with respect to the intensity of the amide-I band.

CONCLUSIONS

The calixarene-based methodology allows a simple and robust functionalization of germanium surfaces. Newly synthesized oEGylated calix[4]arene-tetradiazonium salt **1** was used for the elaboration of ready-to-use surfaces for the immobilization of biomolecules while displaying antifouling properties. For that purpose, the small rim of the calix[4]arene platform was decorated with four oEG chains, one being equipped with a terminal reactive carboxylic group. Monolayers of calix[4]arenes constitute thus versatile molecular platforms that bring simultaneously multiple new properties to a surface. Importantly, the native form of the investigated proteins was maintained upon immobilization on germanium, as evidenced by IR spectroscopy. The oEGylated calixarene-coated germanium surfaces were used for the selective detection by FTIR spectroscopy and fluorescence microscopy of streptavidin in the presence of BSA. This study not only constitutes a proof of concept that FTIR sensing of proteins can be achieved by this approach but also highlights that other analytical techniques, such as fluorescence microscopy, are compatible with the calixarene-based methodology. In addition to qualitative analysis of protein's structures, IR spectroscopy allows quantitative analysis. Calibration models could be developed to quantify the number of receptors on the surface as well as the number of targets to be detected.

EXPERIMENTAL SECTION

Chemicals and Materials All solvents and reagents were at least of reagent grade quality and were purchased either from Alfa Aesar, Sigma-Aldrich, TCI, or Acros Organics. All reactions

were performed under an inert atmosphere. Reactions were magnetically stirred and monitored by thin layer chromatography using Merck Kiesegel 60F254 plates. Flash chromatography was performed with silica gel 60 (particle size 35−70 μm) supplied by Merck. Anhydrous dimethylformamide (DMF) was obtained from Acros Organics. Anhydrous tetrahydrofuran (THF) was obtained from distillation on Na/benzophenone. Anhydrous dichloromethane (DCM) was obtained from distillation on CaH_2. Ultrapure water was obtained via a Millipore Milli-Q system (18.2 MΩ·cm). The fluorescent FITC-BSA (fluorescein isothiocyanate-bovine serum albumin conjugate) was purchased from Sigma-Aldrich as well as the BSA used for ATR-FTIR spectroscopy experiments. The fluorescent streptavidin-ATTO655 was purchased from ATTO-TEC and the streptavidin used for ATR-FTIR spectroscopy experiments was purchased from Alfa Aesar. Both polished germanium squares (10 × 10 × 0.5 mm) and germanium single-crystal triangular prisms (base 6.8 mm × 45 mm length and an internal incident angle of 45°) were purchased from ACM (France). Syntheses of compounds **2** and **4** were achieved as previously described[36,39] and are given in the Supporting Information. The detailed synthesis of compound **1** is given here.

Synthesis of calix[4]arene-tetra-^{t}Bu-tris-oEG$_4$ 3. Commercial *p*-^{t}Bu-calix[4]arene (3.4 g, 5.2 mmol) was dissolved in dry DMF (80 mL). Anhydrous barium hydroxide (2.7 g, 15.8 mmol) and barium oxide (3.8 g, 24.8 mmol) were added, followed by compound **2** (10 g, 36.9 mmol). The reaction mixture was stirred at 75°C for 16 hours under inert atmosphere. After reaction, the mixture was brought to room temperature and 5 mL of H_2O was added. The mixture was filtered, and the filtrate was evaporated under reduced pressure. The resulting oil was dissolved in DCM (100 mL) and washed three times with water (3 x 70 mL) The organic layer was filtered on WA and the filtrate was concentrated under reduced pressure. The crude oil was purified by flash

chromatography on silica gel (EtOAc/MeOH = 100:0 to 97:3) to afford the desired compound **3** as a pale-yellow oil (5.7 g, 4.7 mmol, 89 % yield). ^{1}H NMR (300 MHz, $CDCl_3$, 298 K) δ (ppm): 7.10 (s, 2H), 7.02 (s, 2H), 6.51 (s, 4H), 5.71 (s, 1H), 4.38 (dd, *J* = 19.1, 12.8 Hz, 4H), 4.16 (s, 4H), 4.05 – 4.00 (m, 4H), 3.86 – 3.80 (m, 4H), 3.75 – 3.47 (m, 40H), 3.37 (s, 3H), 3.36 (s, 6H), 3.17 (dd, *J* = 17.6, 13.0 Hz, 4H), 1.32 (d, *J* = 2.8 Hz, 18H), 0.82 (s, 18H). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K), δ (ppm): 153.7, 151.4, 150.7, 145.8, 145.5, 141.4, 135.9, 132.5, 132.1, 129.1, 125.8, 125.1, 125.0, 124.9, 74.5, 72.1, 72.0, 71.6, 70.9, 70.8, 70.7, 70.7, 70.6, 70.1, 70.0, 59.1, 34.2, 34.0, 33.8, 31.9, 31.8, 31.4, 31.2, 31.2. FTIR, ν (cm^{-1}): 2952, 2923, 2902, 2867, 1723, 1478, 1457, 1359, 1296, 1246, 1199, 1107, 1046, 940, 871. HRMS: calcd. for $C_{71}H_{110}O_{16}Na$ $(M+Na)^+$ 1241.77 found 1241.67.

Synthesis of calix[4]arene-tetra-^{t}Bu-tris-oEG$_4$-mono-oEG$_6$-OH 5. Calix[4]arene-tris-oEG$_4$ **3** (1.00 g, 0.82 mmol) was dissolved in dry DMF (20 mL). NaH (60% dispersion in oil, 66 mg, 1.64 mmol) was added and the mixture was stirred at room temperature for 5 minutes under inert atmosphere. Then, compound **4** (0.85 g, 1.64 mmol) was added and the reaction mixture was stirred for 16 hours at 75°C under inert atmosphere. After reaction, the mixture was concentrated under reduced pressure. The resulting oil was dissolved in DCM (50 mL) and washed three times with water (3 x 50 mL). The organic layer was filtered on WA and the filtrate was concentrated under reduced pressure. The resulting oil was dissolved in MeOH (10 mL) and 1 mL of HCl (37%) was added. The reaction mixture was stirred at room temperature for 2 hours. After reaction, the mixture was concentrated under reduced pressure. The resulting oil was dissolved in DCM (25 mL) and washed three times with water (3 x 25 mL). The organic layer was filtered on WA and the filtrate was evaporated under reduced pressure to afford the desired compound **5** as a pale-yellow oil (1.15 g, 7.75 mmol, 94 % yield). ^{1}H NMR (300 MHz, $CDCl_3$, 298 K), δ (ppm): 6.76 (s,

4H), 6.75 (s, 4H), 4.41 (d, *J* = 12.5 Hz, 4H), 4.02-4.19 (m, 8H), 3.86-3.97 (m, 8H), 3.51-3.69 (m, 61H), 3.37 (s, 9H), 3.09 (d, *J* = 12.5 Hz, 4H), 1.07 (s, 18H), 1.06 (s, 18H). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K), δ (ppm): 153.3, 144.6, 133.9, 133.8, 125.0, 72.9, 72.7, 72.0, 70.8, 70.7, 70.6, 70.6, 70.5, 70.4, 61.8, 59.1, 33.8, 31.5, 31.1. FTIR, ν (cm^{-1}): 2951, 2899, 2867, 1480, 1457, 1360, 1300, 1248, 1200, 1113, 941, 870.

Synthesis of calix[4]arene-tetra-nitro-tris-oEG$_4$-mono-oEG$_6$-acid 6. Calix[4]arene-tetra-^{t}Bu-tris-oEG$_4$-mono-oEG$_6$-OH **5** (0.5 g, 0.34 mmol) was dissolved in dry DMF (10 mL). NaH (60% dispersion in oil, 135 mg, 3.37 mmol) was added and the mixture was stirred at room temperature for 5 minutes under inert atmosphere. Then, *tert*-butylbromoacetate (500 µL, 3.37 mmol) was added and the reaction mixture was stirred for 16 hours at 75°C under inert atmosphere. After reaction, the mixture was concentrated under reduced pressure. The resulting oil was dissolved in DCM (50 mL) and washed three times with water (3 x 50 mL). The organic layer was filtered on WA and the filtrate was concentrated under reduced pressure. The resulting oil was dissolved in DCM (15 mL) and cooled to 0°C. A mixture of glacial CH_3COOH/fuming HNO_3 (1:1) (2 mL) was added. The purple mixture was stirred at 0°C for 5 minutes and then at room temperature for 16 hours. After reaction, the mixture was diluted with DCM (35 mL) and the organic layer was washed with water (several times 50 mL) until the pH of the aqueous phase reached 7. The organic layer was filtered on WA and the filtrate was concentrated under reduced pressure. The resulting oil was triturated three times with 5 mL of diethylether. The diethylether was discarded and the remaining oil was dried under vacuum to yield compound **6** as a yellow oil (0.26 g, 1.71 mmol, 51 % yield). ^{1}H NMR (300 MHz, $CDCl_3$, 298 K) δ (ppm): 7.59 (s, 4H), 7.57 (s, 4H), 4.65 (d, *J* = 14.2 Hz, 4H), 4.24 (s, 8H), 4.12 (s, 2H), 3.81 (s, 8H), 3.65 – 3.50 (m, 65H), 3.43 – 3.33 (m, 15H). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K), δ (ppm): 171.5, 161.9, 143.1, 135.9, 124.0, 74.5, 72.0, 70.7,

70.6, 70.5, 70.5, 70.4, 69.2, 59.1, 31.2. FTIR, ν (cm^{-1}): 2920, 2870, 1741, 1585, 1521, 1449, 1345, 1300, 1262, 1212, 1094, 1043, 928. HRMS: calcd. for $C_{69}H_{101}N_4O_{32}$ $(M+H)^+$ 1497.64 found 1497.64.

Synthesis of calix[4]-tetraaniline-tris-oEG$_4$-mono-oEG$_6$-acid 7. Calix[4]arene-tetra-nitro-tris-oEG$_4$-mono-oEG$_6$-acid **6** (1.22 g, 0.81 mmol) and $SnCl_2.2H_2O$ (4.10 g, 18.1 mmol) were suspended in EtOH (50 mL). The reaction mixture was stirred for 16 hours at 60°C under inert atmosphere. After reaction, the mixture was brought to room temperature and an aqueous NaOH solution (1 M) was added until pH 10 was reached. The mixture was concentrated under reduced pressure. The crude residue was suspended in MeOH (10 mL) and filtered. The filtrate was evaporated under reduced pressure and the residue was suspended in DCM (5 mL). The mixture was filtered, and the filtrate was evaporated to yield the compound **7** as a yellow-orange oil (757 mg, 0.54 mmol, 66%). ^{1}H NMR (300 MHz, $CDCl_3$, 298 K), δ (ppm): 6.05 (s, 4H), 6.03 (s, 4H), 4.30 (d, *J* = 13.0 Hz, 4H), 4.14 (s, 2H), 4.02 – 3.92 (m, 8H), 3.85 – 3.78 (m, 10H), 3.69 – 3.49 (m, 87H), 3.37 (s, 14H), 2.90 (d, *J* = 13.0 Hz, 4H). ^{13}C NMR (100 MHz, CD_3OD, 298 K), δ (ppm): 177.4, 149.9, 142.8, 136.9, 117.4, 74.9, 73.0, 71.6, 71.4, 71.3, 71.1, 71.0, 70.7, 70.7, 70.6, 70.4, 59.1, 32.0. FTIR, ν (cm^{-1}): 3352, 2917, 2870, 1607, 1474, 1455, 1349, 1245, 1212, 1104, 1948, 945, 852. HRMS: calcd. for $C_{69}H_{109}N_4O_{24}$ $(M+H)^+$ 1377.74 found 1377.75.

Synthesis of calix[4]arene-tetradiazonium-tris-oEG$_4$-mono-oEG$_6$-acid 1 Calix[4]-tetra-aniline-tris-oEG$_4$-mono-oEG$_6$-acid **7** (200 mg, 0.14 mmol) was dissolved in dry acetonitrile (3 mL). At –40°C, $NOBF_4$ (150 mg, 1.28 mmol) was added and the reaction mixture was stirred at –40°C for 2 hours under inert atmosphere. After reaction, the mixture was evaporated under reduced pressure and the resulting oil was triturated three times with 3 mL of diethylether. The resulting oil was dissolved in dry acetonitrile and evaporated under reduced pressure to yield the desired

compound **6** as a red oil (240 mg, 1.35 mmol, 94% yield). ^{1}H NMR (300 MHz, CD_3CN, 298 K), δ (ppm): 8.08 (s, 4H), 7.99 (s, 4H), 4.72 (d, *J* = 15.7 Hz, 4H), 4.48 (s, 6H), 4.10 (s, 3H), 3.85 – 3.67 (m, 12H), 3.65 – 3.39 (m, 56H), 3.30 (s, 11H). ^{13}C NMR (100 MHz, CD_3CN, 298 K), δ (ppm): 172.7, 168.8, 138.8, 135.1, 107.8, 77.6, 72.5, 71.1, 71.0, 70.9, 70.8, 58.9, 31.1. FTIR, ν (cm^{-1}): 3238, 3086, 2930, 2883, 2268, 1734, 1570, 1441, 1354, 1291, 1263, 1090, 919, 844. HRMS analysis was not performed because of the low stability of compound **1** against temperature.

Chemical grafting of calixarene 1 on germanium surfaces. Germanium surfaces were first immersed in ultrapure water and then in ethanol. Sonication was applied each time for 5 minutes. They were then rinsed with Et_2O and dried under argon atmosphere. The surfaces were dipped in a 5 mM solution of the diazonium salt **1** in aqueous 0.1 M sodium hydroxide for 2 hours, without stirring in order to avoid any mechanical damage of the surface (a minimal volume of solution was used). Once the grafting was achieved, all the surfaces were thoroughly washed with ultrapure water and then acetonitrile. Sonication was applied each time for 5 minutes. The surfaces **Ge-1** were then dried under argon flow.

Preparation of surfaces Ge-1'. Surfaces **Ge-1** were placed in a sealed tube and covered with 80 mM EDC solution in 0.1 M MES buffer at pH 6.0. An equal amount of a 200 mM NHS solution in 0.1 M MES buffer at pH 6.0 was added, giving rise to a final concentration of 40 mM EDC and 100 mM NHS in MES buffer (0.1 M). The mixture was allowed to react for 2 hours under argon atmosphere. The surfaces were then washed with ultrapure water and dried under an argon flow affording surfaces **Ge-1'**.

Preparation of the FITC-BSA microarrays for the fluorescence microscopy studies. The microarrays of fluorescent FITC-BSA were printed with an Arrayjet Marathon non-contact inkjet

Microarrayer (ArrayJet, Roslin, UK) on germanium slides **Ge-1'** or **Ge-1** (control experiment). In practice, drops of *ca.* 100 pL FITC-BSA solutions (0.5, 1, 5 and 10 mg/mL) in 0.1 M PBS buffer at pH 7.4/ethylene glycol 1/1 v/v were deposited to form regular arrays. Each sample was spotted 30 times in X direction (30 replicates). After 3 hours of incubation, the slides were rinsed with ultrapure water and dried under argon flow.

Preparation of surfaces Ge-1-BSA and Ge-1-control. Surfaces **Ge-1'** or **Ge-1** were incubated for 3 hours in a 0.1 mg/mL BSA solution in 0.1 M PBS buffer at pH 7.4. After reaction, the surfaces were thoroughly washed with ultrapure water under sonication. The surfaces were then dried under an argon flow affording surfaces either **Ge-1-BSA** (from **Ge-1'**) or **Ge-1-control** from (**Ge-1**).

Preparation of Ge-1-biotin spotted surfaces. The microarrays of biotin derivative were printed with an Arrayjet Marathon non-contact inkjet Microarrayer (ArrayJet, Roslin, UK) on germanium slides **Ge-1'**. In practice, drops of *ca.* 100 pL biotin-oEG$_2$-NH$_2$ solutions (10 mg/mL) in 0.1 M PBS buffer at pH 7.4/ethylene glycol 1/1 v/v were deposited to form regular arrays. Each sample was spotted 30 times in X direction (30 replicates). After 3 hours of incubation, the slides were rinsed with ultrapure water and dried under argon flow, affording **Ge-1-biotin spotted** surfaces.

Preparation of surfaces Ge-1-biotin. Surfaces **Ge-1'** were incubated for 3 hours in a 10 mg/mL biotin-oEG$_2$-NH$_2$ solution in 0.1 M PBS buffer at pH 7.4. The surfaces were then thoroughly washed with ultrapure water under sonication and dried under argon flow, affording surfaces **Ge-1-biotin**.

Recognition of streptavidin. Before any detection experiment, surfaces **Ge-1-biotin** or **Ge-1-biotin spotted** were first incubated in a PBS buffer solution at pH 9.3 in order to quench the eventual residual activated ester group. The biotinylated surfaces were then locally incubated

under either conditions A or B. ***Conditions A.*** The **Ge-1-biotin spotted** surfaces were incubated with a drop of 0.5 µL of a solution containing 100 µg/mL of fluorescent streptavidin ATTO-655 and 100 µg/mL of fluorescent FITC-BSA in 0.1 M PBS buffer at pH 7.4/ethylene glycol 1/1 v/v. After 2 hours of incubation, the slides were rinsed with ultrapure water and then dried under argon flow. ***Conditions B.*** The surfaces **Ge-1-biotin** were incubated either with drops of 1 µL solution of 100 µg/mL streptavidin in 0.1 M PBS buffer at pH 7.4/ethylene glycol 1/1 v/v or with drops of 1 µL of solution containing 100 µg/mL of streptavidin and 100 µg/mL of BSA in 0.1 M PBS buffer at pH 7.4/ethylene glycol 1/1 v/v. After 3 hours of incubation, the surfaces were rinsed with ultrapure water and then dried under argon flow.

ATR-FTIR spectra were recorded at 22°C on a Bruker Equinox 55 FTIR spectrophotometer equipped with a liquid nitrogen-cooled mercury−cadmium−telluride detector. The spectrophotometer was continuously purged with dried air. The target chemicals were deposited in solution on a germanium single-crystal internal reflection element (triangular prism of 6.8 × 45 mm, with an internal incidence angle of 45°), and the solvent was removed with a flow of nitrogen gas. Bare germanium was used for the background spectrum. Opus software (4.2.37) was used to record 128 scans with a nominal resolution of 4 cm^{-1}. Data were processed and analyzed using the home-written Kinetics package in Matlab R2007a by subtraction of water vapor, baseline correction, and apodization at 8 cm^{-1}. All spectra were equally normalized on the aromatic ring stretching of the calixarenes around 1460 cm^{-1}.

Fluorescence microscopy experiments were conducted on a Nikon Ti-Eclipse inverted microscope. A filter cube (Semrock) with a 628 nm excitation filter (bandpass 40 nm), a dichroic mirror centered at 660 nm and a 692 nm emission filter (bandpass 40 nm) was employed to match the spectral properties of ATTO 655. A filter cube (B-2A) with a 470 nm excitation filter (bandpass

40 nm), a dichroic mirror centered at 505 nm and a 515 nm emission filter (Longpass) was employed to match the spectral properties of FITC. The surface exposure time for image recording varies between 100 ms for bright field images and 5000 ms for fluorescence images. 8 scans are recorded for the fluorescence images for the detection of the ATTO-655 dye and for the other images a single scan is performed. The images were acquired with an Andor Clara camera controlled by the Solis software. A 10× magnification objective (NA = 0.30, working distance 16 mm) was employed, providing an imaging density of 1.7 pixels per µm. The images were processed with the ImageJ software.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge on the ACS Publications website at DOI: XXX.

Supplementary IR Data, Fluorescence image and Variable Angle Spectroscopic Ellipsometric (VASE) Data. Detailed procedure for the preparation and characterization of the synthesized compounds, NMR spectra (PDF).

Conflicts of interest.

L. T.-G. was a postdoctoral researcher for X4C between October 2014 and September 2015. I. J. and C. L. are shareholders of X4C and I. J. is a consultant for X4C. All other authors declare that they have no conflict of interest.

Corresponding Authors

Ivan.Jabin@ulb.be, Vincent.Raussens@ulb.ac.be

ACKNOWLEDGMENT

The authors would like to thank Joëlle De Meutter and Erik Goorgmaghtigh for performing the printing experiences with an Arrayjet Marathon non-contact inkjet Microarrayer (ArrayJet, Roslin, UK) on the germanium slides. The authors acknowledge Thomas Doneux for assistance with fluorescence microscopy experiments. The "Actions de Recherches Concertées" of the Fédération Wallonie-Bruxelles, the Bridge – Green Chemistry Program of Innoviris (postdoctoral grant to R.B.) and the ULB (Ph.D. grant to P.B.) are acknowledged for financial support. L. T.-G. is a postdoctoral researcher and V.R. is senior research associate of the Fonds de la Recherche Scientifique – FNRS.

REFERENCES

1. Andreescu, S.; Sadik, O. A., Trends and challenges in biochemical sensors for clinical and environmental monitoring. *Pure. Appl. Chem.* **2004,** *76*, 861-878.
2. Pearson, J. E.; Gill, A.; Vadgama, P., Analytical aspects of biosensors. *Ann. Clin. Biochem.* **2000,** *37*, 119-145.
3. Kirsch, J.; Siltanen, C.; Zhou, Q.; Revzin, A.; Simonian, A., Biosensor technology: recent advances in threat agent detection and medicine. *Chem. Soc. Rev.* **2013,** *42*, 8733-8768.
4. de Jongh, H. H. J.; Goormaghtigh, E.; Ruysschaert, J.-M., Amide-Proton Exchange of Water-Soluble Proteins of Different Structural Classes Studied at the Submolecular Level by Infrared Spectroscopy. *Biochemistry* **1997,** *36*, 13603-13610.
5. Goormaghtigh, E.; Cabiaux, V.; Ruysschaert, J.-M., Determination of Soluble and Membrane Protein Structure by Fourier Transform Infrared Spectroscopy. In *Physicochemical Methods in the Study of Biomembranes*, Hilderson, H. J.; Ralston, G. B., Eds. Springer US: Boston, MA, 1994; pp 405-450.
6. Goormaghtigh, E.; Gasper, R.; Bénard, A.; Goldsztein, A.; Raussens, V., Protein secondary structure content in solution, films and tissues: Redundancy and complementarity of the information content in circular dichroism, transmission and ATR FTIR spectra. *BBA-Proteins Proteom.* **2009,** *1794*, 1332-1343.
7. Goormaghtigh, E.; Ruysschaert, J.-M.; Raussens, V., Evaluation of the Information Content in Infrared Spectra for Protein Secondary Structure Determination. *Biophys. J.* **2006,** *90*, 2946-2957.
8. Kleiren, E.; Ruysschaert, J.-M.; Goormaghtigh, E.; Raussens, V., Development of a quantitative and conformation-sensitive ATR-FTIR biosensor for Alzheimer's disease: The effect of deuteration on the detection of the AB peptide. *Spectroscopy* **2010,** *24*, 61-66.
9. Gosselin, E.; Vanden Eynde, J. J.; Petit, A.; Conti, J.; De Coninck, J., Designing a high performance, stable spectroscopic biosensor for the binding of large and small molecules. *J. Colloid. Interf. Sci.* **2017,** *508*, 443-454.

10. Voue, M.; Goormaghtigh, E.; Homble, F.; Marchand-Brynaert, J.; Conti, J.; Devouge, S.; De Coninck, J., Biochemical interaction analysis on ATR devices: a wet chemistry approach for surface functionalization. *Langmuir* **2007,** *23*, 949-55.
11. Devouge, S.; Conti, J.; Goldsztein, A.; Gosselin, E.; Brans, A.; Voue, M.; De Coninck, J.; Homble, F.; Goormaghtigh, E.; Marchand-Brynaert, J., Surface functionalization of germanium ATR devices for use in FTIR-biosensors. *J. Colloid. Interf. Sci.* **2009,** *332*, 408-15.
12. Goldzstein, A.; Aamouche, A.; Homblé, F.; Voue, M.; Conti, J.; De Coninck, J.; Devouge, S.; Marchand-Brynaert, J.; Goormaghtigh, E., Ligand–receptor interactions in complex media: A new type of biosensors for the detection of coagulation factor VIII. *Biosens. Bioelectron.* **2009,** *24*, 1831-1836.
13. Nabers, A.; Ollesch, J.; Schartner, J.; Kotting, C.; Genius, J.; Haussmann, U.; Klafki, H.; Wiltfang, J.; Gerwert, K., An infrared sensor analysing label-free the secondary structure of the Abeta peptide in presence of complex fluids. *J. Biophotonics.* **2016,** *9*, 224-34.
14. Schartner, J.; Guldenhaupt, J.; Katharina, G.; Rosga, K.; Kourist, R.; Gerwert, K.; Kotting, C., Highly stable protein immobilization via maleimido-thiol chemistry to monitor enzymatic activity. *Analyst* **2018,** *143*, 2276-2284.
15. Amy, S. R.; Chabal, Y. J., Passivation and Characterization of Germanium Surfaces. In *Advanced Gate Stacks for High-Mobility Semiconductors*, Dimoulas, A.; Gusev, E.; McIntyre, P. C.; Heyns, M., Eds. Springer Berlin Heidelberg: Berlin, Heidelberg, 2007; pp 73-113.
16. Schartner, J.; Gavriljuk, K.; Nabers, A.; Weide, P.; Muhler, M.; Gerwert, K.; Kotting, C., Immobilization of proteins in their physiological active state at functionalized thiol monolayers on ATR-germanium crystals. *Chembiochem* **2014,** *15*, 2529-2534.
17. Collins, G.; Aureau, D.; Holmes, J. D.; Etcheberry, A.; O'Dwyer, C., Germanium Oxide Removal by Citric Acid and Thiol Passivation from Citric Acid-Terminated Ge(100). *Langmuir* **2014,** *30*, 14123-14127.
18. Girard, A.; Geneste, F.; Coulon, N.; Cardinaud, C.; Mohammed-Brahim, T., SiGe derivatization by spontaneous reduction of aryl diazonium salts. *Appl. Surf. Sci.* **2013,** *282*, 146-155.
19. Collins, G.; Fleming, P.; O'Dwyer, C.; Morris, M. A.; Holmes, J. D., Organic Functionalization of Germanium Nanowires using Arenediazonium Salts. *Chem. Mater.* **2011,** *23*, 1883-1891.
20. Combellas, C.; Jiang, D.-e.; Kanoufi, F.; Pinson, J.; Podvorica, F. I., Steric Effects in the Reaction of Aryl Radicals on Surfaces. *Langmuir* **2009,** *25*, 286-293.
21. Breton, T.; J. Downard, A., Controlling Grafting from Aryldiazonium Salts: A Review of Methods for the Preparation of Monolayers. *Aust. J. Chem.* **2017,** *70*, 960-972.
22. Malmos, K.; Dong, M.; Pillai, S.; Kingshott, P.; Besenbacher, F.; Pedersen, S. U.; Daasbjerg, K., Using a Hydrazone-Protected Benzenediazonium Salt to Introduce a Near-Monolayer of Benzaldehyde on Glassy Carbon Surfaces. *J. Am. Chem. Soc.* **2009,** *131*, 4928-4936.
23. Nielsen, L. T.; Vase, K. H.; Dong, M.; Besenbacher, F.; Pedersen, S. U.; Daasbjerg, K., Electrochemical Approach for Constructing a Monolayer of Thiophenolates from Grafted Multilayers of Diaryl Disulfides. *J. Am. Chem. Soc.* **2007,** *129*, 1888-1889.
24. Leroux, Y. R.; Fei, H.; Noël, J.-M.; Roux, C.; Hapiot, P., Efficient Covalent Modification of a Carbon Surface: Use of a Silyl Protecting Group To Form an Active Monolayer. *J. Am. Chem. Soc.* **2010,** *132*, 14039-14041.

25. Liu, G.; Böcking, T.; Gooding, J. J., Diazonium salts: Stable monolayers on gold electrodes for sensing applications. *J. Electroanal. Chem.* **2007,** *600*, 335-344.
26. Jiang, C.; Moraes Silva, S.; Fan, S.; Wu, Y.; Alam, M. T.; Liu, G.; Justin Gooding, J., Aryldiazonium salt derived mixed organic layers: From surface chemistry to their applications. *J. Electroanal. Chem.* **2017,** *785*, 265-278.
27. Mattiuzzi, A.; Jabin, I.; Mangeney, C.; Roux, C.; Reinaud, O.; Santos, L.; Bergamini, J. F.; Hapiot, P.; Lagrost, C., Electrografting of calix[4]arenediazonium salts to form versatile robust platforms for spatially controlled surface functionalization. *Nat. Commun.* **2012,** *3*, 1130-1137.
28. Troian-Gautier, L.; Valkenier, H.; Mattiuzzi, A.; Jabin, I.; den Brande, N. V.; Mele, B. V.; Hubert, J.; Reniers, F.; Bruylants, G.; Lagrost, C.; Leroux, Y., Extremely robust and post-functionalizable gold nanoparticles coated with calix[4]arenes via metal-carbon bonds. *Chem. Commun.* **2016,** *52*, 10493-10496.
29. Troian-Gautier, L.; Martínez-Tong, D. E.; Hubert, J.; Reniers, F.; Sferrazza, M.; Mattiuzzi, A.; Lagrost, C.; Jabin, I., Controlled Modification of Polymer Surfaces through Grafting of Calix[4]arene-Tetradiazoate Salts. *J.Phys. Chem. C* **2016,** *120*, 22936-22945.
30. Troian-Gautier, L.; Mattiuzzi, A.; Reinaud, O.; Lagrost, C.; Jabin, I., Use of calixarenes bearing diazonium groups for the development of robust monolayers with unique tailored properties. *Org. Biomol. Chem.* **2020,** *18*, 3624-3637.
31. Blond, P.; Mattiuzzi, A.; Valkenier, H.; Troian-Gautier, L.; Bergamini, J.-F.; Doneux, T.; Goormaghtigh, E.; Raussens, V.; Jabin, I., Grafting of Oligo(ethylene glycol)-Functionalized Calix[4]arene-Tetradiazonium Salts for Antifouling Germanium and Gold Surfaces. *Langmuir* **2018,** *34*, 6021-6027.
32. Santos, L.; Mattiuzzi, A.; Jabin, I.; Vandencasteele, N.; Reniers, F.; Reinaud, O.; Hapiot, P.; Lhenry, S.; Leroux, Y.; Lagrost, C., One-Pot Electrografting of Mixed Monolayers with Controlled Composition. *J. Phys. Chem. C* **2014,** *118*, 15919-15928.
33. Valkenier, H.; Malytskyi, V.; Blond, P.; Retout, M.; Mattiuzzi, A.; Goole, J.; Raussens, V.; Jabin, I.; Bruylants, G., Controlled Functionalization of Gold Nanoparticles with Mixtures of Calix[4]arenes Revealed by Infrared Spectroscopy. *Langmuir* **2017,** *33*, 8253-8259.
34. Retout, M.; Brunetti, E.; Valkenier, H.; Bruylants, G., Limits of thiol chemistry revealed by quantitative analysis of mixed layers of thiolated-PEG ligands grafted onto gold nanoparticles. *Journal of Colloid and Interface Science* **2019,** *557*, 807-815.
35. Note that these this figures shows ideal situations where the grafted calix[4]arenes forms four covalent bonds with the surface, but the number of links may vary between one to four.
36. Yuan, Z.; Chen, J.; Zeng, Y.; Li, Y.-Y.; Han, Y.; Li, Y., Unsurpassed cage effect for the photolysis of dibenzyl ketones in water-soluble dendrimers. *Org. Biomol. Chem.* **2011,** *9*, 6256-6264.
37. Lavendomme, R.; Zahim, S.; De Leener, G.; Inthasot, A.; Mattiuzzi, A.; Luhmer, M.; Reinaud, O.; Jabin, I., Rational Strategies for the Selective Functionalization of Calixarenes. *Asian J. Org. Chem.* **2015,** *4*, 710-722.
38. Silverstein, R. M.; Webster, F. X.; Kiemle, D., *Spectrometric Identification of Organic Compounds*. 7th ed.; Wiley: **2005**.
39. Loiseau, F. A.; Hii, K. K.; Hill, A. M., Multigram Synthesis of Well-Defined Extended Bifunctional Polyethylene Glycol (PEG) Chains. *J. Org. Chem.* **2004,** *69*, 639-647.

For TOC use only