Grafting of Oligo(ethylene glycol) Functionalized Calix[4]arene-tetra-diazonium Salts for Antifouling Germanium and Gold Surfaces

Pascale Blond,[†,‡] Alice Mattiuzzi,[†,¥] Hennie Valkenier,[⊥] Ludovic Troian-Gautier,[†] Jean-François Bergamini,[§] Thomas Doneux,[Φ] Erik Goormaghtigh,[‡] Vincent Raussens[‡,] and 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, Center for Structural Biology and Bioinformatics, Université Libre de Bruxelles (ULB), avenue F. D. Roosevelt 50, CP206/02, B-1050 Brussels, Belgium.

[⊥] Engineering of Molecular NanoSystems, Ecole Polytechnique de Bruxelles, Université Libre de Bruxelles (ULB), avenue F. D. Roosevelt 50, CP165/64, B-1050 Brussels, Belgium.

[§] Université de Rennes 1, Institut des Sciences Chimiques de Rennes (Equipe MaCSE), CNRS, UMR 6226, Campus de Beaulieu, Bat 10C, 35042 Rennes Cedex, France.

[Φ] Chimie Analytique et Chimie des Interfaces, Université Libre de Bruxelles (ULB), Campus de la Plaine, boulevard du Triomphe, CP255, B-1050 Brussels, Belgium.

[¥] X4C, Rue Chêne Bonnet 128, 6110 Montigny-le-Tilleul, Belgium.

ABSTRACT

Biosensors that can determine protein concentration and structure are highly desired for biomedical applications. For the development of such biosensors, the use of Fourier transformed infra-red (FTIR) spectroscopy with the attenuated internal total reflection (ATR) configuration is particularly attractive but it requires appropriate surface functionalization of the ATR optical element. Indeed, the surface has to specifically interact with a target protein in close contact with the optical element and must display antifouling properties to prevent nonspecific adsorption of other proteins. We here report robust monolayers of calix[4]arenes bearing oEGs chains, which were grafted on germanium and gold surfaces via their tetra-diazonium salts. The formation of monolayers of oEGylated calix[4]arenes was confirmed by AFM, IR and contact angle measurements. The antifouling properties of these modified surfaces were studied by ATR-FTIR spectroscopy and fluorescence microscopy and the non-specific absorption of BSA was found to be reduced by 85% compared to non-modified germanium. In other words, the organic coating by oEGylated calix[4]arenes provides remarkable antifouling properties, opening the way to the design of germanium- or gold-based biosensors.

INTRODUCTION

Proteins are involved in a very large number of biological processes. Even a small change in their concentration, post-translational modifications, or in their native structure can lead to pathologies.[1] Biosensors that enable fast and selective identification and structural characterization

of proteins in complex mixtures represent thus an important target for research.[2,3] A major challenge in the development of such biosensors is the modification of surfaces by a robust organic monolayer able to specifically interact with a protein[4] and displaying antifouling properties to prevent nonspecific adsorption phenomena.

In this context, there is an increasing interest in the use of germanium-based surfaces because this material can be readily used for Fourier transformed infra-red (FTIR) spectroscopy.[5,6,7,8] In contrast with other detection methods, FTIR spectroscopy allows to simultaneously collect a wealth of information such as secondary structure and post-translational modifications.[9,10] Therefore, FTIR spectroscopy with attenuated internal total reflection (ATR) configuration is currently applied to study mono- and multilayers of bioorganic samples.[11] Because the evanescent wave propagates outside of the ATR element on a very short distance only, the ATR configuration allows the detection of analytes in aqueous media when they are brought in close contact with the optical element. This ATR element could for example be a Ge crystal with the appropriate geometry. Ge has the advantages over Si (a much more studied element[12]) to have a larger refractive index (resulting in a better signal-to-noise ratio) and to be transparent in a broader spectral range, allowing the study of more important chemical groups absorbing between 1400 and 800 cm^{-1}.

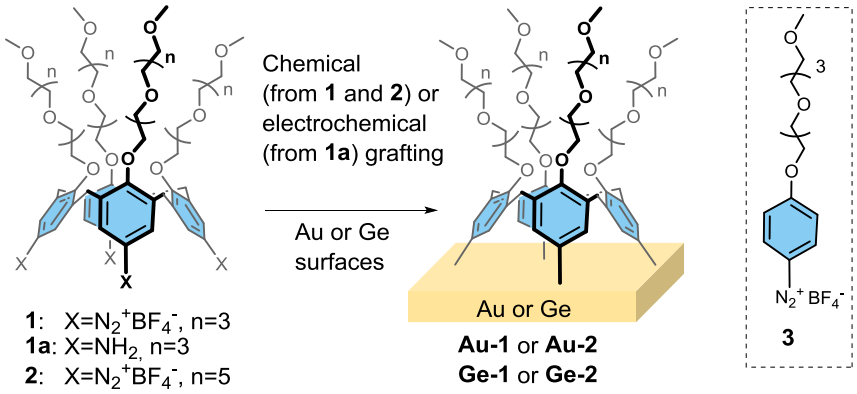
Chemisorbed thiols[6,13] and silane derivatives[5,8,14,15] are generally used for the chemical modification of Ge surfaces. However, the lack of reproducible grafting protocols and of long-term stability in aqueous media of the obtained monolayers are important limitations of these methods. In contrast, the reductive grafting of aryldiazonium salts[3,16,17,18] yields stable layers on Ge.[19,20,21] Nevertheless, this technique generates *in situ* aryl radicals that easily react with the

already grafted aryl groups rendering the formation and organization of monolayers difficult to control.[22,23]

In this regard, we have recently developed a general strategy for the formation of robust, homogeneous and well-organized organic monolayers on various surfaces thanks to the use of calix[4]arene-tetra-diazonium salts.[24,25,26] These compounds can be electrochemically or chemically reduced to produce aryl radicals that can be grafted *via* multiple covalent bonds. The structure and geometry of the calix[4]arenes do not allow any additional grafting, preventing the formation of multilayers. Furthermore, when grafting calix[4]arenes functionalized at the small rim with for instance appending COOH groups, the resulting calixarene layer can be further post-functionalized under mild conditions by various chemical species.[24] An additional feature, in contrast to all the other known methods, is the preparation of binary monolayers of controlled composition in a single step from the grafting of binary mixtures of calix[4]arene-tetra-diazonium salts.[27,28]

Regarding the nonspecific adsorption of proteins and biomacromolecules, this is typically prevented by surface modification with small shielding molecules or polymers. Coatings based on oligo(ethylene glycol) (oEG), polysaccharides, zwitterionic compounds or perfluoroalkyls are some examples.[29,30,31,32,33,34] As oEGs are nontoxic and nonimmunogenic, they are widely used in medical applications.[35,36,37,38] The dominant way through which proteins adhere to surfaces is by hydrophobic effect.[39,40] Uncharged oEGylated surfaces interact with water molecules, preventing this hydrophobic effect and thus effectively shielding the surface from protein fouling. It is noteworthy that the grafting of antifouling coatings on Ge has been scarcely reported.[14,41]

In order to develop a new ATR device suitable for biosensing applications, we have investigated the grafting on Ge of a robust monolayer of calix[4]arenes decorated with multiple oEGs arms. For this, calix[4]arene-tetra-diazonium salts bearing oEGs substituents of different lengths (**1** and **2**) and a reference compound **3**[42] were synthesized (Scheme 1). The feasibility of the grafting was first evaluated on standard gold surfaces through previously developed electrochemical and chemical grafting processes.[26] As Ge is a semi-conducting surface, the conventional electrochemical grafting process is not appropriate and thus only the chemical process was then used. Finally, the antifouling properties of the surfaces that were modified with oEGylated calixarenes were evaluated by FTIR spectroscopy and confocal laser scanning fluorescence microscopy upon exposure to proteins.



Scheme 1. Chemical grafting of calix[4]arenes **1** and **2** or electrochemical grafting of **1a**. Inset: structure of reference diazonium salt **3**. Note that the representation of the grafted calixarene does not necessarily imply that its four aryl units are linked to the surface.

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, Roth 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 DMF was obtained from Acros organics. Anhydrous THF was obtained from distillation on Na/benzophenone. Ultrapure water was obtained via a Millipore Milli-Q system (18.2 MΩ cm). The fluorescent FITC-BSA (fluorescein isothiocyanate BSA conjugate) was purchased from Sigma-Aldrich as well as the BSA used for ATR-FTIR spectroscopy experiments. Gold-coated silicon wafer (1000 Å layer thickness) was purchased from Sigma-Aldrich. Both sides polished germanium squares (10 x 10 x 0.5 mm) and germanium single-crystal triangular prisms (base 6.8 mm x 45 mm length and an internal incident angle of 45°) were purchased from ACM (France).

Caution! Although we have not encountered any problem, it is noted that diazonium salts derivatives are potentially explosive and should be handled with appropriate precautions.

Both calix[4]arene-tetra-oEGs **1** and **2** were prepared following the same strategy. The detailed synthesis of compound **1** (Scheme 2) is given here, while details for the compounds **2** and **3** are reported in the SI.

15,35,55,75-tetra-tert-butyl-12,32,52,72-tetrakis(2-(2-methoxyethoxy)ethoxy)-1,3,5,7(1,3)-tetrabenzenacyclooctaphane 6. Commercial *p*-^{t}Bu-calix[4]arene (2.0 g, 3.1 mmol) was dissolved in dry DMF (50 mL). NaH (60% dispersion in oil, 0.840 g, 21.0 mmol) and 2,5,8,11-tetraoxatridecan-13-yl 4-methylbenzenesulfonate **4** (7.6 g, 21.0 mmol) were added and the reaction mixture was stirred for 24 hours at 75°C under inert atmosphere. The reaction mixture was then brought to room temperature and quenched dropwise with 5 mL of HCl (0.5 M). The mixture was

then concentrated under reduced pressure. The resulting oil was dissolved in CH_2Cl_2 (200 mL), the organic layer was washed with H_2O (3 x 150 mL) and the combined aqueous layers were extracted with CH_2Cl_2 (300 mL). The combined organic layers were concentrated under reduced pressure. The crude oil was purified by flash chromatography on silica gel (EtOAc/MeOH = 85:15). The oil was washed with H_2O to yield the compound **6** as a yellow oil (4.2 g, 3.0 mmol, 96%).

^{1}H NMR (300 MHz, $CDCl_3$, 298K), δ (ppm): 1.06 (s, 36H), 3.09 (d, *J* = 12 Hz, 4H), 3.37 (s, 12H), 3.49-3.71 (m, 48H), 3.93 (t, *J* = 4.5 Hz, 8H), 4.09 (t, *J* = 4.5 Hz, 8H), 4.41 (d, *J* = 12 Hz, 4H), 6.75 (s, 8H). ^{13}C NMR (100 MHz, $CDCl_3$, 298K), δ (ppm): 31.2, 31.6, 33.9, 59.2, 70.5, 70.6, 70.6, 70.7, 70.8, 70.8, 72.1, 73.0, 125.1, 133.9, 144.7, 153.4. FTIR, ν (cm^{-1}): 3412, 2952, 2921, 2899, 2867, 1644, 1480 1457, 1392, 1361, 1300, 1249, 1199, 1107, 1062, 1027, 944, 869. HRMS: calcd. for $C_{80}H_{128}O_{20}$ $(M+H)^+$ 1409.91 found 1409.91.

15,35,55,75-tetra-nitro-12,32,52,72-tetrakis(2-(2-methoxyethoxy)ethoxy)-1,3,5,7(1,3)-tetrabenzenacyclooctaphane 8. Note that compound **8** was already described in the literature[43] but is here prepared *via* another synthetic strategy. Calix[4]arene-tetra-oEG$_4$ **6** (4.2 g, 3.0 mmol) was dissolved in CH_2Cl_2 (100 mL). A mixture of glacial CH_3COOH/fuming HNO_3 (1:1) (22 mL) was added at 0°C and the reaction mixture was stirred for 5 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting oil was dissolved in CH_2Cl_2 (100 mL), the organic layer was washed with H_2O (3 x 70 mL) and the combined aqueous layers were extracted with CH_2Cl_2 (150 mL). The combined organic layers were concentrated under reduced pressure. The crude oil was purified by flash chromatography on silica gel (DCM/Acetone = 65:35) to yield compound **8** as a yellow oil (2.5 g, 1.8 mmol, 61%).

^{1}H NMR spectrum of compound **8** is in accordance with the one reported in the literature.[43] ^{1}H NMR (300 MHz, $CDCl_3$, 298K), δ (ppm): 3.33-3.44 (m, 16H), 3.48-3.63 (m, 48H), 3.80 (t, J = 4.5 Hz, 8H), 4.24 (t, J = 4.5 Hz, 8H), 4.65 (d, J = 12 Hz, 4H), 7.58 (s, 8H).

12,32,52,72-tetrakis(2-(2-methoxyethoxy)ethoxy)-1,3,5,7(1,3)-tetrabenzenacyclooctaphane-15,35,55,75-tetraamine 1a. Note that compound **1a** was already described in the literature[43] but is here prepared *via* another synthetic strategy. Calix[4]arene-tetra-NO_2-tetra-oEG$_4$ **8** (0.901 g, 0.660 mmol) and Pd/C (53 mg, 0.498 mmol) were suspended in EtOH (18 mL). Hydrazine hydrate (3 mL, 61.8 mmol) was added dropwise and the reaction mixture was stirred for 15 hours at reflux. The reaction mixture was filtered on Celite and the Celite was washed with EtOH and CH_2Cl_2. The filtrate was concentrated under reduced pressure to yield the compound **1a** as a yellow oil (0.798 g, 0.641 mmol, 97%).

^{1}H NMR spectrum of compound **1a** is in accordance with the one reported in the literature.[43] ^{1}H NMR (300 MHz, CD_3OD, 298K), δ (ppm): 2.94 (d, J = 13 Hz, 4H), 3.35 (s, 12H), 3.48-3.70 (m, 48H), 3.89 (t, J = 5 Hz, 8H), 4.03 (t, J = 5 Hz, 8H), 4.40 (d, J = 13 Hz, 4H), 6.17 (s, 8H).

12,32,52,72-tetrakis(2-(2-methoxyethoxy)ethoxy)-1,3,5,7(1,3)-tetrabenzenacyclooctaphane-15,35,55,75-tetrakis(diazonium) 1. Calix[4]-tetra-aniline-tetra-oEG$_4$ **1a** (50 mg, 0.040 mmol) was solubilized in dry acetonitrile (0.5 mL). At -40°C, $NOBF_4$ (46 mg, 0.400 mmol) was added and the reaction mixture was stirred for 2 hours at -40°C under inert atmosphere. The reaction mixture was concentrated under reduced pressure at room temperature to yield the compound **1** as a yellow/orange oil. Due to the well-known low stability of diazonium groups, compound **1** was used without further purification for the grafting experiments.

HRMS analysis was not performed because of the low stability of compound **1** against temperature.

^{1}H NMR (600 MHz, CD_3CN, 298K), δ (ppm): 3.30 (s, 12H), 3.43-3.59 (m, 48H), 3.78 (d, J = 14 Hz, 4H), 3.84 (br t, 8H), 4.49 (br t, 8H), 4.72 (d, J = 14 Hz, 4H), 8.02 (br s, 8H). ^{13}C NMR (150 MHz, CD_3CN, 298K), δ (ppm): 31.0, 58.9, 70.4, 70.7, 70.7, 70.9, 71.0, 71.0, 72.5, 77.5, 107.7, 135.0, 138.7, 168.7. FTIR, ν (cm^{-1}): 3083, 2942, 2883, 2267, 1571, 1443, 1355, 1293, 1264, 1096, 1025, 922, 846.

Electrochemical grafting strategy. Electrochemical measurements were conducted in a three-electrode cell using a Ag|AgCl saturated KCl electrode as reference electrode, a large area platinum foil as counter electrode, and a polycrystalline gold disk (1.6 mm in diameter, from Bioanalytical Systems) as working electrode, all connected to an Autolab PGSTAT30 (Metrohm Autolab) potentiostat equipped with a ScanGen module.

Gold electrodes were first polished with alumina paste (1 μm particles) and sonicated in ultrapure water for 10 minutes before being dried under a flux of nitrogen. The diazonium salt **1** was prepared *in situ* in 0.5 M aqueous HCl in the presence of $NaNO_2$ (8 eq.) from the corresponding calix[4]-tetra-aniline **1a** (1.5 mM). The gold substrate was functionalized through the reduction of the *in situ* generated diazonium cations by six voltammetric cycles, the potential being swept between +0.5 V and -0.4 V versus Ag|AgCl electrode. Once the grafting was achieved, the surfaces were thoroughly rinsed with ultrapure water and sonication was applied for 10 minutes.

Chemical grafting strategy. ***Caution!*** *Piranha solution is a very strong oxidant and should be handled very carefully.* Gold surfaces were first immersed in a piranha solution (H_2SO_4/H_2O_2 3:1) for 10 minutes and sonicated for 5 minutes. They were subsequently washed with concentrated sulphuric acid and with ultrapure water and then dried under argon atmosphere. 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 (**1-3**) in aqueous 0.1 M sodium hydroxide for 2 hours, without stirring in order to avoid any mechanical damage of the surface by the magnetic stirrer bar (a minimal volume of solution is 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 were then dried under argon flow.

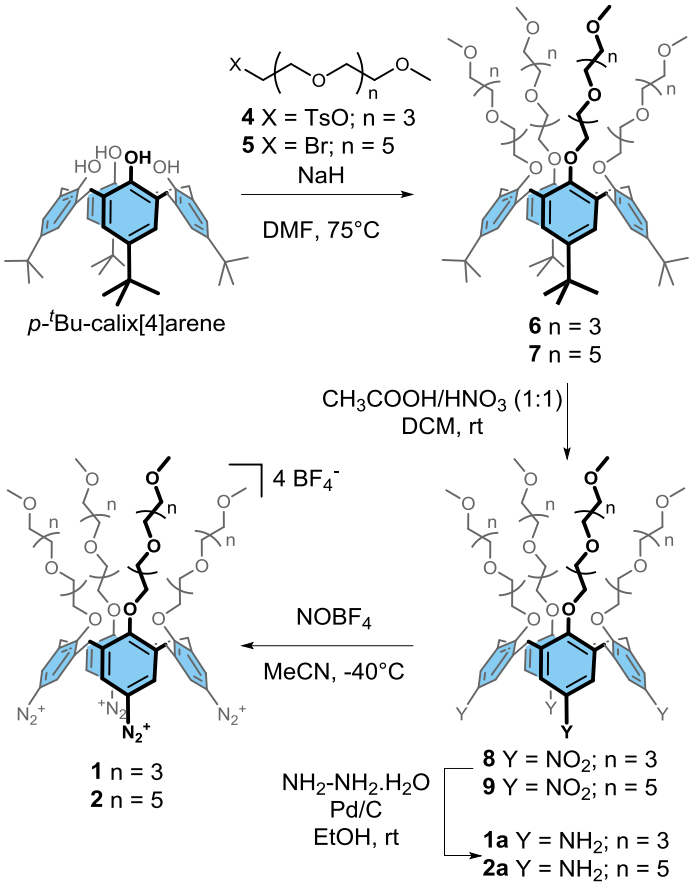
ATR-FTIR spectra were recorded at 22°C on a Bruker Equinox 55 FTIR spectrophotometer equipped with a liquid nitrogen-cooled mercury-cadmium-telluride (MCT) detector. The spectrophotometer was continuously purged with dried air. The target chemicals were deposited in solution on a germanium single-crystal internal reflection element (triangular prism of 6.8 x 45 mm, with an internal incidence angle of 45°) and the solvent was removed with a flow of nitrogen gas. The spectra from the grafted monolayer were obtained following the chemical grafting of the calix[4]arene target directly on the germanium internal reflection element. In each case, bare germanium was used for the background spectrum. The nonspecific adsorption experiment was measured in a flow-through cuvette with a 100 µg/mL solution of BSA in phosphate buffer media in D_2O (PBS-D_2O) and a flow rate of 10 µL/min. Opus software (4.2.37) was used to record 128 scans with a nominal resolution of 2 cm^{-1} for Figures 1 and S54 and 64 scans with a nominal resolution of 4 cm^{-1} for Figures 2, S55 and S56. Data were processed and analysed using the home-written Kinetics package in Matlab R2013a by subtraction of water vapor, baseline correction, apodization at 16 cm^{-1}.

Confocal Laser Scanning Fluorescence Microscopy: fluorescence images were recorded with a Nikon Ti-Eclipse inverted microscope, equipped with a laser emitting at 488 nm. The emitted light was collected in the epi-fluorescence mode and a dichroic mirror was employed to select

wavelengths shorter than 540 nm. A bandpath emission filter (515−530 nm), matching well the emission spectrum of fluorescein, was placed in front of the photo-multiplier tube. A 10× magnification objective (NA = 0.30, working distance 16 mm) was employed. The images were processed with the software ImageJ.

RESULTS AND DISCUSSION

Both calix[4]arene-tetra-oEGs **1** and **2** were prepared following the strategy developed for other calix[4]arene-tetra-diazonium salts (Scheme 2).[24,26] First, a tetra O-alkylation of *p*-^{t}Bu-calix[4]arene constrained the macrocycle in a cone conformation and allowed the introduction of the oEGs arms on the small rim. Then, a sequence of ipso-nitration / reduction afforded the tetra-anilines **1a** and **2a** in respectively 57 % and 52 % overall yields from *p*-^{t}Bu-calix[4]arene. Finally, a diazotation reaction yielded target diazonium salts **1** and **2** whose structures were clearly confirmed by 1D and 2D NMR spectroscopy. Grafting experiments were performed on gold and germanium surfaces with both calix[4]arenes **1** and **2** as well as with the reference compound **3** for comparison (Scheme 1).



Scheme 2. Synthesis of calix[4]arene-tetra-oEG$_n$-tetra-diazonium salts **1** and **2**.

Similarly to previously reported calixarenes,[26] grafting of the new oEGylated calixarenes was first evaluated on a gold substrate, using calix[4]-tetra-aniline precursor **1a**, from which the corresponding tetra-diazonium salt **1** was prepared *in situ* by adding an excess of $NaNO_2$ in acidic conditions. Reduction of the diazonium cations was performed by six voltammetric cycles. The density of the electrografted layer was characterized by analyzing its blocking property towards the electrochemical response of the redox probe $Fe(CN)_6^{3-/4-}$.[24] Complete inhibition of the electrochemical response was found, indicating that a dense organic layer had been formed at the electrode surface (Figure S51).

Having found that the electrochemical grafting methodology was applicable to the newly synthesized calix[4]arene-tetra-oEGs, we subsequently undertook the chemical grafting of the diazonium salts (**1**-**3**) on Au and Ge substrates. The previously reported protocols for the grafting of aryl diazonium salts on Ge in organic solvents requires an oxide-free Ge surface obtained by treatment with an aqueous HF solution.[19,20,21] Here, the grafting procedure on Ge substrate was performed in a basic aqueous solution and involved the *in situ* formation of diazoates.[44,45,46] Under these aqueous conditions, the laborious and hazardous pre-treatment with aqueous HF is not needed, as the Ge oxide at the surface is water soluble.[47] The chemical grafting of diazonium salts **1**, **2** and **3** was performed upon immersion of the Au and Ge substrates in an aqueous 0.1 M NaOH solution containing 5 mM of the desired diazonium salt for 2 hours. This procedure afforded gold surfaces **Au-1**, **Au-2** and **Au-3** and germanium surfaces **Ge-1** and **Ge-2** (Scheme 1). Each grafting experiment was repeated at least three times to assess the reproducibility.

All these modified surfaces were then characterized by contact angle measurements and the results were compared to those obtained with bare surfaces (*i.e.*, surfaces that were treated similarly to the modified ones but in the absence of the diazonium salt). Contact angles of 62 ± 9° and 30 ± 10° were obtained respectively for bare Au and Ge substrates (Table 1). For all the modified oEGylated surfaces, the contact angle was found to be *ca.* 56°. These results clearly confirm the presence of the oEG layer on all the modified surfaces and highlight the reproducibility of the grafting process. Furthermore, the value of *ca.* 56° is in agreement with those found in literature for oEGylated surfaces.[48]

Table 1. Contact angle measurements and thickness estimations of the grafted organic layers.

	Contact angle[a] (°)	Thickness[b] (nm)

Bare Au[c]	62 ± 9	-
Au-1	55 ± 5	2.4 ± 0.4
Au-2	56 ± 4	n.d.[d]
Au-3	56 ± 7	19.0 ± 1.0
Bare Ge[c]	30 ± 10	-
Ge-1	56 ± 1	3.0 ± 0.3
Ge-2	56 ± 3	n.d.[d]

[a] Average values obtained by multiple analyses repeated on several surfaces. [b] Determined by AFM measurement (see text). [c] The bare surfaces were treated similarly to the modified ones but in the absence of the diazonium salt. [d] n.d.: not determined.

Atomic Force Microscopy (AFM) measurements were performed on the surfaces **Au-1**, **Au-3**, **Ge-1** and on the bare gold and germanium substrates. All the modified surfaces exhibited a surface topography similar to that of the corresponding bare substrate, indicating a thin uniform deposit on the surface (Figure S52 and S53). Next, thicknesses of the organic layers were estimated in contact mode through scratching experiments. The AFM tip was used to scratch a square area on the modified samples by exercising a force sufficient to remove the organic part without damaging the substrate. Using the height difference between both areas of the surface profile (Figure S52 and S53), thicknesses of the organic layer of 2.4 and 3.0 nm were found for **Au-1** and **Ge-1** respectively (Table 1). These values correspond well with the height of the calix[4]arene-tetra-oEG$_4$ structure (*ca.* 2.2 nm estimated from MM2 energy minimizations with ChemBio3D software), suggesting that a monolayer of grafted calix[4]arene-tetra-oEGs **1** was obtained on gold and germanium. In the case of the control surface **Au-3**, a thickness of 19.0 nm was estimated, clearly indicating the formation of multilayers. This result highlights the crucial role played by the calixarene structure in the formation of monolayers.

The germanium surfaces **Ge-1** and **Ge-2** were then analyzed by ATR-FTIR spectroscopy. For comparison, the IR absorbance spectra of compounds **1**, **2** and their corresponding anilines **1a** and **2a** were recorded (Figures 1 and S54). Typical bands belonging to the calix[4]arene core and oEG groups were observed both for the compounds and the corresponding monolayers. The asymmetric COC stretching from the oEG chains around 1100 cm^{-1} is clearly visible in all spectra. Other bands can be attributed to the calix[4]arene moieties such as the symmetric COC_{Ar} stretching around 1050 – 1020 cm^{-1} and the aromatic ring stretching around 1460 cm^{-1}. The IR results thus clearly confirm the grafting of calixarenes **1** and **2** on Ge.

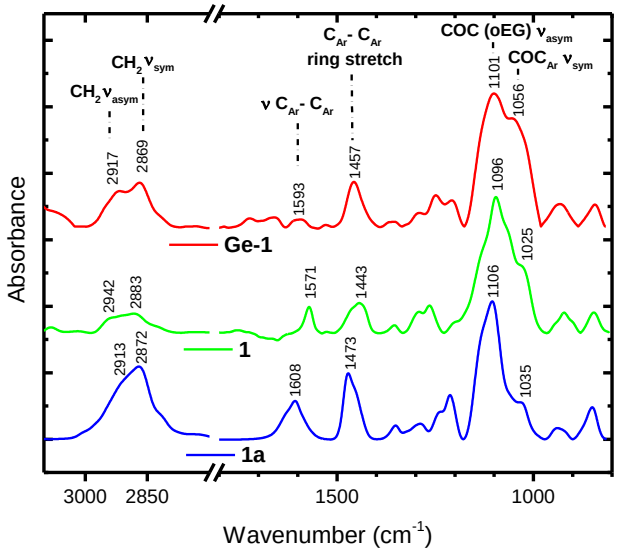


Figure 1. ATR-FTIR absorption spectra from 3100-2700 and 1800-800 cm^{-1} of modified surface **Ge-1** (red), calix[4]arene-tetra-diazonium salt **1** (green) and calix[4]-tetra-aniline **1a** (blue).

The nonspecific adsorption of proteins on bare Ge and on oEGylated Ge substrates was monitored by ATR-FTIR spectroscopy. Surfaces **Ge-1** and **Ge-2** were compared to evaluate the influence of the chain length of oEGs on the protein adsorption. A flow-through cuvette with a

solution of bovine serum albumin (BSA, 100 µg/mL) in phosphate buffer media in D_2O (PBS-D_2O) at 22°C was used to analyze the nonspecific binding of BSA on the surfaces. BSA is used as model protein in many investigations because it is one of the most abundant proteins found in blood and it adheres very well to surfaces. First, the stability of the calixarene layer under the study conditions was evaluated through exposure of the surface **Ge-1** to a flow (10 µL/min) of PBS-D_2O buffer during 16 h. The decomposition of the organic layer should give rise to the appearance of negative absorbance bands in the regions where the calixarene signals are located (*e.g.*, at 1100 cm^{-1}). This was not observed, indicating that the calix[4]arene monolayer is stable under aqueous conditions for at least 16 hours (Figure S55). The surfaces **Ge-1** and **Ge-2** were then placed under a flow (10 µL/min) of the PBS-D_2O medium for 10 min in order to reach an equilibrium state under aqueous conditions. The flow was then switched to the solution of BSA in PBS-D_2O for 16 hours and finally back to PBS-D_2O for 5 hours. Infrared spectra were recorded every minute for the first 20 minutes and then every 10 minutes till the end of the experiment. Adsorbed BSA was characterized by two bands whose intensities were increasing over time: the amide-I' at 1640 cm^{-1}, which is mainly associated with the C=O(ND) stretching vibrations and the amide-II' at 1450 cm^{-1}, which results mainly from in plane ND bending vibrations (Figure 2a and S56). The integral of the amide-I' band at 1640 cm^{-1} was calculated for each spectrum. The graph shows that the binding of BSA to surfaces **Ge-1** and **Ge-2** is reduced by more than 85% in comparison to bare Ge (Figure 2b). No difference was found between **Ge-1** and **Ge-2**, showing that an oEG length of four on the calix[4]arenes is sufficient for the effective prevention of nonspecific protein adsorption on Ge. Such an efficiency in the reduction of nonspecific adsorption has been reported scarcely and, in the case of the previous systems, either a very thick coverage was necessary (*e.g.*, polymer brushes)[48,49] or a limited stability was observed.[36]

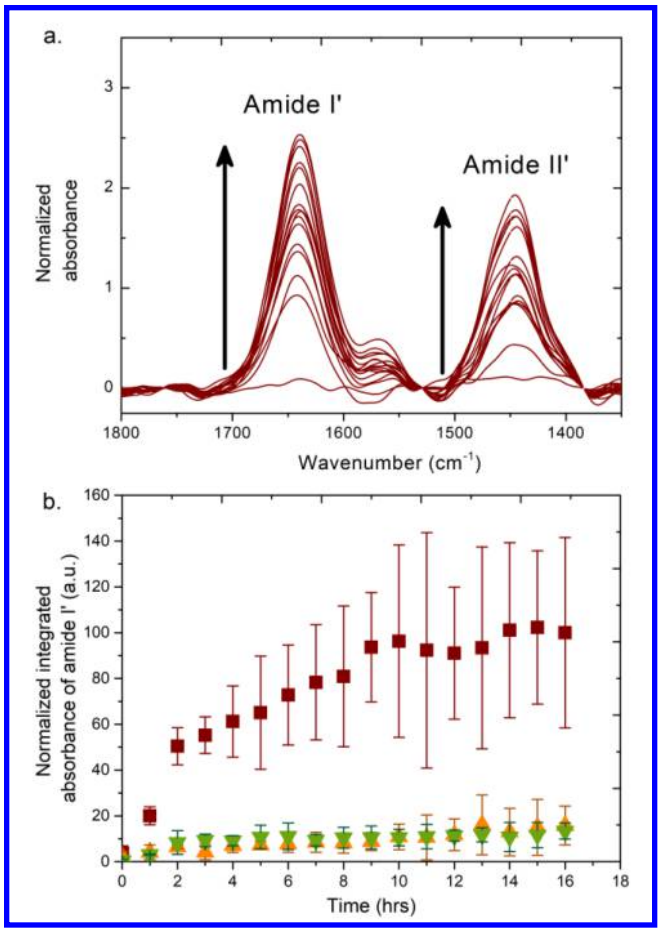


Figure 2. (a) ATR-FTIR absorption spectra from 1800-1350 cm^{-1} reported for every hour during the BSA nonspecific adsorption experiment on bare Ge. (b) Normalized adsorption of BSA on bare Ge substrate (dark red ■), **Ge-1** (orange ▲) and **Ge-2** (green ▼). The error bars correspond to the standard deviation obtained from two or three independent measurements.

The nonspecific adsorption of proteins was also evaluated by confocal laser scanning fluorescence microscopy. A defined area of Au and Ge surfaces was first modified with the calix[4]arene-tetra-oEG$_4$ **1**, the surfaces were then submerged in a 100 µg/mL solution of fluorescent FITC-BSA in PBS buffer for 6 hours, followed by rinsing in PBS buffer for 16 hours. The resulting fluorescence images are shown in Figure 3. On both images, two areas can be clearly

distinguished: the unmodified one serving as control (top) and the one modified with the calix[4]arene (bottom). On the modified zone, the fluorescence is significantly decreased, showing a drastic reduction of the nonspecific adsorption of BSA. This result is not quantitative, but gives a good indication of the remarkable antifouling character of the modified surfaces.

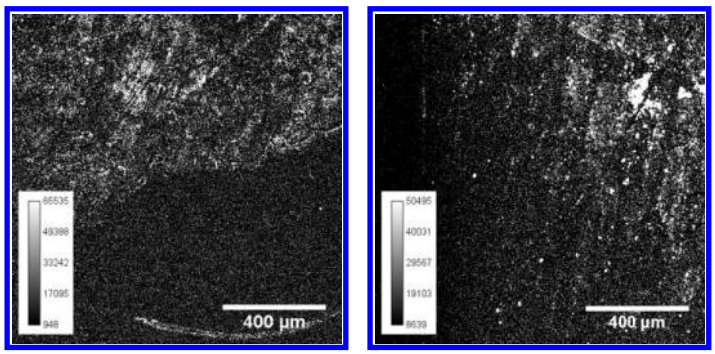


Figure 3. Fluorescence images acquired on gold and germanium surfaces that were partially modified with the calix[4]arene-tetra-oEG$_4$ **1** and subsequently immersed in a 100 µg/mL solution of fluorescently labelled BSA in PBS buffer for 6 hours.

CONCLUSIONS

In conclusion, we have developed a reproducible procedure for the grafting of thin and robust organic monolayers of calix[4]arene-tetra-oEGs on Ge and Au that do not require any laborious or dangerous pre-treatment. The significant antifouling properties of these monolayers were demonstrated. The nonspecific absorption of proteins was decreased by more than 85% compared to bare Ge. Such an efficiency is unique if we consider the thickness (*ca.* 3 nm) and the high stability of the calixarene layer. The modified Ge surfaces thus constitute promising devices for the development of ATR-FTIR-biosensors. Current developments in our laboratories are directed

toward the grafting of calix[4]arene-tetra-oEGs with terminal post-functionalizable groups (*e.g.* COOH, azide or alkyne groups) that can serve for the controlled immobilization of proteins.

ASSOCIATED CONTENT

Supporting Information

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

Detailed procedure for the preparation and characterization of the synthesized compounds, NMR spectra, AFM results, and additional FTIR experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: ijabin@ulb.ac.be; vrauss@ulb.ac.be

ORCID

Pascale Blond: 0000-0001-8515-1737

Alice Mattiuzzi: 0000-0002-3784-8449

Hennie Valkenier: 0000-0002-4409-0154

Ludovic Troian-Gautier: 0000-0002-7690-1361

Thomas Doneux: 0000-0002-9082-8826

Erik Goormaghtigh: 0000-0002-2071-2262

Vincent Raussens: 0000-0002-7507-1845

Ivan Jabin: 0000-0003-2493-2497

Notes

The authors declare no competing financial interest.

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