Strategies for the biofunctionalization of sulfur-based chalcogenide glasses

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Abstract

Effective surface functionalization plays a critical role in the selectivity and sensitivity of sensing devices. While chalcogenide glasses (ChG) have received a lot of attention as promising optical materials for mid-IR biosensors, there have been just a few reported instances of their successful (bio)functionalization. In the present work, we explore three different approaches for the grafting of heterobifunctional derivatives on Ge\textsubscript{25}Sb\textsubscript{65}S\textsubscript{65} substrates. One of these approaches consisted in the use of traditional silane chemistry to functionalize hydroxyl moieties on the ChG, while the other two are novel strategies based on the potential reactivity of maleimide and cyclooctyne derivatives for sulfhydryl moieties at the surface of the inorganic glass matrix. Using these approaches, biotinylated surfaces were prepared and the selective grafting of a fluorescent-labeled protein was then validated via IR spectroscopy, AFM and fluorescence. Our results showed that successful biofunctionalization of Ge-Sb-S ChG was achieved by all three methods. Moreover, the functionalization process can be easily adapted for substrates with any type of geometry and for the grafting of different molecules at the surface, paving the way for the future development of sensing systems.

Introduction

Chalcogenide glasses (ChG) are a family of glassy materials composed by the association of chalcogens (S, Se and/or Te) with Ge, As, Ga and/or Sb in an oxide-free atmosphere\textsuperscript{1,2}. These glasses present unique optical properties such a high non-linear refractive index and a broad transparency on the infrared (IR) spectra. Moreover, some compositions of ChG tend to be highly stable against crystallization, allowing their drawing into optical fibers\textsuperscript{1} as well as photonic circuits.\textsuperscript{3} Such features make these materials especially appealing for the design of IR optical sensing systems, which allows for qualitative and quantitative profiling of the fundamental vibrational absorption bands of organic molecules (located in the 4000 – 400 cm\textsuperscript{-1} region of the spectrum), resulting in a “fingerprint” of the chemical and biological species present on a sample.\textsuperscript{4} In this sense, the inherent molecular selectivity of mid-IR sensors present a great alternative for non-invasive, non-destructive, label-free and fast analysis for a wide range of applications including environmental monitoring, food security, pharmaceutical analysis and medical diagnostics.\textsuperscript{4,5}

However, compared with other sensing systems, mid-IR sensors present lower sensitivity which can be limiting for some biological applications.\textsuperscript{6,7} One way to overcome this issue consist in functionalizing the transducer with specific receptors, which are molecules with high affinity for the analytes of interest. The use of biomolecules such as antibodies or aptamers as receptors can selectively increase the quantity of target molecules near the sensing surface leading to a drastic improvement of the detection limits. While surface functionalization has been extensively studied for a wide set of materials, the particular nature of ChG as well as the heterogeneity of the functional groups available at their surface, which in turn limits the anchoring points for the grafting processes, have hinder progress in the chemical functionalization of these glasses.

A group led by Irudayaraj\textsuperscript{4} bypassed this problem by depositing nanometric-thick islands of gold over the ChG surface, allowing them to biofunctionalized their system via well-known thiol-gold interactions. While this is a practical solution, the deposition of a gold layers can affect optical/electrical features printed on the surface of the glass and possibly its sensitivity, limiting the potential of the system. On the other hand, a recent study employing engineered peptides\textsuperscript{8} with high affinity for Se show an intriguing method for the grafting of molecules via hydrophobic interactions
with Ge–Se–Te ChG glasses. Yet, due to the nature of the grafting process, the stability of the functional surfaces over time or under different conditions might be compromised. Considering all of these reasons, direct chemical functionalization by covalent bonding with the glass surface would be preferable. In this regard, a few examples have been reported in the literature using some heterobifunctional derivatives based on organosilanes, disulfides or arylazides and, more recently, engineered peptides with high affinity for Se. In most of these cases, however, UV light is employed either to promote surface activation or to initiate the coupling reaction at the surface. Due to the photosensitivity of ChG in the UV region, such treatment could induce structural changes in the bulk and not only at surface level, negatively impacting some of the properties of interest of the material. Such effects could be particularly important for sulfur-based ChG whose optical and electrical properties have been tuned for specific applications.

Chemical functionalization of these engineered sulfur-based ChG could pave the way to merge optical/electrical features into novel biosensing devices. However, a novel functionalization approach is required as current strategies pose serious threats to the integrity of its particular features or simply cannot be translated to this material. In the present work, we performed ATR-FTIR characterization of Ge-Sb-S ChG to determine functional groups that can be exploited as anchoring points for the grafting of organic compounds. Then, different strategies for the functionalization of each target moiety were conceived for the grafting of hetero-bifunctional linkers containing terminal biotin groups. Biotinylated surfaces were then used as a proof of concept for surface biofunctionalization, allowing the selective grafting of a fluorescent-labeled protein via Streptavidin-biotin interactions.

**Results**

*Surface analysis of pristine ChG and proposed strategies*

Firstly, we analyzed pristine ChG via ATR-FTIR (Figure 1) in order to elucidate which functional groups available at its surface could be exploited for surface functionalization. Our results indicate that, even without surface activation via UV/O₃ or plasma, hydroxyl moieties (broad band at 3480 cm⁻¹) were present in our samples. Moreover, an important amount of sulphhydril moieties (2500 cm⁻¹) were also available, hinting at several possible pathways for the modification of these materials.

![Figure 1. ATR-FTIR measurements of a pristine sample highlighting the presence of hydroxyl (OH) and sulphhydril (SH) moieties at the surface.](image)

Once we confirmed the presence of the possible targets at the surface of the ChG samples, we envisioned multiple functionalization approaches that adhered to the following constraints: (a) the functionalization method should not be limited by the geometry of the substrate, enabling the
The first strategy (Figure 2, Route I: Silane) corresponded to the classical silane chemistry approach which traditionally requires the use of UV/O₃ or O₂/plasma. The amount of hydroxyl moieties observed by ATR on our pristine samples suggested that surface activation might not be necessary to achieve successful grafting of organosilanes. While popular and commercially available, silane derivatives such as 3-aminopropyltriethoxysilane (APTES) produce irregular multilayers due to its polar end-group and the low carbon number resulting in a weak self-assembly capacity, inducing high variability in subsequent post-chemical modification procedures. For this reason we selected long-chain organosilanes prepared via hydrosilylation of a molecular precursor as reported by the team of Vellutini. Under this strategy, samples are exposed to freshly prepared solution of 11-bromoundecyltrimethoxysilanes under an inert atmosphere, leading to the formation of Bromine-terminated self-assembled monolayer (Br SAM). Then, nucleophilic substitution of the Br moieties is performed by immersion in a saturated solution of NaN₃ to afford the corresponding azide-terminated SAM (N₃ SAM).

Thanks to the azide-alkyne click chemistry further post-functionalization processes can take place without potential cross-reaction with native groups present in biomolecules of interest. To perform this step, we selected dibenzocyclooctyne (DBCO) derivatives which could serve two distinctive goals: (1) to provide fast and reliable coupling with the terminal azides from the organosilane SAM via strain-promoted azide-alkyne cycloaddition (SPAAC), and (2) as a potential novel strategy for direct grafting on the sulphydryl moieties of the ChG (Figure 2, Route II: Cyclooctyne).

Dibenzocyclooctyne has been widely used as a cooper free alternative to traditional alkyne-azide click reactions. Nonetheless, the affinity of cyclooctyne derivatives for thiol moieties, at least in solution, has been reported to occur in the presence of UV light or even sporadically. To the best of our knowledge, no work so far has described their interaction with inorganic sulphydryl moieties embedded in a solid substrate. Therefore, the possibility of using dibenzocyclooctyne derivatives on sulphydryl containing surfaces opens up an interesting venue for their potential use as a modal system where it can allow indirect functionalization (Route I, coupling with pre-existing organo-silane layer) or direct functionalization of a substrate (Route II, coupling with sulphydryl moieties on the ChG).

Similarly, maleimides have been broadly used due to their affinity for thiols in solution. Yet, their use for direct functionalization of sulfur containing materials has not been exploited (Figure 2, Route III: Maleimide). For this reason, we selected two maleimide derivatives as an alternative method for grafting on the sulphydryl moieties present on our ChG. The selected maleimide derivatives differed only in the nature of the linker connecting the functional moieties: one presented a hydrazine (rigid) and the other an ethylene glycol chain (flexible). Differences in the bio-accessibility of biotin between these two maleimide derivatives could provide crucial information for the successful biofunctionalization of the material, allowing further optimization steps for specific applications in the future.

Finally, all the biotinylated samples were exposed to a solution of a fluorescent-labeled protein (Streptavidin-Cy5) in a phosphate buffer containing a surfactant (Tween) as a passivating agent. The role of the passivating agent consisted in blocking non-specific adsorption sites allowing only selective binding of the protein via Streptavidin-biotin interactions. Moreover, the proteins were
labeled with a dye (Cy5: $\lambda_{ex}$ 647 nm and $\lambda_{em}$ 663 nm) leading to further validation of each functionalization strategy by fluorescence-based methods.

Figure 2. Schematic representation of the evaluated functionalization approaches: (Route I) Silane-based functionalization of hydroxyl moieties, (Route II) cyclooctyne-based functionalization of sulfhydryl moieties and (Route III) maleimide-based functionalization of sulfhydryl moieties. Surface biofunctionalization was performed in the presence of Tween®20 as passivating agent with Streptavidin-Cy5* (electron surface and crystal structure of streptavidin showing in red the biotin-binding regions. Image modified, under Creative Commons license, from Weisenburger et al. to show structure of the fluorescent dye.)

Surface characterization

Successful surface biofunctionalization was evaluated by ATR measurements. In order to facilitate visualization of the data, we focused our analysis on the region between 2000-1200 cm$^{-1}$ of pristine and biofunctionalized samples. All spectra were normalized based on the band at 1586 cm$^{-1}$, which can
be assigned to the bending mode of water molecules trapped inside the glass matrix, and a differential spectrum (biofunctionalized minus pristine) for each treatment was calculated (Figure 3).

All the samples presented a band at around 1739 cm\(^{-1}\), corresponding to a carbonyl ester group,\(^{32}\) after the biofunctionalization step. This band can be attributed to the presence of the surfactant Tween\(^{20}\) which was used as passivating agent to limit non-specific protein adsorption. Other bands of relevance are the amide I (1680-1630 cm\(^{-1}\))\(^{32}\) and amide II (1550-1510 cm\(^{-1}\))\(^{33}\) which can be ascribed to the presence of a protein (Streptavidin) and, to a lesser extent, to the contribution of the heterobifunctional biotinylated derivatives used. The silanized samples (Fig.3a and 3b) showed the highest intensity for both amide bands, followed by samples exposed to maleimide-EG\(_3\)-biotin derivative (Fig 3d) and then those immersed in DBCO-EG\(_4\)-Biotin (Fig 3c). The maleimide-hydrazine-biotin derivative (Fig.3e) presented the lowest intensity of amide bands among the functionalized samples, showing a similar profile to the reference (Fig. 3f: pristine ChG exposed to Streptavidin in PBS-Tween\(^{20}\) buffer).

Figure 3. Normalized ATR measurements of each sample, before surface functionalization (pristine) and after exposure to Streptavidin-Cy5, and their difference spectra. (a) ChG functionalized with a Bromide-terminated SAM exposed to DBCO-EG\(_4\)-Biotin, (b) ChG functionalized with a N\(_3\)-terminated SAM exposed to DBCO-EG\(_4\)-Biotin, (c) Pristine ChG exposed to DBCO-EG\(_4\)-Biotin, (d) Pristine ChG exposed to Maleimide-EG\(_3\)-Biotin (Mal-EG\(_3\)-Biotin), (e) Pristine ChG exposed to Maleimide-hydrazine-Biotin (Mal-Biotin), and (f) reference ChG exposed to Streptavidin in PBS-Tween\(^{20}\) buffer.
The biofunctionalized surfaces were then studied via Atomic Force Microscopy (AFM) to determine changes in their topography as well as in the viscoelastic properties induced by the grafting of proteins at their surface (Figure 4). Silanized substrates presented complex topographies with the highest roughness among the evaluated samples (RMS: 1.97 ± 0.08 nm for Br SAM, RMS: 2.5 ± 0.1 nm for N$_3$ SAM), such complex structures could be attributed to potential polycondensation of the silane moieties on our substrate. On the other hand, small particles were observed in the substrates functionalized with Mal-EG$_3$-biotin (RMS: 1.3 ± 0.2 nm), DBCO-EG$_4$-biotin (RMS: 1.0 ± 0.3 nm) and, to a much lesser extent, on Mal-hydrazine-biotin (RMS: 0.89 ± 0.06 nm). The height range of these particles, 3-6 nm, corresponds to previously reported AFM measurements of Streptavidin$^{8,34}$ supporting ATR results regarding the presence of the protein on the functionalized surfaces. Finally, almost none of such particles were observed on the pristine substrate exposed to Streptavidin-Cy5, this possibly due to the effectiveness of Tween®20 in limiting non-specific adsorption.

As a reference, topographic analysis of a pristine sample without any treatment is shown in Figure 4g. Further study of the data obtained by PeakForce Quantitative Nanomechanical Mapping (QNM) AFM shows clear differences in the adhesion profiles on the functionalized surfaces. These changes in the adhesion properties match the localization of the particles observed via topographic analysis, confirming that the composition of these structures is completely different from the background. In the case of the reference samples (pristine ChG with or without exposure to Streptavidin) uniform adhesion profiles can be observed, indicating homogeneity in the mechanical response of the surface.
Figure 4. Topographic and adhesion measurements via AFM of each sample after biofunctionalization. (a) Sample functionalized with a Bromide-terminated SAM exposed to DBCO-EG₄-Biotin, (b) sample functionalized with a N₃-terminated SAM exposed to DBCO-EG₄-Biotin, (c) pristine sample exposed to DBCO-EG₄-Biotin, (d) pristine sample exposed to Maleimide-EG₄-biotin, (e) pristine sample exposed to Maleimide-hydrazine-biotin, (f) reference sample directly exposed to Streptavidin-Cy5 and (g) pristine sample not exposed to Streptavidin-Cy5. (h) Root mean square (RMS) roughness calculated by AFM mapping of three different areas (0.5 x 0.5 µm²) per sample.

Microluminiscence analysis of the surfaces was then used to validate the presence of the fluorescent-labeled protein. For this assay, three different zones of 30 x 30 µm² were scanned for each sample using a confocal microscope coupled with a Raman spectrometer with a 633 nm laser for the
excitation of the Cy5 fluorophore (Figure 5). At micrometric scale, all the functionalized surfaces presented relatively homogenous levels of fluorescence with varying intensities. As with IR analysis, the substrates with an N$_3$ SAM showed the highest levels of fluorescence suggesting the highest quantity of proteins grafted at their surface. Samples functionalized with maleimide-EG$_3$-Biotin had slightly higher fluorescent signal than those with DBCO-EG$_4$-Biotin, which is also consistent with previous ATR and AFM observations. Br SAM surfaces presented significant levels of fluorescence whereas Mal-hydrazine-biotin surfaces barely show any signal. Finally, reference substrates (pristine ChG exposed to Strepta-Cy5, Figure 5f) presented no fluorescence at all.

**Figure 5.** Fluorescence validation of biofunctionalized surfaces. Cartographies of ChG functionalized with (a) Br SAM + DBCO-EG$_4$-biotin, (b) N$_3$ SAM + DBCO-EG$_4$-Biotin, (c) DBCO-EG$_4$-Biotin, (d) Mal-EG$_2$-Biotin, (e) Mal-Biotin and (f) pristine glass after exposure to Streptavidin-Cy5. (g) Median spectra calculated from all recollected data points per sample.
Discussion

Characterization by ATR of sulfur-based ChG (Ge$_{25}$Sb$_{10}$S$_{65}$) suggested the presence of hydroxyl and sulfhydryl groups available at surface level. Based on these results, we proposed multiple strategies based on the reactivity of such functional moieties with either organosilane, maleimide and cyclooctyne derivatives.

For the functionalization of hydroxyl moieties, we adapted a process previously reported by our team$^{21}$ for the silanization of silica substrates. Briefly, bromide-terminated organosilanes are grafted and then a post-functionalization step yields an azide-terminated monolayer that can be further modified via click chemistry. Typically, silanization processes involve a mild oxidation of the surface via UV/O$_2$ or O$_2$ plasma,$^{35}$ leading to an increase in the available hydroxyl moieties. While this surface activation can improve the grafting efficiency of silane-derivatives, such process could result in significant modification of physico-chemical and optical properties of our ChG. Considering ATR results showing that hydroxyl moieties are already present on the surface, no further surface activation was performed. Using the described method, organosilane SAM with inactive (Br) and active (N$_3$) terminal groups were prepared (Route I: Silane).

Silanized ChG (with Br- and N$_3$-terminated SAM) and a pristine ChG were then exposed to a solution containing the cyclooctyne derivative: DBCO-EG$_2$-Biotin. DBCO can bind the N$_3$ moieties from “active” silane SAM via click chemistry while, due to the cross-reactivity of cyclooctynes with thiols, possible direct grafting on sulfhydryl moieties on the ChG could be expected (Route II: Cyclooctyne). The inactive silane SAM (Br-terminated), whose terminal group does not react with the cyclooctyne, was used to observe the effect of a pre-existing organic layer in the accessibility of the sulfhydryl moieties. In this way, we could estimate the contribution of direct functionalization (coupling of cyclooctynes directly on sulfhydryl moieties) in the fluorescence signal observed on “active” silane SAM (N$_3$-terminated). Such information can be valuable in order to better understand effects of mixed SAM using both functionalization strategies.

After the biofunctionalization step, ATR measurements show the presence of the characteristic amide I and II bands on all the three samples (silane Br- and N$_3$-terminated SAM as well as direct grafting of DBCO-derivative), suggesting effective binding of the protein at the surface. AFM measurements showed the presence of particles of 3-6 nm in height, which is the size range of Streptavidin, and with an adhesion profile drastically different from the rest of the ChG surface on the sample exposed directly to DBCO-EG$_2$-Biotin, while complex structures were observed on the substrates with a silanized SAM. These complex structures might be the result of polycondensation of the silanes forming aggregates at the surface of the ChG. While this particular silanization protocol has been proven to form monolayers on silica substrates,$^{21}$ the heterogeneous distribution of surface moieties and the low grafting density of silanes on our ChG might promote the formation of such aggregates under the tested conditions. The high sulfur to germanium ratio of our ChG might result in a relative disparity of the surface distribution and density of hydroxyl and sulfhydryl moieties. After the grafting of organosilanes, this inhomogeneous distribution of functional groups at the surface of the ChG might result in an incomplete layer of silanes with low grafting densities.

Further surface analysis showed that the biofunctionalized sample with an “inactive” silane layer presented a Cy5 fluorescence signal -15% lower than a sample without a silane SAM. This result suggests that zones rich in sulfhydryl moieties remain accessible to be modified by the DBCO heterolinker even in the presence of a pre-existing organic layer. Moreover, such heterolinkers can be further biofunctionalized with only minor losses possibly limited by steric hindrance of the terminal groups. Coherently, biofunctionalization of “active” silane layers resulted in an increase of 53% of Cy5 signal compared to the sample without SAM. Therefore, the functionalization of sulfhydryl and
hydroxyl moieties with biotin-derivatives led to a significant increase in the potential binding sites for Streptavidin. Moreover, possible polycondensation of the silane would lead to higher amount of N\textsubscript{3}-terminal groups heterogeneously distributed in the surface leading to zones richer in biotin and thus, into aggregates of Streptavidin as observed in the AFM topographic and adhesion profile images (Figure 4b).

Regarding our third approach, the use of maleimide derivatives for direct functionalization of sulfhydryl moieties, it is important to highlight that the selective reaction of maleimide derivatives with thiolated compounds, in solution at about neutral pH, has been extensively studied and it is recognized as one of the most efficient Michael-type addition.\textsuperscript{30,36} Yet, no instance of their use on inorganic sulfhydryl containing surfaces has been reported so far. For this strategy, two maleimide-biotin derivatives: Mal-biotin (rigid hydrazine linker) and Mal-EG\textsubscript{3}-Biotin (flexible ethylene glycol linker) were employed in order to evaluate the role of the linker chain in further coupling assays.

When analyzing Mal-biotin treated surfaces via ATR, no significant differences with the reference sample was observed in terms of amide I and II band intensity. However, AFM images showed the presence of particles in the size range of Streptavidin at very low densities. This result was further validated via fluorescence analysis of the surface which show a very weak signal of the Cy5 fluorophore. Interestingly, functionalization of the substrates using the same terminal groups but replacing the hydrazine by a short ethylene glycol chain (Mal-EG\textsubscript{3}-Biotin) showed significantly different results. In this case, high intensity of the characteristic amide I and II bands were observed by ATR denoting the presence of proteins after the biofunctionalization step. AFM images also showed high density of the same type of particles observed on the previous tests, while fluorescence measurements of the substrate presented the second highest Cy5 intensity among all the evaluated samples.

For both treatments with maleimide derivatives, maleimide-sulfhydryl reaction appears to be an effective method for the grafting of organic compounds over sulfurous ChG. However, either due to steric hindrance and/or out-of-plane coupling to the surface, replacing the rigid hydrazine linker by a more flexible ethylene glycol chain seems to greatly improve the availability of the biotin terminal group and thus, the amount of Streptavidin captured. The use of maleimide derivatives with flexible linkers seems to be provide higher levels of biofunctionalization than cyclooctynes as ATR results showed higher intensity of amide I and II bands, AFM images suggest a higher number of proteins per surface area and fluorescence showed higher amounts of Cy5 on the surface for samples modified with Mal-EG\textsubscript{3}-Biotin than for those with DBCO-EG\textsubscript{4}-Biotin.

While the presence of a base/nucleophile, Michael acceptor and the thiol used will affect the kinetics and mechanism of a thiol-Michael reaction,\textsuperscript{36} it is expected that maleimides present higher affinity for thiols than cyclooctynes. In our tests, both treatments (maleimide- and cyclooctyne-based functionalization) were performed under the same conditions (concentration of derivative, solvent, temperature and incubation time) and the maleimide-based approach showed better results either due to its higher affinity for the substrate or other factors such as steric hindrance during the coupling. Such results might be of interest for the use of both approaches in the development of mixed surfaces. Our previous tests with Br\textsuperscript{-} and N\textsubscript{3}-SAM showed the possibility of functionalizing both hydroxyl and sulfhydryl moieties of the ChG. Yet, in this case we employed the same terminal group for the grafting of Streptavidin. One could then envision a different system where saturation of available sulfhydryl moieties is achieved by the use of maleimide-derivatives (allowing the grafting of molecules of interest) while organosilanes with terminal azides can be “clicked” with DBCO derivatives that present affinity for a different (bio)molecule.
Conclusion

Our results prove that Ge-Sb-S ChG can be successfully functionalized by either of our three proposed approaches.

Nevertheless, the highest amount of grafted protein was observed when using the silanization route. This could be explained due to a combined effect of direct and indirect functionalization of the surface by the DBCO-derivative, allowing simultaneous functionalization of the hydroxyl groups (via silanization with N₃-terminal derivatives) and sulphhydryl groups (via direct coupling with the cyclooctyne). While it is unclear the potential effect of polycondensation, this approach could be employed for future development mixed functional layers with varying terminal groups.

Moreover, cyclooctyne and maleimide-based functionalization methods showed to be highly promising for the functionalization of sulfur-based ChG, as both strategies are insensitive to the presence of water largely simplifying the grafting process. To the best of our knowledge, this work represents the first reported result regarding the use of maleimide and cyclooctynes for the biofunctionalization of sulphhydryl moieties embedded in solid state substrates, establishing the bases for the use of these systems in biosensing applications.
**Experimental methods**

**Materials**

ChG glasses were prepared using high purity elemental Ge, Sb and S (Alfa Aesar, 99.999%). Hydrosilylation was performed using the catalyst Platinum (0)-1,3-divinyl-1,1,3,3-tetramethyldisiloxane (Sigma-Aldrich, 2%), trimethoxysilane (Sigma-Aldrich, 95%) and 11-bromoundecene (Sigma-Aldrich, 95%). Substitution of the bromide group was done with sodium azide (TCI, >99.0%). Surface (bio)functionalization required the use of Biotin-dPEG₃-Maleimide (Quanta Biodesign, >90%), Dibenzocyclooctyne-PEG₄-biotin (Sigma-Aldrich), Biotin-maleimide (Sigma-Aldrich, 95%) and Streptavidin Cy₅ Conjugate (Invitrogen, 0.2 mg/mL).

**Synthesis of ChG glasses:**

Chalcogenide glasses were prepared based on the stoichiometric composition Ge₂₅Sb₁₀S₆₅. Raw materials were weighed out in a glove box under a nitrogen atmosphere before being inserted in a quartz ampule. The ampules were then put under vacuum (10⁻² mbar) and sealed with an oxygen-methane torch. To ensure homogeneity of the mixture, the samples were heated on a rocking furnace and a heating rate of 1 °C min⁻¹ up to 850 °C was set. After 12 h of homogenization at this temperature, the furnace was stopped and the system was left to gradually decrease in temperature until 750 °C before quenching in water. Then, the samples were annealed for 6 h at 10 °C below its glass transition temperature (T_g: 355 °C). Finally, the glasses were cut and polished into ~1 mm thick disks of 1 cm of diameter. Before each functionalization approach, all substrates were cleaned by sonication in CHCl₃ for 15 min and dried under a N₂ flow.

**Functionalization via silanization:**

A hydrosilylation and silanization processes were performed as described by the team of Vellutini and Genin.²⁰,²¹ Briefly, 500 µL of acetonitrile are added to a Schlenk tube under an argon atmosphere followed by 11-bromoundecene (5x10⁻² mmol), distilled trimethoxysilane (2.5x10⁻¹ mmol) and a platinum catalyst (Platinum(0)-1,3-divinyl-1,1,3,3-tetramethyldisiloxane, to a final concentration of 5% v/v). The mixture is left at room temperature under constant agitation for 1 h to obtain 11-bromoundecyltrimethoxysilane. Afterwards, the system is put under vacuum for 1 h to evaporate the solvent.

The different ChG substrates were set in the silanization flask under an argon atmosphere. Two different solutions, one of the freshly prepared 11-bromoundecyltrimethoxysilane (5x10⁻⁵ mol) and another of trichloroacetic acid (TCA) (0.8 mg, 5x10⁻⁶ mol) each in 50 mL of anhydrous toluene, were successively added to the silanization flask and kept at 20 °C for 16 h. Afterwards, the substrates were cleaned by subsequent cycles of 5 min sonication in toluene (2x), milliQ water (2x) and CHCl₃ (2x), and dried under N₂ flow.

To prepare N₃-terminated SAM, a nucleophilic substitution of the bromide with azide moieties was performed. Substrates with a bromine-terminated SAM were immersed in a saturated solution of sodium azide in dry DMF for 48 h, r.t. and under an argon atmosphere. Finally, the substrates were sonicated in cycles of 5 min in DMF (2x), milliQ water (2x) and CHCl₃ (2x), and dried under N₂ flow.

**Functionalization via maleimide-sulphydril interaction**

Pristine samples were immersed in a solution of 6.4 mL milliQ water mixed with 1.475 mL DMSO and 125 µL of maleimide-EG₃-Biotin (6x10⁻³ M in DMSO), final concentration of Mal-EG₃-Biotin of 9.4x10⁻⁵ M. After 16 h at room temperature in an orbital shaker, the samples were sonicated in cycles of milliQ water (2x) and CHCl₃ (2x), and then dried under N₂ flow. The same protocol was then employed using another derivative which replaced the ethyleneglycol linker with a hydrazine.
**Functionalization via DBCO-sulfhydryl interaction**

Pristine samples were immersed in a solution containing 6.4 mL milliQ water mixed with 1.35 mL DMSO and 250 µL of DBCO-Biotin (3x10^{-3} M in DMSO), final concentration of DBCO-Biotin of 9.4x10^{-5} M. After incubation for 16 h at room temperature in an orbital shaker, the samples were sonicated in cycles of milliQ water (2x) and CHCl$_3$ (2x), and dried under a N$_2$ flow.

**Strain-promoted alkyne-azide cycloadditions (SPAAC)**

Substrates with a N$_3$-terminated SAM were immersed in the same solution used for evaluating DBCO-sulfhydryl interactions. After incubation for 16 h at room temperature in an orbital shaker, the samples were sonicated in cycles of milliQ water (2x) and CHCl$_3$ (2x), and dried under a N$_2$ flow.

**Biofunctionalization via biotin-streptavidin interaction**

Each substrate was covered with a solution of PBS-T (Phosphate Buffer Saline with Tween$^{®}$20 at 0.05% v/v) containing Streptavidin-Cy5 (10% v/v, 0.1 mg. mL$^{-1}$) and left in a humid chamber (relative humidity ~90%) at room temperature for 1 h. The samples were then immersed in cycles of 5 min (three times per solution) in PBS-T and PBS. Finally, the samples were immersed in milliQ water thrice to avoid formation of salt crystals at the surface.

**Surface evaluation via Infrared spectroscopy**

Attenuated Total Reflectance (ATR) spectra were measured on ThermoScientific FTIR spectrometer at a resolution of 4 cm$^{-1}$ using silver gate accessory (Ge crystal) and a DTGS detector. The Ge crystal accessory was selected due to the high reflective index of the ChG samples as well as to avoid damaging the substrate during the measurement.

**Atomic Force Microscopy (AFM) analysis**

The topography as well as shifts in the viscoelastic properties of the surface were evaluated by AFM. Samples were scanned using a AFM Dimensions Icon (Bruker) on PeakForce QNM mode with PFQNE-AL cantilever and the data was then processed with the open-source software Gwyddion.$^{37}$ For each sample, at least three regions of 1 x 1 µm$^2$ were recorded (digitally zoomed areas of 0.5 x 0.5 µm$^2$ are present in the manuscript to aid the visualization). As the force applied by the tip was automatically adjusted and varied from sample to sample, in order to simplify comparison among the different surfaces, we set the mean adhesion value for each surface to zero.

**Fluorescence evaluation of biofunctionalized surfaces**

Microluminescence images were acquired using a LabRAM HR Evolution (Horiba) spectrometer with a 633 nm laser source focused using a 50x (N.A. 0.9) objective lens. For each treatment, cartographies from 3 different areas were obtained (each consisting of ~256 spectra). In order to compare the fluorescence intensity among samples, all the spectra were normalized on the Raman band of the ChG at 637 nm (635-640 nm region) and an averaged spectrum was calculated based on the median values for each treatment. Cartographies were obtained from the integration of normalized spectra in the 670-680 nm region.
References


