Supporting Information

Enhancing the sensitivity of biotinylated surfaces by tailoring the design of the mixed self-assembled monolayer synthesis

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**Figure S1:** Multistep approach to the in-situ synthesis of the biofunctionalized mixed SAM starting from a 3MPA:11MUA (10:1 molar ratio) solution. In this scheme we are assuming: 1) all the carboxylic groups are activated with the EDC/NHSS, 2) the steric hindrance of the biorecognition element drives the biofunctionalization on the longer chains, while the shorter chains are not involved, 3) all the unreacted succinimide esters react with the ethanolamine leading to the formation of an amodic group.

**Figure S2:** Schematic representation of the two different starting SAMs used in this study.
Synthesis of the N-(2-hydroxyethyl)-3-mercaptopropanamide (NMPA)

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich, Alfa Aesar, or Acros Organics. Thin layer chromatography (TLC) was performed using plates from Merck (silica gel 60 F254). Column chromatography was performed with Merck silica gel 60 Å (63–200 μm) as the stationary phase. Flash chromatographic separations were performed either using Merck silica gel 60 Å (15–40 μm). $^1$H-NMR spectra were recorded in the indicated solvent on an Agilent 500-vnmr 500 spectrometer (499.801 MHz). The following data are reported: chemical shift (δ) in ppm, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, dd = doublet of doublets, dt = doublet of triplets, m = multiplet, br = broad signal), integration, and coupling constant (J) in hertz. $^{13}$C NMR (125 MHz) were recorded on a 500-vnmr 500 spectrometer (499.801 MHz) on novel final compounds: chemical shift (δ) in ppm were reported. HRMS-ESI analyses were performed on a Bruker Daltonics MicrOTOF-Q II mass spectrometer. All spectra were in accordance with the assigned structure.

The synthesis of NMPA was performed according to an already reported procedure [1], as depicted in Figure S3.

Figure S3: Reaction scheme for the synthesis of NMPA.

The commercially available 3-mercaptopropanoic acid was activated on the COOH group and protected on the thiol group by reaction with ethyl chloroformate and N,N-diisopropylethylamine (DIPEA) in tetrahydrofuran (THF). The obtained intermediate A, without further purification, was reacted with ethanolamine obtaining the intermediate B that was deprotected with potassium tert-butoxide (tBuOK), in dry methanol (MeOH) at room temperature, affording the desired NMPA. The crude NMPA was purified by gradient column chromatography DCM (dichloromethane) 100% → DCM/THF/MeOH:90/7/3 obtaining a colorless oil with an overall 51% yield.

$^1$H-NMR (DMSOd$_6$) δ: 7.85 (s broad, 1H), 4.62 (t, 1H, J=5.4 Hz), 3.37 (q, 2H, J=5.8 Hz), 3.10 (q, 2H, J=5.8 Hz), 2.62 (t, 2H, J=7.1 Hz), 2.36 (t, 2H, J=7.1 Hz), 2.25 (s broad, 1H). (Fig. S4). $^{13}$C-NMR (DMSOd$_6$) δ: 20.39, 34.39, 41.90, 60.32, 179.72. (Fig. S5). HRMS-ESI for C$_5$H$_{11}$NO$_2$S (m/z): [M-H] calcd, 148.0438; found, 148.0435.
Figure S4: $^1$H-NMR spectrum of the resulting NMPA.
Figure S5: $^{13}$C-NMR spectrum of the resulting NMPA.
**SPR measurements**

The SPR sample-holder allows the exposure of a mm²-area in a static-flow cell endowed with a 100 µL volume, where the proper solution is slotted. For the real time monitoring of the biotinylation protocols (Fig. S6), the SAM-modified slides (Fig. S2) were mounted and an initial baseline was acquired by water rinsing to check SPR conditions in liquid range. Afterwards, the baseline in the angular scan experiments (Fig. S7) was acquired in 2-(N-morpholino)ethane-sulfonic acid (MES) buffer followed by injection of 1-Ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) and N-hydroxysulfosuccinimide sodium salt (NHSS) (0.2 M/0.05 M) solution in MES to activate the alkanethiol carboxylic terminal groups for 15 min (STEP 1). After a 5 min rinsing with PBS, pentyamine-biotin (0.1 mg/mL in PBS) was injected and left in contact for 90 min (STEP 2). The system was rinsed with PBS before injecting a 1 M Ethanolamine-HCl (EA) solution for 45 min, to ensure the saturation of unreacted NHS esters sites (STEP 3). Typical angular shifts recorded with time during biofunctionalization for both protocol a and b are reported in Fig. S7.
Figure S6: Biofunctionalization protocols for the two different starting SAMs on gold proposed in this study: a) 3MPA:11MUA (10:1), b) NMPA:11MUA (10:1). Schemes describe reactions in a qualitative way.
Figure S7: Functionalization process of starting SAMs immobilized on SPR gold surface. The sensogram reports angle shifts of the plasmonic peak over time for (a) 3MPA:11MUA SAM and (b) NMPA:11MUA SAM.

Monitoring of Streptavidin binding

Before starting with streptavidin (SA) injection, the biotinylated gold slides were first washed with PBS several times monitoring the baseline signal until stability (about 20 min). Nine increasing concentration of SA were tested on SAMs prepared according to the two different procedures, comparing the SPR response of those systems. The dose response was performed by manual injection of 1 mL of each SA solution over the SPR cell (100 µL) in static conditions for at least 30 min in the
concentration range 500 pM - 1.6 µM. The unbound analyte was washed by injecting PBS for 10 min through the cell after each SA solution exposure.

The SPR angle shift (Δθ) observed on the biotinylated SAMs versus SA concentration is reported in Fig. S8. A linear regression fit was performed for both cases in the range of SA 500 pM – 25 nM (red dotted lines in the Figure). Sensitivity was expressed as the slope of the linear plot. In order to calculate the limit of detection (LOD), a response equal to the blank signal + kσ₀, hereby taking k = 3 and σ₀ = the blank standard deviation, was taken. This response was interpolated with the linear regression; extrapolating the corresponding SA concentration (LOD).

Figure S8: Linear fitting of The SPR angle shift vs increasing SA concentrations in the in 500 pM-25 nM range referred to 3MPA:11MUA biotinylated SAM (left), NMPA:11MUA biotinylated SAM (right).
Attenuated Total Reflectance – Infrared Characterization

SPR Navi-200 glass sensor slides covered by a 50 nm gold layer were functionalized using the same protocols reported in Fig. S6. ATR-IR analysis was carried out with a Perkin-Elmer Spectrum-Two instrument mounting the specific ATR module equipped with diamond prism. Substrates were rinsed with HPLC water and dried under nitrogen before the analysis. Background was acquired versus air (resolution 2 cm\(^{-1}\), 64 scans). Each sample was analysed in three different points in the same conditions reported for background in order to evaluate in-plane reproducibility. Spectra were acquired in the range between 4000 and 400 cm\(^{-1}\) reporting transmittance (%) vs wavenumber (cm\(^{-1}\)). The most interesting peaks were identified between 1850 cm\(^{-1}\) and 1400 cm\(^{-1}\) [2]. This range can be divided in three different regions: the carbonyl stretching region (1750-1600 cm\(^{-1}\)), the aliphatic N-H bending region (1600-1500 cm\(^{-1}\)) and the methylene bending region (1500-1400 cm\(^{-1}\)). Pentylamine-biotin spectrum was acquired by analysing a thin film deposited by drop casting of a 0.1 mg/mL solution in PBS on a SiO\(_2\) surface. Both background and sample spectrum were carried out using the same parameters reported above, except for the number of scans (16). Raw data were processed using the Perkin-Elmer Software (Spectrum) and Origin Pro 2015. Savitzky-Golay filter was applied for baseline correction of IR spectra.
**Table S1:** Vibrational assignment and IR positions of signals identified in spectra 1-4 (Fig. 1). Positions signed with an asterisk are referred to broad bands and are reported without error. Aliphatic amides signals in [2] are referred to a 3MPA:11MUA SAM functionalized using ammonia instead of ethanolamine, being responsible for the slight differences in the positions. sh = shoulder

| Vibrational Assignment | Position (cm⁻¹) | Position (cm⁻¹) |
|------------------------|----------------|----------------|
| **Spectrum 1**         | [this work]    | [2-4]          |
| Symmetric stretching COO^- | 1438±2       | 1461           |
| C-H bending            | 1470sh        | 1468           |
| Asymmetric stretching COO^- | 1570*       | 1520/1550      |
| C=O stretching         | 1720*         | 1720/1740      |
| **Spectrum 2**         |                |                |
| C-N stretching         | 1418±1        | 1408           |
| C-H Bending            | 1460*         | 1468           |
| N-H Bending            | 1550*         | 1610 [2]       |
| C=O stretching (Aliphatic amides) | 1651*  | 1675 [2]       |
| N-H Bending (Biotin ring amides) | 1645 |                |
| C=O stretching (Biotin ring amides) | 1699 (component in C=O band) | 1702 |
| C=O stretching         | 1720*         | 1720/1740      |
| **Spectrum 3**         |                |                |
| C-N stretching         | 1416±1        | 1408           |
| C-H Bending            | 1457±1        | 1468           |
| 1474±1                |                |                |
| N-H bending            | 1559±1        | 1610 [2]       |
| C=O stretching (Aliphatic amides) | 1650*  | 1675 [2]       |
| C=O stretching         | 1720*         | 1720/1740      |
| **Spectrum 4**         |                |                |
| C-N stretching         | 1419±1        | 1408           |
| C-H Bending            | 1457±1        | 1468           |
| 1474±1                |                |                |
| N-H bending            | 1560±1        | 1610 [2]       |
| C=O stretching (Aliphatic amides) | 1650*  | 1675 [2]       |
| C=O stretching         | 1720*         | 1720/1740      |
Molecular Dynamics simulations

All the simulated systems were solvated using TIP3P water [5]. 47 Na\(^+\) and 47 Cl\(^-\) were added to each simulated system. MD simulations were performed using the NAMD 2.13 package [6] and the CGenFF [7,8] force-field with RESP charges [9] calculated at the HF/6–31 G(d,p) level of theory using the Gaussian package [10]. Following the protocol adopted by Macchia et al. [11], gold atoms were removed and, to ensure the charge neutrality of the system, hydrogen atoms were added and kept constant during the MD simulations to mimic the bulk constraints. Lennard–Jones interactions were modelled using a cut-off of 12 Å employing a switching function (switching radius of 10 Å). Electrostatic interactions were treated using the Particle–Mesh–Ewald (PME) method [12], with a real-space cut-off of 12 Å and a grid spacing of 1 Å per grid point in each dimension. The Langevin thermostat [13] (damping coefficient 1 ps\(^{-1}\)) was employed to simulate the canonical ensemble. For each system, a 100 ns-long MD simulation at T = 25 °C was performed using a time step of 1 fs and storing the coordinates every 10,000 steps (10 ps). All simulations were performed on the GALILEO supercomputer at CINECA, Italy. The H-bond occupancy, that is the percentage of frames in which a given H-bond is detected, was computed using as thresholds a distance atom acceptor (AA)-atom donor (AD) equal to 3 Å and an angle AD–H–AA equal to 150°. Root Mean Square Fluctuations (RMSF) were calculated for each biotinylated 11-MUA (B-11MUA) of all the simulated systems. For all the considered systems, the equilibration of the structure required less than 10 ns and thus the first 10 ns were removed from the analysis.

**Figure S9:** a) Top view of a simulated system. Notice that the x-y plane is 132 Å x 122 Å wide; b) 2D sketches of the simulated systems. Blue, green and red balls indicate the presence of NMPA, B-11MUA and B-3MPA chains respectively.

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