Antifouling and Antithrombogenic Ultrathin Surface Chemistry for Bioanalytical and Biomedical Applications

by

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Department of Chemistry
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Abstract

Whether the aim is to merely prevent the adsorption and accumulation of biological species, or to inhibit the surface-mediated activation of potentially harmful biological processes, biomaterial research invariably faces the need for man-made, foreign surfaces intimately contacting bodily fluids/tissues to be ‘bioinert’. A popular strategy to address this technological constraint consists in passivating substrate materials with an antifouling (respectively a biocompatible) organic coating. Yet, despite tremendous research activity and progress in recent times, efficient adlayers are scarce, and endowing artificial surfaces with such properties remains, in essence, a difficult task. This PhD Thesis describes recent research contributions in the development of original and versatile stealth coatings, based on novel oligoethylene glycol trichlorosilane surface chemistry, for bioanalytical and biomedical healthcare applications. Surface modification also has the advantage of being straightforward, rapid and inexpensive.

One primary objective was to engineer biosensors capable of selectively and sensitively detecting target analytes in real-world biofluids – exploiting the transducing technology of the ultra-high frequency electromagnetic piezoelectric acoustic sensor (EMPAS) system – as potential clinical assay alternatives to current screening/diagnostic tests. Biosensing platforms featured dual-functional, binary organosilane surface chemistry on quartz combining high
analyte binding capability (for biorecognition) with pronounced antifouling properties (to minimize the otherwise overwhelming interference signal from the biological matrix). Clinical testing performance was successfully demonstrated through the detection of bacterial endotoxin – a potent pathogen associated with the highly-incident, deadly condition of sepsis. EMPAS measurements performed in full human blood plasma (and in a real-time and label-free advanced fashion compared to modern clinical assays that rely on chromogenic reporter molecules) showed that samples at abnormally high concentration (1000 pg/mL) can be readily differentiated from those presenting basal endotoxin level (< 10 pg/mL).

Unimolecular, subnanometric silane adlayers featuring a single type of ultrashort monoethylene glycol-terminated chains (MEG-OH) also displayed pronounced antifouling behaviour, against full serum. Further experimental and computational studies collectively corroborated the mechanistic hypothesis according to which water would play a critical role in this respect – through the formation of a permeant, tightly coordinated hydration network, whose disturbance by foulants was rationalized to constitute a penalty in terms of energy and generate repulsive forces. Another major finding was the key participation of the single, internal ether atom of oxygen in the MEG chains in maintaining such a stable, nanoscale zone of hydration. The scope of derivatizable substrate materials of biotechnological importance was also readily expanded to gold (with adapted thiol anchoring chemistry) and polycarbonate polymer. In the latter work with plastic, the unique MEG-OH ‘nanogel’ ultrathin surface chemistry was also shown to display remarkable antithrombogenic properties, far exceeding those of the bare plastic substrate. More importantly, thrombus growth was nearly non-existent. Such performance was quite outstanding considering the fact that whole human blood did not require any anticoagulant treatment to prevent clotting (besides its standard collection and storage in heparinized tubes) despite being exposed to a foreign surface, in vitro.
To My Family, With Love
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Figure 27. Molecular structure of poly(SBMA-co-NIPAAm) copolymers.

Figure 28. Molecular structure of PLL-g-PEG copolymers.

Figure 29. Molecular structure of two variants of PLL-g-PMOXA copolymers.

Figure 30. Molecular structure of short, non-ionic monomers for ‘grafting from’ polymerization: (A) 3-hydroxypropyl (HPMA), (B) 2-hydroxyethyl (HEMA), and (C) 2-hydroxypropyl (HPM) methacrylate esters; (D) N-(2-hydroxypropyl) methacrylamide (HPMA); (E) 2-hydroxyethyl acrylamide (HEAA); and (F) non-substituted acrylamide.

Figure 31. Molecular structure of the polyCBMA-$bl$-PPO-$bl$-polyCBMA zwitterionic poloxamer derivative ($n = 40$, $m = 48$).

Figure 32. Molecular structure of (A) polyMeOEGMA-$bl$-polyCBAA and (B) polyHEMA-$co$-polyCBAA hybrid copolymer brushes.
Figure 33. Molecular structure of the (A) ammonium/sulfonate and (B) ammonium/carboxylate mixed-charged, pseudozwitterionic (meth)acrylate copolymer brushes.

Figure 34. Molecular structure of the (A) \(N,N,N\)-trimethylammonium (TMA), (B) sulfonic acid (SA), and (C) carboxylic acid (CA) \(\omega\)-functionalized alkylthiol gold surface modifiers. At physiological pH 7.4, SA and CA moieties in the mixed SAMs exist as their negatively-charged sulfonate and carboxylate forms, respectively.

Figure 35. Molecular structure of the poly(vinyl amine)-g-hexanoyl/dextran carbohydrate polymers.

Figure 36. Molecular structure of the poly(L-Lysine)-g-dextran carbohydrate polymers.

Figure 37. Cartoon representation of the generic structure of surface modifiers for SAM formation showing the three customizable parts (‘anchoring function’, ‘backbone’ and ‘head function’).

Figure 38. Schematic representation of alkylthiol-based SAM formation on Au(111) substrate.

Figure 39. Schematic representation of the silanol reversible adsorption/desorption and condensation steps (shown as an example is the case of monomeric trisilanol species).

Figure 40. Schematic representation of the cross-linkage between two neighbouring, surface-bound silanols into a condensed siloxane moiety.

Figure 41. Illustration of the Cartesian axes assigned to quartz crystals, and examples of plate cuts (AT and BT). Figure adapted from reference 354.

Figure 42. Schematic representation of an AT-cut quartz crystal (with thickness \(t_{q}\)) subjected to a perpendicular oscillating electric field \((E)\), the resulting shear oscillating displacement of particles along the x-axis \((u_x)\), and the associated standing wave (at acoustic resonance).

Figure 43. Some examples of the various interfacial factors and liquid properties (such as viscosity, \(\eta_{liq}\), and density, \(\rho_{liq}\)) influencing the composite response of BAW piezoelectric sensors in the liquid phase.
**Figure 44.** Schematic representation of a TSM device architecture showing the flow-through cell, the encased quartz disc plated with gold electrodes on both sides, and the network analyzing system.

**Figure 45.** The simplest form of the Butterworth-van Dyke (BVD) equivalent circuit for TSM resonators.

**Figure 46.** The electromagnetic piezoelectric acoustic sensor (EMPAS). (A) Picture of the EMPAS experimental set-up featuring the electronic instruments and hardware necessary to excite acoustic resonance within quartz discs, the flow-through cell holder wherein the latter are encased with the coil, and the injection system for running buffer and analysis samples (left); as well as (right) the EMPAS working principle. (B) The planar spiral coil used to remotely induce within electrodeless piezoelectric quartz discs a secondary electric field that instigates acoustic resonance. Parts of this figure are adapted from references 413 and 414.

**Experimental**

**Figure 47.** Synthesis of TTTA.

**Figure 48.** Synthesis of PFP-TTTA.

**Figure 49.** Synthesis of OEG-TTTA.

**Figure 50.** Synthesis of MEG-TFA.

**Figure 51.** Synthesis of OTS-TFA.

**Figure 52.** Synthesis of MEG-OMe.

**Figure 53.** Synthesis of HS-MEG-OH.

**Figure 54.** Synthesis of HS-OTS-OH.

**Figure 55.** Synthesis of biotinthiol.

**Figure 56.** Synthesis of biotinamine.

**Figure 57.** Schematic description of the stratified system chosen to study surface hydration showing (from bottom to top) the Si/SiO₂ substrate, the organosilane adlayer (MEG-OH or OTS-OH) as well as transitional and
bulk water. Also shown are the thickness (d), roughness (σ) and scattering length density (SLD) of the various layers.

**Figure 58.** (A) Experimental set-up used to record real-time platelet adhesion, aggregation and thrombus formation. (B) Close-up view of the perfusion chamber showing blood in- and outlets. (C) Computer interface used for data analysis and calculation of thrombus surface coverage.

**Figure 59.** Structure and dimensions of the SiO₂ slab cut from α-quartz for MD simulations. The atom colour code is as follows: yellow (silicon) and red (oxygen).

**Figure 60.** (A) Surface functionalization, (B) full 5 × 5 coverage, and (C) encasing water box models used for MD simulations. Shown, as an example, is the unimolecular MEG-OH system. The atom colour code is as follows: yellow (silicon), turquoise (carbon), red (oxygen), and white (hydrogen).

**Results and Discussion**

**Figure 61.** TTTA/OTS mixed adlayer-based EMPAS biosensor prototype for the real-time and label-free detection of biotin/avidin model interactions in buffer: preparation (steps I & II) and working principle.

**Figure 62.** EMPAS resonant frequency shifts for avidin specific binding (blue) / non-specific adsorption (red), respectively measured with biotinylated and non-biotinylated TTTA/OTS mixed adlayer platforms.

**Figure 63.** Schematic representation of PFP-TTTA/OTS mixed adlayer formation on quartz (step I) and the subsequent, site-specific covalent immobilization of biothiol/ biotinamine probes (step II).

**Figure 64.** (A) XPS surveys for (bottom to top) cleaned bare quartz (red) and PFP-TTTA/OTS mixed adlayers, non-biotinylated (blue) or biotinylated with biothiol (green) or biotinamine (gold). For clarity, the latter three profiles have been shifted upwards. *Note:* the peak for carbon in the XPS survey of cleaned bare quartz is due to contamination by adventitious species. (B-C) Narrow scans for nitrogen N₁s and sulfur S₂p. The takeoff angle shown here is 46° relative to the normal. *Note:* the amide (-CO-NH-) and thioester
(-CO-S-)463 functions that form during biotin attachment (Figure 63 – step II) also respectively contribute to the observed N1s and S2p XPS signals.

**Figure 65.** EMPAS resonant frequency shifts for avidin specific binding (blue) and non-specific adsorption (red), respectively measured with biotinylated (by means of biotin thiol) and non-biotinylated PFP-TTTA/OTS mixed adlayer platforms. Arrows mark the successive injections of sacrificial BSA and target avidin solutions (both 0.1 mg/mL ~ 1.5 μM in PBS buffer). *Note:* for comparative purposes, injection times and initial resonant frequencies have been normalized.

**Figure 66.** EMPAS resonant frequency shifts for avidin specific binding (blue) and non-specific adsorption (red), respectively measured with biotinylated [with biotin thiol (A and C) or with biotamine (B)] and non-biotinylated PFP-TTTA/OTS mixed adlayer platforms: (A-B) with intermediate injection of sacrificial BSA, or (C) without. Both BSA and avidin solutions were 0.1 mg/mL ~ 1.5 μM in PBS buffer.

**Figure 67.** Chemical structures of (A) OEG-TTTA linker and (B) MEG-TFA diluent trichlorosilane surface modifiers.

**Figure 68.** (A) XPS surveys for (bottom to top) cleaned bare quartz (red) and OEG-TTTA/MEG-TFA mixed adlayers – non-biotinylated (blue) or biotinylated with biotin thiol (green). For clarity, the latter two profiles have been shifted upwards. *Note:* the peak for carbon in the XPS survey of cleaned bare quartz is due to contamination by adventitious species. (B-C) Narrow scans for nitrogen N1s and sulfur S2p. The takeoff angle shown here is 72.5° relative to the normal (surface analysis).

**Figure 69.** EMPAS resonant frequency shifts for avidin specific binding (blue) and non-specific adsorption (red), respectively measured with biotinylated and non-biotinylated OEG-TTTA/MEG-TFA mixed adlayer platforms. Measurements were performed at the ultra-high frequency of 1.06 GHz.

**Figure 70.** (A) EMPAS quartz discs photographed next to a Canadian penny. (B) The corresponding spiral coils used to remotely instigate resonance within the quartz substrate. (C) EMPAS resonant frequency shifts for avidin specific...
binding (blue) and non-specific adsorption (red), respectively measured with biotinylated and non-biotinylated OEG-TTTA/MEG-TFA mixed adlayer platforms prepared on (left) d = 13.6 mm and (right) d = 7.0 mm quartz discs. Avidin samples were 1.5 μM in PBS buffer.

**Figure 71.** EMPAS resonant frequency shifts due to fouling on non-biotinylated OEG-TTTA/MEG-TFA mixed adlayer platforms of 1.5 μM avidin samples prepared in (left) PBS buffer and (right) goat serum.

**Figure 72.** EMPAS resonant frequency shifts for avidin specific binding (blue) and non-specific adsorption (red) measured with 1.5 μM avidin samples in serum, respectively on biotinylated and non-biotinylated OEG-TTTA/MEG-TFA mixed adlayer platforms (4-5 replicates per data set). L:D is the volumetric ratio (v/v) of OEG-TTTA linker (L) and MEG-TFA diluent (D) in the silanizing solutions used to prepare the various mixed adlayers (see Experimental Section 2.4.2.B.).

**Figure 73.** EMPAS resonant frequency shifts measured on biotinylated OEG-TTTA/MEG-TFA mixed adlayer platforms (4-5 replicates per data set), upon exposure to serum samples either spiked with 1.5 μM avidin (blue) or unspiked (yellow).

**Figure 74.** EMPAS response to a wide range of avidin serum solutions, measured with OEG-TTTA/MEG-TFA mixed adlayer platforms (L:D = 1:50). Note: the two outliers for the blank experiments (unspiked serum) were not taken into account in the calculation of the cut-off value (|Δf| + SD).

**Figure 75.** Saturation of tetrameric avidin with four biotin ligands (molecular representation taken from www.proteopedia.org).

**Figure 76.** (A) From bacteremia to multi-organ failure: the key role of LPS/endotoxin in the pathogenesis of sepsis. (B) General chemical structure of monomeric bacterial lipopolysaccharide.

**Figure 77.** Chemical structure of polymyxin B.

**Figure 78.** Personalized ‘theranostic’ treatment of endotoxemia combining a therapeutic neutralization cartridge and an in-line EMPAS diagnostic system in an all-integrated ‘theranostic circuit device’.
Figure 79. Fluorine $F_{1s}$ and nitrogen $N_{1s}$ XPS signal intensity (counts per second areas) for OEG-TTTA/MEG-TFA mixed adlayers formed from various L:D silanizing solutions, before (blue) and after (red and green) PMB probe immobilization. Note: counts per second area values are those recorded at the takeoff angle of 72.5° relative to the normal (surface analysis).

Figure 80. Schematic representation (top view) of the hypothesized different packing of OEG-TTTA linker (L) and MEG-TFA diluent (D) residues within mixed adlayers at various L:D compositions.

Figure 81. Realistic representation of OEG-TTTA/MEG-TFA/PMB surface chemistry on quartz.

Figure 82. EMPAS resonant frequency shifts measured with PMB-functionalized L:D = 1:10 and 1:50 OEG-TTTA/MEG-TFA mixed adlayer platforms, upon exposure to human plasma samples spiked with LPS at 1000 pg/mL (blue) or unspiked (yellow). For comparison, also shown is the resonant frequency shift for bare quartz exposed to unspiked donor plasma (left).

Figure 83. EMPAS resonant frequency profiles for bare quartz (red) and a PMB-functionalized L:D = 1:50 OEG-TTTA/MEG-TFA mixed adlayer platform (yellow) both exposed to LPS-unspiked human donor blood plasma; as well as (blue) a PMB-functionalized L:D = 1:50 OEG-TTTA/MEG-TFA mixed adlayer platform exposed to human blood plasma spiked with LPS at 1000 pg/mL. Note: for comparative purposes, injection times (marked by the arrow) and initial resonant frequencies have been normalized.

Figure 84. Systematic surface modification of quartz with unimolecular adlayers.

Figure 85. (A) XPS surveys for (bottom to top) cleaned bare quartz (red), as well as MEG-TFA (blue) and MEG-OH (green) unimolecular silane adlayers. Note: the peak for carbon in the XPS survey of cleaned bare quartz is due to contamination by adventitious species. (B) Corresponding static contact angles measured with water as the test liquid.

Figure 86. EMPAS resonant frequency shifts upon injection of full goat serum onto bare and adlayer-derivatized quartz surfaces. Measurements were performed at the ultra-high frequency of 0.94 GHz. Note: for rigour and comparative
purposes, OTS and MEG-OMe adlayers also underwent the overnight methanol/water treatment through which MEG-OH and OTS-OH coatings were generated.

**Figure 87.** EMPAS response profiles for (A) bare quartz and (B) MEG-OH coating exposed to serum.

**Figure 88.** EMPAS profiles for (A) pre-hydrated and (B) ‘dry’ MEG-OMe adlayers exposed to serum.

**Figure 89.** (A/B) MEG-OH silane/thiol surface chemistries on quartz/gold for EMPAS/TSM sensing.

**Figure 90.** TSM resonant frequency shifts due to 10% serum adsorption on HS-MEG-OH adlayers formed on gold for increasing periods of time (5 to 1080 min). *Note:* ‘t = 0’ means gold is unmodified.

**Figure 91.** TSM profiles for (A) bare gold and (B) a ‘30 min’ MEG-OH film exposed to 10% serum. For comparative purposes, injection times and initial resonant frequencies have been normalized.

**Figure 92.** TSM profiles for pre-hydrated (A) bare gold and (B) a MEG-OH film exposed to 10% serum.

**Figure 93.** TSM response profiles for pre-hydrated bare gold (blue), as well as HS-MEG-OH (red) and HS-OTS-OH (green) ‘30 min’ films exposed to 10% serum.

**Figure 94.** Stratified model chosen to study surface hydration showing (from bottom to top) the Si/SiO₂ substrate, the silane adlayer (MEG-OH or OTS-OH) as well as transitional and bulk water. In the analysis of NR data, silane adlayer and transitional water are treated as one single medium (‘Layer 1’). Also shown are the thickness (d), roughness (σ) and scattering length density (SLD) of the various layers.

**Figure 95.** Reflectivity data measured in CMW and corresponding fits with $\chi^2$ values for bare (red circles/lines) as well as MEG-OH- or OTS-OH-modified (black circles/lines) Si/SiO₂ substrates.
Figure 96. SLD profiles for MEG-OH (dashed red line) and OTS-OH (solid blue line) systems generated using the stratified model presented in Figure 94. Note: the Si wafer is not shown.

Figure 97. Distinct hydration patterns for MEG-OH and OTS-OH silane coatings on Si/SiO$_2$ proposed from the interpretation of NR data. Note: this schematic representation (not to scale) merely depicts MEG-OH and OTS-OH surface chemistry, not the actual surface coverage/patchiness of these adlayers nor the anchorage nature and degree of order/packing of the surface-modifying residues within.

Figure 98. Systematic chain modification for MD simulation of surface hydration. Note: arrows do not represent chemical transformations/synthetic pathways.

Figure 99. Radial distribution function of water for the various silane adlayers on $\alpha$-quartz. Note: the reference here is the full chain.

Figure 100. Innermost radial distribution function of water for the various silane adlayers on $\alpha$-quartz. Note: the reference here is the bottom five atoms (see Figure 98): C1-C5 for OTS and OTS-OH, and C1-C2-C3-O4-C5 for O-OTS, MEG-OH and MEG-OMe.

Figure 101. Outermost radial distribution function of water for the various silane adlayers on $\alpha$-quartz. Note: the reference here is the top two non-hydrogen atoms (see Figure 98): C7-C8 for OTS and O-OTS, C6-O7 for OTS-OH and MEG-OH, and O7-C8 for MEG-OMe.

Figure 102. Coordination of explicit water within the 3 × 3 residue core of the MEG-OH assembly (top view snapshot). Shown are the various possible H-bond interactions of water with the internal ether or/distal hydroxyl moieties. A, B, and C illustrate multi-site, interchain interactions; while D and E show water molecules sharing a single binding site. Not present in this frame, however, is the interaction of intramolecular nature, wherein a molecule of water bridges both internal and distal oxygen atoms of a same EG unit (or interactions involving water as the H-bond acceptor). Note: for clarity, the oxygen atoms from the substrate and silanols, in addition to the hydrogen atoms from the residues, are not represented. The atom colour
code is as follows: yellow/shaded (silicon), blue (carbon), red/pink (oxygen), and white (water’s hydrogen).

**Figure 103.** Water clustering within the MEG-OH assembly. (A) The bottom molecule of water bridges neighbouring chains through the silanol and internal ether moieties. This locked configuration provides an environment favourable for the interaction of a second (top) bridging molecule of water. (B) Water clustering propagates as other molecules arrive. The overall effect is an unexpectedly-long residency time, well into the nanosecond regime, for these water molecules. *Note:* these lifetimes, which not surprisingly increase with burial depth, overlap. The atom colour code is as follows: yellow/shaded (silicon), blue (carbon), red/pink (oxygen), and white (water’s hydrogen).

**Figure 104.** Antifouling and surface hydration: basic requirements.

**Figure 105.** Innermost and outermost (dashed and solid lines) radial distribution functions of water for unimolecular MEG-OH (blue) and mixed OEG-TTTA/MEG-OH (red) assemblies. *Note:* the reference for ‘innermost’ RDFs is the bottom five atoms (C1-C2-C3-O4-C5). The reference for ‘outermost’ RDFs is the top two non-hydrogen atoms (C6-O7) for the MEG-OH residues, and the corresponding two C6-O7 middle atoms for the central OEG-TTTA residue in the mixed assembly.

**Figure 106.** Radial distribution function of water around the TFE ester head function of the central OEG-TTTA residue in the mixed assembly. *Note:* the reference is the terminal eight atoms (C13-O13-O14-C15-C16-F16/1-F16/2-F16/3).

**Figure 107.** Schematic representation of the half sphere-shaped ‘exclusion volume’ hypothesized to contribute in preventing protein adsorption.

**Figure 108.** Schematic representation of the two-step, straightforward surface modification of bisphenol A polycarbonate (BPA-PC) plastic polymer with MEG-OH silane surface chemistry.

**Figure 109.** XPS surveys for (top to bottom) plasma-activated bare BPA-PC (red), as well as MEG-TFA (blue) and MEG-OH (green) adlayers.
Figure 110. Overlapped C_1s narrow scans for bare (red), MEG-TFA-modified (blue) and MEG-OH-modified (green) BPA-PC surfaces showing the various C-C/C=C (and C-Si), C-O, C=O, and C-F bond contributions and relative compositions. Note: the takeoff angle is 90° relative to the surface (bulk analysis).

Figure 111. (A) Indicative percentage of surface coverage due to platelet adhesion, aggregation and thrombus formation on (left to right) collagen-coated, bare and MEG-OH-modified BPA-PC substrates. (B-D) A selection of representative video frames showing platelet adhesion, aggregation and thrombus formation (or lack of) on collagen-coated, bare and MEG-OH-modified BPA-PC substrates (32× magnification) after 5 min of exposure to whole human blood at a shear rate of 1000 s^-1. Replicate pictures for all various surfaces can be found gathered in Appendices K-M.

Future Work

Figure 112. Truly ‘in-line’ EMPAS biosensing set-up for continuous LPS detection in whole blood (left) vs. ‘off-line’ configuration for timed LPS detection in cell-cleared blood plasma (right). Note: not shown in the off-line detection approach is the intermediate centrifugation system to separate blood plasma from cells.

Figure 113. CATD/HTS mixed silane adlayer-based EMPAS immunosensor for the real-time and label-free serological detection of HIV-2 anti-gp36 vs. HIV-1 anti-gp41 antibodies: working principle and preliminary results (Δf ± SD, 5 replicates per set). Note: EMPAS measurements were recorded at the ultra-high operating frequency of 1.06 GHz, with anti-gp36 and anti-gp41 monoclonal antibody samples both prepared as 0.1 mg/mL solutions in full foetal bovine serum.

Figure 114. N-phenyl-N’-piperidinyl-oxalamide HIV entry inhibitors with high affinity for gp120.

Figure 115. Chemical structure of a potential gp120 target molecular probe for HIV detection with OEG-TTTA/MEG-TFA mixed adlayer-based EMPAS sensing platforms.
Figure 116. Phosphonate vs. silanol substrate anchorage for more robust MEG-OH surface chemistry.

Appendix

Figure 117. TTTA $^1$H NMR (400 MHz, CDCl$_3$).

Figure 118. PFP-TTTA $^1$H NMR (400 MHz, CDCl$_3$).

Figure 119. OEG-TTTA $^1$H NMR (400 MHz, CDCl$_3$).

Figure 120. MEG-TFA $^1$H NMR (400 MHz, CDCl$_3$).

Figure 121. OTS-TFA $^1$H NMR (400 MHz, CDCl$_3$).

Figure 122. MEG-OMe $^1$H NMR (400 MHz, CDCl$_3$).

Figure 123. HS-MEG-OH $^1$H NMR (400 MHz, CDCl$_3$).

Figure 124. HS-OTS-OH $^1$H NMR (400 MHz, CDCl$_3$).

Figure 125. Biotinthiol $^1$H NMR (300 MHz, CDCl$_3$).

Figure 126. Biotinamine $^1$H NMR (300 MHz, CD$_2$OD).

Figure 127. Platelet adhesion, aggregation and thrombus formation on collagen-coated BPA-PC after 5 min exposure to whole human blood at a shear rate of 1000 s$^{-1}$.

Figure 128. Platelet adhesion, aggregation and thrombus formation on bare BPA-PC after 5 min exposure to whole human blood at a shear rate of 1000 s$^{-1}$.

Figure 129. Inhibition of platelet adhesion, aggregation and thrombus formation on MEG-OH-modified BPA-PC after 5 min exposure to whole human blood at a shear rate of 1000 s$^{-1}$. Arrows mark the few observable micro-aggregates.

Figure 130. XPS narrow scans for cleaned bare quartz (red) and OEG-TTTA/MEG-TFA mixed adlayers – non-functionalized (blue) and biotinylated with biotinthiol (green) – recorded for the characteristic elements of quartz as well as OEG-TTTA/MEG-TFA and biotinthiol residues. The presence of carbon for bare quartz is due to contamination by adventitious carbon. For clarity, the profiles for sulfur (S) and nitrogen (N) are not normalized with respect to the ‘counts/s’ axis. The takeoff angle shown here is 72.5° relative to the normal (surface analysis).
Figure 131. Estimated population of bystander (199) and available for single binding site (1) PMB molecules, at pH 7.4. *Note:* the location of the non-protonated RNH₂ site was chosen randomly, for representation purposes.

Figure 132. XPS narrow scans for (top to bottom) C₁s, F₁s, Si₂p and O₁s recorded for (left to right) cleaned bare quartz (red) as well as MEG-TFA (blue) and MEG-OH (green) unimolecular adlayers. The takeoff angle shown here is 72.5° relative to the normal (surface analysis).

Figure 133. Variable-angle ellipsometry data (green and blue dashed/dotted lines) and corresponding curve fitting (solid red lines) for the determination of MEG-TFA adlayer thickness on quartz using the Cauchy model.

Figure 134. XPS narrow scans for (top to bottom) S₂p, C₁s, O₁s and Au₄f recorded for cleaned bare gold (left, red) and HS-MEG-OH adlayer (right, blue). The presence of sulfur (at 169 eV), carbon and oxygen signals for bare gold is due to contamination by adventitious species.

Figure 135. Innermost radial distribution functions of water for (left/right) siloxane and silanol coatings.

Figure 136. Outermost radial distribution functions of water for (left/right) siloxane and silanol coatings.
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# List of Abbreviations

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<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>BAW</td>
<td>Bulk acoustic wave</td>
</tr>
<tr>
<td>BPA</td>
<td>Bisphenol A</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BVD</td>
<td>Butterworth-van Dyke</td>
</tr>
<tr>
<td>CA</td>
<td>Carboxylic acid (or contact angle)</td>
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<td>Contact angle goniometry</td>
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<tr>
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<td>EMPAS</td>
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</tr>
<tr>
<td>FBGC</td>
<td>Foreign body giant cells</td>
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<td>FBS</td>
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</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>HS-MEG-OH</td>
<td>2-(2-mercapto-ethoxy)-ethanol</td>
</tr>
<tr>
<td>HS-OTS-OH</td>
<td>5-mercapto-pentanol</td>
</tr>
<tr>
<td>HTS</td>
<td>Hexyltrichlorosilane</td>
</tr>
<tr>
<td>IDT</td>
<td>Interdigital transducer</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>ITO</td>
<td>Indium tin oxide</td>
</tr>
<tr>
<td>L</td>
<td>Linker</td>
</tr>
<tr>
<td>L:D</td>
<td>Linker:diluent ratio</td>
</tr>
<tr>
<td>LAH</td>
<td>Lithium aluminum hydride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>LAL</td>
<td><em>Limulus</em> amebocyte lysate</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>L-3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MD</td>
<td>Molecular dynamics</td>
</tr>
<tr>
<td>MEG</td>
<td>Monoethylene glycol</td>
</tr>
<tr>
<td>MEG-OMe</td>
<td>Ethylene glycol 3-trichlorosilylpropyl methyl ether</td>
</tr>
<tr>
<td>MEG-TFA</td>
<td>2-(3-trichlorosilylpropoxy)-ethyl trifluoroacetate</td>
</tr>
<tr>
<td>MeOEGMA</td>
<td>Methoxy-terminated OEG methacrylate</td>
</tr>
<tr>
<td>MES</td>
<td>2-((N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>3-MPA</td>
<td>3-mercaptopropionic acid</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxy-succinimide</td>
</tr>
<tr>
<td>NIPAAm</td>
<td>N-isopropylacrylamide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NR</td>
<td>Neutron reflectometry</td>
</tr>
<tr>
<td>NSA</td>
<td>Non-specific adsorption</td>
</tr>
<tr>
<td>OEG</td>
<td>Oligoethylene glycol</td>
</tr>
<tr>
<td>OEG-TTTA</td>
<td>3-(2-(2-(3-trichlorosilylpropoxy)-ethoxy)-ethoxy)-propanoic acid 2,2,2-trifluoroethyl ester</td>
</tr>
<tr>
<td>OEO</td>
<td>Oligoethylene oxide</td>
</tr>
<tr>
<td>OTS</td>
<td>Octyltrichlorosilane</td>
</tr>
<tr>
<td>OTS-TFA</td>
<td>6-trichlorosilyl-hexanyl trifluoroacetate</td>
</tr>
<tr>
<td>OWLS</td>
<td>Optical waveguide lightmode spectroscopy</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PC</td>
<td>Polycarbonate</td>
</tr>
<tr>
<td>PCMA</td>
<td>Phosphorylcholine methacrylate</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEO</td>
<td>Polyethylene oxide</td>
</tr>
<tr>
<td>PFP</td>
<td>Pentfluoro phenyl</td>
</tr>
<tr>
<td>PFP-TTTA</td>
<td>13-trichlorosilyl-tridecanoic acid pentfluoro phenyl ester</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(L-Glutamic acid)</td>
</tr>
<tr>
<td>PhMe</td>
<td>Toluene</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly(L-Lysine)</td>
</tr>
<tr>
<td>PMB</td>
<td>Polymyxin B</td>
</tr>
<tr>
<td>PMOXA</td>
<td>Poly(2-methyl-2-oxazoline)</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>PPO</td>
<td>Polypropylene oxide</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet-poor plasma</td>
</tr>
<tr>
<td>PPS</td>
<td>Polypropylene sulfide</td>
</tr>
<tr>
<td>QCM</td>
<td>Quartz crystal microbalance</td>
</tr>
<tr>
<td>RDF</td>
<td>Radial distribution function</td>
</tr>
<tr>
<td>rfx</td>
<td>Reflux</td>
</tr>
<tr>
<td>RMSF</td>
<td>Root-mean-square fluctuation</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>rt</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SA</td>
<td>Sulfonic acid</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-assembled monolayer</td>
</tr>
<tr>
<td>Abbr.</td>
<td>Full Form</td>
</tr>
<tr>
<td>-------</td>
<td>-----------</td>
</tr>
<tr>
<td>SAW</td>
<td>Surface acoustic wave</td>
</tr>
<tr>
<td>SBMA</td>
<td>Sulfo betaine methacrylate</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SLD</td>
<td>Scattering length density</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>TCD</td>
<td>Theranostic circuit device</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetyl</td>
</tr>
<tr>
<td>TFE</td>
<td>Trifluoroethyl</td>
</tr>
<tr>
<td>TMA</td>
<td>Trimethylammonium</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TSM</td>
<td>Thickness shear mode</td>
</tr>
<tr>
<td>TTTA</td>
<td>13-trichlorosilyl-tridecanoic acid 2,2,2-trifluoroethyl ester</td>
</tr>
<tr>
<td>VDW</td>
<td>Van der Waals</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
</tbody>
</table>
Publications

This PhD Thesis research gave rise to a series of peer-reviewed papers, editorials, book chapters, public press articles, and patents. These are listed below in chronological order of publication:


8. Thompson, M.; Sheikh, S.; Sheng, J. C.-C.; Blaszykowski, C. ‘Avoidance of non-specific binding on an acoustic wave biosensor using linker and diluent molecules for device surface modification’ *United States patent US 8,491,958* (issued July 23, **2013**)


1. Introduction

1.1. Overview

The world population currently exceeds 7 billion individuals and is predicted to reach 10 to 11 billion by the end of the 21st century.\(^1\) Average life expectancy, too, is steadily increasing. The consequence will be a tremendous amount of stress continually placed upon the healthcare industry to develop/improve widely accessible, affordable, and safe, biomedical equipment. Contact of artificial materials with biofluids (e.g. blood, urine) is very common in many aspects of modern medicine. Whether applied in vivo or in vitro, the range of equipment, implants or devices is immense and includes, for example: the bypass circuitry used in hemodialysis and coronary surgery, catheters, stents and pacemakers; as well as biosensors for clinical diagnostics and the more recent ‘lab-on-a-chip’ technology. However, in spite of tremendous advances notably in terms of design, these continue to suffer from the bio-incompatibility of the synthetic materials they are manufactured from, which can lead to potentially severe complications caused by blood coagulation and tissue inflammation.\(^2,3\) One major issue with foreign materials contacting biofluids is their tendency to accumulate biological species on their surface (most notably proteins) – a natural, more general phenomenon referred to as fouling that negatively affects the performance, longevity and integrity of biomedical equipment, implants and devices.

In this context, this PhD Thesis presents original research towards the development of new, ultrathin surface chemistry to impart a variety of both organic and inorganic coated substrates with antifouling/biocompatible properties for biomedical and bioanalytical applications in blood and its cell-cleared serum/plasma derivatives. Particular attention is also focused on better understanding the fundamental role of water in surface antifouling. Prior to presenting and discussing results, an introduction will provide concise but solid background covering: (i) blood (its composition, role, importance); (ii) the biomedical industry (and the concepts of ‘biosensor’ and ‘biomaterial’); (iii) blood-material interactions (with the notions of ‘biocompatibility’ and ‘fouling’); (iv) proteins (their structure, adsorption onto surfaces and the complex mechanism associated with it); (v) biological host responses to material-blood contact (i.e. coagulation and ‘foreign body reaction’) and their potential consequences; (vi) surface modification chemistries to combat fouling from highly proteinaceous blood plasma/serum (including a description of the various mechanistic hypotheses behind protein repellence, and a reflection on the link between ‘surface antifouling’ and ‘material biocompatibility’); (vii) ‘self-assembling monolayer’ surface chemistry (concept, variants, use); and (viii) acoustic wave physics (principle, applications).
1. 2. Blood: composition, role and importance

Blood is a complex biofluid in which bathe both cellular and non-cellular species that are essential in sustaining life as they provide a means of delivering vital nutrients and gases to cells (or conversely disposing of metabolic waste products), regulating the internal environment (e.g. pH, osmosis), protecting the organism against foreign bodies (through the action of the immune system), or preventing blood loss (via hemostasis and coagulation).\(^4\)\(^6\) Considering the importance of blood in maintaining a human organism healthy and functional, it is not surprising that the daily production of blood cells reaches approximately 1 trillion.\(^6\) On average, the volume of blood in a healthy adult individual is about 5 liters, or about 7% of the body weight. Normally, blood circulates throughout the body in a forward, laminar fashion via a closed-loop system of channels known as blood vessels (Figure 1).

![Figure 1](image)

**Figure 1.** Blood vessel structure (showing the various layers constituting the vascular wall), and whole blood components (plasma, platelets, white and red cells).

Whole blood is essentially composed of three parts, which easily separate upon centrifugation (Figure 2). The least dense fraction is plasma, a viscous aqueous liquid that accounts for ~54% of the blood volume. The second, thinner phase is called the ‘buffy coat’ (~1% in volume) and contains platelets and white blood cells. Lastly, red blood cells make up the densest fraction, which accounts for the remaining ~45% of the volume of blood.\(^4\)\(^6\) Together, the
buffy coat and red blood cells constitute the cellular portion of blood. On the other hand, plasma gathers the non-cellular components of blood, and is composed of 90% water and 10% solid material, the latter consisting of such elements as ions, lipids, vitamins, and more than 4500 different (types of) proteins \(^7\) including albumin, globulins and fibrinogen. \(^8\) Albumin (MW ~ 66 kDa, 15 × 3.8 × 3.8 nm dimensions, pI = 4.8) \(^4,9\) – the most abundant plasma protein (30-50 g/L) \(^4,8\) – is an osmotic regulator as well as functions as a carrier for various metals, ions, fatty acids, amino acids, enzymes and drugs. \(^4\) Immunoglobulins – which are characterized by great structural diversity \(^4,8\) – are the next most abundant plasma proteins, followed by fibrinogen (MW ~ 340 kDa, 47 × 4.5 × 4.5 nm dimensions, pI = 5.8) \(^4,9\) that has an approximate blood concentration of 1.5-4 g/L \(^4,8\) and is essential for the formation of blood clots. When fibrinogen and other clotting factors are removed from plasma, the remaining medium is known as serum. \(^4\)

**Figure 2.** Blood composition. Upon centrifugation, blood separates into three fractions (from top to bottom): (i) ‘plasma’, which gathers the non-cellular components of blood; (ii) the ‘buffy coat’ made of platelets and white blood cells; and (iii) the ‘red blood cells’. **Note:** buffy coat and red blood cells collectively constitute the cellular or ‘formed’ elements of blood.
The cellular fraction of blood is composed of red blood cells or *erythrocytes* (7.5 μm in diameter and 2 μm-thick, lifespan of ~120 days), white blood cells or *leukocytes* (5 types: neutrophil, lymphocyte, monocyte, eosinophil, and basophil / 5-20 μm in diameter), and non-nucleated platelets or *thrombocytes* (2-4 μm in diameter and 0.5 μm-thick, lifespan of 7-10 days). Each of these formed elements has its own unique purpose. For instance, red blood cells contain hemoglobin (~29 pg) to transport oxygen fuel and carbon dioxide waste, while white blood cells provide protection against pathogens and other foreign entities through the body’s immune response. Platelets, which are fragments of bone marrow cells, are a key factor in *hemostasis* and the formation of blood clots (*thrombosis*) at the site of an injury. Hemostasis is the general term used to describe the process responsible for preventing the loss of blood while ensuring that the latter remains and travels through the vascular system in a fluid state. This is a natural, continuous process that involves several interrelated mechanisms. Hemostasis is instantaneously initiated to repair damaged blood vessels upon wall injury. The response comprises three processes: (i) vascular spasms (to reduce the flow of blood); (ii) the formation of a loose platelet plug to temporarily cover the site of injury in the endothelial lining; and (iii) blood clot formation through *coagulation*. Following vessel repair, the clot is dissolved and normal blood flow is restored.

Paradoxically, the normally desired activation of the immune and coagulation protective mechanisms becomes problematic when blood – whether *in vivo* or *in vitro* – is purposely exposed to exogenous, foreign materials during for instance a medical procedure. As will be discussed later on, adverse effects may as well affect both host and foreign bodies.
1.3. A look into the biomedical industry

As the world population continues to grow, so does the economic and societal pressure to improve the biomedical industry. As an example of tremendous healthcare expenditure, the Province of Ontario (Canada) currently spends over 1 billion Canadian dollars per year on medical assays performed in both private laboratories and public hospitals.\(^{12}\) The use of blood-contacting medical equipment is not new and has actually been around for centuries.\(^{13}\) Nowadays, millions of patients continue to rely on similar devices as a means to save their life or enhance its quality.\(^{14}\) From diagnostics to treatment to management, numerous medical applications require blood to come into contact with a foreign material. One well-known example is the glucose biosensor, which is routinely used by individuals suffering from diabetes mellitus – a metabolic disorder characterized by an abnormally high blood concentration of glucose resulting from a lack in insulin production/response.\(^{15}\) Conceptually, biosensors refer to analytical devices designed to convert biomolecular interactions occurring at the sensor-liquid interface into a measurable signal.\(^{12,16}\) Composed of a biorecognition element intimately interfaced with a transducing component, biosensors are engineered in practice to selectively and sensitively detect in a real-time and ideally label-free manner target analytes that can range in size and complexity from small molecular species to larger biological entities (Figure 3).\(^{12,16}\)

![Figure 3](image.png)

**Figure 3.** Schematic representation of the generic architecture of biosensors featuring the biosensing interface, the transducing component and the output system.

Recent years have seen these analytical structures draw considerable attention, especially from a commercial stance,\(^{17}\) as potential clinical screening/diagnostic alternatives offering several possible advantages in terms of convenience, reduced costs, speed of analysis, and/or portability for (remote) point-of-care testing.\(^{12}\) For a biosensor to be fully functional, reliable and
commercially viable, however, a number of critical requirements need to be addressed. First, the biorecognition element (made of biomolecular receptor probes) must be securely attached to the transducer, with proper spatial orientation and loading density as well as homogeneous distribution to ensure optimal analyte binding capability.\textsuperscript{12,18} Furthermore, molecular affinity must be maximally retained upon immobilization in order for the specific analyte to effectively interact with surface-attached receptor probes. Second, the device should ideally display both high selectivity and sensitivity, as well as provide reliable and reproducible measurements – even in the presence of potentially interfering species. This latter issue is particularly relevant to biosensors intended to perform detection in complex biological matrices such as blood (or its cell-cleared plasma/serum derivatives). Some of these specifications are not solely restricted to biosensor technology but also apply to the very closely related field of research known as biomaterials.

There are several definitions of ‘biomaterial’ in use.\textsuperscript{19} Nevertheless, the word biomaterials was recently redefined to refer to materials (other than food and drugs) engineered to be used, independently or as part of a larger system, in human or veterinary medicine for diagnostic or therapeutic procedures involving continuous contact with biological fluids.\textsuperscript{20-22} A glimpse into the past reveals that biomaterials have always had a role in the medical field and in disease treatment. For instance, a corpse dating back to 200 AD was found in Europe bearing an iron dental implant.\textsuperscript{2} Although there was certainly no established understanding of the science and related biological processes surrounding the practice of medical implantation, it is evident that, even in such ancient times, there was a desire to strengthen or replace diseased/damaged anatomy.\textsuperscript{2} In modern society, synthetic plastic polymers are ubiquitous and may/have replaced the conventional materials that were wood, glass and metal in nearly all imaginable applications.\textsuperscript{23} Nowadays, it is common practice to use biomaterials in a wide range of applications aiming to: replace diseased or damaged body parts (e.g. artificial hip/knee joints, dental implants); assist in healing (e.g. bone plates and screws); improve function (e.g. intraocular lenses); correct abnormalities (e.g. cardiac pacemakers); or aid in treatment (e.g. catheters).\textsuperscript{24} The duration of biomaterial exposure to blood varies greatly, depending on the application or procedure, and may range from a few minutes to an individual’s entire lifetime – the latter situation being encountered in the case of implants. A concept inevitably associated with biomaterials is that of hemocompatibility, or that more general of biocompatibility. With regards to implants, the latter term refers to the ability for constituent materials to co-exist in
harmony with the surrounding biological host environment (fluids, tissue) without causing local or systemic adverse responses (i.e. to be biologically ‘inert’). Examples of host responses and potential complications that can be triggered as a result of material ‘bio-incompatibility’ include: inflammation (acute or chronic), complement system activation, embolization, cell/tissue impairment, infections, and tumorigenesis. As well, to various extents, artificial surfaces cause thrombogenesis – the initiation or promotion of thrombosis, itself colloquially also known as ‘blood clotting’.

Each year, hundreds of thousands of patients resort to biomedical equipment, implants or devices made of various synthetic biomaterials (Table 1). It is thus not surprising that the global biomaterial market, which includes equipment manufactured for both diagnostic and therapeutic purposes, was estimated to have reached the staggering amount of $150-200 billion USD in 2012.

<table>
<thead>
<tr>
<th>Equipment/implant/device</th>
<th>Number in the USA (approx. per year)</th>
<th>Biomaterial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraocular lens</td>
<td>2,500,000</td>
<td>Poly(methyl methacrylate) – PMMA</td>
</tr>
<tr>
<td>Contact lenses</td>
<td>30,000,000</td>
<td>Silicone-acrylate</td>
</tr>
<tr>
<td>Vascular graft</td>
<td>300,000</td>
<td>Poly(tetrafluoroethylene) – PTFE, Poly(ethylene terephthalate) – PET</td>
</tr>
<tr>
<td>Heart valve</td>
<td>100,000</td>
<td>Treated pig valve, carbon</td>
</tr>
<tr>
<td>Pacemaker</td>
<td>400,000</td>
<td>Polyurethane</td>
</tr>
<tr>
<td>Blood bag</td>
<td>40,000,000</td>
<td>Poly(vinyl chloride) – PVC</td>
</tr>
<tr>
<td>Catheter</td>
<td>200,000,000</td>
<td>Silicone rubber, polyurethane, Teflon</td>
</tr>
<tr>
<td>Heart-lung oxygenator</td>
<td>300,000</td>
<td>PVC, polyurethane, polypropylene, silicone, PET</td>
</tr>
<tr>
<td>Coronary stent</td>
<td>1,500,000</td>
<td>Stainless steel</td>
</tr>
</tbody>
</table>

(Table 1 to be continued)
(Table 1 continued)

<table>
<thead>
<tr>
<th>Equipment/implant/device</th>
<th>Number in the USA (approx. per year)</th>
<th>Biomaterial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal dialysis</td>
<td>320,000</td>
<td>Cellulose</td>
</tr>
<tr>
<td>Hip/knee prostheses</td>
<td>500,000</td>
<td>Titanium, polyethylene – PE</td>
</tr>
<tr>
<td>Dental implant</td>
<td>910,000</td>
<td>Titanium, stainless steel, alumina, calcium phosphate</td>
</tr>
</tbody>
</table>

Table 1. Estimated numbers of common biomedical equipment, implants and devices used on a yearly basis in the USA, together with the biomaterials they are/may be manufactured from.

For different reasons, the exogenous materials used in these medical interventions unfortunately do not always satisfy or sustain the required, contextual level of biocompatibility, which may potentially lead to the occurrence of short- or long-term complications for patients.\textsuperscript{2,3,32} For instance, artificial heart valves and catheters are susceptible to thrombosis and, if large enough, circulating thromboemboli can obstruct capillaries and restrict blood flow leading to tissue damage/infarct and even patient death.\textsuperscript{2,3,33} Similarly, blood oxygenator and thermoregulator apparatuses, which are commonly used for several hours during cardiopulmonary bypass surgery, may cause blood coagulation and thrombus formation.\textsuperscript{34} Much later, post-operative complications may also occur following the original, problem-free procedure. An example with relative severity would be the mechanical loosening of artificial knee/hip joints that requires reparative surgery 10-15 years after the initial replacement.\textsuperscript{2,3} Organ dysfunction – causing, for example, incapacitating neuropsychological disorders (e.g. cognitive impairment) in the case of the brain – is also unfortunately a real occurrence.\textsuperscript{35} Furthermore, large doses of anticoagulants (e.g. heparin) need to be repeatedly administered in many cases – either during or after a surgical/implant procedure – to prevent clotting issues; these drugs carry however a significant risk to induce adverse effects such as an increased probability of hemorrhage requiring patients to undergo blood transfusion.\textsuperscript{34,36,37} As demonstrated through this limited list of examples, biomaterials for biomedical applications are still prone to failure despite years of intense research. The outcome – whether a biomaterial will be recognized as ‘foreign’ and trigger a plethora of complex processes that can severely interfere with a patient’s diagnosis/treatment, or worse endanger their life – depends on the interaction of the biomaterial surface with the components of blood.
1. 4. Blood-material interaction

Most biomedical equipment, implants and devices are fabricated from materials foreign to the human body such as titanium, stainless steel, ceramic or plastic. A commonality among these exogenous materials is the invariable, spontaneous deposition of a layer of proteins on their surface upon exposure to blood – whether in vivo or in vitro. This initial phenomenon of protein adsorption would then be followed by the adhesion and aggregation of platelets, the adhesion of white blood cells, the activation of the complement system, blood coagulation and various other, often interconnected mechanisms (Figure 4).

**Figure 4.** Schematic representation of the highly involved nature of the biological processes triggered by the contact of blood with foreign materials. As can be seen (far left), protein adsorption is the first occurring phenomenon. The resulting layer of adhered proteins (orange boxes) is deemed to mediate subsequent biological processes and be the precursor to cellular adhesion. Figure adapted from reference 42.

It is indeed generally acknowledged that the deleterious biological processes orchestrated by the immune and coagulation systems are triggered by fouling – a natural, more general adsorption phenomenon where exogenous surfaces become (in this case) irreversibly covered with the biological species present in the matrix of blood, most notably proteins. Being
able to reliably prevent this sequence of events from occurring is paramount however in devising biocompatible materials (i.e. biomaterials able to properly perform an intended function without inducing adverse effects on the host organism), as well as biosensor platforms with full functionality and extended life expectancy.

Fouling by proteins is a ubiquitous phenomenon – a genuine biotechnological plague – that impairs the performance of biomedical equipment, implants and devices. As discussed earlier, serious complications can arise when an exogenous surface comes into contact with blood (Figure 5).

![Figure 5. The recurrent biotechnological concerns associated with the interaction of blood with exogenous materials. (A) The foreign body reaction (for in vivo biomaterial applications), wherein artificial surfaces become covered with successive layers of proteins, ‘foreign body giant cells’ and a final fibrous capsule made of collagen. The end result is the isolation of the implanted object from the surrounding host biological environment. (B) The non-specific adsorption of interfering species (i.e. proteins) in biosensor technology leading to erroneous readings.](image)

For instance, the formation of harmful thromboemboli may result from using multicomponent, multicomposite artificial circuitry (tubing, filters, oxygenator, pump) to circulate blood extracorporeally during such procedures as coronary bypass surgery or hemodialysis. Another case is that of the foreign body reaction – a protective tissue response
that accompanies normal wound healing following the implantation of non-degradable synthetic materials, and finally leads to the function- and fate-altering fibrous encapsulation of implanted objects.\textsuperscript{53,54} Similar issues are observed in biosensor technology where fouling – more often referred to as non-specific adsorption (NSA) – is of particular concern for detection platforms intended to perform clinical analysis in blood, its cell-cleared plasma/serum derivatives, or other biological fluids.\textsuperscript{12,50} With a total protein concentration ranging between 60-80 g/L,\textsuperscript{12,50,55} these blood-based fluids are incidentally considered to constitute the most challenging proteinaceous media of analysis. In fact, these proteins have the natural tendency to adsorb non-specifically to sensing interfaces thereby altering the integrity of the sensors’ response by generating an often overwhelming interference signal that prevents the detection – let alone the quantification – of target analytes present at considerably lower concentration (down to the ng/L region, or a difference of nine orders of magnitude).\textsuperscript{12,39} This unfortunately may lead to the occurrence of ‘false positive’ results due to the incorrect interpretation of the biosensor response as originating from a genuine binding event.\textsuperscript{12,56} On the other hand, ‘false negative’ results might also result if analyte binding is prevented. Both these situations, understandably, pose serious clinical dangers.\textsuperscript{12,57}

It has become abundantly clear that the educated design and development of antifouling/biocompatible materials for bioanalytical and biomedical applications require a prior detailed understanding on how proteins adsorb onto surfaces. Let us now take a closer look at the phenomenon and mechanism of protein adsorption.
1. 5. Protein adsorption

1. 5. 1. Protein structure

Proteins are intricate biopolymers constructed from a series of various amino acid monomers that, due to the unique nature of their side-chain, present varied properties in terms of hydrophobicity/hydrophilicity, polarity, acidity/basicity and charge (Figure 6).\textsuperscript{2,9,30,38,58,59} The amino acid sequence, and three-dimensional arrangement, determine protein function (e.g. metabolic, signalling, architectural) and interaction with the external environment.

![Chemical structure of amino acids](image)

**Figure 6.** Chemical structure of amino acids in their natural L-configuration.

Structurally, proteins can be described through four levels of increasing complexity. The first and simplest is the primary structure, which refers to the linear sequence of amino acids linked via covalent amide bonds. Next is the secondary structure level that describes the α-helix, β-sheet and random loop structures formed by polypeptide chains through intramolecular hydrogen bonding. These defined secondary structures coordinate with each other to establish the tertiary structure, wherein proteins adopt a folded, more compact conformation stabilized
through non-covalent interactions [e.g. van der Waals (< 5 kJ/mol), hydrogen bonding (< 5-40 kJ/mol), electrostatic/dipole (5 to > 190 kJ/mol), hydrophobic (5-40 kJ/mol)] and chemical linkages (i.e. disulfide bridges). Finally, the quaternary structure level describes the association of multiple polypeptide chains with defined tertiary structure into multi-subunit protein complexes. Protein conformation in the lowest free-energy state is induced and stabilized by the aqueous environment in which folding took place.30,58,61

Proteins can be further described with respect to their morphology and classified into one of the three following main categories: membrane, fibrous and globular. Membrane proteins, as the name suggests, are proteins associated with the membrane of cells or organelles. ‘Integral membrane proteins’ that are found attached to/embedded in phospholipid bilayers would be an example. Fibrous proteins, such as collagen and keratin, present asymmetrical and elongated rod-like features and are typically insoluble in water. In contrast, globular proteins such as albumin and hemoglobin have more of a roughly spherical shape. These proteins generally fold in such a way that hydrophilic/charged amino acid residues are exposed on the outer surface, while hydrophobic ones are buried in the inner core of the protein held through hydrogen bonding, weak acid-base and/or hydrophobic interactions. The aim of such concealment is to minimize the energetic cost associated with less-favourable non-polar/polar interactions between hydrophobic protein domains and the surrounding aqueous environment.58 Still, proteins generally display on their surface a variable assortment of hydrophilic and hydrophobic regions, giving rise to their amphipathic nature.38,62 As a result, globular proteins present variable solubility in aqueous media. Finally, it is also possible to group proteins into families according to their functional role (e.g. metabolic, signalling, architectural).

1.5.2. Protein-surface interaction

Proteins are amphipathic, surface-active macromolecules with heterogeneous, adaptable shell that can, as discussed above, display high affinity for virtually any type of man-made surface.62-64 Several comprehensive reviews have been published to cover this topic of protein-material interaction from both a theoretical and experimental stance.38,58,59,65-69 A concise summary found below compiles the key factors at play.

When the surface of an exogenous (artificial) material is exposed to proteins, such as those found in biological milieux (e.g. blood), a slew of interactions take place.38,70,71 One of the first
events, which is often overlooked or simply ignored, is the spontaneous adsorption (nanoseconds timescale)\textsuperscript{72} of water molecules and ions to form a water/electrical double layer.\textsuperscript{58,73} Shortly thereafter (within seconds),\textsuperscript{30,41,74} proteins migrate from the bulk solution towards the surface \textit{via} convection/diffusion, where they adsorb through a combination of multiple van der Waals, hydrogen bonding, electrostatic and hydrophobic interactions (\textbf{Figure 7}).\textsuperscript{58,65,72-79} It has been postulated that proteins initially adsorb to surfaces in their native state\textsuperscript{58,59,80} and, for adsorption to take place, that both protein and material surfaces must be dehydrated.\textsuperscript{38,43,58} In any event, it is likely the case that flexible, adsorbed proteins – to optimize the energy of interaction\textsuperscript{38,81} and in response to their environment – eventually assume altered conformational states and interact with surfaces in multiple ways of various strength, some of which are depicted in \textbf{Figure 7}.\textsuperscript{58,68,75,76,82} The adhesion of the much larger, less motile entities that are cells happens last, through the adsorbed layer of proteins.\textsuperscript{43}

\textbf{Figure 7.} A look into protein adsorption onto artificial surfaces. Non-exhaustive, schematic representation of the various possible scenarios that can occur upon the exposure of exogenous materials to proteinaceous media. Figure adapted from references 75 and 76.

It is safe to say that protein adsorption onto surfaces is a complex process. The latter is dynamic in nature and hinges around the following time-dependent stages: (i) migration from the
bulk phase towards the surface; (ii) random surface adsorption/desorption; (iii) spreading through conformational alteration (accompanied by a decrease in the rate of desorption); and (iv) exchange or irreversible adhesion and denaturation

Besides surface affinity, it is thus clear that the final composition of an adsorbed layer of proteins will be greatly influenced by the various kinetic aspects of adsorption, which are unique to each (type of) protein competing for a surface spot. One important related parameter that describes the ability of proteins to transport from the bulk phase towards the interface (and depends on the protein size as well as bulk concentration) is the diffusion coefficient, values of which are given in Table 2 for the main proteins of blood plasma.

<table>
<thead>
<tr>
<th>Plasma protein</th>
<th>Molecular weight (kDa)</th>
<th>Plasma concentration (mg/mL)</th>
<th>Diffusion coefficient $\left(10^{-7}\text{cm}^2/\text{s}\right)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>66</td>
<td>40</td>
<td>6.1</td>
</tr>
<tr>
<td>IgG</td>
<td>150</td>
<td>8-17</td>
<td>4.0</td>
</tr>
<tr>
<td>Transferrin</td>
<td>77</td>
<td>2.3</td>
<td>5.0</td>
</tr>
<tr>
<td>HDL</td>
<td>170</td>
<td>18</td>
<td>4.6</td>
</tr>
<tr>
<td>IgA</td>
<td>150</td>
<td>1-4</td>
<td>4.0</td>
</tr>
<tr>
<td>Complement 3</td>
<td>180</td>
<td>1.6</td>
<td>4.5</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>340</td>
<td>2-3</td>
<td>2.0</td>
</tr>
<tr>
<td>LDL</td>
<td>2000</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Table 2. Molecular weight, concentration and diffusion coefficient of the main proteins in blood plasma.

During the early, diffusion-controlled\textsuperscript{9,65} stage of protein adsorption, surface coverage can be mathematically expressed as equation 1:

$$\theta = 2C_0\sqrt{\frac{D \times t}{\pi}}$$

where $\theta$ is the surface coverage, $D$ is the diffusion coefficient, $C_0$ is the protein bulk concentration, and $t$ is time.\textsuperscript{58,59}
As time progresses, adsorbed proteins are then displaced by those with higher surface affinity. This phenomenon is known as the ‘Vroman effect’ and will be further discussed in Section 1.5. 3. B.\textsuperscript{43,64,68,84,86,87} In this time-dependent exchange process, the surface concentration and composition of proteins do not necessarily match those found in the bulk solution.\textsuperscript{3,38,59,72,85,88}

Surveying the literature, it seems as though there still exists a lack of consensus regarding the exact mechanistic nature of protein adsorption in spite of tremendous research efforts over the last few decades, and that a thorough theory is still pending.\textsuperscript{89,90} Nevertheless, it is generally agreed upon that the phenomenon can be described through the following thermodynamic expression (equation 2):\textsuperscript{9,59,67,74,91}

\[
\Delta G_{\text{ads}} = \Delta H_{\text{ads}} - T\Delta S_{\text{ads}}
\]  

where $\Delta G_{\text{ads}}$, $\Delta H_{\text{ads}}$ and $\Delta S_{\text{ads}}$ are respectively the changes in Gibbs free energy, enthalpy and entropy of the surface-protein-water system upon protein adsorption, and $T$ is the temperature. Adsorption is energetically favoured when $\Delta G_{\text{ads}} < 0$.\textsuperscript{61,67}

Additionally, it was recently proposed that the term $\Delta G_{\text{ads}}$ could be further divided into the following three components (equation 3):\textsuperscript{73}

\[
\Delta G_{\text{ads}} = \Delta G_{\text{hydrophobic}} + \Delta G_{\text{dehydration}} + \Delta G_{\text{interaction}}
\]

where $\Delta G_{\text{hydrophobic}}$ is the free energy gain resulting from the expulsion of the ‘hydrophobic’ proteins from the aqueous medium upon adsorption; $\Delta G_{\text{dehydration}}$ is the free energy cost due to the resulting displacement of interfacial water; and $\Delta G_{\text{interaction}}$ takes into account protein-surface and protein-protein interactions (free energy gain).

In order for a protein to adsorb, it would thus appear that both material and protein must undergo surface dehydration,\textsuperscript{43,61,73} a chaotropic process transferring into the bulk aqueous solution the molecules of water that were once coordinated within the material’s and protein’s layer/shell of hydration.\textsuperscript{43,58,59,61,67,73,74,92} The associated cost in energy would sharply rise with material surface hydrophilicity and need to be overcome in order to promote protein
adsorption.\textsuperscript{73} Energetically favourable situations (\textit{i.e.} \(\Delta H_{\text{ads}} < 0\) and \(\Delta S_{\text{ads}} > 0\)) include non-covalent\textsuperscript{38,64} and covalent bond formation, structural alteration of the protein (\textit{i.e.} unfolding) or the material surface (\textit{e.g.} interfacial reorganization of charged groups),\textsuperscript{67} as well as solvation effects.\textsuperscript{89,93} Although these factors may seem somewhat simple to identify, it is in actuality tremendously challenging to account for each and every enthalpic and entropic term (and their relative contribution) involved in the overall change in free energy – and thus difficult to fully grasp the phenomenon of protein adsorption.\textsuperscript{74} With that being said, there exists a relatively limited series of parameters known to influence protein adsorption, which relate to: (i) the nature of proteins with respect to their morphology and flexibility, size and molecular weight, and charge/polarity strength; (ii) the surface physicochemical properties (\textit{e.g.} topography, electrostatic potential, surface energy); and (iii) the environmental conditions (\textit{e.g.} pH, ionic strength and temperature).\textsuperscript{3,38,64,94,95}

Some empirical ‘principles’ have even emerged in the literature.\textsuperscript{58,73} With respect to the nature of proteins for instance, it seems as though the larger a protein, the more likely it is to possess multiple adhesion sites hence readily adsorb to a surface.\textsuperscript{64,88} The same would hold true for proteins near their isoelectric point (the pH value at which a protein is electrically neutral).\textsuperscript{88} Vis-à-vis surface properties, proteins would tend to display higher affinity for hydrophobic surfaces than hydrophilic ones.\textsuperscript{38,43,70,81,93,96,97} Furthermore, it has been shown that surface roughness also impacts protein adsorption, greater amounts of proteins adsorbing on rougher surfaces.\textsuperscript{98} Regarding environmental conditions, it would appear that the higher the temperature, the greater the rate of protein adsorption and the resulting amount of adsorbed proteins.\textsuperscript{99} The complexity of the mechanism of protein adsorption as well as the multiplicity of influencing parameters (and their interconnection) discussed earlier call for these ‘generalizations’ and others to be handled with extreme caution, as there always exists the possibility of finding equally compelling counter-examples. An example would be how the literature is replete with opposing views as to whether proteins adsorb in a mono- or multi-layer fashion.\textsuperscript{73,83} A reason for this apparent disagreement is that it is the tendency of small proteins – with which most protein adsorption studies are performed – to form monolayers, whereas less investigated larger proteins have been experimentally shown to produce multilayers.\textsuperscript{73} Furthermore, to add another level of complexity, it is important to note that blood proteins from different mammalian species may display different adsorption behaviour.\textsuperscript{73} Finally to consider as well is the possibility that a given protein-surface system may not always produce the same final organization in terms of adsorbed
protein orientation, conformation and packing\textsuperscript{74} owing to even subtle discrepancies in the multivariate, complex mechanism of protein adsorption.\textsuperscript{38}

1. 5. 3. Protein adsorption models

1. 5. 3. A. Single-protein solutions

Studies of protein adsorption date back to the early 1900s and have really gained in popularity since the 1930s.\textsuperscript{59,80} By allowing sufficient time for a protein and a surface to interact, it can be assumed that an equilibrium will be established between the amount of protein adsorbed on the surface and that of protein remaining in the bulk solution.\textsuperscript{100} This equilibrium relationship is commonly referred to as an \textit{adsorption isotherm}, which can be supplemented with other empirical data to help understand protein-surface interactions and even the dynamic composition of the layer of adsorbed protein.\textsuperscript{100} In general, adsorption isotherms are represented graphically by plotting the amount of adsorbed material as a function of non-adsorbed material, at a constant temperature and pH.\textsuperscript{100} The resulting adsorption isotherm plot can then be fitted with a suitable adsorption model to provide information regarding the thermodynamics of protein adsorption along with other factors related to the adsorption process.\textsuperscript{101} There are several different adsorption isotherm models used to describe the process of protein adsorption. For instance, one model developed by Freundlich describes monolayer protein adsorption onto heterogeneous surfaces displaying binding sites that vary with respect to the rate and energy of protein adsorption.\textsuperscript{101,102} Another example is the Brunauer-Emmett-Teller (BET) model, which depicts multilayer protein adsorption.\textsuperscript{101,103} However, the most frequently used and reported\textsuperscript{101} model to address protein adsorption (from solution) is that of Langmuir\textsuperscript{104} – even though it was originally developed to explain gas-to-solid phase adsorption.\textsuperscript{58,70,79,100} The \textit{Langmuir isotherm} expression can be derived by treating the adsorption/desorption of a material (the ‘adsorbate’) onto a surface (the ‘adsorbent’) as a state of equilibrium. In the case of protein adsorption from a solution to a solid surface, this can be represented as \textbf{equation 4}:

\[
P + S \xrightarrow{\text{adsorption}} PS \xleftarrow{\text{desorption}}
\]

where \(P\) is the adsorbing protein, \(S\) is the surface (available binding sites), and \(PS\) represents the protein bound to the surface.\textsuperscript{101}
The adsorption equilibrium constant, \( K_{\text{ads}} \), can then be defined as follows (equation 5):

\[
K_{\text{ads}} = \frac{[PS]}{[P][S]} \quad (5)
\]

Considering that the amount of protein bound to the surface ([PS]) is proportional to the surface coverage of adsorbed proteins (\( \theta \)), that [S] is proportional to the number of vacant binding sites (1 – \( \theta \)), and that [P] is proportional to the protein bulk concentration (\( C_0 \)), another equilibrium constant – the adsorption constant (A), which measures the capability of adsorption – can next be defined through the following expression (equation 6):

\[
A = \frac{\theta}{(1-\theta)C_0} \quad (6)
\]

Rearrangement of equation 6 isolating for surface coverage provides the familiar mathematical form of the Langmuir adsorption isotherm (equation 7):

\[
\theta = \frac{AC_0}{1 + AC_0} \quad (7)
\]

Graphically – when surface coverage (\( \theta \)) is plotted against the bulk concentration of protein (\( C_0 \)), as shown in Figure 8 – a steep linear segment is first observed, followed by a plateau at saturation. Surface saturation typically occurs between 0.1-0.5 \( \mu \)g/cm\(^2\) (depending on the protein dimension and orientation) – a value characteristic of a close-packed, monolayer-like coverage.\(^7\)

---

**Figure 8.** General graphical form of Langmuir isotherm, and schematic representation of the process of protein surface adsorption/desorption onto available binding sites until saturation. Figure adapted from reference 70.
The Langmuir isotherm model makes several assumptions: (i) there is only one type of molecule adsorbing from solution; (ii) the latter is dilute; (iii) there are a finite number of available binding sites; (iv) there is single occupancy per site (i.e. monolayer coverage); (v) the surface is energetically uniform; (vi) adsorption sites are independent of each other (i.e. lateral interactions between adsorbed/adsorbing molecules are insignificant); and (vii) adsorption is reversible.\textsuperscript{58,101} In practice however, most of these conditions are usually violated.\textsuperscript{70,101} For instance, the manner an incoming protein adsorbs onto a surface surely is dictated by its interaction with pre-adsorbed proteins. Not only that, but also something Langmuir himself recognized is the fact that surfaces are not energetically homogeneous.\textsuperscript{70} Moreover, very large proteins can occupy more than one binding site.\textsuperscript{64} This undoubtedly simple model still continues to be used to fit experimental adsorption data despite the fact that the assumptions made may not be satisfied;\textsuperscript{101} however, more sophisticated variants have since been developed to account for the more complex nature of the process of protein adsorption, including adsorption irreversibility,\textsuperscript{58,105} adsorbed protein conformational changes,\textsuperscript{97} as well as protein rearrangement over time\textsuperscript{80} resulting in a completely irreversible protein layer.\textsuperscript{58,68}

1. 5. 3. B. Multi-protein solutions

Adsorption studies and models have traditionally been focused on simple single-protein solutions. Attempts have been made however to model the more complex competitive adsorption of two or more proteins. One example is the two-protein model developed by Bessinger and Leonard with albumin and IgG.\textsuperscript{97} Nevertheless, this model is highly impractical to predict protein adsorption behaviour as it involves 12 variable parameters.\textsuperscript{58,97} Not surprisingly, modelling complexity to predict protein adsorption increases along with the number of proteins included in the model. Empirically however, it has been observed that mixtures of proteins interact with artificial surfaces in a sequential manner. As discussed in Section 1. 5. 2., the various types of protein initially diffuse towards the surface at a rate depending on their diffusion coefficient and bulk concentration.\textsuperscript{58} A temporal adsorption pattern thus takes place, wherein higher mobility, more abundant proteins first adsorb transiently before being gradually replaced by less motile, scarcer ones with higher surface affinity (Figure 9).\textsuperscript{12,38,87,93} This general, well-established phenomenon of sequential protein adsorption was first observed in the late 1960s by Vroman and Adams through ellipsometry studies, and is generally termed the ‘Vroman effect’.\textsuperscript{29,43,64,68,84,86,87,106,107} What was eventually found is that blood plasma proteins adsorb onto
glass and some metal oxide surfaces in the following order: albumin, γ-globulin, fibrinogen, fibronectin, clotting Factor XII, and high-molecular weight kininogen (HMWK).\textsuperscript{58,108}

![Protein Adsorption Diagram](image)

**Figure 9.** Schematic representation of the dynamic ‘Vroman effect’ for a three-protein mixture. Initial surface adsorption is determined by protein mobility and concentration. However, as time progresses, proteins sequentially exchange with respect to their affinity for the surface.

Additionally, diffusion from the bulk phase towards the surface entails an environmental change, which may induce proteins to conformationally rearrange. As a consequence, hydrophobic domains – once buried within the protein and hidden from the aqueous medium – may be revealed and adsorb onto the sorbent material. Further, surface-induced conformational restructuration may ensue and lead to the exposure of cryptic bioactive sites able to initiate biological processes upon binding with cell transmembrane integrin receptors (for instance).\textsuperscript{38,61,109,110} [Indeed, it would be through cells interrogating the first-adsorbed layer of proteins – and not the substrate material itself – that surface-induced cellular activation may be triggered.\textsuperscript{38}] It has been recognized that the presence of fibrinogen as part of an adsorbed layer of proteins stimulates platelet and leukocyte adhesion, and thus plays a role in the surface-activation of the coagulation and immune responses.\textsuperscript{49,111-115} Similarly, it has also been reported that high thrombogenicity can be triggered by the presence of adsorbed γ-globulins, which enhance platelet\textsuperscript{116} and leukocyte\textsuperscript{117} adhesion.\textsuperscript{97} Research has made it quite evident that host biological responses to artificial material exposure are mediated by ‘surface-active/activated’, adsorbed proteins.\textsuperscript{34,49,54,109}
1. 6. Biological processes/responses induced by blood-material interactions: a closer look

Biomaterials have found a prominent place in the medical field through their implementation in various types of equipment, implants and devices used to improve or even save the life of an ever-increasing number of individuals. As discussed earlier, the interaction of these exogenous surfaces with blood may unfortunately trigger biological processes/responses with harmful effects, which by no means are restricted to the health of the recipients of a medical intervention but also may compromise the performance and integrity/longevity of blood-contacting objects. As no perfect biocompatible artificial surface is available as of yet, it is important that biological responses to biomaterials be at least managed so that the quality of a patient’s life is not impacted. For instance, blood coagulation via clotting – which is normally activated following injury to prevent the organism from losing blood – may also be stimulated during various medical procedures such as coronary bypass surgery and renal dialysis. A problem with such undesired clot formation is the risk for patients to experience a cerebrovascular accident (i.e. a stroke) if circulating thromboemboli obstruct blood vessels and consequently restrict blood supply (ischemia) in the brain. Another case is that of the foreign body reaction, an immune response that accompanies normal wound healing following the implantation of foreign materials and eventually leads to the encapsulation and deterioration of implanted objects. Both these biological processes/responses are discussed in greater detail next.

1. 6. 1. Blood coagulation and thrombosis

1. 6. 1. A. Mechanism: history, clotting factors and pathways

There are, within blood and tissue, over 50 recognized essential substances – respectively termed procoagulants and anticoagulants – that, as their name suggests, either promote or inhibit coagulation. Their balance dictates whether blood will clot or not. In a healthy individual, blood interacting with normal, uninjured endothelium (the cellular lining making up the inner wall of blood vessels – Figure 1) does not elicit coagulation. This is mainly due to the fact that endothelial cells release (nitric oxide, prostacyclin) as well as expose (heparan sulfate – an analogue of heparin, thrombomodulin) anticoagulant species. Blood vessel injury, on the other hand, does trigger coagulation, as does the contact of blood with exogenous materials. Surface-induced thrombosis is triggered by the initial, spontaneous acquisition of a layer of adsorbed blood proteins onto which platelets and leukocytes adhere to activate the coagulation and complement systems. Study of the influence of interfacial adsorption on blood clotting can be dated back to Johlin’s 1929 investigation of the formation of interfaces between plasma and
various organic media. A number of theories were proposed in the decades that followed to explain the formation of blood clots, but it was not until 1964 that the cascade or ‘waterfall’ models emerged to explain the role of various protein clotting factors through a series of enzymatic reactions, and thus aid in clarifying the confusion surrounding earlier theories. As was the case for protein adsorption, it is also safe to say that the mechanism behind blood coagulation is also highly involved. The latter hinges on a sequence of proteolytic reactions involving the chain-reaction activation of zymogens (i.e. inactive enzyme precursors) – the so-called coagulation factors. Among the various factors involved in the clotting mechanism (Table 3), at least a dozen plasma proteins normally circulate throughout the body in their inactive form. The Roman numeral labelling system used for most clotting factors (Table 3) correlates with the order in which these were discovered, not the order in which they are actually involved in the coagulation cascade. Their active form is denoted by the addition of the suffix ‘a’ next to their Roman numeral. For instance, activated Factor X will be noted as ‘Xa’.

<table>
<thead>
<tr>
<th>Roman numeral</th>
<th>Synonymous names</th>
<th>Normal plasma concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Fibrinogen</td>
<td>2500</td>
</tr>
<tr>
<td>II</td>
<td>Prothrombin</td>
<td>100</td>
</tr>
<tr>
<td>III</td>
<td>Tissue factor, tissue thromboplastin</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>Ionized calcium (Ca^{2+})</td>
<td>45-56</td>
</tr>
<tr>
<td>V</td>
<td>Proaccelerin, labile factor, Ac-globulin (Ac-G)</td>
<td>7</td>
</tr>
<tr>
<td>VII</td>
<td>Serum prothrombin conversion accelerator (SPCA), proconvertin, stable factor</td>
<td>0.5</td>
</tr>
<tr>
<td>VIII*</td>
<td>Antihemophilic factor (AHF), antihemophilic globulin (AHG), antihemophilic factor A</td>
<td>0.1</td>
</tr>
<tr>
<td>IX</td>
<td>Plasma thromboplastin component (PTC), Christmas factor, antihemophilic factor B</td>
<td>6</td>
</tr>
<tr>
<td>X</td>
<td>Stuart-Prower factor</td>
<td>10</td>
</tr>
</tbody>
</table>

(Table 3 to be continued)
(Table 3 continued)

<table>
<thead>
<tr>
<th>Roman numeral</th>
<th>Synonymous names</th>
<th>Normal plasma concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XI</td>
<td>Plasma thromboplastin antecedent (PTA), antihemophilic factor C</td>
<td>4</td>
</tr>
<tr>
<td>XII</td>
<td>Hageman factor</td>
<td>30</td>
</tr>
<tr>
<td>XIII</td>
<td>Fibrin-stabilizing factor</td>
<td>15</td>
</tr>
<tr>
<td>Prekallikrein</td>
<td>Fletcher factor</td>
<td>50</td>
</tr>
<tr>
<td>High-molecular weight kininogen</td>
<td>HMWK, Fitzgerald factor</td>
<td>70</td>
</tr>
<tr>
<td>von Willebrand factor (platelet adhesion cofactor)*</td>
<td>VWF</td>
<td>7</td>
</tr>
<tr>
<td>Platelet/tissue phospholipid</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

Table 3. Blood clotting factors. *In plasma, Factor VIII and von Willebrand factor are complexed.

The clotting mechanism can be divided into two pathways (Figure 10), which can be initiated simultaneously or separately.\(^4\,5\,49\) On the one hand, the *intrinsic pathway* is activated when: (i) blood is exposed to collagen following the lesion of a blood vessel; (ii) substances within blood are damaged; or (iii) blood contacts a negatively-charged, foreign surface.\(^5\,26\,49\) On the other hand, the *extrinsic pathway* is activated by the rupture of the vascular wall and exposure of blood to damaged tissue.\(^3\,5\,26\,49\) Both processes involve phospholipids – ‘platelet phospholipids’ for the intrinsic pathway, ‘tissue phospholipids’ for the extrinsic pathway – with which certain activated coagulation factors complex, bridged by Ca\(^{2+}\) – a necessary factor in clot formation.\(^3\,5\,49\) In fact, with the exception of factor activation through contact, Ca\(^{2+}\) is required in most steps of blood coagulation (Figure 10). Finally, although the intrinsic and extrinsic coagulation pathways are initiated differently, both merge into a *common pathway* that eventually leads to the formation of a stabilized fibrin clot (Figure 10).\(^3\,5\,26\,49\)
Figure 10. Simplified representation of the intricate blood coagulation cascade. Two differently-activated pathways exist – the intrinsic pathway (pink panel) and the extrinsic pathway (blue panel). Both eventually merge into a common pathway (purple panel) at the end of which a stabilized fibrin clot is formed. Figure adapted from reference 49.

Although a more recent, widely acknowledged model has emerged (wherein coagulation is mediated and regulated by Tissue factor-bearing cells rather than phospholipid vesicles), the earlier model presented herein (Figure 10) continues to serve as a reference to describe coagulation and will be further discussed next.
1. 6. 1. B. A closer look at the coagulation pathways

The initiation of the extrinsic pathway involves the release of Tissue factor (Factor III) – an intracellular protein found in tissue but not in plasma that activates Factor VII (VIIa). In the presence of Ca$^{2+}$, the Tissue factor-VIIa complex next activates Factor X (Xa), entering as such the common coagulation pathway (Figure 10). On the other hand, the intrinsic pathway is initiated through the action of Factor XII, prekallikrein and high-molecular weight kininogen (HMWK) upon contact with collagen following blood vessel wall damage, or contact with a foreign surface. In essence, activation starts with the surface adsorption-induced conformational restructuration of Factor XII into its activated form XIIa, which converts inactive prekallikrein into kallikrein. Kallikrein accelerates the activation of Factor XII. Along with HMWK, XIIa activates Factor XI (XIa), which in turn activates Factor IX (IXa). The latter complexes with activated Factor VIII (VIIIa) and platelet phospholipids to activate Factor X, entering as such the common coagulation pathway (Figure 10).

Upon activation either through the intrinsic or extrinsic pathway (Figure 10), Factor X (i.e. Xa) merges with activated Factor V (Va) and phospholipids to form a complex known as prothrombin activator that, in the presence of Ca$^{2+}$, cleaves and converts prothrombin (Factor II) into thrombin (IIa), the activator of Factor V. In the early stages of coagulation, Factor V exists in the prothrombin activator complex in its inactivated form; however, once the production of thrombin starts, so does the activation of Factor V (into Va) – triggering an ‘autocatalytic’ process that greatly accelerates prothrombin activation. In the prothrombin activator complex, it is activated Factor X (Xa) that cleaves prothrombin into thrombin, phospholipids and activated Factor V (Va) accelerating the process. Continuing onto the last steps of the coagulation mechanism, another role of thrombin is to transform fibrinogen (Factor I) into fibrin monomers that automatically polymerize into a loose mesh. Additionally, thrombin also activates Factor XIII (XIIIa), which stabilizes the mesh via the covalent cross-linking of fibrin strands into a dense insoluble aggregate. Blood clots are also composed of blood cells, platelets, plasminogen and other plasma proteins trapped within the fibrin network. With respect to time, it takes approximately 1-6 min for the intrinsic pathway to produce a clot once it has been activated. On the other hand, depending on the severity of tissue trauma, clot formation through the extrinsic pathway may take as little as 15 seconds.
The body also naturally possesses different mechanisms and feedback loops to prevent excessive blood coagulation and the formation of large, abnormal thrombi.\textsuperscript{26} For instance, anticoagulants are normally found in the body, such as antithrombin III (within plasma) that binds and inactivates thrombin and other coagulation promoters such as Factors IXa, Xa and XIa. Endothelial cells also play an important regulatory role, either through the release of substances such as Protein C (which prevents clotting by inactivating Factors Va and VIIIa) or by displaying inhibitory receptors.\textsuperscript{126} \textit{Fibrinolysis} is another manner through which the organism regulates coagulation. In this mechanism, inactive plasminogen is converted to active plasmin \textit{via} activators such as tissue plasminogen activator or urokinase plasminogen activator.\textsuperscript{3,5,26} Plasmin digests the fibrin mesh within the clot, producing soluble fibrin-degradation products that function as thrombin inhibitors.\textsuperscript{3,5,26} Furthermore, plasmin also digests fibrinogen as well as inactivates Factors Va, VIIIa and XIIa.\textsuperscript{3,26} Once bleeding has stopped, the \textit{fibrinolytic system} plays the important role of dissolving obsolete clots to restore blood flow. Otherwise, the obstruction of healed blood vessels (and the resultant restriction of blood supply) could lead to their destruction along with that of surrounding tissue.
1.6.2. Inflammation, wound healing and foreign body responses

The classical view of host responses to in vivo applications of synthetic materials consists of a series of interlinked events that include: blood-material interactions, inflammation (first acute then possibly chronic), and the foreign body reaction with final fibrous encapsulation (Figure 11). The degree and extent of these responses depend on various factors such as the severity of the tissue injury caused by the implantation procedure, or the physicochemical properties of the implanted object.

Figure 11. Cartoon illustration of the host response caused by the implantation of artificial materials. Figure adapted from references 3 and 53.
Tissue injury (incision wound and associated trauma) is the first event to trigger a host response. As discussed in previous sections, the spontaneous adsorption of proteins such as fibrinogen, fibronectin, IgG and C3b (a product of the activated complement system) onto the inserted object is also an early occurrence,\textsuperscript{54,127} of the actual act of implantation (Figure 11). The composition of the protein layer plays a crucial role in the activation of both the coagulation and complement systems, as well as in the interaction between platelets and immune cells to form the \textit{provisional matrix} and initiate the inflammatory response.\textsuperscript{54,128} The provisional matrix also encompasses a plethora of other substances such as fibrin (from coagulation) as well as inflammatory products. The matrix aids and mediates – both structurally and chemically – the inflammatory and wound healing processes by modulating macrophage activity as well as cellular propagation \textit{via} activator/inhibitor molecules. The nature of the primary immune cells interacting with the provisional matrix varies with time (Figure 11). Neutrophils first dominate the acute phase of inflammation. After approximately two days, monocytes (which differentiate into macrophages) take over and attack the material. Neutrophils and macrophages attach to surface-adsorbed IgG and C3b proteins \textit{via} specific surface receptors that induce intracellular signalling cascades.\textsuperscript{54,127} Other molecules such as the C5b-9 complex – a product of the activated complement system – have also been shown to be able to recruit leukocytes.\textsuperscript{27} Usually, macrophages dispose of foreign invaders through \textit{phagocytosis} – a process that can be summarized into three main steps that are: invader engulfment, destruction and exocytosis as waste products. Considering the relative size of macrophages \textit{vs.} foreign objects (much larger), individual phagocytosis is not an option. Instead, macrophages fuse together to form multinucleated \textit{foreign body giant cells} (FBGC), as shown in Figure 11.\textsuperscript{129} [The mechanism through which FBGC form is extremely complex and has not been fully explained as of yet.\textsuperscript{14,54}] Cellular fusion still is insufficient to surround the implant; as a consequence, FBGC release cytokines (chemical messengers) in an attempt to recruit fibroblast cells, which will initiate the synthesis of collagen (the main structural protein of connective tissue). The end result is the generation of a fibrotic capsule (roughly 50-200 μm-thick)\textsuperscript{3} that isolates the implanted object from the surrounding host environment. With time, the environment within the fibrotic capsule will cause the implant to degrade.\textsuperscript{128} The resulting alteration of the implant’s integrity (as well as functionality and longevity) may require patients to undergo costly and invasive elimination/replacement surgery.\textsuperscript{75,130} Even though a comprehensive description and full understanding of host responses to artificial materials are still to come, the mechanism schematically depicted in Figure 11 is generally agreed upon.\textsuperscript{3,14,53}
If there was only one message to take away from previous sections, it would be that the spontaneous adsorption of proteins onto artificial materials is a genuine biotechnological plague that affects the performance and fate of both bioanalytical and biomedical equipment, implants or devices contacting blood or other biofluids. The next section discusses recent strategies to combat protein fouling from arguably the most challenging proteinaceous media: blood plasma and serum.

1. 7. Surface modification to minimize fouling from blood-based fluids

As discussed in previous sections, exogenous materials coming into contact with blood (or other proteinaceous media such as its plasma/serum derivatives) spontaneously acquire a layer of proteins on their surface. The concern with respect to biomedical equipment, implants and devices is for biological processes with potentially deleterious effects to ensue. For biosensors, the issue is that of non-specific adsorption on the sensing interface, which generates an often overwhelming interference signal that lowers device sensitivity (i.e. decrease the signal-to-noise ratio) to clinically irrelevant levels. It is thus not surprising that over the last few decades considerable effort has been devoted to engineering protein-resistant (or antifouling) surfaces – most traditionally through the application of organic films onto bulk substrates. Extensive literature has been published that describes numerous types of stealth organic coatings able to minimize protein adsorption down to a few ng/cm$^2$. Among these, oligo- and polyethylene glycol (OEG and PEG)-based constructs have been historically the most studied. Other antifouling surface chemistries, for example, rely on polysaccharide (e.g. dextran) or biomacromolecule (e.g. bovine serum albumin) building blocks. However, praised antifouling properties are almost always reported to be displayed in experiments performed with single – or extremely simple mixtures of – model proteins in buffer. Obviously, such testing protocol has no practical relevance clinically, as these sample solutions do not meet the more stringent conditions imposed by highly complex, real-life biological milieux such as blood plasma and serum. Unfortunately, there are much fewer examples of coatings that attain/retain in actuality such level of performance when challenged by these biofluids. The following section concisely reviews such recently-developed antifouling surface chemistry, which features thin self-assembled monolayers (SAM) and thicker polymer brushes/films. Unless otherwise specified, sample solutions contain ≥ 10 vol. % of blood plasma/serum and fouling amounts do not exceed $\Gamma = 30$ ng/cm$^2$. 

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1.7.1. ‘Bio-inspired’ constructs: single amino acids, peptides and peptoids

One of the first studies to investigate the use of amino acid-based coatings for antifouling purposes was conducted in 1995 by Lestelius et al. In that work, 0.6 nm-thick L-Cysteine (Figure 12A) monolayers were directly immobilized on gold substrates, then exposed to 10% human plasma diluted in PBS for 10 min followed by successive PBS/water rinsing. Using in situ ellipsometry, the surface coverage due to protein adsorption was estimated to be $\Gamma \sim 200$ ng/cm$^2$ – i.e. only slightly lower than that observed for unmodified gold. However, when the investigators tested 1.3 nm-thick monolayers made of glutathione tripeptide (Figure 12B), considerable reduction in protein fouling was observed ($\Gamma < 50$ ng/cm$^2$).

Figure 12. Molecular structure of (A) L-Cysteine amino acid and (B) glutathione tripeptide.

Further investigations on the antifouling properties of single amino acid-based monolayers – attached to gold substrates through $N$-3-mercaptopropionic acid (3-MPA) graft residues – were conducted by Masson’s group in 2008. Each coating was exposed to 76 mg/mL, undiluted bovine serum for 20 min and protein NSA/fouling was quantified using surface plasmon resonance (SPR). Among the 19 natural amino acids studied, the lowest amount of NSA ($\Gamma \sim 418$ ng/cm$^2$) was observed for SAMs made of polar (L-Asparagine, L-Serine) or ionic (L-Aspartic acid) amino acids (Figure 13A). Conversely, the highest amount of fouling ($\Gamma \sim 808$ ng/cm$^2$) was seen for the hydrophobic L-Tyrosine-based SAM. NSA for the entire series of amino acids increased in the following order: Asp < Asn < Ser < Met < Glu < Gln < Thr < Gly < His < Cys < Arg < Phe < Trp < Val < Pro < Ile < Leu < Ala < Tyr. The following year, the study was extended to short 3-MPA-homopeptide SAMs with the general structure 3-MPA-(AA)$_n$-OH – where AA is Leu, Phe, Ser, Asp or His; and $n = 2$ to 5. Surface coverage due to NSA was lowest for pentapeptide SAMs composed of L-Serine building blocks ($\Gamma = 132 \pm 33$ ng/cm$^2$ for 3-MPA-Ser$_5$-OH – Figure 13B). In this study too, it was concluded that monolayers made of polar and ionic amino acids were the most efficient at reducing NSA. Continuing on, Masson and co-workers next investigated coatings made of 3-MPA-pentapeptides presenting binary sequences of amino acids with the
general 3-MPA-A_x-B_y-OH formula – where A was either His, Asp, Ser or Leu; B was either His, Asp or Ser; and x + y = 5.\textsuperscript{145,146} For the nine different designs investigated, NSA was consistently lower than 80 ng/cm\textsuperscript{2}, the best resistance to protein adsorption belonging to 3-MPA-Ser\textsubscript{3}-Asp\textsubscript{2}-OH (Figure 13C) SAM with \( \Gamma = 23 \pm 10 \) ng/cm\textsuperscript{2}. Most recently, a third generation of ternary patterned hexapeptide constructs was investigated, of which 3-MPA-(Leu-His-Asp)\textsubscript{2}-OH (Figure 13D) SAMs exhibited the least amount of NSA (\( \Gamma = 12 \pm 11 \) ng/cm\textsuperscript{2}).\textsuperscript{147}

\textit{(A) Single amino acid study (\( \Gamma \sim 418 \) ng/cm\textsuperscript{2})}

\textit{(B) Homopentapeptide study (\( \Gamma \sim 132 \) ng/cm\textsuperscript{2})}

\textit{(C) Binary-patterned pentapeptide study (\( \Gamma \sim 23 \) ng/cm\textsuperscript{2})}

\textit{(D) Ternary-patterned hexapeptide study (\( \Gamma \sim 12 \) ng/cm\textsuperscript{2})}

\textbf{Figure 13.} Molecular structure of (A) 3-MPA-Asn-OH, 3-MPA-Ser-OH and 3-MPA-Asp-OH single amino acids, (B) 3-MPA-Ser\textsubscript{3}-OH homopentapeptide, (C) 3-MPA-Ser\textsubscript{3}-Asp\textsubscript{2}-OH binary-patterned pentapeptide, and (D) 3-MPA-(Leu-His-Asp)\textsubscript{2}-OH ternary-patterned hexapeptide.
In 2002, Picart and co-workers reported their findings on the antifouling properties of polyelectrolyte multilayered constructs made of alternating strata of poly(L-Lysine), PLL, and poly(L-Glutamic acid), PGA, polypeptides (Figures 14A and 14B, respectively). The films, which ranged in thickness from 1 to 153 nm, were exposed for 60 min to 10% serum diluted in MES-Tris buffer, and optical waveguide lightmode spectroscopy (OWLS) was used to determine the amount of fouling. The authors observed that films exposing a terminal PLL layer invariably adsorbed a significant amount of serum proteins ($\Gamma > 300$ ng/cm$^2$), regardless of film thickness (i.e. irrespective of the number of alternating layers beneath). In contrast, protein adsorption on PGA-terminated coatings was negligible, at least up to six layers of polyelectrolytes for ~16 nm-thick (PLL/PGA)$_3$ films.

![Molecular structure](image)

**Figure 14.** Molecular structure of (A) poly(L-Lysine), PLL, and (B) poly(L-Glutamic acid), PGA.

More recently, in 2013, Liu and co-workers prepared zwitterionic poly(L-Serine) brushes on gold through a ‘grafting from’ photochemical process – known as ‘surface-initiated photoiniferter-mediated polymerization’ – that involved a pre-coated SAM of thiol/dithiocarbamoyl photoiniferter (Figure 15A) and L-Serine methacrylate monomer (Figure 15B). Considering different film thicknesses could be readily obtained by varying the UV-irradiation time, the authors were able to determine the relationship existing between this parameter and fouling from full human blood serum/plasma. Interestingly, results showed that serum fouling was minimal ($\Gamma = 9.2 \pm 1.3$ ng/cm$^2$) for an intermediate thickness of 37 nm, as measured upon 10 min of exposure using SPR as the detection technique. Both thinner and thicker brushes (approximately 5-55 nm range) were indeed less resistant to serum adsorption ($10 < \Gamma < 50$ ng/cm$^2$). Gratifyingly, when the optimal 37 nm-thick surface coating was tested against full plasma, fouling was also limited ($\Gamma = 12.9 \pm 2.1$ ng/cm$^2$). In contrast, both bare gold and photoiniferter precursor SAM surfaces were substantially fouled by both serum and plasma with $\Gamma > 150$ ng/cm$^2$. 

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In 2005, Messersmith and co-workers reported the synthesis of a new peptidomimetic polymer to modify Ti surfaces for antifouling purposes.\textsuperscript{150} The polymeric structure features two distinct domains: (i) a short biomimetic substrate-anchoring pentapeptide made of alternating L-DOPA and L-Lysine residues, \textit{N}-coupled to (ii) a \textit{N}-(2-methoxyethyl)-substituted 20-mer peptoid (\textbf{Figure 16A}). Polymer coating exposed to whole human serum for 20 min demonstrated excellent resistance to protein adsorption (\(\Gamma \approx 4\) ng/cm\(^2\), as opposed to \(\Gamma \approx 435\) ng/cm\(^2\) for unmodified Ti). The project was later extended to investigate the influence of the nature of the peptoid side-chains on antifouling. In the new study, three different side-chains would be employed (2-methoxyethyl-, 2-hydroxyethyl- and 2-hydroxypropyl-) while keeping identical the anchoring pentapeptide sequence (\textbf{Figures 16A-C}).\textsuperscript{151} Although the authors encountered mixed success among polymers, all three \(\approx 4.3\) nm-thick coatings displayed superior resistance to protein adsorption compared to the unmodified TiO\(_2\) substrate of the study (\(\Gamma = 15-83\) ng/cm\(^2\) \textit{v.s.} \(\Gamma = 342 \pm 21\) ng/cm\(^2\)), when exposed to whole human serum for 20 min.\textsuperscript{151} Varying the repeat length of \textit{N}-(2-methoxyethyl)-substituted peptoid segments (\(n = 10\) to 50, for a coating thickness ranging from 2.8 to 4.2 nm) had statistically no effect on protein adsorption (\(\Gamma = 15-53\) ng/cm\(^2\)).\textsuperscript{152}
Figure 16. Molecular structure of the (A) N-(2-methoxyethyl)-, (B) N-(2-hydroxyethyl)-, and (C) N-(2-hydroxypropyl)-substituted 20-mer peptoids C-coupled to a substrate-anchoring pentapeptide sequence made of alternating L-DOPA and L-Lysine residues.

Another study on peptoid-based antifouling coatings introduced a new type of polymer with N-ethyl-β-alanine units (Figure 17). Poly(β-peptoid)s were tethered to gold via C-terminal cysteamine graft residues. Fouling was assessed by SPR upon contact for 10 min with dilute (10% in PBS) as well as full human serum and plasma. Gold substrates coated with PEtA20 – a 20-mer of N-ethyl-β-alanine – exhibited excellent antifouling properties against both 10% serum ($\Gamma = 10.7 \pm 3.6 \text{ ng/cm}^2$) and 10% plasma ($\Gamma = 5.2 \pm 6.2 \text{ ng/cm}^2$). Comparable levels of performance were achieved for full serum ($\Gamma = 10.8 \pm 14.8 \text{ ng/cm}^2$) and full plasma ($\Gamma = 9.8 \pm 12.7 \text{ ng/cm}^2$). Surfaces built with longer PEtA40 poly(β-peptoid) displayed superior protein resistance with fouling amounts from 10% serum and 10% plasma being respectively $\Gamma = 3.9 \pm 1.2$ and $5.7 \pm 1.5 \text{ ng/cm}^2$; while those for 100% serum and 100% plasma were $\Gamma = 5.9 \pm 3.4$ and $9.7 \pm 3.2 \text{ ng/cm}^2$. 

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Figure 17. Molecular structure of PEtA20 (n = 20) and PEtA40 (n = 40) poly(N-ethyl-β-alanine) polypeptoids.

1.7.2. Oligo- and polyethylene oxides/glycols

Coatings featuring oligo- or polyethylene oxide (OEO and PEO) – or their derivatives – building block molecules constitute perhaps the most studied/used type of antifouling surfaces over the years. One example is tetruglyme (Figure 18), whose ~100 nm-thick films on gold have been shown using SPR to significantly resist protein adsorption whether from 10, 50 or 100% human plasma, protein fouling being respectively $\Gamma = 4.8, 17.3$ and 24.1 ng/cm$^2$ after exposure for 10-20 min. In comparison, bare gold adsorbed a considerably greater amount of proteins ($\Gamma \sim 244$ ng/cm$^2$), even from 1% plasma. At this dilution, protein adsorption on tetruglyme films was calculated to be $\Gamma = 1.3$ ng/cm$^2$.

Figure 18. Molecular structure of tetruglyme.

This short tetrameric OEG chain was integrated into an OEG$_4$-terminated alkylthiol linear molecule (Figure 19 – top) with which OEG$_4$-SAMs were formed on gold. These exhibited different antifouling behaviour depending on the nature and dilution of the testing medium. Thus, exposure for 15 min to 10% human plasma triggered significant fouling ($\Gamma \sim 200$ ng/cm$^2$) in contrast to 10% human serum ($\Gamma \sim 15$ ng/cm$^2$), 100% human serum ($\Gamma \sim 85$ ng/cm$^2$) or 20% human platelet-poor plasma (PPP, $\Gamma = 38.3$ ng/cm$^2$). In 2012, Rodriguez-Emmenegger et al. reported that OEG$_6$-SAMs (9 Å-thick), prepared with a slightly longer thiol molecule (Figure 19 – bottom), performed reasonably well against undiluted foetal bovine serum (FBS) but not against an undiluted human fluid (blood plasma), fouling being respectively $\Gamma = 26.1 \pm 2.7$ and 71.0 $\pm 8.0$ ng/cm$^2$.
Figure 19. Molecular structure of the OEG$_n$-terminated alkylthiol building block molecules (n = 4, 6) for antifouling SAM formation on gold.

Higher-molecular weight PEG molecules (MW = 2-20 kDa range) were also used to coat gold substrates (film thickness 1-5 nm) for antifouling purposes.$^{161}$ Resistance to protein adsorption – against 10% FBS for 30 min – was measured using SPR to be $\Gamma = 6$-10 ng/cm$^2$, the best performance being displayed by intermediately dense/thick PEG coatings formed with MW = 5 kDa polymer molecules.$^{161}$

An investigation involving derivatives of poloxamers/Pluronic$^{\text{TM}}$ – which are amphiphilic PEO-$bl$-PPO-$bl$-PEO triblock copolymers consisting of a central hydrophobic polypropylene oxide (PPO) segment flanked by two hydrophilic PEO chains – was carried out by Tosatti and co-workers.$^{162}$ These ‘sulfur’ PEO-$bl$-PPS-$bl$-PEO analogues (Figure 20A) were directly anchored to gold substrates – through multisite polysulfide chemisorption of the central PPS block – as ~34 Å-thick adlayers that were next exposed to whole human serum for 30 min.$^{162,163}$ Using SPR, protein fouling was measured to be $\Gamma = 16 \pm 5$ ng/cm$^2$, a value significantly lower than that of bare gold ($\Gamma = 380 \pm 14$ ng/cm$^2$).$^{162}$ Similar performance ($\Gamma = 25 \pm 17$ ng/cm$^2$) was observed for another variety of PEG-$bl$-PPS-$bl$-PEG-based coating (Figure 20B).$^{163}$

Figure 20. Molecular structure of two varieties of sulfur-based poloxamer analogues (PEO-$bl$-PPS-$bl$-PEO triblock copolymers).
Another kind of multidentate copolymeric chemisorbate – the terpolymer shown in Figure 21 that features undecylphosphonate, PEG and n-butyl side-chains branching out a central methacrylate backbone in a 1/1/8 ratio – has been shown to be able to self-organize onto TiO$_2$ substrates into 30 Å-thick films exhibiting excellent antifouling properties (Γ = 4 ± 1 ng/cm$^2$), as measured using OWLS upon exposure to full human serum for 15 min.$^{164,165}$

![Molecular structure of the PEG-polyalkyl phosphonate terpolymer.](image)

**Figure 21.** Molecular structure of the PEG-polyalkyl phosphonate terpolymer.

Further, complex OEG dendritic structures were studied by Gillich et al.$^{166}$ These macromolecules – which are composed of OEG dendrons conjugated to substrate-anchoring multidentate oligomers of L-DOPA/dopamine (Figure 22) – were designed to self-assemble onto TiO$_2$ surfaces.
Using ellipsometry, it was concluded that the amount of fouling strongly depended on coating surface coverage. After exposure to full blood serum for 20 min, the lowest protein adsorption – actually below the detection limit of the technique, and that of OWLS (~2 ng/cm²) – was obtained for monomolecular adlayers at saturation coverage. Films prepared with a linear PEG adsorbate of similar molecular weight (Figure 23) also exhibited such remarkable antifouling properties. This work constituted the latest addition to several previous studies, wherein linear PEG adsorbates possessing various L-DOPA-, dopamine- or derivatives-based anchors had also been successfully tested for their resistance to protein adsorption from full human serum.¹⁶⁷-¹⁷⁰
In 2011, Huck and co-workers published a systematic structural study investigating the influence on protein adsorption of the architecture of various OEO polymer brushes built on gold from the ‘grafting from’ polymerization of regular (Figure 24A), linear (Figure 24B) or dendritic (Figure 24C) glycerol-based monomeric substructures onto a precursor SAM. Using SPR, it was found that 17 nm-thick first-generation dendronic brushes (Figure 24C – left) performed best after exposure to undiluted human serum for 5 min ($\Gamma = 20 \pm 10$ ng/cm$^2$). Linear hydroxylated ‘bottle’ brushes (Figure 24B – $R = H$) 6 nm-thick also displayed antifouling properties with $\Gamma = 53 \pm 12$ ng/cm$^2$. On the other hand, second-generation dendritic brushes (Figure 24C – right), as well as regular$^{172}$ and linear methoxylated (Figure 24B – $R = \text{Me}$) coatings performed poorly with $\Gamma > 100$ ng/cm$^2$, regardless of brush thickness (3 to 22 nm). Similar architectural dependence and trends of protein adsorption were observed when antifouling experiments were conducted with plasma, the highest resistance to protein adsorption ($\Gamma = 8 \pm 6$ ng/cm$^2$) still belonging to the first-generation dendritic brushes.$^{171}$ Regardless, all polymer brushes did display superior antifouling performance – against both undiluted human serum and plasma – compared to bare gold for which fouling was respectively $\Gamma = 217 \pm 5$ and 385 $\pm 5$ ng/cm$^2$.

![Molecular structure](image)

**Figure 24.** Molecular structure of the (A) regular, monoglycerol- and (B) linear, oligoglycerol-based methacrylate monomers; as well as the (C) first- (left) and second-generation (right) dendritic oligoglycerol acrylate monomers.
Other types of bottle brushes were prepared on gold via this polymerization strategy using the linear, hydroxy- (HOEGMA) or methoxy-terminated (MeOEGMA) OEG methacrylate monomers shown in Figure 25. In 2008, Zhang et al. prepared 20-25 nm-thick poly(MeOEGMA) brushes that were able to limit fouling from 100% human plasma to $\Gamma = 9.2 \pm 6.5 \text{ ng/cm}^2$, as measured by SPR. In a separate investigation, Rodriguez-Emmenegger et al. reported similar low-fouling for both poly(HOEGMA) and poly(MeOEGMA) brushes with $\Gamma = 16.3$ and 18.9 ng/cm$^2$, respectively. Another study showed that poly(MeOEGMA) brushes ~30-40 nm-thick had remarkable antifouling properties against full FBS ($\Gamma < 0.6 \text{ ng/cm}^2$ upon 15 min of exposure). With regards to stability, Huck and co-workers demonstrated that, after one month of storage under ambient conditions, poly(MeOEGMA) brushes still exhibited excellent protein resistance against full FBS ($\Gamma \sim 12 \text{ ng/cm}^2$), fouling against 10% FBS being undetectable.

![Molecular structure of (A) HOEGMA and (B) MeOEGMA monomers.](image)

Figure 25. Molecular structure of (A) HOEGMA and (B) MeOEGMA monomers.

Furthermore, many research groups reported that poly(HOEGMA) and poly(MeOEGMA) brushes exhibiting excellent antifouling properties could also be prepared onto various types of substrate material, other than gold, using this ‘grafting from’ polymerization strategy. For instance, when ~95 Å-thick poly(MeOEGMA) brushes polymerized onto silicon oxide were exposed to undiluted FBS for 60 min, fouling was found to be below the LOD of the ellipsometer apparatus used to measure protein adsorption (1 Å). Another example involves ~135 Å-thick poly(HOEGMA) brushes that were prepared on titanium and shown to limit protein adsorption to a maximum of $\Gamma = 20 \text{ ng/cm}^2$, as measured by SPR upon exposure to 10% FBS diluted in DMEM. In a recent study, Rodriguez-Emmenegger et al. reported the growth of both poly(HOEGMA) and poly(MeOEGMA) brushes onto nylon-6/6, itself pre-coated onto various inorganic (gold, silicon, TiAlV alloy) or organic (polypropylene) substrates. Protein adsorption from full FBS (15 min of exposure) on poly(HOEGMA) brushes grown on nylon/Au substrates was measured by SPR to be $\Gamma = 9.7 \text{ ng/cm}^2$. Conversely, analogous poly(MeOEGMA) coatings were not fouled by full FBS ($\Gamma < 0.03 \text{ ng/cm}^2$, the LOD of the SPR instrument). With respect to undiluted human plasma, fouling amounts were respectively $\Gamma = 28.3$ and 19.5 ng/cm$^2$. 

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for poly(HOEGMA) and poly(MeOEGMA). Whether brushes were exposed to full FBS or full human plasma, fouling was much lower than that recorded for bare (Γ = 307 ng/cm²) or nylon-coated gold surfaces (Γ = 177 ng/cm²). This study also showed that poly(MeOEGMA) brushes were able to maintain their antifouling performance against both types of biological fluid after storage in PBS for 5 months (sheltered from light).

1. 7. 3. Zwitterionic sulfo- and carboxybetaines

Zwitterionic monomers – globally neutral molecules that contain formal positive and negative charged moieties at separate locations – have also been used to prepare protein-resistant coatings through the ‘grafting from’ polymerization method. Examples include sulfo betaine and carboxy betaine methacrylates (SBMA and CBMA – Figures 26A and 26B), as well as the carboxybetaine acrylamide (CBAA) variant shown in Figure 26C. Together with the polyOEGMA brushes described earlier, these recently-established polySBMA, polyCBMA and polyCBAA zwitterionic brushes rank among the coatings with highest antifouling performance known against blood serum and plasma.

A fourth kind of zwitterionic brush, constructed from bio-inspired phosphorylcholine methacrylate (PCMA – Figure 26D), has also been described but shown to be unable to prevent fouling from human plasma, even if diluted to 33% in PBS (Γ > 345 ng/cm², 30 min of exposure, SPR detection). Acceptable antifouling performance was only displayed against 10% FBS (Γ = 13–45 ng/cm²).

Figure 26. Molecular structure of the (A) SBMA and (B) CBMA methacrylate, (C) CBAA acrylamide, and (D) PCMA methacrylate zwitterionic monomers.
With respect to SBMA, a 2008 study conducted by Horbett and co-workers showed using SPR that 10-15 nm-thick polySBMA brushes performed fairly well against undiluted human plasma (\(\Gamma = 9.1 \pm 0.6 \text{ ng/cm}^2\)).\(^{173}\) Jiang and co-workers published a more systematic study, wherein polySBMA brushes of various thickness (15-90 nm range) were tested for their ability to withstand protein adsorption from full human serum upon 10 min of exposure.\(^{183}\) This work concluded that fouling was minimal (\(\Gamma = 6.1 \text{ ng/cm}^2\)) at the intermediate thickness of 62 nm. In fact, both thinner and thicker brushes adsorbed greater amounts of serum proteins, always below \(\Gamma \sim 20 \text{ ng/cm}^2\) however. Bailey’s group showed that effective polySBMA brushes can also be grown on substrates other than gold (unlike the above scenarios) such as silicon oxide.\(^{184}\) Fouling from undiluted FBS (\(\Gamma = 26 \text{ ng/cm}^2\)) was found to be much lower than that recorded for bare silicon oxide surfaces (\(\Gamma = 300 \text{ ng/cm}^2\)). Finally, there is also the approach by Chang et al. who, in their research for hemocompatible materials, devised copolymer coatings combining the excellent antifouling characteristics of zwitterionic polySBMA with the attractive thermo-responsive properties of non-ionic poly(N-isopropylacrylamide) (polyNIPAAm).\(^{185}\) The authors prepared a series of poly(SBMA-co-NIPAAm) hybrid copolymers (Figure 27) that were next physisorbed through hydrophobic-hydrophobic interactions onto CH₃-terminated SAMs pre-coated on gold. Using SPR, it was shown that copolymer brushes with a 15 mol% minimum content of SBMA systematically displayed high protein resistance against 10% PPP (\(\Gamma < 5 \text{ ng/cm}^2\)). Conversely, uncoated SAMs adsorbed a considerably greater amount of plasma proteins (\(\Gamma \sim 150 \text{ ng/cm}^2\)), as did polyNIPAAm homopolymer coatings (\(\Gamma \sim 90 \text{ ng/cm}^2\)).

![Figure 27. Molecular structure of poly(SBMA-co-NIPAAm) copolymers.](image)

Along with studying the antifouling behaviour of polySBMA coatings, Zhang et al. also examined in 2008 the antifouling properties of 10-15 nm-thick polyCBMA brushes exposed to undiluted human plasma.\(^{173}\) The result was a minimization of protein adsorption to \(\Gamma = 0.4 \pm 0.9 \text{ ng/cm}^2\). Another study investigated the influence of the incubation temperature on the amount of
protein adsorption from undiluted human plasma (10 min of exposure). This work revealed that 29 nm-thick polyCBMA brushes exhibited ultralow fouling properties ($\Gamma < 0.3$ ng/cm$^2$, the LOD of the SPR sensor) at all examined temperatures (25, 30 and 37°C). That same year, Rodriguez-Emmenegger et al. also described polyCBMA brushes able to efficaciously resist protein adsorption; however, only from 33% human plasma diluted in PBS. The amount of plasma proteins deposited after 30 min of exposure was below the LOD of the SPR instrument used ($\Gamma < 0.6$ ng/cm$^2$). In stark contrast, fouling on uncoated gold was much greater ($\Gamma = 400 \pm 98$ ng/cm$^2$). This study also explored the effect of polySBMA brushes and showed that, unlike the polyCBMA system, these brushes adsorbed substantial amounts of plasma proteins ($\Gamma = 320 \pm 78$ ng/cm$^2$) almost equivalent to that observed for unmodified gold. When CBMA and SBMA monomers were randomly copolymerized, protein adsorption decreased ($\Gamma = 180 \pm 26$ ng/cm$^2$). However, when polyCBMA was sequentially grown over polySBMA in a diblock layered copolymer configuration, fouling decreased to a much more acceptable level ($\Gamma = 24 \pm 10$ ng/cm$^2$). More recently, in 2012, Michálek and his team were able to construct polyCBMA brushes (20 nm-thick) on which protein fouling from full plasma (not diluted at 33% in PBS) was simply undetectable by SPR ($\Gamma < 0.03$ ng/cm$^2$, 30 min of exposure).

The last variant of zwitterionic brushes to be discussed – based on CBAA monomer – has been the object of multiple studies. One such work was conducted by Jiang and co-workers, who prepared polyCBAA brushes (15-20 nm-thick) onto which fouling from either 10, 50 or 100% human plasma (7 min of exposure) was below or near the LOD of their SPR sensor (0.2 ng/cm$^2$). The following year, the authors presented a more comprehensive study, wherein the effects on fouling of film thickness (10-55 nm range) and incubation temperature (25 or 37°C) were determined using SPR for both full human serum and plasma (10 min of exposure). This work revealed the existence of an intermediate, optimal thickness (~21 nm) at which protein adsorption was minimal (in fact undetectable), regardless of the incubation temperature or the nature of the contacting fluid. Moreover, the range in thickness for which $\Gamma < 5$ ng/cm$^2$ was 15-26 nm, at 25°C. Gratifyingly, these polyCBAA coatings maintained their high resistance to protein adsorption at the more relevant body temperature of 37°C, for an even wider range of thicknesses (15-40 nm). Soon after, Jiang and co-workers supplemented this remarkable work with a study in which CBAA monomers of various spacing length between the two charged moieties were investigated. Brushes 15-20 nm-thick, prepared with either regular-ethylene CBAA-2 (the structure actually shown in Figure 26C) or shorter-methylene CBAA-1
monomers, reduced protein adsorption from full human plasma and serum below $\Gamma = 5$ ng/cm$^2$, as measured with SPR upon 10 min of exposure. Conversely, polymerization of CBAA-3 monomer, which bears a longer propylene spacer, yielded slightly thicker brushes (15-25 nm) onto which fouling from undiluted fluids was higher, especially for serum ($\Gamma \sim 70$ ng/cm$^2$). In 2011, Rodriguez-Emmenegger et al. also reported polyCBAA brushes able to resist fouling from 100% FBS for 15 min ($\Gamma < 0.03$ ng/cm$^2$, the LOD of the SPR sensor). Unfortunately, with respectively $\Gamma = 15$ and 16 ng/cm$^2$, SPR measurements for undiluted human plasma and serum were not as satisfactory. Shortly after however, the authors reported 18 nm-thick polyCBAA brushes able to suppress fouling from undiluted human plasma ($\Gamma < 0.03$ ng/cm$^2$). Similar resistance to protein adsorption was observed with foetal bovine and calf sera in a more comprehensive study, which also aimed to assess fouling from five other human and animal biofluids (human cerebrospinal fluid, saliva and urine as well as chicken egg and whole cow milk). In comparison, plasma proteins avidly adsorbed on bare gold ($\Gamma = 307$ ng/cm$^2$). Lastly, the authors also showed that fouling on these polyCBAA brushes was still undetectable after longer exposure (120 vs. 15 min).

1.7.4. Hybrid, derivative and biomimetic materials

The remaining surface-modifying (macro)molecules to be discussed may not necessarily belong to any of the three families presented thus far. Rather, the following are combinations thereof (i.e. hybrids), derivatives or totally unrelated materials. Nonetheless, these surface modifiers are no less important and form, as a matter of fact, some of the best antifouling coatings known against blood serum and plasma.

1.7.4. A. PLL-graft-PEG hybrids and PLL-graft-PMOXA analogues

In a series of studies published between 2000 and 2012, several groups described hybrid surface modifiers consisting of a PLL polypeptide backbone partially grafted with PEG side-chains through amine residues (Figure 28). At pH 7.4, these PLL-g-PEG molecules were shown to spontaneously adsorb onto a variety of metal oxide surfaces made of niobium, titanium, silicon/titanium, or tantalum. The adsorption mechanism involves multiple electrostatic interactions between the residual, positively-charged PLL side-chains and the negatively-charged oxide surface. As a result, the polymer backbone lies parallel to the substrate and PEG side-chains stretch perpendicularly into the liquid environment in a comb-like overall structure. For optimized copolymer architectures, protein adsorption upon exposure
to human serum (15-60 min) was near/lower than the LOD of the OWLS sensor (2 ng/cm$^2$).$^{190-192,196}$ This represented a dramatic decrease compared to uncoated oxide surfaces for which serum adsorption ranged between $\Gamma = 223-596$ ng/cm$^2$. $^{190-192,196}$ One study also showed that antifouling properties were maintained whether PLL-g-PEG coatings were exposed to multiple successive serum injections over several hours, or stored dry for several months.$^{190}$

![Figure 28. Molecular structure of PLL-g-PEG copolymers.](image)

The surface of poly(dimethylsiloxane) (PDMS) substrates was also modified with PLL-g-PEG copolymers. Unlike bare PDMS however ($\Gamma = 385 \pm 18$ ng/cm$^2$), PLL-g-PEG coatings were able to prevent protein adsorption from serum ($\Gamma \sim 1.0$ ng/cm$^2$), as assessed using OWLS.$^{198}$ Long-term resistance (up to 12 weeks) was also demonstrated. Finally, Konradi et al. reported an analogous PLL-graft copolymer presenting poly(2-methyl-2-oxazoline) (PMOXA) side-chains (Figure 29A).$^{199,200}$ Similarly, PLL-g-PMOXA coatings exhibited excellent antifouling properties against full human serum ($\Gamma < 2$ ng/cm$^2$), even after repeated 15 min exposures (as measured with OWLS). A copolymer variant introduced shortly after (Figure 29B) performed as efficiently,$^{196,201}$ even following prolonged exposure (up to 2 weeks) to simulated physiological conditions of ionic strength or/and an oxidative environment.$^{196}$ These particular PLL-g-PMOXA films were also shown to be significantly more stable under these physiological conditions than the PLL-g-PEG parent coatings.$^{196}$
1. 7. 4. B. Short, non-zwitterionic monomers for ‘grafting from’ polymerization

Other types of short hydroxyalkyl monomers – related to monoglycerol methacrylate presented in Figure 24A – have also been used to prepare ‘grafting from’ polymer brushes on gold, which have actually proven to be more efficient at resisting fouling from plasma/serum. In 2011, Zheng (in collaboration with Yu) used 3-hydroxypropyl and 2-hydroxyethyl methacrylate monomers (HPMA and HEMA – Figures 30A and 30B) to build polymer brushes of various thickness (~10-60 nm) and assessed their antifouling properties upon 10 min of exposure to both diluted and undiluted human serum and plasma. Interestingly, this study also revealed that optimal antifouling was achieved for intermediate ranges of film thickness (20-45 nm), corroborating the observations made by Jiang’s team for both polySBMA and polyCBAA systems. For both serum and plasma diluted at 10% in PBS, polyHEMA and polyHPMA brushes exhibited remarkable antifouling properties with nearly undetectable protein adsorption (Γ < 0.3 ng/cm²). With respect to undiluted serum and plasma, antifouling performances were still excellent for polyHEMA brushes with Γ ~ 3.0 and 3.5 ng/cm², respectively. In sharp contrast, polyHPMA brushes adsorbed much greater amounts of proteins (Γ ~ 13.5 and 50.0 ng/cm² for serum and plasma, respectively). Another type of non-fouling polymer brush, introduced by Rodriguez-Emmenegger et al., was constructed from N-(2-hydroxypropyl) methacrylamide monomer (also abbreviated as HPMA – Figure 30D). These polyHPMA brushes (17 nm-thick) were able to resist fouling from undiluted human plasma, FBS and calf serum for 15 min. Indeed, for all tested biological samples, readings were below the limit of detection of the SPR sensor apparatus used for the study (0.03 ng/cm²). Similar non-fouling was observed for
five other human and animal biofluids (human cerebrospinal fluid, saliva and urine as well as chicken egg and whole cow milk).\textsuperscript{160} Interestingly, polyHPM brushes (30 nm-thick) resulting from the polymerization of the corresponding 2-hydroxypropyl methacrylate \textit{ester} monomer (HPM – \textbf{Figure 30C}) adsorbed a considerably greater amount of plasma proteins with $\Gamma = 40.5$ ng/cm\textsuperscript{2}.\textsuperscript{51} Importantly for practical applications, protein adsorption on polyHPMA coatings was still undetectable upon long-term contact (120 vs. 15 min) with 100\% plasma. In addition, multiple exposures to plasma and storage in PBS over 2 years barely affected the non-fouling characteristics of the coatings, demonstrating their excellent re-usability and shelf-life stability. In this regard, these non-ionic polyHPMA brushes easily outperformed the otherwise equally as non-fouling zwitterionic polyCBAA ones ($\Gamma = 17$ vs. 125 ng/cm\textsuperscript{2} after 2 years). To our knowledge, the latest addition to this family of short, non-ionic hydroxyalkyl monomers, reported by Zheng’s group in 2011, is N-(2-hydroxyethyl) acrylamide (HEAA – \textbf{Figure 30E}).\textsuperscript{204,205} All polymer brushes grown within a wide range of thicknesses (~10-40 nm) were shown by SPR to be nearly non-fouling with almost undetectable protein adsorption ($\Gamma < 0.3$ ng/cm\textsuperscript{2}), when exposed for 10 min to either undiluted human serum or plasma. Additionally, it was observed that a polyHEAA brush only 12 nm-thick was able to retain such remarkable antifouling properties for a longer period of time (60 min). Lastly, there is also the shortest monomer of them all – the non-substituted acrylamide (\textbf{Figure 30F}) – that was used by Liu and co-workers to prepare ~39 nm-thick polyacrylamide films, which were shown to resist protein adsorption (10 min of exposure) from both full human serum and plasma ($\Gamma = 2.8 \pm 0.9$ and 1.7 $\pm$ 0.7 ng/cm\textsuperscript{2}, respectively).\textsuperscript{206} In comparison, unmodified gold substrates invariably adsorbed proteins $\Gamma > 140$ ng/cm\textsuperscript{2}, regardless of the nature of the fluid.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure30}
\caption{Molecular structure of short, non-ionic monomers for ‘grafting from’ polymerization: (A) 3-hydroxypropyl (HPMA), (B) 2-hydroxyethyl (HEMA), and (C) 2-hydroxypropyl (HPM) methacrylate esters; (D) N-(2-hydroxypropyl) methacrylamide (HPMA); (E) 2-hydroxyethyl acrylamide (HEAA); and (F) non-substituted acrylamide.}
\end{figure}
1. 7. 4. C. Zwitterionic poloxamer derivatives

Using their expertise with zwitterionic materials to expand the scope of antifouling coating strategies, Jiang and co-workers recently reported the synthesis of polyCBMA-\textit{bl}-PPO-\textit{bl}-polyCBMA, a triblock copolymer poloxamer derivative (Figure 31).\textsuperscript{207} Antifouling surfaces were prepared on gold upon physisorption of the middle PPO segment onto a pre-coated hydrophobic SAM of 1-undecane-thiol, thus compelling the superhydrophilic polyCBMA zwitterionic side-chains to face the aqueous environment. Fouling from undiluted blood plasma, as measured using SPR upon 10 min of exposure, was low at $\Gamma = 5.2 \pm 0.2$ ng/cm$^2$.

![Figure 31. Molecular structure of the polyCBMA-\textit{bl}-PPO-\textit{bl}-polyCBMA zwitterionic poloxamer derivative (n = 40, m = 48).](image)

1. 7. 4. D. PolyCBAA hybrid copolymers

Recently (2012), Rodriguez-Emmenegger et al. reported on their pioneering work with hybrid polyMeOEGMA-\textit{bl}-polyCBAA diblock copolymer brushes (Figure 32A) that were sequentially grown on gold (2 $\times$ 15 nm thickness) using the ‘grafting from’ polymerization method.\textsuperscript{175} These brushes were able to suppress fouling from various undiluted biological media (FBS, calf serum and human plasma) below the LOD of the SPR sensor apparatus used in the study ($\Gamma < 0.03$ ng/cm$^2$, 30 min of exposure). In comparison, bare gold adsorbed considerably greater amounts of proteins with $\Gamma = 261 \pm 9$, $280 \pm 7$ and $307 \pm 11$ ng/cm$^2$ for FBS, calf serum and human plasma, respectively. In order to improve the water absorptivity and antifouling character of pure polyHEMA hydrogels while maintaining their excellent mechanical properties, the same group soon after prepared a series of polyHEMA-co-polyCBAA hydrogel films (Figure 32B) for which the resistance to fouling from undiluted human plasma (3h of incubation) was estimated using Fourier transform infrared-attenuated total reflectance (FTIR-ATR) and the relative intensity of amide I ($\sim1650$ cm$^{-1}$) and II ($\sim1550$ cm$^{-1}$) bands.\textsuperscript{181} Gradually varying the composition of CBAA zwitterionic co-
monomer in the polymerization solution, the authors showed that fouling became undetectable (*i.e.* below the LOD of 20 ng/cm²) when the polymer CBAA content reached 10 mol%.

![Figure 32](image)

**Figure 32.** Molecular structure of (A) polyMeOEGMA-bl-polyCBAA and (B) polyHEMA-co-polyCBAA hybrid copolymer brushes.

### 1.7.4. E. Pseudozwitterionic materials

As shown earlier in *Section 1.7.3.*, zwitterionic polymer brushes exhibit excellent protein repellence. However, these do not constitute the only type of ‘grafted from’ mixed-charged polymer coatings with antifouling properties. In fact, ‘pseudozwitterionic’ copolymer films, wherein the positively- and negatively-charged moieties are now located on two separate subunits, are also able to resist protein adsorption. This was confirmed in 2010 by Chang and Chen with 30-45 nm-thick methacrylate copolymer brushes presenting positively-charged *N*,*N*,*N*-trimethylammonium and negatively-charged sulfonate side-chains (*Figure 33A*).\textsuperscript{157} Protein adsorption from 20% human PPP in PBS (pH 7.4) was measured with SPR upon ~15 min of exposure at 37°C and found to be very low (\(\Gamma = 7.65\) ng/cm²). As well, Bernards’ group also reported in 2012 that ~18 nm-thick acrylate copolymer brushes – with positively-charged *N*,*N*,*N*-trimethylammonium and negatively-charged carboxylate subunits (*Figure 33B*) – were able to reduce fouling from both 10% and undiluted FBS to respectively \(\Gamma = 2.1 \pm 2.0\) and \(4.3 \pm 1.7\) ng/cm², as measured with SPR after exposure for 10 min.\textsuperscript{208}
Figure 33. Molecular structure of the (A) ammonium/sulfonate and (B) ammonium/carboxylate mixed-charged, pseudozwitterionic (meth)acrylate copolymer brushes.

Earlier investigations by Jiang’s group had already demonstrated the concept of mixed-charged coating material for antifouling purposes.\textsuperscript{57} In this study, combinations of \textit{N,N,N}-trimethylammonium (TMA), sulfonic acid (SA) or carboxylic acid (CA) \textit{ω}-functionalized alkylthiol gold surface modifiers (Figure 34) were investigated by means of SPR for their resistance to human serum/plasma protein adsorption.

Figure 34. Molecular structure of the (A) \textit{N,N,N}-trimethylammonium (TMA), (B) sulfonic acid (SA), and (C) carboxylic acid (CA) \textit{ω}-functionalized alkylthiol gold surface modifiers. At physiological pH 7.4, SA and CA moieties in the mixed SAMs exist as their negatively-charged sulfonate and carboxylate forms, respectively.

At physiological pH 7.4, the best antifouling performance recorded for the TMA/SA system was achieved against 10\% human serum (\(\Gamma \sim 15 \text{ ng/cm}^2\), 10 min of exposure).\textsuperscript{57} This was also the case for the TMA/CA system (\(\Gamma \sim 25 \text{ ng/cm}^2\)). Unfortunately, however, neither of these combinations were able to resist adsorption from 100\% serum (\(\Gamma \sim 90-180 \text{ ng/cm}^2\)). Fouling from human plasma was even greater, even if the latter was diluted to 10\% with PBS (\(\Gamma \sim 190-950 \text{ ng/cm}^2\)).\textsuperscript{57,173}
1. 7. 4. F. Carbohydrates: glycocalyx mimics

Finally, there is an important class of biomimetic materials inspired by the outer surface of biological cell membranes. This interfacial region (known as ‘glycocalyx’) consists of a non-adhesive mesh of highly hydrated carbohydrate-rich macromolecules (proteoglycans and glycoproteins) and functions as a barrier that regulates molecular/cellular interactions.\textsuperscript{209,210} In 1998, Marchant and co-workers set out to mimic such supramolecular assembly by engineering oligosaccharide polymers for the surface modification of highly-oriented pyrolytic graphite (HOPG).\textsuperscript{210} These polymeric structures consisted of a poly(vinyl amine) backbone onto which hydrophobic hexanoyl and hydrophilic dextran side-chains were simultaneously grafted \textit{via} the pendant amine groups (\textbf{Figure 35}).

\textbf{Figure 35.} Molecular structure of the poly(vinyl amine)-g-hexanoyl/dextran carbohydrate polymers.

On HOPG, these graft-polymers were shown to grow monomolecular films 7-12 Å-thick. In this process, hexanoyl chains assemble onto the underlying HOPG substrate through hydrophobic-hydrophobic interactions thereby compelling the flexible poly(vinyl amine) backbone to lie parallel to the surface and the hydrophilic dextran side-chains to stretch outwards in the aqueous medium, in an overall three-dimensional comb-like structure. Although not quantified \textit{per se}, the level of protein adsorption on these glycocalyx-like assemblies, upon incubation for 30 min at 37°C in 50% human PPP diluted in PBS, was shown to be considerably lower than that recorded on unmodified HOPG. Using FTIR-ATR following the relative absorbance of the characteristic amide I (\textasciitilde{}1650 cm\textsuperscript{-1}) and II (\textasciitilde{}1550 cm\textsuperscript{-1}) protein bands, the authors in fact estimated plasma protein adsorption to be reduced by at least 90%.

In 2001, a follow-up study was conducted by Marchant’s group with a more systematic investigation, wherein a series of oligomaltose-based analogous polymer films with varying
saccharide side-chain length were tested for their antifouling properties – under the same experimental conditions \(i.e\). against 50% PPP for 30 min at 37°C.\textsuperscript{211} Still using FTIR-ATR, the authors demonstrated in this work that plasma protein adsorption steadily decreased with increasing film thickness, the best result (a 95.8% reduction) being obtained for the thickest (42 Å) films studied. Gratifyingly, Spencer and co-workers did later quantify with OWLS the antifouling properties of a series of analogous poly(L-Lysine)-\emph{graft}-dextran polymers (\textbf{Figure 36}) immobilized onto silicon/titanium oxide surfaces.\textsuperscript{212} The best resistance to protein adsorption recorded against human serum (30 min of exposure) was \(\Gamma = 13 \pm 8\) ng/cm\(^2\).

\textbf{Figure 36}. Molecular structure of the poly(L-Lysine)-\emph{g}-dextran carbohydrate polymers.

\textbf{1. 7. 5. Summary}

Literature review revealed that only a relatively limited number of antifouling coating strategies exist to reduce protein adsorption from the highly complex, real-world biofluids that are blood plasma and serum (even if diluted) down to biotechnologically relevant levels \(i.e\). a few ng/cm\(^2\), as summarized in \textbf{Table 4}. These remarkable antifouling properties were imparted to a wide variety of both inorganic and organic underlying substrates, through the use of (sub)nanometrically-thin SAMs or thicker polymer brushes/films \textbf{(Table 4)}. Surface-modifying building block molecules could be organized into three main families including the popular OEO/PEO-based benchmark constructs as well as the emerging classes of zwitterionic sulfo/carboxybietaines and bio-inspired peptides/peptoids. Coatings prepared from hybrid or derivative surface modifiers also displayed excellent performance, so did those biomimicking the non-adhesive outer layer of biological cell membranes \(i.e\). the glycocalyx).
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**Table 4.** Antifouling performance of various surface chemistries against blood-based fluids. *Note:* regardless of the nature of the biofluid and substrate, fouling on bare (uncoated) surfaces was reported to be greater than 140 ng/cm² (even at 10% dilution).
1. Resistance of OEO/PEO-based coatings to protein adsorption: schools of thought

1. 8. 1. Overview

As mentioned previously, PEO and OEO constructs have been widely studied as practical protein-resistant coatings, with both low toxicity and immunogenicity, to be implemented in real-world applications. Despite years of research however, the mechanism behind the antifouling properties of such surface chemistry still remains somewhat obscure, and the object of great interest for both fundamental and applied physical chemistry research. The origin of the ‘PEO/OEO effect’ has long been debated, and two main schools of thought – that treat protein repellence from a ‘physical’ or ‘chemical’ point of view – have emerged. There have been several contributions to both views of protein resistance; however, only the key ones will be discussed below.

1. 8. 2. The ‘physical’ view

This view heavily hinges on notions developed in the Alexander–de Gennes theory of polymer interfaces. The theory, which focuses on steric effects, was applied by Andrade and Jeon to build a theoretical model, wherein the effect of long and flexible PEO chains on protein adsorption was studied. This work essentially showed that, as a protein approaches the surface, PEO chains would experience compression – a situation entropically unfavourable resulting in the generation of repulsive forces. This ‘steric repulsion’ (or ‘excluded volume’) effect is considered the main mechanism responsible for protein repellence. Although this highly influential work by Andrade and Jeon continues to be heavily referred to, it has been pointed out notably by Szleifer et al. that such a simplistic model suffers from some limitations. For instance, it would only be valid for very long, heavy PEO chains (n > 1000, MW > 44 000 g/mol) as well as for high surface density. Another shortcoming would be that proteins are modelled as spheres or as having infinite size. Szleifer and co-workers, using the single chain mean field theory, worked at developing an improved model taking into account all interactions PEO chains may experience. This study, which assumed protein-PEO, protein-solvent and PEO-solvent interactions to be identical, concluded that – unlike surface grafting density – the length of PEO chains had little effect on protein resistance. Another milestone in the ‘physical’ view of protein repellence was reached by Halperin, who pursued Andrade–Jeon’s and Szleifer’s efforts. In Halperin’s model, the effective interaction experienced by a protein approaching a PEO brush-coated surface is described as being the sum of: (i) a purely repulsive force between protein and PEO brush; and (ii) a purely attractive force
between protein and bare surface. Protein adsorption is discussed as a function of PEO chain grafting density, brush thickness, and protein size.

1.8.3. The ‘chemical’ view

The ‘physical’ models discussed thus far relied on steric repulsion and entropic effects to explain the resistance of PEO coatings to protein adsorption. Although these models were built in a hydrated state (rather than in vacuum), the effect of water was neglected. In fact, molecules of water were represented as non-interacting spheres, which clearly does not accurately describe the reality of hydrated systems in which hydrogen bonding interaction with water is an important aspect.\textsuperscript{214} This naturally led to the ‘chemical’ view of protein resistance in which the general consensus – according to which water through surface hydration would play a key role in antifouling – has been reached; the hypothesis being relevant not only to PEG/OEG coatings (hydration \textit{via} hydrogen bonding) but also zwitterionic films (hydration \textit{via} ionic solvation).\textsuperscript{73,91,135,136,226-235} Even though the effect of surface hydration would affect coatings made of either long PEG or short OEG chains, its relative contribution to antifouling would be much greater for the latter kind of construct as the role of entropy due to chain flexibility/compression would be, in this case, less significant.\textsuperscript{135} Regardless, the precise underlying mechanism still is a matter of some debate, owing to the sheer number of influential parameters on both surface and protein ends.\textsuperscript{73,91,226,230-235} There have been as well several milestones in the development of the chemical view of protein repellence. One example is the theory proposed by Besseling \textit{et al.}, which takes into account hydration forces between surfaces and the dependence of water molecules’ orientation on interactions.\textsuperscript{236-238} Besseling \textit{et al.} suggested that the surface, water molecules and incoming protein communicate through hydrogen bonding interactions, whose magnitude would depend on the number of electron donor (\textit{i.e.} oxygen atoms) and electron acceptor (\textit{i.e.} hydrogen atoms) sites involved. The model suggested that incoming proteins would be repelled from highly hydrated PEG or OEG surfaces considering the enthalpic cost associated with the removal of water molecules hydrogen-bonded to the PEG/OEG chains. Grunze and co-workers also aided in the understanding of this chemical view of protein resistance by investigating the effect of chain conformation through both experimental and theoretical means.\textsuperscript{239-241} The authors prepared, on both gold and silver substrates, OEG-SAMs containing three ethylene glycol (EG) units, and used Fourier transform infrared reflection (FT-IR) spectroscopy to look at chain conformation while assessing fibrinogen adsorption through force measurements.\textsuperscript{239,241} It was found that OEG-SAMs prepared
on gold substrates displayed amorphous and helical chain conformations, and were resistant to fibrinogen adsorption. Conversely, OEG-SAMs prepared on silver substrates formed tightly packed assemblies with high surface density and preferred ‘all-trans’ chain conformation, and adsorbed fibrinogen. All-trans OEG molecules were driven into such conformation as a result of their perpendicular packing orientation with respect to the silver substrate, as opposed to the 30° chain tilt (relative to the normal) observed in the case of gold. Another consequence was a smaller cross-sectional area per molecule on silver (19.1 Å²) than on gold (21.4 Å²).\(^{239}\) To understand the underlying raison d'être behind their experimental observation, Grunze and co-workers conducted a complementary study using computer simulations.\(^{240,242}\) The authors concluded that, unlike trans OEG chains, OEG chains adopting a helical conformation offer a conducive environment for interchain water absorption – the corresponding OEG-SAMs on gold displaying as such a tighter interfacial layer of water. In 2003, Herrwerth et al. expanded on this model and demonstrated that – along with OEG chain conformation – the hydrophilicity of both head function and inner chain units, as well as chain lateral packing density, influenced the ability of OEG-SAMs to coordinate water and thus resist protein adsorption.\(^{241}\) The authors found that protein adsorption on OEG-SAMs increased as internal or interfacial water coordination decreased.\(^ {241}\) This led to the argument of a ‘water barrier’, wherein embedded and interfacial water molecules are tightly bound and organized into permeated structures that have an energy cost in terms of perturbation.\(^ {135}\) Another argument rather puts forth the notion of ‘interfacial energy matching’, in which there is no net energy gain for biological solutes – that reside fully solvated in the bulk aqueous medium – to adsorb on hydrated surfaces.\(^ {62,226,230}\) In both situations, water in contact with surfaces may form a phase physically distinct from ordinary bulk water,\(^ {226,227,230,235}\) as supported by experimental evidence.\(^ {243-245}\) Another source of debate lies in the nature and extent of this special zone of hydration.\(^ {226,230,245-247}\) For instance, while some have described physically-distinct water interphases projecting up to several hundred microns into the contiguous aqueous medium,\(^ {245}\) others – in stark contrast – reported surface kosmotropicity\(^ {248}\) to be much more limited in range, to a few layers of water or less.\(^ {226,230,243,244}\) In view of these contradictory accounts, it is not surprising that the contentious question of surface hydration – and its connection to antifouling – continues to be relentlessly researched.\(^ {135,226}\)
1. 9. Surface antifouling and material biocompatibility

Scouring the literature, it seems as though the ability of a surface to resist fouling would constitute a requirement to devise biocompatible materials, which intuitively is a quite reasonable assumption to make. As discussed earlier, deleterious biological processes triggered by contact of blood with exogenous materials are more than likely mediated by ‘surface-activated’, adsorbed proteins (i.e. activated upon adsorption). It is thus natural to believe that minimizing the total amount of adsorbed proteins with antifouling coatings may be an adequate strategy to achieve biocompatibility – however, only if the proteins responsible for triggering the harmful thrombogenic and immune responses are prevented from adsorbing. Nevertheless, it can be argued that the key to achieving biocompatibility is not necessarily to minimize the amount but to control the dynamics of protein adsorption, more specifically the degree of unfolding the proteins that are able to initiate the aforementioned biological processes may experience upon co-adsorption with other spectator proteins. The reason lies in the fact that the process of adsorption may lead to the exposure of previously hidden bioactive sites through surface-induced conformational protein restructuration, i.e. lead to surface-induced protein activation. Although admittedly difficult to achieve on an individual basis, the ability to control the conformational state of relevant adhered proteins – rather than combating the total amount of fouling – would likely constitute a much more pertinent parameter to focus on in the engineering of biocompatible materials.

In any event, coatings will certainly have to be carefully designed and customized, an option ‘self-assembling monolayer’ (SAM) surface chemistry discussed next offers. SAMs have been the object of tremendous attention over several decades now, as demonstrated by the extensive literature that has appeared on the matter. In the following section, focus will be given to presenting the salient features of this surface modification technology.
1. 10. Self-assembling monolayer surface chemistry

Self-assembling monolayer (SAM) surface chemistry has been reported on numerous occasions to constitute a convenient strategy to modify/tailor the surface properties of a material without altering those of the bulk substrate.\textsuperscript{250-252} This unique feature endows SAM chemistry with a privileged position to study surface interaction phenomena (\textit{e.g.} wetting, adhesion, adsorption),\textsuperscript{60} and with great potential in many technological applications including bioanalytical devices (\textit{e.g.} sensors and biochips),\textsuperscript{76,253-255} biomaterials,\textsuperscript{139,256} and photovoltaic cells,\textsuperscript{257} among other examples.\textsuperscript{257-259} The first SAM was reported in 1946 by Bigelow \textit{et al.}, who formed oleophobic monolayer films onto clean metal surfaces.\textsuperscript{260} However, it was not until about 40 years later that the interest in the field actually grew with the introduction of thiol- and silane-based SAMs,\textsuperscript{261-264} as evidenced by the vast number of publications that have appeared in the literature ever since.\textsuperscript{252,265} SAM chemistry relies on the use of long organic molecules that spontaneously form ordered molecular assemblies onto solid, more often than not, inorganic substrates.\textsuperscript{12,252,260} SAM formation can be carried out in the liquid or gas phase, the former scenario being most commonly employed.\textsuperscript{252} Substrates are typically chosen for an inherent property to be exploited in an intended application (\textbf{Figure 37}),\textsuperscript{258} while surface-modifying molecules are designed, in turn, depending on the nature of the substrate to be modified (\textit{e.g.} metal, metal oxide). For example, thiol molecules will be deposited onto metals such as gold, silver, platinum, and copper; while silane-based molecules will be used to modify the oxide/hydroxylated surface of quartz, glass, oxidized polyethylene or indium-tin oxide (ITO), for instance.\textsuperscript{76,258,265,267-270} The great diversity encountered in SAM chemistry stems from the fact that the surface-modifying molecules from which they are built are essentially composed of three customizable parts (\textbf{Figure 37}): (i) an anchoring or ‘tail function’ that interacts with the substrate; (ii) a backbone of variable length that spaces the ‘head’ group (see next) from the anchoring function, and provides stability to the assembly \textit{via} intermolecular interactions; and (iii) a distal ‘head function’ that influences the surface properties of the resulting assembly, and that can be chemically inert (\textit{e.g.} CH\textsubscript{3}→), or reactive and thus be modified (\textit{e.g.} –O–C(O)–CF\textsubscript{3} → –OH) or site-specifically functionalized (\textit{e.g.} to attach biosensor receptor probes), post-assembly.\textsuperscript{251,252,258,271-274} In the latter case, these bifunctional surface modifiers are called ‘linkers’. Even though there are virtually countless possibilities in terms of chemical composition, it must be ensured in the design of surface-modifying molecules that anchoring, backbone and head groups are chemically compatible. Taking the trichlorosilyl moiety (Cl\textsubscript{3}Si→) anchoring function as an example, it only tolerates the presence of a limited number of functional
groups. This accordingly restricts the diversity of such surface modifiers, especially with respect to the nature of functionalizable head groups. For instance, this highly reactive trichlorosilyl electrophilic moiety will not tolerate the presence of nucleophilic head groups (e.g. alcohol, carboxylic acid, amine) that, as a consequence, must either be protected during SAM formation (then deprotected) or introduced post-assembly.\textsuperscript{272}

![Figure 37. Cartoon representation of the generic structure of surface modifiers for SAM formation showing the three customizable parts ('anchoring function', 'backbone' and 'head function').]

The self-assembly process to form an ordered system is normally driven both by the spontaneous adsorption of surface-modifying molecules onto the substrate and the lateral, non-covalent intermolecular interactions (e.g. van der Waals) between neighbouring chains.\textsuperscript{268,272,275} The degree of ordering in a SAM depends on intermolecular chain-chain interactions and naturally on any factor that may influence them.\textsuperscript{258} By using longer molecules (> 10 atoms), stronger intermolecular forces are established that lead to SAM structures possessing greater stability and ordering.\textsuperscript{258,276} Finally, the substrate’s topography and head function’s physicochemical properties (e.g. bulky vs. small) also play a role on the ordering of SAMs.\textsuperscript{258}
As discussed earlier, SAM chemistry is highly versatile and diversified due to the fact that there exists an immense range of possible surface modifier/substrate pairing combinations, which allows, in principle, for the surface properties (e.g. adhesibility, wettability, work function, antifouling) of virtually any substrate material to be finely tuned. In the specific case of linkers, another unique advantage is the possibility to covalently and site-specifically tether biological entities to (in)organic substrates, in a durable and controlled manner. Other benefits of SAM surface chemistry include: (i) its simplicity (dip-and-rinse procedures); (ii) the need for only a small quantity of surface modifier to coat large substrate areas (~10^{14} molecules/cm^2, or ~1 nmol/cm^2); and (iii) the low cost associated with reagents. Although there have been a plethora of reports on a variety of SAMs prepared on various substrates, alkylthiols on gold and alkyltrichlorosilanes on oxide/hydroxylated surfaces seem to have captured the most interest within the scientific community, the former being far more documented than the latter.

1. 10. 1. Alkylthiol-based SAMs

These SAMs are routinely prepared on various metal surfaces. Gold, however, is a substrate of choice due to its remarkable chemical (resistant to oxidation) and physical (e.g. electrical conductivity, optical reflectivity) properties, which endow this metal with a unique position in a variety of analytical techniques such as quartz crystal microbalance (QCM) and surface plasmon resonance (SPR). Thiol-based SAMs on gold can be prepared in a straightforward fashion employing different kinds of organosulfur compounds such as di-alkyl sulfides (R–S–R), di-alkyl disulfides (R–S–S–R), and Cysteine; however, alkylthiols (R–S–H), in particular on gold Au(111) substrates, have been the most commonly and thoroughly studied system, and yield SAMs with highest density and uniformity. When a gold substrate is immersed into a solution of thiol surface modifier (10–1000 μM) – more often than not in ethanol – approximately 80-90% of the monolayer is achieved within the first few minutes. However, attaining a well-packed, ordered assembly can take up to several hours or even a couple of days. Adsorption kinetic studies have shown that there are two distinct phases for alkylthiol SAM formation on gold surfaces. The first phase is rapid and results in the random deposition of thiol molecules onto the gold substrate. This process is dependent on the concentration of thiol in solution, and is driven by the coordination of the thiol anchoring function to the gold substrate.
via a gold-thiolate pseudocovalent bond of 40-50 kcal/mol (167-209 kJ/mol) approximate strength.\textsuperscript{252,258,265,269,284,289} The exact mechanism governing thiol chemisorption is still unknown; however, it is generally described to occur through a formal redox reaction, as depicted in the following equation 8:\textsuperscript{258,265,267}

\[
R\text{-SH} + \text{Au}_n^0 \rightarrow R\text{-S}^- \text{Au}^+ \cdot \text{Au}_n^0 + \frac{1}{2} \text{H}_2
\]  

(8)

where R represents the rest of the thiol surface-modifying molecule.

Once thiol molecules have adsorbed and surface coverage is close to saturation (80-90%), a second – slow – reorganization phase takes place, in which thiol molecules start re-orienting in an upright position, densely covering the substrate – a process that is driven by intermolecular forces between chains and can last from hours to days. Although the gold-thiolate interaction is relatively strong, adsorbed molecules are still mobile and able to spread towards surface defects to increase surface coverage and density within the film.\textsuperscript{255} The end result for SAMs formed on Au(111) substrates – as shown through scanning tunneling microscopy studies – is a well-ordered and close-packed hexagonally-arranged assembly with minimized surface defects and neighbouring sulfur atoms separated by a distance of 4.97 Å.\textsuperscript{289-292} Furthermore, within this molecular construct, the area occupied per thiolate residue would be 21.4 Å\textsuperscript{2} with a maximum theoretical surface density of approximately 4.7 residues per nm\textsuperscript{2}.\textsuperscript{289-292} In addition, studies have shown that alkyl chains [R–(CH\textsubscript{2})\textsubscript{n}–, n > 10] within these assemblies are in an all-trans conformation with a chain tilt of approximately 30° relative to the normal (Figure 38).\textsuperscript{76,252,258,269,280,289} The mechanism of SAM formation on other metallic substrates is similar; however, there may be structural discrepancies with respect to packing and density as a result of a different chain tilt – the latter being dependent on the spacing between chains (and consequently on intermolecular interactions) caused by the morphology of the substrate and the metal/sulfur lattice (e.g. chain tilts relative to the normal are: ~0-12° on Ag, ~12° on Cu, and ~14-18° on Pd).\textsuperscript{277,293,294} Additionally, by altering the backbone from the hydrocarbon to the EG variety, the time required for molecules to rearrange and reach surface saturation increases.\textsuperscript{295} The quality of SAMs, and the ability to reproduce them in a reliable manner, depend on several factors related for instance to the substrate features (e.g. morphology, contamination), the surface modifier concentration, the deposition time, and temperature.\textsuperscript{288,296,297}
Once prepared, thiolate-SAMs on gold are rather stable and not moisture sensitive. Nevertheless, such attractive SAM surface chemistry possesses some limitations such as: (i) its restriction to the surface modification of certain metals; and (ii) its susceptibility to oxidative damage at the sulfur atom that results in weaker interactions with the metal substrate and SAM etching.\textsuperscript{288,297} The degree of oxidation is influenced by the quality of the SAMs, the amount of light SAMs are exposed to, and the atmospheric level of ozone.\textsuperscript{297-299} Silane-based SAMs do not suffer from such shortcomings. Although alkythiol-based SAMs are known to exhibit higher order, packing and homogeneity compared to alkyltrichlorosilane-based analogues, the latter offer superior chemical stability as they involve the formation of strong, covalent bonds (~370 kJ/mol in strength\textsuperscript{300} vs. < 209 kJ/mol for the S→Au bond) with the underlying hydroxylated/oxide substrate.\textsuperscript{76,272}

1. 10. 2. Alkyltrichlorosilane-based SAMs

Hydroxylated/oxide surfaces – natural (\textit{e.g.} quartz) or not (\textit{e.g.} ITO, oxidized plastic polymers) – constitute another important type of substrate material SAM surface chemistry can be implemented on.\textsuperscript{301} The formation of trichlorosilane-based SAMs onto such surfaces (also called ‘silanization’) is routinely carried out in dilute organic solutions (\textit{e.g.} in toluene) and is a more complicated process than that of thiolate SAM formation on gold.\textsuperscript{302} Reproducibly constructing defined trichlorosilane-based SAMs is by no means an easy task and requires
protocol conditions to be strictly controlled and respected, as years of experience have confirmed. The mechanism of SAM formation is still under debate; however, it is generally accepted that silanization is a multi-step process. The first step is the hydrolysis of the trichlorosilyl tail function (–SiCl₃) into a trisilanol species [–Si(OH)₃], by water present in the solvent or adsorbed on the substrate (equation 9):

$$\text{R–SiCl}_3 + 3 \text{H}_2\text{O} \rightarrow \text{R–Si(OH)}_3 + 3 \text{HCl}$$ (9)

where R represents the rest of the trichlorosilane surface-modifying molecule. It is important to note that the actual degree of hydrolysis depends on the amount of water present in solution/on the substrate, and that (partially) hydrolyzed silanol molecules can cross-polymerize in solution into a mixed population of siloxane oligomers prior to chemisorbing onto the substrate – an example of which being: 2 R–Si(OH)₃ → R(HO)₂Si–O–Si(OH)₂R + H₂O.

In a second step, silanol species diffuse from the bulk solution towards the substrate where they undergo a reversible process of adsorption/desorption during which they can randomly condense with surface hydroxyls to form surface-bound silanols, as shown in Figure 39.

![Figure 39. Schematic representation of the silanol reversible adsorption/desorption and condensation steps (shown as an example is the case of monomeric trisilanol species).](image)

This condensation occasionally results in the formation of molecular islands that nucleate over the surface of the substrate, slowly grow during a reorganization phase, and finally aggregate to form the SAM. It was initially reported that trace amount of water was necessary to obtain well-packed films; however, recent accounts have shown that increasing the amount of water (or not using a fresh solution of surface modifier) favours island-type growth. Siloxane cross-linkage can also occur on-surface between neighbouring silanol residues (Figure 40), and enhance the robustness of the assembly.
Figure 40. Schematic representation of the cross-linkage between two neighbouring, surface-bound silanols into a condensed siloxane moiety.

Computer simulations have shown however that, due to chain steric hindrance/density effects and other geometric constraints, siloxane cross-linkage is incompatible with the formation of full-coverage SAMs.\textsuperscript{312} In terms of order and packing, SAMs consisting of long hydrocarbon chains (8 to 18 atoms of carbon) form monolayer-like coatings, in which alkyl chains are in an upright position and closely packed with a cross-sectional area per residue of approximately 20 Å\textsuperscript{2}.\textsuperscript{266,313-316} Alkyl chains orient themselves to maximize neighbouring intermolecular forces (\textit{i.e.} van der Waals interactions) within the assembly, resulting in tighter packing and higher ordering. As it is also the case for thiol-based SAMs on gold, being able to reliably control and reproduce trichlorosilane-based SAM surface chemistry in terms of quality and interfacial properties depends on several factors, which include for instance: the features of the substrate (\textit{e.g.} morphology, surface hydroxyl density, contamination), the water content within the solvent\textsuperscript{265,308} as well as adsorbed on the substrate,\textsuperscript{303,317} the type of solvent used,\textsuperscript{308} the age of the surface modifier solution, the deposition time, and temperature.\textsuperscript{258,265,305,308} Furthermore, pH can also affect the integrity/stability of SAMs, one example being that, under basic (or acidic) conditions, Si–O bonds undergo hydrolysis leading to SAM damage/etching.\textsuperscript{258,318}

1. 10. 3. Mixed adlayers: binary blends of surface modifiers

While \textit{unimolecular} SAMs (\textit{i.e.} SAMs formed from a \textit{single} type of surface modifier) have been well and widely documented throughout the literature,\textsuperscript{251} less attention has been given to the more sophisticated ‘mixed SAM’ surface chemistry, which incidentally constitutes another strategy to finely tune material surface properties.\textsuperscript{265,319} In essence, mixed SAMs offer the possibility to construct heterogeneous surfaces in terms of chemical functionalities, and are simply prepared from solutions consisting of surface modifiers varying in length and/or chemical structure. One example of such combination would be how a bifunctional linker (for the subsequent attachment of biomolecules, for instance) is used in conjunction with a shorter, monofunctional (or not) ‘\textit{diluent’}. In this scenario, the diluent is incorporated with the intent of
spacing out linker molecules within an otherwise congested ‘undiluted’ assembly, thereby reducing steric hindrance around neighbouring, functionalizable linker head functions. As a result, mixed SAMs are anticipated to offer enhanced attachment capability (better reactivity and accessibility) and to facilitate access with regard to target analyte binding. In the latter case, diluents for biosensor applications should also be designed to provide/produce an antifouling background for protruding analyte receptors. Mixed molecular constructs can be generated by various means such as stepwise or co-deposition. The former situation involves a sequential adsorption process, wherein one type of surface modifier is first deposited and partially covers the substrate while the second surface-modifying partner then, ideally, fills any leftover bare substrate. Conversely, co-deposition, as the name suggests, describes a situation in which the substrate comes in contact with both types of surface modifier simultaneously. Whichever means is used to generate a mixed coating, studies have shown that the surface composition of adsorbates does not necessarily reflect that found initially in solution. The exact process through which different surface modifiers assemble remains unknown, however, it has been observed that surface modifiers differing significantly in chain length or possessing bulky head functions tend to form segregated phases; whereas mixtures containing molecules of similar chain length tend to produce more homogeneous assemblies.

A review of relevant literature quite clearly reveals that the concept of SAM surface chemistry is implicitly associated with a certain, inherent degree of packing and order in the resulting molecular constructs. Nevertheless, as discussed within this section, there are many, different factors that may affect the assembly process and final organization – may the case be that of thiol surface modifiers on metals, trichlorosilanes on hydroxylated/oxide substrates, or other situations. As a result, the more general term ‘adlayer’ is likely more appropriate to describe such assemblies.

1. 10. 4. Biomolecule immobilization onto organic adlayers

The ability to functionalize organic adlayers once assembled onto desired substrates – through the subsequent attachment of biomolecules – endows such assemblies with great potential in various detection/biosensing applications. However, as touched upon in Section 1.3., achieving maximum device performance necessitates the immobilization process to fulfill a number of requirements. Firstly, molecular affinity must be maximally retained upon
Several factors may cause a probe biomolecule to lose its binding affinity for a target such as the properties of the surface or those of the immobilization solvent, the alteration of the biomolecule’s conformation (with potentially accompanying loss in spatial target complementarity), or a drastic change in its micro-environment due to interactions with the underlying surface. Some biomolecules may also be more prone to structural alterations upon attachment (e.g. RNA strands) compared to other, more robust ones (e.g. antibodies). Additionally, it is crucial for biomolecules to properly orient at the surface of detection platforms without concealing binding sites for target analyte capture. With respect to device performance, biomolecules should be distributed homogeneously in order to obtain maximum density per surface area unit without steric hindrance (a situation corresponding to the so-called ‘optimal loading’). Other requirements for this transformation are for the integrity of the adlayer to be respected, and the resulting assembly to be stable and durable.

Among the various possible strategies that have been developed for biomolecule immobilization onto adlayers, covalent binding is widely used and appreciated as it offers stable and durable attachment. In fact, this site-specific immobilization technique results in the formation of strong covalent bonds (~200-800 kJ/mol) between biomolecules and adlayers’ head functions. In the case of adlayers prepared on gold with alkylthiol linkers bearing a carboxylic acid head function, a pre-activation step is necessary for attachment however. This is traditionally accomplished through the well-established ‘EDC/NHS’ coupling chemistry, which intermediately transforms carboxyl acid moieties into more reactive NHS ester functions that in turn can react with amine nucleophiles to form covalent amide bonds. In the case of trichlorosilane-based coatings, it is possible to avoid this pre-activation extra step, and install – directly during adlayer assembly – highly reactive electrophilic head functions able to efficiently bind thiol- or amine-bearing molecules.
1. 11. Detection of biomolecular interactions: acoustic wave (bio)sensors

1. 11. 1. The ‘enzyme-linked immunosorbent assay’ (ELISA) gold standard

Many detection techniques have been developed over the years to monitor biomolecular interactions in such different fields as clinical diagnostics, food safety, environmental monitoring, and defense and security.\textsuperscript{339,340} With respect to clinical diagnostics, there are a number of criteria that should be taken into consideration when devising a detection platform, including: high throughput, rapid analysis (especially for emergency situations), high selectivity and sensitivity for the targeted analyte, and cost-efficiency.\textsuperscript{12} For decades, the label-based \textit{enzyme-linked immunosorbent assay} (ELISA) has been successfully used in many various screening/diagnosis test applications, which has endowed the technique with the status of clinical benchmark for the detection and quantification of biomarkers in biological samples.\textsuperscript{341,342} The ELISA relies on the basic immunology concept of a specific antibody recognizing a target antigen (\textit{e.g.} peptide, hormones) and monitoring the interaction \textit{via} an enzyme-substrate labelled reaction. Irrespective of its variant (‘direct’, ‘indirect’, or ‘sandwich’), the ELISA procedure invariably involves the following basic steps: (i) coating each of a 96-well polystyrene plate with an antibody (or antigen) specific to the targeted analyte; (ii) passivating non-functionalized areas with a blocking agent (\textit{e.g.} a protein such as bovine serum albumin) to prevent the non-specific adsorption of reagents and sample interferents; (iii) adding the test sample and subsequent reagents; (iv) incubating these reactants; (v) washing the wells to remove non-specifically bound material; and (vi) adding a specific enzyme-linked antibody to bind to the captured target analyte and subsequently react with an added chromogenic substrate to produce a colorimetric signal, which is proportional to the amount of bound target analyte.\textsuperscript{342} There are several benefits to ELISA testing such as high sample handling capability, remarkable sensitivity (down to the low pM range), and relatively long shelf-life for reagents;\textsuperscript{342,343} however, these assays are not without their drawbacks.\textsuperscript{341} Limitations include the following: (i) the procedure is time-consuming (hours to days); (ii) the complementary antibody (or antigen) to the targeted analyte must be available; (iii) the activity of the enzyme may be affected by the sample matrix; and (iv) the assay is susceptible to generate false positive and false negative results.\textsuperscript{341,344,345}

Until recently, few truly innovative alternative techniques for bioanalytical detection have been proposed. One such type of technology, which has received considerable attention, is based on \textit{acoustic wave physics}. Acoustic wave (bio)sensors have indeed been suggested as analytical tools to detect and monitor biomolecular interactions, whereby changes in device parameters –

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e.g., the resonant frequency (see Section 1.11.4.) – can be related to physicochemical changes occurring at the sensor-liquid interface (e.g., thickness, viscoelasticity and/or interfacial coupling) during the binding event between surface-attached receptor probes and target analyte molecules. Fundamentally, these sensors rely on the phenomenon of piezoelectricity (from the Greek ‘piezein-elektro’ meaning ‘pressure-electric’) – or piezoelectric effect – and employ piezoelectric materials as (part of) their transducing element to measure mechanical and/or electrical changes occurring near/on the sensor surface.

1.11.2. The piezoelectric effect and the prevalence of quartz as piezoelectric material

Discovered in 1880 by brothers Pierre and Jacques Curie, the piezoelectric effect is a reversible electromechanical phenomenon founded on the coupling relationship existing between the electrical polarization and the mechanical load of a crystal lacking a center of symmetry. The direct piezoelectric effect involves the generation of a proportional electrical polarization (and thus an electromotive force, voltage) in response to mechanical strain – the latter being the deformation experienced by crystals as a result of applied stress (may it be bending, shear, torsion, tension, or compression). The reverse or converse piezoelectric effect – predicted in 1881 by Lippmann and confirmed the same year by the Curies – is also true; that is, piezoelectric crystals can exhibit a mechanical strain when subjected to an external electric field.

There exists a number of natural and synthetic piezoelectric materials; however, quartz – a crystalline form of silica (SiO$_2$) – is technologically the most prevalent due to its unique physicochemical properties. There are two polymorphic forms of quartz: α- and β-quartz. Below 573ºC, quartz exists in its α form; however, at this temperature occurs a structural phase transition (or inversion), whereby the more symmetrical β-quartz structure is produced (the latter is stable between 573 and 870ºC). Due to the anisotropic nature of quartz, crystals can be cut at specific angles/along certain planes (Figure 41) thus producing wafers with different electromechanical properties. One such cut is the ‘AT-cut’ (a cut normal to the y-axis but rotated +35º15’ with respect to the z-axis, as shown in Figure 41), which is frequently used as the transducing element of piezoelectric (bio)sensors due to its resonant frequency stability over a wide range of temperatures.
1.11.3. Two families of acoustic wave-based devices: SAW and BAW sensors

Although acoustic wave-based devices can be categorized into different classes (see below), they all essentially operate through the same following principle. Upon application of an oscillating electric field, stress is induced into the piezoelectric crystal and generates a periodical motion of particles restored by elastic forces.\textsuperscript{12} This displacement launches acoustic waves in the material, on which the phenomenon of piezoelectric resonance hinges. The electromechanical coupling and consequential stress are related to the crystal symmetry, the configuration and orientation of the applied electric field, and the angle/plane along which the crystal was cut relative to the crystallographic axes.\textsuperscript{348} There are, in essence, three types of motion particles can follow (linear, elliptical, circular), which in turn gives rise to different types of acoustic wave (compressional, shear).\textsuperscript{357} In a compressional wave (also referred to as ‘longitudinal wave’), particle motion is parallel to wave propagation; whereas, in a shear wave (also referred to as ‘transverse wave’), displacement is perpendicular to the direction of wave propagation.\textsuperscript{358} Depending on the nature of the acoustic wave and the vibration mode, devices can be classified into two main families known as surface acoustic wave and bulk acoustic wave (SAW and BAW) sensors.\textsuperscript{359} SAW devices commonly consist of interdigital transducer (IDT) metal electrodes, which are mounted on the surface of the piezoelectric substrate. The aim of this IDT
arrangement is to create, guide and confine acoustic waves at the ‘near’ surface of the sensor.\textsuperscript{12,357,359} Surface particles move in an elliptical manner resulting in the so-called ‘Rayleigh wave’ that comprises both a shear and compressional component, and propagates near the sensing surface (approximately one acoustic wavelength away).\textsuperscript{346,349,358,360} Conversely, in BAW devices, acoustic waves travel unguided through the volume of the material, whereby particle displacement is parallel to the surface (\textit{i.e.} in a shear motion).\textsuperscript{349,357,360} Both types of acoustic wave sensors are able to detect and monitor molecular interactions occurring at the sensor-gas interface; however, BAW devices tend to operate more effectively in liquids.\textsuperscript{360,361} The reason for such behavioural discrepancy between sensor architectures resides in the fact that – although SAW devices do offer the possibility to operate at high frequencies (typically 100-500 MHz) – such capability is impaired in liquid media as, in these conditions, the compressional component of the Rayleigh wave is greatly compromised by attenuation effects.\textsuperscript{357,361,362} On the other hand, the shear wave propagation throughout the bulk in BAW devices allows for these to be operated in liquids without excessive damping.\textsuperscript{361} As a result, BAW sensors such as the \textit{thickness shear mode} (TSM) device have been by far the most employed type of acoustic wave sensor for monitoring molecular interactions in the liquid phase.\textsuperscript{12,363-371} BAW sensors will be discussed in greater detail next.

1.11.4. A closer look at bulk acoustic wave sensors: the ‘thickness shear mode’ sensor

Piezoelectric BAW sensors provide a sensitive, real-time and label-free method to monitor biomolecular interactions. Additionally, different surface chemistries can be applied onto the piezoelectric substrate for specific (bio)molecular probe immobilization and subsequent target analyte binding, thus making BAW devices highly suitable in a wide range of (bio)sensor applications. The benchmark in piezoelectric BAW device is the \textit{thickness shear mode} (TSM) sensor, also commonly referred to as the ‘quartz crystal microbalance’ (QCM).\textsuperscript{12,347,372,373} These sensors typically consist of a thin, AT-cut piezoelectric quartz wafer sandwiched between two metal electrodes typically made of gold.\textsuperscript{340} It is through the application of an oscillating electrical potential \textit{via} these plated electrodes that the mechanical motion of particles within the substrate is actuated and acoustic resonance instigated, as per the ‘converse piezoelectric effect’ principle discussed in \textit{Section 1.11.2}.\textsuperscript{12,348} As such, a shear wave is generated, which propagates through the bulk of the piezoelectric material (with very little dissipation) and is reflected at the device-surroundings interface. In order to actually achieve acoustic resonance however, a standing wave must be generated and maintained between the opposite faces of the crystal (\textbf{Figure 42}), that is
incident and reflected waves undergo constructive interference producing motion antinodes (or maximum displacement) at both crystal faces.\textsuperscript{346,374} Any change occurring at the surface of the piezoelectric crystal (such as the addition of mass) will alter its acoustic properties, which will transpire as a monitorable change in the parameters associated with the standing shear wave (\textit{e.g.} a shift in its resonant frequency).\textsuperscript{374}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure42.png}
\caption{Schematic representation of an AT-cut quartz crystal (with thickness $t_q$) subjected to a perpendicular oscillating electric field ($E$), the resulting shear oscillating displacement of particles along the x-axis ($u_x$), and the associated standing wave (at acoustic resonance).}
\end{figure}

A standing wave occurs when the acoustic wavelength ($\lambda$) is twice the thickness of the quartz crystal ($t_q$), a relationship that can be mathematically expressed as \textbf{equation 10}:

$$\lambda = \frac{2t_q}{N}$$  \hspace{1cm} (10)

where $N$ is the resonance mode (an integer).\textsuperscript{346}

The resonant frequency of the generated acoustic wave (\textit{i.e.} the frequency at which acoustic resonance occurs) is inversely proportional to its wavelength ($\lambda$), and dependent on the velocity at which the wave travels through quartz ($v_s$), as shown through the following \textbf{equation 11}:\textsuperscript{348}

$$f = \frac{v_s}{\lambda}$$  \hspace{1cm} (11)

where $v_s = 3.34 \times 10^3$ m/s (in AT-cut quartz).\textsuperscript{375}
Combining both previous expressions gives equation 12:

\[ f_N = \frac{N v_s}{2t_q} \]  

(12)

In practice, acoustic resonance can only be excited at odd harmonics (i.e. when N is an odd integer) of the fundamental resonant frequency of the crystal (for which N = 1, but usually found noted as ‘\(f_0\)’). 

346,348,360,376

The shear wave velocity is dependent on the physical properties of quartz, notably its shear modulus (\(\mu_q\)) and density (\(\rho_q\)), as shown in the following equation 13:

\[ v_s = \frac{\sqrt{\mu_q}}{\sqrt{\rho_q}} \]  

(13)

where \(\mu_q = 2.947 \times 10^{11} \text{ g/(cm}^2 \text{s)}\), for AT-cut quartz, and \(\rho_q = 2.648 \text{ g/cm}^3\). 346,348,377

The application of TSM devices for chemical sensing largely stems from the work of Sauerbrey,378 who while conducting micro-gravimetric measurements in the gas phase noticed that changes in resonant frequency were proportional to the amount of foreign mass deposited on the quartz surface.377,379 This observation led to the formulation of the famous ‘Sauerbrey equation’ (equation 14):

\[ \Delta f = -\frac{2f_0^2}{\sqrt{\rho_q\mu_q}} \frac{\Delta m}{A} = -\frac{2f_0^2}{Z_q} \frac{\Delta m}{A} \]  

(14)

where \(\Delta f\) is the frequency shift due to mass addition (Hz), \(f_0\) is the fundamental resonant frequency of the sensor (i.e. the quartz crystal), \(A\) is the effective surface sensing area (cm\(^2\)), \(\rho_q\) is the density of quartz, \(\mu_q\) is the shear modulus of the particular cut of quartz employed, \(Z_q\) is the acoustic impedance of quartz [\(8.80 \times 10^5 \text{ g/(cm}^2 \text{s)}\) for AT-cut quartz], and \(\Delta m\) is the adsorbed mass (g). 346,348

This gravimetric relationship constitutes the foundation on which quartz piezoelectric sensing relies to quantify mass adsorption (with a detection limit estimated to be of the order of \(10^{-12} \text{ g}\)).377 Thus, this equation predicts, for example, that a mass loading of 5.5 ng/cm\(^2\) will result in a frequency shift of 1 Hz (when using a \(f_0 = 9 \text{ MHz quartz crystal}\)).380 Sauerbrey’s model
assumes that mass deposits from the gas phase as a thin, rigid and uniform layer having the same density and acoustic properties as quartz; in other words, the adsorbed mass perfectly couples to the oscillatory motion of the quartz wafer without friction.\textsuperscript{346,348,357,379} Under these conditions, the added mass constitutes a simple extension of the underlying quartz substrate – resulting in an increase of the acoustic wavelength and, consequently as per equation 11, a decrease in the resonant frequency of the shear oscillation.\textsuperscript{370} Another (potential) shortcoming of Sauerbrey’s equation is that its validity is limited to small mass loads; the relationship diverging from linearity when mass addition results in a frequency shift greater than approximately 2\% of the unloaded resonant frequency.\textsuperscript{346} This is due to the fact that the acoustic delay caused by the addition of the deposited layer is no longer negligible.\textsuperscript{381} The equation is also not valid when: (i) the viscoelastic properties of the deposited mass significantly differ from that of quartz; or (ii) an acoustic wave sensor is operated in the liquid phase – as may be the case for (bio)sensing applications.\textsuperscript{340,346,379} In the latter scenario, even though it does provide reasonable results for dilute solutions, Sauerbrey’s equation does not take into consideration the loss of acoustic energy into the surrounding liquid environment.\textsuperscript{379} In reality, when an acoustic wave sensor is operated in liquid, the energy of the acoustic wave is transferred (and eventually lost) into the liquid medium, the effect being a damping of the propagating wave due to viscous coupling.\textsuperscript{12,382} This simple fact clearly shows that the response of acoustic wave sensors operated in the liquid phase is not merely governed by mass addition/loss, but there also exists in fact many other influential factors related to the liquid medium, sensor surface and interfacial phenomena (Figure 43).\textsuperscript{12,346

\textbf{Figure 43.} Some examples of the various interfacial factors and liquid properties (such as viscosity, $\eta_{\text{liq}}$, and density, $\rho_{\text{liq}}$) influencing the composite response of BAW piezoelectric sensors in the liquid phase.
A consequence, in terms of terminology, of the composite nature of the response of these acoustic wave sensors is that the name ‘quartz crystal microbalance’ – although ubiquitously used – is not appropriate to describe the non-gravimetric nature of the measurements made in the liquid phase; rather, the recommendation was made that such devices be termed with the aforementioned, less questionable name ‘thickness shear mode’ sensors.

Over the years, several models have been developed to account for the various parameters affecting the overall frequency response of TSM sensors in liquids (shown in Figure 43). Nomura and Minemura were the first, for instance, to present an empirical formulation linking frequency shift with fluid density and specific conductivity. Another empirical formulation was submitted by Nomura and Okuhara, who considered the effects of viscosity and density on resonant frequency; while Yao and Zhou used a similar model to consider the influence of the liquid’s dielectric constant. In 1985, Kanazawa and Gordon introduced a theory for the operation of quartz resonators in non-conducting viscous fluids, wherein the shear wave generated in the quartz resonator was matched to the damped, propagating shear wave in the liquid describing a no-slip coupling process (i.e. the velocities of the substrate and the liquid are equal at the interface). The model describes the influence of the density and viscosity of a liquid on the resonant frequency through the following expression (equation 15):

\[ \Delta f = \frac{-f_0^{3/2}}{Z_q} \sqrt{\rho_{liq} \eta_{liq}} = \frac{-2f_0^2}{Z_q} \rho_{liq} \delta \]  (15)

where \( f_0 \) is the fundamental resonant frequency of the quartz resonator, \( \rho_{liq} \) is the density of the liquid, \( \eta_{liq} \) is its viscosity, \( Z_q \) is the acoustic impedance of quartz, and \( \delta \) is the shear wave penetration depth (or decay length) given by equation 16:

\[ \delta = \frac{\eta_{liq}}{\pi \rho_{liq} f_0} \]  (16)

According to this model, when one face of a quartz crystal resonator is placed in a Newtonian liquid (i.e. a fluid whose viscosity does not change even when external stress is applied to it) and interfacial effects are disregarded, the quartz resonator will not drive the entire bulk of the liquid into motion considering the transverse displacement decays exponentially into the liquid, with a characteristic length \( \delta \) described by equation 16.
also shows that shear waves propagate farther into fluids with higher kinematic viscosity \((i.e. \text{greater } \eta_{liq}/\rho_{liq} \text{ ratio})\).\(^{390}\) The decay length \(\delta\) corresponds to the depth within which the vibration amplitude is attenuated \(e\) times (~63\%) and most of a sensor’s response originates from.\(^{375,389}\) As an example, the decay length for a \(f_0 = 9\) MHz quartz crystal operating in water is approximately 180 nm.\(^{376}\) Many of these initial models ignored interfacial properties/phenomena, which have been clearly shown to affect the acoustic signal. Later models were developed however to account for these interfacial effects, such as: surface roughness,\(^{391-393}\) surface stress,\(^{394}\) hydrodynamic coupling,\(^{395-397}\) viscoelasticity,\(^{398}\) surface free energy, interfacial viscosity, and interfacial slippage.\(^{370,371}\) The fact of the matter is that, although several more or less sophisticated models have been proposed over the years, there is no universal model available as of yet able to account for all the many different factors affecting the response of TSM sensors in liquid.\(^{346}\) Additionally, it is essential to note that, due to acoustic coupling effects and interfacial slip at the liquid-sensor interface, an increase in resonant frequency may be observed despite mass addition.\(^{398}\) Equally as important, the alteration of acoustic coupling due to interfacial chemistry offers a valuable method to detect and monitor on-surface biomolecular interactions, and associated conformational changes or restructuration.\(^{346,357,400-403}\)

Experimentally, a number of configurations have been employed in order to try and ‘fully’ characterize – through the investigation of various factors affecting its response – the TSM sensor system under liquid loading conditions.\(^{404}\) Devices are operated at high frequencies, typically in the megahertz range (5-20 MHz),\(^{346,361}\) and measurements can be conducted in a static fashion or with flowing liquid dispensed \(via\) a flow-through injection system (Figure 44), the latter arrangement allowing for kinetic processes occurring at the sensor-liquid interface to be studied.\(^{12,346}\) The most thorough approach to characterize the TSM sensor response in liquids is provided by the \textit{acoustic network analysis} method (Figure 44).\(^{12}\)
Figure 44. Schematic representation of a TSM device architecture showing the flow-through cell, the encased quartz disc plated with gold electrodes on both sides, and the network analyzing system.

The heart of this analysis method lies in the fact that it is possible to describe the mechanical behaviour and acoustic properties of piezoelectric resonators in electrical terms. First developed by Kipling and Thompson, acoustic network analysis provides a real-time method to calculate various parameters including resonant frequency and corresponding energy dissipation factors, thus providing information on sensor behaviour in the liquid phase. In essence, the network analysis method consists in applying a sinusoidal voltage to the piezoelectric crystal (at a set of frequencies under resonance conditions) and measuring the resulting impedance of the sensor (i.e. its magnitude and phase); and fitting the continuously collected impedance data to an equivalent electrical circuit, whose electrical parameters can be related to the physical properties of the sensor. The Butterworth-van Dyke (BVD) equivalent circuit is most commonly used to describe acoustic resonators. While the BVD circuit can be modified or extended to depict different load conditions, its simplest form (Figure 45) combines a static capacitor \( C_0 \) in parallel with a series circuit containing a resistor \( R_m \), an inductor \( L_m \) and a capacitor \( C_m \).

Figure 45. The simplest form of the Butterworth-van Dyke (BVD) equivalent circuit for TSM resonators.
The acoustic branch of the circuit consists of the motional resistance \( R_m \), the motional inductance \( L_m \) and the motional capacitance \( C_m \), and represents the mechanical oscillation of a resonating quartz crystal.\(^{409}\) The relationship between the equivalent circuit parameters and the physical characteristics of the quartz crystal can be expressed as follows (equations 17-20):

\[
R_m = \frac{t_q^3 r}{8A\varepsilon^2} \\
L_m = \frac{t_q^3 \rho}{8A\varepsilon^2} \\
C_m = \frac{8A\varepsilon^2}{\pi^2 t_q c} \\
C_0 = \frac{k\varepsilon_0 A}{t_q}
\]  

where \( t_q \) is the thickness of the quartz crystal, \( r \) is the dissipation coefficient, \( A \) is the area of the quartz crystal, \( \varepsilon \) is the piezoelectric stress constant for quartz, \( c \) is the elastic constant for quartz, \( \rho \) is the density of quartz, \( k \) is the dielectric constant of quartz, and \( \varepsilon_0 \) is the dielectric permittivity of free space.\(^{405}\) In brief, \( R_m \), \( L_m \), and \( C_m \) respectively represent the acoustic energy loss, inertial mass and elastic properties of the vibrating quartz crystal.\(^{354}\) The static capacitance \( C_0 \) relates to the dielectric nature of the device (i.e. the dielectric quartz disc placed in-between two electrodes).\(^{354}\) Additionally, due to the parallel configuration of the equivalent circuit, two resonant frequencies are produced if damping is negligible (that is \( R_m \) tends to 0) and the impedance phase shift is equal to zero. These two resonant frequencies are: the series resonant frequency that occurs at minimum impedance, and the parallel resonant frequency (or anti-resonant frequency) that occurs at maximum impedance.\(^{357,377,390,407}\) The series resonant frequency \( f_s \) is the real resonant frequency that will yield the highest amplitude of oscillation and measures energy storage; while the parallel resonant frequency \( f_p \) is purely electrical and will not generate mechanical oscillation.\(^{390,407}\) Another important parameter that can be obtained from impedance data is the quality factor \( (Q) \), which for any resonant system is defined as the ratio between stored and dissipated/lost energy per resonant cycle (i.e. oscillation). It is expressed as (equation 21):
where \( \Gamma \) is the bandwidth (i.e. the width of the resonant envelope measured in Hz), and \( D \) is the dissipation. \( \Gamma, D, \) and \( R_m \) are all parameters, which provide information regarding acoustic energy dissipation.\(^{34,390}\) When the aim is to investigate physicochemical events at the device-liquid interface, the two parameters of most interest are \( f_s \) and \( R_m \) considering that a shift in \( f_s \) can be associated with changes in energy storage (e.g. deposition of mass, biomolecular conformational rearrangement), and that an increase in \( R_m \) can be associated with energy loss through friction, heat or viscous means.\(^{410}\) With regards to applications, recent times have seen a rise in the implementation of TSM technology\(^{347,372,373}\) to study various phenomena from on-surface protein conformational restructuration,\(^{400}\) to nucleic acid hybridization,\(^{411}\) to cell-surface attachment.\(^{412}\)

Although TSM devices have been successfully used for various purposes, there are some limitations with this technology. Firstly, the application of a voltage between the plated electrodes – that are necessary to instigate acoustic resonance within the sandwiched piezoelectric quartz crystal – generates fringing electric fields at the electrodes’ edges that can propagate into the surrounding solution, thus influencing the response of TSM devices.\(^{407}\) Additionally, the use of electrodes not only limits the type of surface chemistry that can be used (i.e. to thiol SAM surface chemistry), but also necessitates hardwire connections that may interfere with liquid flowing through the analysis chamber. Next, the TSM technology has a relatively modest on-surface limit of detection of approximately 1 ng/cm\(^2\).\(^{413}\) A strategy to lower the detection limit and enhance sensitivity is to operate quartz acoustic wave-based devices at higher frequencies. This has been attempted with limited success by working either with thinner quartz resonators or at higher harmonics (as per equation 12).\(^{407,413}\) Recently, an innovative acoustic wave sensor device – that, incidentally, can provide both these options simultaneously (i.e. thinner piezoelectric quartz wafer and higher operating frequency) – was introduced in an effort to resolve the various problems encountered by the TSM technology and improve the quality of detection.\(^{407,413}\)
1.11.5. A novel type of BAW device: the electromagnetic piezoelectric acoustic sensor

A relatively recent addition to the family of BAW-based devices is the electromagnetic piezoelectric acoustic sensor (EMPAS), a highly sensitive flow-through device also capable of performing measurements in a real-time and label-free manner (Figure 46A). The EMPAS is a hybrid system resulting from the fusion between the TSM and the magnetic acoustic resonance sensor technologies, and relies on the electromagnetic excitation of acoustic resonance at higher harmonics in the piezoelectric quartz substrate. In its configuration, the EMPAS operates by remotely instigating ultra-high frequency acoustic shear waves (up to 1.06 GHz – the 53rd harmonic for \( f_0 = 20 \) MHz) within electrode-free AT-cut quartz discs upon the action of an external electromagnetic field emanating from a planar spiral coil (Figure 46B – left) placed at close proximity underneath the quartz substrate (~30 μm). The applied electromagnetic field will induce a secondary electric field within the quartz crystal, which drives the converse piezoelectric effect discussed in Section 1.11.2. and instigates acoustic resonance (Figure 46B – right).

Figure 46. The electromagnetic piezoelectric acoustic sensor (EMPAS). (A) Picture of the EMPAS experimental set-up featuring the electronic instruments and hardware necessary to excite acoustic resonance within quartz discs, the flow-through cell holder wherein the latter are encased with the coil, and the injection system for running buffer and analysis samples (left); as well as (right) the EMPAS working principle. (B) The planar spiral coil used to remotely induce within electrodeless piezoelectric quartz discs a secondary electric field that instigates acoustic resonance. Parts of this figure are adapted from references 413 and 414.
Any changes, even subtle, at/near the sensor-liquid interface – whether caused by the adsorption/desorption (i.e. mass addition/loss) of species (e.g. target analytes, foulants) and/or due to interfacial effects such as an alteration in viscoelasticity and slip phenomena – will cause a shift in the resonant frequency of the sensor that, in electrical terms, corresponds to a change in the impedance of the coupled coil.\textsuperscript{39,50,374,407,413,416}

In practice, the EMPAS system offers a number of important advantages over the more conventional acoustic wave sensors based on TSM technology, such as:\textsuperscript{12,340,357,407,413} (i) an electrode-free environment (no hardwire connections required and no associated stray electric fields); (ii) the possibility to operate the sensor at ultra-high frequencies (e.g. 1.06 GHz), via the launch of odd bulk acoustic wave overtones, to attain higher analytical sensitivity\textsuperscript{407} and an on-surface detection limit of approximately $10^{-15}$ mol/cm$^2$;\textsuperscript{340} and, (iii) frequency tunability, which offers in theory the possibility to probe the sensor’s interfacial region at different depths – as per equation 16, where $f_0$ would become $f_N = N \times f_0$ (N being an odd overtone integer) – and notably to focus the evanescent acoustic wave, hence EMPAS measurements, onto the interfacial chemistry/phenomena of interest, with the hope of collecting further information and gaining deeper insight into the structural features of adsorbed (bio)films.\textsuperscript{417-419} This concept of differential probing was coined ‘acoustic fingerprinting’.\textsuperscript{418} Additional advantages include: (iv) the opportunity to directly modify quartz sensing platforms with the more versatile and robust silane chemistry (or other types of surface chemistries capable of coating hydroxylated/oxide substrates); and, (v) the possibility to miniaturize the electrode-free, monolithic quartz discs with respect to their thickness and diameter. In this respect, there may be plenty of room to maneuver as shown in a recent account that reported the use of 2 mm-wide, 9.7 μm-thick AT-cut quartz resonator plates ($f_0 = 170$ MHz) in a wireless-electrodeless BAW biosensor having a sensitivity of 15 pg/(cm$^2$ Hz).\textsuperscript{420} In comparison, the EMPAS device operated at 1.06 GHz (53$^{\text{rd}}$ harmonic)\textsuperscript{18,402} with electrode-free quartz discs ~83 μm-thick and ~13 mm-wide ($f_0 = 20$ MHz) has a theoretical sensitivity of ~21 pg/(cm$^2$ Hz) at this frequency, which incidentally corresponds to an increase in sensitivity of approximately three orders of magnitude relative to a more conventional TSM device operated at 5 MHz.\textsuperscript{421}
With respect to applications, the EMPAS system has been used to investigate biomolecular interactions in buffer\textsuperscript{18} and multi-protein surface adsorption phenomena (\textit{i.e.} fouling from full serum),\textsuperscript{50} and recently successfully applied to the serological detection of human immunodeficiency virus (HIV) antibodies.\textsuperscript{402} This immunosensor constituted the first real-world application of the EMPAS technology in the bioanalytical field.

Admittedly, the EMPAS system also has shortcomings, the main one probably being that – as a member of the BAW family – its response in the liquid phase is not purely gravimetric in nature (as discussed above and in \textit{Section 1. 11. 4.}, as well as shown in \textbf{Figure 43}). Another issue would be how sensitive the EMPAS electronics (\textit{i.e.} the coil) is to external electromagnetic interference (which increases background noise), and stimuli that may erroneously cause the resonant frequency to shift.\textsuperscript{407,422} In practice, the composite nature of the EMPAS signal implies that the implementation of such device for the detection of target analytes should not be expected to yield dose-response curves exhibiting a quantitative relationship of proportionality such as that observed in the linear ‘dynamic range’ of traditional calibration curves. This by no means constitutes however an obstacle to analyte detection with the EMPAS system as the latter can very well be promoted as a qualitative screening/diagnostic technique providing a rapid and cost-effective ‘yes or no’ response to the question as to whether an individual is afflicted with a suspected disease/condition – a positive result being followed-up, if possible/desired, by quantitative analysis with another technique. Demonstration will be made in the ‘Results and Discussion’ section of this PhD Thesis that the highly sensitive EMPAS sensor indeed allows for serological ‘cut-off’ assays with pM limit of detection to be developed.

1. 12. 1. Research background

It has become abundantly clear from the literature review presented in the previous sections of this Introduction that fouling of artificial surfaces coming in contact with blood or its plasma/serum derivatives is a major clinical problem, a true Achilles Heel of Biotechnology, which has stimulated a great amount of research – not only from a practical point of view to produce antifouling surface chemistries for a variety of substrate materials, but also from a more fundamental standpoint to try and understand by which mechanism(s) these coatings actually exhibit their repellent properties. Despite considerable research efforts however, materials commonly used for biomedical and bioanalytical applications are arguably still quite primitive and certainly not truly inert, as they still are susceptible to fouling, a natural adsorption phenomenon that can activate host responses with potentially deleterious effects (as in the case of implants with the foreign body reaction) or/and interfere with target analyte detection (as in the case of biosensors).

1. 12. 2. Research motivation

Our laboratory has long been interested in developing ultrathin organic coatings for bioanalytical and biomedical applications. This PhD Thesis naturally evolved in line with work by predecessors but still set out to conduct original, both fundamental and applied, research. Particular attention was notably given to developing novel ultrathin surface chemistry to be combined with ultra-high frequency acoustic wave physics – the intent being to develop biosensors with advanced features (i.e. real-time and label-free measurement capability) as potential clinical testing alternatives for cost-effective and rapid biomarker analysis in blood plasma/serum. Another closely related project focused on devising novel antithrombogenic coatings for biomedical applications (e.g. for the circuitry used to circulate blood extracorporeally during renal dialysis and cardiopulmonary bypass procedures). To summarize, this PhD Thesis reports innovative and exciting work relevant to the bioanalytical and biomedical fields; however, the surface chemistry presented herein is by no means restricted to the clinical world and may very well find applications in any domain plagued by fouling in general. The following paragraphs succinctly outline the various research projects conducted during this PhD Thesis that will be detailed in the ‘Results and Discussion’ section.
1. Research objectives

The first objective of research, discussed in Section 3.1., was to engineer real-time and label-free biosensors based on EMPAS technology able to detect target analytes with high selectivity and sensitivity. Biosensing platforms consisted of quartz discs coated with dual-functional, binary organosilane surface chemistry that combined both antifouling and biorecognition capability. We began our investigation employing the biotin/avidin couple as a test system, our choice being motivated by the fact that avidin displays for biotin very high affinity with a dissociation constant $K_D$ in the order of $10^{-15}$ M. We initially performed EMPAS measurements in buffer, and then moved on to full serum creating, as such, a real-world detection scenario. Also assessed was the effect on the sensor response, notably with respect to non-specific adsorption, through using a blocking agent (bovine serum albumin) or surface-modifying molecules with different backbones (oligoethylene glycol vs. alkyl). Miniaturization of the sensing platforms for future potential point-of-care applications was also accomplished. The initial biotin/avidin prototype paved the way for a real-life biosensor able to detect in full human plasma abnormally high level of bacterial endotoxin, a pathogen associated with sepsis.

The second objective of research, discussed in Section 3.2., was to investigate with the EMPAS the antifouling behaviour against full serum of a series of ultrathin, unimolecular adlayer coatings prepared from a variety of monoethylene glycol (MEG)-based organosilane molecules (and alkylated derivatives). Experimental (neutron reflectometry) together with computational (molecular dynamics simulations) data provided further insight into the debated relationship hypothesized to exist between surface hydration and antifouling, and gave rise to a list of basic requirements for coatings to exhibit such a property. Also demonstrated was the adaptability of the most effective MEG chemistry to the surface modification of gold, a substrate material widely used for detection purposes in piezoelectric (TSM) and optical (SPR) biosensor devices; as well as that of polycarbonate, a plastic polymer increasingly employed in the biomedical industry. Another highlight of this latter study was the remarkable antithrombogenic properties displayed by the applied surface chemistry – platelet adhesion, aggregation and thrombus formation from whole, non-anticoagulated human blood being nearly non-existent.
2. Experimental

2.1. General remarks

This experimental section includes procedures for:

- the synthesis of surface modifiers and immobilization probes (Section 2.3);
- substrate cleaning and activation (Section 2.4.1);
- the preparation of unimolecular and mixed adlayers (Section 2.4.2);
- probe immobilization (Section 2.4.3);
- surface characterization (Sections 2.5.1-2) and analysis (Sections 2.5.3-6);
- molecular dynamics simulations (Section 2.6).

2.2. Materials and methods

**Chemical synthesis:** reagents were purchased from Sigma-Aldrich (and used as received), chloroplatinic acid hexahydrate catalyst (99.9%) from Strem Chemicals. Heavy-walled round bottom pressure tubes (d = 2.5 cm, h = 11.4 cm) for the solvent-free synthesis of trichlorosilane surface modifiers were purchased from VWR. $^1$H and $^{13}$C NMR spectra were recorded at room temperature on Varian (or Mercury) 300 or 400 MHz spectrometers using CDCl$_3$ or CD$_3$OD as the NMR solvents. $^1$H and $^{13}$C NMR spectra are referenced to the residual solvent peak (CDCl$_3$: 7.27 and 77.23 ppm, respectively; CD$_3$OD: 3.31 ppm). The abbreviations s, d, t, q, dd, td and m respectively stand for singlet, doublet, triplet, quadruplet, doublet of doublet, triplet of doublet, and multiplet. The term ‘br’ means the signal is broad.

**Substrates:** piezoelectric quartz discs for EMPAS measurements (AT-cut, d = 0.537” ~ 13.6 mm or 0.275” ~ 7.0 mm, 83 μm-thick, $f_0$ = 20.0 MHz) and TSM measurements (AT-cut, d = 0.537” ~ 13.6 mm, $f_0$ = 9.0 MHz – plated with symmetric gold electrodes 0.196” ~ 4.9 mm in diameter) were purchased from Laptech Precision Inc. Silicon wafers [(111) orientation, d = 100 mm, 5000 μm-thick] were purchased from University Wafers. BPA-PC slides for X-ray photoelectron spectroscopy (XPS) and contact angle goniometry (CAG) analysis (1.4 × 1.4 cm and 1/16” ~ 1.6 mm-thick) and sheets for thrombogenicity experiments (7.5 × 2.5 cm and 0.005” ~ 0.13 mm-thick) were respectively purchased from SABIC Polymersshapes and McMaster-Carr.
**Substrate cleaning/activation, adlayer formation and probe immobilization:** Surfaces were systematically handled with thoroughly pre-cleaned stainless steel tweezers to avoid external contamination. The furnace (model F–A1620) used for Si wafer oxidation was manufactured by Thermolyne Corp., the plasma chamber (model PDC–3XG) used for activating BPA-PC and cleaning substrates from Harrick. Silanization reactions with trichlorosilane surface modifiers were prepared in a glovebox maintained under inert (N$_2$) and anhydrous (P$_2$O$_5$) atmosphere, using glassware that had been pre-treated overnight with a 1/20 (v/v) solution of octadecyltrichlorosilane in anhydrous toluene. Anhydrous toluene (Sigma-Aldrich) or anhydrous ethanol (Commercial Alcohols Inc.) were systematically used for adlayer formation, unless otherwise specified. Commercially available octyltrichlorosilane (OTS) surface modifier was purchased from Sigma-Aldrich and redistilled before use. Anhydrous dimethylformamide (DMF) – freshly distilled from calcium hydride under high vacuum – was systematically used for biotin-thiol and biotinamine probe immobilization. Attachment of polymyxin B (PMB – sulfate salt from Sigma-Aldrich) was carried out in ‘endotoxin-free’ (EA = 0.31) Dulbecco’s phosphate buffered saline (PBS, CaCl$_2$ and MgCl$_2$ free – pH 7.4), imperatively using sterile glassware and laboratory equipment. If required, sterilization was achieved upon overnight baking in an oven maintained at 200°C.

**Surface analysis:** EMPAS measurements were performed at the ultra-high frequency of 0.74, 0.82, 0.90, 0.94 or 1.06 GHz, at room temperature using *LabView 6.0* interface. Resonant frequency data were acquired every 10 s. TSM measurements were performed at 9.0 MHz, at room temperature using a Hewlett Packard (model HP4395A) network analyzer. Resonant frequency and motional resistance data were acquired every 12 s. Avidin (from egg white, lyophilized powder), bovine serum albumin (lyophilized powder), lipopolysaccharide (from *Escherichia coli* serotype O55:B5, lyophilized powder) and goat serum (45-75 mg protein/mL) were purchased from Sigma-Aldrich. Dulbecco’s phosphate buffered saline (PBS, CaCl$_2$ and MgCl$_2$ free – pH 7.4) was also obtained from Sigma-Aldrich and made ‘endotoxin-free’ (EA = 0.31, from 0.54) – when required (*i.e.* in the PMB/LPS detection EMPAS study) – upon filtration through a 0.22 μm syringe driven filter unit. Human blood and blood plasma were collected from apparently healthy donors at St. Michael’s Hospital (Toronto, Ontario – Canada). D$_2$O used to prepare contrast-matched water for the neutron reflectometry measurements was provided by Chalk River Labs – Atomic Energy Canada Ltd.

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1 As determined using the endotoxin activity assay (EAA). $^{424,425}$
2. 3. Chemical synthesis

2. 3. 1. Surface modifiers

2. 3. 1. A. TTTA

TTTA linker 6 was synthesized in five steps from 11-bromo-undecene 1 in 31% overall yield (Figure 47):

**Figure 47.** Synthesis of TTTA.

12-tridecenoic acid 2,2,2-trifluoroethyl ester 5. To a stirred solution of 60% sodium hydride (0.48 g, 12.1 mmol, 1.1 equiv.) in tetrahydrofuran (50 mL) was added dropwise diethylmalonate (2.02 mL, 13.2 mmol, 1.2 equiv.) at 0°C. After addition, the reaction was allowed to warm to room temperature then stirred for 1h. 11-bromo-undecene 1 (2.54 mL, 11.0 mmol, 1.0 equiv.) and anhydrous sodium iodide (1.65 g, 11.0 mmol, 1.0 equiv.) were then successively added. After refluxing overnight, the reaction was quenched with brine then extracted with ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and then evaporated under reduced pressure to provide diethyl malonate compound 2. The latter was diluted with a 1/1 (v/v) mixture of ethanol (20 mL) and 2.6 M aqueous potassium hydroxide (20 mL). The reaction was vigorously stirred at room temperature overnight then the solvents were evaporated under reduced pressure to provide malonic acid dipotassium salt 3. This residue was then submitted to a dichloromethane/water extraction. The combined aqueous layers were concentrated under reduced pressure to about 100 mL then cautiously acidified with concentrated sulfuric acid. The resulting solution was refluxed overnight then submitted to a dichloromethane/water extraction. The combined organic layers
were dried over anhydrous sodium sulfate, filtered, and then evaporated under reduced pressure to provide carboxylic acid 4: $^1$H NMR (400 MHz, CDCl$_3$) δ ~11 (br, 1H), 5.82 (m, 1H), 5.00 (m, 1H), 4.93 (m, 1H), 2.36 (t, $J = 7.4$ Hz, 2H), 2.05 (m, 2H), 1.64 (m, 2H), 1.45-1.23 (m, 14H). The latter (2.06 g, 9.7 mmol, 1.0 equiv.) was dissolved in dichloromethane (70 mL) then N,N'-dicyclohexyl-carbodiimide (DCC: 2.22 g, 10.7 mmol, 1.1 equiv.), 2,2,2-trifluoroethanol (0.78 mL, 10.7 mmol, 1.1 equiv.) and 4-dimethylamino-pyridine (4-DMAP: 0.12 g, 1.0 mmol, 0.1 equiv.) were successively added. The reaction was stirred at room temperature overnight then filtered through a short plug of Celite. After evaporation of the filtrate under reduced pressure, purification by column chromatography (hexanes/ethyl acetate: 100/0 to 99/1) finally provided titled ester 5 as a pale yellow oil (1.53 g, 47% yield – 4 steps); $^1$H NMR (400 MHz, CDCl$_3$) δ 5.81 (m, 1H), 4.99 (m, 1H), 4.93 (m, 1H), 4.47 (q, $J = 8.4$ Hz, 2H), 2.41 (t, $J = 7.5$ Hz, 2H), 2.04 (m, 2H), 1.64 (m, 2H), 1.45-1.21 (m, 14H); $^{13}$C NMR (75 MHz, CDCl$_3$) 172.3, 139.4, 123.2 (q, $J = 275.5$ Hz), 114.3, 60.3 (q, $J = 36.4$ Hz), 34.0, 33.8, 29.8, 29.7, 29.6, 29.4, 29.3, 29.2, 29.1, 24.9; IR (neat) 1760 cm$^{-1}$; HRMS (EI, m/z) calcd. for C$_{15}$H$_{25}$O$_2$F$_3$ (M$^+$) 294.1807, found 294.1806.

13-trichlorosilyl-tridecanoic acid 2,2,2-trifluoroethyl ester 6 (TTTA). In a heavy-walled pressure tube equipped with a magnetic stir bar, ester 5 (1.18 g, 4.0 mmol, 1.0 equiv.) and chloroplatinic acid hexahydrate (21 mg, 0.14 mmol, 1.0 mol%) were loaded. The tube was transferred into a glovebox and trichlorosilane (0.82 mL, 8.0 mmol, 2.0 equiv.) was added to the solution. The tube was tightly fastened then removed from the glovebox. The resulting solution was stirred at 80°C for 22h behind a protecting shield. Purification by Kugelrohr distillation under vacuum finally provided TTTA 6 as a colourless oil (1.16 g, 67% yield); bp = 170-180°C (0.15 Torr); $^1$H NMR (400 MHz, CDCl$_3$ – Appendix A) $^{272}$ δ 4.45 (q, $J = 8.5$ Hz, 2H), 2.41 (t, $J = 7.4$ Hz, 2H), 1.72-1.55 (m, 4H), 1.45-1.22 (m, 18H); $^{13}$C NMR (100 MHz, CDCl$_3$) $^{272}$ 172.4, 123.3 (q, $J = 275.8$ Hz), 60.3 (q, $J = 36.4$ Hz), 33.9, 32.0, 29.8, 29.7, 29.6, 29.5, 29.4, 29.2, 29.1, 24.9, 24.5, 22.5.
PFP-TTTA linker 8 was synthesized in five steps from 11-bromo-undecene 1 in 73% overall yield (Figure 48):

12-tridecenoic acid 4. To a stirred solution of 60% sodium hydride (0.48 g, 12.1 mmol, 1.1 equiv.) in tetrahydrofuran (50 mL) was added dropwise diethylmalonate (2.02 mL, 13.2 mmol, 1.2 equiv.) at 0°C. After addition, the reaction was allowed to warm to room temperature then stirred for 1h. 11-bromo-undecene 1 (2.54 mL, 11.0 mmol, 1.0 equiv.) and anhydrous sodium iodide (1.65 g, 11.0 mmol, 1.0 equiv.) were then successively added. After refluxing overnight, the reaction was quenched with brine then extracted with ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and then evaporated under reduced pressure to provide diethyl malonate compound 2. The latter was diluted with a 1/1 (v/v) mixture of ethanol (20 mL) and 2.6 M aqueous potassium hydroxide (20 mL). The reaction was vigorously stirred at room temperature overnight then the solvents were evaporated under reduced pressure to provide malonic acid dipotassium salt 3. The residue was then submitted to a dichloromethane/water extraction. The combined aqueous layers were concentrated under reduced pressure to about 100 mL then cautiously acidified with concentrated sulfuric acid. The resulting solution was refluxed overnight then submitted to a dichloromethane/water extraction. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and then
evaporated under reduced pressure to provide titled carboxylic acid 4 as a beige solid (1.91 g, 82% yield – 3 steps); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta ~11\) (br, 1H), 5.82 (m, 1H), 5.00 (m, 1H), 4.93 (m, 1H), 2.36 (t, \(J = 7.4\) Hz, 2H), 2.05 (m, 2H), 1.64 (m, 2H), 1.45-1.23 (m, 14H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) 180.7, 139.4, 114.3, 34.3, 34.0, 29.7 (2 \(^{13}\)C), 29.6, 29.4, 29.3, 29.2, 29.1, 24.9; IR (neat) 2900-2400 (br), 1700 cm\(^{-1}\); HRMS (EI, m/z) calcd. for C\(_{13}\)H\(_{24}\)O\(_2\) (M\(^+\)) 212.1776, found 212.1779.

12-tridecenoic acid pentafluorophenyl ester 7. To a solution of carboxylic acid 4 (1.00 g, 4.5 mmol, 1.0 equiv.) and triethylamine (1.32 mL, 9.5 mmol, 2.1 equiv.) in dichloromethane (25 mL) was added dropwise pentafluorophenyl trifluoroacetate (1.24 mL, 7.1 mmol, 1.6 equiv.). The reaction was stirred overnight at room temperature then submitted to a dichloromethane/water extraction. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and then evaporated under reduced pressure. Purification by column chromatography (hexanes/ethyl acetate: 100/0 to 90/10) finally provided titled ester 7 as a low melting point, pale yellow solid (1.51 g, 89% yield); \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta 5.82\) (m, 1H), 5.00 (m, 1H), 4.94 (m, 1H), 2.66 (t, \(J = 7.5\) Hz, 2H), 2.05 (m, 2H), 1.78 (m, 2H), 1.48-1.26 (m, 14H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) 169.7, 143-140 (m), 141-138 (m), 139.4, 140-137 (m), 125.3 (m), 114.3, 34.0, 33.5, 29.7 (2 \(^{13}\)C), 29.6, 29.3 (2 \(^{13}\)C), 29.2, 29.1, 25.0; IR (neat) 1791 cm\(^{-1}\); Elemental analysis: calcd. for C\(_{19}\)H\(_{23}\)O\(_2\)F\(_5\): C, 60.31; H, 6.13; found: C, 60.08; H, 6.03.

13-trichlorosilyl-tridecanoic acid pentafluorophenyl ester 8 (PFP-TTTA). In a heavy-walled pressure tube equipped with a magnetic stir bar, ester 7 (0.38 g, 1.0 mmol, 1.0 equiv.) and chloroplatinic acid hexahydrate (6 mg, 0.01 mmol, 1.0 mol%) were loaded. The tube was transferred into a glovebox and trichlorosilane (0.62 mL, 6.0 mmol, 6.0 equiv.) was added to the solution. The tube was tightly fastened then removed from the glovebox. The resulting solution was stirred at room temperature for 22h behind a protecting shield. The excess of trichlorosilane was finally removed under high vacuum to provide PFP-TTTA 8 as a thick yellow-greenish oil (0.54 g, quantitative yield); \(^1\)H NMR (400 MHz, CDCl\(_3\) – Appendix B) \(\delta 2.67\) (t, \(J = 7.4\) Hz, 2H), 1.78 (m, 2H), 1.59 (m, 2H), 1.47-1.26 (m, 18H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) 169.8, 143-140 (m), 141-138 (m), 125.5 (m), 33.5, 32.0, 29.8, 29.7, 29.6, 29.5, 29.3, 29.2, 29.1, 25.0, 24.5, 22.5.
2. 3. 1. C. OEG-TTTA\textsuperscript{18}

OEG-TTTA linker 15 was synthesized in six steps from 2-allyloxy-ethanol 9 in 18% overall yield (Figure 49):

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{synthesis.png}
\caption{Synthesis of OEG-TTTA.}
\end{figure}

(2-allyloxy-ethoxy)-acetic acid methyl ester 10. To a stirred solution of 2-allyloxy-ethanol 9 (10.9 mL, 99.9 mmol, 1.0 equiv.) in tetrahydrofuran (200 mL) was carefully added 60% sodium hydride (4.8 g, 120.0 mmol, 1.2 equiv.) in small portions at room temperature. The reaction was then refluxed for 1h then cooled to 0°C. Methyl bromoacetate (11.4 mL, 120.3 mmol, 1.2 equiv.) was then added dropwise. After 15 min at 0°C, the reaction was submitted to an ethyl acetate/water extraction. The combined organic layers were washed with brine, dried over anhydrous magnesium sulfate, filtered, and then evaporated under reduced pressure. Purification by Kugelrohr distillation under reduced pressure finally provided titled ester 10 as a colourless oil (9.77 g, 55% yield); \textbf{bp} = 130-145°C (tap water vacuum); \textbf{\textsuperscript{1}H NMR} (400 MHz, CDCl\textsubscript{3}) \delta 5.91 (m, 1H), 5.28 (m, 1H), 4.99 (m, 1H), 4.19 (s, 2H), 4.02 (m, 2H), 3.76 (s, 3H), 3.75 (m, 2H), 3.64 (m, 2H); \textbf{\textsuperscript{13}C NMR} (100 MHz, CDCl\textsubscript{3}) 171.1, 134.8, 117.3, 72.4, 71.2, 69.7, 68.9, 51.9; \textbf{IR} (neat) 1755 cm\textsuperscript{-1}; \textbf{HRMS} (ESI, m/z) calcd. for C\textsubscript{8}H\textsubscript{15}O\textsubscript{4} (MH\textsuperscript{+}) 175.0964, found 175.0960.
2-(2-allyloxy-ethoxy)-ethanol 11. To a stirred solution of methyl ester 10 (9.77 g, 55.1 mmol, 1.0 equiv.) in tetrahydrofuran (100 mL) was carefully added one portion of lithium aluminum hydride (LAH: 95%, 1.10 g, 27.5 mmol, 0.5 equiv.) at 0ºC. After 30 min, another portion of LAH was carefully added, and the reaction was stirred for an additional 30 min before being carefully quenched with a saturated aqueous solution of sodium sulfate. The resulting white aluminum salts were then filtered over a short plug of Celite, and the filtrate was evaporated under reduced pressure. Purification by Kugelrohr distillation under reduced pressure finally provided titled alcohol 11 as a colourless oil (7.99 g, 99% yield); bp > 200ºC (tap water vacuum). Spectroscopic data were consistent with those reported in the literature: 427,428 ¹H NMR (400 MHz, CDCl₃) δ 5.92 (m, 1H), 5.28 (m, 1H), 5.19 (m, 1H), 4.04 (m, 2H), 3.73 (m, 2H), 3.68 (m, 2H), 3.62 (m, 4H), 2.36 (brs, 1H).

3-(2-(2-allyloxy-ethoxy)-ethoxy)-propanoic acid 2,2,2-trifluoroethyl ester 14. To a stirred solution of alcohol 11 (8.77 g, 60.0 mmol, 2.2 equiv.) in tetrahydrofuran (100 mL) was carefully added freshly hexanes-degreased sodium metal (0.2 g, 8.7 mmol, 0.3 equiv.) in small portions at room temperature. The reaction was then stirred at room temperature for 1h (until the sodium metal chunks disappeared). A solution of ethyl acrylate (2.97 mL, 27.3 mmol, 1.0 equiv.) in tetrahydrofuran (30 mL) was then added dropwise (30 min) through an addition funnel. After 2h at room temperature, the reaction was quenched with 10 drops of glacial acetic acid then submitted to a chloroform/water extraction. The combined organic layers were dried over anhydrous magnesium sulfate, filtered, and then evaporated under reduced pressure to provide ethyl ester 12. The latter was diluted with a 1/1 (v/v) mixture of methanol (120 mL) and 2.5 M aqueous potassium hydroxide (120 mL). The reaction was vigorously stirred at room temperature overnight then extracted with chloroform. The aqueous layer was cautiously acidified with concentrated (37%) hydrochloric acid then extracted with chloroform. The aqueous layer was cautiously acidified with dichloromethane (120 mL) then N,N'-dicyclohexyl-carbodiimide (DCC: 4.25 g, 20.4 mmol, 1.1 equiv.), 2,2,2-trifluoroethanol (1.50 mL, 20.4 mmol, 1.1 equiv.) and 4-dimethylamino-pyridine (4-DMAP: 0.23 g, 1.9 mmol, 0.1 equiv.) were successively added. The reaction was stirred at room temperature overnight then filtered through a short plug of Celite. After evaporation of the
filtrate under reduced pressure, purification by column chromatography (hexanes/ethyl acetate: 95/5 to 50/50) finally provided titled ester 14 as a pale yellow oil (3.59 g, 44% yield – 3 steps); ¹H NMR (400 MHz, CDCl₃) δ 5.92 (m, 1H), 5.28 (m, 1H), 5.18 (m, 1H), 4.49 (q, J = 8.4 Hz, 2H), 4.03 (m, 2H), 3.79 (t, J = 6.4 Hz, 2H), 3.63 (m, 8H), 2.71 (t, J = 6.4 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃) 170.2, 134.9, 123.1 (q, J = 275.7 Hz), 117.2, 72.4, 70.9, 70.8, 70.7, 69.6, 66.3, 60.5 (q, J = 36.5 Hz), 34.8; IR (neat) 1760 cm⁻¹; HRMS (ESI, m/z) calcd. for C₁₂H₂₀O₅F₃ (MH⁺) 301.1257, found 301.1258.

3-(2-(2-(3-trichlorosilylpropyloxy)-ethoxy)-ethoxy)-propanoic acid 2,2,2-trifluoroethyl ester 15 (OEG-TTTA). In a heavy-walled pressure tube equipped with a magnetic stir bar, ester 14 (1.65 g, 5.5 mmol, 1.0 equiv.) and chloroplatinic acid hexahydrate (28 mg, 0.06 mmol, 1.0 mol%) were loaded. The tube was transferred into a glovebox and trichlorosilane (1.12 mL, 11.0 mmol, 2.0 equiv.) was added to the solution. The tube was tightly fastened then removed from the glovebox. The resulting solution was stirred at room temperature for 20h behind a protecting shield. Purification by Kugelrohr distillation under vacuum finally provided OEG-TTTA 15 as a colourless oil (1.85 g, 77% yield); bp = 175-185°C (0.19 Torr); ¹H NMR (400 MHz, CDCl₃ – Appendix C) δ 4.47 (q, J = 8.4 Hz, 2H), 3.78 (t, J = 6.4 Hz, 2H), 3.61 (m, 8H), 3.51 (t, J = 6.4 Hz, 2H), 2.70 (t, J = 6.4 Hz, 2H), 1.85 (m, 2H), 1.48 (m, 2H); ¹³C NMR (100 MHz, CDCl₃) 170.1, 123.1 (q, J = 275.7 Hz), 71.7, 70.8, 70.7, 70.6, 70.3, 66.3, 60.4 (q, J = 36.6 Hz), 34.7, 22.7, 21.1.

2. 3. 1. D. MEG-TFA¹⁸,⁵⁰

MEG-TFA 17 was synthesized in two steps from 2-allyloxy-ethanol 9 in 59% overall yield (Figure 50):

![Figure 50. Synthesis of MEG-TFA.](image)

2-allyloxy-ethyl trifluoroacetate 16. To a stirred solution of 2-allyloxy-ethanol 9 (4.36 mL, 40.0 mmol, 1.0 equiv.), triethylamine (11.2 mL, 80.0 mmol, 2.0 equiv.) and 4-dimethylamino-pyridine (4-DMAP: 0.49 g, 4.0 mmol, 0.1 equiv.) in dichloromethane (80 mL)
was added dropwise trifluoroacetic anhydride (TFAA: 6.74 mL, 48.0 mmol, 1.2 equiv.) at 0°C. After addition, the reaction was allowed to warm to room temperature then stirred overnight. The reaction was then submitted to a dichloromethane/saturated aqueous ammonium chloride extraction. The combined organic layers were dried over anhydrous magnesium sulfate, filtered, and then evaporated under reduced pressure. Purification by distillation under reduced pressure finally provided titled trifluoroacetate 16 as a colourless oil (5.76 g, 72% yield); bp = 72-74°C (tap water vacuum); ¹H NMR (300 MHz, CDCl₃) δ 5.88 (m, 1H), 5.29 (m, 1H), 5.21 (m, 1H), 4.52 (t, J = 4.8 Hz, 2H), 4.03 (m, 2H), 3.76 (t, J = 4.8 Hz, 2H); ¹³C NMR (75 MHz, CDCl₃) 157.7 (q, J = 42.1 Hz), 134.2, 117.8, 114.7 (q, J = 283.9 Hz), 72.4, 67.2, 67.0.

2-(3-trichlorosilylpropyloxy)-ethyl trifluoroacetate 17 (MEG-TFA). In a heavy-walled pressure tube equipped with a magnetic stir bar, trifluoroacetate 16 (3.97 g, 20.0 mmol, 1.0 equiv.) and chloroplatinic acid hexahydrate (104 mg, 0.20 mmol, 1.0 mol%) were loaded. The tube was transferred into a glovebox and trichlorosilane (4.10 mL, 40.2 mmol, 2.0 equiv.) was added to the solution. The tube was tightly fastened then removed from the glovebox. The resulting solution was stirred at room temperature for 20h behind a protecting shield. Purification by Kugelrohr distillation under vacuum finally provided MEG-TFA 17 as a colourless oil (5.46 g, 82% yield); bp = 115-120°C (0.09 Torr); ¹H NMR (400 MHz, CDCl₃ – Appendix D) δ 4.52 (m, 2H), 3.76 (m, 2H), 3.56 (t, J = 6.2 Hz, 2H), 1.85 (m, 2H), 1.48 (m, 2H); ¹³C NMR (100 MHz, CDCl₃) 157.7 (q, J = 42.3 Hz), 114.7 (q, J = 284.1 Hz), 71.8, 67.9, 67.0, 22.8, 21.0.

2. 3. 1. E. OTS-TFA

OTS-TFA 20 was synthesized in two steps from 5-hexenol 18 in 52% overall yield (Figure 51):

![Figure 51. Synthesis of OTS-TFA.](image)

5-hexenyl trifluoroacetate 19. To a stirred solution of 5-hexenol 18 (1.84 mL, 15.0 mmol, 1.0 equiv.), triethylamine (4.18 mL, 30.0 mmol, 2.0 equiv.) and 4-dimethylamino-pyridine (4-DMAP: 0.19 g, 1.5 mmol, 0.1 equiv.) in dichloromethane (40 mL) was added dropwise
trifluoroacetic anhydride (TFAA: 2.53 mL, 18.0 mmol, 1.2 equiv.) at 0ºC. After addition, the reaction was allowed to warm to room temperature then stirred overnight. The reaction was then submitted to a dichloromethane/saturated aqueous ammonium chloride extraction. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and then evaporated under reduced pressure (no heating). Purification by column chromatography (hexanes/dichloromethane: 100/0 to 90/10) finally provided titled trifluoroacetate 19 as a colourless volatile oil (1.99 g, 67% yield); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 5.79 (m, 1H), 5.04 (m, 1H), 5.00 (m, 1H), 4.37 (t, $J$ = 6.8 Hz, 2H), 2.11 (m, 2H), 1.77 (m, 2H), 1.51 (m, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$) 157.8 (q, $J$ = 41.9 Hz), 138.0, 115.4, 114.8 (q, $J$ = 284.4 Hz), 68.3, 33.2, 27.7, 24.9; IR (neat) 1785 cm$^{-1}$.

6-trichlorosilyl-hexanyl trifluoroacetate 20 (OTS-TFA). In a heavy-walled pressure tube equipped with a magnetic stir bar, trifluoroacetate 19 (0.98 g, 5.0 mmol, 1.0 equiv.) and chloroplatinic acid hexahydrate (26 mg, 0.05 mmol, 1.0 mol%) were loaded. The tube was transferred into a glovebox and trichlorosilane (1.02 mL, 10.0 mmol, 2.0 equiv.) was added to the solution. The tube was tightly fastened then removed from the glovebox. The resulting solution was stirred at room temperature for 20h behind a protecting shield. Purification by Kugelrohr distillation under vacuum finally provided OTS-TFA 20 as a colourless oil (1.29 g, 78% yield); bp = 130-140ºC (20 Torr); $^1$H NMR (400 MHz, CDCl$_3$ – Appendix E) $\delta$ 4.36 (t, $J$ = 6.6 Hz, 2H), 1.77 (m, 2H), 1.62 (m, 2H), 1.52-1.38 (m, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) 157.8 (q, $J$ = 42.0 Hz), 114.8 (q, $J$ = 284.1 Hz), 68.2, 31.4, 28.1, 25.3, 24.4, 22.4.

2.3.1. F. MEG-OMe$^{50}$

MEG-OMe 22 was synthesized in two steps from 2-allyloxy-ethanol 9 in 27% overall yield (Figure 52):

![Figure 52] Synthesis of MEG-OMe.

Ethylene glycol allyl methyl ether 21. To a stirred solution of 2-allyloxy-ethanol 9 (1.64 mL, 15.0 mmol, 1.0 equiv.) in diethyl ether (50 mL) was carefully added 60% sodium hydride
(0.78 g, 19.5 mmol, 1.3 equiv.) in small portions at 0°C. The reaction was stirred for 1h then methyl iodide (2.19 mL, 34.9 mmol, 2.3 equiv.) was added dropwise. The reaction was allowed to warm to room temperature then stirred overnight. The reaction was submitted to a diethyl ether/water extraction, after which the combined organic layers were dried over anhydrous sodium sulfate, filtered, and then evaporated under reduced pressure (no heating). Purification by column chromatography (pentane/diethyl ether: 100/0 to 70/30) finally provided titled ether 21 as a colourless volatile oil (1.04 g, 59% yield). Spectroscopic data were consistent with those reported in the literature: 429 \( ^1H \text{NMR} \) (400 MHz, CDCl\(_3\)) \( \delta \) 5.92 (m, 1H), 5.27 (m, 1H), 5.18 (m, 1H), 4.03 (m, 2H), 3.58 (m, 4H), 3.39 (s, 3H).

**Ethylene glycol 3-trichlorosilylpropyl methyl ether 22 (MEG-OMe).** In a heavy-walled pressure tube equipped with a magnetic stir bar, ether 21 (0.52 g, 4.5 mmol, 1.0 equiv.) and chloroplatinic acid hexahydrate (24 mg, 0.05 mmol, 1.0 mol%) were loaded. The tube was transferred into a glovebox and trichlorosilane (0.91 mL, 8.9 mmol, 2.0 equiv.) was added to the solution. The tube was tightly fastened then removed from the glovebox. The resulting solution was stirred at room temperature for 20h behind a protecting shield. Purification by Kugelrohr distillation under vacuum finally provided MEG-OMe 22 as a colourless oil (0.54 g, 47% yield); bp = 90-100°C (20 Torr); \( ^1H \text{NMR} \) (400 MHz, CDCl\(_3\)) – Appendix F) \( \delta \) 3.55 (m, 6H), 3.39 (s, 3H), 1.87 (m, 2H), 1.49 (m, 2H); \( ^{13}C \text{NMR} \) (100 MHz, CDCl\(_3\)) 72.1, 71.9, 70.3, 59.3, 22.8, 21.2.

2. 3. 1. G. HS-MEG-OH\(^{248}\)

**HS-MEG-OH 24** was synthesized in two steps from 2-(2-chloro-ethoxy)-ethanol 23 in 95% overall yield (Figure 53):

![Chemical structure of HS-MEG-OH](image)

**Figure 53.** Synthesis of HS-MEG-OH.

**2-(2-mercapto-ethoxy)-ethanol (HS-MEG-OH) 24.** To a stirred solution of 2-(2-chloro-ethoxy)-ethanol 23 (4.28 mL, 40.0 mmol, 1.0 equiv.) in acetonitrile (120 mL) were successively added thiourea (15.4 g, 200.0 mmol, 5.0 equiv.) and sodium iodide (6.0 g, 40.0 mmol, 1.0 equiv.) at room temperature. The reaction was then refluxed for three days, after which volatiles were
evaporated under reduced pressure. The residue was dissolved in a 1/2 (v/v) mixture of ethanol (50 mL) and water (100 mL) to which was added, cautiously portion-wise, powdered sodium hydroxide (40.0 g, 1.0 mol, 25 equiv.) at room temperature. The reaction was then refluxed for one day. The resulting solution was cautiously acidified (at 0°C) to pH ~ 2 by addition of concentrated (37%) hydrochloric acid, then repeatedly extracted with copious amounts of dichloromethane. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and then evaporated under reduced pressure. Distillation under vacuum finally provided HS-MEG-OH 24 as a colourless oil (4.64 g, 95% yield – 2 steps); bp ~150°C (~20 Torr). Spectroscopic data were consistent with those reported in the literature: \(^{430}\) H NMR (400 MHz, CDCl\(_3\) – Appendix G) \(\delta\) 3.75 (t, \(J = 4.6\) Hz, 2H), 3.64 (t, \(J = 6.2\) Hz, 2H), 3.59 (t, \(J = 4.6\) Hz, 2H), 2.72 (td, \(J = 8.2, 6.2\) Hz, 2H), 2.4-2.0 (brs, 1H), 1.57 (t, \(J = 8.2\) Hz, 1H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) 72.6, 72.0, 61.5, 24.2.

2. 3. 1. H. HS-OTS-OH

HS-OTS-OH 27 was synthesized in three steps from 5-bromo-pentanoic acid 25 in 15% overall yield (Figure 54):

![Synthesis of HS-OTS-OH](image-url)

**Figure 54.** Synthesis of HS-OTS-OH.

5-mercapto-pentanol (HS-OTS-OH) 27. To a stirred solution of 5-bromo-pentanoic acid 25 (2.80 g, 15.0 mmol, 1.0 equiv.) in anhydrous dimethylformamide (25 mL) was added potassium thioacetate (3.50 g, 30.0 mmol, 2.0 equiv.) at room temperature. The reaction was stirred overnight then submitted to a chloroform/10% aqueous hydrochloric acid extraction. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and then evaporated under reduced pressure to provide thioester 26. To a stirred solution of the latter in tetrahydrofuran (60 mL) was carefully added one portion of lithium aluminum hydride (LAH: 95%, 0.60 g, 15.0 mmol, 1.0 equiv.) at 0°C. After 30 min, another portion of LAH was carefully added and the reaction stirred for an additional 30 min. This operation was repeated once more, then the reaction finally allowed to warm to room temperature. After 2h of stirring, the reaction
was carefully quenched with a saturated aqueous solution of sodium sulfate. The resulting white aluminum salts were then filtered over a short plug of Celite, and the filtrate was evaporated under reduced pressure to provide HS-OTS-OH 27 along with its disulfide dimer. This mixture was dissolved in tetrahydrofuran (30 mL) then triethylamine (4.2 mL, 30.0 mmol, 2.0 equiv.) and dithiothreitol (DTT: 4.68 g, 30.0 mmol, 2.0 equiv.) were successively added at room temperature. The reaction was stirred overnight, after which volatiles were evaporated under reduced pressure. Purification by column chromatography (hexanes/ethyl acetate: 100/0 to 50/50) finally provided HS-OTS-OH 27 as an orange oil (0.28 g, 15% yield – 3 steps). Spectroscopic data were consistent with those reported in the literature:$^{431}$ $^1$H NMR (400 MHz, CDCl$_3$ – Appendix H) δ 3.66 (t, $J = 6.4$ Hz, 2H), 2.55 (td, $J = 7.6$, 7.2 Hz, 2H), 1.70-1.53 (m, 4H), 1.53-1.43 (m, 2H), 1.36 (brs, 1H), 1.35 (t, $J = 7.6$ Hz, 1H).
2. 3. 2. Probes

2. 3. 2. A. Biotinthiol

Biotinthiol 33 was synthesized in five steps from biotin 28 in 33% overall yield (Figure 55):

Figure 55. Synthesis of biotinthiol.

**Biotin ethyl ester 29.** To a stirred solution of biotin 28 (0.90 g, 3.7 mmol, 1.0 equiv.) in absolute ethanol (30 mL) were added few drops of concentrated sulfuric acid at room temperature. After stirring overnight, the reaction was submitted to a dichloromethane/saturated aqueous sodium carbonate extraction. The combined organic layers were then evaporated under reduced pressure to provide biotin ethyl ester 29 as a white solid (0.96 g, 97% yield). Spectroscopic data were consistent with those reported in the literature: \(^{432}\) \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 5.55 (brs, 1H), 5.17 (brs, 1H), 4.54 (m, 1H), 4.34 (m, 1H), 4.15 (q, \(J = 7.2\) Hz, 2H), 3.18 (m, 1H), 2.93 (dd, \(J = 12.8, 4.8\) Hz, 1H), 2.75 (d, \(J = 12.8\) Hz, 1H), 2.36 (t, \(J = 7.6\) Hz, 2H), 1.69 (m, 4H), 1.45 (m, 2H), 1.24 (t, \(J = 7.2\) Hz, 3H).

**Biotinol 30.** To a stirred solution of biotin ethyl ester 29 (0.96 g, 3.5 mmol, 1.0 equiv.) in dichloromethane (10 mL) was added dropwise diisobutylaluminum hydride (DIBAL-H: 1.0 M in hexanes, 12.4 mL, 12.4 mmol, 3.5 equiv.) at -78°C. After addition, the reaction was allowed to warm to room temperature then stirred for 2h. The reaction was then carefully quenched, at
-78°C, by dropwise addition of methanol then water. After evaporation of the solvents under reduced pressure, purification by overnight Soxhlett extraction (absolute ethanol) finally provided biotinol 30 as a white solid (0.80 g, 98% yield). Spectroscopic data were consistent with those reported in the literature:\textsuperscript{433} \textsuperscript{1}H NMR (400 MHz, CD\textsubscript{3}OD) δ 4.49 (dd, J = 7.8, 4.8 Hz, 1H), 4.30 (dd, J = 7.8, 4.8 Hz, 1H), 3.55 (t, J = 6.6 Hz, 2H), 3.21 (m, 1H), 2.93 (dd, J = 12.6, 4.8 Hz, 1H), 2.71 (d, J = 12.6 Hz, 1H), 2.16 (s, 1H), 1.74 (m, 1H), 1.57 (m, 3H), 1.45 (m, 4H).

**Biotin tosylate 31 and biotin thioacetate 32.** To a stirred solution of biotinol 30 (0.80 g, 3.5 mmol, 1.0 equiv.) in pyridine (20 mL) was added tosyl chloride (1.75 g, 9.1 mmol, 2.6 equiv.) at 0°C. After addition, the reaction was allowed to warm to room temperature then stirred for 2h. The reaction was then submitted to a dichloromethane/1 M aqueous sulfuric acid extraction. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and then evaporated under reduced pressure. The residue was rapidly purified by column chromatography (ethyl acetate/methanol: 100/0 to 75/25) to provide 0.70 g of an off-white solid composed of a mixture of biotin tosylate 31 and biotin chloride byproduct [\textsuperscript{1}H NMR (tosylate – 400 MHz, CDCl\textsubscript{3})\textsuperscript{434} δ 7.79 (d, J = 8.2 Hz, 2H), 7.35 (d, J = 8.2 Hz, 2H), 5.91 (brs, 1H), 5.24 (brs, 1H), 4.51 (m, 1H), 4.30 (m, 1H), 4.03 (t, J = 6.4 Hz, 2H), 3.12 (m, 1H), 2.91 (dd, J = 12.8, 5.2 Hz, 1H), 2.74 (d, J = 12.8 Hz, 1H), 2.46 (s, 3H), 1.75-1.54 (m, 4H), 1.46-1.28 (m, 4H); \textsuperscript{1}H NMR (chloride – 400 MHz, CDCl\textsubscript{3})\textsuperscript{435} δ 5.60 (brs, 1H), 5.25 (brs, 1H), 4.52 (m, 1H), 4.31 (m, 1H), 3.54 (t, J = 6.6 Hz, 2H), 3.17 (m, 1H), 2.93 (dd, J = 12.8, 5.2 Hz, 1H), 2.76 (d, J = 12.8 Hz, 1H), 1.84-1.54 (m, 4H), 1.54-1.40 (m, 4H)]. The latter was dissolved in acetonitrile (30 mL) then anhydrous sodium iodide (2.65 g, 17.7 mmol) and potassium thioacetate (2.06 g, 17.7 mmol) were successively added at room temperature. The reaction was refluxed overnight then submitted to a dichloromethane/water extraction. The combined organic layers were washed with brine, dried over anhydrous sodium sulfate, filtered, and then evaporated under reduced pressure. Purification by column chromatography (dichloromethane/methanol: 99/1 to 95/5) finally provided biotin thioacetate 32 as a beige solid (0.42 g, 42% yield – 2 steps). Spectroscopic data were consistent with those reported in the literature:\textsuperscript{436} \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) δ 5.22 (brs, 1H), 4.86 (brs, 1H), 4.55 (m, 1H), 4.34 (m, 1H), 3.17 (m, 1H), 2.94 (dd, J = 12.8, 5.2 Hz, 1H), 2.87 (t, J = 7.4 Hz, 2H), 2.76 (d, J = 12.8 Hz, 1H), 2.36 (s, 3H), 1.74-1.57 (m, 4H), 1.50-1.37 (m, 4H).
**Biotinthiol 33.** To a stirred solution of biotin thioacetate 32 (0.41 g, 1.4 mmol, 1.0 equiv.) in tetrahydrofuran (40 mL) was added lithium aluminum hydride (LAH: 95%, 0.45 g, 11.4 mmol, 8.0 equiv.) in small portions at 0°C. After addition, the reaction was allowed to warm to room temperature then stirred for 1 h. The reaction was carefully quenched with a 1 M aqueous solution of hydrochloric acid, and then the resulting aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous sodium sulfate, filtered, and then evaporated under reduced pressure. Purification by column chromatography (ethyl acetate/methanol: 100/0 to 80/20) finally provided biotinthiol 33 as a white solid (0.29 g, 83% yield). Spectroscopic data were consistent with those reported in the literature: \(^{436}\) \(^{1}\)H NMR (300 MHz, CDCl\(_3\) – Appendix I) \(\delta\) 5.00 (brs, 1H), 4.84 (brs, 1H), 4.55 (m, 1H), 4.35 (m, 1H), 3.20 (m, 1H), 2.95 (dd, \(J = 12.8, 5.2\) Hz, 1H), 2.76 (d, \(J = 12.8\) Hz, 1H), 2.56 (q, \(J = 7.3\) Hz, 2H), 1.76-1.59 (m, 4H), 1.53-1.40 (m, 4H), 1.37 (t, \(J = 7.3\) Hz, 1H).

2.3.2. B. Biotinamine\(^{416}\)

Biotinamine 36 was synthesized in eight steps from biotin 28 in 23% overall yield (Figure 56):

![Synthesis of Biotinamine](image)

**Figure 56.** Synthesis of biotinamine.
**Biotin ethyl ester 29.** To a stirred solution of biotin 28 (0.90 g, 3.7 mmol, 1.0 equiv.) in absolute ethanol (30 mL) were added few drops of concentrated sulfuric acid at room temperature. After stirring overnight, the reaction was submitted to a dichloromethane/saturated aqueous sodium carbonate extraction. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and then evaporated under reduced pressure to provide biotin ethyl ester 29 as a white solid (0.96 g, 97% yield). Spectroscopic data were consistent with those reported in the literature:

\[ ^1H \text{NMR} \ (400 \text{ MHz, CDCl}_3) \delta 5.55 \ (\text{brs, 1H}), 5.17 \ (\text{brs, 1H}), 4.54 \ (\text{m, 1H}), 4.34 \ (\text{m, 1H}), 4.15 \ (\text{q, } J = 7.2 \text{ Hz, 2H}), 3.18 \ (\text{m, 1H}), 2.93 \ (\text{dd, } J = 12.8, 4.8 \text{ Hz, 1H}), 2.75 \ (\text{d, } J = 12.8 \text{ Hz, 1H}), 2.36 \ (\text{t, } J = 7.6 \text{ Hz, 2H}), 1.69 \ (\text{m, 4H}), 1.45 \ (\text{m, 2H}), 1.24 \ (\text{t, } J = 7.2 \text{ Hz, 3H}). \]

**Biotinol 30.** To a stirred solution of biotin ethyl ester 29 (0.96 g, 3.5 mmol, 1.0 equiv.) in dichloromethane (10 mL) was added dropwise diisobutylaluminum hydride (DIBAL-H: 1.0 M in hexanes, 12.4 mL, 12.4 mmol, 3.5 equiv.) at -78°C. After addition, the reaction was allowed to warm to room temperature then stirred for 2h. The reaction was then carefully quenched, at -78°C, by dropwise addition of methanol then water. After evaporation of the solvents under reduced pressure, purification by overnight Soxhlett extraction (absolute ethanol) finally provided biotinol 30 as a white solid (0.80 g, 98% yield). Spectroscopic data were consistent with those reported in the literature:

\[ ^1H \text{NMR} \ (400 \text{ MHz, CD}_3\text{OD}) \delta 4.49 \ (\text{dd, } J = 7.8, 4.8 \text{ Hz, 1H}), 4.30 \ (\text{dd, } J = 7.8, 4.8 \text{ Hz, 1H}), 3.55 \ (\text{t, } J = 6.6 \text{ Hz, 2H}), 3.21 \ (\text{m, 1H}), 2.93 \ (\text{dd, } J = 12.6, 4.8 \text{ Hz, 1H}), 2.71 \ (\text{d, } J = 12.6 \text{ Hz, 1H}), 2.16 \ (\text{s, 1H}), 1.74 \ (\text{m, 1H}), 1.57 \ (\text{m, 3H}), 1.45 \ (\text{m, 4H}). \]

**Biotin tosylate 31 and biotin iodide 34.** To a stirred solution of biotinol 30 (0.69 g, 3.0 mmol, 1.0 equiv.) in pyridine (20 mL) was added tosyl chloride (2.88 g, 15.0 mmol, 5.0 equiv.) at 0°C. After addition, the reaction was allowed to warm to room temperature then stirred for 2h. The reaction was then submitted to a dichloromethane/1 M aqueous sulfuric acid extraction. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and then evaporated under reduced pressure. The residue was purified by column chromatography (dichloromethane/methanol: 100/0 to 90/10) to provide 0.83 g of a bright orange solid composed of a ~9:1 mixture of biotin tosylate 31 and biotin chloride byproduct \[ ^1H \text{NMR} \ (\text{tosylate – 400 MHz, CDCl}_3) \delta 7.79 \ (\text{d, } J = 8.2 \text{ Hz, 2H}), 7.35 \ (\text{d, } J = 8.2 \text{ Hz, 2H}), 5.91 \ (\text{brs, 1H}), 5.24 \ (\text{brs, 1H}), 4.51 \ (\text{m, 1H}), 4.30 \ (\text{m, 1H}), 4.03 \ (\text{t, } J = 6.4 \text{ Hz, 2H}), 3.12 \ (\text{m, 1H}), 2.91 \ (\text{dd, } J = 12.8, 5.2 \text{ Hz, 1H}), 2.74 \ (\text{d, } J = 12.8 \text{ Hz, 1H}), 2.46 \ (\text{s, 3H}), 1.75-1.54 \ (\text{m, 4H}), 1.46-1.28 \ (\text{m, 4H}); \]

\[ ^1H \text{NMR} \ (\text{chloride – 400 MHz, CDCl}_3) \delta 5.60 \ (\text{brs, 1H}), 5.25 \ (\text{brs, 1H}), 4.52 \ (\text{m, 1H}), 4.31 \ (\text{m, 1H}), 3.71 \ (\text{m, 1H}), 2.92 \ (\text{d, } J = 12.8 \text{ Hz, 1H}), 2.81 \ (\text{t, } J = 7.2 \text{ Hz, 2H}), 2.15 \ (\text{s, 1H}), 1.80 \ (\text{m, 1H}), 1.65 \ (\text{m, 1H}), 1.54 \ (\text{m, 1H}), 1.34 \ (\text{m, 1H}), 1.27 \ (\text{m, 1H}). \]
3.54 (t, $J = 6.6$ Hz, 2H), 3.17 (m, 1H), 2.93 (dd, $J = 12.8$, 5.2 Hz, 1H), 2.76 (d, $J = 12.8$ Hz, 1H), 1.84-1.54 (m, 4H), 1.54-1.40 (m, 4H)]. The latter was dissolved in acetone (20 mL) then anhydrous sodium iodide (3.24 g, 21.6 mmol, 10.0 equiv.) was added. The reaction was then stirred at room temperature overnight, sheltered from light. After evaporation of acetone, the residue was submitted to a dichloromethane/aqueous sodium thiosulfate extraction. The combined organic layers were washed with brine, dried over anhydrous sodium sulfate, filtered, and then evaporated under reduced pressure to provide biotin iodide 34 as a pale yellow solid (0.71 g, 69% yield – 2 steps). Spectroscopic data were consistent with those reported in the literature: $^{432}$ $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 4.77 (brs, 1H), 4.66 (brs, 1H), 4.54 (m, 1H), 4.33 (m, 1H), 3.20 (t, $J = 6.9$ Hz, 2H), 3.19 (m, 1H), 2.95 (dd, $J = 12.6$, 5.1 Hz, 1H), 2.75 (d, $J = 12.6$ Hz, 1H), 1.83 (m, 2H), 1.70 (m, 2H), 1.46 (m, 4H).

**Biotin azide 35.** To a stirred solution of biotin iodide 34 (0.71 g, 2.1 mmol, 1.0 equiv.) in dichloromethane (25 mL) were successively added triethylamine (1.18 mL, 8.4 mmol, 4.0 equiv.), di-tert-butyl dicarbonate (Boc$_2$O: 1.84 g, 8.4 mmol, 4.0 equiv.) and 4-dimethylamino-pyridine (4-DMAP: 26 mg, 0.2 mmol, 0.1 equiv.). The reaction was stirred at room temperature overnight, after which volatiles were evaporated under reduced pressure. The residue was rapidly purified by column chromatography (hexanes/ethyl acetate: 100/0 to 80/20) to provide 0.88 g of a pale yellow solid. The latter (0.27 g) was dissolved in anhydrous dimethylformamide (4 mL) then sodium azide (0.13 g, 2.0 mmol, 4.0 equiv.) was added. The reaction was stirred at 100°C for 2h then submitted to an ethyl acetate/brine extraction. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and then evaporated under reduced pressure to provide 0.26 g of a pale yellow solid. The latter was dissolved in dichloromethane (4 mL) then trifluoroacetic acid (TFA: 1 mL) was added. The reaction was stirred at room temperature for 1h, after which volatiles were evaporated under reduced pressure. Purification by column chromatography (ethyl acetate/methanol: 100/0 to 90/10) finally provided biotin azide 35 as an off-white solid (0.11 g, 66% yield – 3 steps). Spectroscopic data were consistent with those reported in the literature: $^{433,437}$ $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 5.47 (brs, 1H), 5.29 (brs, 1H), 4.52 (m, 1H), 4.33 (m, 1H), 3.29 (t, $J = 6.8$ Hz, 2H), 3.17 (m, 1H), 2.94 (dd, $J = 12.8$, 5.0 Hz, 1H), 2.75 (d, $J = 12.8$ Hz, 1H), 1.84-1.54 (m, 4H), 1.54-1.34 (m, 4H).
**Biotinamine 36.** To a stirred solution of biotin azide 35 (0.11 g, 0.4 mmol, 1.0 equiv.) in tetrahydrofuran (2 mL) were added triphenylphosphine (0.23 g, 0.8 mmol, 2.0 equiv.) and water (0.2 mL). The reaction was stirred at room temperature overnight, after which volatiles were evaporated under reduced pressure. Purification by preparative thin layer chromatography (ethyl acetate/methanol: 80/20) finally provided biotinamine 36 as an off-white solid (54 mg, 54% yield). Spectroscopic data were consistent with those reported in the literature: \(^{438}\) \(^1\)H NMR (300 MHz, CD\(_3\)OD – Appendix J) \(\delta\) 4.49 (m, 1H), 4.30 (m, 1H), 3.21 (m, 1H), 2.93 (dd, \(J = 12.6, 4.8\) Hz, 1H), 2.71 (d, \(J = 12.6\) Hz, 1H), 2.63 (brt, 2H), 1.82-1.26 (m, 8H).
2. 4. Surface preparation

2. 4. 1. Substrate cleaning and activation

2. 4. 1. A. Quartz discs

Quartz discs were first sonicated in 20 mL of concentrated dishwashing soap for 30 min. The discs were then copiously rinsed with hot tap water, followed by distilled water, before being dried under a gentle stream of nitrogen. Next, the discs were individually soaked in 6 mL of Piranha solution (3:1 v/v mixture of 98% H₂SO₄ and 30% H₂O₂) pre-heated to 90°C using a water bath \([\text{Caution: Piranha solutions are corrosive. Handle with extreme care}]\). After 30 min, the discs were thoroughly rinsed with distilled water (×3) followed by methanol (×3). The discs were next sonicated in another portion of methanol for 2 min, followed by a rinse with methanol and then individually transferred into glass vials, which were subsequently placed in an oven maintained at 150°C for drying. After 2h, the vials were immediately transferred into a humidity chamber (70-80% relative humidity, room temperature) for overnight surface moisturization.

2. 4. 1. B. Si/SiO₂ wafers

Silicon wafers were first heated in a furnace at 850°C then allowed to cool to room temperature (30-45 min) to prepare oxide films approximately 160-180 Å-thick. Next, the Si/SiO₂ wafers were copiously rinsed with deionized water then dried under a gentle stream of nitrogen. The Si/SiO₂ wafers were rinsed with toluene (×3), followed by chloroform (×3), and then thoroughly dried with nitrogen. Lastly, the Si/SiO₂ wafers were rinsed with methanol, dried with nitrogen, and then transferred into a humidity chamber (70-80% relative humidity, room temperature) for overnight surface moisturization.

2. 4. 1. C. Gold TSM discs

Gold TSM discs were first soaked in a 1% aqueous solution of sodium dodecyl sulfate, gently wiped with a clean cotton swab while in solution, and then placed on a spinning plate for 20 min. Next, the TSM discs were copiously rinsed with hot tap water, followed by distilled water, before being soaked in acetone on a spinning plate for 10 min. This latter step was repeated with absolute ethanol then methanol, both steps including cotton swabbing. The TSM discs were dried under a gentle stream of nitrogen then plasma-cleaned (N₂) for 20 min. To minimize surface contamination of gold by air pollutants, cleaned TSM discs were immediately soaked in freshly prepared solutions of surface modifier (see Section 2. 4. 2. A. 3.).
2.4.1. D. Bisphenol A polycarbonate (BPA-PC) slides and sheets

BPA-PC slides/sheets were first copiously rinsed with warm tap water followed by distilled water, and then washed under gentle spinning in an aqueous solution of sodium dodecyl sulfate (1%). After 5 min, BPA-PC substrates were thoroughly rinsed with this surfactant solution followed by tap water, distilled water then ethanol. After drying under a gentle stream of nitrogen, BPA-PC substrates were air-plasma cleaned/activated for 20 min then immediately transferred into a humidity chamber (70-80% relative humidity, room temperature) for overnight surface moisturization.

2.4.2. Adlayer preparation

*Note:* for procedures involving trichlorosilane surface modifiers (TTTA, PFP-TTTA, OEG-TTTA, OTS, OTS-TFA, MEG-OMe, MEG-TFA), silanizing solutions and silanization reactions were prepared in a glovebox maintained under inert (N$_2$) and anhydrous (P$_2$O$_5$) atmosphere. To prevent the undesired reaction of these trichlorosilane molecules with the silanization glassware, the latter must be pre-treated overnight with a 1/20 (v/v) solution of octadecyltrichlorosilane in anhydrous toluene.

2.4.2. A. Unimolecular adlayers

2.4.2. A. 1. On quartz discs

OTS, OTS-TFA, MEG-TFA or MEG-OMe (10 µL) was first diluted with anhydrous toluene (10 mL). The resulting solutions were portioned (1000 µL) in test tubes into which cleaned quartz discs were then individually soaked. The test tubes were sealed with rubber stoppers, removed from the glovebox, and then placed on a spinning plate for 60 min at room temperature. Next, the discs were thoroughly rinsed with toluene (×3) then sonicated in another portion of this solvent for 5 min. After a final rinse with toluene, the previous rinsing procedure was repeated with chloroform. Finally, the discs were rinsed with chloroform (×2) then dried under a gentle stream of nitrogen.

To generate the OTS-OH and MEG-OH adlayers, freshly prepared OTS-TFA- and MEG-TFA-coated quartz discs were individually soaked in 3 mL of a 1/1 (v/v) solution of methanol and Milli-Q water. After overnight exposure at room temperature on a spinning plate, the discs were finally rinsed with methanol (×3) then dried under a gentle stream of nitrogen. Unless otherwise specified (*Section 3.2.1. C. 3.*), other unimolecular adlayers (*i.e.* OTS and MEG-OMe) were also soaked overnight in 3 mL of a 1/1 (v/v) solution of methanol and Milli-Q water.
2.4.2. A. 2. On Si/SiO₂ wafers

MEG-TFA or OTS-TFA (75 µL) was first diluted with anhydrous toluene (75 mL). This solution was poured onto a Si/SiO₂ wafer placed in a Petri dish (d = 12.5 cm, h = 6.5 cm), which was then covered with a watch glass. After sealing around its circumference with Parafilm, the Petri dish was removed from the glovebox and placed on a spinning plate, for 60 min at room temperature. Next, the wafers were thoroughly rinsed with toluene (×3) then sonicated in another portion of this solvent for 5 min. After a final rinse with toluene, the previous rinsing procedure was repeated with chloroform. The wafers were dried with nitrogen then individually soaked in 75 mL of a 1/1 (v/v) solution of methanol and deionized water. After overnight exposure at room temperature on a spinning plate, the wafers were finally rinsed with methanol (×3) then dried under a gentle stream of nitrogen.

2.4.2. A. 3. On gold TSM discs

Note: in order to use freshly cleaned gold surfaces (to minimize airborne contamination), solutions of HS-MEG-OH thiol surface modifier should be prepared while TSM discs are being cleaned (Section 2.4.1.C.).

HS-MEG-OH (6.1 mg, 0.05 mmol) was first diluted with anhydrous ethanol (10 mL). The resulting 5 mM solution was portioned (1000 µL) in test tubes into which freshly cleaned TSM discs were then individually soaked. The test tubes were sealed with rubber stoppers then placed on a spinning plate for increasing periods of time (5, 15, 20, 30, 40, 50, 60 and 1080 min) at room temperature. Finally, TSM discs were thoroughly rinsed with anhydrous ethanol (×3) then dried under a gentle stream of nitrogen.

For the surface hydration experiments (performed with HS-MEG-OH adlayers prepared in anhydrous ethanol for 30 min), TSM discs were individually soaked in deionized water (2 mL), immediately following adlayer formation, overnight at room temperature on a spinning plate. Finally, TSM discs were immediately transferred, wet, into the TSM cell holder (the bottom face of the TSM disc being, however, carefully dried under a gentle stream of nitrogen).
2.4.2. A. 4. On bisphenol A polycarbonate (BPA-PC) slides and sheets

MEG-TFA (10 μL) was first diluted with hexanes (10 mL). The resulting solution was portioned (1000 μL) in vials into which cleaned/activated BPA-PC slides were then individually soaked. The vials were capped, removed from the glovebox, and then placed on a spinning plate for 60 min at room temperature. Next, the slides were thoroughly rinsed with hexanes (×3) then sonicated in another portion of this solvent for 5 min. This rinsing procedure was repeated with 95% ethanol. The slides were dried under a gentle stream of nitrogen then individually soaked in 1 mL of a 1/1 (v/v) solution of 95% ethanol and Milli-Q water. After overnight treatment at room temperature on a spinning plate, the slides were finally rinsed with 95% ethanol (×3) then dried under a gentle stream of nitrogen.

For MEG-TFA adlayer formation on BPA-PC sheets, the silanizing solution per sample was 20 μL of MEG-TFA surface modifier diluted with 20 mL of hexanes. The volume of the 95% ethanol/water solution used for the subsequent conversion of MEG-TFA to MEG-OH adlayer was 6 mL per sample.
2. 4. 2. B. Mixed adlayers (on quartz discs)

Linkers (TTTA, PFP-TTTA or OEG-TTTA) and diluents (OTS or MEG-TFA) were first separately diluted with anhydrous toluene, in proportions indicated in Table 5. Appropriate volumes of these linker/diluent stock solutions (see Table 5) were then mixed in separate test tubes (i.e. portioned) into which cleaned quartz discs were then individually soaked. The test tubes were sealed with rubber stoppers, removed from the glovebox, and then placed on a spinning plate for 120 min at room temperature. Next, the discs were thoroughly rinsed with toluene (×2) then sonicated in another portion of this solvent for 5 min. After a final rinse with toluene, the previous rinsing procedure was repeated with chloroform. Finally, the discs were rinsed with chloroform (×2), gently dried with nitrogen, and then either: (i) transferred into glass vials for storage (awaiting XPS or EMPAS analysis); or (ii) immediately engaged in the subsequent procedure of probe immobilization (Section 2. 4. 3.).

### Table 5. Volumes of neat linker (TTTA, PFP-TTTA or OEG-TTTA), neat diluent (OTS or MEG-TFA) and anhydrous toluene used to prepare stock solutions from which samples are mixed in various proportions (L:D ratio) for the formation of TTTA/OTS, PFP-TTTA/OTS and OEG-TTTA/MEG-TFA mixed adlayers on quartz discs for EMPAS experiments.

<table>
<thead>
<tr>
<th>EMPAS study</th>
<th>Surface modifying system (Linker : Diluent)</th>
<th>L:D ratio (v/v)</th>
<th>Stock solutions</th>
<th>Silanizing solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avidin (in PBS buffer)</td>
<td>TTTA : OTS</td>
<td>1:1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>PFP-TTTA : OTS</td>
<td>1:1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>OEG-TTTA : MEG-TFA</td>
<td>1:1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Avidin (in serum)</td>
<td>OEG-TTTA : MEG-TFA</td>
<td>1:1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>OEG-TTTA : MEG-TFA</td>
<td>1:10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>OEG-TTTA : MEG-TFA</td>
<td>1:50</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>OEG-TTTA : MEG-TFA</td>
<td>1:100</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>LPS (in plasma)</td>
<td>OEG-TTTA : MEG-TFA</td>
<td>1:1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>OEG-TTTA : MEG-TFA</td>
<td>1:10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>OEG-TTTA : MEG-TFA</td>
<td>1:50</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>
2. 4. 3. Probe immobilization

2. 4. 3. A. Biotinthiol and biotinamine

Probe solutions (1.0 mg/mL) were prepared by dissolving biotinthiol or biotinamine into freshly distilled, anhydrous DMF. These solutions were portioned (950 μL) in dry test tubes into which freshly prepared TTTA/OTS, PFP-TTTA/OTS, or OEG-TTTA/MEG-TFA mixed adlayer-coated quartz discs were then individually soaked. Next, the test tubes were sealed with rubber stoppers, removed from the glovebox, and then placed on a spinning plate overnight at room temperature. Finally, the discs were thoroughly rinsed with spectrograde methanol (×3), dried under a gentle stream of nitrogen, and then stored in glass vials awaiting XPS or EMPAS analysis.

2. 4. 3. B. Polymyxin B (PMB)

Note: quartz discs must imperatively be handled in sterilized glassware (baked overnight in an oven maintained at 200°C). To avoid re-contamination upon cooling to room temperature, glassware is wrapped in aluminum foil. Other necessary laboratory equipment must also be sterile/sterilized. Endotoxin-free PBS buffer is obtained upon filtration through a 0.22 μm syringe driven filter unit.

Probe solution (5.6 mg/mL) was prepared by dissolving PMB powder into endotoxin-free PBS. Sonication was used to aid dissolution. The resulting solution was portioned (950 μL) in test tubes into which freshly prepared OEG-TTTA/MEG-TFA mixed adlayer-coated quartz discs were then individually soaked. Next, the test tubes were sealed with rubber stoppers, and then placed on a spinning plate overnight at room temperature. Finally, the discs were thoroughly rinsed with sterile water (×4), gently dried with nitrogen, and then individually stored in sterilized glass vials awaiting XPS or EMPAS analysis.
2. 5. Surface characterization and analysis

2. 5. 1. Contact angle goniometry

Static contact angles were measured with a CAM101 goniometer (KSV Instruments Ltd.) and Milli-Q water as the test liquid at the Department of Chemistry – University of Toronto (Toronto, Ontario – Canada). Once the sessile droplets were gently deposited onto the surfaces, 5 frames were recorded at 1s intervals. The (averaged) contact angle values were generated using the CAM101 program provided with the instrument.

2. 5. 2. Angle-resolved X-ray photoelectron spectroscopy

Angle-resolved XPS was performed with a Theta Probe Instrument (ThermoFisher Scientific) located at Surface Interface Ontario (Toronto, Ontario – Canada). Samples were analyzed with monochromated Al Kα X-rays focused to elliptical spots with a dimension of ~400 μm along the long axis. Takeoff angles of analysis can be found in the various Tables/Figures. The binding energy scale was calibrated to the main C 1s signal at 285 eV. Data processing and analysis of characteristic elements was performed using Avantage software.

2. 5. 3. Electromagnetic piezoelectric acoustic sensor

2. 5. 3. A. Unimolecular adlayers

EMPAS measurements were performed at 0.94 GHz, with a typical noise < 500 Hz. After the standard set-up of the EMPAS device, the adlayer-coated (OTS, MEG-TFA, MEG-OH, OTS-OH or MEG-OMe) or bare quartz discs were individually inserted into the flow-through Plexiglass cell holder, securely fastened atop a 30 μm-thick Teflon O-ring. PBS buffer was then flowed over the disc surface at a rate of 50 μL/min, ensuring the chamber is completely filled without air bubbles. Once the resonant frequency signal stabilized, 50 μL of undiluted, full goat serum were introduced into the flow-through system using an injection loop equipped with a low-pressure chromatography valve. Once the serum sample completely passed over the surface, the uninterrupted PBS buffer flow rinsed the latter of any loosely bound material. When the resonant frequency signal stabilized again, the experiment was stopped and the frequency shift calculated.
2. 5. 3. B. Mixed adlayers

2. 5. 3. B. 1. Avidin analyte

*Avidin samples:* stock solutions of avidin (1.0 mg/mL ~ 15 μM) were freshly prepared by dissolving lyophilized avidin (1.0 mg) into PBS buffer (1 mL). When not in use, these solutions were sealed and kept in a fridge. Fresh avidin samples for EMPAS measurements (0.1 mg/mL ~ 1.5 μM) were finally prepared by diluting 10 μL of stock solution (allowed to warm to room temperature) with 90 μL of PBS, or goat serum. For the development of the qualitative cut-off assay, 150 nM, 15 nM, 1.5 nM, 150 pM and 1.5 pM solutions of avidin in serum were also prepared through serial dilution.

*Sacrificial BSA:* for EMPAS experiments involving a preliminary injection of sacrificial BSA (PFP-TTTA/OTS system), stock solutions of BSA (1.0 mg/mL) were freshly prepared by dissolving lyophilized BSA into PBS buffer. When not in use, these solutions were sealed and kept in a fridge. Fresh BSA solutions for injection (0.1 mg/mL) were finally prepared by diluting 10 μL of stock solution (allowed to warm to room temperature) with 90 μL of PBS.

EMPAS measurements were performed at 0.74, 0.82, 0.90, 0.94 or 1.06 GHz (see Sections 3. 1. & 3. 2. 1. for actual operating frequency), with a typical noise < 500 Hz. After the standard set-up of the EMPAS device, biotinylated (or non-biotinylated) mixed adlayer-coated (TTTA/OTS, PFP-TTTA/OTS or OEG-TTTA/MEG-TFA) quartz discs were individually inserted into the flow-through Plexiglass cell holder, securely fastened atop a 30 μm-thick Teflon O-ring. PBS buffer was then flowed over the disc surface at a rate of 50 μL/min, ensuring the chamber is completely filled without air bubbles. Once the resonant frequency signal stabilized, 50 μL of avidin-spiked PBS buffer or serum solution (at a chosen concentration) were introduced into the flow-through system using an injection loop equipped with a low-pressure chromatography valve. Once the avidin sample completely passed over the surface, the uninterrupted PBS buffer flow rinsed off any loosely bound material. When the resonant frequency signal stabilized again, the experiment was stopped and the frequency shift calculated.

For EMPAS experiments, wherein sacrificial BSA is introduced prior to avidin (PFP-TTTA/OTS system), the injection/frequency stabilization protocol followed for BSA was identical to that aforementioned for avidin.
2.5.3. B. 2. Lipopolysaccharide (LPS) analyte

*Note:* sterile laboratory equipment must be used. Endotoxin-free PBS buffer is obtained upon filtration through a 0.22 μm syringe driven filter unit. Prior to running EMPAS experiments, the injection system (including tubing) must be sterilized by flowing a 0.1 M NaOH aqueous solution \(^{426}\) for 15 min then rinsed with sterile water for 15 min as well, both at a rate of 800 μL/min.

**LPS samples:** stock solutions of LPS (10\(^4\) pg/mL) were freshly prepared by first dissolving lyophilized LPS (1.0 mg) into endotoxin-free PBS buffer (10 mL). The resulting 10\(^8\) pg/mL LPS solution was brought to 10\(^4\) pg/mL through 1/100 serial dilution (×2) with endotoxin-free PBS buffer (10 μL of LPS solution into 990 μL of buffer). When not in use, these 10\(^4\) pg/mL stock solutions were sealed and kept in a fridge. Fresh LPS samples for EMPAS measurements (10\(^3\) pg/mL) were finally prepared by diluting 10 μL of stock solution (allowed to warm to room temperature) with 90 μL of human blood plasma.\(^{ii}\)

EMPAS measurements were performed at 0.94 GHz, with a typical noise < 500 Hz. After the standard set-up of the EMPAS device,\(^{407,422}\) PMB-functionalized OEG-TTTA/MEG-TFA mixed adlayer-coated or bare quartz discs were individually inserted into the flow-through Plexiglass cell holder, securely fastened atop a 30 μm-thick Teflon O-ring. Endotoxin-free PBS buffer was then flowed over the disc surface at a rate of 50 μL/min, ensuring the chamber is completely filled without air bubbles. Once the resonant frequency signal stabilized, 50 μL of LPS-spiked (10\(^3\) pg/mL) or unspiked blood plasma were introduced into the flow-through system using an injection loop equipped with a low-pressure chromatography valve. Once the blood plasma sample completely passed over the surface, the uninterrupted endotoxin-free PBS buffer flow rinsed the latter of any loosely bound material. When the resonant frequency signal stabilized again, the experiment was stopped and the frequency shift calculated.

2.5.4. **Thickness shear mode sensor**\(^{248}\) [supervised Ms. Ceren Avci]

TSM measurements were performed at 9.0 MHz, with a typical noise of ~5 Hz for the resonant frequency and ~0.2 Ω for motional resistance. After the standard set-up of the TSM device,\(^{439}\) which included a systematic calibration of the network analyzer before each run, TSM discs were individually inserted and securely fastened into the flow-through chamber. Once the

\(^{ii}\) Donor blood plasma was ‘endotoxin-free’ (EA = 0.26), as assessed using the endotoxin activity assay (EAA).\(^{424,425}\)
resonant frequency signal stabilized in air, PBS buffer was flowed over the disc surface using a syringe-pump, at an initial rate of 300 µL/min that was decreased to 100 µL/min once the chamber was filled and cleared of air bubbles. The resonant frequency was left to stabilize, and then a 10% solution of goat serum in PBS was injected at the same 100 µL/min rate for exactly 1 min before final re-introduction of the PBS buffer-only flow. Once the resonant frequency and motional resistance signals stabilized again, the experiment was stopped and the shifts in frequency and motional resistance calculated.

2.5. Neutron reflectometry \({}^{440}\) [performed by Ms. Natalia Pawlowska] \({}^{441}\)

NR experiments were performed on the D3 neutron reflectometer located at the Canadian Neutron Beam Centre (Chalk River, Ontario – Canada). Measurements were carried out for 8-10h on areas approximately 60 × 50 mm\(^2\). To enhance the scattering contrast between adlayers and interfacial regions, bulk water (a 175/125 v/v mixture of D\(_2\)O and H\(_2\)O) was contrast-matched (SLD = 3.54 \times 10^{-6} \text{ Å}^{-2}\) with the SiO\(_2\) substrate (SLD = 3.48 \times 10^{-6} \text{ Å}^{-2}\), as shown in Figure 57. Reflectivity data fitting – through adjustment of the various layers’ thickness (d), interfacial roughness (\(\sigma\)) and scattering length density (SLD) for the chosen stratified model (Figure 57) – was performed using MOTOFIT program running on IGOR Pro 6.32A software (Wavemetrics Inc.),\(^{442}\) and assessed for quality using the chi-squared method (\(\chi^2\)). A complete, detailed description of the neutron reflectometer set-up, the scanning geometries and procedures (measurements in air and water, with or without adlayer), as well as the parameters and steps of computer modelling/data fitting can be found in references 440 and 441.

![Figure 57. Schematic description of the stratified system chosen to study surface hydration showing (from bottom to top) the Si/SiO\(_2\) substrate, the organosilane adlayer (MEG-OH or OTS-OH) as well as transitional and bulk water. Also shown are the thickness (d), roughness (\(\sigma\)) and scattering length density (SLD) of the various layers.](image-url)
2. 5. 6. Thrombogenicity dynamic assay\textsuperscript{443,444} [performed by Mr. Kiril Fedorov]

Blood was collected in heparinized Vacutainers from apparently healthy human donors at St. Michael’s Hospital (Toronto, Ontario – Canada) and used within a few hours. No additional anticoagulant treatment was given to prevent clotting. Whole blood was freshly labelled with 3,3’-dihexyloxacarbocyanine iodide fluorescent dye (1 µM, 10 min at 37°C) prior to use. Thrombogenicity experiments were performed at room temperature at a shear rate of 1000 s\(^{-1}\) using a rectangular perfusion chamber (GlycoTech) shown in Figure 58. Real-time platelet adhesion, aggregation and thrombus formation was visualized and monitored under an Axiovert 135 inverted fluorescent microscope (Carl Zeiss) equipped with a DP70 digital camera (Olympus) using Slidebook software (Intelligent Imaging Innovations) under 32× magnification (Figure 58). Background fluorescence intensity was defined by this program and subtracted for the entire course of recorded images, only showing positive signals from adhered platelets/aggregates. Surface coverage due to platelet adhesion, aggregation and thrombus formation on type I collagen-coated, bare, and MEG-OH-modified BPA-PC surfaces (Appendices K–M) was assessed using ImageJ software on still images at 5 min.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure58.png}
\caption{Figure 58. (A) Experimental set-up used to record real-time platelet adhesion, aggregation and thrombus formation. (B) Close-up view of the perfusion chamber showing blood in- and outlets. (C) Computer interface used for data analysis and calculation of thrombus surface coverage.}
\end{figure}
2.6. Molecular dynamics simulations: model details and simulation protocol

MD simulations were performed with the VMD software\textsuperscript{445} (version 1.9.1) and NAMD program\textsuperscript{446} at the Department of Chemistry – University of Toronto (Toronto, Ontario – Canada), and the Tyndall National Institute – University College Cork (Cork – Ireland) where adlayer models in water were built by Dr. Damien Thompson. The substrate was a SiO$_2$ slab cut from $\alpha$-quartz, with (001) orientation and 1.97 $\times$ 1.97 $\times$ 0.90 nm dimensions (Figure 59).\textsuperscript{iii}

\begin{figure}[h]
  \centering
  \includegraphics[width=\textwidth]{si-o2_slab.png}
  \caption{Structure and dimensions of the SiO$_2$ slab cut from $\alpha$-quartz for MD simulations. The atom colour code is as follows: yellow (silicon) and red (oxygen).}
  \label{fig:si-o2_slab}
\end{figure}

Full-coverage coatings encompassed 5 $\times$ 5 molecular residues on one face, the other side being left unfunctionalized (Figures 60A and 60B). Chain separation and lateral packing density were respectively $\sim$0.49 nm and $\sim$4.2 residue/nm$^2$ (or $\sim$6.9x10$^{-10}$ mol/cm$^2$).\textsuperscript{441,448,449} Unless MD simulations were intended to study dry coatings, each adlayer model was encased in a 4.85 $\times$ 4.85 $\times$ 9.70 nm tetragonal box of water, producing as such a total cell size of approximately 23,000 atoms (Figure 60C). The TIP3P model was used for water.\textsuperscript{450}

\textsuperscript{iii} $\alpha$-Quartz is the most stable polymorph of silica (SiO$_2$) at ambient conditions, and the (001) surface is the most stable type.\textsuperscript{447}
Before starting to record MD simulation data, each system was first relaxed using 2000 steps of steepest descent minimization with respect to the CHARMM force field\cite{451,452}, then allowed to equilibrate to a stable room temperature by gradually raising the temperature from 0 to 295 K over 2.5 ns. MD simulations were then run for > 30 ns with a sampling frequency of 20 ps, unless otherwise specified. To model the adlayers obtained empirically, the flexible residues were not subjected to any positional constraints (other than their attachment site), unlike the substrate’s silicon and oxygen atoms that were restrained to their crystallographic positions throughout the simulations. This resulted in a mixed population of chain conformations for > 1500 different coating structures over which data points and statistical error bars were averaged for the central residue and its nearest neighbours (the 3 × 3 inner core), unless otherwise noted.
3. Results and Discussion

3. 1. Mixed adlayer surface chemistry

3. 1. 1. EMPAS biosensor: prototype in buffer (biotin/avidin model)

3. 1. 1. A. Working principle and background research: TT TA/OTS system\textsuperscript{18,453}

In previous work,\textsuperscript{453} we described the implementation of trichlorosilane surface chemistry with EMPAS technology for the development of a label-free biosensor prototype able to detect, in real time, biotin/avidin model interactions in buffer (Figure 61). Biosensing platforms consisted of piezoelectric quartz discs onto which functionalizable mixed organosilane adlayers were prepared using TTTA linker in combination with shorter, monofunctional OTS diluent (Figure 61 – step I). Subsequently biotinylated adlayers (step II) were dedicated to detect the specific binding of avidin analyte. On the other hand, non-functionalized mixed assemblies constituted control surfaces to assess avidin NSA (Figure 61).

![Figure 61](image)

**Figure 61.** TTTA/OTS mixed adlayer-based EMPAS biosensor prototype for the real-time and label-free detection of biotin/avidin model interactions in buffer: preparation (steps I & II) and working principle.

EMPAS measurements were performed at 1.06 GHz using 1.5 μM avidin samples in PBS buffer (Figure 62A). The average frequency shift due to avidin specific binding ($\Delta f = -15.3 \pm 2.2$ kHz, n = 4) was substantially larger than that caused by avidin NSA ($\Delta f = -4.0 \pm 1.6$ kHz, n = 4) (Figure 62B). Biosensor performance, assessed through the calculation of ‘specific binding vs. non-specific adsorption’ frequency shift ratio $R_{SB/NSA}$, was promising (3.8:1). Reproducibility was acceptable for specific binding (14% RSD) but rather poor for NSA (39% RSD).
Figure 62. EMPAS resonant frequency shifts for avidin specific binding (blue) / non-specific adsorption (red), respectively measured with biotinylated and non-biotinylated TTTA/OTS mixed adlayer platforms.

3.1.1. B. PFP-TTTA/OTS system: PFP vs. TFE head function

We now study the PFP-TTTA/OTS system (Figure 63), wherein linker residues now possess a pentafluorophenyl (PFP) functionalizable ester head group in place of the trifluoroethyl (TFE) one, as in the case of TTTA (Figure 61). Mixed adlayers were prepared upon immersion of cleaned quartz discs into a 1/1/2000 (v/v/v) solution of PFP-TTTA linker and OTS diluent in anhydrous toluene, for 2h at room temperature (Figure 63 – step I). Compared to other work, our strategy to prepare functionalizable PFP coatings is original in the sense that PFP ester moieties, as part of the surface-modifying linker molecules, are directly installed during adlayer formation – not stepwise, post-assembly.

Figure 63. Schematic representation of PFP-TTTA/OTS mixed adlayer formation on quartz (step I) and the subsequent, site-specific covalent immobilization of biotin-thiol/biotinamine probes (step II).
Subsequent biotinylation was then readily performed in a single, preactivation-free second step upon exposure to biotinthiol in anhydrous DMF, overnight at room temperature (Figure 63 – step II). The immobilization of biotinamine – the analogous aminated probe – was also considered (Figure 63).457-460

Both successful types of surface modification (adlayer formation and biotinylation) were characterized using angle-resolved XPS (Appendix N) following the appearance/loss of a fluorine peak (F_{1s} at 688 eV), the one element unambiguously attributable to PFP-TTTA linker/surface residues (Figure 64A). With respect to biotinylation, peaks for nitrogen (N_{1s} at 400 eV, ureyl moiety RNH-CO-NHR461,462) and sulfur (S_{2p} at 164 eV, sulfide -S-) also appeared as can be seen in Figures 64B and 64C, respectively. The ability of PFP-TTTA linker to modify the surface of quartz and the resulting adlayers to react with both thiolated and aminated molecules are well in line with those we recently reported for a closely related PFP system.338

![Figure 64](image-url)

**Figure 64.** (A) XPS surveys for (bottom to top) cleaned bare quartz (red) and PFP-TTTA/OTS mixed adlayers, non-biotinylated (blue) or biotinylated with biotinthiol (green) or biotinamine (gold). For clarity, the latter three profiles have been shifted upwards. **Note:** the peak for carbon in the XPS survey of cleaned bare quartz is due to contamination by adventitious species. (B-C) Narrow scans for nitrogen N_{1s} and sulfur S_{2p}. The takeoff angle shown here is 46° relative to the normal. **Note:** the amide (-CO-NH-) and thioester (-CO-S-)463 functions that form during biotin attachment (Figure 63 – step II) also respectively contribute to the observed N_{1s} and S_{2p} XPS signals.
Initial EMPAS experiments (conducted at 0.74 GHz) showed no statistical difference in resonant frequency shift between platforms biotinylated with biotinthiol \((\text{avidin specific binding: } \Delta f = -12.7 \pm 1.5 \text{ kHz, } n = 6)\) and non-biotinylated ones \((\text{avidin NSA: } \Delta f = -13.3 \pm 1.3 \text{ kHz, } n = 5)\). Reason for satisfaction however was the good reproducibility of the measurements, respectively 12% and 10% RSD. In an attempt to alleviate the high level of NSA, we implemented a technique routinely used in assay technologies such as the enzyme-linked immunosorbent assay (ELISA),\(^{464}\) which consists in passivating non-functional areas with a blocking agent. A popular sacrificial agent of the sort – whose remarkable passivating properties have been recently described in the context of piezoelectric sensing\(^{465}\) – is biomacromolecular bovine serum albumin (BSA), a flexible globular protein that presents as well the advantages of being easily accessible, abundant and relatively cheap.\(^{140}\) Our next set of EMPAS experiments (performed at 0.90 GHz) then consisted in sequentially injecting BSA over both types of biosensing platforms, followed by avidin once the resonant frequency stabilized (Figure 65). The ‘ingenuity’ of this approach was to actually take advantage of the fouling behaviour of our adlayers to try and generate antifouling background biofilms, prior to analyte introduction.

![Figure 65](image.png)

**Figure 65.** EMPAS resonant frequency shifts for avidin specific binding (blue) and non-specific adsorption (red), respectively measured with biotinylated (by means of biotinthiol) and non-biotinylated PFP-TTTA/OTS mixed adlayer platforms. Arrows mark the successive injections of sacrificial BSA and target avidin solutions (both 0.1 mg/mL \(~ 1.5 \mu\text{M}\) in PBS buffer). *Note:* for comparative purposes, injection times and initial resonant frequencies have been normalized.
This ‘on-line’ approach of preliminarily blocking NSA with BSA proved successful since, under these conditions, the average ‘avidin’ resonant frequency shift for non-biotinylated platforms (i.e. NSA) was considerably smaller ($\Delta f = -2.4 \pm 1.1$ kHz, $n = 4$) than that measured for biotinylated ones ($\Delta f = -9.2 \pm 1.0$ kHz, $n = 4$). This corresponded to $R_{SB/NSA} = 3.8:1$ (Figure 66A). Biosensing platforms biotinylated with biotinamine led to an even higher specific binding signal ($\Delta f = -11.4 \pm 1.3$ kHz, $n = 4$) – a possible cause of which could be higher probe loading (see Appendix N) – hence an improved $R_{SB/NSA}$ of 4.7:1 (Figure 66B). To note, the average frequency shifts due to BSA adsorption on non-biotinylated and biotinylated (with biotinthiol or biotinamine) platforms were respectively $\Delta f = -6.4 \pm 1.1$, $-6.0 \pm 1.0$ and $-6.9 \pm 1.3$ kHz.

![Graph](image)

**Figure 66.** EMPAS resonant frequency shifts for avidin specific binding (blue) and non-specific adsorption (red), respectively measured with biotinylated [with biotinthiol (A and C) or with biotinamine (B)] and non-biotinylated PFP-TTTA/OTS mixed adlayer platforms: (A-B) with intermediate injection of sacrificial BSA, or (C) without. Both BSA and avidin solutions were 0.1 mg/mL ~ 1.5 μM in PBS buffer.

Overall, this PFP-TTTA/OTS mixed system study proved successful as EMPAS measurements generally displayed good and improved reproducibility (10-15% RSD). Additionally, the work demonstrated that both thiolated and aminated probes could be attached through PFP ester moieties. However, the improvement of biosensor performance (as assessed via $R_{SB/NSA}$) using an intermediate injection of sacrificial BSA to block NSA was not drastic enough – in view of the results presented earlier for the analogous TTTA/OTS system having TFE functionalizable ester groups – to justify the implementation of such an extra step of operation for future applications. Another cost- and time-effective strategy, featuring an inherently antifouling adlayer, would be preferable. We revisited the TFE system.
3.1.1. C. OEG-TTTA/MEG-TFA system: ethylene glycol vs. alkyl chains

As discussed in introductory Sections 1.7.2. & 1.8., ethylene glycol (EG)-based coatings constitute an extremely popular type of construct to impart underlying substrates with protein-repellent properties. In the context of our study, the next logical step was thus to prepare mixed EG adlayers exposing TFE ester binding sites for biotin probe attachment. For this purpose, we synthesized OEG-TTTA linker, TTTA’s analogue with an EG backbone (Figure 67A). The diluent molecule – MEG-TFA (Figure 67B) – was of the monoethylene glycol type and possessed a labile trifluoroacetyl (TFA) terminal group. Biosensing platforms were prepared following the usual protocol upon first immersion of cleaned quartz discs into 1/1/2000 (v/v/v) solutions of OEG-TTTA and MEG-TFA in anhydrous toluene, for 2h at room temperature, followed by biotinylation with biotinthiol in anhydrous DMF (for specific binding measurements), overnight at room temperature.

Figure 67. Chemical structures of (A) OEG-TTTA linker and (B) MEG-TFA diluent trichlorosilane surface modifiers.

The effective silanization of the quartz substrate was demonstrated using XPS, primarily following the appearance of a peak for fluorine F$_{1s}$ at 687 eV (Figure 68A). Further analysis (narrow scans) of the other elements characteristic of quartz and OEG-TTTA/MEG-TFA surface modifiers/residues (Si, O and C) – which can be found in Appendix O – confirmed successful adlayer formation. With respect to the biotinylation step with biotinthiol, peaks for nitrogen (N$_{1s}$ at ~399 eV, ureyl moiety RNH-CO-NHR$^{461,462}$) and sulfur (S$_{2p}$ at ~163 eV, biotin sulfide -S- and thioester$^{463}$ attachment -CO-S-) appeared as can be seen in Figures 68B and 68C, respectively. This was accompanied by a decrease (not a total loss) of the F$_{1s}$ signal, as expected upon adlayer functionalization with TFE leaving group removal. It is not clear however whether this residual F$_{1s}$ peak reflected the presence of MEG-TFA diluent residues still bearing their cleavable TFA moiety, or that of untouched functionalizable TFE ester groups from OEG-TTTA residues. In the latter case, such a situation of incomplete probe loading – a hypothesis, which the weakness of the N$_{1s}$ and S$_{2p}$ signals would appear to support (Figures 68B and 68C) – would not necessarily
be detrimental to analyte detection; the reason being that partially functionalized biosensing platforms with spaced-out, protruding biotin binding sites would be more easily accessible to incoming, bulky molecules of avidin (approximately 56 × 50 × 40 Å in size)\textsuperscript{423} in terms of interfacial and lateral steric hindrance.\textsuperscript{423,467} This issue is associated with the important notion of ‘optimal probe loading’ at which largest analyte binding occurs.\textsuperscript{467} Repulsion by the antifouling mixed adlayer (see upcoming EMPAS experiments) – a phenomenon that reasonably should affect (to various extents) any approaching species – could help explain why biotinthiol immobilization would not proceed to completion.

Figure 68. (A) XPS surveys for (bottom to top) cleaned bare quartz (red) and OEG-TTTA/MEG-TFA mixed adlayers – non-biotinylated (blue) or biotinylated with biotinthiol (green). For clarity, the latter two profiles have been shifted upwards. Note: the peak for carbon in the XPS survey of cleaned bare quartz is due to contamination by adventitious species. (B-C) Narrow scans for nitrogen N\textsubscript{1s} and sulfur S\textsubscript{2p}. The takeoff angle shown here is 72.5° relative to the normal (surface analysis).

This strategy to reduce NSA with a mixed EG coating proved highly successful since the average EMPAS resonant frequency shift caused by avidin NSA on non-biotinylated OEG-TTTA/MEG-TFA platforms was only \(\Delta f = -0.9 \pm 0.3\) kHz (\(n = 3\)), close to noise level (< 0.5 kHz – Figure 69A). In comparison, avidin NSA on alkylated TTTA/OTS (Section 3.1.1.A.)
and PFP-TTTA/OTS (*Section 3. 1. 1. B.*) mixed adlayers was significantly larger at respectively $\Delta f = -4.0 \pm 1.6$ and $-2.4 \pm 1.1$ kHz; the latter system even requiring an extra blocking step to display such performance. This result with the novel OEG-TTTA/MEG-TFA system is well in line with the general observation found in the literature that OEGylated films exhibit pronounced protein-repellent properties.\textsuperscript{32,62,135,466} With respect to avidin specific binding, the average EMPAS resonant frequency shift was still healthy ($\Delta f = -6.1 \pm 1.0$ kHz, $n = 3$), which resulted in a $R_{SB/NSA}$ of 6.3:1 (Figure 69B). This represented a substantial improvement in terms of the biosensor’s analytical performance, without the need for an extra step to block NSA.

![Figure 69. EMPAS resonant frequency shifts for avidin specific binding (blue) and non-specific adsorption (red), respectively measured with biotinylated and non-biotinylated OEG-TTTA/MEG-TFA mixed adlayer platforms. Measurements were performed at the ultra-high frequency of 1.06 GHz.](image)

3. 1. 1. D. OEG-TTTA/MEG-TFA system: biosensing platform miniaturization\textsuperscript{18,453}

The world of biosensors is expanding at a rapid pace with an ever-increasing demand for more sensitive portable devices for point-of-care testing.\textsuperscript{468} One obvious step towards device miniaturization – which also includes that of the signal transduction microelectronics and that of the sample delivery system (microfluidics) – is to reduce the biosensing platform size, a task which we accomplished in previous EMPAS work.\textsuperscript{453} In view of this on-going trend towards biosensor miniaturization and in order to make our device more attractive for future applications, we had thus prepared OEG-TTTA/MEG-TFA mixed adlayers on $d = 0.275'' \sim 7.0$ mm quartz discs ($f_0 = 20.0$ MHz) – i. e. on a surface area approximately four times smaller (Figure 70A) than that of the previous $d = 0.537'' \sim 13.6$ mm larger discs (38 vs. 146 mm$^2$). The spiral coil used to remotely instigate resonance within the quartz substrate had also been resized accordingly (Figure 70B). This new set of EMPAS experiments conducted at 0.82 GHz proved...
successful (Figure 70C), resulting in a good $R_{SB/NSA}$ of 4.1:1 with a still extremely low NSA level ($\Delta f = -0.8 \pm 0.3 \text{ kHz}, n = 6$). The average resonant frequency shift for avidin specific binding was $\Delta f = -3.4 \pm 0.8 \text{ kHz} (n = 6)$. Overall, this project had shown that both the EMPAS transducing circuit electronics and the OEG-TTTA/MEG-TFA mixed adlayer-based biosensing platforms could be significantly miniaturized without altering analytical performance.

**Figure 70.** (A) EMPAS quartz discs photographed next to a Canadian penny. (B) The corresponding spiral coils used to remotely instigate resonance within the quartz substrate. (C) EMPAS resonant frequency shifts for avidin specific binding (blue) and non-specific adsorption (red), respectively measured with biotinylated and non-biotinylated OEG-TTTA/MEG-TFA mixed adlayer platforms prepared on (left) $d = 13.6 \text{ mm}$ and (right) $d = 7.0 \text{ mm}$ quartz discs. Avidin samples were 1.5 μM in PBS buffer.
In the field of biosensor technology, successfully miniaturizing detection platforms certainly constitute an achievement, even if measurements are performed with clinically irrelevant ‘analyte-only’ buffer solutions in the absence of any potentially interfering species. Although this situation is legitimate in early stages of development, the objective for a biosensor application – where the greatest difficulty arguably resides – still remains however the ability to detect and quantify target analytes in complex, real-world biofluid samples, such as those made of blood serum. Moving beyond PBS buffer as the carrier solution and performing measurements in full serum – with the ultimate aim of building a dose-response curve against which to test future samples at unknown concentration – is thus what we next set out to accomplish.

3. 1. 2. Real-world EMPAS biosensor: serological detection (biotin/avidin model)

3. 1. 2. A. Context

There exists in the literature thousands, perhaps even tens of thousands, of publications describing biosensing applications. Most of them are used for detection in buffer with ‘analyte-only’ samples. From a strict practical point of view however, these are of no clinical relevance since target analytes in real-world biofluid samples must be distinguished from potentially interfering species present in the biological matrix. A serum sample for instance, even cleared of the cellular components of blood, still consists of a highly complex mixture of various types of proteins at high concentration (60–80 g/L). These proteins have the propensity to adsorb non-spectively to the sensing surface of devices thereby preventing the detection – not to mention the quantification – of target analytes present at considerably lower concentration (down to the ng/L level, or a difference of nine orders of magnitude). Indeed, these adsorbing species also generate physicochemical stimuli that are indiscriminately detected by the biosensor and interfere with the specific response of the target analyte. From an analytical point of view, an unfortunate consequence of this undesired fouling phenomenon is the occurrence of ‘false positives’, i.e. a response incorrectly interpreted as a genuine binding event, not to mention that this often overwhelming background signal lowers sensitivity (poor signal-to-noise ratio) to clinically irrelevant levels. This issue of signal interference from the biological matrix, understandably, renders such biosensing interfaces inadequate for real-world bioanalytical applications. Incidentally, it constitutes arguably the single most important reason why biosensors still have not found a prominent place as alternative screening/diagnostic tools in clinical analysis despite tremendous promise notably in terms of cost/ease of operation and the aforementioned miniaturization capability for point-of-care testing.
3. 1. 2. B. Dose-response curve

3. 1. 2. B. 1. Surface chemistry optimization

Our first ‘real-world’ EMPAS experiment (conducted at 0.94 GHz) consisted in exposing non-biotinylated OEG-TTTA/MEG-TFA biosensing platforms to 1.5 μM avidin samples – not prepared in PBS buffer but rather in full serum – then monitor the level of fouling generated by these serum solutions. Not surprisingly for such a challenging proteinaceous medium, the EMPAS signal for serum was considerably larger (14-fold) than that recorded for PBS buffer, at Δf = –14.2 ± 3.5 (n = 5) vs. –0.9 ± 0.3 (n = 3) kHz, as can be seen in Figure 71.

![Figure 71](image_url)

**Figure 71.** EMPAS resonant frequency shifts due to fouling on non-biotinylated OEG-TTTA/MEG-TFA mixed adlayer platforms of 1.5 μM avidin samples prepared in (left) PBS buffer and (right) goat serum.

A rationale behind such a large(r) fouling signal is that albumin – the most abundant protein in serum – has been shown to display high affinity for TFE-terminated mixed SAMs. In light of this account and the incomplete nature of probe loading on our OEG-TTTA/MEG-TFA system (Section 3. 1. 1. C.), we hypothesized that the unreacted, residual TFE ester moieties of OEG-TTTA linker residues acted as nucleation sites for protein fouling (see also Section 3. 2. 3. H.). We thus next investigated whether decreasing the relative linker-to-diluent (L:D) composition in the silanizing solutions used to prepare OEG-TTTA/MEG-TFA mixed adlayers would have an impact on the magnitude of the EMPAS signal. As shown in Figure 72, the EMPAS response (red bars) did decrease along with the silanizing solutions’ L:D ratio, convincingly supporting our hypothesis. Worth noting in particular was the extremely low average frequency shift measured for L:D = 1:50 (Δf = –0.7 ± 0.3 kHz, n = 4). With respect to avidin specific binding recorded with biotinylated platforms (blue bars), a similar trend was observed, the signals being satisfyingly much larger than their respective controls (Figure 72).
Figure 72. EMPAS resonant frequency shifts for avidin specific binding (blue) and non-specific adsorption (red) measured with 1.5 μM avidin samples in serum, respectively on biotinylated and non-biotinylated OEG-TTTA/MEG-TFA mixed adlayer platforms (4-5 replicates per data set). L:D is the volumetric ratio (v/v) of OEG-TTTA linker (L) and MEG-TFA diluent (D) in the silanizing solutions used to prepare the various mixed adlayers (see Experimental Section 2.4.2.B.).

3.1.2.B.2. Detection threshold/cut-off

Considering that EMPAS responses are not purely gravimetric in nature since they are also governed by viscoelastic and slip phenomena, for instance (as discussed in Section 1.11.4.) – and that a dose-response curve should therefore not be expected to exhibit a quantitative proportional relationship (such as that observed in the dynamic range of traditional calibration curves) – the next step towards the generation of a dose-response curve was to run ‘blank’ experiments, wherein biotinylated platforms would be exposed to unspiked serum samples lacking avidin target analyte. The aim is to define a qualitative ‘detection threshold’ (or cut-off) above which EMPAS responses from future unknown samples would be considered positive signals. Blank experiments (Figure 73) were performed for the two L:D systems that had given us the best ratio in terms of ‘specific vs. non-specific’ signal (i.e. L:D = 1:10 and 1:50, Figure 72). The results were compared to those obtained previously from the injection of 1.5 μM avidin serum samples, also run on biotinylated platforms. Of the two 1:10 and 1:50 L:D systems, it is the latter that provided the largest discrepancy between avidin-spiked and unspiked serum samples (R = 2.0:1), with a ‘blank’ resonant frequency shift of Δf = −1.7 ± 0.1 kHz (n = 4), as seen in Figure 73.
Figure 73. EMPAS resonant frequency shifts measured on biotinylated OEG-TTTA/MEG-TFA mixed adlayer platforms (4-5 replicates per data set), upon exposure to serum samples either spiked with 1.5 μM avidin (blue) or unspiked (yellow).

As a result, we selected this ‘optimal’ L:D = 1:50 ratio to further build our dose-response curve, running EMPAS experiments for a wide range of avidin concentrations (Figure 74).

Figure 74. EMPAS response to a wide range of avidin serum solutions, measured with OEG-TTTA/MEG-TFA mixed adlayer platforms (L:D = 1:50). Note: the two outliers for the blank experiments (unspiked serum) were not taken into account in the calculation of the cut-off value (|Δf<sub>blank</sub>| + SD).
Immediately evident in Figure 74 is the non-linear relationship between EMPAS responses and injected doses of avidin. This observation corroborates the non-quantitative nature of EMPAS measurements. Regardless, as shown in Figure 74, a qualitative detection cut-off could be set at $|\Delta f| = 1.8$ kHz ($|\Delta f_{\text{blank}}| + \text{SD}$), with a limit of detection in the pM range for the assay.

3. 1. 2. B. 3. Special assay interferents

The excellent analytical performance of our biosensor in serum was tarnished however by the occurrence of false negative and false positive results (Figure 74), which we tentatively hypothesize to be caused by special types of interfering species endogenous to serum. In the former case, saturation during sample preparation of avidin’s four binding sites by biotin (Figure 75) – whose likely presence in supplied goat serum was confirmed by our provider (Appendix P) – could explain the observation of false negatives at low avidin concentration.\(^{471}\) The pM range at which interference occurs matches well with the concentration of biotin previously reported for goat serum (~72 pg/mL or ~295 pM, i.e. ~74 pM per binding site as per the 1:4 avidin/biotin binding stoichiometry).\(^{472}\) False positives for unspiked serum samples, on the other hand, could be explained by the (abnormally high) cross-reactivity of platform-bound biotinyl residues with biotinidase, a biotin-binding protein present in serum and responsible for the bioavailability of this vitamin (H, reclassified as B7).\(^{473}\) We note incidentally that the presumed biotinidase cross-reactivity could also potentially interfere with avidin detection in spiked serum samples.

Figure 75. Saturation of tetrameric avidin with four biotin ligands (molecular representation taken from www.proteopedia.org).
3.1.2. B.4. Concluding remarks

By moving beyond buffer and conducting EMPAS experiments in serum, we created a real-world detection scenario, wherein the analyte of interest (avidin) was present at low concentration (down to ~1.5 pM) and had to be distinguished from an excess of interfering species in a highly complex mixture (serum matrix). This overall successful project – that led to the development of: (i) a new dual-functional organosilane mixed surface chemistry that combined antifouling and biorecognition abilities; and (ii) a qualitative cut-off assay with pM limit of detection (which validated the EMPAS as a qualitative analysis technique) – was tempered however by the occurrence of false positive and false negative artifacts, which we suspected to be caused by special types of interfering species endogenous to serum. These results are a reminder that, even with the model biotin/avidin test system chosen for its unmatched strong affinity ($K_D \sim 10^{-15}$ M), serological detection in biosensor technology is far from being an easy task as there may be several, unsuspected facets to signal interference. In the particular case of our study, it may be the situation that our target analyte (avidin) and cross-reactive, endogenous biotinidase interacted with platform-bound biotinyl residues and serum ‘free’ biotin (and other metabolites), in a complex overall interplay of equilibria governed by relative concentrations and affinities.

This dual-functional OEG-TTTA/MEG-TFA mixed surface chemistry is not devoid of any interest however. A potential application in biosensor technology would be in the construction of supramolecular ‘sandwich’ assays, wherein multivalent avidin or its sister molecules (streptavidin and neutravidin) – strongly bioconjugated to mixed adlayer platforms through bound biotinyl residues – would serve as capture/docking sites for an extra layer of biotin-labelled probes. Another project, currently underway, which uses OEG-TTTA/MEG-TFA mixed surface chemistry, relates to the detection and neutralization of lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria and a central player in the pathogenesis of sepsis. Sepsis is a detrimental condition, wherein the original appropriate host response to an infection becomes amplified and dysregulated. Being able to detect and neutralize LPS molecules in a timely manner is vital as systemic inflammation can rapidly lead to multiple-organ dysfunction and death.
3. 1. 3. A. Sepsis and endotoxemia: overview, pathogenesis and treatment

An estimated 750,000 individuals are affected by sepsis annually in the United States (similar incidence is reported in Europe and around the world), with ~200,000 casualties. Sepsis is a life-threatening condition, actually one of the leading causes of death worldwide responsible for tremendous medical expenditure. Sepsis occurs when a local infection overcomes the host natural defense mechanism, triggering a vigorous and dysregulated inflammatory host response that will cause infected patients to display the generic symptoms of ‘systemic inflammatory response syndrome’ (SIRS). If not treated promptly and properly, this generalized, self-injurious state of sepsis can worsen into different stages of various severity, the last of which eventually resulting in multiple organ dysfunction and failure (‘septic shock’). At acute states of the disease, mortality is extremely high.

The pathogenesis of sepsis is relatively well known, one of the most potent immunomodulator being bacterial lipopolysaccharide (LPS) – also known as ‘endotoxin’ – which is a major constituent of the outer membrane of Gram-negative bacteria (e.g. Escherichia coli). One source of LPS release into the bloodstream (causing ‘endotoxemia’) occurs upon the action of antibacterial host factors when bacteria enter the circulatory system – a condition known as ‘bacteremia’ (Figure 7A). Due to bacteria’s ubiquity in Nature, LPS contamination is not limited to blood, but actually represents a genuine environmental hazard (and an issue in biotechnology) everyone is exposed to on a daily basis, to various extents. LPS is an amphipathic molecule with a general structure characterized by a central, diphosphorylated glucosamine disaccharide that is flanked by a hydrophobic lipid domain encompassing several fatty acid chains (together forming the epitope ‘Lipid A’ region), and a long hydrophilic polysaccharide tail (Figure 7B). Among bacteria serotypes, there exists chemical variability for LPS, especially at the terminal region known as ‘O-antigen’ (Figure 7B), the epitope Lipid A region being the most conserved. To this structural heterogeneity may also add a complex supramolecular chemistry of polymorphic aggregates, micelles and vesicles on which LPS bioactivity appears to depend.
Figure 76. (A) From bacteremia to multi-organ failure: the key role of LPS/endotoxin in the pathogenesis of sepsis. (B) General chemical structure of monomeric bacterial lipopolysaccharide.

Clinical management of sepsis\(^4^8^5\) is possible as bacterial infection can be handled with antibiotics but is limited since the bactericidal process may result in the liberation of more endotoxin (as such worsening the situation),\(^4^8^5,4^8^6,4^9^3\) not to mention that bacteria can develop multidrug-resistance.\(^4^8^5\) One strong, broad-spectrum antibiotic with high affinity for endotoxin is polymyxin B (PMB – Figure 77),\(^4^8^5,4^8^7,4^9^4,4^9^5\) a cyclic peptide whose neuro- and nephrotoxicity are unfortunately major drawbacks that contraindicate its systemic use.\(^4^2^6,4^8^0,4^8^5,4^9^3,4^9^6\) A ‘safer’ approach to take advantage of this molecular affinity is to immobilize PMB securely\(^4^2^6,4^8^7,4^9^3,4^9^6,4^9^7\) (i.e. covalently to prevent leaching) to the surface of a polymer adsorbent material\(^4^2^6,4^8^8,4^9^3,4^9^8,4^9^9\) in order to remove LPS from blood circulated extracorporeally.\(^4^8^5,4^8^7,4^9^5\) This \textit{in vitro}, ‘drug-free’ method has been implemented with success since 1994 in the \textit{Toraymyxin} hemoperfusion cartridge developed by the Japanese company \textit{Toray Industries Inc.}.\(^4^8^1,4^8^5,4^9^5,4^9^6,5^0^0\) Other endotoxin neutralization strategies have encountered various success.\(^5^0^1\)

Figure 77. Chemical structure of polymyxin B.
3.1.3. B. Motivation: developing an alternative test for endotoxin detection

Clinical tests – such as the ‘Limulus amebocyte lysate’ (LAL) assay\textsuperscript{502,503} or the more recent ‘endotoxin activity assay’ (EAA)\textsuperscript{424,425,504} – exist to detect endotoxin in bodily fluids; nevertheless, they are expensive, relatively fastidious to implement and (may) require reporter molecules (\textit{i.e.} the EAA requires a chemiluminescent agent,\textsuperscript{424,425,504} modern variants of the LAL assay rely on a chromogenic substrate\textsuperscript{502,503}). In this context emerged the idea that immobilizing PMB receptors on our newly developed dual-functional OEG-TTTA/MEG-TFA silane surface chemistry\textsuperscript{497} could potentially lead to the development of a biosensor alternative based on EMPAS technology for the cost-effective, user-friendly and label-free detection of LPS.\textsuperscript{505}

Another exciting component of the project came from the realization that: (i) the same surface chemistry could be implemented in a hemofiltration cartridge to actually neutralize LPS; and (ii) both these diagnostic and therapeutic features could eventually be used in tandem/combined in an all-integrated ‘theranostic circuit device’ (TCD) configuration (\textbf{Figure 78}) for the personalized treatment\textsuperscript{506} of endotoxemia/sepsis. Due to its confidential character, the second (therapeutic) aspect of the work will not be presented herein.

\textbf{Figure 78.} Personalized ‘theranostic’ treatment of endotoxemia combining a therapeutic neutralization cartridge and an in-line EMPAS diagnostic system in an all-integrated ‘theranostic circuit device’.
3.1.3. C. Biosensing platform preparation and characterization

Learning from our previous experiments with the biotin/avidin system, we directly produced a series of OEG-TTTA/MEG-TFA mixed adlayers with varying composition – upon immersion of cleaned quartz discs into silanizing solutions with different L:D ratio (see Experimental Section 2.4.2.B.), in anhydrous toluene for 2h at room temperature. Subsequent PMB immobilization to complete the biosensing platform was performed under mild aqueous conditions, in PBS buffer (pH 7.4) overnight at room temperature. We chose this neutral pH environment in order to minimize: (i) siloxane adlayer etching from the substrate;\textsuperscript{318,507} and (ii) the population of reactive PMB amine moieties\textsuperscript{272,454-456} to prevent multi-amine site attachment of PMB (Appendix Q), since maximizing the number of exposed free amine groups has been shown to correlate with higher endotoxin removal(binding capacity).\textsuperscript{496}

Using XPS, the success of both mixed adlayer formation and subsequent PMB attachment was readily demonstrated for all ratios through the monitoring of peaks for fluorine F\textsubscript{1s} (from OEG-TTTA and MEG-TFA residues) and nitrogen N\textsubscript{1s} (from the PMB probe), as shown in Appendix R. There is, nonetheless, an interesting point that deserves to be discussed here in greater detail: the (relative) evolution of these XPS signals as a function of the L:D ratio (Figure 79).

**Figure 79.** Fluorine F\textsubscript{1s} and nitrogen N\textsubscript{1s} XPS signal intensity (counts per second areas) for OEG-TTTA/MEG-TFA mixed adlayers formed from various L:D silanizing solutions, before (blue) and after (red and green) PMB probe immobilization. \textit{Note:} counts per second area values are those recorded at the takeoff angle of 72.5° relative to the normal (surface analysis).
As seen for the attachment of biotinthiol (Figure 68), there also remained, after PMB immobilization, a peak for fluorine (whose relative intensity decreased along with L:D), as shown in Figure 79. Considering (i) that MEG-TFA residues are quantitatively converted into MEG-OH chains upon overnight hydrolysis of their labile TFA terminal group (as shown in upcoming Sections 3. 2. 1. & 3. 2. 4.); and (ii) that PMB immobilization was performed in aqueous conditions (PBS buffer) overnight, it is reasonably the case that the residual F\textsubscript{1s} peaks solely originated from the unreacted TFE ester function of OEG-TTTA linker residues. Assuming that ‘in-solution’ L:D compositions remained unchanged ‘on-surface’ upon adlayer formation, it would therefore make sense that the lower the L:D ratio, the lower the relative intensity of the residual F\textsubscript{1s} peak (Figure 79 – red vs. blue bars) – the latter being almost non-existent at L:D = 1:50 (a decrease of 93%).

When looking more specifically at the evolution of the F\textsubscript{1s} signal intensity upon adlayer formation through the L:D series, another interesting pattern emerged: the lower the amount of OEG-TTTA linker (L) in the silanizing solution, for a constant content of MEG-TFA diluent (D) (see Experimental Section 2. 4. 2. B.), the higher the magnitude of the fluorine peak in the resulting mixed adlayer (Figure 79 – blue bars). As MEG-TFA and OEG-TTTA molecules carry the same number of three fluorine atoms (Figure 67) – and assuming again same ‘in-solution’ and ‘on-surface’ L:D composition – this observation would seem to suggest that shorter MEG-TFA surface-modifying residues pack denser than longer OEG-TTTA ones do. As such, it is likely that more of these OEG-TTTA linker residues would bind to the substrate at L:D = 1:50 (comparatively to L:D = 1:10) and be available for PMB attachment, with higher accessibility as well since likely less sterically hindered within the mixed assembly (Figure 80). Together, these features would explain very well the higher nitrogen content from the PMB probe observed for this 1:50 ratio compared to L:D = 1:10 (Figure 79 – corresponding green bars). In this respect, the lower residual F\textsubscript{1s} peak observed for L:D = 1:50 is not contradictory with higher original content of OEG-TTTA linker residues (than L:D = 1:10 – Figure 80), as fewer of these more reactive residues would remain after PMB immobilization. Lastly, ‘L:D = 1:1’ mixed adlayers would be the least packed but comparatively incorporate the highest amount of OEG-TTTA linker residues (Figure 80), explaining why the largest nitrogen content is observed at this ratio (Figure 79) despite the probability of greater steric hindrance/congestion to probe attachment.
Figure 80. Schematic representation (top view) of the hypothesized different packing of OEG-TTTA linker (L) and MEG-TFA diluent (D) residues within mixed adlayers at various L:D compositions. 

As already discussed for the biotin/avidin system in Section 3.1.1. C., partial probe loading, of PMB in this case, is not necessarily surprising considering the pronounced antifouling properties displayed by this OEGylated mixed adlayer system – not to forget that PMB cyclic peptide molecules are large(r) entities to accommodate on a surface. Several examples of unimolecular (i.e. undiluted) TFE ester-terminated coatings being functionalized to completion with primary amine-bearing molecules have already been described in the literature. We note however that: (i) these reported coatings did not incorporate OEG but alkyl chains; and/or, (ii) aminated molecules were not as sterically demanding as PMB. Nevertheless, it can be argued that partial probe loading may be beneficial for the binding of bulky analytes (LPS is a large biomacromolecule with ~ 2 × 2 × 6 nm dimensions).

All things considered, the following representation of OEG-TTTA/MEG-TFA/PMB mixed adlayer surface chemistry can reasonably be proposed (Figure 81): 

Figure 81. Realistic representation of OEG-TTTA/MEG-TFA/PMB surface chemistry on quartz.
3.1.3. D. EMPAS measurements

LPS detection was performed at 0.94 GHz with L:D = 1:10 and 1:50 PMB-functionalized biosensing platforms using full human blood plasma. The average ‘blank’ resonant frequency shifts due to fouling by LPS-unspiked donor plasma were $\Delta f = -7.6 \pm 1.0 \text{ (n = 3)}$ and $-7.7 \pm 2.5 \text{ (n = 4)} \text{ kHz for L:D = 1:10 and 1:50, respectively (Figure 82).}$ In comparison, the corresponding frequency shift on bare quartz was much greater ($\Delta f \sim -28 \text{ kHz}$), and incidentally close to the value recorded for full serum as will be shown in upcoming Section 3.2.1. C. 1. ($\Delta f \sim -31 \text{ kHz}$).

The interesting observation here is not so much the similar, pronounced antifouling behaviour displayed by both types of L:D biosensing platform coating (compared to the bare substrate – Figures 82 and 83), or the comparable value obtained for specific LPS binding experiments at L:D = 1:10 ($\Delta f = -6.5 \pm 2.1 \text{ kHz, n = 2 – Figure 82}$), but the entirely different specific response profile recorded for L:D = 1:50 biosensing platforms exposed to plasma samples at high LPS/endotoxemia level (1000 pg/mL). Indeed, as can be seen in Figure 83, the resonant frequency in the case of L:D = 1:50 was higher after contact with LPS-spiked plasma, hence the resonant frequency shift positive ($\Delta f = +4.1 \pm 2.2 \text{ kHz, n = 2 – Figure 82}$), a behaviour in stark contrast to that observed for biosensing platforms exposed to unspiked donor plasma.

![Figure 82. EMPAS resonant frequency shifts measured with PMB-functionalized L:D = 1:10 and 1:50 OEG-TTTA/MEG-TFA mixed adlayer platforms, upon exposure to human plasma samples spiked with LPS at 1000 pg/mL (blue) or unspiked (yellow). For comparison, also shown is the resonant frequency shift for bare quartz exposed to unspiked donor plasma (left).](image)

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iv Donor blood plasma was ‘endotoxin-free’ (EA = 0.26), as assessed using the endotoxin activity assay (EAA).424,425,504

v ‘Low’, ‘intermediate’ and ‘high’ endotoxin levels can be respectively defined as < ~50 pg/mL (EAA < 0.4), ~50-400 pg/mL (0.4 < EAA < 0.6), and > ~400 pg/mL (EAA > 0.6).
Figure 83. EMPAS resonant frequency profiles for bare quartz (red) and a PMB-functionalized L:D = 1:50 OEG-TTTA/MEG-TFA mixed adlayer platform (yellow) both exposed to LPS-unspiked human donor blood plasma; as well as (blue) a PMB-functionalized L:D = 1:50 OEG-TTTA/MEG-TFA mixed adlayer platform exposed to human blood plasma spiked with LPS at 1000 pg/mL. Note: for comparative purposes, injection times (marked by the arrow) and initial resonant frequencies have been normalized.

3.1.3. E. Results interpretation and significance

Resonant frequency increase upon analyte interaction with a piezoelectric sensing surface is perhaps counter-intuitive. Nevertheless, there exists a rational explanation for this phenomenon as well as some literature precedents to which this latest example adds. In fact, this observation likely reflects the occurrence of stiffening phenomena that result from the PMB/LPS interaction event and are reminiscent of those observed during binding experiments involving bovine submaxillary gland mucin, calcium-binding calmodulin and HIV envelope glycoprotein gp120 immobilized on the surface of more conventional bulk acoustic wave sensors. On a speculative level, it is possible that the interaction caused the surface-bound PMB receptors to conformationally rearrange and lock with LPS ligands into a rigid biomolecular complex that ‘froze’ the coating. [At present, it is not entirely clear whether LPS monomer/aggregate supramolecular chemistry has some degree of involvement.] This viscoelastic phenomenon, which relates to the more fundamental concept of ‘slip’ in acoustic wave physics, would in turn cause the imposed coating to eventually oscillate with better synchronicity with the underlying piezoelectric quartz substrate, hence resulting in an increase in
resonant frequency despite mass addition.\textsuperscript{399} This EMPAS experiment is another example of acoustic wave devices being sensitive to both added mass and a variety of phenomena occurring at the sensor-liquid interface. From a practical point of view, this unexpected result – which constitutes a stimulating enigma to be solved in terms of the physical chemistry at play – does not represent however a set-back to the development of an EMPAS biosensor for the detection of LPS in blood plasma. Quite to the contrary, these initial experiments are significant as they show that plasma samples at elevated LPS concentration (1000 pg/mL) can be easily distinguished with the EMPAS from those presenting basal level (< 10 pg/mL).\textsuperscript{510,511} The abnormally high former value is of the order of magnitude that can be found in patients in dire need of treatment,\textsuperscript{511} such as those admitted to intensive care units.\textsuperscript{504}

\textbf{3. 1. 3. F. Concluding remarks}

The versatility of the dual-functional OEG-TTTA/MEG-TFA mixed adlayer surface chemistry for the development of real-world EMPAS biosensors was demonstrated. As for the case in point, the analyte of interest was one of the most potent activators of sepsis, bacterial LPS (also known as endotoxin), whose presence in full human blood plasma was detected in a real-time and label-free manner unlike current clinical assays that rely on chromogenic reporter molecules. Biosensing platforms were functionalized with PMB receptors, a cyclic peptide antibiotic molecule with high affinity for LPS. EMPAS experiments showed that high endotoxemia plasma samples can be easily and rapidly distinguished from those presenting basal endotoxin level. Remaining to be done, for the biosensor component of our theranostic approach to combat sepsis, is the construction of a dose-response curve (or cut-off assay) with a range of clinically relevant LPS concentrations. Being able to diagnose endotoxemia, and its degree of severity, in a timely and expeditious manner in patients displaying SIRS symptoms (and with suspected sepsis) will allow for them to be properly triaged and taken care of/treated.\textsuperscript{483,512}

Up to now, research was focused on engineering biosensing platforms with combined antifouling and biorecognition capabilities. Dual-functionality was achieved through new mixed adlayer silane surface chemistry. In the remainder of this PhD Thesis, special attention will be given to the bioinert aspect of surface modification for biomaterial applications. The antifouling and antithrombogenic properties of simpler, unimolecular adlayer systems will be investigated.
3. 2. Unimolecular adlayer surface chemistry

3. 2. 1. Antifouling behaviour against serum

3. 2. 1. A. Context and motivation

There exists extensive literature praising the antifouling properties of stealth organic coatings against samples containing single, or extremely simple mixtures of, model proteins in a buffer solution. Much more limited in actuality (see introductory Section 1. 7.) is the technologically relevant consideration of otherwise more challenging, real-world biological milieux, such as those constituted by blood serum/plasma. Key issues with fouling by these highly complex proteinaceous media are not only the nature, strength and amount but also dynamics of adsorption of the various proteins present within blood on artificial surfaces. However, considering the difficulty of the task at hand, it is therefore not surprising that the prevalent focus of research on antifouling coatings merely lies in trying to minimize the amount of protein fouling, little to no attention being given to the more fundamental dynamic aspect of the phenomenon. Being highly sensitive to both gravimetric and interfacial effects, the label-free EMPAS device is capable of probing the adsorption dynamics – hence the fouling behaviour – of global biological systems interacting with any antifouling coating imposed on quartz. As seen in previous Sections 3. 1. 2-3., this was exemplified with full serum and the OEG-TTTA linker/MEG-TFA diluent mixed silane adlayer system. An interesting question that arose from this study was whether simpler, unimolecular MEG-TFA adlayers would also display pronounced antifouling properties against serum. This project was part of a larger investigation, wherein the influence of chemical functionalities on the antifouling behaviour of varied unimolecular adlayers was methodically studied through systematic structural modification (Figure 84). Noteworthy in the series of adlayers studied is the monoethylene glycol (MEG) family that incorporates a single, internal ether atom of oxygen in the chains (Figure 84).

Figure 84. Systematic surface modification of quartz with unimolecular adlayers.
3. 2. 1. B. Adlayer preparation and characterization on quartz

Unimolecular adlayers were prepared in a straightforward and cost-effective manner through simple dip-and-rinse procedures. MEG-TFA, MEG-OMe and OTS were prepared upon immersion of cleaned quartz discs into 1/1000 (v/v) solutions of the appropriate trichlorosilane surface modifier in anhydrous toluene, for 60 min at room temperature. MEG-OH coatings were readily generated in a second step from MEG-TFA adlayers (Figure 84) upon overnight solvolysis of the labile trifluoroacetyl (TFA) terminal groups in 1/1 (v/v) methanol/water. In the same manner, OTS-TFA adlayers (not shown) were converted into OTS-OH. Both successful surface modifications (adlayer formation and subsequent complete TFA group removal) were characterized for the MEG system using angle-resolved XPS (Figure 85A and Appendix S), following the appearance (respectively the total loss) of a peak for fluorine at 689 eV, the one element unambiguously attributable to MEG-TFA surface modifier/residues. Unlike fluorine however, the signal for carbon (at 285 eV) was not affected by the mild, yet lengthy, overnight aqueous treatment – clearly demonstrating that the latter had effectively cleaved the terminal TFA groups without etching the residual MEG-OH film from the quartz substrate (Figure 85A). If not a proof of chemisorption (i.e. of strong covalent anchorage), this certainly is an indicator of great robustness for the MEG-OH coating. MEG adlayer characterization was also supplemented with contact angle (Figure 85B) and ellipsometry measurements, the latter giving a subnanometric thickness of ~5 Å for the ultrathin MEG-TFA coating (Appendix T).

Figure 85. (A) XPS surveys for (bottom to top) cleaned bare quartz (red), as well as MEG-TFA (blue) and MEG-OH (green) unimolecular silane adlayers. Note: the peak for carbon in the XPS survey of cleaned bare quartz is due to contamination by adventitious species. (B) Corresponding static contact angles measured with water as the test liquid.
3. 2. 1. C. EMPAS experiments

3. 2. 1. C. 1. Measurements

The antifouling behaviour of bare and adlayer-derivatized quartz surfaces against full (goat) serum is summarized in Figure 86, where it is immediately evident that all organic adlayers – irrespective of their nature – caused a smaller net frequency shift compared to the value observed for unmodified quartz ($\Delta f = -30.9 \pm 3.7$ kHz, $n = 4$). Fully alkylated OTS adlayers performed poorly ($\Delta f = -22.1 \pm 5.4$ kHz, $n = 8$). Substitution for distal hydroxyl groups in OTS-OH provided no improvement ($\Delta f = -22.8 \pm 5.5$ kHz, $n = 5$). Remarkably, the incorporation of an internal, single ether atom of oxygen in MEG-OH resulted in a highly significant reduction in the net frequency shift associated with serum fouling ($\Delta f = -2.1 \pm 0.9$ kHz, $n = 6$). This represented for the MEG-OH system a ~15-fold decrease compared to bare quartz. It is also worth noting that, in a number of other experiments, no net frequency shift was observed. Other MEG adlayers (MEG-TFA and MEG-OMe) also exhibited an antifouling effect, although to a lesser extent with $\Delta f = -6.5 \pm 2.8$ (n = 4) and $-7.3 \pm 1.1$ (n = 6) kHz, respectively. These results corroborate with previous literature accounts on the antifouling properties of EG-based coatings (OEG/PEG) against serum/plasma. However, our surface chemistry is unique in the sense that antifouling adlayers are of the simplest monoethylene glycol (MEG) kind, incorporating building blocks with a single EG unit in the chain (Figure 84).

Figure 86. EMPAS resonant frequency shifts upon injection of full goat serum onto bare and adlayer-derivatized quartz surfaces. Measurements were performed at the ultra-high frequency of 0.94 GHz. Note: for rigour and comparative purposes, OTS and MEG-OMe adlayers also underwent the overnight methanol/water treatment through which MEG-OH and OTS-OH coatings were generated.

*vi* It is worth noting that the MEG-TFA system displayed erratic behaviour in terms of EMPAS response profiles (both shape and magnitude). We tentatively hypothesize it to be the result of the random removal of the labile TFA groups during the EMPAS experiments, by the action of the running PBS buffer or/and nucleophilic serum species.
3.2.1. C. 2. Adsorption dynamics\textsuperscript{50}

Beside the difference in the magnitude of the frequency shifts (Figure 86), the difference in the shape of the EMPAS response profiles is also notable. For bare quartz, the resonant frequency first dropped sharply (~ -25 kHz) then gradually decreased (~ -7 kHz in ~600 s) before final stabilization (Figure 87A). While the former phase was undoubtedly due to serum reaching the quartz surface, producing a high level of fouling; the second phase likely reflected the passage of the viscous sample over the surface, in the flow-through configuration of the EMPAS device. Negligible final rinse-off by the uninterrupted buffer flow was observed, suggesting that species responsible for fouling accumulated irreversibly. In stark contrast, the EMPAS profile for the MEG-OH adlayer displayed radically different behaviour (Figure 87B), with a progressive and comparatively limited initial drop in resonant frequency (> -5 kHz in ~250 s) typically followed by a gradual and extensive rinse-off (up to 1500 s and +2 kHz). Such dynamic behaviour of serum – which has a literature precedent in the context of acoustic wave physics and EG surface chemistry\textsuperscript{513} – seemed to indicate that, in this case, the majority of serum species were adsorbed in a reversible fashion involving transient interaction with the surface.

\textbf{Figure 87.} EMPAS response profiles for (A) bare quartz and (B) MEG-OH coating exposed to serum.
Interestingly, the shape of the MEG-OH profile (post injection) could be the signature of a sequential adsorption process reminiscent of that discovered by L. Vroman (see Section 1. 5. 3. B.), wherein higher mobility, more abundant serum proteins first adsorbed transiently before being gradually displaced by less motile, scarcer ones with higher surface affinity. It is also possible that the final increase in resonant frequency may have reflected the occurrence of rigidifying viscoelastic phenomena within the formed fouling layer – examples of which would be the surface-matching three-dimensional restructuration (unfolding) of individual proteins through conformational rearrangement, and their collective settlement into a more compact biofilm. Stiffening events witnessed through BAW sensing have already been documented several times in the literature during binding experiments involving surface-immobilized submaxillary gland mucin, calcium-binding calmodulin and HIV envelop-glycoprotein gp120, and herein during EMPAS detection of bacterial LPS (Sections 3. 1. 3. D-E.).

3. 2. 1. C. 3. Antifouling and surface hydration

From a mechanistic point of view, antifouling properties are generally acknowledged to correlate with (the state of) surface hydration. Two straightforward EMPAS experiments – that consisted in exposing to full serum MEG-OMe and OTS adlayers that had not been pre-hydrated overnight – supported this widely proposed hypothesis. In the former case, the resonant frequency shift for ‘dry’ MEG-OMe adlayers was much larger than that previously observed for pre-hydrated coatings, with \( \Delta f = -12.8 \pm 0.9 \) (n = 5) vs. \(-7.3 \pm 1.1 \) (n = 6) kHz (Figure 88). In comparison, pre-hydration had essentially no effect on the fully alkylated OTS system that still performed poorly with \( \Delta f = -22.1 \pm 5.4 \) kHz (n = 8), vs. \(-20.8 \pm 4.6 \) kHz (n = 4) pre-treatment.

![Figure 88](image.png)

**Figure 88.** EMPAS profiles for (A) pre-hydrated and (B) ‘dry’ MEG-OMe adlayers exposed to serum.
In addition to depending on the chemical nature of the exposed adlayer (MEG-OME vs. OTS), not surprisingly, these EMPAS experiments also suggested that proper surface hydration for full antifouling performance (of the MEG-OME system) may be a lengthy process requiring a longer period of time than the initial, < 30 min EMPAS stabilization phase in buffer. Surface hydration – and its intimate connection to antifouling – will be discussed in upcoming sections, both from an empirical (3.2.2.) and computational (3.2.3.) point of view.

3.2.1. D. MEG-OH surface chemistry on gold

Given the evident success encountered with quartz on the EMPAS, we next set out to expand the scope of MEG-OH antifouling surface chemistry by adapting the latter to another technologically important substrate: gold (Figure 89). Our choice for this material was notably motivated by its widespread use in more conventional piezoelectric detection technology (and surface plasmon resonance), on which many highly sensitive commercial (bio)sensing systems rely. The dynamics of serum adsorption (10% in PBS) was probed with the conventional acoustic wave device, the TSM sensor discussed in Section 1.11.4., which relies on plated gold electrodes – upon which surface functionalization is carried out – to excite acoustic resonance within the piezoelectric quartz disc. A series of MEG-OH adlayers on gold was prepared upon immersion of cleaned TSM discs into 5 mM solutions of HS-MEG-OH surface modifier in anhydrous ethanol, for increasing periods of time at room temperature (Figure 89).

Figure 89. (A/B) MEG-OH silane/thiol surface chemistries on quartz/gold for EMPAS/TSM sensing.

vii The MEG-OH silane surface chemistry developed for hydroxylated quartz (Figure 89A) is not suitable for gold. Surface modification of gold is achieved through use of the appropriate HS-MEG-OH thiol molecule (Figure 89B).
This time trial experiment (Figure 90) revealed that resonant frequency was least affected upon serum injection for HS-MEG-OH adlayers prepared for 30 min ($\Delta f = -121 \pm 16$ Hz, n = 3). XPS characterization of these adlayers is provided in Appendix U.

Figure 90. TSM resonant frequency shifts due to 10% serum adsorption on HS-MEG-OH adlayers formed on gold for increasing periods of time (5 to 1080 min). Note: ‘t = 0’ means gold is unmodified.

Such perhaps counter-intuitive observation – that lowest fouling (as assessed using acoustic wave physics) occurs at intermediate time of film formation – has literature precedents, wherein parameters such as surface coverage/order$^{514}$ or film thickness$^{183,188,202}$ were tuned over a wide range to study their effect on protein adsorption. The former factor is possibly at play in our case.

As can be seen in Figure 91, this optimal ‘30 min’ HS-MEG-OH surface modification resulted in a modest but visible decrease in frequency shift compared to unmodified gold for which $\Delta f = -147 \pm 23$ Hz (n = 3).

Figure 91. TSM profiles for (A) bare gold and (B) a ‘30 min’ MEG-OH film exposed to 10% serum. For comparative purposes, injection times and initial resonant frequencies have been normalized.
The critical role of surface hydration on the dynamics of serum adsorption witnessed with the EMPAS was also evidenced with gold in a next set of TSM experiments (Figure 92).\textsuperscript{248} In fact, we observed that the net frequency shift observed for ‘30 min’ HS-MEG-OH adlayers pre-hydrated overnight was significantly reduced to $\Delta f = -87 \pm 8$ Hz ($n = 3$), from $\Delta f = -121 \pm 16$ Hz ($n = 3$) for dry coatings. In contrast, serum adsorption on bare gold was unaffected ($\Delta f = -150 \pm 12$ Hz, $n = 3$ vs. $\Delta f = -147 \pm 23$ Hz, $n = 3$ pre-treatment).

\textbf{Figure 92.} TSM profiles for pre-hydrated (A) bare gold and (B) a MEG-OH film exposed to 10% serum.

For reasons that will become evident in the following discussion on ‘surface hydration’ (Sections 3.2.2-3.), it is important to note that pre-hydrated HS-OTS-OH thiol adlayers on gold (Figure 93) displayed the same fouling behaviour against serum as their silane analogues on quartz – relative to the bare substrate and corresponding MEG-OH adlayer (Figures 86 and 93).

\textbf{Figure 93.} TSM response profiles for pre-hydrated bare gold (blue), as well as HS-MEG-OH (red) and HS-OTS-OH (green) ‘30 min’ films exposed to 10% serum.
3. 2. 1. E. Concluding remarks

Empirical evidence was presented through EMPAS experiments that ultrathin MEG unimolecular silane adlayers – prepared from structurally simple surface modifiers in a straightforward manner – are able to radically alter the fouling behaviour of bare quartz against full serum, the effect being the most dramatic for MEG-OH adlayers displaying terminal hydroxyl moieties. Conversely, antifouling properties were much less pronounced for alkylated adlayers lacking internal ether atoms of oxygen in the chains. Also demonstrated was the adaptability of MEG-OH chemistry for the surface modification of gold, another technologically important substrate material. Another key feature of the work was the vastly different global dynamics of serum adsorption observed between bare and MEG-OH-derivatized substrates: while serum proteins adsorbed avidly onto unmodified quartz (gold) in a largely irreversible fashion, the adsorption process on MEG-OH coatings appeared much more limited and transient in nature. Surface hydration experiments revealed that water would play a critical role in this respect, likely through a mechanism involving the key participation of the internal ether atoms of oxygen in the instigation of a special intrafilm zone of hydration.
3. 2. 2. Probing surface hydration using neutron reflectometry

3. 2. 2. A. Context and motivation

Water is essential to Life, mediating a wide variety of biological and other processes thanks to its many unique dissolving, dissociating/lysing, solvating, lubricating, etc properties. Not surprisingly, this ubiquitous fluid is widely hypothesized to also play a key role in the antifouling of surfaces, although the precise underlying mechanism is still a matter of debate. For instance, one argument puts forth the ‘water barrier’ concept, wherein embedded and interfacial water molecules are tightly bound and organized into permeated structures that have an energy cost in terms of disturbance. Another school of thought favours rather the notion of ‘interfacial energy matching’ according to which there is no net energy gain for biological solutes – that are fully solvated in the bulk aqueous medium where they reside – to adsorb on hydrated surfaces. In both cases, water in contact with surfaces may form a phase physically distinct from ordinary bulk water, as supported by experimental evidence. The nature and extent of this special zone of hydration are, also, debated. Indeed, while some describe physically-distinct water interphases to project up to several hundred microns into the contiguous aqueous medium, others – in stark contrast – report surface kosmotropicity (defined as the ability of a surface to structure water at the molecular level) to be much more limited in range, to a few layers of water or less. Understandably, the contentious question of surface hydration – with respect to its connection with antifouling – has been and continues to be relentlessly researched by many.

As seen in previous Section 3. 2. 1., we also have contributed to the field and showed using acoustic wave physics that surface hydration is indeed intimately involved in the mechanism of antifouling, for thiol and silane monoethylene glycol (MEG) adlayers prepared on gold and quartz, respectively. In the latter work, a key observation was that the internal ether atom of oxygen in the single EG unit is necessary to dramatically alter the dynamics of serum adsorption, the synergy being strongest for MEG-OH silane adlayers possessing terminal hydroxyl moieties. In contrast, the effect was considerably less pronounced for alkylated adlayers lacking internal atoms of oxygen. Similar behaviour was observed for the analogous thiol surface chemistry imposed on gold. It was then hypothesized that the unique antifouling properties of the MEG system were rooted in a special intrafilm zone of hydration involving the key participation of the internal ether atoms of oxygen in the chains, a feature the less effective alkylated system lacks.

Using neutron reflectometry, we now experimentally examine this theory by probing the state of hydration of MEG-OH (glycol) vs. OTS-OH (alkyl) unimolecular silane coatings.
3. 2. 2. B. Neutron reflectometry: principle and experimental/model considerations

Neutron reflectometry (NR) is one of various techniques available to investigate the properties of water at surfaces. In practice, NR probes the specular reflection of neutrons off atomic nuclei and, being sensitive to scattering by light isotopes (i.e. H and D), indeed allows for the assessment of interfacial water density as well as film water absorptivity. An important part of NR analysis relies on computer modelling during which an experimental reflectivity curve is compared to simulated curves that assume a stratified structure for the substrate/surface chemistry under investigation. Data fitting for a selected ‘n-layer’ model is optimized by adjusting the thickness (d), interfacial roughness (σ) and scattering length density (SLD) of the various layers, and assessed for quality using the least-squares method ($\chi^2$).

Figure 94 schematically depicts the stratified model we chose to study the hydration of MEG-OH and OTS-OH silane adlayers. This model is composed of three main parts: (i) the Si/SiO$_2$ substrate; (ii) bulk water; and (iii) ‘Layer 1’, the latter treating the silane adlayer and transitional water as one single medium considering that ultrathin films such MEG-OH and OTS-OH are difficult to resolve experimentally. To enhance the scattering contrast of ‘Layer 1’, the technique of ‘contrast variation’ was used, wherein the SLD of bulk water was matched to that of the underlying SiO$_2$ substrate (Figure 94). This D$_2$O/H$_2$O solution is referred to as ‘contrast-matched water’ (CMW). Finally, to exploit the scattering of the distinctive oxide layer, MEG-OH and OTS-OH adlayers were not prepared on monolithic quartz but on oxidized silicon wafers (Si/SiO$_2$).

![Figure 94. Stratified model chosen to study surface hydration showing (from bottom to top) the Si/SiO$_2$ substrate, the silane adlayer (MEG-OH or OTS-OH) as well as transitional and bulk water. In the analysis of NR data, silane adlayer and transitional water are treated as one single medium (‘Layer 1’). Also shown are the thickness (d), roughness (σ) and scattering length density (SLD) of the various layers.](image-url)
3. 2. 2. C. Two distinct patterns of surface hydration\textsuperscript{440,441}

Experimental reflectivity curves (and corresponding fits) for MEG-OH and OTS-OH silane coatings are gathered in Figure 95, together with that of the unmodified Si/SiO\textsubscript{2} substrate. This latter curve is noticeably featureless (no fringe) demonstrating that no distinguishable ‘medium’ existed, in this case, between the SiO\textsubscript{2} layer and the ‘contrast-matched’ bulk water.

![Figure 95](image)

**Figure 95.** Reflectivity data measured in CMW and corresponding fits with $\chi^2$ values for bare (red circles/lines) as well as MEG-OH- or OTS-OH-modified (black circles/lines) Si/SiO\textsubscript{2} substrates.

**Figure 96** shows the corresponding SLD profiles generated upon adjustment of the various thickness, interfacial roughness and scattering length density parameters.

![Figure 96](image)

**Figure 96.** SLD profiles for MEG-OH (dashed red line) and OTS-OH (solid blue line) systems generated using the stratified model presented in Figure 94. Note: the Si wafer is not shown.
3.2.2. D. NR data interpretation

For the MEG-OH system, the low amplitude and smooth minima of the fringes – whose very existence revealed/confirmed the presence of a distinguishable layer sandwiched between the two contrast-matched media (i.e. SiO₂ and bulk CMW) – indicated that this intercalated medium was not well defined. The corresponding SLD profile showed the latter to be ~40 Å-thick with a minimum SLD of ~3.2 x 10⁻⁶ Å⁻² and to encompass ‘Layer 1’ (~20 Å) and an additional water gradient spanning over ~20 Å until bulk water resumed (Figure 96). With respect to ‘Layer 1’, the SLD value near the SiO₂ interface – which was very close to that of CMW (~3.3 x 10⁻⁶ vs. 3.54 x 10⁻⁶ Å⁻²) – strongly suggested that the MEG-OH adlayer absorbed water. This provided convincing proof for the existence of a special intrafilm zone of hydration involving the internal ether atom of oxygen in the MEG chains, which was previously hypothesized in the EMPAS study (Section 3.2.1.E.) and also supported by a recent account on water penetration into EG films. Equally as interesting is the long-range effect of this ultrathin MEG-OH adlayer, which was demonstrated by the existence of the additional, physically-distinct interphase of water following the SLD minimum (Figure 96).

To determine whether the internal ether atoms of oxygen played a role in the existence of the continuous transition zone of water stemming from within the MEG-OH adlayer, we next performed NR measurements with the OTS-OH system that lacks such feature (i.e. the internal ether atoms of oxygen in the chains, as shown in Figure 84). When comparing both reflectivity curves (Figure 95), it is immediately visible that the interaction of water with the OTS-OH adlayer was measurably different. In particular, the sharper, higher-amplitude fringes suggested in this case the existence of a better-defined intercalated medium now differing substantially from the surrounding, contrast-matched media. The effect is more evident in the corresponding SLD profile in Figure 96. For the OTS-OH system, NR data fitting revealed that transitional water also existed but was confined to ~20 Å and structured differently (minimum SLDs ~ 1.2 x 10⁻⁶ vs. 3.2 x 10⁻⁶ Å⁻²) than that of MEG-OH, with as well a much sharper transition towards bulk water (Figure 96). Another key difference is the SLD value near the SiO₂ interface (~2.0 x 10⁻⁶ Å⁻²), which now significantly deviates from that of CMW (3.54 x 10⁻⁶ Å⁻²), suggesting that the OTS-OH adlayer may be impenetrable by water.
For the OTS-OH system that lacks internal ether atoms of oxygen, water organization appeared to be interfacial in nature, with shorter-range but higher structuration up to the bulk water interface. This is to be compared/contrasted with the continuous, long-range and diffuse transition zone of water interpreted from NR data for the MEG-OH system. We propose in Figure 97 a schematic representation of these two different patterns of surface hydration.

![Figure 97. Distinct hydration patterns for MEG-OH and OTS-OH silane coatings on Si/SiO₂ proposed from the interpretation of NR data. Note: this schematic representation (not to scale) merely depicts MEG-OH and OTS-OH surface chemistry, not the actual surface coverage/patchiness of these adlayers nor the anchorage nature and degree of order/packing of the surface-modifying residues within.](image)

A final comment must be made regarding adlayer surface coverage since this parameter surely plays a role in (the state of) surface hydration, hence SLDs. Whether or not MEG-OH and OTS-OH adlayers present similar surface coverage, it is reasonable to assume that water would infiltrate a patchy assembly at defected/damaged sites. This argument can very well contribute in explaining the water-like SLD value computed for the MEG-OH coating (Figure 96), and the water absorption properties attributed to this system. This behaviour is to be contrasted with that...
of the impermeable OTS-OH coating, which as mentioned earlier displayed near the SiO$_2$ interface a SLD value substantially different from that of CMW. In this light, it is likely to be the case that the internal ether atoms of oxygen in MEG-OH, which OTS-OH lacks, were involved with maintaining embedded water molecules within the assembly through H-bonding – irrespective of whether the interactions occurred ‘intermolecularly’ in coated areas, or in interstitial, uncovered ones. In this regard, whether water reached down to the substrate (as depicted in Figure 97) and the latter participated in the instigation of the intrafilm zone of hydration is an interesting question we will provide an answer to in upcoming Section 3. 2. 3. F.

3. 2. 2. E. Concluding remarks

Neutron reflectometry revealed distinct hydration patterns between MEG-OH and OTS-OH silane coatings (on Si/SiO$_2$) and supported our previous hypothesis according to which the unique/pronounced antifouling properties observed for the MEG system in EMPAS experiments stemmed from a special intrafilm zone of hydration involving the key participation of the internal ether atom of oxygen in the chains, a feature the less effective alkylated system lacks. For MEG-OH, interpretation of NR data was consistent with the existence of a relatively thick, diffuse interphase of water stemming from within the adlayer. Conversely, this physically-distinct (from bulk) transition zone of water appeared to be thinner for the OTS-OH system and confined between the adlayer and bulk water. A key difference between both systems was in fact their ability to uptake water: while OTS-OH seemed to be impenetrable by water, MEG-OH presented hydrogel-like characteristics.

To gain further insight into the hydration of our ultrathin silane adlayers, we complemented our experimental neutron reflectometry study with a series of molecular dynamics simulations. This computational approach to investigate surface hydration is discussed next.
3. 2. 3. Computational study of surface hydration: molecular dynamics simulations

3. 2. 3. A. Systematic approach

As seen in previous Section 3. 2. 1., the connection between antifouling and surface hydration is a fascinating but daunting question to answer, which constitutes a topic of great current interest in both fundamental and applied physical chemistry. Following our EMPAS/TSM and NR experimental studies on the matter, we now use molecular dynamics (MD) computer simulations to try and gain a deeper understanding of the role of surface functionalities in the molecular-level structuration of water (surface kosmotropicity) – within and atop our, to various degrees, antifouling ultrathin organosilane adlayers depicted in Figure 84. As was the case for our original EMPAS work (Section 3. 2. 1. C.), we set out to perform the computational analysis of surface hydration in a methodical fashion, modifying step-by-step the chains’ chemical nature – from fully alkylated OTS to MEG-OH and MEG-OME monoethylene glycols through partly ‘oxygenated’ OTS-OH and O-OTS chains (Figure 98). This systematic comparative approach was intended to single out the influence each functional modification may have, but also to unravel any collaborative or antagonistic effects.

Figure 98. Systematic chain modification for MD simulation of surface hydration. Note: arrows do not represent chemical transformations/synthetic pathways.

Full-coverage coatings were modelled on α-quartz substrates and encompassed 5 × 5 molecular residues with a chain separation of ~0.49 nm and a lateral packing density of ~4.2

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viii In reality, organosilane coatings likely anchor to substrates as a mixture of silanol (Si-OH) and condensed siloxane (Si-O-Si) residues. MD simulations data (2 ns) for siloxane-based coatings are compiled in Appendix V.
residue/nm² (or ~6.9 x 10⁻¹⁰ mol/cm²),⁴⁴⁸,⁴⁴⁹ leaving reasonable room for chain motion and water embedment (the van der Waals diameter of water is ~0.3 nm).⁵²³,⁵²⁴ Unless otherwise noted, MD simulations (or ‘trajectories’) lasted > 30 ns, and the presented data are those computed for the 3 × 3 inner core of residues.

3. 2. 3. B. Water distribution

We began our computational study of surface hydration investigating whether and, if so, how water organizes within and atop the various adlayers. For this purpose, we used the ‘radial distribution function’ (RDF), which describes the probability of finding water molecules (in this case) organized at a certain distance from a reference (herein taken on the adlayers). On a ‘RDF vs. distance’ graph, this materializes as a distinct peak with proportional magnitude. In Figure 99, it is therefore immediately visible that only the fully alkylated OTS adlayer would be unable to constructively interact with water,⁵²¹,⁵²² as the pre-hydration EMPAS experiment presented in Section 3. 2. 1. C. 3. already hinted. In stark contrast, the MEG-OH adlayer would possess the highest likelihood to do so among the other coatings – in a ‘multilayered’ manner moreover.⁵²¹,⁵²² Additionally, we note that the OTS and MEG-OH adlayers, incidentally, displayed complete opposite antifouling behaviour when exposed to full serum (MEG-OH being vastly superior, as shown in Figure 86).

![Radial distribution function of water for the various silane adlayers on α-quartz. Note: the reference here is the full chain.](image)

**Figure 99.** Radial distribution function of water for the various silane adlayers on α-quartz. Note: the reference here is the full chain.
Determining the actual distribution of water within/atop an adlayer required however localized RDFs to be generated. Figure 100, which overlaps ‘innermost’ RDFs where the reference was taken at the base of the assemblies (see caption), revealed that adlayers lacking internal atoms of oxygen (i.e. OTS and OTS-OH) do not present peaks for water at ~2.5 and ~3.5 Å.231 In other words, water would not be able to penetrate into these alkylated adlayers. This was demonstrated empirically for the OTS-OH system in the NR study presented in Section 3. 2. 2. Conversely, O-OTS, MEG-OMe and MEG-OH adlayers would be able to absorb and organize water around their internal ether oxygen atoms (Figure 100),231 confirming the previously hypothesized key participation of these atoms in surface hydration (Section 3. 2. 2. E.). Also, with the exception of OTS,231,522 all adlayers would appear to possess the ability to coordinate interfacial water – to various extents – as the farther peaks at ~4.6 Å would suggest. Both water absorptivity and structuring properties were evidenced experimentally for the MEG-OH adlayer in the aforementioned NR study, wherein a continuous transition zone of water distinct from bulk was shown to stem from within the coating.

![Figure 100. Innermost radial distribution function of water for the various silane adlayers on α-quartz. Note: the reference here is the bottom five atoms (see Figure 98): C1-C5 for OTS and OTS-OH, and C1-C2-C3-O4-C5 for O-OTS, MEG-OH and MEG-OMe.](image)

As well, ‘outermost’ RDFs (Figure 101) clearly showed the ability for both OH-terminated MEG-OH and OTS-OH adlayers to arrange interfacial water in their direct vicinity (peaks at
This is again in accordance with our previous experimental NR data, which showed that interfacial water behaves differently than bulk water for both systems (Section 3.2.2).\textsuperscript{440} MEG-OMe, which exposes less hydrophilic methoxy groups (Figure 98), would be less prone to do so but would appear to interact in a slightly closer fashion (~2.6 vs. ~2.9 Å). Due to the radial nature of the distribution function, the probability discussed earlier of finding water organized within the MEG-OH adlayer (near the internal atoms of oxygen) is also computed in Figure 101, as the peaks located at ~3.3 (shoulder) and ~4.6 Å. Similar interpretation can be made for both MEG-OMe and O-OTS systems. Interestingly, (a fraction of) these peaks may also very well reflect longer-range structuration of water into the contiguous aqueous phase – especially for the MEG systems, which incidentally also appear to present an extra, smaller peak farther at ~7 Å. This long-range effect was shown experimentally in the case of MEG-OH (and OTS-OH) using NR, as described in Section 3.2.2. [Due to the aforementioned radial nature of the RDF and chain-chain separation of ~5 Å, it is likely that water ordering laterally within a MEG adlayer (as opposed to perpendicularly into the adjacent aqueous phase) also contributes to the smaller peaks at ~7 Å (Figure 101).] When comparing with the OH-terminated MEG-OH system, one cannot help but notice the profound impact a ‘mere’ methylation of the distal OH groups has on the water wetting, absorbing and structuring properties – not to mention on the antifouling behaviour – of the resulting MEG-OMe adlayer. Other substantial discrepancies are discussed hereafter.

![Figure 101. Outermost radial distribution function of water for the various silane adlayers on α-quartz. Note: the reference here is the top two non-hydrogen atoms (see Figure 98): C7-C8 for OTS and O-OTS, C6-O7 for OTS-OH and MEG-OH, and O7-C8 for MEG-OMe.](image-url)
From solely analyzing water RDFs, it can be confidently concluded that: (i) only the adlayers possessing internal ether atoms of oxygen (MEG-OH, MEG-OMe and O-OTS) are prone to absorb and arrange water within the assembly; and (ii) OH-terminated adlayers (MEG-OH and OTS-OH) have high(er) probability to organize interfacial water, whether or not they are of the MEG variety. Comparing these simulated RDF data of surface hydration with observed (anti)fouling behaviour using the EMPAS, it appears that the ability for an adlayer to organize interfacial water is important, only if water coordination also occurs internally within the assembly. Similar conclusions were drawn by others working with oligoether thiol SAMs. We continue the analysis of surface hydration, and of its link to antifouling, with respect to another important parameter: energy.

3.2.3. C. Adlayer energy

3.2.3. C. 1. In the presence of water (wet state)

Table 6 gathers the adlayers’ chain-chain packing energy and chain-water solvation energy, with their corresponding electrostatic and van der Waals (VDW) components. As expected, the fully alkylated OTS system, which was essentially shown to be unable to either absorb or adsorb water (Figures 99-101, Table 6 – E2),\textsuperscript{231,522} is almost exclusively stabilized through chain-chain interactions of VDW nature (E1 & 3). The addition of an internal atom of oxygen in water-permeable O-OTS slightly decreases interchain stabilization (−10.9 vs. −13.5 kcal/mol, E7 & 1) but allows for favourable, largely counterbalancing chain-water interactions to take place (E8 & 9). These, of electrostatic nature, presumably are the signature of water engaging in H-bond interactions with the internal oxygen atoms. Both phenomena greatly intensify with the incorporation of a second (distal) ether atom of oxygen as seen when comparing MEG-OMe with O-OTS (−1.4 vs. −10.9 and −31.6 vs. −11.3 kcal/mol, respectively – E13 & 7 / E14 & 8), the net effect on the system energy still being a beneficial one (−33.0 vs. −22.2 kcal/mol, E15 & 9). Such an antagonistic effect of hydration is exacerbated in the case of MEG-OH (Table 6 – E10 & 11 vs. E13 & 14) – which overall turns out to be the most stable of all systems (−71.3 kcal/mol, E12) – and peaks for OTS-OH (E4 & 5). We tentatively hypothesize chain-chain destabilization to reflect the occurrence of repulsive dipole-dipole (e.g. C–O–C, C–O–H) interactions induced in the assembly as a result of adlayer distortion\textsuperscript{235} due to water embedment and H-bonding with the various internal and/or distal oxygen atoms. We note that, in the absence of water in the ‘dry’ state, chain-chain interactions are comparatively more favourable (Table 7 – E4, 10 & 13 vs. Table 6 – E4, 10 & 13).
Table 6. Computed chain-chain and chain-water interaction energies. *Calculated over 2 ns for 500 frames (4 ps sampling). Standard deviations are time- and structure-averaged.

<table>
<thead>
<tr>
<th>Adlayer</th>
<th>Entry</th>
<th>Type of interaction</th>
<th>Energy (kcal/mol)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Electrostatic</td>
</tr>
<tr>
<td>OTS</td>
<td>E1</td>
<td>chain-chain</td>
<td>−0.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>E2</td>
<td>chain-water</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>E3</td>
<td>Total</td>
<td>0.0 ± 0.4</td>
</tr>
<tr>
<td>OTS-OH</td>
<td>E4</td>
<td>chain-chain</td>
<td>35.5 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>E5</td>
<td>chain-water</td>
<td>−89.0 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>E6</td>
<td>Total</td>
<td>−53.5 ± 5.5</td>
</tr>
<tr>
<td>O-OTS</td>
<td>E7</td>
<td>chain-chain</td>
<td>−0.7 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>E8</td>
<td>chain-water</td>
<td>−8.8 ± 6.6</td>
</tr>
<tr>
<td></td>
<td>E9</td>
<td>Total</td>
<td>−9.5 ± 7.8</td>
</tr>
<tr>
<td>MEG-OH</td>
<td>E10</td>
<td>chain-chain</td>
<td>17.0 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>E11</td>
<td>chain-water</td>
<td>−85.2 ± 8.8</td>
</tr>
<tr>
<td></td>
<td>E12</td>
<td>Total</td>
<td>−68.2 ± 9.7</td>
</tr>
<tr>
<td>MEG-OMe</td>
<td>E13</td>
<td>chain-chain</td>
<td>8.2 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>E14</td>
<td>chain-water</td>
<td>−30.3 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>E15</td>
<td>Total</td>
<td>−22.1 ± 5.5</td>
</tr>
</tbody>
</table>

3. 2. 3. C. 2. Surface dehydration energy: wet vs. dry state

More information, notably with respect to the overall stabilizing effect of hydration, can be extracted from calculating energy values for adlayers in the ‘dry’ state (Table 7), wherein MD simulations look at adlayer systems in vacuum (i.e. in the absence of water). Better yet – despite the admittedly hypothetical nature of this state – simulating such ‘dry’ films also allows to calculate an ‘energy of surface dehydration’\(^{38,73}\) (its upper limit), a determining parameter in the process of protein adsorption. Thus, little stabilization through hydration was computed for OTS (−2.1 kcal/mol, Table 7 – E1-3), as expected for such a fully alkylated system that was shown earlier to barely interact with water (Figures 99-101 and Table 6 – E2). Stabilization increasing along with chain oxygen content and adlayer interactivity with water [from −2.1 to −12.6 to −23.6 kcal/mol for respectively OTS, O-OTS and MEG-OMe (Table 7 – E3, 9 & 15)] was also not surprising as chain-water interactions are favourable ones (Table 6). The largest stabilizing
The effect of hydration was computed for the OH-terminated OTS-OH and MEG-OH systems (< −62 kcal/mol, E6 & 12). Interestingly, these MD simulations entertained the idea that any surface dehydration process – for example during protein adsorption\textsuperscript{32,38,47,522,525} – would be an expensive one thermodynamically.\textsuperscript{38,73,91,166,231} In this regard, we remark from Table 7 that it would be considerably less difficult to (fully) dehydrate MEG-OMe than MEG-OH adlayers (E15 vs. E12), explaining well why MEG-OMe coatings are more fouled than MEG-OH ones (Figure 86).

<table>
<thead>
<tr>
<th>Adlayer</th>
<th>Entry</th>
<th>State</th>
<th>Energy (kcal/mol)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Electrostatic</td>
</tr>
<tr>
<td>OTS</td>
<td>E1</td>
<td>Dry</td>
<td>−0.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>E2</td>
<td>Wet</td>
<td>0.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>E3</td>
<td>(E2 − E1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>OTS-OH</td>
<td>E4</td>
<td>Dry</td>
<td>8.5 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>E5</td>
<td>Wet</td>
<td>−53.5 ± 5.5</td>
</tr>
<tr>
<td></td>
<td>E6</td>
<td>(E5 − E4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>−62.0 ± 5.8</td>
</tr>
<tr>
<td>O-OTS</td>
<td>E7</td>
<td>Dry</td>
<td>−1.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>E8</td>
<td>Wet</td>
<td>−9.5 ± 7.8</td>
</tr>
<tr>
<td></td>
<td>E9</td>
<td>(E8 − E7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>−8.0 ± 7.8</td>
</tr>
<tr>
<td>MEG-OH</td>
<td>E10</td>
<td>Dry</td>
<td>0.9 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>E11</td>
<td>Wet</td>
<td>−68.2 ± 9.7</td>
</tr>
<tr>
<td></td>
<td>E12</td>
<td>(E11 − E10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>−69.1 ± 10.0</td>
</tr>
<tr>
<td>MEG-OMe</td>
<td>E13</td>
<td>Dry</td>
<td>−2.3 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>E14</td>
<td>Wet</td>
<td>−22.1 ± 5.5</td>
</tr>
<tr>
<td></td>
<td>E15</td>
<td>(E14 − E13)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>−19.8 ± 6.3</td>
</tr>
</tbody>
</table>

Table 7. Computed energy for adlayers in the dry and wet state. *Calculated over 2 ns for 500 frames (4 ps sampling). Standard deviations are time- and structure-averaged. Note: energy values for the wet state are retrieved from Table 6.
All these energy values, together with the previous RDF profiles, allowed for a better understanding of surface hydration and its link to antifouling to emerge. Notably, the ability for (i) an adlayer to coordinate water both internally and interfacially (MEG-OH and MEG-OMe vs. OTS-OH, O-OTS and OTS);\textsuperscript{241} and (ii) the resulting hydrated structure to exhibit a large energy of dehydration (MEG-OH vs. MEG-OMe),\textsuperscript{38,73,229,525,526} appeared to be determining prerequisites, only the unique MEG-OH system would be able to satisfy.

3.2.3. D. Adlayer flexibility

Another parameter habitually discussed to rationalize the protein-repellent properties of surfaces is flexibility/compressibility.\textsuperscript{32,47,135,525} Herein, adlayer flexibility was assessed through calculation of the chains’ root-mean-square fluctuation (RMSF). Computations revealed that there is little to no statistical difference in terms of flexibility between adlayers (Table 8) and that this parameter, if any, would only play a secondary role to hydration in the antifouling mechanism. Indeed, as can be seen in Table 8, the less antifouling MEG-OMe system (Figure 86) displays higher flexibility than MEG-OH (RMSF = 1.95 vs. 1.44 Å) – that is it would offer more conformational degrees of freedom to deform when compressed by adsorbing proteins (decreasing entropy)\textsuperscript{47} – however, as discussed earlier, it is less prone to interacting with and retaining water molecules. The non/less-influential role of adlayer flexibility was to be expected for such ultrathin structures made of short EG residues, for which antifouling is indeed believed to be primarily governed by chain hydration.\textsuperscript{32,47,135,203,525} Another example would come from comparing the antifouling, water-permeated MEG-OH adlayer with the ‘non-antifouling’, inappropriately- or non-hydrated OTS-OH and OTS ones,\textsuperscript{228} all of which essentially present identical flexibility (1.44, 1.48 and 1.53 Å – Table 8).

<table>
<thead>
<tr>
<th>Adlayer</th>
<th>Flexibility (Å)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTS</td>
<td>1.53 ± 0.46</td>
</tr>
<tr>
<td>OTS-OH</td>
<td>1.48 ± 0.27</td>
</tr>
<tr>
<td>O-OTS</td>
<td>1.79 ± 0.35</td>
</tr>
<tr>
<td>MEG-OH</td>
<td>1.44 ± 0.27</td>
</tr>
<tr>
<td>MEG-OMe</td>
<td>1.95 ± 0.23</td>
</tr>
</tbody>
</table>

Table 8. Computed adlayer flexibility. *Assessed using the root-mean-square fluctuation method. RMSF values are calculated over all adlayer non-hydrogen atoms.
3.2.3. E. Water dynamicity

We now turn our attention to another very important aspect of surface hydration: water dynamicity (lability and mobility). When viewing the trajectory generated for the best antifouling MEG-OH system, we observed that multiple molecules of water could simultaneously absorb around the internal ether atoms of oxygen (Figure 102), and remain trapped in such an ultrathin structure for several nanoseconds with limited mobility (assessed through calculation of positional RMSF). [Similar observations were made for MEG-OMe\textsuperscript{235} and O-OTS systems.]\textsuperscript{ix} Astonishingly, the highest residency time was over 27 ns (RMSF = 0.5 ± 0.4 Å), which corresponds to an eternity at the molecular level. This unanticipated nanosecond timescale is in fact of the kind that would rather be expected for water molecules caged in superficial protein clefts.\textsuperscript{527-529} Absorbed water displayed an assortment of H-bonding interactions, as can be seen in Figure 102. A single molecule of water could either bridge two neighbouring internal or distal atoms of oxygen, or a combination thereof (Figure 102, A-C). Situations were also found where internal ether and distal hydroxyl moieties in a cis-arrangement (\emph{i.e.} from a same EG unit) would act in tandem to intramolecularly ‘pinch’ a water molecule,\textsuperscript{231,240,526} well into the nanosecond regime as well (~3 ns, RMSF ~ 0.7 Å). In this regard, literature teaches us that chain conformation, and the level of freedom associated with it, have also been proposed to play a role in the degree and strength of chain/surface hydration.\textsuperscript{239,240,518,526} Accordingly, the multi-conformational, amorphous nature of the MEG-OH coating seen in Figure 102 may constitute one of the reasons why this film would be able to maintain such a stable and extensive state of hydration.\textsuperscript{239,240,518,526} Sharing of oxygen binding sites, whether of the internal or distal nature, between water molecules was also observed (Figure 102, D & E). Closer to the top of the film, interfacial water was more labile (~0.5 ns, RMSF ~ 0.8 Å). In this interphase, some molecules could also be found hovering over several residues for ~0.3 ns.\textsuperscript{228} Their mobility, as a result, was greater (RMSF ~ 3.2 Å). Finally, bulk water molecules diffusing freely (rapidly exchanging positions on a picosecond scale) were expectedly the most motile (RMSF > 10 Å).\textsuperscript{227,228} Not surprisingly, water lability and mobility correlated with burial depth and outward distance from the adlayer.\textsuperscript{230} Recalling that the release of water would energetically be an expensive process – as proposed earlier herein (Section 3.2.3. C. 2.) and elsewhere\textsuperscript{32,38,73,91,166,229,522,525,526} – one can understand how these stably-bound molecules of water with limited dynamicity would play a crucial role in the endowment of MEG-OH with antifouling properties. We note in this regard that interfacial water molecules in the

\textsuperscript{ix} Rare molecules of water could also be found penetrating the OTS-OH assembly for ~0.5 ns (RMSF ~ 0.8 Å).
case of the less antifouling MEG-OMe adlayer were generally more dynamic than those interacting with MEG-OH (~0.2 vs. 0.5 ns and RMSF ~2.3 vs. 0.8 Å).\textsuperscript{47,228,229,522}

\textbf{Figure 102.} Coordination of explicit water within the $3 \times 3$ residue core of the MEG-OH assembly (top view snapshot). Shown are the various possible H-bond interactions of water with the internal ether or/distal hydroxyl moieties. A, B, and C illustrate multi-site, interchain interactions; while D and E show water molecules sharing a single binding site. Not present in this frame, however, is the interaction of intramolecular nature, wherein a molecule of water bridges both internal and distal oxygen atoms of a same EG unit (or interactions involving water as the H-bond acceptor). \textit{Note:} for clarity, the oxygen atoms from the substrate and silanols, in addition to the hydrogen atoms from the residues, are not represented. The atom colour code is as follows: yellow/shaded (silicon), blue (carbon), red/pink (oxygen), and white (water’s hydrogen).

\textbf{3. 2. 3. F. Substrate effect / MEG-OH ‘nanogel’}

A closer look at the water network within the MEG-OH assembly is rich in information. Specifically, it was observed that water molecules can H-bond with the deeply buried silanol moieties in an interaction that also involves the internal ether oxygen of a neighbouring residue (\textbf{Figure 103A} – bottom situation). As such, this MEG-OH chain is compelled to adopt a kinked, semi-helical \textit{cis}-arrangement that locks the distal end. In this configuration, the terminal OH group is properly positioned to bind to another molecule of water, together with the internal ether oxygen of the adjacent chain (\textbf{Figure 103A} – top situation). The effect further propagates with the arrival of other molecules of water (\textbf{Figure 103B}). Such water clustering might very well explain the magnitude of the energy of surface dehydration computed for MEG-OH (\textbf{Table 7 – E12}) and the unexpectedly-long lifetime of water molecules confined within this assembly
(Figure 103B) – that is it might explain the surprising ability of this ultrathin adlayer to maintain such a tight, nanoscale layer of hydration.

**Figure 103.** Water clustering within the MEG-OH assembly. (A) The bottom molecule of water bridges neighbouring chains through the silanol and internal ether moieties. This locked configuration provides an environment favourable for the interaction of a second (top) bridging molecule of water. (B) Water clustering propagates as other molecules arrive. The overall effect is an unexpectedly-long residency time, well into the nanosecond regime, for these water molecules. *Note:* these lifetimes, which not surprisingly increase with burial depth, overlap. The atom colour code is as follows: yellow/shaded (silicon), blue (carbon), red/pink (oxygen), and white (water’s hydrogen).
We note that water clustering would, in practice, also be triggered by the silica substrate (SiO$_2$) at defected/damaged spots,$^{520}$ in which case partial adlayer surface coverage should be beneficial and thoughtfully considered in the development of antifouling coatings for such and other hydroxylated/oxide material surfaces. Regardless of whether water seeding is residue or substrate silanol-mediated, we speculate the end result to be an overall strengthening of an expanded water network, wherein the MEG-OH chains would act as molecular pillars around which water clusters could nucleate and grow.$^x$ An equivalent in the macroscopic world would be how reinforced concrete settles and structures around a supporting mesh of steel. In some aspects, the MEG-OH system resembles hydrogels.$^{531}$

3.2.3 G. Hydration in the antifouling mechanism: a molecular-level rationalization

The parameters studied in our MD simulations are interconnected in a subtle overall manner, which renders the rationalization of the antifouling mechanism with respect to surface hydration difficult. Nonetheless, basic requirements have appeared (Figure 104). Firstly, adlayers must display both internal and interfacial hydrophilicity$^{241}$ and kosmotropicity (the ability to structure water at the molecular level).$^{231}$ Secondly, hydration water must be tightly bound$^{47,135,231,522,525}$ with limited dynamicity (lability and mobility).$^{521}$ In contrast, we assign no significant role to entropy linked to adlayer flexibility/compressibility.$^{47}$ This antifouling mechanism, for ultrathin structures made of short EG residues, concurs with the one generally invoked in the literature,$^{47,135,525}$ and accounts for the uniqueness of the MEG-OH system. These attributes all together create a robust hydration network, whose disturbance by adsorbing proteins would constitute a penalty in terms of energy and result in repulsive forces.$^{228,234,243,521,522}$ To add a kinetic component to the picture, proteins would not be able to reside at close proximity long enough$^{47}$ to unfold and conformationally adopt the adequate geometry to fit the surface, optimize the energy of interaction and irreversibly adsorb.$^{12}$ As mentioned in Section 1. 5. 2., this process would be accompanied by the release of water from the proteins hydration shell.$^{38,73}$ From the perspective of both the surface and proteins, dehydration plays a major role in the mechanism of antifouling.

$^x$ Quartz also displays water structuring properties$^{530}$ but does not benefit from the strengthening effect on the water network provided by the MEG-OH support chains. This could explain why quartz presents a surface that is easily fouled by serum proteins in comparison to the MEG-OH coating (Figure 86).
Before concluding this computational study of surface hydration, the results of another very informative series of MD simulations will be presented below, wherein the central MEG-OH residue was replaced with an OEG-TTTA linker residue (Figure 105 – top vs. bottom right). In terms of water distribution, this substitution had a modest but notable impact on the ‘outermost’ RDF profile (Figure 105 – solid lines). Little effect, however, was observed for its ‘innermost’ counterpart (Figure 105 – dashed lines). As the lower section of the OEG-TTTA chain matches neighbouring MEG-OH residues (Figure 105 – bottom right), such little discrepancy was not surprising. As for dynamicity, water molecules within the mixed assembly were also observed to reside and cluster for several nanoseconds with limited mobility (e.g. ~12 ns with RMSF = 1.00 ± 0.97 Å; ~10 ns with RMSF = 0.64 ± 0.53 Å).

**Figure 104.** Antifouling and surface hydration: basic requirements.

### 3. 2. 3. H. Mixed assembly hydration

Before concluding this computational study of surface hydration, the results of another very informative series of MD simulations will be presented below, wherein the central MEG-OH residue was replaced with an OEG-TTTA linker residue (Figure 105 – top vs. bottom right). In terms of water distribution, this substitution had a modest but notable impact on the ‘outermost’ RDF profile (Figure 105 – solid lines). Little effect, however, was observed for its ‘innermost’ counterpart (Figure 105 – dashed lines). As the lower section of the OEG-TTTA chain matches neighbouring MEG-OH residues (Figure 105 – bottom right), such little discrepancy was not surprising. As for dynamicity, water molecules within the mixed assembly were also observed to reside and cluster for several nanoseconds with limited mobility (e.g. ~12 ns with RMSF = 1.00 ± 0.97 Å; ~10 ns with RMSF = 0.64 ± 0.53 Å).
Figure 105. Innermost and outermost (dashed and solid lines) radial distribution functions of water for unimolecular MEG-OH (blue) and mixed OEG-TTTA/MEG-OH (red) assemblies. Note: the reference for ‘innermost’ RDFs is the bottom five atoms (C1-C2-C3-O4-C5). The reference for ‘outermost’ RDFs is the top two non-hydrogen atoms (C6-O7) for the MEG-OH residues, and the corresponding two C6-O7 middle atoms for the central OEG-TTTA residue in the mixed assembly.

Computing water RDF around the TFE ester head group was also rich in information as it revealed that the probability for water to closely organize in this region is low (Δa.u. ~ 0.1 at ~3.5 Å), as can be seen in Figure 106. The hydrophobic CF₃ group is likely responsible for such an effect/observation. [This peak at ~3.5 Å is probably due to hydration water around the lower, protruding EG section of the OEG-TTTA residue. The hydrated MEG-OH background is most likely responsible for the other two peaks observed farther at ~5 and ~7.5 Å.] Recalling that protein repellence may be intimately linked to the ability of a coating to tightly coordinate and structure interfacial (and embedded) water molecules, as discussed in Section 3. 2. 3. G., the existence of such a low-structured pocket of water around the terminal TFE ester group would perhaps explain why proteins (i.e. albumin) display high affinity for TFE-terminated mixed self-assembled monolayers. This would also very well support our related hypothesis of Section 3 1. 2. B. 1. according to which unreacted TFE ester groups would act as nucleation spots for protein fouling.
Figure 106. Radial distribution function of water around the TFE ester head function of the central OEG-TTTA residue in the mixed assembly. Note: the reference is the terminal eight atoms (C13-O13-O14-C15-C16-F16/1-F16/2-F16/3).

Finally, using the RMSF method, it also appeared that the chains in the OEG-TTTA/MEG-OH mixed assembly would be, on average, slightly more flexible than those in the unimolecular MEG-OH adlayer (RMSF = 2.04 ± 0.40 vs. 1.44 ± 0.27 Å). Interestingly, when singled out in the RMSF calculation, the central OEG-TTTA chain displayed higher flexibility (RMSF = 3.03 ± 0.91 vs. 2.04 ± 0.40 Å). This residue protruding unhindered from the shorter, surrounding MEG-OH-packed background is a likely reason for such a result. Whether a sweeping motion exists that would create an ‘exclusion volume’ over the surface (Figure 107) preventing proteins from adsorbing (decreasing NSA) is unclear however. So would be: (i) the effect on probe loading content and distribution (which could be expected to be amplified for longer, probe-functionalized linker residues); and (ii) the contribution to antifouling by the loss in entropy due to the compression of this flexible chain by incoming proteins. These issues are relevant to our earlier biosensor studies with OEG-TTTA/MEG-TFA mixed adlayers and biomolecular probes (Section 3.1.).
Figure 107. Schematic representation of the half sphere-shaped ‘exclusion volume’ hypothesized to contribute in preventing protein adsorption.

3.2.3.1. Concluding remarks

Surface hydration constitutes a cornerstone in the mechanism traditionally postulated to rationalize the antifouling/protein-repellent properties of thin coatings made of short EG residues. In this section, following our EMPAS and NR experimental studies on the matter, we used MD computer simulations to gain further insight into the role of surface hydration in the remarkable antifouling properties previously observed for MEG-OH adlayers. A methodical approach – wherein several related organosilane adlayers displaying varied chemical functionalities and antifouling behaviour were systematically simulated – allowed for crucial prerequisites to emerge, those which make the MEG-OH system unique over all others. Notably, it was shown using the radial distribution function that both internal and interfacial hydrophilicity and kosmotropicity (the ability of a surface to structure water at the molecular level) must be displayed, in agreement with conclusions previously drawn in the literature. In contrast, adlayer flexibility – as a gauge of compressibility – was assigned, if any, a secondary role in the antifouling mechanism of such ultrathin structures made of short building blocks for which the role of entropy linked to chain flexibility/compression is believed to be less significant. The antifouling mechanism was also discussed in terms of surface dehydration energy and water dynamicity (lability and mobility), and notably the crucial requirement for clustered water molecules to remain tightly bound for extensive periods of time (i.e. exhibit slow exchange dynamics). In this regard, a contribution of the substrate to surface hydration – which
would also participate in endowing antifouling adlayers with hydrogel-like characteristics – was also proposed and might very well be behind the unique antifouling properties of the MEG-OH system. All things considered, the release of water from such a hydrated adlayer during protein adsorption would constitute a penalty in terms of energy.

The pronounced antifouling behaviour against a complex biological medium such as serum is only one remarkable feature of MEG-OH surface chemistry. As will be described next, ultrathin MEG-OH coatings also exhibit outstanding antithrombogenicity. By performing new real-world experiments with whole blood as opposed to cell-cleared serum, the highest level of bodily fluid complexity with which to challenge MEG-OH coatings was reached. This study, with which we set foot into the not necessarily related\textsuperscript{xi} realm of biomaterial hemocompatibility, was also the occasion to demonstrate once more – after quartz and gold (Section 3. 2. 1.) – the versatility of MEG-OH chemistry through the modification of the surface of another biotechnologically important material: plastic.

\textsuperscript{xi} As argued in reference 40, ‘antifouling’ refers to the ability of a material surface to prevent biological (protein) adsorption and accumulation. Subtly, on the other hand, ‘hemocompatible’ (antithrombogenic in the present case) refers to the ability of a material not to trigger a biological process/response (\textit{i.e.} thrombosis) upon surface-induced activation of adsorbed proteins. These are two different, not necessarily related, surface properties.
3. 2. 4. Antithrombogenicity of MEG-OH surface chemistry

3. 2. 4. A. Context and motivation

Contact of blood with artificial materials made of titanium, stainless steel, ceramics or plastic polymers is very common in many aspects of modern medicine. Whether applied in vivo or in vitro, the range of equipment, implant and devices is immense and includes – for instance – catheters, stents, pacemakers, and the circuitry used for extracorporeal circulation during dialysis or coronary bypass surgery. As seen in introductory Sections 1. 4. & 1. 6. however, the interaction of blood components (i.e. proteins) with exogenous surfaces may potentially stimulate deleterious biological processes orchestrated by the immune and coagulation systems; and post-operative complications, unfortunately, become a real issue. Incapacitating neuropsychological brain disorder (e.g. cognitive impairment) would be an example of relative severity. To avoid having to ‘trade’ a medical condition with another, the search for hemocompatible materials has been and continues to be, understandably, the object of intense efforts.

In contemporary society, synthetic plastic polymers are ubiquitous and may/have replaced the conventional materials that were wood, metal and glass in nearly all imaginable applications. Polycarbonates (PC), more often than not based on bisphenol A (BPA), are not spared from this trend and actually rank among the fastest prospering plastic polymers. This also is true in the biomedical industry where thermoplastic BPA-PC resins are highly praised for their many attractive properties. Commercial BPA-PC for a given healthcare application is required to be certified ‘biocompatible’ (i.e. ‘medical-grade’) by suppliers in compliance with governmental regulations/international standards (ISO 10993). It should not come as a surprise however that, for diverse reasons, there always exists room for improvement/innovation in this domain; and that novel, permanent surface modification strategies are always welcome.

It is in this context that we set out to derivatize the surface of BPA-PC plastic substrates with MEG-OH silane chemistry (Figure 108) and expose the resulting coatings to whole blood.

\[\text{xii} \quad \text{Recurrently raised however with this variety of PC plastic is the question of BPA leaching from the polymer, which constitutes a societal concern since BPA has well characterized endocrine-disrupting activity with strongly suspected effects on human health. However, considering that BPA contamination comes from prolonged/repeated contact with the polymer (mainly from food packaging), BPA leaching from single-use, disposable biomedical equipment is not likely to constitute a significant health threat since exposure in this case is generally limited in time.}\]
in order to assess their antithrombogenic properties. It is important to point out that this work by no means constitutes the first example of chemical surface modification of BPA-PC to improve antithrombogenicity since one strategy, bio-inspired, has already been described elsewhere.\textsuperscript{139,542}

In that work,\textsuperscript{139} the authors mimicked the glycocalyx – the outer surface of biological cell membranes that consists of a highly hydrated carbohydrate-rich mesh\textsuperscript{209,210} – with a polysaccharide coating (dextran) to reduce platelet adhesion on medical-grade Lexan, a BPA-based PC plastic polymer.\textsuperscript{543} We, conversely, employ non-mimetic, ultrathin MEG silane surface chemistry – the first account of its kind to our knowledge.

![Figure 108. Schematic representation of the two-step, straightforward surface modification of bisphenol A polycarbonate (BPA-PC) plastic polymer with MEG-OH silane surface chemistry.](image)

### 3. 2. 4. B. Adlayer preparation and characterization\textsuperscript{443}

Surface modification of BPA-PC plastic with MEG-OH silane chemistry was as straightforward as that of quartz presented in Section 3. 2. 1. B. MEG-TFA adlayers were first readily prepared in a simple dip-and-rinse procedure upon immersion of cleaned BPA-PC substrates – pre-activated upon exposure to a plasma of highly reactive ionized/radical oxygen species to generate surface hydroxyls\textsuperscript{544} – into a 1/1000 (v/v) solution of MEG-TFA surface modifier in hexanes, for 1h at room temperature (Figure 108 – step I). Subsequent solvolysis of the labile TFA terminal groups – with a 1/1 (v/v) solution of 95% ethanol and water, overnight – readily provided the desired MEG-OH coating (Figure 108 – step II). Both successful transformations were characterized with XPS following the appearance/loss of peaks for fluorine (F\textsubscript{1s} at 688 eV) and silicon (Si\textsubscript{2p} at 103 eV), the two elements unambiguously attributable to MEG-TFA/MEG-OH adlayers (Figures 108 and 109, Appendix W). Unlike fluorine however – whose peak completely disappeared – the signal for silicon was not affected by the subsequent solvolytic treatment (Figure 109, Appendix W), clearly demonstrating that the latter had
effectively cleaved all terminal TFA groups without etching the residual MEG-OH coating from the BPA-PC substrate (see also Section 3.2.1.B.). If not a proof of chemisorption – i.e. of strong covalent anchorage to the substrate (Figure 108) – this certainly is an indicator of great robustness for the MEG-OH adlayer, a definitely desirable attribute for biomaterial coatings.

**Figure 109.** XPS surveys for (top to bottom) plasma-activated bare BPA-PC (red), as well as MEG-TFA (blue) and MEG-OH (green) adlayers.

With respect to carbon, the XPS signal decreased substantially upon formation of the MEG-TFA adlayer (Figure 109, Appendix W) – as expected for a now buried, underlying plastic substrate from which most of the carbon signal originates. Looking closer at its narrow scan (Figure 110), it can be seen that – depending on the type of surface – the C$_{1s}$ peak is actually composed of up to four different kinds of carbon bonds: C-C/C=C (and C-Si), C-O, C=O, and C-F. Expectedly (Figure 108), bare BPA-PC only shows XPS peaks for C-C/C=C (at 285 eV), C-O (at 287 eV), and C=O (at 291 eV) bonds. For the MEG-TFA adlayer, two new peaks characteristic of the TFA group appeared (C=O at 290.5 eV and C-F at 293.5 eV – Figure 110). Also, the relative intensity of the C-O signal at 287 eV significantly increased as a result of the incorporation of multiple MEG backbones with several ether bonds within the newly formed coating (Figures 108 and 110). We note as well that, as anticipated, the C=O peaks for the carbonate moiety of the substrate and the TFA ester moiety of the MEG-TFA coating have slightly different binding energies (291 vs. 290.5 eV, respectively), and actually overlap in the
C\textsubscript{1s} narrow scan of the MEG-TFA adlayer (Figure 110). Upon TFA solvolysis to form MEG-OH, both C-F and C=O peaks (at 290.5 eV) completely disappeared whereas the C-O signal from the MEG backbones remained untouched (Figure 110) confirming that only the TFA terminal groups were cleaved during the aqueous treatment, as concluded earlier from the observation of the Si\textsubscript{2p} signal. Also, we note here that the substrate’s C=O signal at 291 eV is the only ‘carbonyl’ peak remaining upon conversion of MEG-TFA to MEG-OH (expectedly attenuated as well, compared to bare BPA-PC). Overall, these XPS data for BPA-PC surface modification with MEG-TFA/MEG-OH silane chemistry are well in line with those obtained in the aforementioned study with quartz substrates (Section 3. 2. 1. B.).

![Figure 110](image)

**Figure 110.** Overlapped C\textsubscript{1s} narrow scans for bare (red), MEG-TFA-modified (blue) and MEG-OH-modified (green) BPA-PC surfaces showing the various C-C/C=O (and C-Si), C-O, C=O, and C-F bond contributions and relative compositions. **Note:** the takeoff angle is 90° relative to the surface (bulk analysis).

Both stages of surface modification (MEG-TFA and MEG-OH adlayers) as well as the bare BPA-PC surface were further characterized with static contact angle goniometry (CAG) using water as the test liquid (Table 9). As received (i.e. non-cleaned and non-activated), bare BPA-PC presented a rather hydrophobic surface with a contact angle (CA) of 94 ± 3°, n = 3. Upon plasma cleaning and activation, the CA significantly decreased to 45 ± 1° (n = 3), a value consistent with the formation of a more hydrophilic surface presenting hydroxyls and other polar groups.\textsuperscript{544,546}
Pronounced hydrophobicity (CA = 85 ± 2°, n = 5) was displayed by the MEG-TFA coating presumably owing to the exposure of CF₃ groups to the outer environment. [This CA value is consistent with that previously measured for MEG-TFA adlayers on quartz (CA = 90° – Figure 85).] Finally, as expected upon removal of these hydrophobic CF₃ moieties during TFA solvolysis to form polar hydroxyls in MEG-OH coating, some hydrophilicity was recovered as the decreased CA value revealed (CA = 55 ± 4°, n = 3). Overall, these supplementary CAG data match well with the expected changes in wettability associated with the various modifications of surface functionalities.

<table>
<thead>
<tr>
<th>BPA-PC surface</th>
<th>Static contact angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare (as received)</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>Bare (plasma-activated)</td>
<td>45 ± 1</td>
</tr>
<tr>
<td>MEG-TFA adlayer</td>
<td>85 ± 2</td>
</tr>
<tr>
<td>MEG-OH adlayer</td>
<td>55 ± 4</td>
</tr>
</tbody>
</table>

Table 9. Static contact angle values for bare BPA-PC (non- and plasma-activated) as well as MEG-TFA and MEG-OH adlayers, measured with water as the test liquid.

3.2.4. C. Antithrombogenicity assessment

Antithrombogenic properties were next evaluated in real time using a perfusion chamber and whole human blood, labelled with cell-permeant 3,3′-dihexyloxacarbocyanine iodide (DiOC₆) fluorescent dye, dispensed for 5 min at a (high) shear rate of 1000 s⁻¹. These conditions of hemodynamics provided an environment favourable for platelet accumulation/thrombus growth and, hence, appropriate to assess the (short-term) antithrombogenic properties of our exogenous MEG-OH coating. In practice, our experimental set-up (see Section 2.5.6.) allowed to record videos from which it was possible to extract and process frames to access surface coverage due to platelet adhesion, aggregation and thrombus formation.

As a positive control, in order to test our experimental protocol, we first exposed blood to BPA-PC pre-coated with type I collagen, a protein revealed on the wall of injured blood vessels that triggers coagulation (see Section 1.6.1.). As can be seen in Figures 111A and 111B, this
control experiment was successful since thrombosis on these collagen surfaces was quite pronounced with a surface coverage calculated to be $\sim 8.2 \pm 2.3 \%$ ($n = 4$). Bare BPA-PC exposed to labelled blood over a course of 5 min also triggered significant thrombus growth ($\sim 5.7 \pm 3.6 \%$ surface coverage, $n = 8$ – Figure 111A), as can be observed in Figure 111C. In stark contrast, MEG-OH surface chemistry evidently displayed otherwise more remarkable antithrombogenic properties (Figure 111D), with a barely quantifiable surface coverage of $\sim 0.1 \pm 0.1 \%$ ($n = 8$ – Figure 111A), frames essentially showing blank areas (Appendix M). This represented for the MEG-OH coating a substantial decrease in platelet adhesion, aggregation and thrombus formation of over 97% compared to the bare, non-derivatized BPA-PC substrate (Figure 111A). More importantly, thrombogenesis was nearly non-existent (Figure 111D).

![Figure 111.](image)

**Figure 111.** (A) Indicative percentage of surface coverage due to platelet adhesion, aggregation and thrombus formation on (left to right) collagen-coated, bare and MEG-OH-modified BPA-PC substrates. (B-D) A selection of representative video frames showing platelet adhesion, aggregation and thrombus formation (or lack of) on collagen-coated, bare and MEG-OH-modified BPA-PC substrates ($32 \times$ magnification) after 5 min of exposure to whole human blood at a shear rate of 1000 s$^{-1}$. Replicate pictures for all various surfaces can be found gathered in Appendices K-M.
Also, considering the scale of the images (10 μm) and the size of individual platelets (2-4 μm), the few observable low-sized micro-aggregates (marked by arrows) appeared to only encompass a limited number of platelets (Figure 111D, Appendix M). Even if these aggregates happened to detach and circulate in the bloodstream as thromboemboli – should MEG-OH surface chemistry on BPA-PC be implemented in a biomedical application – their small size (< 5 μm across) should lower the risk of blood vessel occlusion and associated injuries. Not to forget also is the possibility for these ‘micro-clots’ to be managed (i.e. dissolved) by the regulatory fibrinolytic system.

3.2.4. D. Concluding remarks

To address the potentially harmful consequences of surface-induced/mediated thrombosis resulting from the contact of blood with materials foreign to the human body, an ultrathin antithrombogenic coating based on monoethylene glycol silane surface chemistry (MEG-OH) was developed. The strategy was exemplified with polycarbonate – a plastic polymer increasingly employed in the biomedical industry. Antithrombogenicity was assessed after 5 min of exposure to fluorescently-labelled whole human blood dispensed through a perfusion chamber at a shear rate of 1000 s⁻¹, a condition of hemodynamics conducive to platelet accumulation/thrombus growth. Remarkably, platelet adhesion, aggregation and thrombus formation on MEG-OH surface chemistry was greatly inhibited (> 97% decrease in surface coverage) compared to the bare substrate. In other words, the MEG-OH coating displayed excellent antithrombogenic properties, far exceeding those of the non-derivatized plastic material. More importantly, thrombogenesis was nearly non-existent. These results are quite remarkable considering: (i) that blood was neither given nor did require any additional anticoagulant treatment to prevent clotting (besides its standard collection and storage in heparinized Vacutainers) despite the high shear rate conditions used within these in vitro blood experiments; and (ii) the structural simplicity of the MEG-TFA surface modifier used to prepare the MEG-OH coating, the latter constructed in a straightforward and inexpensive manner.

In summary, the ultrathin MEG-OH silane surface chemistry possesses two highly praised and sought-after attributes in a bioinert material coating: antifouling behaviour against blood serum, and antithrombogenicity when exposed to blood.

Moreover – even though cell labelling with DiOC₆ fluorescent dye is not specific to platelets – the probability is low for these few observable ‘spots’ to correspond to other types of larger blood cells, given their size (less than 5 μm across). A compilation of blood cell dimensions is provided in introductory Section 1.2.

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4. Summary and Conclusions

The functionality and life expectancy of biomedical/bioanalytical healthcare equipment, implants or devices critically depend on the interactivity of their exogenous surface with the protein components of the bodily fluid they are exposed to. For artificial implants and other biomaterial applications, concerns arise from the possibility for biological processes/responses with deleterious effects (on both foreign and host bodies) to ensue from the initial adsorption of proteins. For biosensor platforms, this natural adhesion phenomenon is synonymous with the occurrence of an often overwhelming interference signal that prevents the detection – not to mention the quantification – of target analytes present at considerably lower concentration. In all situations, the outcome largely hinges on the physicochemical makeup of the artificial surface contacting the biological environment. To address these recurrent and ubiquitous biotechnological issues, a popular strategy that is the object of tremendous research effort consists in passivating substrate materials with an antifouling/biocompatible organic coating. This PhD Thesis described our recent, both fundamental and applied, research contributions to these fields of biotechnology.

One primary area of research aimed to devise piezoelectric biosensors able to selectively and sensitively detect target analytes in a real-time and label-free manner in biofluids (e.g. blood serum and plasma), as potential clinical assay alternatives with advanced features to current screening/diagnostic tests. One requirement was for biosensing interfaces to be operational upon exposure to complex mixtures of potentially interfering species present in the biological matrix at considerably greater concentration (several orders of magnitude). Analyte detection was to rely on the electromagnetic piezoelectric acoustic sensor (EMPAS) ultra-high frequency transducing technology; biosensing platforms to feature new, dual-functional organosilane coating combining both high antifouling and biorecognition capabilities. Methodical research into such surface chemistry led to the identification of functionalizable ‘mixed adlayers’ readily prepared on quartz resonator discs from a binary blend of unprecedented, EG-based trichlorosilane building block molecules (OEG-TTTA and MEG-TFA surface modifiers – Figure 67). Equally as straightforward was the subsequent immobilization of analyte-binding biomolecular receptor probes – a transformation that had the merit of being performed in a preactivation-free single step, under mild conditions, and of being compatible with both thiol and amine anchoring moieties. We first exemplified our clinical testing method with a biosensor
prototype dedicated to the detection of biotin/avidin model interactions in full serum. This project proved to be successful overall as a qualitative cut-off assay could be developed in serum with a limit of detection in the pM range, validating the EMPAS as a qualitative analysis technique. This excellent analytical performance was tempered however by the occurrence of false negative and false positive results likely caused by special kinds of interfering species endogenous to serum. The versatility of OEG-TTTA/MEG-TFA mixed adlayer-based EMPAS biosensing platforms was next demonstrated through the detection of bacterial lipopolysaccharide (also known as ‘endotoxin’), a biomarker analyte of true clinical significance that plays a central role in the pathogenesis of sepsis – a potentially lethal condition affecting countless individuals worldwide. Similarly, endotoxin detection was performed in a real-world complex biofluid (full human blood plasma), and in a real-time and label-free manner unlike current clinical assays that rely on chromogenic reporter molecules. Initial EMPAS experiments showed that plasma samples at abnormally high endotoxin concentration (1000 pg/mL) can be easily and rapidly distinguished from those presenting basal level (10 pg/mL).

Research activity was also focused on determining whether simpler coatings constructed from a single type of ultrashort trichlorosilane surface modifier would also display pronounced antifouling properties against full serum. This new EMPAS project additionally constituted an opportunity to methodically study the influence of the nature of surface functionalities in this respect. A key result was the observation that, among a series of ultrathin unimolecular adlayers with systematically-varied chemical structure (Figure 84), only the monoethylene glycol (MEG) variety incorporating a single, internal ether atom of oxygen in the chains was able to dramatically alter serum adsorption, the synergy being strongest for the MEG-OH system presenting terminal hydroxyl moieties. On this particular antifouling coating, most serum proteins appeared to adsorb in a transient, reversible fashion. Conversely, the antifouling behaviour was less pronounced for alkylated adlayers lacking the internal ether oxygen atom in the chains. Further comparative analysis suggested that antifouling properties would also be intimately connected to (the state of) surface hydration, wherein the internal ether atom of oxygen in the MEG backbones – a feature alkylated systems lack – is hypothesized to play a key role in this matter through the instigation of a special intrafilm zone of hydration. Ensuing neutron reflectometry experiments conducted with the antifouling MEG-OH system and its less effective OTS-OH alkylated analogue (Figures 84 & 86) corroborated this mechanistic hypothesis by revealing the existence of distinct hydration patterns. For the OTS-OH system,
water appeared unable to penetrate the adlayer, but formed a short-ranged interphase physically distinct from bulk water. This region also seemed to be characterized by a highly-structured molecular organization with a sharp transition towards ordinary bulk water. This was in contrast to the continuous, longer-range and diffusive transition zone of water stemming from within the highly-absorbent MEG-OH adlayer. Collectively, acoustic wave sensing and neutron reflectometry experiments provided convincing empirical evidence that the internal ether atom of oxygen in the MEG chains would play a determining role in (the state of) surface hydration, hence antifouling. Finally, also demonstrated was the adaptability of the antifouling MEG-OH ‘nanogel’ surface chemistry for the derivatization of gold, another biotechnologically important substrate material.

The experimental probing of surface hydration was next complemented with a series of molecular dynamics simulations (run with a similar set of unimolecular silane adlayers with methodically-varied chemical structure) that allowed for a better understanding of antifouling with respect to surface hydration to emerge; and for a molecular-level mechanism – well in line with that generally invoked in the literature – to be rationalized in terms of a set of basic requirements, all of which only the unique MEG-OH system was able to fulfil. Thus, this computational study revealed that, in order to be pronouncedly antifouling, an adlayer must display both internal and interfacial hydrophilicity and kosmotropicity (the ability to structure water at the molecular level). Furthermore, hydration water must be tightly coordinated with limited dynamicity (lability and mobility). The satisfaction of all these criteria would give rise to a stable permeant network of water, whose disturbance via dehydration upon protein adsorption would constitute an energetic penalty and generate repulsive forces. An unprecedented ‘substrate effect’ to surface hydration (to our knowledge) – that would take place at defected/damaged sites and participate in endowing antifouling adlayers with hydrogel-like characteristics – was also proposed, together with the controversial argument according to which partial surface coverage should therefore be thoughtfully considered in the construction of antifouling coatings.

In addition to its pronounced antifouling properties, the unique and versatile MEG-OH surface chemistry was finally shown to also display outstanding antithrombogenicity, through a study that further expanded to plastic the scope of derivatizable substrate materials of biotechnological importance. Surface modification was readily exemplified with polycarbonate, a plastic polymer widely used in the healthcare industry. Antithrombogenic properties were
assessed using a perfusion chamber and fluorescently-labelled whole human blood dispensed for 5 minutes under hemodynamic conditions favourable for platelet accumulation/thrombus growth. Control experiments showed that platelet adhesion, aggregation and thrombus formation on bare polycarbonate was quite pronounced with a surface coverage calculated to be ~6%. In stark contrast, ultrathin MEG-OH surface chemistry displayed otherwise more remarkable antithrombogenicity with a barely quantifiable surface coverage of ~0.1% – or a decrease of over 97% compared to non-derivatized polycarbonate. More importantly, thrombogenesis was nearly non-existent. Moreover, the few observable micro-aggregates appeared to only comprise a limited number of platelets, making them unlikely to cause any harm associated with blood vessel occlusion – should they detach as circulating thromboemboli. These results with MEG-OH silane surface chemistry were quite remarkable considering that blood was not given, nor did require, any anticoagulant treatment to prevent clotting (besides its standard collection and storage in heparinized Vacutainers) in spite of being exposed in vitro to a foreign surface.

The main drive of this PhD Thesis research was not only to try and engineer original stealth surface chemistry, but also to ensure: (i) that the desired antifouling/biocompatible properties would be displayed upon exposure to real-world biofluids; (ii) that the surface chemistry would be versatile for widespread use in diverse biomedical and bioanalytical applications, and easily adaptable to many different types of substrate material; and (iii) that surface modification would be straightforward, rapid and cost-effective. All these technologically and economically critical specifications were met successfully.
5. Future Work

5.1. Preamble

Scientific research is driven by an insatiable quest for knowledge and potential technological applications. It is therefore often the case that a successful study does not constitute an end in itself but rather evolves/ramifies into multiple related new projects. The work presented in this PhD Thesis is no different. Hereinafter is thus proposed a non-exhaustive list of possible future areas of research and development for the unique OEG-TTTA/MEG-TFA and MEG-OH surface chemistries, whose tremendous potentials – as will be demonstrated – still await to be explored. Both fundamental and applied aspects of research will be discussed.

5.2. Dual-functional OEG-TTTA/MEG-TFA mixed adlayer surface chemistry

5.2.1. Mixed assembly characterization for optimal binding affinity

Even though our work with OEG-TTTA/MEG-TFA mixed adlayer surface chemistry is highly application-oriented, it is always beneficial when derivatizing a substrate with a functional coating to try and gain deeper insight into the applied surface chemistry. For instance, alternately labelling OEG-TTTA linker and MEG-TFA diluent molecules with a radioisotope tracer may prove useful in determining the packing density and relative composition of both surface modifiers within a resulting mixed assembly. In doing so, the interesting question as to whether the composition of binary OEG-TTTA/MEG-TFA silanizing solutions translates ‘on-surface’ may potentially also be answered. More importantly for molecular recognition, radionuclide labelling may also serve to quantify subsequent probe loading on OEG-TTTA/MEG-TFA mixed adlayers. All these proposed radiolabelling experiments could be part of a multi-parameter trial study aiming to determine the conditions of ‘optimal probe loading’ for highest binding affinity/capture performance in biosensor and other molecular recognition applications.

5.2.2. Biosensor and other molecular recognition applications

5.2.2. A. Biomarker detection in bodily fluids

Demonstration was made in this PhD Thesis that the ultra-high frequency EMPAS device – together with the dual-functional OEG-TTTA/MEG-TFA mixed adlayer-based surface chemistry that combines both recognition and antifouling abilities – is sensitive enough to detect target analytes at pM concentration directly in the most challenging biological media that are full blood
plasma and serum. A detection limit of this order of magnitude is important for early disease/condition screening/diagnosis.\textsuperscript{550} The real-time and label-free measurement capabilities offered by our biosensing interface are other beneficial qualities a potential clinical assay alternative should present. Its versatility in terms of subsequent probe functionalization is another advantage of OEG-TTTA/MEG-TFA mixed adlayer surface chemistry that, in principle, should allow for any disease/condition biomarker or pathogen analyte to be detected in any given full bodily fluid through simple biosensing platform customization – provided that a probe binding partner is available and can be immobilized with maximally retained affinity.\textsuperscript{320,334} Such multi-purpose biosensing platform can virtually be functionalized with any receptor probes ranging in size and complexity from small organic molecules (e.g. inhibitors) to medium-sized aptamers or peptide immunodominant epitopes, to large biomacromolecules (e.g. whole antibodies, or paratope fragments). Although there is also interest in developing EMPAS biosensors for the less publicized but equally as important rare diseases,\textsuperscript{551} there exists a couple of afflictions with tremendous societal impact that we are committed to develop a sensitive biosensing assay for: endotoxin-induced sepsis and human immunodeficiency virus infection. As will also be discussed next, the applicability of the dual-functional OEG-TTTA/MEG-TFA mixed adlayer surface chemistry is not limited to (bio)sensing.

5.2.2. B. Endotoxin detection (and neutralization)

An immediate objective would be to finish constructing the dose-response curve for the EMPAS detection of LPS/endotoxin in full human blood plasma presented in Section 3.1.3. This would also be the occasion to try and better understand the physical chemistry at play behind the unusual EMPAS response profile (a net increase in resonant frequency) observed for PMB-functionalized OEG-TTTA/MEG-TFA mixed adlayer-based biosensing platforms exposed to LPS-spiked plasma samples. As discussed earlier, being able to diagnose endotoxemia (and its degree of severity) in a timely and expeditious manner in patients with suspected sepsis is crucial as it would allow for them to be promptly/properly triaged and treated (especially for emergency situations). Also offering a prompt and personalized treatment should result in improved prognosis and contribute to eradicate this dreadful condition of sepsis that affects millions of individuals around the world and claims the life of many. As discussed in Section 3.1.3. B., we propose for this purpose to engineer a separate LPS neutralization cartridge that would also rely on the key PMB-functionalized OEG-TTTA/MEG-TFA mixed adlayer surface chemistry, the ultimate aim being to actually combine both diagnostic and therapeutic features into an all-
integrated ‘theranostic circuit device’ (TCD). In this respect, a truly ‘in-line’ configuration with continuous LPS monitoring (Figure 112 – left) will require the EMPAS biosensing interface to actually be operational in whole blood, for prolonged periods of time as well. If not, an ‘off-line’ TCD design, wherein LPS detection would be performed in a pseudosimultaneous manner with plasma samples intermediately cleared of blood cells, is always possible (Figure 112 – right).

Figure 112. Truly ‘in-line’ EMPAS biosensing set-up for continuous LPS detection in whole blood (left) vs. ‘off-line’ configuration for timed LPS detection in cell-cleared blood plasma (right). Note: not shown in the off-line detection approach is the intermediate centrifugation system to separate blood plasma from cells.

Finally, it is important to note that this tandem strategy to treat an illness with TCDs based on dual-functional OEG-TTTA/MEG-TFA mixed adlayer surface chemistry is evidently not limited to the case of endotoxin-induced sepsis but is, in principle, eminently applicable to the combined detection and neutralization of any pathogen present in blood. This fundamentally universal scope of application is another unique and attractive characteristic of such an innovative biomedical device.
5.2.2. C. Human immunodeficiency virus (HIV) detection

5.2.2. C.1. Background research

EMPAS detection of HIV biomarkers – a notorious infection that needs no introduction – is a project we initiated a few years ago. In that study, anti-HIV-2 monoclonal antibodies were detected in full serum using EMPAS biosensing platforms functionalized with immunodominant peptide epitopes of HIV-2 transmembrane glycoprotein gp36 through halogenoacetyl-based mixed silane adlayers (Figure 113). Preliminary results showed that this EMPAS immunosensor was effective in detecting and distinguishing HIV-2 anti-gp36 from HIV-1 anti-gp41 antibodies with good selectivity, in a real-world detection scenario. The average resonant frequency shifts were respectively Δf = −15.9 ± 3.6 (n = 5) and −5.2 ± 0.5 (n = 5) kHz, for a R_{gp36/gp41} selectivity ratio of ~3.0:1 (Figure 113).

![Figure 113](image-url)

**Figure 113.** CATD/HTS mixed silane adlayer-based EMPAS immunosensor for the real-time and label-free serological detection of HIV-2 anti-gp36 vs. HIV-1 anti-gp41 antibodies: working principle and preliminary results (Δf ± SD, 5 replicates per set). **Note:** EMPAS measurements were recorded at the ultra-high operating frequency of 1.06 GHz, with anti-gp36 and anti-gp41 monoclonal antibody samples both prepared as 0.1 mg/mL solutions in full foetal bovine serum.

Although HIV immunosensing with the EMPAS system was performed in a real-time and label-free preferable manner, the level of antibody detection (100 μg/mL) was not near that of clinical relevance (pg/mL) displayed by modern, state-of-the-art laboratory tests that also offer
combined HIV-1 and HIV-2 antibody as well as HIV antigen screening capabilities. Another downside to our method – this one linked to the biosensing platform preparation – was the elevated cost associated with the purchase/synthesis of the HIV-2 antigen peptide probe. For all these reasons, our EMPAS strategy to detect HIV needed to be revisited.

5.2.2. Alternative approach to HIV detection: gp120 biomarker

One strategy to prevent lymphocyte depletion by HIV – and the resulting development of the acquired immunodeficiency syndrome (AIDS) – is to block the entry of the virion into host cells. The mechanism of cell infection involves an early binding interaction between CD4 host cell receptors and HIV envelope glycoprotein gp120, whose ensuing conformational rearrangement triggers a cascade of events that eventually result in the HIV transmembrane glycoprotein gp41-mediated fusion of viral and host cell membranes. Research towards HIV entry inhibitors recently resulted in the identification of small organic molecules with high affinity for gp120 (K_D ~ μM) that possess the generic N-phenyl-N’-piperidinyl-oxalamide structure shown in Figure 114.

![Figure 114. N-phenyl-N’-piperidinyl-oxalamide HIV entry inhibitors with high affinity for gp120.](image_url)

What interested us in this family of inhibitor molecules – beside their attractive structural simplicity – was the possibility to design a HIV gp120 target probe that could be tethered to OEG-TTTA/MEG-TFA mixed adlayers through a spacer arm attached to the non-substituted nitrogen atom of the piperidine ring (Figure 114). Our enthusiasm was rapidly tempered however upon the realization that the gem-dimethyl substitutions around this atom, and the tremendous steric hindrance associated with it, would preclude easy synthetic modification. Fortunately, a recent structure-activity relationship study with inhibitor analogues suggested that these methyl groups may not be necessary for the binding and conformational rearrangement of gp120 to occur. It then became possible for us to propose the simple chemical structure shown in Figure 115 as a potential affinity probe molecule to be immobilized on OEG-TTTA/MEG-TFA mixed adlayers for the EMPAS detection of HIV through its envelope glycoprotein gp120.
Figure 115. Chemical structure of a potential gp120 target molecular probe for HIV detection with OEG-TTTA/MEG-TFA mixed adlayer-based EMPAS sensing platforms.

A thiol anchoring moiety and/or an EG-based spacer arm are also envisioned, so is the affinity-altering installation of/substitution for other substituents on the aromatic ring (Figure 114). This latter modification could also prove useful to ensure, if necessary, that all polymorphic forms of HIV gp120 are detected. A longer spacer arm might also be needed to allow for the affinity section of the immobilized receptor probe to reach gp120’s deep binding cavity.

The thermodynamics of interaction between gp120 and oxalamide CD4 mimics (Figure 114) has been thoroughly studied in recent years. The key result was that gp120 indeed typically undergoes extensive conformational restructuration upon binding, which is characterized by a loss in entropy (–TΔS > 0 kcal/mol) largely compensated by a favourable change in enthalpy (ΔH < 0 kcal/mol). Another study later confirmed this restructuration phenomenon by means of acoustic wave physics. In the latter work, exposure of surface-attached gp120 to a CD4 mimic oxalamide ligand resulted in a net increase in the resonant frequency of the piezoelectric device, a loss in viscous character (i.e. a stiffening phenomenon) the authors found to be in good qualitative agreement with the loss in entropy previously reported for this interaction. In this regard, it may thus be possible that the EMPAS response signal due to HIV gp120 binding also relied on a rigidifying conformational change experienced by the protein, as it was hypothesized earlier to be the case for the PMB/LPS interaction (Section 3.1.3.E.).

We finally note that our new EMPAS strategy for direct and early detection of the virion through its envelope glycoprotein gp120 — rather than the later detection of anti-HIV antibodies produced at sufficiently-high, detectable concentration by the immune system in response to the
infection (during the so-called ‘serological latency’ or ‘diagnostic window’ of the affliction) would allow, in principle, for the disease to be screened/diagnosed in its very early stages of development, and therefore be managed with increased efficacy.

5. 2. 2. D. Detection in aqueous media

The initial EMPAS work performed in buffer that led to the subsequent studies with clinically-relevant blood serum/plasma samples is not devoid of any interest however. Indeed, it could actually prove extremely useful in the development of other clinical biosensing assays intending to perform analysis in considerably less proteinaceous and challenging bodily fluids such as urine or saliva. Environmental detection of toxic chemicals or other pollutants/pathogens in water/aqueous samples is another among many possibilities.

5. 3. Unimolecular MEG-OH surface chemistry for biomaterial applications

5. 3. 1. Surface modification of other (bio)technologically-important substrate materials

We have shown in this PhD Thesis that unimolecular MEG-OH surface chemistry could not only be implemented to derivatize both organic and inorganic substrates presenting a hydroxyl/oxide surface but could also be adapted to modify metal substrates, essentially demonstrating the potential for such chemistry to be imposed on any type of material. Although they should readily find an application with the current popular substrate materials that are aluminum nitride, indium-tin oxide or graphene, it is certainly in the field of biomedical equipment, implants and devices with the surface modification of stainless steel or other plastic polymers (than polycarbonate) – for instance poly(ethylene terephthalate), poly(vinyl chloride), polyurethane, or PDMS – that MEG-OH coatings should blossom thanks to their remarkable antifouling and antithrombogenic properties. Regardless of the chemical nature of the substrate material, MEG-OH chemistry could also very well be used to coat the surface of microfluidic channels, microarray assays, separation membranes, colloidal drug delivery systems, or nanoparticle/quantum dots, for instance.

5. 3. 2. Protein adsorption: amount and conformational state

Another potential objective with unimolecular MEG-OH surface chemistry would be to try and actually quantify (ng/cm²) the amount of blood-borne protein adsorption on this type of antifouling coating, using a ‘total concentration’ protein assay (e.g. biuret test) after surface desorption. In fact, even so-called ‘antifouling’ or ‘protein-resistant’ surfaces adsorb measurable
amounts of proteins, as recently shown by others.\textsuperscript{582} [In this respect, it seems unfortunate that the terms ‘antifouling’ and ‘non-fouling’ – the latter corresponding to the arguably elusive, perfect antifouling situation where no protein adsorption occurs\textsuperscript{40} – have acquired a confusing and misleading status of synonyms in the literature.] This project would also be the occasion to try and identify these fouling proteins, as was done in the aforementioned study through electrophoresis separation and subsequent LC-MS/MS analysis.\textsuperscript{582} Recalling that material-induced deleterious biological processes are initiated by surface-activated proteins that restructured upon adsorption to expose cryptic bioactive sites,\textsuperscript{40} determining the conformational state (native vs. unfolded) of these identified adsorbed proteins should also prove extremely helpful in explaining why MEG-OH coatings display such outstanding antithrombogenicity. The task is certainly difficult but yet definitely worth it for the educated design of future hemocompatible coatings.

5.3.3 Sterilization and long-term antithrombogenicity

Another requirement for MEG-OH silane chemistry – should it be implemented in a biomedical application – is the ability to withstand the drastic conditions used to sterilize medical equipment.\textsuperscript{583} One concern with such surface chemistry is the possibility of hydrolytic etching from the substrate.\textsuperscript{318} Experiments have been planned to assess MEG-OH coating stability on polycarbonate in the near future. If it turns out that effective sterilization is indeed damaging, an alternative for a more robust MEG-OH surface chemistry would be to rely on a more stable phosphonate anchorage rather than the current, more labile silanol attachment (Figure 116).\textsuperscript{301,318,507,584}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{Figure116.png}
\caption{Phosphonate vs. silanol substrate anchorage for more robust MEG-OH surface chemistry.}
\end{figure}

From both integrity and functionality point of views, whether MEG-OH coatings can withstand long-term exposure to blood (without the latter needing to be anticoagulated, ideally)
in implant applications or lengthy surgery procedures is another important question that needs to be answered, as is that related to the possible detachment of the few observable platelet aggregates into potentially harmful circulating thromboemboli.\textsuperscript{35,52}

5.3.4. Anti-biofouling

Another domain of research where the bioinert MEG-OH surface chemistry could prove useful is anti-biofouling.\textsuperscript{585} Thus, MEG-OH coatings could be employed for instance to try and prevent marine organisms from colonizing ship hulls. A successful outcome would offer ship-owners the possibility to alleviate the biofouling financial burden associated with anti-corrosion maintenance and the drag-induced extra consumption of fuel.\textsuperscript{32,526,586} Decreasing the related greenhouse gas and particle emissions, or the release of the toxic chemicals used in biocidal coatings/paints, would also have a direct, positive impact on the environment.\textsuperscript{526,586} A commercially available, environmentally-friendly method to combat marine biofouling consists in equipping ship hulls with an electronic device, which launches ultrasonic waves that mechanically prevent barnacles and other marine animals from settling through vibration.\textsuperscript{586} In this regard, a related interesting question would be to determine the actual contribution, if any, of the piezoelectric EMPAS actuation in the reduction of fouling by MEG-OH surface chemistry.\textsuperscript{587-589}

Finally, one can also imagine MEG-OH surface chemistry to be implemented in antimicrobial/antibacterial applications,\textsuperscript{32,585} such as is the case for the emergent class of antifouling sulfo- and carboxybetaine zwitterionic polymer brushes.\textsuperscript{590} Should MEG-OH coatings indeed display antimicrobial/antibacterial properties, the conditions of the aforementioned sterilization process could be less drastic – if necessary not to etch/damage the imposed surface chemistry (Section 5.3.3.).

5.3.5. Molecular dynamics simulations

Although the antifouling mechanism proposed for the unique MEG-OH ‘nanogel’ surface chemistry is already well in line with the one traditionally postulated for assemblies made of short EG residues, a more detailed interpretation of the role of water through surface hydration could emerge from the computation of other important parameters related to the coating (\textit{e.g.} packing density, surface coverage/defects) and environment (\textit{e.g.} temperature, ionic strength). A complete picture, however, would require proteins (and their interaction with the coating) to actually be incorporated in the MD simulations.\textsuperscript{38,228,231} All these computations could also be
accompanied by actual experiments. For instance, a time trial study – wherein MEG-OH (and other related coatings) would be prepared for varied periods of time – could shed light on the actual influence of adlayer surface coverage on surface hydration, and hence antifouling. The hypothesized role played by the underlying substrate in this respect could also be elucidated.

5.4. Concluding remarks

In conclusion, it is our hope that the work presented in this PhD Thesis, and its future extensions, will positively impact the fields of bioanalytical and biomaterial research, and materialize into concrete healthcare applications many people around the world may benefit from.
Appendix
Appendix A

Figure 117. TTTA $^1$H NMR (400 MHz, CDCl$_3$).

TTTA

$^1$H NMR (400 MHz, CDCl$_3$)
Appendix B

Figure 118. PFP-TTTA $^1$H NMR (400 MHz, CDCl$_3$).
Appendix C

Figure 119. OEG-TTTA $^1$H NMR (400 MHz, CDCl$_3$).

- 198 -
Appendix D

Figure 120. MEG-TFA $^1$H NMR (400 MHz, CDCl$_3$).
Appendix E

Figure 121. OTS-TFA $^1$H NMR (400 MHz, CDCl$_3$).
Appendix F

Figure 122. MEG-OMe $^1$H NMR (400 MHz, CDCl$_3$).
Appendix G

Figure 123. HS-MEG-OH $^1$H NMR (400 MHz, CDCl$_3$).
Appendix H

Figure 124. HS-OTS-OH $^1$H NMR (400 MHz, CDCl$_3$).
Appendix I

Biotin thiol

$^1$H NMR (300 MHz, CDCl$_3$)

**Figure 125.** Biotin thiol $^1$H NMR (300 MHz, CDCl$_3$).
Biotinamine

$^1$H NMR (300 MHz, CD$_3$OD)

**Figure 126.** Biotinamine $^1$H NMR (300 MHz, CD$_3$OD).
Appendix K

**Thrombogenicity of collagen-coated BPA-PC**

*(positive control)*

*Figure 127.* Platelet adhesion, aggregation and thrombus formation on collagen-coated BPA-PC after 5 min exposure to whole human blood at a shear rate of 1000 s\(^{-1}\).*
Appendix L

Thrombogenicity of bare BPA-PC

Figure 128. Platelet adhesion, aggregation and thrombus formation on bare BPA-PC after 5 min exposure to whole human blood at a shear rate of 1000 s\(^{-1}\).
Appendix M

Antithrombogenicity of MEG-OH-modified BPA-PC

Figure 129. Inhibition of platelet adhesion, aggregation and thrombus formation on MEG-OH-modified BPA-PC after 5 min exposure to whole human blood at a shear rate of 1000 s$^{-1}$. Arrows mark the few observable micro-aggregates.
### Table 10. Angle-resolved XPS relative atomic percentages for the characteristic elements of quartz and PFP-TT/T/OTS mixed adlayers, non-functionalized and biotinylated with biotin thiol or biotin amine probes. *Duplicate analysis. **Triplet analysis. †This signal is due to contamination by adventitious carbon. ‡Residual $F_{1s}$ signal: biotinylation with biotin thiol did not always proceed to completion. Note: takeoff angles are relative to the normal.
**Figure 130.** XPS narrow scans for cleaned bare quartz (red) and OEG-TTTA/MEG-TFA mixed adlayers – non-functionalized (blue) and biotinylated with biotin thiol (green) – recorded for the characteristic elements of quartz as well as OEG-TTTA/MEG-TFA and biotin thiol residues. The presence of carbon for bare quartz is due to contamination by adventitious carbon. For clarity, the profiles for sulfur (S) and nitrogen (N) are not normalized with respect to the ‘counts/s’ axis. The takeoff angle shown here is 72.5° relative to the normal (surface analysis).
Appendix P

Serum endogenous biotin – supplier response

Date: Mon, 9 Jul 2012 14:16:07 -0500 (CDT)
From: Sigma-Aldrich Technical Service <sigma_aldrich@mailnj.custhelp.com>
Reply-To: Sigma-Aldrich Technical Service <sigma_aldrich@mailnj.custhelp.com>
Subject: G9023, Goat serum [Incident: 120709-000293]
To: 

Subject
---------------------------------------------------------------
G9023, Goat serum

Sigma-Aldrich Technical Service Answer
---------------------------------------------------------------
Scientist's Response Via Email(Audrey Fleming) - 07/09/2012 02:16 PM
Hello,

Thank you for contacting Sigma-Aldrich Technical Service.

We do not determine the biotin content of goat serum. It is likely that there is some biotin present as the product has not been dialyzed to remove it.

Please click here (http://www.surveymonkey.com/s.asp?u=919792443639_) to complete a short one question survey about your experience with Sigma-Aldrich Technical Service and suggest how we can increase the value of our partnership. Thank you for contacting Sigma-Aldrich.

I hope that you find this information helpful. If you have further questions, please reply to this email.

Sincerely,
Audrey Fleming
Sigma-Aldrich Technical Service

Your Question By Web Form - 07/09/2012 12:13 PM
Is biotin (and its derivatives) present in goat serum? if so, in what concentration(s)?

[---001:000913:18530---]
Appendix Q

**PMB immobilization – pH considerations**

To determine the relative number/concentration of PMB amine groups available for attachment in their reactive RNH$_2$ basic form *versus* their unreactive RNH$_3^+$ acidic form, the following equation 22 can be used:\textsuperscript{xiv}

\[
\text{pH} = \text{pKa} + \log \frac{[\text{RNH}_2]}{[\text{RNH}_3^+]} \quad (22)
\]

Since pH = 7.4 (PBS buffer) and taking pKa = 10.4 (to facilitate calculations), equation 22 becomes:

\[
7.4 = 10.4 + \log \frac{[\text{RNH}_2]}{[\text{RNH}_3^+]} \quad (23)
\]

Rearrangement of equation 23 then provides:

\[
\frac{[\text{RNH}_2]}{[\text{RNH}_3^+]} = 10^{-3} \quad (24)
\]

In other words, at pH 7.4, there would exist 1 reactive amine site in its basic RNH$_2$ form available for attachment for every 1000 unreactive amine groups in their acidic RNH$_3^+$ form. In terms of molecular composition, this would translate into 199 bystander, fully-protonated PMB molecules, and 1 PMB molecule available for attachment presenting 1 out of 5 randomly non-protonated amine function, as schematically illustrated in Figure 131.

**Figure 131.** Estimated population of bystander (199) and available for single binding site (1) PMB molecules, at pH 7.4. *Note:* the location of the non-protonated RNH$_2$ site was chosen randomly, for representation purposes.

In conclusion, this neutral pH environment should allow for PMB receptor molecules to be controllably attached through a single amine group while respecting the integrity of the underlying siloxane mixed adlayer.

\textsuperscript{xiv} It is assumed that all five amine sites (Figure 77) – the only functions in the PMB molecule that possess acid/base properties in water – act as individual entities with identical pKa values.
Appendix R

**OEG-TTTA/MEG-TFA/PMB system – XPS characterization**

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<td></td>
<td>42.5</td>
<td>27.1</td>
<td>3.2</td>
<td>23.4</td>
<td>46.3</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>57.5</td>
<td>30.6</td>
<td>3.5</td>
<td>21.6</td>
<td>44.3</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72.5 (surf.)</td>
<td>33.6</td>
<td>4.0</td>
<td>19.1</td>
<td>43.3</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>PMB</td>
<td>27.5 (bulk)</td>
<td>11.9</td>
<td>0.0</td>
<td>33.4</td>
<td>54.2</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>42.5</td>
<td>15.0</td>
<td>0.2</td>
<td>30.4</td>
<td>54.0</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>57.5</td>
<td>22.0</td>
<td>0.0</td>
<td>27.0</td>
<td>50.4</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72.5 (surf.)</td>
<td>31.6</td>
<td>0.3</td>
<td>20.9</td>
<td>46.0</td>
<td>1.2</td>
<td></td>
</tr>
</tbody>
</table>

**Table 11.** Angle-resolved XPS relative atomic percentages for the characteristic elements of quartz and OEG-TTTA/MEG-TFA mixed adlayers, non-functionalized (‘no PMB’) and functionalized with PMB. † This signal is due to contamination by adventitious carbon. Note: takeoff angles are relative to the normal.
Appendix S

MEG-OH chemistry on quartz – XPS characterization

<table>
<thead>
<tr>
<th>Surface</th>
<th>Takeoff angle (°)</th>
<th>% C₁s 281-295 eV</th>
<th>% F₁s 685-693 eV</th>
<th>% O₁s 529-537 eV</th>
<th>% Si₂p 99-107 eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaned bare quartz</td>
<td>27.5 (bulk)</td>
<td>2.6 †</td>
<td>0.1</td>
<td>61.1</td>
<td>36.2</td>
</tr>
<tr>
<td></td>
<td>42.5</td>
<td>2.5 †</td>
<td>0.1</td>
<td>63.2</td>
<td>34.2</td>
</tr>
<tr>
<td></td>
<td>57.5</td>
<td>3.9 †</td>
<td>0.1</td>
<td>65.1</td>
<td>30.9</td>
</tr>
<tr>
<td></td>
<td>72.5 (surf.)</td>
<td>6.9 †</td>
<td>0.0</td>
<td>62.8</td>
<td>30.3</td>
</tr>
<tr>
<td>MEG-TFA adlayer</td>
<td>27.5 (bulk)</td>
<td>20.9</td>
<td>4.2</td>
<td>49.5</td>
<td>25.4</td>
</tr>
<tr>
<td></td>
<td>42.5</td>
<td>22.6</td>
<td>4.8</td>
<td>49.7</td>
<td>22.9</td>
</tr>
<tr>
<td></td>
<td>57.5</td>
<td>23.9</td>
<td>4.9</td>
<td>51.2</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td>72.5 (surf.)</td>
<td>23.5</td>
<td>5.9</td>
<td>51.0</td>
<td>19.6</td>
</tr>
<tr>
<td>MEG-OH adlayer</td>
<td>27.5 (bulk)</td>
<td>19.9</td>
<td>0.1</td>
<td>52.3</td>
<td>27.7</td>
</tr>
<tr>
<td></td>
<td>42.5</td>
<td>22.2</td>
<td>0.0</td>
<td>52.8</td>
<td>25.0</td>
</tr>
<tr>
<td></td>
<td>57.5</td>
<td>24.4</td>
<td>0.6</td>
<td>52.1</td>
<td>22.9</td>
</tr>
<tr>
<td></td>
<td>72.5 (surf.)</td>
<td>25.8</td>
<td>0.0</td>
<td>52.9</td>
<td>21.3</td>
</tr>
</tbody>
</table>

Table 12. Angle-resolved XPS relative atomic percentages for the characteristic elements of quartz as well as MEG-TFA and MEG-OH unimolecular adlayers. †This signal is due to contamination by adventitious carbon. Note: takeoff angles are relative to the normal.

As can be seen in Figure 132, bare quartz only exhibited XPS peaks for silicon (Si₂p at 104 eV) and oxygen (O₁s at 533.5 eV), beside that for adventitious carbon (C₁s at 285.5 eV). Upon MEG-TFA adlayer formation, new signals for fluorine (F₁s at 689 eV), silicon (102.5 eV) and oxygen (532.5 eV) appeared, those for quartz’s silicon and oxygen conversely decreasing as expected for a now buried, underlying substrate (Figure 132). With respect to carbon, the peaks expected for the TFA terminal groups (C=O and CF₃) are clearly visible for the MEG-TFA adlayer at 288-295 eV, but absent for the MEG-OH film. Upon conversion to MEG-OH, the fluorine peak also completely disappeared (Figure 132). In comparison, the adlayers’ silicon and oxygen signals were not affected during the transformation, demonstrating that the TFA groups were cleaved without the residual MEG-OH coating to be etched from the quartz substrate.
**Carbon**

- **Bare quartz**
- **MEG-TFA adlayer**
- **MEG-OH adlayer**

**Fluorine**

**Silicon**

**Oxygen**

**Figure 132.** XPS narrow scans for (top to bottom) C\(_1s\), F\(_1s\), Si\(_2p\) and O\(_1s\) recorded for (left to right) cleaned bare quartz (red) as well as MEG-TFA (blue) and MEG-OH (green) unimolecular adlayers. The takeoff angle shown here is 72.5° relative to the normal (surface analysis).
Appendix T

MEG-TFA adlayer thickness on quartz

The thickness of unimolecular MEG-TFA adlayer on quartz was determined using ellipsometry measurements performed by Yao Tian (Laboratory of Professor Kenneth S. Burch) at the McLennan Department of Physics and Institute for Optical Sciences – University of Toronto (Toronto, Ontario – Canada). Samples were analyzed using a HS-190 VASE ellipsometer (J. A. Woollam Co.) with a 75W light source high-speed monochromator system at 35°, 55° and 75° angles. Curve fitting was performed using the Cauchy model (Figure 133), which provided a thickness of 0.51 ± 0.01 nm for the MEG-TFA adlayer on quartz, with a MSE of 0.47.

![Graph](image)

**Figure 133.** Variable-angle ellipsometry data (green and blue dashed/dotted lines) and corresponding curve fitting (solid red lines) for the determination of MEG-TFA adlayer thickness on quartz using the Cauchy model.
Appendix U

**MEG-OH chemistry on gold – XPS characterization**

As can be seen in Table 13 (relative atomic percentages) and Figure 134 (narrow scans), the derivatization of gold with HS-MEG-OH thiol surface modifier (30 min adlayer formation) was characterized by the appearance of a peak for sulfide sulfur at 164 eV. This signal for sulfur was expectedly absent for bare gold, which displayed however another peak for sulfur at 169 eV due to contamination by adventitious sulfur oxide species (Figure 134). The presence of carbon and oxygen on bare gold was also due to contamination.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Takeoff angle (°)</th>
<th>% S$_{2p}$ 162-166 eV</th>
<th>% C$_{1s}$ 283-291 eV</th>
<th>% O$_{1s}$ 528-536 eV</th>
<th>% Au$_{4f}$ 82-92 eV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cleaned bare gold</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27.5 (bulk)</td>
<td>0.0</td>
<td>28.4†</td>
<td>36.4†</td>
<td>35.2</td>
<td></td>
</tr>
<tr>
<td>42.5</td>
<td>0.3</td>
<td>32.9†</td>
<td>38.9†</td>
<td>27.9</td>
<td></td>
</tr>
<tr>
<td>57.5</td>
<td>0.0</td>
<td>40.1†</td>
<td>40.0†</td>
<td>19.9</td>
<td></td>
</tr>
<tr>
<td>72.5 (surf.)</td>
<td>0.0</td>
<td>47.5†</td>
<td>40.9†</td>
<td>11.6</td>
<td></td>
</tr>
<tr>
<td><strong>HS-MEG-OH adlayer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27.5 (bulk)</td>
<td>1.4</td>
<td>12.9</td>
<td>4.0</td>
<td>81.7</td>
<td></td>
</tr>
<tr>
<td>42.5</td>
<td>0.9</td>
<td>19.4</td>
<td>4.9</td>
<td>74.8</td>
<td></td>
</tr>
<tr>
<td>57.5</td>
<td>1.9</td>
<td>24.5</td>
<td>7.2</td>
<td>66.4</td>
<td></td>
</tr>
<tr>
<td>72.5 (surf.)</td>
<td>1.7</td>
<td>42.5</td>
<td>9.1</td>
<td>46.7</td>
<td></td>
</tr>
</tbody>
</table>

Table 13. Angle-resolved XPS relative atomic percentages for gold and the characteristic elements of HS-MEG-OH adlayer. †These signals are due to contamination by adventitious species. Note: takeoff angles are relative to the normal.
Figure 134. XPS narrow scans for (top to bottom) S$_{2p}$, C$_{1s}$, O$_{1s}$ and Au$_{4f}$ recorded for cleaned bare gold (left, red) and HS-MEG-OH adlayer (right, blue). The presence of sulfur (at 169 eV), carbon and oxygen signals for bare gold is due to contamination by adventitious species.
Appendix V

**MD simulations – siloxane vs. silanol models**

I. Water distribution

**Siloxane coating**

**Silanol coating**

![Figure 135. Innermost radial distribution functions of water for (left/right) siloxane and silanol coatings.](image1)

![Figure 136. Outermost radial distribution functions of water for (left/right) siloxane and silanol coatings.](image2)

As can be seen in Figures 135 and 136, there is no fundamental difference between siloxane- and silanol-based coatings in their (in)ability to organize water. Only the magnitude may vary. This holds true for silanol-based coatings whether the MD simulation is run for 2 ns (this appendix) or > 30 ns (Section 3. 2. 3. B.).
Table 14. Electrostatic, van der Waals and total energies of chain-chain and chain-water interaction for siloxane- and silanol-based coatings.

As well, as seen in Table 14, there is no fundamental difference in terms of trend in energy between the silanol and siloxane series, the latter coatings being generally more stable. The following order of stability, OTS < O-OTS < MEG-OMe < OTS-OH < MEG-OH, still stands.
III. Flexibility

<table>
<thead>
<tr>
<th>Coating</th>
<th>Type of anchorage</th>
<th>Flexibility (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTS</td>
<td>Siloxane</td>
<td>1.45 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>Silanol</td>
<td>1.36 ± 0.30</td>
</tr>
<tr>
<td>OTS-OH</td>
<td>Siloxane</td>
<td>1.16 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>Silanol</td>
<td>1.03 ± 0.17</td>
</tr>
<tr>
<td>O-OTS</td>
<td>Siloxane</td>
<td>1.22 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>Silanol</td>
<td>1.17 ± 0.26</td>
</tr>
<tr>
<td>MEG-OH</td>
<td>Siloxane</td>
<td>1.17 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>Silanol</td>
<td>1.11 ± 0.15</td>
</tr>
<tr>
<td>MEG-OMe</td>
<td>Siloxane</td>
<td>1.25 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>Silanol</td>
<td>1.22 ± 0.31</td>
</tr>
</tbody>
</table>

*Table 15.* Siloxane- and silanol-based coating flexibility assessed using the root-mean-square fluctuation (RMSF) method. RMSF values are calculated over all adlayers non-hydrogen atoms.

Again, as can be seen in *Table 15*, there is little to no statistical difference in terms of flexibility between siloxane- and silanol-based coatings – or, incidentally, between coatings displaying the same type of anchorage to the substrate. This holds true for silanol-based coatings whether the MD simulation is run for 2 ns (this appendix) or > 30 ns (*Section 3.2.3.D.*).
Appendix W

**MEG-OH chemistry on BPA-PC – XPS characterization**

<table>
<thead>
<tr>
<th>BPA-PC surface</th>
<th>% C₁s 283-295 eV</th>
<th>% O₁s 529-537 eV</th>
<th>% F₁s 685-693 eV</th>
<th>% Si₂p 100-106 eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare (activated)</td>
<td>76.3</td>
<td>22.8</td>
<td>0.0</td>
<td>0.9</td>
</tr>
<tr>
<td>MEG-TFA adlayer</td>
<td>44.7</td>
<td>36.8</td>
<td>5.9</td>
<td>12.6</td>
</tr>
<tr>
<td>MEG-OH adlayer</td>
<td>47.5</td>
<td>39.0</td>
<td>0.0</td>
<td>13.5</td>
</tr>
</tbody>
</table>

Table 16. XPS relative atomic percentages for the characteristic elements of the BPA-PC substrate as well as MEG-TFA and MEG-OH adlayers. *Note:* the takeoff angle is 90° relative to the surface (bulk analysis).

For all elements, the variations in XPS signal intensity were consistent with those expected to be observed for the various stages of surface modification, as described in detail in the main text (*Section 3. 2. 4. B.*). However, a comment needs to be made on the magnitude of the Si₂p signal, which was seemingly a little bit high in regards to the substrate vs. coating elemental compositions. A possible explanation would be that the exposure of the BPA-PC substrate to plasma – a procedure whose aim is to (clean and) degrade the polymer surface to reveal hydroxyls and other polar groups for subsequent adlayer siloxane anchorage – increased its roughness and porosity. As such, the relative silicon content from MEG-TFA/MEG-OH adlayers would appear artificially high because XPS would have essentially probed a voided/less dense substrate in terms of carbon and oxygen atoms.
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