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Surface chemistry for *in situ* sample desalting and dynamic MALDI targets

Mohammed KAJJOUT, Christian ROLANDO, Séverine LE GAC

Université des Sciences et Technologies de Lille
Organic and Macromolecular Chemistry Laboratory, COM UMR 8009,
Proteomics, Post-translational Modifications and Glycobiology, IFR 118
Institut Michel Eugène CHEVREUL (IMMCL), FR 2638
59655 Villeneuve d'Ascq CEDEX, FRANCE

E-mail: Mohammed.Kajjout@ed.univ-lille1.fr; Christian.Rolando@univ-lille1.fr; Severine.Le-Gac@univ-lille1.fr.

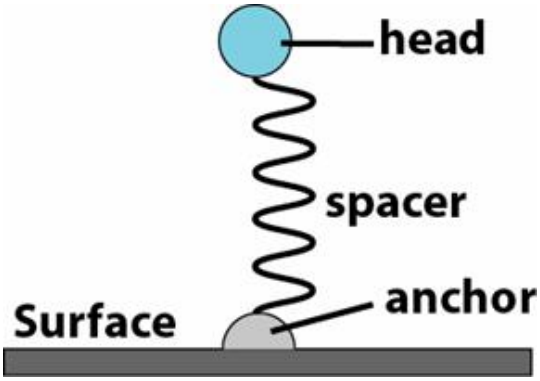
Introduction

The growth of the fields of microsystems and nanotechnology leads to a need for reliable protocols of surface chemistry [1, 2]. An overall decrease of dimensions results in an increase of the surface-to-volume ratio as a function of 1/r: surfaces become relatively more important and surface phenomena preponderant. Consequently, miniaturisation of devices and analytical tools (e.g. screening chips or microsensors) goes together with a need for surface treatment. This aims at (i) avoiding not desired effects such as non specific adsorption of molecules and (ii) benefiting from this relative importance of surfaces. This surface treatment may be achieved using two strategies, either a static or a dynamic coating. The former one consists on a simple immobilisation of molecules whereas the latter is based on species which are sensitive to external stimuli (temperature, pH change, application of a tension…), leading subsequently to a change of the surface properties.

When such a functionalisation [3] is combined to microtechnology techniques, it is possible to pattern the surface immobilized monolayer. For example, different types of molecules can be successively deposited and immobilized on the surface. Microtechnology processes use a PDMS stamp, micro-contact printing or photolithography techniques together with a photoresist.

This work deals with gold-coated surfaces on which thiol compounds are immobilized. Nonetheless, the herein presented chemistry could be applied to other types of materials providing the anchor is changed and adapted to another type of material. Two approaches were tested here: (i) a surface treatment based on self-assembled monolayers e.g. so as to afford a hydrophobic/hydrophilic balance (ii) a « thick» or in-volume functionalization [4] based on a polymer layer grown on a self-assembled monolayer immobilized on the surface. The polymer chemistry we used for this thick functionalization on surface is derived from the chemistry we used for developing monoliths in microfluidic microsystems dedicated to proteomics [5, 6].

Immobilized molecules consist of three parts: (i) an anchor in direct interaction/contact with the surface (thiols or silane), (ii) a spacer and (ii) a head or surface moiety which gives the final properties to the surface. It is thus possible to separately change the nature of the different parts of the molecules depending on the nature of the material to be functionalized and on the desired functionality for the monolayer immobilized onto the. surface (Scheme 1).



Scheme1. Composition of immobilised molecules

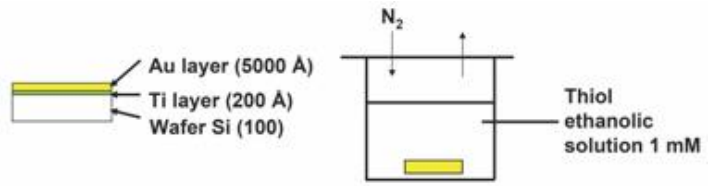
Anchor on the surface: its nature depends on the surface to be functionalized. «Thiol» reagents are used for gold-coated surface, alkoxy or «chlorosilane » for silicon surface (or glass or fused-silica).

Spacer: its nature is chosen depending on the applications (rigidity, hydrophilicity). Either an unfunctionnalized aliphatic chain or a polyethyleneglycol

chain that aims to prevent non specific adsorption of proteins may be used.
Head: OH: hydrophilic surface; CH_3: hydrophobic surface; reactive moiety (-COOH, $-NH_2$): further anchoring of molecules.

Surface treatment

Surface treatment consists in simply immobilizing simple compounds on a surface, for instance thiols on a gold-coated surface here. Thereby we wanted to (i) validate the change of properties of the surface and (ii) develop a reliable protocol for thiol immobilization on a gold surface using commercially available thiol compounds. The herein immobilized molecules present a hydrophilic or a hydrophobic head so as to afford hydrophilic or hydrophobic surfaces. For that purpose, we used various commercially available thiols which were adsorbed on a gold-coated surface: ca fait repetition. The surface consists of a silicon or Pyrex wafer which had been covered with a thin layer of titanium (200 Å) and a layer of gold (5000 Å). Adsorption was validated using hexadecanethiol and mercaptoundecanol.
Molecule adsorption is achieved using a standard protocol [4] (Scheme 2): the piece of gold-coated material is incubated overnight in a 1 mM thiol solution in ethanol, once the solution has been degassed using inert gas. Thereafter surfaces are characterized for their hydrophobicity/hydrophilicity by measuring the contact angle of a DI water droplet on the functionalized surface (Figure 1).

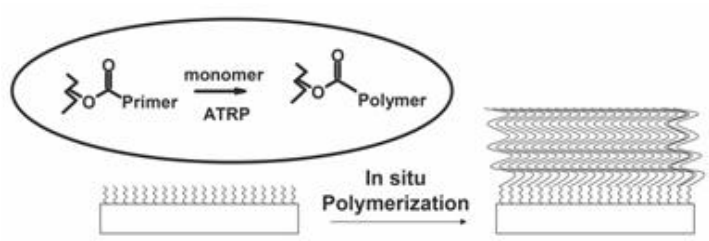


Scheme 2. Protocol for molecule adsorption

Figure 1. Variation of the contact angle according to the structure of the adsorbed thiol

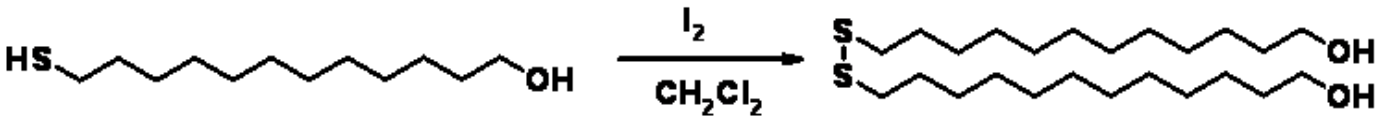
Volume functionalization

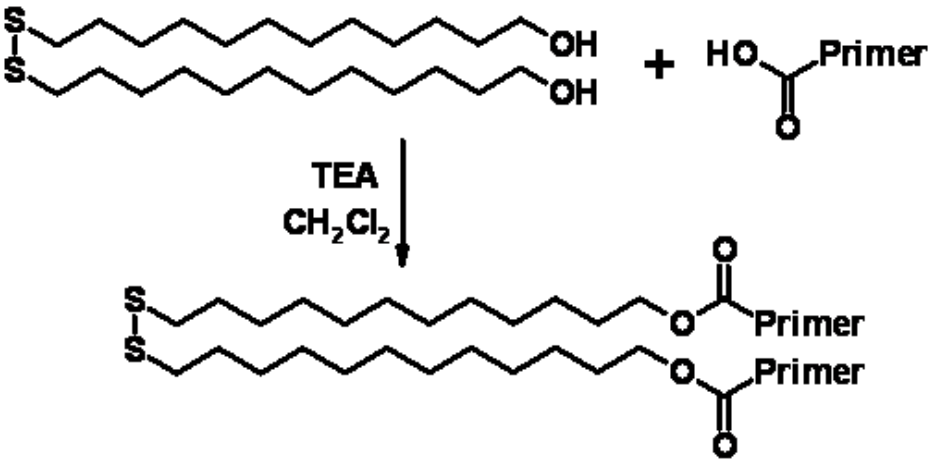
This approach aims at creating an in-volume functionalization of the surface using linear brush-like polymer. Polymer composition is chosen regarding the targeted application. We used here polymer having hydrophobic moieties for desalting purposes or polymer having reactive moieties (epoxy ring). The latter enable a further anchoring of proteins on the surface e.g. proteolytic enzyme (trypsin), antibodies for digestion applications and selective trapping of proteins respectively.
The first step consists in synthetizing the molecule to be immobilized on the surface. For that purpose, we decided to use a protocol described in the literature. The molecule includes a double-anchor, i.e. a disulfide bond, an aliphatic spacer and a reactive head which consists of a bromoester moiety, as illustrated in Scheme 3. The bromoester moiety enables a further surface polymerization after the immobilization of the thiol onto the surface.



Scheme 3. Principle of brush polymer synthesis

The synthesis of the molecule to be immobilized on the surface proceeds in two steps. The first step corresponds to the formation of the disulfide bond between two molecules of mercapto-alcohol. During the second step, the bromoester head is added on the resulting disulfide compound (Scheme 4).





Scheme 4. Chemical synthesis of the reactive anchor

The final compound is then immobilized on the surface using the protocol described above: a piece of gold-coated material is incubated overnight in a 1 mM ethanolic solution of the disulfide compound. As before, the resulting surface is characterized so as to validate the adsorption of the molecule.

The polymerization [7] is then carried out following a protocol described in the literature. It proceeds at room temperature for several hours in a 50:50 solution based on a 50/50 methanol-water mixture and on monomers, to which bipyridine and Cu(II) salts are added.

The polymerization was first carried out for 4 hours using various methacrylate monomers: Glycidyl (epoxy), methyl (-CH_3), butyl (-C_4H_9), lauryl (-$C_{12}H_{25}$) methacrylate. Contact angle measurement (Table 1) shows that the more hydrophobic the monomers lead to the more hydrophobic the surface.

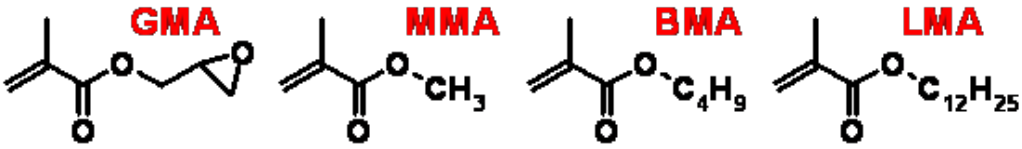
Surface	**Native surface**Æ				
		GMA	MMA	BMA	LMA
Contact angle θ°	**55°**	**53°**	**70°**	**85°**	**95°**

Table 1. Contact angle according to monomer structure

Results and discussions

Tests on a cytochrome *c digest*

We used here monomers bearing hydrophobic moieties (e.g. lauryl methacrylate or LMA) for desalting purpose. The resulting hydrophobic surfaces were prepared on a MALDI target for purifying peptide mixtures before their analysis using MALDI-MS techniques (for a review see [8]). Once the peptide solution (tryptic digest of cytochrome *c* at 80 femtomole per microliter) has been deposited onto the functionalized surface and let for drying, the surface is washed. The matrix (DHB) is then added on washed spots for analysis by MALDI-MS. These test experiments have demonstrated that peptidic fragments are retained on the surface polymer phase and that the polymer was not released from the surface during surface washing or laser irradiation: it remains anchored on the surface. In addition to this, it has been seen that desalting was successful and efficient for salt concentrations as high as 1 M. This desalting efficiency is 100-fold more than on a conventional MALDI target during a routine experiment. On insert of each figure (Figure 2a to 2d) the corresponding MALDI spectrum obtained in routine conditions (stainless steel target) with the same salt concentration is displayed.

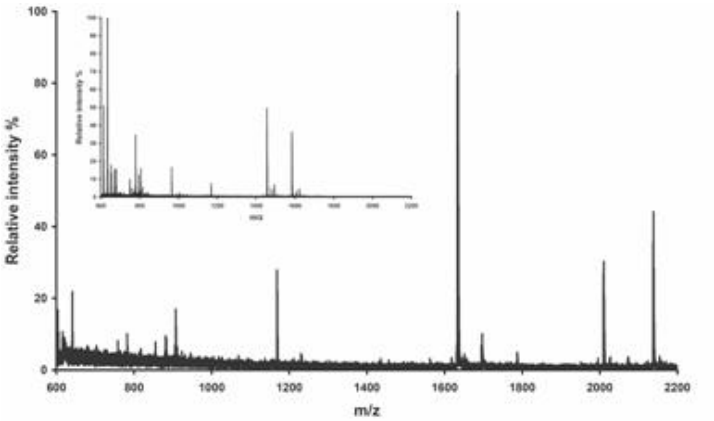


Figure 2a. Peptidic digest (80 fmol); NaCl at 10^{-3} M

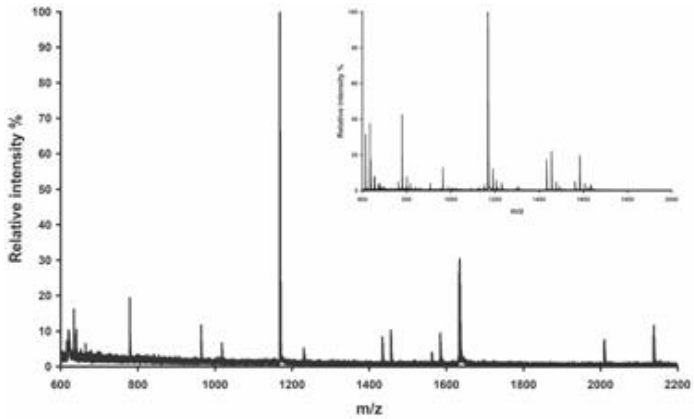


Figure 2b. Peptidic digest (80 fmol); NaCl at 10^{-2} M

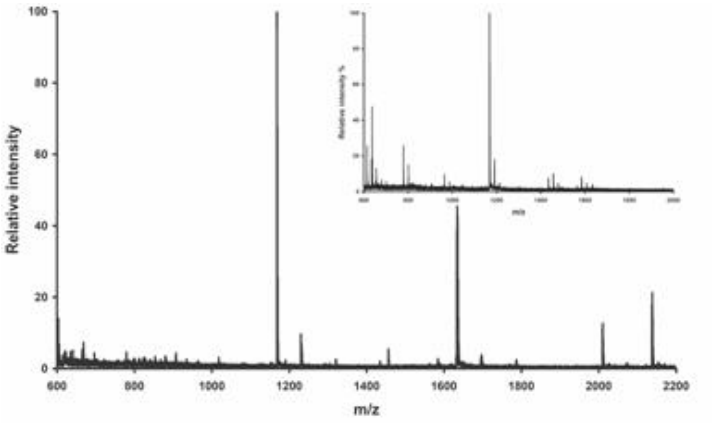


Figure 2c. Peptidic digest (80 fmol); NaCl at 10^{-1} M

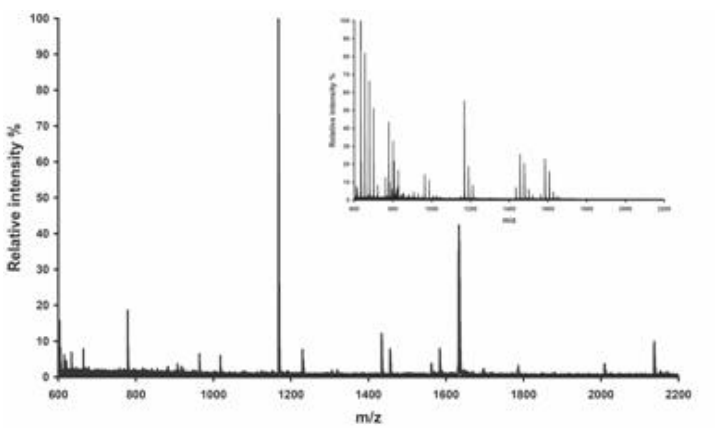


Figure 2d. Peptidic digest (80 fmol); NaCl at 1 M

Figure 2. MALDI spectra of a tryptic digest of cytochrome c at 80 femtomole per microliter at increasing NaCl concentration. In insert; corresponding non desalted spectra obtained on a stainless steel MALDI target using conventional conditions.

Analysis of crude human plasma

The dramatic results we obtained on a standard peptide digest containing 1 M NaCl prompted us to test the same analysis on crude plasma which has a global salt concentration of 0.9 % i.e. roughly 0.5 M NaCl.

For digested samples, samples corresponding to 10 mg/mL total protein of plasma were diluted 10-fold in 6M guanidine, 50mM Tris-HCl, 4mM DTT. The samples were kept at 70 °C for one hour. Then 10 % of the final volume of a solution of 50 mM iodoacetamide was added, followed by incubation at 0°C in the dark for 2 hours. 6 µl of a 10 µg/µL trypsin solution from bovine pancreas were added to each sample. Tryptic digestion took place overnight at 37 °C [9, 10]. For analysis performed whithout digestion native plasma samples was used without any dilution or treatment. Peptides were analyzed by MALDI in reflectron mode using DHB as matrix whereas proteins were analyzed in linear mode using sinapinic acid. We were delighted that the major proteins of plasma may be identified using our MALDI target without any treatment of the sample (Figure 3).

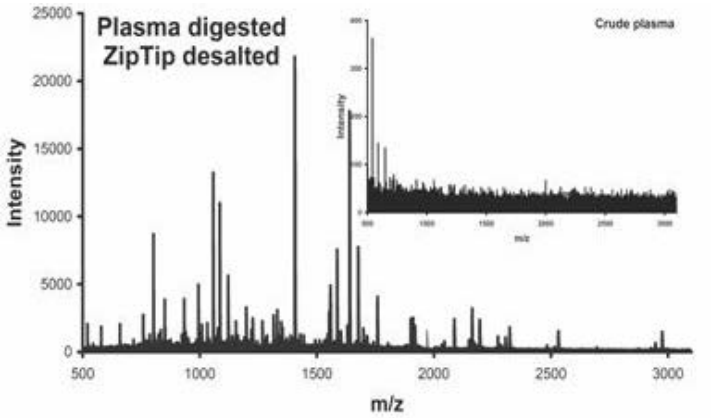


Figure 3a. Digested plasma, desalted using a ZipTip, in insert crude plasma (undigested, undesalted).
Analysis on a conventional MALDI target

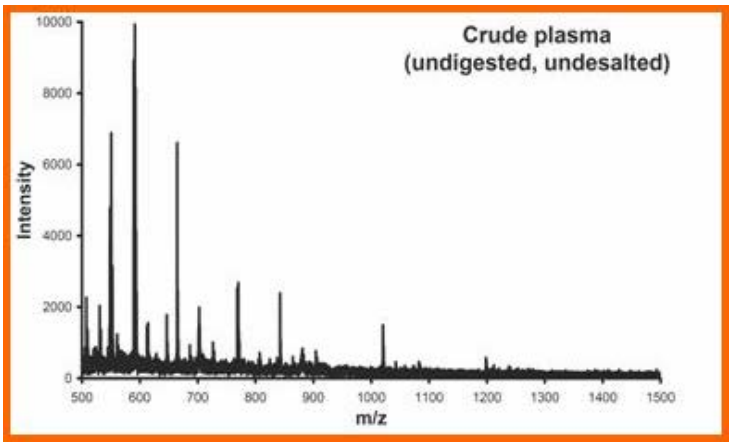


Figure 3b. Crude plasma (undigested, undesalted), low mass
Analysis on our MALDI dynamic surface

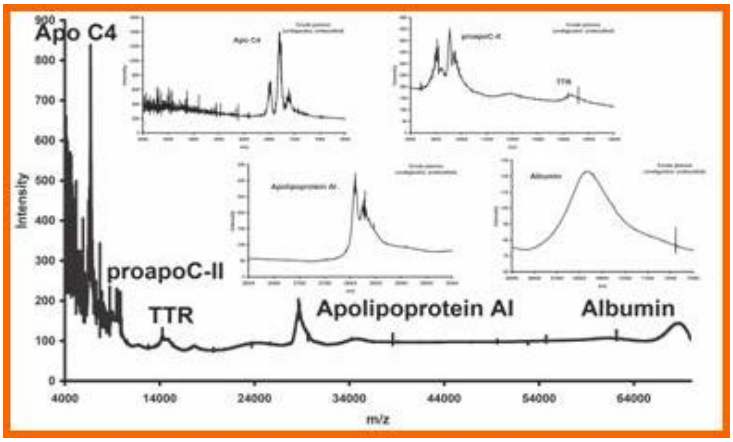


Figure 3c. Crude plasma (undigested, undesalted), high mass
Analysis on our MALDI dynamic surface

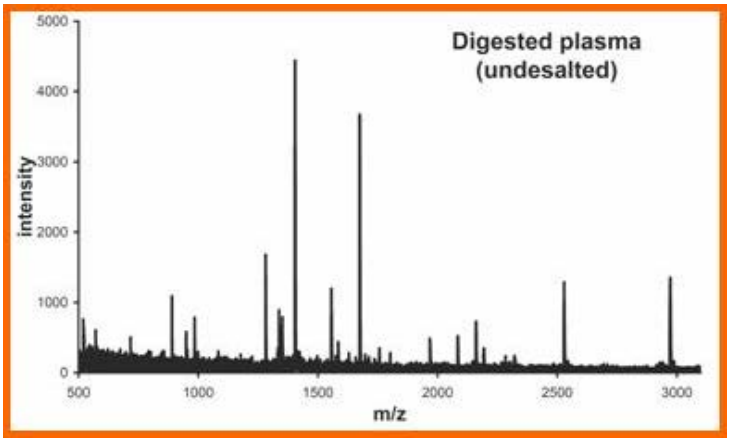


Figure 3d. Digested plasma, undesalted,
Analysis on our dynamic MALDI surface

Figure 3. MALDI analysis of crude human plasma

Conclusion

We describe here functionalized surfaces for single droplet handling. In many cases the analytical chemist must face the manipulation of single droplet samples. The traditional/conventional strategy consists in dissolving the droplet in additional solvent so as to perform the preparation of sample with devices working with a continuous flow of liquid. At the final stage the sample must be concentrated again to the state of a droplet. Therefore, the development of surface chemistry for the purification of single liquid droplets is of a strong interest in order to minimize sample handling and loss.

Unfortunately, monolayer surface chemistry is not compatible with the concentration required for real world analysis using most techniques including mass spectrometry except fluorescence and surface plasmon resonance. Consequently, we decided to use a polymer-based chemistry that we developed previously for capillary columns dedicated to proteomics applications, giving access to 1 micron thick film. Such a thickness allows for handling sample quantities compatible with most analytical detection methods and especially mass spectrometry. The methacrylate-based brush polymer were anchored on a gold surface by using a linker bearing at one end a disulfide and at the other end a bromoester then prepared using an original living polymerisation method. Both non-reactive monomers like butylmethacrylate (BMA) and reactive ones like glycidyl methacrylate (GMA) may be used and mixed together in any proportion.

Plain gold surface on silicon wafer were used as starting surfaces. We first tested the exchange efficiency between droplet and surface by using a hydrophobic laurymethacrylate phase. A one microliter droplet of an intentionally salted (20%) solution containing various peptides (1 picomolar) was placed on the surface either manually or using a dispensing robot and the exchange forced by several aspiration. The spot was washed with water, and analyzed by MALDI. In both cases, peptides were detected with the same intensity as in the starting unsalted solution. Furthermore no adducts were detected in the mass spectrum, showing a very efficient cleaning of the mixture. Analysis on crude biological samples like human plasma shows dramatic results. We were delighted that the major proteins of plasma may be identified using our MALDI target without any treatment of the sample.

The following parameters are currently under investigation, monolith porosity, monolith thickness, nature of the monomers.

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