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Unravelling Nonspecific Adsorption of Complex Protein Mixture on Surfaces with SPR and MS

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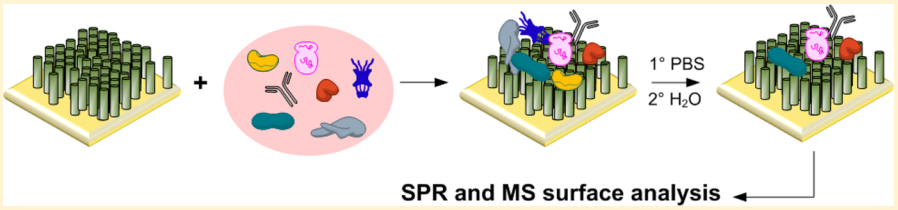
Supporting Information



ABSTRACT: Characterization of protein adsorption to surfaces has implications from biosensing to protective biocoatings. While research studies have principally focused on determining the magnitude of protein adsorption to surfaces, the proteins involved in the process remains only broadly identified and has not been investigated on several surfaces. To further elucidate the nonspecific adsorption process of serum to surfaces, surface plasmon resonance (SPR) and matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) were used in combination to obtain quantitative and qualitative information about the process of protein adsorption to surfaces. To validate the technique, crude serum was nonspecifically adsorbed on four self-assembled monolayer (SAM) on gold: 16-mercaptohexadecanoic acid (16-MHA), 11-mercaptoundecane(ethylene glycol)$_3$-COOH (PEG), 3-MPA-LHDLHD-OH, and 3-MPA-HHHDD-OH. Direct MS analysis of the nonspecifically adsorbed proteins suggested the presence of a variety of protein (BSA, IgG, and apolipoprotein A-1). Performing a trypsin digestion of the nonspecifically adsorbed proteins confirmed the presence of BSA and apolipoprotein A-1 and further revealed the complexity of the process by detecting the presence of complement C3, SHC-transforming protein 1, and kininogen 2. The level of nonspecific adsorption on different surfaces measured by SPR sensing directly correlated with the intensity of the serum protein and indirectly with the tryptic peptides measured by MS. Detailed analysis of the BSA peptides digested on 16-MHA and for BSA digested in solution was used to investigate the orientation of BSA on this surface. The combination of SPR and MS allows the quantitative and qualitative understanding of protein adsorption processes to surfaces.

Understanding the processes involved in nonspecific adsorption is essential for biosensing in crude biofluids. For example, screening for diseases or medical conditions is commonly done by the detection of a specific marker present in serum. Quantification of protein biomarkers remains the premier source of information for the diagnostic of different medical conditions such has cardiomyopathy or cancer.[1,2] The analysis of the biomarkers directly in crude biofluids would be a great improvement in providing rapid, sensitive point-of-care biosensors. However, the complex mixture of background biomolecules in biofluids can lead to important adsorption to the surface of a biosensor, resulting in false positive or deviation of the analytical response. These interferences from the biological matrix have limited direct analysis with biosensors in such complex samples.

Different analytical techniques are used for detection of biomolecules in biological samples. Among them, surface plasmon resonance (SPR) biosensors[3−5] are a promising label-free tool relying on the interaction of the electric field of light undergoing total internal reflection with the electrons oscillating at the surface of a thin film of metal.[6] The plasmon resonance is sensitive to the refractive index at the vicinity of the metallic film and tracking the time-response of the plasmon resonance provides information about the bulk refractive index change and binding of molecules to the surface of the SPR sensor. The surface of the SPR sensor becomes selective to a specific analyte by functionalization of the sensor with a molecular receptor. Among the different classes of biomolecules that can be targeted by a biosensor, proteins are especially suited to SPR sensing due to the important refractive index change of proteins, resulting in high sensitivity of SPR sensors for proteins. In addition, proteins play a major biological role and thus are important biomedical targets. SPR sensing has thus been extensively used to detect many proteins with low nanogram per milliliter sensitivity.[3]

SPR sensors are particularly adapted to perform analysis in biofluids, as the light beam indirectly probes the surfaces of the

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SPR sensors and avoids the issues associated with absorption of biomolecules contained in biofluids and the turbidity of biofluids. However, nonspecific adsorption of proteins to the SPR sensor remains an issue for quantification of biomolecules directly in biofluids. To reduce the nonspecific adsorption, multiple strategies were developed to purify the sample by extraction/separation[7] or to reduce the contribution of interferences on the signal. Solving nonspecific adsorption also requires ultralow fouling surfaces to minimize the background signal coming from the sample. Different classes of surfaces have been proposed to decrease nonspecific adsorption of many blood-based biofluids. Polyethylene glycol (PEG) and its derivatives have been used with undiluted serum to reduce nonspecific adsorption.[8,9] Surface chemistry based on zwitterionic molecules,[10] peptides,[11,12] and many others classes of molecule have also been used for that purpose and were discussed in a recent review.[13] Among the zwitterionic surfaces proposed as ultralow fouling surfaces, polysulfobetaine methacrylic acid (pSBMA), polycarboxybetaine methacrylic (pCBMA) and polyoligoethylene glycol methacrylic pOEGMA significantly reduced nonspecific adsorption.[14,15] For the peptide based surfaces, 3-MPA-LHDLHD-OH showed the greatest reduction of nonspecific adsorption of crude serum.[16] SAMs based on peptides have also been proposed to reduce nonspecific adsorption of other fluids, such as cell lysate.[11] While these surfaces are effective in reducing nonspecific adsorption, the mechanism for the reduction is still debated.

Proteins were identified as the primary source of nonspecific adsorption in serum. It is generally accepted that the surface must be hydrophilic, have hydrogen bond acceptors and electrically neutral for the reduction of nonspecific adsorption of proteins.[17] Surface wetting is also suspected to play a role in minimizing nonspecific adsorption of proteins. However, proteins involved in nonspecific adsorption are poorly identified and the correlation of surface chemistry with the nonspecifically adsorbed proteins remains poorly understood. It is suspected that albumin (66.4 kDa), immunoglobulin-G (IgG) (150 kDa) and fibrinogen (340 kDa) are mainly involved in the nonspecific adsorption process of plasma.[18] Their high concentration in biofluids makes them likely to be involved in nonspecific adsorption. IgG and fibrinogens are present in plasma at concentrations in the micrograms per milliliter range, while albumin can be found in the milligrams per milliliter range.[19] SPR sensing was employed to provide evidence that nonspecific adsorption follows a competitive process with successive adsorption/desorption events to surfaces. This sequential process known as the Vroman cascade is believed to occur rapidly for small protein of high abundance before being replaced by larger protein diffusing slowly but with stronger affinity for adsorption on the surface.[18] For example, albumin can be displaced from the surface by IgG, a larger protein that has more affinity for adsorption on the surface. Then, IgG can be displaced by fibrinogen using a similar process. While previously published studies characterized the adsorption process for pure solution of protein or mixture of proteins, nonspecific adsorption in plasma or serum involves a more complex mixture or protein and little is known about the synergic effect of a complex mixture on nonspecific adsorption. While SPR sensing is effective to quantify proteins, it remains a poor qualitative tool to identify molecules binding to the surface of the sensor. Thus, SPR sensing must be combined with a characterization tool capable of identifying proteins adsorbed to surfaces.

Mass spectrometry (MS) is a state of the art technique for protein analysis and identification.[20] Absorbed proteins can be identified after digestion on a surface using trypsin to generate sets of specific peptides.[21] The MS and MS/MS analysis of these peptides fragments is commonly used for sequencing and identifying proteins. Matrix assisted laser desorption ionization MS (MALDI-MS) is well suited for surface analysis with ionization taking place on the surface.[22] The possibility of performing SPR and MALDI-MS directly on the SPR sensor, the sensitivity of SPR sensing and MALDI-MS for proteins, the complementarity of these surface techniques and the simplicity of adapting analysis of SPR sensors on MALDI-MS makes it advantageous to combine these techniques as previously demonstrated by Nedelkov et al.[23−25] The combination of SPR with mass spectrometry is a promising approach that should enable a rapid identification of biomolecules adsorbed on a surface.[14]

EXPERIMENTAL DETAILS

Preparation of the SPR Sensors. The provenance of the materials is provided as electronic Supporting Information. Microscope slides of 25 × 75 × 1.0 mm or coverslips of 22 × 22 × 0.17 mm were cleaned in warm piranha solution (sulfuric acid + hydrogen peroxide −3:1 at approx.. 80 °C) for at least 90 min. ***Caution:*** *Piranha solution is highly corrosive!* The glass slides were then washed abundantly with deionized water to remove any traces of piranha solution. Cleaned and dried glass slides were covered with 0.5 nm chromium and 45 nm gold using sputtering (Cressington 308R sputter coater, Ted Pella Inc. Redding, CA) with an argon pressure of 0.02 mbar and a current set at 80 mA during deposition. The thickness of the metallic layer was calibrated by AFM. Monolayers of 16-MHA, PEG, 3-MPA-LHDLHD-OH, and 3-MPA-HHHDD-OH were formed overnight on the SPR sensors in DMF solutions at a concentration of 1 mM. The samples were abundantly rinsed with ethanol to remove unbound molecules before drying the SPR sensor with a stream of nitrogen.

Serum Adsorption and Enzymatic Digestion. The different surfaces formed on 25 × 75 × 1.0 mm gold coated microscope glass slide were exposed to bovine serum for 10 min. The SPR sensors with the nonspecifically adsorbed serum were then immersed in PBS for 2 min and in distilled water for 2 min to wash off loosely bound proteins and excess salt. Enzymatic digestion of the proteins was performed directly on the surface at 37 °C by deposition of a solution of 0.045 μg/μL of trypsin. The enzyme was solubilized and activated in a solution of 50 mM acetic acid/100 mM ammonium bicarbonate (1:10). The digestion setup consists of a heating plate on which a glass Petri dish was filled with humidified cotton to maintain a constant temperature and humidity in the chamber during digestion. The samples were deposited directly on the humidified cotton. To monitor the temperature of the chamber during the digestion, a temperature probe was dipped in a droplet of water on a glass slide, also located in the chamber. To compensate evaporation of the solution, small volumes of buffer solution (50 mM acetic acid and 100 mM ammonium bicarbonate with a 1:10 ratio) were added over the course of the digestion to maintain the enzyme in solution.

Bovine Serum Albumin (BSA) Digestion in Solution. A 1 mg/mL solution of BSA was prepared in 100 mM NH_4HCO_3 at pH 8 and 9 μg of trypsin were added for the enzymatic digestion. The mixture was heated at 37 °C in an oil bath for 18

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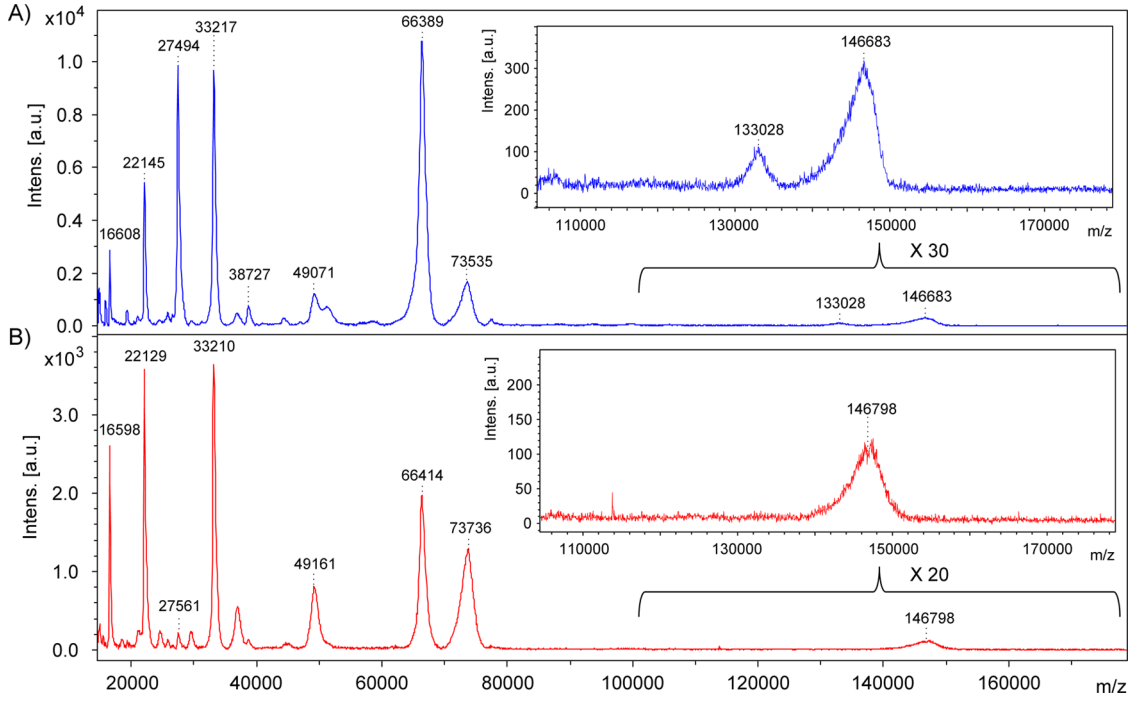

Figure 1. MALDI mass spectra of proteins from whole bovine serum adsorbed on 16-MHA before (A) and after rinsing the surface with PBS (B).

h and the digested solution was placed at −80 °C before analysis.

MS Analysis. Solutions of 10 mg/mL α-CHCA and SA were prepared in 1:1 ACN/H_2O + 0.2% TFA. These matrices were spotted directly on the sample where proteins from bovine serum were adsorbed. The α-CHCA was used to study peptides generated by enzymatic digestion while SA was used to study undigested proteins. Depending on the analysis (protein or peptide), two successive volumes of 0.5 μL of the matrix were spotted on the sample, with enough time between the depositions for the matrix to dry (at room temperature). The total amount of matrix added on the surface was thus 10 μg. Analyses of proteins and tryptic peptides on surfaces were performed with a MALDI-TOF MS UltrafleXtreme mass spectrometer using an Nd:YAG/355 nm laser and operated by Flex Control 3.0 software (Bruker Daltonics, Billerica, MA). Data acquisition of peptides was carried out in reflectron geometry under optimized delayed extraction conditions with a nominal source accelerating voltage of +25 kV in the m/z range between 60−5000. For proteins, data acquisition was done with the linear geometry with optimized sources potentials for the m/z range between 10 000−200 000. Profiling data were acquired by summing 10 × 500 laser shots on a single sample. Calibration of the instrument for lower m/z range was done using a mix of known peptides (Bradikinin, Angiotensin II, ACTH (18−39), Insulin B chain) and a BSA solution was used to calibrate the instrument at higher m/z range. Flex Analysis software (Bruker Daltonics) was then used for mass spectrum analysis. For peptide characterization, MS/MS measurements were performed by using the LIFT/TOF/TOF mode. Protein identification was performed using MASCOT search engine and Swissprot database.

SPR Measurements and Instrumentation. SPR measurements were performed on a custom miniature instrument based on a dove prism.[26] The sensors consisted of gold-coated microscope coverslips of 22 × 22 mm. Nonspecific adsorption of serum was measured on different monolayers. The SPR sensors were mounted using a RI-matching fluid to ensure optical contact with the prism and a custom flow cell was fitted on the system to inject the samples on the SPR sensor. The dark spectrum measurement was acquired with a total of 100 data points with the light source turned off. Then, 100 spectra with s-polarized light were recorded for the reference signal and finally the p-polarized spectra were recorded at different time intervals with a rate of one spectrum per second for the kinetic measurement. A portable spectrometer (Ocean optic, Dunedin, FL) was used to collect data and MatLab was used to process data. Static flow conditions were used for all experiments. The kinetic curves consisted of a PBS baseline trace for a period of 5 min followed by bovine serum (2 mL) injected rapidly and nonspecific adsorption was monitored during 20 min for each monolayer.

RESULTS AND DISCUSSION

Proteins Adsorbed to Surfaces. In recent years, the increasing interest for the direct analysis of biomolecules in complex biofluids and the issue of nonspecific adsorption of biofluids to surfaces has led to the development of surface chemistries providing low fouling. It is also important to understand biofouling of serum on SPR sensors, due to the prevalence of serum samples in bioanalysis. Proteins are expected to mainly participate in nonspecific adsorption because of the high concentration of proteins in biofluids.

SPR sensing was used here to provide quantitative information about the surface concentration of proteins nonspecifically adsorbed from bovine serum on different monolayers. While being very sensitive and quantitative, SPR sensing remains a poor qualitative technique to identify molecules adsorbing to surfaces. Hence, the proteins involved in nonspecific adsorption remain poorly identified. In addition, it remains unknown whether the surface chemistry plays a role on the identity of nonspecifically adsorbed proteins. To address

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this question, three types of SAM were investigated: an alkanethiol (16-MHA), an ethylene glycol derivative (SH-PEG) and two short thiolated peptides (3-MPA-HHHDD-OH and 3-MPA-LHDLHD-OH). These molecules each possess a thiol at the distal end to form a SAM and a carboxylic acid group (COOH) in the α position that allows the binding of a biomolecular receptors using standard coupling chemistry in SPR sensing.

SPR sensors monitored quantitatively and in real time the adsorption of proteins from bovine serum on the modified gold surface. Nonspecific adsorption was calculated for the different SAM from the SPR data and the equation of Jung et al.,[27] using the experimental parameters previously reported.[28] The level of nonspecific binding was greater for 16-MHA, which had 236 ± 63 ng/cm^2 of nonspecifically adsorbed proteins. In comparison, PEG showed lesser nonspecific adsorption than 16-MHA with 100 ± 23 ng/cm^2 as reported previously by our group in the literature using identical conditions.[10,28] The two peptide monolayers further reduced nonspecific adsorption of serum. Nonspecific adsorption was respectively 29 ± 1 and 12 ± 11 ng/cm^2 for 3-MPA-HHHDD-OH and for 3-MPA-LHDLHD-OH.[16] Thus, nonspecific adsorption followed the trend of 16-MHA < PEG < 3-MPA-HHHDD-OH < 3-MPA-LHDLHD-OH, showing the potential of peptide based SAMs for biosensing directly in crude bovine serum.

The MS of undigested proteins provided valuable clues about the identity of proteins adsorbed on the surface (Figure 1). A monolayer of 16-MHA was exposed to bovine serum and proteins nonspecifically adsorbed on the SAM were analyzed by MALDI-TOF. Peak ratios of different ions before and after washing with PBS suggest that a fraction of proteins are loosely bound on the SPR sensor modified with 16-MHA (Figure 1). PBS containing 0.05% of Tween 20 was also used to wash proteins bound on the SAM. Tween 20 is a detergent used to disrupt protein−protein interactions and to limit protein aggregation[29,30] and is commonly used in SPR sensing. Using this detergent should disturb proteins aggregated on adsorbed material on the monolayer[30] leaving mainly protein directly adsorbed on the surface. No noticeable changes in the MS spectra were observed upon the addition of Tween 20 to the wash buffer. This indicated that PBS was appropriate to remove loosely bound material from the surface, as the addition of Tween 20 did not further removed proteins from the surface.

According to the Vroman cascade,[18] adsorption of albumin, IgG, and fibrinogen should be expected for blood or plasma. In our case, fibrinogen should not be observed since it is present in plasma and blood, but absent from serum. As the principal proteins in serum are known[19] and by using the Uniprot database, peaks at 66.4, 33.2, 22.1, and 16.6 kDa are likely due to BSA with various charge states (+1 to +4, respectively), while peaks at 146.7, 73.5, 49.1, and 36.7 kDa could indicate the presence of IgG charged +1, +2, +3, and +4, respectively. Another peak at 27.5 kDa could possibly be attributed to the singly charged apolipoprotein A-1 (confirmed by the sequencing of several tryptic peptides). It is not surprising to observe apolipoprotein A-1, as it was previously reported by Cedervall et al. to adsorb on acrylamide copolymer from plasma.[31] By comparing the intensity of MS peaks for BSA (66.4 kDa) and apolipoprotein A-1 (27.5 kDa), we observed a more significant decrease after washing the SPR sensor for apolipoprotein A-1 (27.5 kDa) than for BSA (66.4 kDa) (Figure 1). This suggests that apolipoprotein (27.5 kDa) is more loosely bound to the surface than BSA (66.4 kDa). In addition to these proteins, a significant number of MS peaks were observed in the mass spectrum, indicating that other proteins were adsorbed on 16-MHA. A few peaks were observed at a lower mass range between 5 and 15 kDa (Supporting Informaton Figure SI1). The main peak at 8.5 kDa may be tentatively assigned to ubiquitin based on mass concordance in Uniprot database. Otherwise, the MS spectra were exempt of significant peaks and thus, the region at higher m/z was the focus of the following experiments.

Apolipoprotein A-1 was reported to adsorb favorably on the surface of nanoparticles of increasing hydrophobicity.[31] Thus, the ratio of the peak intensity of apolipoprotein A-1 (27.5 kDa) and BSA (66.4 kDa) after the PBS wash can provide molecular information about the interaction of serum with different surface chemistries. The apolipoprotein A-1/BSA ratios were calculated at 0.11, 0.42, and 1.40 for PEG, 16-MHA and 3-MPA-HHHDD-OH, respectively. The ratio of apolipoprotein A-1 to BSA follows quite well the trend of hydrophobicity of the surface. Indeed, contact angle values of 25°, 27° and 38° were obtained respectively for PEG, 16-MHA and 3-MPA-HHHDD-OH.[11,12] These results indicate that apolipoprotein A-1 adsorb more favorably to gold surfaces of increasing hydrophobicity, similarly to the polymeric surface previously reported.[31] The nonspecific adsorption of serum does not follow the same trend, since 16-MHA has the larger nonspecific adsorption followed by PEG and 3-MPA-HHHDD-OH. Thus, while hydrophobicity is definitely a factor of protein adsorption, apolipoprotein A-1 does not constitute a predictor for the overall level of nonspecific adsorption and it does not explain the trend of nonspecific adsorption.

It must also be pointed out that 3-MPA-LHDLHD-OH, the most hydrophobic surface tested (contact angle of 53°) and also the surface with the lesser nonspecific adsorption, led to a relatively low ratio of apolipoprotein A-1 to BSA of 0.31, which is not following the trend of apolipoprotein A-1 adsorption in relation to surface hydrophobicity. A negative charge was expected at physiological pHs due to the presence of carboxylate groups. Thus, another factor than hydrophobicity and charge may explain why a smaller apolipoprotein A-1 to BSA ratio for 3-MPA-LHDLHD-OH was observed. The structural conformation adopted by the molecule forming the monolayer may explain this result. Peptides can adopt a secondary structure such as an α helix or a β sheet. Previous structure analysis by IR spectroscopy had shown that 3-MPA-HHHDD-OH will have an elongated structure similar to β-sheets,[12] while 3-MPA-LHDLHD-OH will adopt an α-helix conformation on the surface of the SPR sensor.[16] 3-MPA-LHDLHD-OH might provide a less favorable surface for apolipoprotein A-1 adsorption. Our previous study on 3-MPA-LHDLHD-OH revealed that the structure adopted by molecules forming the monolayer varies with the surface potential applied and changed significantly the nonspecific adsorption behavior.[32] Thus, the hydrophobicity and the structure of the SAM are factors involved in the nature of the protein nonspecifically adsorbed.

Protein Digestion on Surfaces. Enzymatic digestion was necessary to confirm the identification of the protein adsorbed on the SPR sensor from bovine serum. Trypsin generated a variety of peptides due to the presence of several lysine and arginine cleavage sites available in proteins. Tandem MS was used for the selection and fragmentation of peptides of a single m/z ratio to elucidate the amino acid sequence of the corresponding protein after database interrogation. For each

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SAM investigated, monitoring the number and relative intensity of peptides generated was used to optimize the digestion conditions. The optimal proteolysis time was reduced for surfaces with lesser nonspecific adsorption with 45, 40, 20, and 10 min being optimal for 16-MHA, PEG, 3-MPA-HHHDD-OH, and 3-MPA-LHDLHD-OH, respectively. The proteolysis time in that case provided complementary information about the level of nonspecific adsorption and was in agreement with SPR sensing. In addition to proteolysis time, the optimal amounts of MALDI matrix deposited on the SPR sensors correlated with the amount of nonspecifically adsorbed proteins. For example, the amount of CHCA used for analysis on 3-MPA-LHDLHD-OH was only of 5 μg to optimize the intensity for the peptide generated by the proteolysis. This is because a molar ratio of matrix/protein between 500:1 and 5000:1 is normally used to have the best signal-to-noise ratio.[33−35] Nevertheless, other parameters such as the crystallization behavior on each SAM because of their respective hydrophobicity should be considered and could impact the optimal amount of CHCA that should be used.

The number of peptide generated by proteolysis was also proportional to the nonspecific adsorption on a surface (Table 1). Peptides with lower intensities in MS were among the first to be no longer observed for surfaces exhibiting lower nonspecific adsorption. The absence of some peptide in the MS spectrum for low fouling surfaces should not be considered as evidence of the absence of the protein from the surface, but as a decrease of the intensity of these peptides below detection limit of the instrument. In these cases, the peptide might be generated by proteolysis of the protein on the surface, but remained undetected by MS. Thus, the absolute number of peaks generated by enzymatic digestion attributed to proteins (other than trypsin) was reduced from 25 peptides for 16-MHA and SH-PEG-COOH to 18 for 3-MPA-HHHDD-OH and 12 for 3-MPA-LHDLHD-OH. Again, values obtained by MS validate that nonspecific adsorption decreases in that order 16-MHA and SH-PEG > 3-MPA-HHHDD-OH > 3-MPA-LHDLHD-OH, which is in good agreement with SPR data.

Table 1. Peptides Observed by MALDI MS on Different SAM after Tryptic Digestion of Adsorbed Proteins Initially Contained in Crude Bovine Serum and Identification of the Corresponding Protein

$[M + H]^+$ (Da)	16-MHA	PEG	3-MPA-HHHDD-OH	3-MPA-LHDLHD-OH	protein
1017.519	X	X	X	X	apolipoprotein A-1
1163.630	X	X	X		bovine serum albumin
1221.537	X	X	X		complement C3
1301.728	X	X	X	X	SHC-transforming protein 1
1305.654	X	X	X	X	bovine serum albumin
1345.631			X		kininogen 2
1439.760	X	X			bovine serum albumin
1479.744	X	X	X	X	bovine serum albumin
1511.792	X	X	X	X	bovine serum albumin
1567.700	X	X	X	X	bovine serum albumin
1639.886	X	X	X		bovine serum albumin
1791.053	X				complement C3
2045.373	X				bovine serum albumin

The pattern of tryptic peptides is quite complex and varies for each surface (Supporting Information Figure SI2 and Table 1). While the major proteins remained the same, different proteins were observed on the surfaces and are thus also responsible for the nonspecific adsorption. A Mascot database search identified several proteins from bovine serum as nonspecifically adsorbed on the SPR sensor (Supporting Information Table SI1). Peptides are mainly associated with four proteins: BSA, apolipoprotein A-1, complement C3, and SHC-transforming protein 1. These proteins represent the strongly bound fraction of proteins responsible for the nonspecific adsorption and (except SHC-transforming protein 1) are "classical plasma proteins", present at high level in serum[19] (mg/mL for BSA, μg/mL for apolipoprotein A-1 and complement C3). The impossibility of assigning all MS peaks generated from enzymatic digestion was due to low abundance signal for some peptides and their fragmentation spectrum generated afterward by MS/MS was thus inconclusive. Nevertheless, it was possible to associate some unsequenced peptides with the four major proteins identified based on the comparison of the m/z obtained experimentally by proteolysis with the theoretical m/z ratio obtained with in silico digestion (using the MS-digest tool of Protein prospector). To ensure a good confidence in the results obtained (Supporting Information Table SI2), only peptide with a m/z difference between the theoretical and experimental value lower than 0.05 Da were considered. These results help to consolidate the identification of BSA, apolipoprotein A-1, complement C3, and SHC-transforming protein 1 on the SAMs investigated.

Three of the four proteins identified on the SPR surface possessed a molecular weight that match proteins reported previously in the literature as "unknowns" for adsorption of plasma on polystyrene surface (apolipoprotein A-1, 28 kDa; complement C3, 180 kDa; SHC-transforming protein 1, 50 kDa).[31] Nonspecific adsorption of plasma reported by Cedervall et al.[31] was carried with gel filtration chromatography, SDS-PAGE and MS to identify nonspecifically adsorbed proteins. This lengthy sequence of preparation steps can be simplified using MALDI-MS directly on the SPR sensor to identify and quantify nonspecifically adsorbed proteins. Our results for serum proteins nonspecifically adsorbed proteins on the different SPR surfaces complement well literature that was inconclusive. Our SPR-MS approach could be advantageous to identify proteins adsorbed on different surfaces.

While the Vroman cascade has put a large emphasis on BSA and IgG as the major causes of nonspecific adsorption in serum, other proteins (apolipoprotein A-1, complement C3, and SHC-transforming protein 1) are also present on the various surfaces tested and thus play a role in nonspecific adsorption. In addition, it would have been expected to observe tryptic peptides belonging to IgG. However, no peptides associated with IgG were identified on any surface analyzed. Since IgG is found in serum at $\mu g/\mu L$[19] and that a peak at 146.7 kDa was observed for protein after the washing procedure (inset Figure 1), a few factors can explain the absence of peptide associated with IgG. The peak at 146.7 kDa (Figure 1) can possibly be attributed to IgG, but it must be considered that IgG is a class of biomolecule regrouping many different paratopes with

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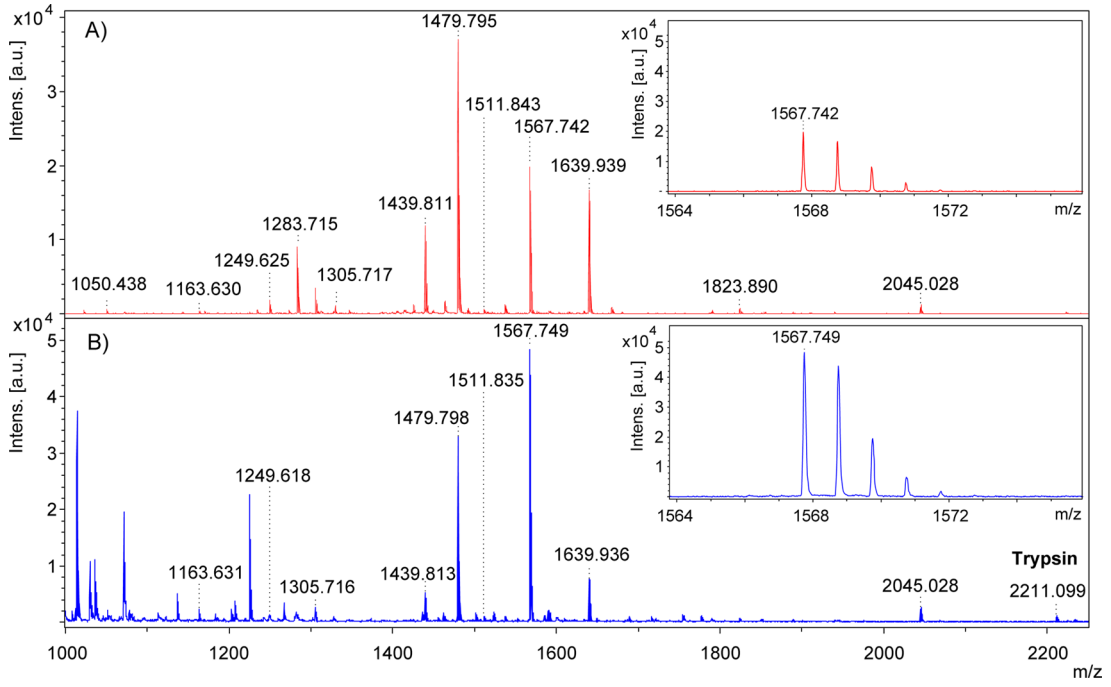

Figure 2. MALDI mass spectra for BSA after trypsin digestion in solution (A) and after its adsorption on a monolayer of 16-MHA (B) with insets displaying the peptide at m/z 1568. Note: The peak at m/z 2211 was identified as an autolysis product of trypsin.

roughly the same m/z ratio. Since the variable chains forming IgGs paratopes will be different from one species to another, different amino acid sequences will be expected and observed after enzymatic digestion. Thus, a small concentration of peptides specific to each IgG would be generated by enzymatic digestion, which would lead to intensities below the detection limit of the MS method. In addition, the quality of the MS/MS spectrum for weak signals may not be sufficient for sequencing and identification of the protein. Finally, the orientation of IgG may be the cause of the weak response to trypsin digestion. Trypsin must access the cleavage sites of IgG adsorbed at the surface and the orientation of IgG on the SPR sensor may be prohibitive for enzyme digestion.

The nature of adsorbed proteins varied on different surfaces. For example, no peptides identified on 3-MPA-LHDLHD−OH were related to complement C3 possibly due to a low abundance of this protein on the surface (Table 1). Nonspecifically adsorbed proteins on the surfaces partly represent the major proteins found in serum. Complements C5−C9, alpha-1-antitrypsin, or transferrin were not detected, even if these proteins are present in serum at concentration level similar to apolipoprotein A-1 or complement C3.[19] The opposite situation where a SAM could have a particular affinity toward a protein might also be possible. For example, a peptide at 1345.631 Da was attributed to the protein kininogen 2 (Table 1) and was only identified on 3-MPA-HHHDD-OH. Another peptide (1542.646 Da) could potentially be linked to this protein based on its m/z and the in silico digestion performed and was only present on the 3-MPA-HHHDD-OH monolayer (Supporting Information Table SI2). Kininogen 2 is a glycoprotein that can be found in serum at concentration in the micrograms per milliliter.[36,37] This protein is known to play an important role in liberating bradykinin,[38] a vasoactive peptide, and to be a thiol protease inhibitor.[39] The fact that kininogen 2 inhibits this type of protease might explained why it has an affinity for the 3-MPA-HHHDD-OH monolayer. Thiol proteases is a class of enzyme that possesses an active site made of a catalytic triad usually composed of three partners: a nucleophile, a base and an acid.[40,41] For a thiol protease, the nucleophile will be a cysteine residue while the base and acid residue might vary. Among the different examples of triad found for proteases, the Ser-His-Asp and the Cys-His-Asp combination are common. Since the 3-MPA-HHHDD-OH is entirely made of amino acid that can be found in the catalytic triad, this monolayer might display affinity for the active site of a thiol protease. This pentapeptide could interact with the catalytic triad of the thiol protease. 3-MPA-LHDLHD-OH did not seem to promote the adsorption of kininogen 2. The presence of an hydrophobic amino acid with His and Asp, or the different configuration of the two peptide monolayers may explain the lower affinity of kininogen 2 to 3-MPA-LHDLHD-OH. Thus, the combination of SPR and MS can further elucidate the proteins nonspecifically adsorbing on surfaces and provide information about the nonspecific adsorption process.

Orientation of BSA on Surfaces. Proteolysis will occur only at certain sites of the protein that are accessible to the enzyme. This selective cleavage is especially the case when proteolysis is carried out for proteins adsorbed on the SPR sensor, where steric hindrance is more important than in solution because of the presence of the surface. MS spectra obtained for digestion of BSA showed differences in intensity for digestion carried in solution and on the SPR sensor (Figure 2). For example, a 300% increase in peak area from digestion on a surface compared to digestion in solution was observed for the peptide at 1568 Da (inset Figure 2). According to the protein structure generated by Protein Homology/analogY Recognition Engine V 2.0 (PHYRE2), this peptide is located on the outer portion of the protein and should be readily accessible for digestion in solution. After adsorption of BSA on 16-MHA, the cleavage sites located on the side of BSA exposed to the solution could be more accessible for proteolysis rather than facing the surface. A small but not negligible change of

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conformation of BSA could also possibly explain the increased signal observed for the peptide after absorption of the protein on 16-MHA.[42]

The ternary structure of BSA is divided into three different domains. The intensities of the trypsic peptides varied from the different domains, but also for single peptides within a domain (Table 2). The peptides generated in solution from domains 2 and 3 were highly represented. They account for respectively 61% and 32% of the total MS peak area for domain 2 and 3 compared to only 6% for domain 1. When the digestion was performed for BSA adsorbed on a surface, the fraction of the total MS peak area for each domain obtained was different with 8%, 79%, and 12%, respectively, for domain 1, 2, and 3. The reduction of the relative intensity observed for domain 3 can possibly come from the adsorption of BSA with this domain predominantly facing the surface. The increase in intensity of domain 2 could be explained by the orientation of this domain toward the solution. Thus, the orientation of the protein adsorbed on a monolayer can be roughly estimated by comparing the relative intensity of the peptides produced enzymatically in solution and for a protein immobilized on a surface.

Table 2. Changes in the MALDI MS Peak Areas of Tryptic Peptides for BSA Digested in Solution and on the SPR Sensor

$[M + H]^+$ (Da)	amino acid sequence	domain	variation (% peak area)[a]
1050.4925[b]	564−573	3	
1163.6307	42−51	1	381
1249.6212[b]	11−20	1	−20
1283.7106[b]	337−347	2	
1305.7161	378−388	2	24
1347.7123[b]	174−185	1	
1439.8118	336−347	2	−32
1479.7954	397−409	2 + 3	23
1511.8428	414−427	3	181
1567.7427	323−335	2	291
1639.9377	413−427	3	−33
1667.8131[b]	445−458	3	
1824.206[b]	484−499	3	
2045.0280	144−159	1	162

[a]The variation represents the change in peak area from digestion performed in solution and on the surface of the SPR sensor. [b]These MS peaks were not sequenced by MS/MS, but correspond to known BSA tryptic peptides based on the m/z ratio. This was confirmed after in silico digestion of the albumin sequence (using the MS-digest tool of Protein prospector).

The integration of SPR sensing and MS experiments on a single substrate have several potential applications. The field of nonspecific adsorption is currently maturing with the discovery of several surface chemistries competent to reduce nonspecific binding and perform bioanalysis directly in crude biofluids. Identifying the molecules still nonspecifically adsorbing to biosensors' surfaces will be important to further reduce nonspecific adsorption. It is hypothesized that mixed monolayers with different nonspecific adsorption profiles may further reduce overall nonspecific adsorption of biofluids. The quantitative nature of SPR sensing can be combined with the high quality data of MS to provide quantitative information to MS data. For example, imaging MS has gained significant attention for providing high quality information about the localization of molecules in tissue sections.[43] However, quantitation has proven difficult and the combination of imaging SPR with imaging MS may solve this challenge. We are currently applying these techniques to selectively transfer the proteome contained with thin tissue sections to imaging SPR chips for quantitation and detecting the different molecules adsorbed to the surface (data not shown). Identifying the proper surface chemistries and understanding the nonspecific adsorption profiles in biofluids will be key in succeeding with the combination of imaging MS and imaging SPR for the qualitative and quantitative analysis of tissue sections.

CONCLUSIONS

Combining SPR and MALDI-MS provides a powerful tool to analyze nonspecific adsorption of proteins on surfaces. The combination of SPR and MS made possible the quantification of nonspecifically adsorbed proteins on a surface with SPR and the identification of these with MALDI-MS. This combination of analytical tools has permitted to further improve the understanding of serum nonspecific adsorption. The results reported here reveal the complexity of the nonspecific adsorption process of a variety of proteins (BSA, apolipoprotein A-1, complement C3, SHC-transforming protein 1, and kininogen 2). Also, nonspecific adsorption of serum involves a greater number of proteins than the Vroman cascade suggests. Different proteomes were identified for different SAM, even revealing that certain monolayer might have some affinity for given proteins. Investigating the affinity of SAM for proteins could be an interesting axis of research where monolayer would be used to promote adsorption of certain protein while preserving low fouling. Lastly, MS was suited to study the orientation of proteins at the surface. By comparing the relative intensity of BSA peaks from a digestion in solution and on the surface of the SPR sensor, it was revealed that BSA preferentially adsorb from domain 3, exposing domain 2 to the solution. Therefore, SPR and MS provide complementary information to further understand nonspecific adsorption processes.

ASSOCIATED CONTENT

Supporting Information

Additional tables and figures as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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