Monolayers of 3-Mercaptopropyl-amino Acid to Reduce the Nonspecific Adsorption of Serum Proteins on the Surface of Biosensors

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Monolayers prepared with polar or ionic amino acids with short side chains have a reduced nonspecific adsorption of serum proteins compared to that of hydrophobic amino acids and organic monolayers immobilized on the gold surface of surface plasmon resonance (SPR) biosensors. Proteins contained in biological samples adsorb on most surfaces, which in the case of biosensors causes a nonspecific response that hinders the quantification of biomarkers in these biological samples. To circumvent this problem, self-assembled monolayers (SAM) of *N*-3-mercaptopropyl-amino acids (3-MPA-amino acids) were prepared from 19 natural amino acids. These SAM were investigated to limit the nonspecific adsorption of proteins contained in biological fluids and to immobilize molecular receptors (i.e., antibodies) that are necessary in the construction of biosensors. SPR and Ge attenuated total reflection (GATR) FTIR spectroscopy were employed to characterize the formation of the amino acid SAMs. Monolayers of 3-MPA-amino acids densely packed on the surface of the SPR biosensors result in a surface concentration of approximately 10^{15} molecules/cm^2. SPR also quantifies the surface concentration of serum proteins nonspecifically adsorbed on 3-MPA-amino acids following the exposure of the biosensor to undiluted bovine serum. The concentration of nonspecifically bound proteins ranged from approximately 400 ng/cm^2 with polar and ionic amino acids to approximately 800 ng/cm^2 with amino acids of increased hydrophobicity. The nonspecific adsorption of serum proteins on the 3-MPA-amino acids increases in the following order: Asp < Asn < Ser < Met < Glu < Gln < Thr < Gly < His < Cys < Arg < Phe < Trp < Val < Pro < Ile < Leu < Ala < Tyr. The analysis of the adsorption and desorption curves for serum proteins on the SPR sensorgram has demonstrated the strong irreversibility of the protein adsorption on each surface. The effective hydrophilicity of the SAMs was measured from the contact angle with a saline buffer and has demonstrated that surfaces minimizing the contact angle with PBS performed better in serum. The antibody for β-lactamase was immobilized on a 3-MPA-glycine SAM, and β-lactamase was detected in the nanomolar range. The presence of β-lactamase is an indicator of antibiotic resistance.

Introduction

Surface plasmon resonance (SPR) is a promising detection technique for fulfilling current needs in the healthcare industry for the rapid, inexpensive diagnostic measurement of biomarkers present in biological samples.[1−3] SPR measures biomarkers (e.g., antigens, DNA, RNA, or enzymes) at concentrations on the order of picomolar or nanomolar in saline solutions. However, biological samples typically consist of a relatively complex mixture of compounds, including a high concentration of nonspecific proteins (e.g., cell culture media contains 1−10 mg/mL protein[4] and serum contains 40−80 mg/mL protein[5]). Moreover, proteins contained in biological samples tend to adsorb nonspecifically to surfaces,[6,7] a process initiating a cascade of phenomenon leading to the rejection of foreign objects in the human body such as prostheses. This problem is not only confined to prosthesis or implant rejection but also limits the use of many biosensing platforms for analyses in biological samples. The origin of this problem is due to the adsorption of proteins on the surface of biosensors, leading to a nonspecific response of the biosensor, which is undistinguishable from the response resulting from the binding of a specific biomarker. In the case of SPR biosensors, the molecular receptor immobilized on the surface of the SPR biosensor recognizes a unique unlabeled biomarker in the solution. Binding of the biomarker to the molecular receptor changes the refractive index of the solution near the biosensor, resulting in a measurable change in the optical response with SPR. This RI change is recorded in real time to monitor the specific binding of the biomarker and to determine the concentration of biomarkers in solutions. However, the adsorption of nonspecific proteins contained in biological samples also causes a change in the refractive index, masking the response from the biomarker.[8] This effect has currently limited SPR biosensing and other biosensing technologies to mostly saline solutions or relatively dilute biological solutions.[5] The dilution of biological samples is usually not an option in bioanalysis because of a relatively low concentration of biomarkers in the biological solutions. Therefore, the reduction of protein adsorption on metallic surfaces is important for many medical applications, especially in extending the life of prostheses and improving the utility of biosensors with biological samples.

SPR biosensors are constructed from a gold thin film immobilized on a dielectric material. Thiol chemistry forms a stable chemical linker on Au surfaces, and it is typically used to immobilize antibodies on Au films.[9−11] Several approaches have been designed to immobilize antibodies on surfaces,

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including but not limited to immobilization on a thiol,[12] on a polymer derived with a thiol,[13,14] or on a polysaccharide immobilized on a thiol[15] or using streptavidin immobilized on a thiol.[16] With each methodology used to immobilize the antibody, the chemical layer serves a dual function on the surface: it must immobilize a maximal concentration of antibodies to increase the signal of the biomarker and it must eliminate the nonspecific adsorption of proteins. Many SAMs have been successfully developed for each purpose individually. However, there is no unique monolayer exhibiting satisfactory functionality for both purposes simultaneously. As an example, a 3D matrix of CM-dextran exhibits a large response from biomarkers as a result of the immobilization of a large concentration of antibodies on the CM-dextran matrix.[15] However, CM-dextran does not perform well in complex solutions.[13] Blocking vacant sites on the surface using bovine serum albumin (BSA) may limit the interactions between the biosensor and biological samples. Otherwise, various approaches were exploited to reduce nonspecific protein adsorption, with the most promising one using poly(ethylene oxide) (PEO) to coat surfaces.[17−19] Among the strategies explored, Whitesides et al. used zwitterionic[20] molecules and explored a vast library of functional groups as terminal groups at the surface of biosensors.[21] They found that hydrophilic monolayers with hydrogen bond donors and no hydrogen bond acceptors and neutral monolayers exhibited improved reduction of the nonspecific adsorption of fibrinogen and lysozyme.[21−25] It was also proposed to use alkanethiols of different lengths to limit the effect of serum protein on SPR biosensors.[26] A membrane-cloaking methodology was recently developed to remove nonspecific adsorption after a sample was exposed to biological fluid, without disrupting the biomarker bound to the molecular receptor.[27] In spite of these results, the mechanism of protein adsorption is not clearly understood.[28] It is suggested that different proteins consecutively adsorb at surfaces, starting with the most prominent protein in serum, albumin, followed by larger proteins such as IgG and fibrinogen.[6,7] This protein cascade at the surface is the first step toward cell adhesion on surfaces. This plenitude of species contributing to nonspecific adsorption requires a model system with protein content similar to that of human serum. Therefore, the nonspecific adsorption of serum protein must be investigated using undiluted serum, such that nonspecific adsorption is measured under conditions similar to those of biological samples.

A novel approach uses biological building blocks to build monolayers and has been the focus of research in mitigating the nonspecific adsorption from proteins contained in biological samples. Of particular interest, Statz et al. synthesized peptidomimetic polymers of alternating L-3,4-dihydroxyphenylalanine (DOPA) and lysine residues to minimize cell adhesion.[29] Thereby, they showed a reduction of cell adhesion by 2 orders of magnitude compared to that of current state-of-the-art methodologies. Otherwise, it was demonstrated that a pentapeptide inhibits the aggregation of gold nanoparticles and also serves as a chemical layer to build a biosensor.[30,31] Hence, amino acid or peptide monolayers have great potential for biosensing: they are biocompatible and a wide selection of functional groups is available, such that they have tunable chemical properties and can be used in the construction of biosensors with the immobilization of antibodies on the terminal carboxylic acid of amino acids or short peptides. Natural amino acids have a broad range of structural and chemical properties, with amino acids having either short or long side chains, hydrophobic or polar side chains, and neutral or ionic side chains. These properties of amino acids make them highly suitable candidates for the determination of the relationship between the chemical structure of monolayers and the nonspecific adsorption of serum on sensors. 3-MPA-amino acids also possess the chemical characteristics necessary for biosensing purposes. An amino acid monolayer may improve the activity retention of immobilized molecular receptors by providing a protein-like environment at the surface. These characteristics are essential for the development of functional biosensors in real biological samples and may avoid the use of blocking agents such as BSA on the surface of biosensors to minimize interactions with biological samples.

This article demonstrates that polar or ionic amino acids attached to 3-mercaptopropionic acid significantly limit nonspecific adsorption from serum proteins. The hydrophilic properties of each SAM were measured using the contact angle, which demonstrates that the SAM with larger hydrophilic character reduces the nonspecific response from bovine serum. The potential of such SAMs for SPR biosensing is demonstrated with an affinity biosensor using an antibody specific to β-lactamase attached to a 3-MPA-Gly monolayer. The presence of β-lactamase is one of the most common factors in antibiotic resistance.[32] Standard detection techniques for β-lactamase involve cell culture that makes these tests unsuitable for the analysis of a large number of samples and for obtaining a rapid response of the β-lactamase concentration. Therefore, it is imperative to improve the time required to perform a β-lactamase assay in order to rapidly diagnose patients exhibiting resistance to traditional antibiotics. Direct detection of this enzyme in cell culture media, serum, or blood samples using SPR biosensors would offer improved methodology to quantify β-lactamase compared to actual detection techniques.[33]

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Experimental Section

Preparation of 3-MPA-amino Acids. Glu, Asp, Asn, and Gln were purchased in the acidic form and reacted to form the methylester on the free carboxylic acids using thionyl chloride ($SOCl_2$, Fluka, purity >99%) in methanol (Fisher Scientific).[34] This operation was necessary to avoid the polymerization of the amino acids. 3-Mercaptopropionic acid (3-MPA) was purchased from Sigma-Aldrich with a purity exceeding 99% and used without further purification. Careful handling of 3-MPA must be undertaken when it is added to solution because this liquid has a very strong odor. The reaction mixture to form the 3-MPA amino acids contained 1 equiv of 3-MPA (2.4 mmol), 1.2 equiv (2.9 mmol) of 4-(dimethylamino)pyridine (DMAP, Fluka purity >98%), 1.1 equiv (2.6 mmol) of *N*,*N*′-dicyclohexylcarbodiimide (DCC, Sigma-Aldrich, purity >99%), and 1.1 equiv (2.6 mmol) of the amino acid. These reagents were dissolved with 15 mL of *N*,*N*-dimethylformamide (DMF, EMD).[35] Sonication was required to dissolve the reaction mixture completely. The reaction mixtures were stored overnight at room temperature, away from excessive light and without agitation. The amino acids were used in the methylester hydrochloride form (Sigma-Aldrich or Fluka), hence requiring >1 equiv of DMAP for the reaction. One equivalent of DMAP neutralized the hydrochloride, and the excess, up to 0.1 equiv of DMAP, catalyzed the coupling reaction between 3-MPA and the amino acid. After the completion of the reaction, each reaction mixture was thereafter filtered to remove a white precipitate of DCC-urea. The filtrate was then evaporated to obtain the 3-MPA-amino acids, generally a white solid with a creamy texture. The FTIR spectrum for each 3-MPA-amino acid was acquired using FTIR with Ge attenuated total reflection (GATR) (Bruker optics) to ensure the completeness of the reaction.

Preparation of 3-MPA-amino Acid Monolayer. Solutions of 5 mM of 3-MPA-amino acid were prepared in absolute ethanol to form a monolayer on microscope slides (BK7, 22 × 22 mm^2, Fisher Scientific) coated with a 5 nm Cr adhesion layer and 48 nm of Au (ESPI metals) using a Cressington 208HR sputter coater. Each amino acid was tested with four replicates. The solution of 3-MPA-amino acid was reacted for at least 16 h to form a well-ordered monolayer.[14] Thereafter, each sample was rinsed with Millipore water and was submerged in a 0.01 M HCl solution for 2 h to hydrolyze the methylester, resulting in the unprotected carboxylic acid on the surface of the SAM. Absolute ethanol was then used to wash each sample. The reaction was followed using SPR spectroscopy and FTIR-GATR to ensure completeness.

SPR and Contact Angle Measurements. The advancing contact angle was measured for each sample with a contact angle instrument built in-house. The contact angle was obtained from 300 μL of PBS (CellGro, Mediatech Inc.). PBS was used to measure the contact angle in a solution of salinity and pH similar to those in serum or other biological samples. The slides were then mounted on a custom-made SPR instrument constructed with modified prism-based geometry using wavelength interrogation. The SPR sensors were stabilized in PBS buffer for 5 min before the spectral reference in s polarization was acquired. Real-time measurement then started in p polarization for 5 min before replacing the PBS buffer with bovine serum (Sigma) containing 76 mg/mL protein for 20 min. The serum was then replaced with PBS for another 5 min to quantify the amount of protein still attached to the surface of the SAM. Raw data were then processed with MatLab. This procedure was repeated for each of the 19 natural amino acids to obtain at least 3 experimental curves for each amino acid.

Measurement of Bovine Serum Protein Desorption with High-Salinity PBS. The irreversibly adsorbed proteins immobilized on 3-MPA-Ser were exposed to PBS enriched with 1 M sodium chloride (Sigma-Aldrich, purity >99.5%). Increasing the ionic strength may remove adsorbed proteins.[21] This experiment was conducted on 3-MPA-Ser that was exposed to bovine serum using the same conditions as described above. After 5 min in unspiked PBS, the solution was replaced with NaCl-enriched PBS for 5 min and then exposed again with unspiked PBS for another 5 min to obtain the amount of protein desorbed.

Immobilization of Anti-β-lactamase. The antibody specific for β-lactamase (QED Bioscience Inc.) was covalently immobilized on an NHS-derived 3-MPA-Gly to demonstrate the use of 3-MPA-amino acid SAMs for the construction of a biosensing layer. β-Lactamase was prepared according to Pelletier et al.[36,37] The 3-MPA-Gly monolayer (four replicates) was prepared as described above. Two different approaches were used to immobilize the antibody, and both are based on the reaction of 3-MPA-Gly with *N*-hydroxysuccinimide (NHS, Sigma-Aldrich, purity >98%).[38−40] This results in the activation of the free carboxylic acid of 3-MPA-Gly in the form of 3-MPA-Gly-NHS. The first approach allowed 3-MPA-Gly to react for 20 min in an aqueous solution composed of 25 mM NHS and 25 mM *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide (EDC, Fluka, purity >97%).[41] The second approach involved the reaction of 3-MPA-Gly with 75 mM of DCC and 75 mM of NHS overnight. NHS and EDC or DCC were all used in large excess compared to 3-MPA-Gly to ensure that an optimal number of binding sites would be available for antibody attachment. Thereafter, the antibody specific to β-lactamase was diluted in sterile PBS to a concentration of 37 μg/mL and reacted overnight with 3-MPA-Gly-NHS on samples previously rinsed with PBS. Each step involving the antibody or an antibody-derived sample was accomplished in a refrigerated laboratory at 4 °C unless otherwise indicated.[42] Thereafter, the samples were rinsed with PBS and reacted for 10 min in a 1 M aqueous solution of ethanolamine hydrochloride (Sigma-Aldrich) adjusted to a pH of 8.5 with 10 M NaOH (Fluka, purity >98%). This step deactivates unreacted NHS remaining on the surface after antibody immobilization.[43] The slides are stored in PBS at 4 °C for at least 60 min prior to use.

Detection of β-Lactamase. A solution of 909 nM β-lactamase was prepared in PBS at 4 °C by the dilution of a stock solution. This solution is kept at 4 °C until 20 min prior to use and then equilibrated at room temperature. A sensor with the β-lactamase-specific monolayer was mounted on the SPR instrument, and room-temperature PBS was placed on the sample for 10 min to stabilize the sensor. A spectral reference (s-polarized light) was acquired immediately before the real-time measurement started. PBS was measured for 5 min to acquire the baseline response and was thereafter replaced with the β-lactamase solution for 20 min. Finally, the sensor was again placed in PBS for 5 min to verify whether the binding between the anti-β-lactamase and β-lactamase was reversible.

Results and Discussion

FTIR Characterization of the 3-MPA-amino Acid SAMs. The preparation of 3-MPA-amino acids was performed in a DMF solution using DCC coupling and DMAP catalysis. DMAP was added at >1 equiv; the first equivalent serves to neutralize the hydrochloride of the methylester amino acid, and the small excess catalyzes the reaction. The reaction is performed with the methylester of the amino acid to avoid the formation of an oligopeptide. However, lysine has yielded a mixed oligopeptide, but it will not be included in these experiments. The synthesis

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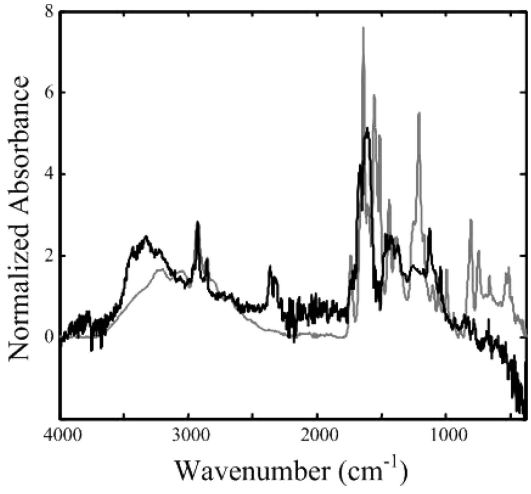

Figure 1. GATR FTIR obtained for a 3-MPA-Tyr complex in solution (thin line) and on a microscope slide coated with 48 nm of gold (thick line).

of 3-MPA-amino acids resulted in excellent yields (>90%). The IR spectra for the product of the reaction between 3-MPA and the amino acid were obtained to confirm the formation of 3-MPA-amino acids. The carbonyl band was the focus of the FTIR analysis because it clearly shows the formation of an amide bond (typically 1630−1680 cm^{-1}) from the carboxylic acid of 3-MPA (typically 1740−1800 cm^{-1}). The C═O stretching band originally at 1720 cm^{-1} for the carboxylic acid shifted to 1640 cm^{-1}, confirming the formation of the amide bond expected for the 3-MPA-amino acids. A C═O band is also observed at 1740 cm^{-1}, corresponding to the methylester form of the amino acid (typically 1730−1750 cm^{-1}). Because all IR spectra were confirmatory for the preparation of 3-MPA-amino acid in the methylester form, a monolayer was formed for each of these products on the Au surfaces of SPR biosensors. The formation of each monolayer was also confirmed by GATR FTIR. The surface of the sensor, onto which the monolayer is immobilized, is placed in direct contact with the GATR crystal to measure the FTIR spectra of the 3-MPA-amino acids. Figure 1 shows the spectra obtained for 3-MPA-Tyr in the methylester form in solution and for the monolayer. Both spectra overlay well, with the bands at 1740 and 1640 cm^{-1} being present in both spectra, confirming the formation of the 3-MPA-Tyr monolayer. The relative intensity of each band usually varies between that of an FTIR spectrum acquired in solution compared to that of the FTIR spectrum acquired in the solid phase of a monolayer, as observed in this case. The absorbance signal around 2400 cm^{-1} was a characteristic of CO_2 present during the measurement. The noise present in the spectrum of a 3-MPA-Tyr in the methylester form of the monolayer is mainly due to water absorption. Lastly, the spectrum of the 3-MPA-amino acid following hydrolysis was acquired as a monolayer on Au to confirm the completeness of the reaction of the 3-MPA-amino acid in the methylester form with HCl. The CH_3 stretching band at 2852 cm^{-1} observed in Figure 1 is characteristic of a CH_3 attached to an oxygen atom such as in the methylester form of 3-MPA-amino acid. Following the reaction with HCl, this band is no longer present. confirming the hydrolysis of the methylester to the carboxylic acid form. Therefore, the FTIR spectra are confirming the presence of each 3-MPA-amino acid on the Au surface of SPR biosensors.

SPR Characterization of the Formation of the 3-MPA-amino Acid SAMs. Monitoring the change of the SPR response ($\Delta\lambda_{SPR}$) during the formation of 3-MPA-amino acids monolayer on the sensor measures the surface concentration of the SAM. The SPR response is calculated from the SPR wavelength in PBS of a bare gold surface and the SPR wavelength in PBS with the 3-MPA-amino acids SAM immobilized on the gold surface. The difference in the SPR response from these measurements results in $\Delta\lambda_{SPR}$. To obtain experimentally the surface concentration (Γ) for each SAM from $\Delta\lambda_{SPR}$, the equation from Jung et al. was employed.[13,44]

$$\Gamma = \rho\left(\frac{-1l_d}{2}\right)\ln\left(1 - \frac{\Delta\lambda_{SPR}}{m(\eta_{SAM} - \eta_{PBS})}\right) \quad (1)$$

The sensitivity (m) of the current SPR biosensor was measured at 1765 ± 100 nm/RIU using a calibration of the sensor with sucrose solutions. The refractive index of PBS buffer (η_{PBS}) was experimentally measured at 1.33476 ± 0.00002 RIU with a high-resolution refractometer. The penetration depth of plasmons (l_d) is approximately 230 nm (use 2.3×10^{-5} cm in eq 1) at the experimental SPR wavelength (λ = 630 nm), and the refractive index of thiols (n_{SAM}) is 1.45 RIU and their density (ρ) is 0.9 g/cm^3.[9] Hence, the surface concentration for each 3-MPA-amino acid is obtained in ng/cm^2 and is thereafter converted to molecules/cm^2 with the molar mass and Avogadro's number (Table 1). The surface concentration of the 3-MPA-amino acid SAM varies between 0.18×10^{15} and 1.80×10^{15} molecules/cm^2, indicating that dense monolayers are formed on the surface of the SPR biosensors. Denser monolayers are mostly obtained from aliphatic amino acids with shorter side chains and aromatic amino acids capable of forming hydrogen bonds. Otherwise, sulfur-containing aliphatic amino acids with long side chains, alcohols, and amine amino acids form the less dense monolayer among 3-MPA-amino acids. Amide 3-MPA-amino acids form denser SAMs than do the corresponding acids (Gln > Glu and Asn > Asp). Thus, various steric and physicochemical factors dictating the packing efficiency of 3-MPA-amino acid monolayers.

Nonspecific Adsorption of Serum Proteins on Surfaces. The adsorption of serum proteins to surfaces is a critical problem to solve in order to develop biosensors that work in biological fluids. The model system used in this study is bovine serum as previously reported elsewhere.[13,26] Bovine serum has a total protein concentration (76 mg/mL) near that of human serum with a protein composition similar to that of human serum, therefore providing a good model system for the use of biosensors in human biological fluid. Thus, the adsorption of bovine serum protein was measured for each 3-MPA-amino acid monolayer and with 16-mercaptohexadecanoic acid (MHA). MHA will serve as a comparison point with previous studies. MHA had previously exhibited low nonspecific adsorption.[26]

The surface concentration for the total protein concentration (Γ_{TOT}) bound to each surface, the fraction irreversibly (Γ_{IRR}) bound to each surface, and the fraction reversibly (Γ_{REV}) bound were extracted from the SPR kinetic curves (Figure 2). The difference between $\Delta\lambda_{TOT}$ and $\Delta\lambda_{IRR}$ results in $\Delta\lambda_{REV}$. The shift $\Delta\lambda_{RI}$ was due to the refractive index difference of PBS and bovine serum. The end point for $\Delta\lambda_{TOT}$ is consistently measured from the SPR wavelength at the 20th minute of exposition to serum. The surface concentration of bound proteins (Γ_{TOT}) is obtained from the change in SPR wavelength measured during serum exposure to the SPR sensors using eq 1. The parameters required to calculate Γ_{TOT} of an adsorbed protein layer are identical to those described in eq 1, except for the refractive index of proteins ($n_{protein}$), which is 1.57 RIU, and the density (ρ) is 1.3 g/mL for proteins.[44]

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Table 1. Surface Concentration of 3-MPA-amino Acids on the SPR Biosensors

	amino acid	Γ_{SAM} (10^{15} molecules/cm^2)	polar	amino acid	Γ_{SAM} (10^{15} molecules/cm^2)
hydrophobic	Gly	1.39	**polar**	Met	0.63
aliphatic and aromatics	Ala	1.80	sulfur-containing amides and alcohols	Cys	0.35
	Val	1.00		Ser	0.29
	Leu	0.79		Thr	0.18
	Ile	0.68		Gln	1.47
	Pro	0.58		Asn	0.72
	Phe	0.52	**ionic**	Asp	0.61
	Tyr	1.12	acids and bases	Glu	1.05
	Trp	1.25		His	0.34
				Arg	0.19

Most interactions occurring on 3-MPA-amino acid monolayers are irreversible with 95% of nonspecifically bound proteins remaining on the surface after a wash with PBS (Figure 2). This value is obtained from the average response for every 3-MPA-amino acid, and no trend could be extracted from comparison of the fraction of irreversible adsorption between amino acids. Increasing the ionic strength of PBS exposed to the 3-MPA-amino acids partially dislodges the irreversibly bound proteins. A significant fraction (approximately 50%) of the irreversibly adsorbed proteins is removed from a highly ionic solution consisting of PBS spiked with 1 M NaCl. The proteins removed from high ionic strength PBS are most likely to be bound with ionic interactions to the surface. Hence, irreversible protein adsorption predominates at the surface of 3-MPA-amino acids irrespective of the amino acid composition, and ionic interactions between the serum proteins and 3-MPA-amino acid contribute significantly to the nonspecific adsorption process.

Adsorption of Serum Protein on 3-MPA-amino Acids. The chemical composition of the side chain for each amino acid greatly influences the properties of the monolayer immobilized on the SPR biosensors. The surface concentration of nonspecifically adsorbed protein varies from 416 ng/cm^2 for 3-MPA-Asp to 808 ng/cm^2 for 3-MPA-Tyr, with a mean of 551 $\pm$ 59 ng/cm^2 (Table 2). The results are reported for four replicate measurements, and the error indicates two standard deviations about the mean. The amino acids are classified in Table 2 according to the hydrophobic, polar, and ionic groups. The hydrophobic amino acids are composed of the aliphatic and aromatic subgroups, the polar amino acids include sulfur-containing alcohols and amides, and the ionic groups contain the acids and bases. Differences in the surface concentration of nonspecifically adsorbed proteins are observed between each group. Hydrophobic amino acids adsorb on average 656 $\pm$ 68 ng/cm^2 of nonspecific proteins from serum, a value significantly larger than for polar (446 $\pm$ 31 ng/cm^2) or for ionic (474 $\pm$ 67 ng/cm^2) amino acids. Although there are no differences in surface coverage for the nonspecifically adsorbed proteins between aliphatic (656 $\pm$ 151 ng/cm^2) and aromatic (657 $\pm$ 81 ng/cm^2) amino acids and within the polar group (Table 2), the ionic amino acids show a distinct pattern within the group. The negatively charged acids have a significantly lower surface coverage of nonspecifically bound proteins at 423 ng/cm^2 (average for Asp and Glu) compared to 524 ng/cm^2 (average for Arg and His) for the positively charged amines. The difference in the charge of the side chain is not the only variable physicochemical property between these groups. The acidic amino acids also have a much smaller side chain than do the amine amino acids. Hence, this notable difference in the surface coverage of nonspecifically adsorbed proteins may be due to a product of various physicochemical differences between acids and amines.

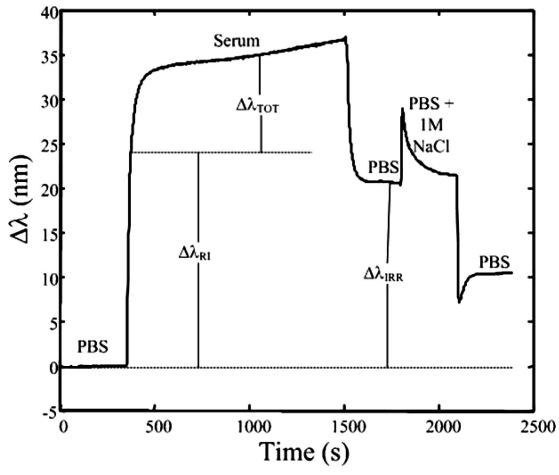

Figure 2. Kinetic curve obtained for a 20 min exposure of bovine serum followed by a 5 min exposure of enriched PBS buffer on a 3-MPA-Ser SAM. Additional sensorgrams are available in Supporting Information for different amino acids.

To determine if the size of the side chain of 3-MPA-amino acids is a factor in the number of nonspecific proteins adsorbing to the SPR sensors, a comparison is required between amino acids with identical functionality but different alkyl chain lengths. Acidic amino acids 3-MPA-Asp and 3-MPA-Glu differ by one methyl group on the side chain. 3-MPA-Asp has the shortest side chain and exhibits a lower surface coverage of nonspecifically bound proteins than does 3-MPA-Glu. Similarly, amides 3-MPA-Asn and 3-MPA-Gln follow the same trend, with 3-MPA-Asn exhibiting a lower surface coverage than 3-MPA-Gln. 3-MPA-Ser is an alcohol with a side chain shorter by one methyl group than that of 3-MPA-Thr. 3-MPA-Ser also shows a nonspecific adsorption lower than for 3-MPA-Thr. Hence, a trend is emerging from these observations; a smaller side chain results in lower nonspecific adsorption from amino acids bearing identical chemical functionality. This trend may also explain the significantly lower surface coverage of 3-MPA-Gly compared to that of all hydrophobic amino acids. Hence, a multitude of physicochemical properties including the size, hydrophobicity, and charge are influencing the nonspecific adsorption of serum proteins on the surface of biosensors covered with 3-MPA-amino acid SAMs.

Physicochemical Properties Limiting Nonspecific Adsorption. To adequately represent the factors influencing the nonspecific adsorption properties of 3-MPA-amino acids, a model must account for each physicochemical property of the 3-MPA-amino acids. A group theory was previously developed for the classification of amino acids with similar physicochemical properties of the side chain.[45] With the theory developed by Taylor, amino acids can be classified using one or more of three primary physicochemical properties: hydrophobic, polar, and small.[45] Thereafter, the amino acids are classified with a secondary set of physicochemical properties including ionic, aromatic,

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Table 2. Nonspecific Binding of Bovine Serum Proteins and Advancing Contact Angle on 3-MPA-AAs

	amino acid	Γ_{total}(ng/cm^2)	$\theta_{c\_advancing}$(deg)		amino acid	Γ_{total}(ng/cm^2)	$\theta_{c\_advancing}$(deg)
hydrophobic	Gly	473 ± 133	52 ± 3	**polar**	Met	422 ± 76	43 ± 2
aliphatic and aromatics	Ala	740 ± 75	60 ± 1	alcohols, sulfur-containing and amides	Cys	515 ± 146	46 ± 1
	Val	614 ± 95	56 ± 3		Ser	419 ± 33	38 ± 4
	Leu	706 ± 19	63 ± 1		Thr	462 ± 135	42 ± 3
	Ile	703 ± 57	58 ± 2		Gln	442 ± 70	53 ± 5
	Pro	702 ± 92	61 ± 4		Asn	417 ± 39	40 ± 4
	Phe	575 ± 202	67 ± 3	**ionic**	Asp	416 ± 121	32 ± 2
	Tyr	808 ± 51	55 ± 3	acids and bases	Glu	431 ± 24	38 ± 2
	Trp	588 ± 91	65 ± 2		His	486 ± 149	37.3 ± 0.3
					Arg	563 ± 140	33 ± 2

aliphatic, tiny, and the proline subgroups. The distinct form of the proline side chain necessitates a specific subgroup in the Taylor theory. The ionic secondary level of physicochemical properties is divided into negative and positive tertiary levels of physicochemical properties. All amino acids can be described in terms of one or more of these physicochemical properties. These sets of physicochemical properties best describing the behavior of the side chain of each natural amino acid were previously determined.[45]

A Venn diagram uses the set theory to show all of the relationships between groups of related properties graphically. The primary, secondary, and tertiary sets, representing physicochemical properties, overlap with other sets to regroup all physicochemical properties held by each individual amino acid. Hence, the Venn diagram provides an adequate visual representation of the various physicochemical properties for all side chains of natural amino acids, and it is represented here according to Livingstone et al.[46] (Figure 3). The close proximity of amino acids in the Venn diagram signifies that they exhibit similar physicochemical properties. Hence, a Venn diagram was constructed for the surface concentration of nonspecific proteins adsorbed on 3-MPA-amino acids in order to extract common physicochemical properties limiting nonspecific adsorption. 3-MPA-amino acids resulting in nonspecific adsorption below the mean value of 551 ng/cm^2 are shown in bold in Figure 3.

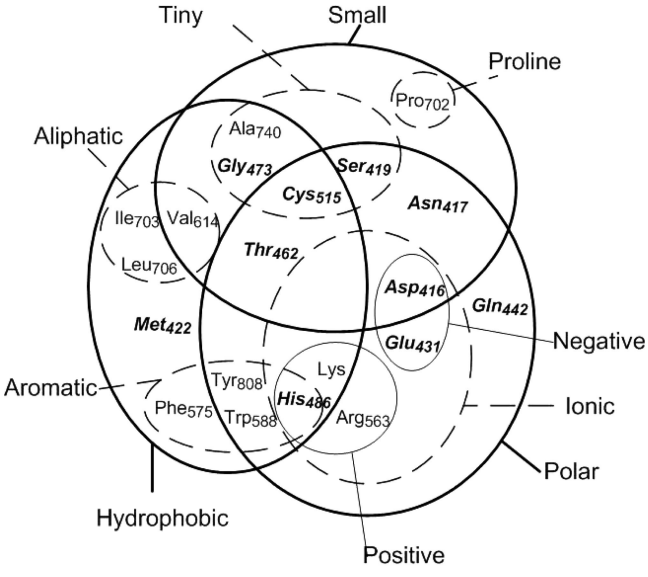

Figure 3. Clustering of nonspecific adsorption on a 3-MPA-amino acid monolayer around a few physicochemical properties represented by the Venn diagram. The surface coverage induced by bovine serum proteins is represented by the subscript next to each amino acid (ng/cm^2). Amino acids in bold have a surface coverage below the average of 551 ng/cm^2, whereas others have a surface coverage above the average.

These 3-MPA-amino acids exhibiting improved resistance to nonspecific protein adsorption are clustered around a few physicochemical properties, mainly located near the center of the Venn diagram. Thus, it is observed that nonspecific adsorption is most improved by 3-MPA-amino acids including at least one of these physicochemical properties: small, tiny, polar, negatively charged, or dual hydrophobic−polar character of the side chain. However, amino acids exhibiting multiple physicochemical properties tend to perform better. As an example, Asp resulted in the lowest nonspecific adsorption of all amino acids and is part of the negative, ionic, polar, and small groups. 3-MPA-amino acids, which have mainly hydrophobic character and include the aliphatic, aromatic, and proline groups did not perform well in bovine serum.

Advancing Contact Angle Relationship with Nonspecific Adsorption. The advancing contact angles vary depending on the chemical composition of the side chain. With the measurement of the contact angle in PBS, the values reported are an indication of the effective hydrophilicity of the surface. Lower contact angles indicate a surface with higher hydrophilicity. It is observed that polar 3-MPA-amino acids have lower contact angles in PBS than do hydrophobic amino acids. A lower contact angle is generally observed for side chains bearing identical chemical functionality (i.e., acids) but with shorter alkyl side chain (i.e., Glu and Asp). Figure 4 shows clustering of the surface coverage measured from the nonspecific adsorption of bovine serum and the contact angle properties of amino acids. Ionic amino acids (squares) and polar amino acids (circles) that generally performed best in resisting nonspecific adsorption are clustered around a lower contact angle, whereas aliphatic and aromatic amino acids, which have generally performed poorly in serum are clustered around higher contact angles. This observation is similar to the

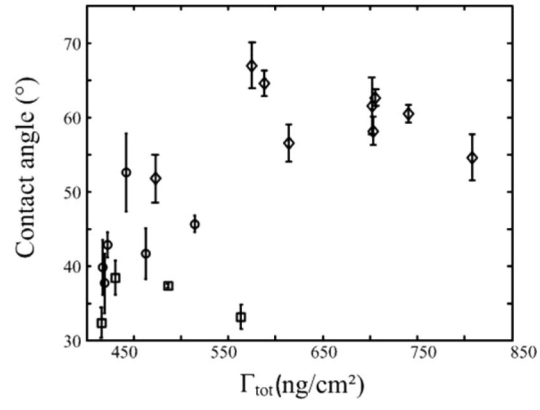

Figure 4. Relationship between surface coverage and the advancing angles of amino acids, where hydrophobic (◊), polar (○), and ionic (□) amino acids are represented.

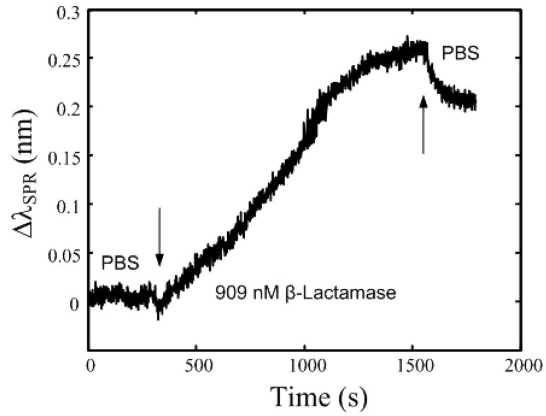

Figure 5. Response resulting from the binding of 909 nM β-lactamase on an SPR biosensor that is specific to β-lactamase, a monolayer of 3-MPA-Gly with anti-β-lactamase. The arrows show when PBS was replaced with β-lactamase and when PBS was contacted with the SPR biosensor following β-lactamase exposition.

conclusions of Xu and Siedlecki.[47] Although the contact angle and nonspecific adsorption properties cannot be characterized as colinear, it is likely that the contact angle must be minimized in order to develop a monolayer, which will resist nonspecific adsorption. Hence, the contact angle may be used as a quick measurement to estimate the resistance of a surface to the nonspecific adsorption of serum proteins.

Nonspecific Adsorption to 16-Mercaptohexadecanoic Acid. Previous studies reported an improved resistance to the adsorption of bovine serum proteins for SPR biosensors using 16-mercaptohexyldecanoic acid (MHA).[26,48] MHA has been used for its ability to reduce nonspecific protein adsorption. Thus, MHA is a comparison point between previously reported work and the results reported herein.[22,49] A level of nonspecific adsorption equal to or below the level of MHA would indicate that 3-MPA-amino acids are performing well in relation to other strategies. A SAM of MHA results in a surface concentration of adsorbed serum proteins of 428 ± 16 ng/cm^2. This demonstrates that the best 3-MPA-amino acids have the same capability to resist nonspecific adsorption compared to MHA because their surface concentrations of bound proteins are similar. One interesting characteristic of MHA is the value of its advancing contact angle with PBS tending toward zero. In fact, the PBS buffer added to the surface of this SAM immediately formed a film on the surface. This confirms once again that better resistance to nonspecific adsorption tends to be on surfaces of increased hydrophilicity.

Immunoassay for β-Lactamase Using 3-MPA-Gly. To demonstrate the possibility of using 3-MPA-amino acid SAMs as a chemical linker for antibodies, a biosensor for β-lactamase was constructed with the immobilization of anti-β-lactamase on the free carboxylic acid of 3-MPA-Gly. Both approaches (using EDC or DCC coupling of NHS) produced efficient biosensors, which resulted in a signal for β-lactamase when exposed to a solution containing 909 nM of this antigen. The β-lactamase solution was prepared in PBS. Figure 5 shows a typical sensorgram obtained from antibody immobilization using the optimal EDC conditions. This demonstrates that 3-MPA-amino acids not only reduce nonspecific adsorption but also can immobilize a large number of antibodies for sensitive biosensing.

Conclusions

This article presents the use of amino acid monolayers based on 3-mercaptopropyl-amino acid for improved resistance to nonspecific adsorption. The chemical preparation and the characterization using FITR, SPR, and the contact angle are reported for these monolayers. FTIR studies demonstrate that a monolayer of 3-MPA-amino acids forms on the Au surface of SPR biosensors and confirms that the deprotection of the methylester on the SAM is complete. The SAMs are generally densely packed on the Au surface, with varying degrees of surface concentration between 0.18×10^{15} and 1.80×10^{15} molecules/cm^2. Aliphatic amino acids with a short side chain, aromatic amino acids, Gln, and Glu form denser monolayers. The study of 19 natural amino acids has determined the characteristics for improved efficiency in limiting the nonspecific protein adsorption from bovine serum. Monolayers with polar and ionic amino acids with the shortest chain length were the most effective in reducing nonspecific adsorption, regardless of the packing density of the SAMs. It has also been shown that there is a relationship between the hydrophilicity of a surface, as measured with advancing contact angle, and its ability to reduce nonspecific binding. These 3-MPA-amino acids exhibit a reduction of the nonspecific adsorption of proteins from serum samples to a level matching the best organic monolayers. Moreover, the possibility of using 3-mercaptopropyl-amino acid as an immobilization layer for antibodies was demonstrated by an immunoassay carried out with β-lactamase. The 3-MPA-amino acid monolayers are a very interesting option for developing monolayers that combine good resistance to nonspecific binding and also immobilize a large number of antibodies for biosensing, as demonstrated by the low adsorption of serum proteins and nanomolar sensitivity for β-lactamase.

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Supporting Information Available: Sensorgrams demonstrating the adsorption of bovine serum on various 3-MPA-amino acid monolayers. This material is available free of charge via the Internet at http://pubs.acs.org.

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