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Covalent Attachment and Derivatization of Poly(L-lysine) Monolayers on Gold Surfaces As Characterized by Polarization−Modulation FT-IR Spectroscopy

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A monolayer of poly(L-lysine) (PL) is attached covalently via amide bonds to an alkanethiol self-assembled monolayer (SAM) on a gold surface. The amide bond is formed in two steps: the terminal carboxylic acid groups of an 11-mercaptoundecanoic acid (MUA) SAM are first activated to the *N*-hydroxysulfosuccinimide (NHSS) ester, followed by reaction of this MUA-NHSS ester monolayer with the amino groups of PL to create multiple amide bond linkages to the surface. The reactivity and packing density of the MUA-NHSS esters are investigated in detail by reacting these intermediates with ammonia (NH_3). In the NH_3 experiments, approximately 50% of the carboxylic acids in the MUA monolayer are converted to amides during the first cycle of this two-step surface reaction. This reaction yield of 50% is limited by the steric packing of the NHSS ester intermediate. However, after three cycles of MUA activation to the NHSS ester and reaction with NH_3, nearly all of the MUA molecules (∼80%) are converted to amides. Polarization−modulation Fourier transform infrared reflection−absorption spectroscopy (PM-FT-IRRAS) is employed to study both the NH_3 and PL reactions on the gold surface. The PM-FT-IRRAS spectrum of a covalently attached PL monolayer indicates that amide bonds are formed with the underlying MUA molecules. This conclusion is confirmed by the fact that the covalent PL monolayer resists desorption despite immersion into solutions of pH < 2 or pH > 12. Finally, the PL is derivatized with a bifunctional NHSS ester−maleimide molecule either by reaction in solution prior to covalent attachment or by reaction with PL already adsorbed to the surface. Up to 50% of the total number of lysine amino groups are converted to maleimide groups, which can be used for the subsequent attachment of sulfhydryl-containing biomolecules.

The preparation of adsorption biosensors, affinity chromatography supports, enzyme-coated electrodes, and other bioanalytical devices often relies on the controlled chemical modification of surfaces.[1−4] Self-assembled monolayers (SAMs) offer a simple yet effective method for modifying both glass and metal surfaces,[5−9] and, in particular, alkanethiol SAMs on gold have been used frequently for controlling the adsorption of biomolecules.[3,10−12] We have shown previously that a monolayer of the polypeptide poly(L-lysine) (PL) electrostatically adsorbs onto a SAM of 11-mercaptoundecanoic acid (MUA) via ion pair formation between the MUA carboxylate and the lysine ammonium groups.[13] Derivatization of this electrostatically adsorbed PL with specific adsorption sites provides control over the surface coverage of an adsorbed protein.[14] In addition, the electrostatic PL and the adsorbed protein are removed from the surface by simply rinsing with a solution of pH < 5 or pH > 12, which disrupts the ion pairs between the MUA and PL. This reversibility of electrostatic PL adsorption is advantageous for both the regeneration of an adsorption biosensor and for obtaining simple solution-phase fluorescence measurements of surface coverage.[14] In some instances, however, a more robust attachment is desirable. Covalent attachment of PL rather than electrostatically adsorbed PL will resist desorption under conditions of high or low pH or high ionic strength, or when a large number of the PL amino groups are derivatized with a neutral or negatively charged moiety.

In this paper, we present a method for the covalent attachment of poly(L-lysine) onto alkanethiol-modified gold surfaces by forming amide bonds via an *N*-hydroxysulfosuccinimide (NHSS) ester intermediate. Other examples of amide bond formation on gold surfaces have been reported which utilize aqueous,[15−18] nonaqueous,[19,20] or gas-phase reactions.[8] For example, the gas-phase

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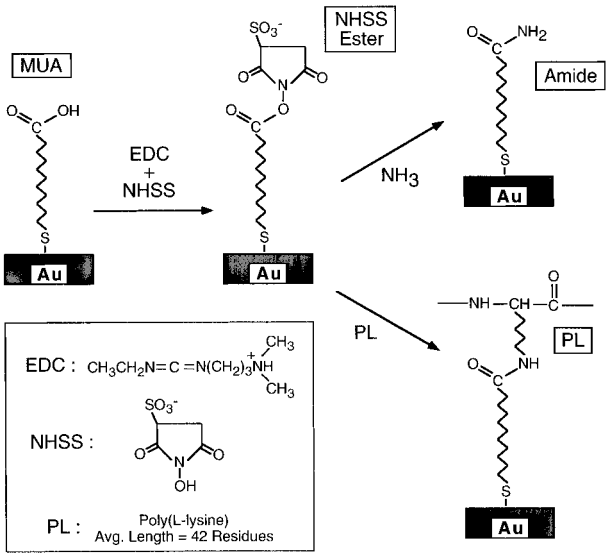

Figure 1. Reaction scheme for forming amide bonds with a self-assembled monolayer of 11-mercaptoundecanoic acid (MUA) on a gold surface. In the first step, the MUA carboxylic acid groups are reacted with the carbodiimide, EDC, and then *N*-hydroxysulfosuccinimide (NHSS) to form the NHSS ester. Subsequent reaction of this actived intermediate with an aqueous solution of either ammonia (NH_3) or poly(L-lysine) (PL) results in the attachment of these amines to the surface by formation of an amide bond.

reaction of thionyl chloride with a MUA SAM yields an activated acid chloride intermediate which subsequently produces an amide (or ester) by reacting with an amine (or alcohol), also in the gas phase.[8] Crooks et al. have recently reported amide bond formation[19,20] and thioester formation[21] via activated MUA SAMs in nonaqueous solvents. However, neither the gas-phase method nor those involving organic solvents are well suited to biomolecule attachment. Thus, in this paper, a water-soluble carbodiimide coupling agent, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), and NHSS are used to convert the carboxylic acids of the MUA monolayer to NHSS esters (see Figure 1). Reaction of this activated MUA-NHSS ester monolayer with an aqueous solution of an amine, either ammonia or PL, creates an amide bond with the surface.

Ammonia was chosen as a test reactant in order to verify the formation of amide bonds with the surface and to quantitate the reactivity of the intermediate NHSS ester monolayer. Formation of the NHSS ester and amide monolayers is followed with the surface-sensitive and surface-selective technique of polarization−modulation Fourier transform infrared reflection−absorption spectroscopy (PM-FT-IRRAS).[8,22−25] This spectroscopic characterization allowed evaluation of various reaction conditions by observing the percent conversion of the MUA carboxylic acids to amide functional groups.

After the initial investigations with NH_3, the NHSS surface attachment strategy is used to covalently attach a monolayer of PL to the MUA-coated gold surface with amide bonds. The significant differences between the PM-FT-IRRAS spectrum of this covalent PL monolayer and that of an electrostatically bound PL monolayer indicate different adsorption mechanisms for these two systems. For instance, the carboxylate band previously attributed to ion pair formation for the electrostatic PL is absent in the covalent attachment of PL. Moreover, the covalent PL monolayer resists desorption upon exposure to acidic and basic solutions, verifying attachment of the PL to the surface via amide bond formation.

Since the covalent attachment of PL converts only a fraction of the lysine residues to amide bonds, many remain available for derivatization. This derivatization is accomplished by forming an amide bond between the amino groups of PL and an NHSS ester-containing species. Previously, poly(L-lysine) has been reacted with NHSS−biotin in order to incorporate specific adsorption sties for the protein avidin.[14] This modification of PL was performed in solution prior to electrostatic adsorption in order to control the spacing of biotin moieties and the resulting avidin surface coverage. In this paper, we demonstrate the derivatization of PL both before and after covalent attachment. The surface reaction of covalent PL with an NHSS−maleimide molecule yields a high density of maleimide functional groups on the gold surface. These maleimide sites offer more versatility than the biotin sites because they will allow for the subsequent attachment of any sulfhydryl-containing biomolecule.[9,26−28]

MATERIALS AND METHODS

Materials. 11-Mercaptoundecanoic acid (MUA) (Aldrich), poly(L-lysine) hydrobromide (PL) (Sigma), lysine hydrochloride (Aldrich), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) (Sigma), *N*-hydroxysulfosuccinimide (NHSS) (Pierce), sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC) (Pierce), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) (Aldrich), triethanolamine hydrochloride (TEA) (Sigma), NH_4Cl (Fluka), NaCl (Fluka), $NaHCO_3$ (Fluka), NaOH (Fluka), HCl (Fisher), and absolute ethanol (Pharmco) were all used as received. Millipore filtered water was used for all aqueous solutions and rinsing.

Surface Preparation and Monolayer Formation. Thin gold films (47 nm) were vapor-deposited onto microscope slide covers (No. 2, 18 × 18 mm^2) that had been silanized with (3-mercaptopropyl)trimethoxysilane [Aldrich] as described previously.[7,23] These samples were immersed into 1 mM ethanolic MUA solutions for at least 24 h before being rinsed with ethanol and then soaked in water for 5 min. The NHSS ester was formed by exposure of the MUA SAM to an aqueous solution of 75 mM EDC and 15 mM NHSS for 1 h.

These MUA-NHSS ester monolayers were reacted for 1−2 h with either (i) an NH_3 solution prepared from 0.5 M NH_4Cl adjusted to pH 7.0 with NaOH or (ii) a solution of 1 mg/mL PL hydrobromide (5 mM lysine residue concentration) in TEA buffer (0.05 M, pH 8.0) with 0.25 M NaCl added. A higher pH was required for the PL reaction than for the NH_3 reaction due to the lower concentration of amino groups (0.005 versus 0.5 M) and the higher pK_a of the PL amino groups (10.5 versus 9.2); NHSS esters only react with the deprotonated form of the amine.[27] Also

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note that the NaCl is necessary in the PL reaction to overcome the electrostatic repulsion of the ammonium groups so that a complete PL monolayer is formed. The PL used in these experiments had an average molecular weight of 9000, corresponding to an average length of 42 lysine residues; similar PL monolayers also were obtained with shorter (six residues) and longer (114 residues) PL. After reaction with PL, some of the NHSS byproduct remained adsorbed to the surface (probably due to electrostatic attraction between the NHSS sulfonate groups and the ammonium groups of the PL). This NHSS was removed by soaking either in 0.5 M NaCl in TEA buffer (0.05 M, pH 8.7) for 12 h or in pH 12.2 NaOH for 15 min. As previously described, the electrostatic PL adsorption occurred onto a MUA surface (without NHSS esters) from a solution of 0.2 mg/mL PL hydrobromide in $NaHCO_3$ buffer (5 mM, pH 8.5).[13,14]

The covalent PL monolayers were derivatized with maleimide groups by immersion of the samples into a 1 mM solution of SSMCC in TEA buffer (0.1 M, pH 7.0). The 15% maleimide-modified PL (Mal-PL) was prepared in solution by the addition of 4 mM SSMCC in water (0.04 mL, 0.16 μmol) to a vortexing solution of 0.5 mg/mL PL hydrobromide (0.4 mL, 0.96 μmol of lysine residues) in TEA buffer (0.1 M, pH 8.0) with 0.25 M NaCl. A 90% yield is assumed for this solution reaction on the basis of similar previous experiments.[14] All monolayer films were rinsed thoroughly with water and dried with nitrogen before PM-FT-IRRAS spectra were obtained.

PM-FT-IRRAS Measurements. PM-FT-IRRAS spectra of 1000 scans at 2 cm^{-1} resolution were obtained with a Mattson RS-1 spectrometer and a narrow-band HgCdTe detector. The optical layout and previously developed real-time interferogram sampling methods have been reported elsewhere.[7,22,23] The PM-FT-IRRAS differential reflectance (%$\Delta R/R$) values were converted to absorbance units for comparison with conventional IRRAS or FTIRRAS data.

Surface Plasmon Resonance (SPR) Measurements. The thicknesses of the NHSS ester monolayers and the covalent PL monolayers were determined with the optical technique of surface plasmon resonance (SPR).[29−31] The SPR experiment and thickness calculation have been described in detail previously.[13,14] Briefly, the reflectivity of a p-polarized HeNe laser beam (λ = 632.8, 1 mW) from a sample assembly (BK7 prism/Au/thin film) is monitored as a function of the incident angle. A steep drop in the reflectivity is observed at an angle just past the critical angle; the position of this SPR angle depends strongly upon the thickness and index of refraction of any material adsorbed onto the gold surface. A five-phase complex Fresnel calculation was used to calculate the thickness of the NHSS ester and PL monolayers; indices of refraction of 1.45 and 1.52, respectively, were assumed.[13]

UV−Visible Spectrophotometry. These measurements were employed to follow the solution-phase reaction of maleimide functional groups with thiols and with the amines, lysine and PL. The presence of thiols was detected by their reaction with Ellman's reagent at pH 8 to give an absorption band at 412 nm. In addition, maleimides were monitored indirectly with this Ellman's test because they react quantitatively with thiols. The UV−visible spectra were obtained with a Hewlett-Packard Model 8452A diode array spectrophotometer.

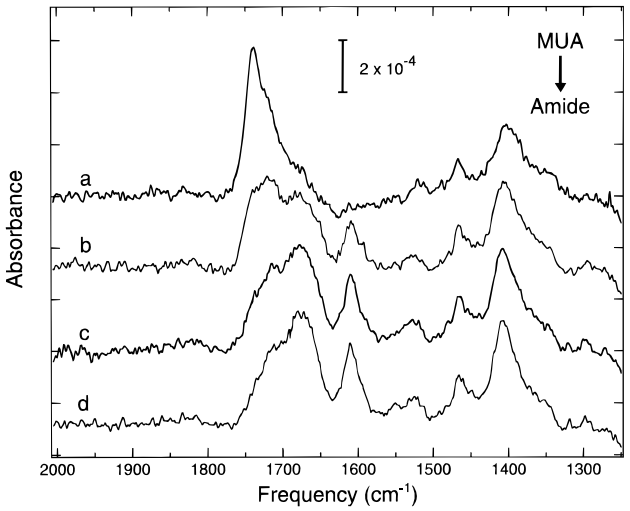

Figure 2. PM-FT-IRRAS spectra showing the gradual conversion of a MUA self-assembled monolayer to an amide monolayer. (a) A MUA SAM with the characteristic carboxylic acid (COOH) band at 1740 cm^{-1}.[48] (b) After reaction of the MUA with EDC/NHSS and then NH_3, partial conversion to the amide is observed. (c) A second cycle of EDC/NHSS and NH_3 converts even more COOH to amides. (d) After the third reaction cycle, nearly complete conversion to the amide is achieved, as indicated by the loss of the COOH band along with increases in the amide bands (see Table 1).

RESULTS AND DISCUSSION

Conversion of a MUA SAM to an Amide Monolayer with NH_3. A two-step reaction scheme for forming amide bonds with a MUA self-assembled monolayer is depicted in Figure 1. In the first step, the carboxylic acid groups of MUA are activated by the carbodiimide, EDC, and then react with NHSS to form NHSS esters of MUA. The amide bond is formed in the second step by reaction with an aqueous solution of an amine. Reaction of PL amino groups with these MUA-NHSS esters leads to an amide bond attachment of a PL monolayer onto the MUA-modified gold surface. Alternatively, when the amine is ammonia (NH_3), an 11-mercaptoundecanamide monolayer, referred to as the "amide monolayer", is created. Observing the formation of this amide monolayer with PM-FT-IRRAS provides insights into the packing and reactivity of the MUA and NHSS ester monolayers.

The PM-FT-IRRAS spectrum of a MUA SAM is shown in Figure 2a, and the band positions are given in Table 1. This carboxylic acid-terminated MUA SAM has been characterized extensively.[8,13,32−38] The most prominent feature is the carbonyl stretch of the free carboxylic acid group (COOH) at 1740 cm^{-1}, which has a shoulder at 1722 cm^{-1} due to hydrogen-bonded COOH.[35,38−41] The carboxylate (COO^-) band at 1400 cm^{-1}

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Table 1. Infrared Frequencies and Vibrational Assignments for Monolayers of 11-Mercaptoundecanoic acid (MUA),[13,33,37–39] 11-Mercaptoundecanamide (Amide),[39] and the *N*-Hydroxysulfosuccinimide (NHSS) Ester of MUA[42,46,49–52]

frequency (cm^{-1})[a]	vibrational assignment	
	MUA Monolayer (Figure 2a)	
1740 ± 2	ν(C═O)	free carboxylic acid stretch
1722sh	ν(C═O)	H-bonded carboxylic acid stretch
1468	$\delta(CH_2)$	methylene scissors deformation
1400	$\nu_s(COO^-)$	symmetric carboxylate stretch
	Amide Monolayer (Figure 2d)	
1715sh	ν(C═O)	residual carboxylic acid stretch
1675	ν(C═O)	amide carbonyl stretch
1610	δ(N—H)	amide N—H bend
1468	$\delta(CH_2)$	methylene scissors deformation
1408	ν(C—N)	amide C—N stretch
	NHSS Ester Monolayer (Figure 3a)	
1820	ν(C═O)	carbonyl stretch of the NHSS ester of MUA
1792sh	ν_s(C═O)	symmetric stretch of the NHS carbonyls
1747	ν_{as}(C═O)	asymmetric stretch of the NHS carbonyls
1653		
1469	$\delta(CH_2)$	methylene scissors deformation of MUA
1370	ν_s(C—N—C)	symmetric CNC stretch of the NHS
1254	$\nu_{as}(SO_3^-)$	asymmetric sulfonate stretch
1222	ν_{as}(C—N—C)	asymmetric CNC stretch of the NHS
1082	ν(N—C—O)	NCO stretch of the NHS
1040	$\nu_s(SO_3^-)$	symmetric sulfonate stretch

[a] sh, shoulder.

indicates that a small number of the MUA molecules are in the deprotonated form.[13,34] This MUA monolayer is reacted with an aqueous solution of EDC and NHSS, followed by reaction with 0.5 M NH_3 at pH 7. The resulting spectrum, plotted in Figure 2b, shows increases in amide bands and a significant decrease in the COOH band at 1740 cm^{-1}, which indicates that many of the MUA molecules have been converted to amides. Further conversion was accomplished by two more repetitions of the two-step cycle of EDC/NHSS and NH_3 (see Figure 2c and d). Spectrum 2d contains three bands due to the primary amide at 1675, 1610, and 1408 cm^{-1} (see Table 1), which agree with those observed by Nuzzo et al. for a monolayer of 16-mercaptohexadecanamide that had been self-assembled onto a gold surface.[39] The residual COOH peak in Figure 2d (shoulder at 1715 cm^{-1}) indicates that some of the MUA molecules remain unreacted after three reaction cycles with EDC/NHSS and NH_3. A fourth cycle caused no further change in the spectrum, suggesting that about 20% of the MUA carboxylic acid groups are not reactive in the ordered MUA SAM. When a less ordered MUA monolayer was prepared by a shorter self-assembly time of only 1 h, essentially the same percentage conversion to amides was observed. Therefore, regardless of the degree of order in the monolayer, approximately 80% of the MUA molecules are accessible for reaction with species from solution.

This conclusion about the number of accessible MUA carboxylic acids is confirmed by the infrared spectra of the intermediate NHSS ester monolayers. Reaction of the MUA SAM with EDC and NHSS yields the MUA-NHSS ester monolayer whose PM-FT-IRRAS spectrum is shown in Figure 3a. The numerous

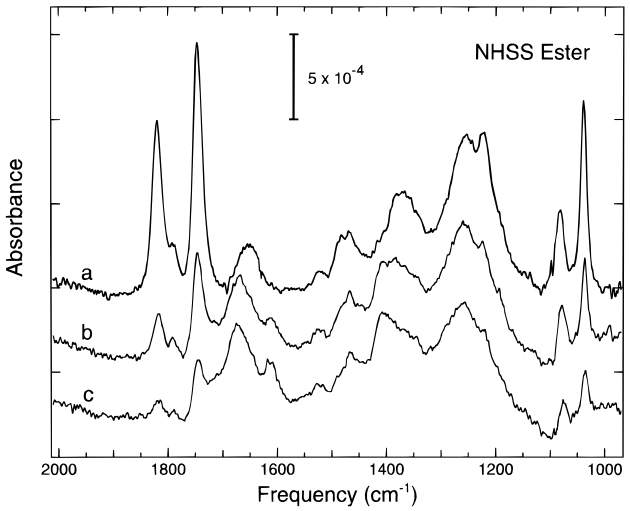

Figure 3. PM-FT-IRRAS spectra of the intermediate NHSS ester layers for the same sample used in Figure 2. (a) NHSS ester monolayer obtained in the first reaction cycle of the MUA SAM with EDC/NHSS. (b) NHSS ester layer from the second cycle. (c) NHSS ester layer from the third cycle. The 1820 cm^{-1} band signifies the formation of MUA-NHSS esters as opposed to merely adsorption of NHSS onto the surface (see Table 1 for band assignments).

prominent bands are tabulated and assigned in Table 1. The strong band at 1820 cm^{-1} is due to the stretching vibration of the MUA carbonyl when it is in the form of an NHSS ester. This band affirms that many NHSS esters of MUA have been formed, because this band is not present in the spectrum of either MUA or NHSS itself. Notice that this band, along with the other NHSS bands, decreases in Figure 3b and c; these spectra correspond to the intermediate NHSS ester layers obtained for the second and third cycles of the EDC/NHSS and NH_3 reaction scheme. A fourth cycle produced no detectable band at 1820 cm^{-1}. Thus, as mentioned above, the three reaction cycles have converted all of the accessible MUA COOH to amides.

In the first reaction cycle of MUA with EDC/NHSS and then NH_3, not all of the accessible MUA carboxylic acid groups are converted to amides; this limitation is attributed to steric packing of the intermediate NHSS ester moieties. The intensity of 1×10^{-3} AU for the 1820 cm^{-1} band (spectrum 3a) obtained for the first NHSS ester layer of MUA did not change significantly, regardless of the concentration of EDC or NHSS or the time of reaction. This observation implies that formation of the NHSS ester monolayer is complete. In addition, surface plasmon resonance (SPR) thickness measurements showed an increase of 9 ± 1.5 Å upon conversion of the MUA SAM to the NHSS ester monolayer. Based on the molecular size of an NHSS ester moiety, this 9 Å thickness indicates a packed monolayer of NHSS esters on the surface. Does this packed NHSS monolayer correspond to all of the accessible MUA molecules (~80%) or to some smaller value? If all 80% are in the form of NHSS esters, then one would expect an 80% conversion to amides in that first cycle, unless some of these MUA-NHSS esters hydrolyze. To estimate the amount of hydrolysis, an NHSS ester monolayer was placed into a pH 7 solution for 1 h with no primary amines present; the 1820 cm^{-1} band decreased by only 25%. Adding a high concentration of amines (0.5 M NH_3) will favor reaction of the NHSS esters with these amines and will reduce the amount of hydrolysis even further. Thus, few NHSS esters are hydrolyzing during the reaction with NH_3 at pH 7, and so the packing density

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of the NHSS moieties must be the limiting factor. Due to these steric effects, approximately 50% of the accessible MUA molecules are converted to amides in the first EDC/NHSS and NH_3 cycle. The second reaction cycle converts most of the remaining accessible COOH to amides, with the third cycle getting the last few to give a total conversion of about 80%.

A one-step amide bond-forming reaction can be performed without the NHSS ester intermediate by placing the MUA-coated gold surface into a solution containing both the EDC and the amine. In this reaction, the carbodiimide activates the MUA carboxylic acid just as before, but then instead of reacting with NHSS, it reacts directly with the amine to form the amide bond. This one-step scheme has the advantage of repeated activation of the same MUA molecule if it hydrolyzes before reaction with the amine, but EDC-activated carboxylic acids hydrolyze much more quickly than NHSS esters.[27] Exposure of a MUA SAM to a solution of 0.2 M EDC and 0.5 M NH_3 at pH 5.5 was able to convert most, but again not all, of the MUA carboxylic acids to amides. However, the two-step reaction involving the NHSS ester intermediate is preferred in the covalent attachment of PL for two reasons: (i) the amine concentration is relatively low in the PL solutions, and the NHSS ester provides more time for the reaction because it hydrolyzes more slowly than the EDC-activated MUA, and (ii) PL, as with all peptides and proteins, has both amino groups and carboxylic acid groups, which in the one-step reaction would be linked together by the EDC.

Covalent Attachment of Poly(L-lysine) Monolayers. Covalent attachment of PL to a MUA monolayer provides a robust irreversible adsorption of this polypeptide onto the gold surface. This attachment is accomplished by the reaction scheme shown in Figure 1, which employs the NHSS ester intermediate to form amide bonds between the carboxylic acid groups of MUA and the ϵ-amino groups of PL. The MUA SAM is first transformed to a MUA-NHSS ester monolayer, as described above, followed by exposure to a solution of 1 mg/mL PL in 0.25 M NaCl at pH 8 for 1−2 h. The investigation of this two-step strategy with NH_3 in the previous section verifies the formation of amide bonds and indicates that each PL chain will likely form several bonds with the MUA molecules.

The attachment of PL onto the MUA-modified gold surface is evidenced by the large amide bands in the PM-FT-IRRAS spectrum plotted in Figure 4a (solid line). These amide I and amide II bands at 1674 and 1545 cm^{-1}, respectively, are due primarily to the amide bonds within poly(L-lysine) itself. A small contribution to these bands (estimated at <15%) results from amide bonds formed between the MUA and PL during the covalent attachment. In addition, approximately 20% of the area of the 1674 cm^{-1} amide I band is due to residual MUA COOH.

Significant differences are observed for the amide bands between the covalent PL spectrum in Figure 4a and the electrostatically adsorbed PL spectrum shown in Figure 4b. The amide I and amide II band positions have changed by 12 and 21 cm^{-1}, respectively. Furthermore, the relative intensities of the amide bands have changed between the two spectra. These differences might be caused, in part, by contributions from the amide bonds formed during the covalent attachment, but it is unlikely that is the only cause of these large changes in the spectra. Other possible contributions include the degree of hydrogen bonding of the PL and the conformation of the polypeptide. Since hydrogen bonding typically shifts amide bands to lower frequencies,[42,43] it might account for the change in either the amide I or the amide II band but not both (notice that these bands shift in opposite directions). The amide band positions for the electrostatic PL have been attributed previously to a random coil conformation,[13] and despite 12 and 21 cm^{-1} differences for the covalent PL, these band positions remain within the limits of a random coil as opposed to those of an α-helix or β-sheet.[43−45] Nonetheless, some conformational changes such as occasional turns or helical segments in the polypeptide chain could cause shifts in the amide bands, even though the PL remains predominantly as a random coil.[43] Overall, the observed spectral differences probably result from a combination of all three contributions: amide bonds between MUA and PL, hydrogen bonding differences, and some conformational changes. These differences in the amide bands of electrostatic PL and covalent PL strongly suggest different adsorption mechanisms for these two types of PL monolayers.

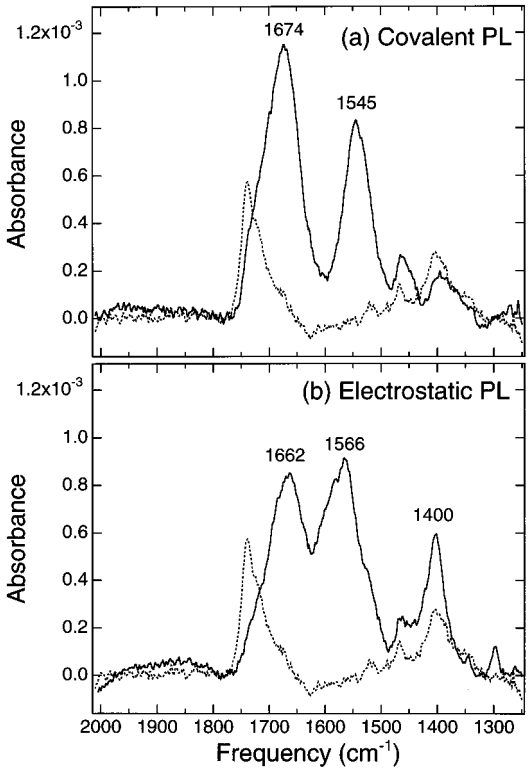

Figure 4. PM-FT-IRRAS spectra of poly(L-lysine) monolayers (solid lines) that are (a) covalently attached and (b) electrostatically adsorbed to MUA SAMs (dotted lines).[48] The amide I and amide II bands have significantly different positions and intensities for these two methods of adsorption. In addition, the large increase of the carboxylate (COO^-) band at 1400 cm^{-1} in (b) indicates electrostatic adsorption via ion pair formation, whereas the absence of this band in (a) verifies the covalent attachment of the PL to the MUA SAM.

More conclusive evidence of covalent attachment is given by the changes in the MUA bands at 1740 (COOH) and 1400 cm^{-1} (COO^-) upon comparison of the PL spectra (solid lines) with the

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MUA spectra (dotted lines) in Figure 4a and b. The electrostatic PL spectrum shows a decrease only for the COOH band but a large increase in COO^- as the carboxylic acids are deprotonated during the ion pair formation between MUA and the ammonium groups of PL.[13] The covalent PL spectrum, however, exhibits decreases for both the COOH and COO^- bands. The reduction in area of these two bands indicates that at least 20% of the MUA carboxylic acids have formed amide bonds with PL.

Some chemical desorption experiments performed on the electrostatic PL and covalent PL samples give further evidence for the covalent attachment mechanism. As previously reported, the electrostatic PL sample completely desorbs upon exposure to either high pH or low pH solutions due to disruption of the ion pairs.[13] In contrast, immersion of the covalent PL into pH 1.8 HCl or pH 12.2 NaOH does not change the amide band intensity in the infrared spectrum or the PL layer thickness, as determined by SPR (see below). This stability over a wide pH range confirms the robust covalent attachment of PL to the MUA monolayer.

The PM-FT-IRRAS spectrum unambiguously demonstrates that the PL is attached to the MUA monolayer, but it does not give a reliable quantitative estimate of the amount of adsorbed PL. Therefore, surface plasmon resonance (SPR) measurements[29−31] were employed to determine the thickness of the covalently attached PL layer. The SPR experiment has been described in detail previously, where it was used to measure a thickness of 10.5 ± 1.7 Å for an electrostatically adsorbed poly(L-lysine) monolayer on a MUA-modified gold surface.[13,14] A similar thickness of 10 ± 2 Å was obtained from SPR measurements of the covalent PL layers, which indicates that a complete but single monolayer of PL is attached to the MUA monolayer. Furthermore, this thickness suggests that the PL chain lies parallel to the surface in an extended configuration. This result, combined with the estimate that 20% of the MUA molecules form amide bonds, leads to the conclusion that multiple amide bonds hold each PL chain on the surface.

Maleimide Attachment to the Poly(L-lysine) Monolayers. Derivatization of the covalent PL monolayers will provide control of biomolecule adsorption onto gold surfaces. A large number of the lysine residues should remain available for derivatization reactions because only some of them have formed amide bonds with the underlying MUA SAM. Modification is accomplished by simply reacting an NHSS ester molecule from solution with the PL amino groups to form more amide bonds. As a test of the derivatizability of PL, we have chosen the maleimide−NHSS ester cross-linking molecule (SSMCC), which produces a maleimide-modified poly(L-lysine) (Mal-PL) monolayer as depicted in Figure 5. Maleimide functional groups react readily with thiols (sulfhydryl groups), and therefore they will be useful for the subsequent attachment of biomolecules, including cysteine-containing peptides, proteins, and antibodies, along with sulfhydryl-tagged proteins, or oligonucleotides.[9,26−28]

The PM-FT-IRRAS spectrum of a Mal-PL sample is shown as the solid line in Figure 6, and the dotted line spectrum corresponds to the covalent PL monolayer before its immersion into the aqueous solution of 1 mM SSMCC in pH 7 buffer for 15 min. The very strong band at 1708 cm^{-1} is an in-phase stretching vibration of the two maleimide carbonyls;[46] the other infrared bands of this Mal-PL monolayer are listed in Table 2. In addition to this *surface* preparation of Mal-PL, a *solution* reaction between

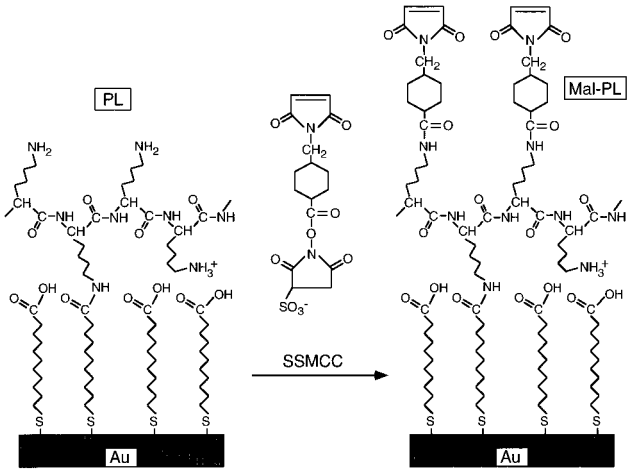

Figure 5. Reaction scheme showing the attachment of maleimide groups to the PL-coated surface. The covalently attached PL monolayer is reacted with an NHSS−maleimide molecule, SSMCC, to form a maleimide-modified poly(L-lysine) (Mal-PL) layer.

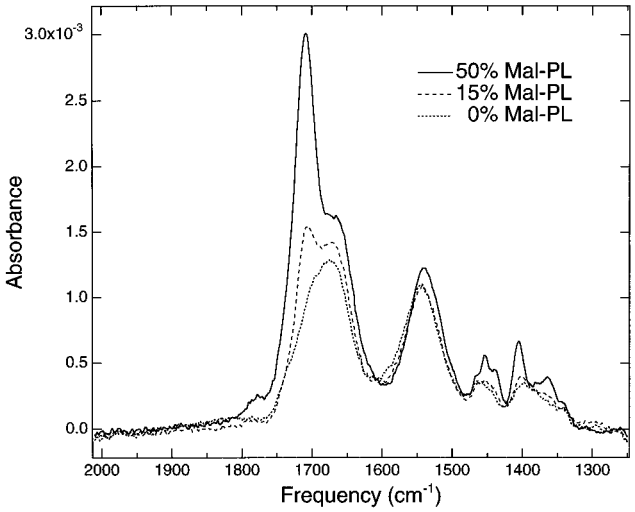

Figure 6. PM-FT-IRRAS spectra for PL monolayers with various percentages of the lysine residues derivatized with maleimide groups. The 0% Mal-PL is simply covalently attached PL. The solid line resulted from the surface reaction between this covalent PL monolayer and SSMCC as shown in Figure 5. The 15% Mal-PL, however, was prepared in solution with known stoichiometry and then attached to a MUA-modified gold surface. By comparing the intensity of the 1708 cm^{-1} maleimide band for the surface reaction with that of the known 15% Mal-PL, a value of 50% maleimide modification of the lysine residues was calculated for the surface reaction.

SSMCC and PL was employed to produce a Mal-PL sample, in which 15% of the lysines are derivatized with maleimides. Covalent attachment of this 15% Mal-PL to a MUA-modified gold surface resulted in the dashed line spectrum in Figure 6. By comparing the absorbance at 1708 cm^{-1} for the 15% Mal-PL with that from the surface reaction, a value of 50% modification of the lysine residues was estimated for the surface derivatization. This result corresponds to approximately 2×10^{14} maleimides/cm^2, based on previous fluorescence studies that determined a surface coverage of 4×10^{14} lysines/cm^2 for PL.[14] This high density of maleimide sites requires the strong covalent attachment of PL, since modification of this many lysine residues of electrostatically adsorbed PL might lead to its desorption from the surface.

Obtaining a high density of active maleimide sites on the surface requires optimization of the SSMCC solution pH. A high

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Table 2. Infrared Frequencies and Vibrational Assignments for a Monolayer of Maleimide-Modified Poly(L-lysine) (Mal-PL, Figure 6)[46,51]

frequency (cm^{-1})	vibrational assignment	
1777 ± 2	ν_s(C═O)	symmetric stretch of maleimide carbonyls
1708	ν_{as}(C═O)	asymmetric stretch of maleimide carbonyls
1676	ν(C═O)	amide I (carbonyl stretch)
1540	ν(C−N) + δ(N−H)	amide II (CN stretch and NH bend)
1454	$\delta(CH_2)$	methylene scissors deformation
1405		
1364	ν_s(C−N−C)	symmetric CNC stretch of maleimide

pH is desirable because the NHSS ester moiety of the SSMCC reacts only with the deprotonated form of the PL amino groups.[27] However, two deleterious side reactions can occur at high pH, rendering the maleimide groups inactive. The first of these is hydrolysis. Maleimides are more stable than NHSS esters, and so hydrolysis usually is not a problem, especially at pH < 8.[27,47] Furthermore, the large 1708 cm^{-1} band for Mal-PL in Figure 6 indicates that the maleimide groups have not hydrolyzed. This band, due to the coupled in-phase stretching of the two maleimide carbonyls, would be absent (or at least decreased) if hydrolysis had occurred, since hydrolysis breaks open the maleimide ring to form the maleamic acid.[47] The second possible side reaction is that between the maleimide and amino groups, such as those in the PL.[27] Inactivation of the maleimides by this route is more difficult to follow since it has no apparent effect on the PM-FT-IRRAS spectrum. Therefore, some preliminary experiments in solution at pH 7.5 and 8 examined the reaction between maleimides and amines using UV−visible spectrophotometry. The reaction between maleimides and a lysine *monomer* was observed to proceed quite slowly at these pH values. However, maleimide groups attached to PL reacted readily with a neighboring lysine amino group via an intramolecular reaction. Based on these results, we chose the relatively low pH of 7 for the surface reaction between SSMCC and the covalent PL monolayer in order to decrease the rate of this side reaction. This reaction pH of 7 still achieves a high density of maleimide sites on the surface, as evidenced by the spectrum in Figure 6, and these sites are expected to remain active toward sulfhydryl-containing molecules.

CONCLUSIONS

Poly(L-lysine) has been covalently attached to a carboxylic acid-terminated alkanethiol self-assembled monolayer on a gold surface via a two-step reaction scheme employing an NHSS ester intermediate. In addition to forming amide bonds between the amino groups of PL and the carboxylic acids of MUA, we explored the reactivity of the MUA-NHSS esters with the simpler amine, NH_3. These experiments with two vastly different amines, PL and NH_3, demonstrate that this two-step reaction scheme can serve as a general method for attaching amines to gold surfaces.

Both the covalently attached PL and the previously examined electrostatically adsorbed PL monolayers offer several advantages for the subsequent attachment of biomolecules onto gold surfaces. For example, both types of PL monolayers are easily derivatized with commercially available NHSS ester molecules. Performing the derivatization in solution prior to adsorption of the PL provides precise control over the number of incorporated adsorption sites and their spacing on the surface, as demonstrated previously with biotin moieties[14] and in the current studies with maleimide functional groups. Derivatizing PL already attached to the surface yields a high density of these adsorption sites and is best performed with the more robust covalent PL monolayers to prevent desorption. The electrostatically adsorbed PL, however, is recommended for applications requiring a reversible adsorption, such as in the construction of biosensors that need to be regenerated. The electrostatic PL monolayer and any attached molecules will desorb upon exposure to either high or low pH solutions,[13] whereas the covalent PL monolayer is irreversibly adsorbed even after immersion into solutions at pH < 2 or pH > 12. We are currently pursuing the use of these poly(L-lysine) monolayers and especially the maleimide-modified PL in the attachment of peptides, proteins, and oligonucleotides onto gold surfaces.

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