Chiral Discrimination of a Proline-Based Stationary Phase: Adhesion Forces and Calculated Selectivity Factors

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ABSTRACT: As early as 1992, proline was examined as a potential chiral selector for high-performance liquid chromatography. In recent years, brush-type selectors with up to 10 proline units have been examined, and the longer peptides were found to be competitive with commercial chiral stationary phases (CSPs). In this article, we report on a comprehensive examination of a t-butoxycarbonyl- (t-Boc-) terminated monoproline selector. This selector was grafted through an amide linkage to an aminopropyl siloxane-terminated Si(111) wafer and to a silicon atomic force microscopy tip. Chemical force spectrometry measurements were performed for interaction forces between two D- or L-monoproline monolayers in water and in the presence of various amino acid solutions. When exposed to amino acids, the adhesion forces measured between the proline layers were reduced. Amino acids containing hydrophilic side chains were found to exhibit a selectivity opposite to that seen for those containing hydrophobic side chains. Molecular dynamics simulations of the monoproline interfaces in the presence of racemic alanine and serine identified the importance of hydrogen-bonding interactions between the amino acids and the monoproline selectors. We found that, when amino acids are bound to the proline selector, their side chains protrude into the bulk solution, explaining the strong impact of side-chain hydrophobicity on the selectivity. Taken together, the experiments and simulations show that hydrogen-bonding interactions are key to effective chiral discrimination for proline-based CSPs.

1. INTRODUCTION

During the past two decades, there has been intense interest in the development and application of enantiomeric resolution techniques, particularly in the pharmaceutical industry. The development of a single enantiomer drug target from a racemate, the “racemic switch”, is now in high demand, with six of the 10 top-selling drugs in the United States in 2009 being single enantiomers. Among enantioseparation techniques, the use of efficient chiral stationary phases (CSPs) in high-performance liquid chromatography (HPLC) is considered one of the fastest-growing areas of chiral separation. Pirkle and others developed a promising class of CSPs, popularly known as brush-type CSPs, in which the chiral moiety is joined to a hydrocarbon tether, with an ionic or covalent linkage to a siloxane group that bonds to the underlying substrate. Some brush-type CSPs exhibit good chiral selectivities in polar and apolar solvents and for diverse analytes. Chiral resolution on these CSPs is realized through the formation of noncovalent diastereomeric complexes between the chiral selector and the enantiomers from a racemic analyte. Each diastereomeric complex is distinct, and the enantiomer associated with the more stable complex elutes from the HPLC column last.

Although a number of CSPs have been prepared, only a few have demonstrated both broad chiral selectivity and effectiveness in a range of solvent environments. Amino acids are well-recognized as a naturally occurring chiral pool; many amino acids or their derivatives have been the basis for chiral CSPs. Proline is, of course, a unique amino acid because of the inclusion of the α-carbon in a pyrrolidine ring. Proline-based selectors have been studied in both enantioselective separation and ligand-exchange chromatography. Recently, a class of CSPs based on short-chain proline peptides has drawn attention because of promising enantioselectivity. Huang and co-workers have reported on a number of factors that impact the performance of these proline-based CSPs, including the identity of the protecting group, the peptide chain length, the length of the hydrocarbon linker between the substrate and the peptide chain, the nature of the peptide linker, substitution on the peptide chain, and the composition of the mobile-phase solvent. Even a relatively short diproline selector was found to lead to selectivity factors comparable to those of commercial...
columns such as Whelk O2.15 The impact of the protecting group was examined for diproline selectors in hexane/2-propanol and CH₂Cl₂/hexane/2-propanol solvent mixtures: Trimethylacetyl groups led to superior sensitivity relative to 9-fluorenylmethoxycarbonyl, t-butoxycarbonyl (t-Boc), benzoxycarbonyl, and acyl groups.9,14,17 Chiral recognition effects have been observed using other methods, surface as the measurement of surface pressure isotherms.18 For example, using a Langmuir monolayer consisting of a helical complex of cholesterol moieties linked to a tetraacyclododecane core, Michinobu et al.19 demonstrated chiral selectivity toward a range of amino acids by means of this technique.

Here, we report on the selectivity of a monoproline-based chiral stationary phase. Self-selectivity and selectivity toward a series of amino acids were assessed using a combination of chemical force spectrometry (CFS) measurements and molecular dynamics (MD) simulations. The chiral selector under consideration consisted of a t-Boc protecting group, monoproline, and an amide linkage to an aminopropyl siloxane-terminated Si(111) wafer or Si atomic force microscopy (AFM) tip. This particular selector was also the subject of an early investigation by Harou et al.17 in which N-acid dinitrobenzoyl racemates were successfully separated. The selection mechanism for proline-based selectors is not obvious, as proline offers only H-bond acceptor sites and no aromatic rings for \( \pi-\pi \) stacking. Indeed, one of the challenges of chromatographic separations is the necessity of inferring the selection mechanism based on characteristics of successfully separated analytes. In this work, we analyzed the composition of the experimental surface using X-ray photoelectron spectroscopy and applied molecular dynamics simulations to obtain a molecular description of the factors that give rise to binding of chiral analytes at the CSP/solvent interface.

We have previously reported on the self-selectivity of \( N-(3,5\text{-dinitrobenzoyl})\text{phenylglycine} \) and \( N-(3,5\text{-dinitrobenzoyl})\text{leucine CSPs in a series of protic and aprotic solvents}.20 dinitrobenzoyl)phenylglycine and terminated Si(111) wafer or Si atomic force microscopy (AFM) monoproline, and an amide linkage to an aminopropyl siloxane-under consideration consisted of a molecular dynamics (MD) simulations. The chiral selector chemical force spectrometry (CFS) measurements and series of amino acids were assessed using a combination of chiral stationary phase. Self-selectivity and selectivity toward a range of amino acids by means of this technique.

2. EXPERIMENTAL AND SIMULATION DETAILS

2.1. Proline Overlayer Preparation. Single-crystal Si-(111) substrates (Wafernet, San Jose, CA) cut into 1 cm \( \times \) 1 cm pieces were precleaned by sonication in hexane followed by acetone and then ethanol and dried under a \( N_2 \) stream. This was followed by immersion in a piranha solution [7:3 (v/v) \( H_2SO_4/30\% \text{H}_{2}O_2 \), 85 °C for 1 h]. Caution! Piranha solution is an extremely dangerous oxidizing agent and should be handled with care using appropriate personal protection. The substrates were then rinsed with deionized water and subjected to the RCA cleaning protocol [1:5:1 (v/v) \( \text{NH}_4\text{OH/H}_2O/30\% \text{H}_2O_2 \) at 70 °C for 20–25 min]. The substrates were subsequently washed with deionized water and dried under a \( N_2 \) stream and then dried in an oven for 2 h at 130 °C.

The AFM tips used were silicon oxide sharpened silicon cantilevers (MikroMasch CSC38/AlIBS) with a tip radius of \( \sim 10 \) nm and a nominal force constant of 0.03–0.08 N/m. AFM tips were imaged using scanning electron microscopy, and the force constants of the cantilevers were determined using the cantilever geometry.25,26

The substrates and AFM tips were amine-terminated by immersion in a solution of 5 mM (3-aminopropyl)-triethoxysilane (APTES, 99%, Aldrich) for 2 h in dry toluene under an inert atmosphere. The wafers were subsequently sonicated in toluene for 20 min and then rinsed with ethanol and dried under a \( N_2 \) stream. Prior to silanization, all glassware used in chemical modification of either substrates or AFM tips was passivated by immersion in an octadecyltrichlorosilane solution for 24 h to avoid any competition for APTES adsorption by silanol sites on the walls of the glassware.

To form a chiral surface, the aminated Si substrates and AFM tips were reacted with either 4-\( N\text{-t-butoxycarbonyl-protected} \)proline (t-Boc-D-Pro) or 4-\( N\text{-t-butoxycarbonyl-protected} \)proline (t-Boc-L-Pro), with 1 equiv of the peptide coupling agent 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinone (EEDQ) in dry tetrahydrofuran (THF) for 12 h. After being removed from solution, the substrates were rinsed with diethyl ether, sonicated in THF for 20 min, and dried in a \( N_2 \) stream. The synthesis of t-Boc-L-Pro and t-Boc-D-Pro is detailed in the Supporting Information.

2.2. Surface Analytical Methods. X-ray photoelectron spectroscopy (XPS) experiments were conducted using a Thermo Instruments Microlab 310F surface analysis system (Hastings, U.K.). The analysis was performed at \( 10^{-9}–10^{-10} \) Torr using a Mg Kα X-ray source (1253.6 eV) at an anode potential of 15 kV and an emission current of 20 mA. The fixed analyzer transmission mode was used to obtain the photoelectron signal using a pass energy of 20 eV. The system was calibrated to the bulk Si 2p line at 99.3 eV as an internal standard. The Shirley algorithm was used as the background subtraction method for all peaks. The Powell peak-fitting algorithm was used with peak areas normalized between different elements using relative XPS sensitivity factors.27

Contact angles were measured at ambient temperature on a VCAOptima goniometer (AST Products, Inc., Billerica, MA) by the sessile drop technique against 1 \( \mu \)L of water purified with a Milli-Q water purification system (Millipore, Billerica, MA). At least three drops were evaluated for each substrate. Standard deviations for contact angles were 1–2°.

AFM force–distance curves were acquired using a PicoSPM instrument (Molecular Imaging, Tempe, AZ) and a Nanoscope IIE controller (Digital Instruments, Santa Barbara, CA). All experiments were carried out at 25 °C. The force between the tip of the AFM and the substrate surface was monitored as a function of the displacement of the substrate relative to the tip. The measurements were conducted in a drop of freshly prepared solution. For force titration experiments, unbuffered solutions of varying pH were used. The pH values were adjusted using NaOH and HCl solutions. Unbuffered solutions were employed to avoid potential competitive adsorption

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effects of buffer ions from solution on the probe–substrate interactions. An ionic strength of 10^{-3} M was maintained for all solutions, except those at pH 2 and 12, for which the ionic strength was 10^{-2} M. Experiments were conducted as quickly as possible and solutions were changed frequently to minimize the possibility that the pH would change as a result of adsorption of atmospheric CO2. Amino acid solutions of 0.5 M concentration were prepared by dissolving the appropriate l- or (when available) d-amino acid (in all cases, Sigma-Aldrich, purity > 99.0%) in water and adjusting the pH to 7.0, using NaOH or HCl as appropriate.

The adhesive force between the tip and substrate was measured from the average of the adhesive well depth from 200 to 300 force–distance curves at each pH value. The reported errors reflect the standard deviation of the data. In the case of force titration data, each experimental data set was run from low to high pH and then from high to low pH. The adhesive force between the tip and substrate was also remeasured several times between the same tip and different surface sites. In the force titration data, the same AFM tip was used at all pH values. In the case of the adhesive force data from the amino acid solutions, the same AFM tip and substrate were used for all t-Boc-l-Pro or t-Boc-d-Pro data sets, with both tip and substrate rinsed in deionized water before the next experiment.

2.3. Molecular Dynamics Simulations. An extensive series of ab initio calculations was performed to capture the structure, relative stability, and flexibility of individual t-Boc-protected l-proline selectors. Initial geometry optimizations were performed at the B3LYP/6-311G(d,p) level, and the cis- and trans-amide conformers were further optimized with mPW2PLYP-D/6-311G(d,p). Single-point energies for the optimized structures were calculated with LPNO–CCSD/6-311G(d,p). Stretches, bends, and dihedral and improper torsional potentials were obtained by fitting to B3LYP/6-311G(d,p) energies, as described in detail elsewhere.

Short-range repulsion and dispersion were represented with a Lennard-Jones (LJ) potential with parameters taken from the OPLS force field. Partial atomic charges were obtained using the CHELPG algorithm applied to the lowest-energy conformer. A similar approach was applied to obtain force fields for zwitterionic alanine and serine: Geometry optimization and flexibility calculations were performed at the B3LYP/6-311G(d,p) level of theory, LJ parameters were obtained from OPLS, and atomic charges were calculated using the CHELPG algorithm.

The simulation cells consisted of two parallel Si surfaces covered by 224 silanols and 32 Boc-l-Pro selectors, with 1400 water molecules between the chiral surfaces. A snapshot is provided in Figure 1. The silanol charges were taken from those previously reported by Zhang et al. The distance between the surfaces was adjusted so that the solvent density near the midpoint of the cell was within 2% of that of bulk water. Equilibration of the simulation cell occurred over the first 1500 ps of the simulation. The cis and trans conformers of monoproline are close in energy, but the torsional barrier for interconversion is high. Therefore, torsional scaling was introduced to expedite selector conformational equilibration: The energy barrier of each backbone torsion was reduced to zero at the start of the simulation and gradually increased to its full value over a 600-ps period. This allowed for rapid cis–trans conformer equilibration at the interface during the equilibration period. Full details on the equilibration procedure are available elsewhere.

The molecular dynamics simulations were performed in the canonical ensemble (NVT) using the MDMC program. Nosé–Hoover thermostats were employed with a simulation temperature of 298 K, and Ewald summations were introduced for long-range interactions. Each simulation consisted of a 1.0-ns equilibration period followed by a 2.0-ns collection period. Fifteen independent simulations were performed for each amino acid, and snapshots were collected for postsimulation analysis.

Hydrogen bonding between the amino acid, alanine or serine, and the t-Boc-l-Pro selectors was assessed through the application of geometric criteria. A hydrogen bond was defined by the simultaneous occurrence of an O···H distance of less than 2.6 Å, an O···H···O angle of greater than 150°, and an O···O separation of less than 3.5 Å. Each snapshot was analyzed to yield overall hydrogen-bonding probabilities at the interface.

3. RESULTS AND DISCUSSION

3.1. Characterization of the Proline-Modified Surfaces. Before exploring chiral discrimination at Boc/monoproline surfaces, we characterized the surfaces themselves, and their precursors, using a range of surface analytical methods. The overall goal was to ensure a flat, homogeneous overlayer of t-Boc-protected monoproline suitable for force spectrometric measurements. Achiral hydrogen-bonding sites, such as any residual amine or siloxane sites arising from the various surface modification processes, should be minimized. Figure 2 outlines the various steps used in the preparation of the t-Boc/monoproline surfaces, together with contact angle measurements, AFM images, and C 1s or N 1s XPS data associated with each stage of the deposition process.

Piranha solution was used to oxidize any organic contaminants from the Si(111) surface and RCA solutions to subsequently form silanol (Si—OH) sites. Water contact angle measurements demonstrated that the surface became highly hydrophilic, with a contact angle of 3° ± 2° following hydroxylation, compared to 45° ± 3° for the unmodified Si(111) wafer. AFM images also showed a homogeneous surface with a low root-mean-square (rms) roughness of 0.16 nm. Si 2p XPS spectra (not shown) also demonstrated the presence of a thick silicon oxide overlayer. Amine functionalization of the surface was then carried out using hydrolysis of
APTES to form an amine-terminated overlayer. A range of APTES solution concentrations and exposure times were explored to minimize formation of polymerized aggregates from solution (as determined using AFM imaging), which were seen to settle as larger particles on the surface, leading to high surface roughness and heterogeneity. Conditions were also chosen to minimize the number of unreacted surface silanol sites that could subsequently protonate the terminal amine group and to ensure a fully cross-linked APTES overlayer, as determined using XPS and contact angle measurements. Under the optimized reaction conditions of 2-h exposure to a solution of 5 mM APTES in toluene, the water contact angle increased to 40° ± 3°, consistent with that previously reported by Lee et al. The surface rms roughness increased slightly to 0.26 nm, with no evidence of the deposition of polymerized aggregates. The C 1s/N 1s XPS area ratio was 4.0 (±0.5):1, consistent with the expected stoichiometric ratio of 3:1. A ratio greater than 3:1 would suggest that the APTES molecules were not completely hydrolyzed, leaving residual ethoxy groups. A 4:1 ratio indicates that, on average, 50% of APTES sites retained a single unreacted ethoxy group. The true fraction is likely even less, because of residual C contamination from the underlying Si substrate and exposure of the sample during transfer to the XPS analysis chamber. Thus, the C 1s spectra indicate the formation of a well-cross-linked APTES layer at the surface. The N 1s peak (lower right panel in Figure 2) indicates the presence of two N species, one at 399.3 eV and the other at 401.1 eV, that can be assigned to the unprotonated (NH₂) and protonated (NH₃⁺) groups, respectively. However, the NH₃⁺ peak area was minimized under these conditions, representing less than 16% of the total N species at the surface.

To form the t-Boc-monoproline chiral stationary phase, either t-Boc-L-Pro or t-Boc-D-Pro was coupled to the amine-terminated surface using the peptide coupling agent EEDQ. Both L and D versions showed identical features, as expected, and only data for t-Boc-L-Pro are presented in Figure 2. Following reaction, the rms roughness of the surface increased somewhat to 0.45 nm. The water contact angle also increased, to 65° ± 3°. As demonstrated in a later section, our molecular dynamics simulations indicate that both the t-butyl group of t-Boc and the carbonyl oxygen between the pyrroline ring and carboxylate group are exposed at the surface. Thus, this increase in contact angle is consistent with replacement of a hydrophilic NH₂ terminal layer with a mixture of hydrophilic carbonyl and hydrophobic t-butyl head groups of the t-Boc-protected proline layer. The surface immobilization of t-Boc-L-Pro was confirmed using XPS: Both C 1s and N1s XPS are well-established in demonstrating the formation of amide linkages on surfaces, in both polymer and siloxane-type systems, as well as for protein adsorption on surfaces. The C 1s/N 1s area ratio was found to be 7.4 (±0.5):1 (stoichiometric ratio of 6.5:1). This is consistent with the enrichment in C observed in the original APTES-modified surface from which the Boc-Pro-modified surface was derived. The C 1s spectrum (lower left panel in Figure 2) shows the presence of two C species, one at 285 eV and the other at 286.5 eV, that can be assigned to the t-Boc group and the aromatic backbone, respectively.
leading to lower but nonzero forces.

The solution-phase analogue, propylamine, at 10.60.49 This is to protonated amine (\(\text{NH}_3^+\)) measured surface p

Above pH 6.0, both tip and substrate are deprotonated, and electrostatic repulsion and little or no adhesive interactions. Covered with surface-bound alkylammonium groups, leading to pH values below 4.0, both tip and substrate are predominantly protonated amine sites on the surface might affect any experiments involving chiral discrimination, we first carried out a series of chemical force titrations: measurements of the tip/substrate adhesive interaction as a function of pH. Experiments were carried out using combinations in which the tip and substrate were representative of each stage of the deposition process: oxidized silicon (silanol), APTES-terminated (amine), and Boc-L-Pro-terminated. The adhesion forces as a function of pH are presented in Figure 3. In each case, force titration experiments were carried out first in low-pH solutions and then at increasing pH. The experiments were then repeated starting at high pH and decreasing to low pH. Data from both sets of experiments (2 to 12, blue solid triangles for low to high pH; 12 to 2, red open squares for high to low pH in Figure 3) were highly reproducible.

Figure 3a shows force titration data when both AFM tip and substrate were reacted with APTES, resulting in amine-terminated surfaces. At pH values of 4.0 and below, no adhesion forces were observed. The adhesion forces increased for pH values above 4 and reached a maximum at pH 6.0, whereas a lower, nonzero force was seen at pH values above 8.0. The maximum adhesion force corresponds to the surface pK\(_{1/2}\) of the amine (i.e., the pH of the bulk solution at which one-half of the surface amine sites have been protonated); under these conditions, the maximum number of tip–substrate amine–ammonium ionic hydrogen-bonding interactions occurs. As ionic H-bonds are known to be up to 30 times stronger than regular H-bonds, the pH at which the maximum adhesion force arises should correspond to the surface pK\(_{1/2}\). At pH values below 4.0, both tip and substrate are predominantly covered with surface-bound alkylammonium groups, leading to electrostatic repulsion and little or no adhesive interactions. Above pH 6.0, both tip and substrate are deprotonated, and neutral H-bonding dominates the tip–substrate interaction, leading to lower but nonzero forces.

The pK\(_{1/2}\) value of 6.0 is slightly different from the true surface pK\(_{a}\) of the amine, as any surface potential can perturb the local surface pH from that of the bulk solution (which was measured here) by up to 0.5 pH units. Nonetheless, the measured surface pK\(_{1/2}\) differs considerably from the pK\(_{a}\) for the solution-phase analogue, propylamine, at 10.60.9 This is to be expected, because the proximity of the alkylamines on the surface, the repulsive energy between surface-bound alkylammonium ions, and the limited ability of the solvent to shield these charged species at the surface all act to destabilize the presence of alkylammonium at the surface and shift the surface pK\(_{a}\) strongly downward from the solution-phase value. Although the difference between the solution pK\(_{a}\) and the surface pK\(_{1/2}\) measured here was large, this result is consistent with the force titration profile that was previously reported for the combination of an AFM tip and poly(dimethyldioxane) substrate both modified with APTES.

Figure 3c shows the force titration data between the silicon oxide sharpened AFM tip and hydroxylated substrate, (d) t-Boc-L-Pro-terminated tip and hydroxylated substrate, and (e) t-Boc-L-Pro-terminated AFM tip and substrate.
occurred at the surface pK$_{1/2}$ of the silanol, at pH 3.0. This value is consistent with that previously observed for a silanol surface pK$_a$.$^{50}$ At higher pH, both tip and substrate are deprotonated, leading to repulsive interactions between negatively charged Si—O$^-$ sites. A similar titration profile is seen in Figure 3d, in which the Boc-L-Pro-terminated tip was titrated against a hydroxylated substrate, although the magnitude of the adhesive interaction at low pH was sharply reduced. At lower pH values, at which the substrate is in the form of Si—OH, the main interaction is between the amide group of t-Boc-L-Pro as a H-bond acceptor and silanol as a H-bond donor. By a pH of 6.0, the silanols are fully deprotonated and can no longer act as H-bond donors, only acceptors. As the substrate remains highly hydrophilic, no interaction is observed between tip and substrate.

Figure 3b shows force titration data in which the substrate was amine-terminated and the AFM tip was terminated with t-Boc-L-Pro. In this case, adhesive interactions were observed at all pH values, presumably because of a combination of H-bonding between amine or ammonium species and H-bond-accepting carbonyl groups of t-Boc-L-Pro and hydrophobic interactions between the t-butyl protecting group on the tip and the hydrocarbon chain of APTES at the surface. However, the hydrophilic interactions dominated in this case and were found to be strongly pH-dependent. The presence of a maximum adhesion interaction at a pH of 6.0, the surface pK$_{1/2}$ of the amine, indicates that unreacted amine sites remained on the AFM tip surface.

Figure 3e shows the force titration profile for the t-Boc-L-Pro-terminated tip and substrate. Adhesive interactions were observed at all pH values, with a relatively weak peak at pH 6.0. Although both tip and substrate are H-bond acceptors and not subject to protonation over the pH range studied here, we should expect the adhesion interactions to be largely independent of pH. The peak at pH 6.0 presumably arises as a result of residual —NH$_3^+$ species on the surface. Although the XPS data did not show any evidence for protonated —NH$_2^-$, these observations indicate that residual unreacted amine sites remained on the surface. Nonetheless, the effect was relatively weak and restricted to the pH 5.0—6.5 range. No evidence was seen for residual SiOH sites in the force titration experiments.

### 3.3. Chemical Force Spectrometry of Amino Acid Solutions

Figure 4 presents data for the adhesion interactions observed at pH 6.0 between a t-Boc-L-Pro- or t-Boc-D-Pro-modified AFM tip and silicon substrate, both in water or both in 0.5 M aqueous solutions of various amino acids. The data represent the averages of at least 300 force—displacement curves acquired at three different points on the substrate surface; the error bars indicate the standard deviation of the data. Figure 5 provides four histograms showing the distribution of adhesive interactions observed for the t-Boc-L-Pro and t-Boc-D-Pro tip/substrate pairs in 0.5 M aqueous solutions of L- or D-alanine. The agreement between forces for mirror-image tip/amine acid/substrate combinations is evident. Some typical force—displacement curves are also shown in Figure 5.

Several trends can be observed in Figure 4. First, in a solution of the achiral amino acid glycine, the interaction forces between a D/D or L/L tip/substrate pairing (light or dark red, respectively) were found to be identical, within experimental error. This is consistent with the fact that these pairings are mirror images, with expected identical physical properties. Furthermore, for the three solutions in which we measured interactions between both D- and L-amino acids (alanine, leucine, and aspartic acid), we could clearly distinguish between enantiomeric and diastereomeric systems. As indicated in Figure 4, for alanine, leucine, and aspartic acid solutions, the diastereomeric pairings of tip/(amine acid)/substrate D/L/D and L/L/L (light and dark blue, respectively) or D/D/D and L/D/L (light and dark green, respectively) had dissimilar adhesive interactions, within experimental error. At the same time, the enantiomeric pairs L/L/L and D/D/D (dark blue and light green, respectively) or D/L/D and L/D/L (light blue and dark green, respectively) exhibited identical interaction forces, within experimental error. The histogram data for alanine, shown in Figure 5, also support this distinction. These results demonstrate that we were able to measure chiral discrimination in this system and to distinguish between enantiomeric and diastereomeric systems.

In the absence of any amino acid, only hydrophobic interactions are expected to occur. We$^{50}$ and others$^{47}$ have found that strongly hydrophobic surfaces can lead to highly variable and irreproducible force—displacement curves when
acquired in water. The measured values for the t-Boc-D-Pro and t-Boc-L-Pro enantiomer pairs in water (i.e., in the absence of amino acid) were similar but slightly outside the error bars. As demonstrated below, the presence of amino acids reintroduces H-bonding interactions, indicating that the remaining systems can be more reproducibly measured.

The second important trend is in the relative magnitudes of the adhesive interactions observed. We found the strongest adhesive interaction between the t-Boc-Pro-terminated tip and substrate in water. As proline contains only H-bond acceptor sites, the tip and sample presumably form strong adhesive interactions by forming a H-bonding network with water at the interface that is disrupted as the tip and sample are pulled apart. This is consistent with the force titration experiments, which showed that the adhesive interactions are relatively independent of pH, other than H-bonding from residual ammonium sites at the surface. The reduced forces observed when solution-phase amino acids are present suggest that these bind to the proline interface, disrupting any H-bonding network and concomitantly reducing the tip/substrate interaction. Glycine, the amino acid with the least sterically hindered side chain, appears to show the greatest binding to the t-Boc-Pro-terminated surfaces, because the tip/substrate interaction is most reduced in this case. Furthermore, for the amino acids with hydrophobic side chains, namely, alanine, valine, leucine, and phenylalanine, the “like” amino acid to that on the tip (i.e., the pairings D/D/D or L/L/L) show lower forces than the “non-like” pairings D/L/D or L/D/L. This suggests that, for the hydrophobic amino acids, like pairings favor a more strongly bound complex.

Amino acids containing polar side chains, namely, aspartic acid, glutamic acid, serine, threonine, asparagine, and glutamine, have higher forces for like pairings, D/D/D or L/L/L. In other words, the like pairings correspond to weakly bound complex between the amino acid and the t-Boc-Pro, whereas the non-like pairs favor a strongly bound complex, interrupting the tip−substrate interaction. Again, the data are not precise enough to indicate whether the size of the side chain leads to any secondary effects. Under the pH conditions used here, all of the amino acids, except glutamic and aspartic acid, will be in the form of zwitterions in solution, with both —COO− and —NH3+ groups present. Glutamic and aspartic acids will be present as anionic species. Note that the t-Boc-Pro-terminated interfaces contain only amide linkages: they will not be ionized under these pH conditions. Therefore, because the pls of glutamic and aspartic acids are much lower than those of any other amino acid examined and yet they behave similarly to the other H-bonding counterparts of higher pl, the impact of side-chain hydrophobicity is not a pH effect, nor is it associated with the ionization state of the amino acid itself.

3.4. Molecular Dynamics Simulations. To explore the connection between side-chain hydrophobicity and differences in binding characteristics between amino acids and the t-Boc-Pro interface, we chose to study two amino acids, namely, alanine and serine, in greater detail using molecular dynamics simulations. As discussed previously, these two amino acids showed opposite binding behaviors between the like and non-like pairings (see Figure 4), but have very similar side chains, the difference being that the hydrophobic —CH3 terminus in alanine is replaced with a hydrophilic —CH2OH group in serine.

In each simulation cell, eight L and eight D zwitterionic amino acid molecules were added to the solvent. Fifteen independent simulations were performed for each amino acid, with a total simulation time of about 45 ns. Selectivity factors were calculated by considering the two equilibria

\[
\text{L-amino acid} + \text{t-Boc-L-Pro} \rightleftharpoons (\text{L-amino acid·t-Boc-L-Pro})
\]

\[
K_L
\]

\[
\text{D-amino acid} + \text{t-Boc-L-Pro} \rightleftharpoons (\text{D-amino acid·t-Boc-L-Pro})
\]

\[
K_D
\]
where a complex is defined by the presence of a hydrogen bond between the amino acid and the proline selector. In this case, because the amino acids were zwitterions, the NH$_3^+$ groups of alanine and either the NH$_3^+$ or alcoholic —OH groups of serine can act as H-bond donors, forming a H-bonding complex to any of the two carbonyl oxygen atoms [O(1) and O(2), Figure 6] or the etheric oxygen atom [O(3), Figure 6] of the Boc-L-Pro selector. The selectivity factor, $\alpha$, is the ratio of the two equilibrium constants associated with these equilibria

$$\alpha = \frac{K_L}{K_D} = \frac{[\text{L-amino acid-t-Boc-L-Pro}][\text{D-amino acid}]}{[\text{(D-amino acid-t-Boc-L-Pro)}][\text{L-amino acid}]} \quad (1)$$

As selectivity factors are always defined as being $\geq 1$, if the $\alpha$ enantiomer was to be more strongly bound than the $\beta$ enantiomer, this equation would be inverted.

Each simulation trajectory was analyzed to identify occurrences of hydrogen bonding between an amino acid and a t-Boc-L-Pro selector. The first three entries in Table 1 give the percentages of oxygen atom sites in the selector that are bound to the amino acids D- or L-serine or D- or L-alanine. Figure 6 shows the surface distributions of the three oxygens sites of the t-Boc-L-Pro selectors. The atom labels are given in Figure 6, which also shows the minimum-energy configuration of the t-Boc-L-Pro selector. The carbonyl of the t-Boc group lies farthest from the surface and is oriented toward the bulk solvent, as seen in the inset in Figure 6. H-bonding occurs frequently at this accessible O(2) site. O(1) of the carbonyl group linked to the APTES tether lies closest at some 0.6 nm from the surface and is likewise accessible to solvent and frequently forms H-bonds. By contrast, O(3), although farther from the surface than O(1), is hindered from H-bonding by the adjacent bulky t-butyl group. It is an insignificant source of H-bonding.

The last three entries in Table 1 list the percentages of amino acids that are either bound to form a complex with the t-Boc-l-Pro selector or are free in solution. These values, in turn, can be used in eq 1 to provide the selectivity factor, $\alpha$. The simulations produced a selectivity factor of 1.5 for alanine, indicating that the L enantiomer is preferentially bound to t-Boc-l-Pro. This is consistent with the adhesion force data for alanine in Figure 4:

The adhesion force between the t-Boc-l-Pro tip and the substrate is lower in the presence of l-alanine than in the presence of d-alanine. As we postulate that binding of the amino acid disrupts the tip/substrate interaction, the more strongly bound l-alanine should lead to a reduced adhesion force. Thus, the simulations indicate that d- and l-alanine can be distinguished by the CSP and properly reproduce the experimental observation that the like l/l/l interaction is preferred.

For serine, the calculated selectivity factor is 1.0. Thus, within the statistics of the MD simulation, we did not observe chiral selectivity with serine. The force spectrometry data in Figure 4 indicate that, whereas the difference in adhesion force between l- and d-serine is statistically significant, the absolute difference is less than for alanine. Indeed, we estimated the selectivity factor using the forces instead. In this case

$$\alpha = \frac{F_{\text{water}} - F_{\text{l-amino acid}}}{F_{\text{water}} - F_{\text{d-amino acid}}} \quad (2)$$

Using the average force of 2.9 nN observed in water (Figure 4), the selectivity factor using the force measurements was 2.1 for alanine and 1.2 for serine. In this case, the additivity of the absolute errors from the force measurements meant that these values had errors of approximately 30%. However, the smaller selectivity factor observed experimentally for serine is still consistent with the molecular dynamics data. More significantly, the MD simulations indicate that both —NH$_3^+$ and —OH sites of serine participate significantly in H-bonding interactions with the Boc-Pro selector.

The molecular dynamics simulations also suggest a mechanism for this differing behavior. Figure 7 shows a histogram comparing the position of the alanine and serine side chains above the surface layer for those amino acids forming H-bonds with the t-Boc-l-Pro selector. l-Alanine, which shows the

![Figure 6. Surface distribution showing the distances of the three O atoms of the boc-l-Pro chiral stationary phase above the silicon oxide surface, as determined from molecular dynamics simulations. The assignment of O atom labels (also employed in Table 1) is indicated on the right, which shows the most stable configuration of t-Boc-l-Pro at the surface.](image_url)

### Table 1. Analysis of the Probability for Diastereomeric Complexes between d-/l-Alanine or d-/l-Serine and t-Boc-l-Proline Selector, As Determined from MD Simulations

<table>
<thead>
<tr>
<th>species</th>
<th>content of t-Boc-l-proline selectors with a hydrogen bond at the specified oxygen atom$^a$ (%)</th>
<th>content of amino acid analytes$^b$ (%)</th>
<th>selectivity factor$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[amino acid-t-Boc-l-Pro] complex</td>
<td>free</td>
<td></td>
</tr>
<tr>
<td>l-alanine (NH$_3^+$)</td>
<td>0.71 ± 0.11 0.69 ± 0.17 0.00 ± 0.00</td>
<td>5.6 ± 0.1 94.4 ± 0.2 1.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>d-alanine (NH$_3^+$)</td>
<td>0.42 ± 0.05 0.49 ± 0.09 0.01 ± 0.00</td>
<td>3.7 ± 0.1 96.3 ± 0.1 1.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>l-serine (NH$_3^+$)</td>
<td>0.38 ± 0.06 0.23 ± 0.04 0.00 ± 0.00</td>
<td>5.9 ± 0.1 94.1 ± 0.1 1.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>l-serine (OH)</td>
<td>0.49 ± 0.08 0.35 ± 0.06 0.01 ± 0.00</td>
<td>5.8 ± 0.1 94.2 ± 0.1 1.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>d-serine (NH$_3^+$)</td>
<td>0.35 ± 0.05 0.25 ± 0.04 0.01 ± 0.00</td>
<td>5.8 ± 0.1 94.2 ± 0.1 1.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>d-serine (OH)</td>
<td>0.48 ± 0.12 0.38 ± 0.10 0.00 ± 0.00</td>
<td>5.8 ± 0.1 94.2 ± 0.1 1.0 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Serine can donate a H-bond through the ammonium or through the alcohol group, and statistics for both groups are provided. $^b$See Figure 6 for atom numbering. $^c$Defined by eq 1.
smaller adhesion force and is hence more tightly bound, holds the hydrophobic side chain closer to the surface (at about 1.2 nm) when bound to the t-Boc-L-Pro selector. d-Alanine, less tightly bound and hence exhibiting higher tip/sample adhesion forces, directs the hydrophobic side chain some 0.1 nm farther from the surface and hence into the solution phase. As solvating these hydrophobic side chains in water is energetically unfavorable, this will lead to a less tightly bound complex. This is consistent with our observation of a greater reduction in adhesive force for those hydrophobic amino acids of like configuration to the proline CSP. It also suggests that the shorter the hydrophobic side chain, the greater should be the reduction in adhesive force compared to water: this is indeed the case for achiral glycine, which has the shortest side chain of all, as can be seen in Figure 4. The evidence for side-chain effects from force data for the remaining series of alanine, leucine, valine, and phenylalanine was less convincing, because of the experimental errors involved; however, the very bulky phenylalanine group does appear to show a lesser reduction compared to the remaining amino acids with alkyl side chains.

Figure 7 indicates that l-serine in particular, when bound to the t-Boc-L-Pro selector, shows a much broader distribution of side-chain carbon atom from the silicon oxide surface in the amino acids l- or d-alanine [CH(NH2)(CO2H)—CH3] and l- or d-serine [CH(NH2)(CO2H)—CH2(OH)] for those amino acids exhibiting H-bonding interactions to the t-Boc-L-Pro chiral stationary phase, as determined from molecular dynamics simulations. The position of oxygen atom O(2) (see Figure 6), the H-bonding O atom of the t-Boc-L-Pro chiral stationary phase, most distant from the silicon oxide surface, is indicated by the dashed line.

The substrate was characterized using surface analysis methods to ensure a flat, homogeneous overlayer of selectors. Using force spectrometric measurements, the adhesive interactions between the t-Boc-proline-terminated tip and substrate were measured in aqueous solution under varying pH conditions and in the presence of a series of amino acids. In a series of chemical force titration experiments, we demonstrated that the CSP showed no evidence of residual SiOH sites and minimal unreacted amine. Using force spectrometric experiments in amino acid solutions, we demonstrated that our approach of using chirally modified AFM tips successfully distinguishes enantiomeric and diastereomeric amino acid complexes. We also found that, in all cases, amino acids in solution form complexes to the proline through H-bonding interactions, disrupting the interactions between proline molecules on the tip and substrate. The nature of the binding is strongly dependent on the amino acid side chain. Amino acids containing hydrophilic side chains showed that the like l/l or d/d proline/(amino acid)/proline complex was more strongly bound. Molecular dynamics simulations indicated that, in such a case, the hydrophobic side chain is held closer to the surface and, hence, unfavorable solvation of the hydrophobic groups by water is minimized. By contrast, the non-like l/d or d/l complexes are preferred when the side chain contains hydrophilic groups, which can exhibit a range of H-bonding interactions with the proline. Whereas most of the amino acids studied were zwitterionic under the experimental conditions studied, both glutamic and aspartic acid, which are anions under these pH conditions, showed no difference in behavior compared with the other amino acids containing hydrophilic side chains, indicating that the pI of the amino acid does not seem to be an important factor in controlling the binding to proline.

4. CONCLUSIONS

We have reported herein on the preparation, characterization, and testing of a brush-type chiral stationary phase, based on a t-Boc-protected proline selector tethered through an aminopropyl silane linkage to Si. The CSP was formed on both an oxidized Si(111) substrate and an oxide-sharpened Si AFM tip. The evidence for side-chain interactions observed when the non-like l-Pro/d-serine combination occurs.

**REFERENCES**
