

# Covalent Attachment of a Nickel Nitrilotriacetic Acid Group to a Germanium Attenuated Total Reflectance Element

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The surface of a germanium internal reflectance element (IRE) was modified to bind 6X-histidine (his)-tagged biomolecules. The step-by-step surface modification was monitored via single-pass attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FT-IR). Initially an adlayer of 7-octenyltrimethoxysilane (7-OTMS) was formed on the Ge crystal through the surface hydroxyl groups, which were produced via ozonolysis of the Ge surface. The vinyl moiety of 7-OTMS was oxidized to a carboxylic acid, which was activated by 1,1'-carbonyldiimidazole (CDI) to produce a labile imidazole. The labile imidazole that resulted from the CDI coupling was then displaced by the primary amine of nitrilotriacetic acid (NTA). Nickel sulfate was added to the system, and it coordinated with the three carbonyl groups and the nitrogen on NTA, thus leaving the ability of Ni to coordinate with two adjacent histidine residues. Binding of his-tagged biotin to nickel nitrilotriacetic acid (Ni-NTA) was observed by ATR-FT-IR spectroscopy. The surface modification method presented in this paper had minimal nonspecific binding, the Ni-NTA surface was reusable if stored properly, and complete removal of the organic surface was achievable.

## Introduction

Protein secondary structure analysis will become a powerful tool for proteomics if it can be combined with tests of protein function in a single spectroscopic assay. For proteomic studies an approach is needed that rapidly identifies the overall fold of a protein and the binding partners of that protein. Such an approach would yield valuable information on biological pathways and the mechanism of disease.<sup>1,2</sup> Several Fourier transform infrared (FT-IR) spectroscopic methods exist for protein secondary structure determination.<sup>3–7</sup> We have recently demonstrated the utility of single-pass attenuated total reflectance (ATR) FT-IR spectroscopy for the rapid determination of protein secondary structure.<sup>8–10</sup> However, there are no such secondary structure determination methods for protein mixtures due to the complexity of the resulting FT-IR spectrum. Perhaps the only feasible way to analyze the secondary structure of proteins in mixtures is to selectively bind a single protein to a surface. Furthermore, the observation of secondary structure changes of an immobilized protein when challenged with a binding partner generates valuable information concerning protein-binding interactions. The present study

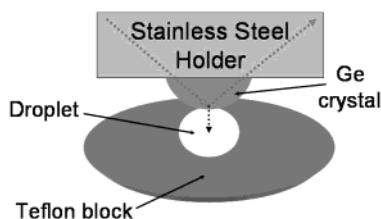
represents a first step in this direction by functionalization of a germanium (Ge) internal reflective element (IRE) to selectively bind histidine (his)-tagged biomolecules.

Surface attachment using self-assembled monolayers (SAMs) is a commonly used strategy for the study of biological systems,<sup>11–13</sup> for instance, the functionalization of Ge surfaces has been studied as a substrate for molecular recognition.<sup>14</sup> SAMs have also been successfully applied to a Ge multipass IRE coated with a thin layer of gold.<sup>15</sup> Our research focuses on the covalent attachment of an adlayer to a Ge single-pass IRE with nickel nitrilotriacetic acid (Ni-NTA) to bind polyhistidine-tagged (his-tagged) biomolecules. Ni-NTA columns are commercially available for the purification of his-tagged proteins, and the binding chemistry is well understood.<sup>16</sup> In addition, the Ni-NTA moiety has been used for binding his-tagged proteins to gold.<sup>17,18</sup> In the present study a surface attachment strategy was used to bind Ni-NTA to a Ge surface. Each chemical modification step was observed spectroscopically by single-pass ATR-FT-IR. The goal of our research is to develop reusable surface-modified single-pass Ge IREs for the study of protein-binding interactions. There were three objectives of the work discussed in this paper. The first is to observe the step-by-step modification of a Ge IRE via single-pass ATR-FT-IR. The second is to observe the binding of his-tagged biotin to the given surface. The third objective concerns the reproducibility of the surface modification method. Due to the expense of Ge IREs, the method is not practical if the surface cannot be removed

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(1) Pelton, J. T.; McLean, L. R. *Anal. Biochem.* **2000**, *277*, 167–176.  
 (2) Maggio, E. T.; Ramnarayan, K. *Drug Discovery Today* **2001**, *6*, 996–1004.  
 (3) Dousseau, F.; Pezolet, M. *Biochemistry* **1990**, *29*, 8771–8779.  
 (4) Baumruk, V.; Pancoska, P.; Keiderling, T. *J. Mol. Biol.* **1996**, *259*, 774–791.  
 (5) Lee, D. C.; Haris, P. I.; Chapman, D.; Mitchell, R. C. *Biochemistry* **1990**, *29*, 9185–9193.  
 (6) Pribic, R.; van Stokkum, I. H. M.; Chapman, D.; Haris, P. I.; Bloemendal, M. *Anal. Biochem.* **1993**, *32*, 366–378.  
 (7) Wi, S.; Pancoska, P.; Keiderling, T. A. *Biospectroscopy* **1998**, *4*, 93–106.  
 (8) Smith, B. M.; Oswald, L.; Franzen, S. *Anal. Chem.* **2002**, *74*, 3386–3391.  
 (9) Smith, B. M.; Franzen, S. *Anal. Chem.* **2002**, *74*, 4076–4080.  
 (10) Pop, C.; Chen, Y.; Smith, B.; Bose, K.; Bobay, B.; Tripathy, A.; Franzen, S.; Clark, C. *Biochemistry* **2001**, *40*, 14224–14235.

(11) Kasemo, B. *Surf. Sci.* **2002**, *500*, 656–677.  
 (12) Castner, D. G.; Ratner, B. D. *Surf. Sci.* **2002**, *500*, 28–60.  
 (13) Whitesides, G. M.; Grzybowski, B. *Science* **2002**, *295*, 2418–2421.  
 (14) Bent, S. F. *Surf. Sci.* **2002**, *500*, 879–903.  
 (15) Liley, M.; Keller, T. A.; Duschl, C.; Vogel, H. *Langmuir* **1997**, *13*, 4190–4192.  
 (16) Wilchek, M.; Bayer, E. A. *Biomol. Eng.* **1999**, *16*, 1–4.  
 (17) Sigal, G. B.; Bamdad, C.; Barberis, A.; Strominger, J.; Whitesides, G. M. *Anal. Chem.* **1996**, *68*, 490–497.  
 (18) Kada, G.; Riener, C. K.; Hinterdorfer, P.; Kienberger, F.; Stoh, C. M.; Gruber, H. J. *Single Mol.* **2002**, *3*, 119–125.



**Figure 1.** The single-pass Ge ATR element used in the ATR-FT-IR microscope is represented schematically. The dashed arrows indicate the infrared beam and the small vertical dashed arrow represents the evanescent wave that penetrates the sample. The sample is a droplet (white circle) that is in contact with the Ge ATR crystal and held in place by the Teflon sample holder. The Teflon sample holder (dark gray) is designed to keep sample in contact with the Ge crystal while minimizing evaporation.

and formed repeatedly on the same Ge crystal. In addition, the preparation of the Ni-NTA surface on Ge is time-consuming. Thus, multiple use of a single Ni-NTA surface is desirable and will be investigated in this study.

### Experimental Details

A Ge single-pass ATR element was modified for the purpose of analyzing histidine-tagged biomolecules via single-pass ATR-FT-IR. As shown in Figure 1, the Ge crystal is centered on a stainless steel holder that slides into a UMA500 microscope (Digilab). The crystal faces down as shown in Figure 1, and a Teflon sample holder with a small depression is used to bring the sample droplet into contact with the Ge ATR crystal. Ozonolysis (Jelight Company, Inc., UVO-60, model #42) was carried out on the entire Ge ATR objective for a period of no less than 20 min in order to produce the necessary hydroxyl groups for the silane reaction. Immediately following ozonolysis, a spectrum of the cleaned Ge crystal was acquired to use as a background spectrum. A Teflon sample holder was made that has a 20- $\mu$ L reservoir such that sample can be in contact with the Ge crystal but the holder also seals the sample so evaporation is minimized. A 1% 7-octenyltrimethoxysilane (7-OTMS, Aldrich, 45,281-S) in toluene solution was deposited into the sample holder and left overnight. The Ge crystal was washed thoroughly with toluene and then placed in contact with 20  $\mu$ L of an oxidizing solution. Placing the Ge crystal in oxidizing solution overnight resulted in the conversion of the vinyl group to a carboxylic acid. The oxidizing solution used consisted of 100  $\mu$ L of 5 mM  $\text{KMnO}_4$ , 100  $\mu$ L of 18 mM  $\text{K}_2\text{CO}_3$ , 100  $\mu$ L of 195 mM  $\text{NaIO}_4$ , and 700  $\mu$ L of  $\text{H}_2\text{O}$ .<sup>19</sup> After 24 h, the Ge crystal was washed with three 1-mL aliquots of 0.3 M  $\text{NaHSO}_3$ , three 1-mL aliquots of 0.1 N HCl, 5 mL of ethanol, and 5 mL of  $\text{H}_2\text{O}$ , respectively. Usually placing the crystal in oxidizing solution overnight yielded a significant carboxylic acid signature; however, there were some instances where the Ge crystal had to be placed in the oxidizing solution up to 48 h. It is important to note that a new oxidizing solution was made for each 24-h period.

The next step in the surface functionalization procedure entailed a 1,1'-carbonyldiimidazole (Sigma C-7625) coupling. A 10 mM solution of 1,1'-carbonyldiimidazole (CDI) in DMSO was placed in the Teflon sample holder overnight. The Ge crystal was rinsed thoroughly with dimethyl sulfoxide (DMSO) and was then placed overnight in a 0.1 M solution of nitrilotriacetic acid (NTA)-amine in water. NTA-amine was synthesized in our laboratory, and details of the preparation can be found elsewhere.<sup>20</sup> The Ge crystal was placed in contact with 20 mM NaOH for 10 min and then placed in a 40 mM  $\text{NiSO}_4$  (Fisher N73-100) solution for 2 h. Afterward, the Ni-NTA surface was ready to selectively bind histidine-tagged biomolecules. In this experiment 6X-histidine tagged biotin was the analyte. The 6X-histidine tagged biotin (his-tagged biotin) was synthesized at the Protein Sequencing and Peptide Synthesis Facility at UNC Chapel Hill

and consisted of the sequence His-His-His-His-His-His-Biotin. After binding of the his-tagged biotin, the surface was washed with 20 mM imidazole (Sigma I-2399) to remove any nonselectively bound molecules. To displace the his-tagged biotin from the surface, the surface was allowed to soak in 100 mM imidazole. To ultimately remove the bound imidazole, the surface was allowed to soak in an equilibrating buffer (20 mM Tris-Cl, 10 mM glucose, 10 mM imidazole, 0.3 M NaCl, pH 8.0) overnight.<sup>21</sup> The Ni-NTA surface was reusable if stored in the equilibrating buffer. The longest a surface remained active was in excess of 2 months. Once the functionality of the surface (as detected by single-pass ATR-FT-IR) decreased significantly, the surface was cleaned either by ozonolysis or by polishing the Ge crystal. This restored the Ge crystal back to its original state. The attachment of the described surface was performed a total of five times on two separate Ge crystals; thus the surface spectra shown throughout this paper are representative of the five surface replications.

In the experimental apparatus, the Ge crystal is at the focus of a Cassagranian objective in a UMA500 microscope (Digilab). The spectra were recorded at ambient temperature in a  $\text{N}_2$  environment and averaged over 1024 scans on a Digilab FTS 6000 FTIR spectrometer equipped with a liquid-nitrogen-cooled MCT detector in a UMA500 microscope. The spectra were recorded with a resolution of 2  $\text{cm}^{-1}$  in the range of 600–4200  $\text{cm}^{-1}$ . Background spectra were obtained subsequently. In addition to the surface spectra, reference spectra were also acquired for solutions of 7-OTMS, CDI, NTA-amine,  $\text{NiSO}_4$ , Ni-NTA, and his-tagged biotin. Reference solutions were brought into contact with an unmodified Ge crystal and allowed to slowly dehydrate in the  $\text{N}_2$  environment in which they were placed. Once a detailed spectrum with minimal solvent interference was obtained, the Ge crystal was gently washed with solvent and dried thoroughly. All spectral data were acquired using the software package Win-IR-Pro v2.97 (Digilab), and data analysis was performed using the software package Igor-Pro v3.12.

### Results and Discussion

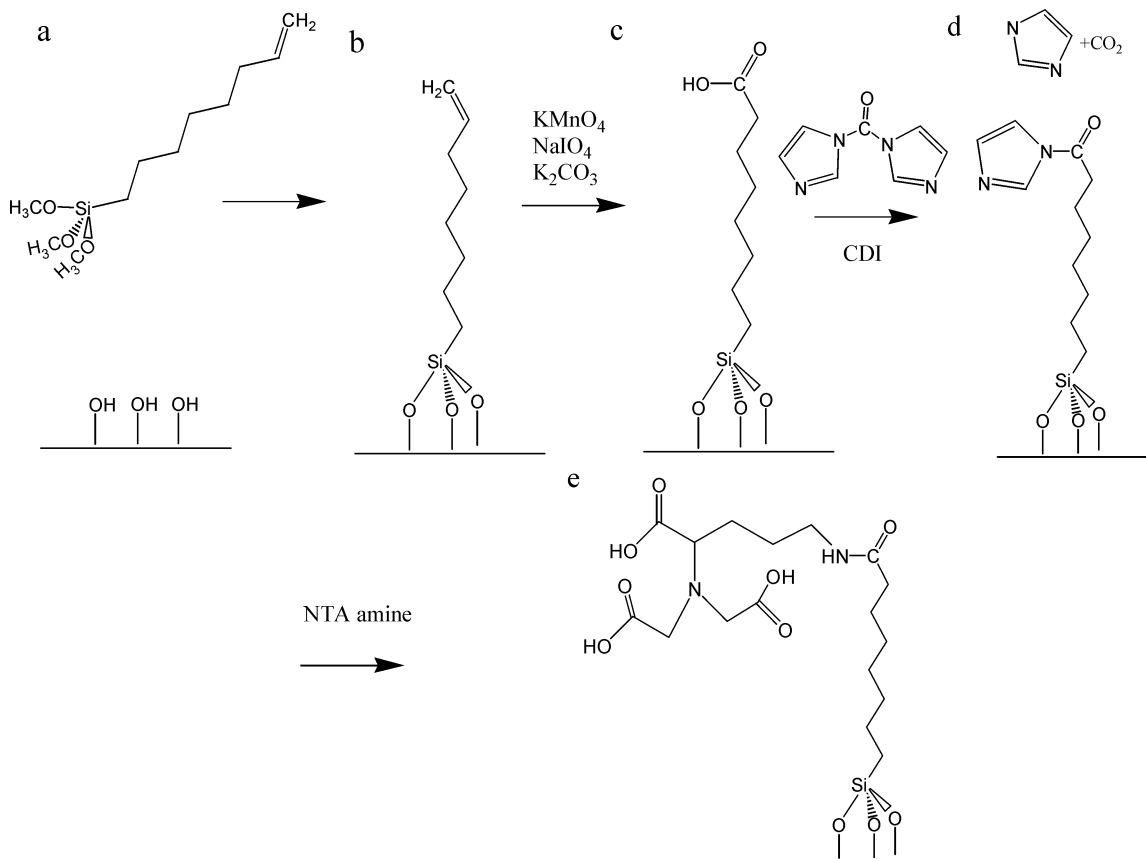
The surface of the germanium crystal was subjected to ozonolysis in preparation for surface attachment chemistry. ATR spectra of the ozonolyzed Ge surface revealed the hydroxyl groups that were not present on the Ge surface prior to ozonolysis (data not shown). The surface attachment scheme for 7-OTMS shown in Figure 2 makes use of the hydroxyl groups (i.e., Ge-OH) for covalent attachment to the Ge surface as shown in Figure 2b. The single-pass ATR-FT-IR spectrum of 7-OTMS on the Ge surface and a reference spectrum of bulk 7-OTMS are presented in Figure 3. From this comparison, it is apparent that successful silanization occurred. The only peaks that do not match are at 1550 and 1700  $\text{cm}^{-1}$ . Medium-sized peaks at 1550 and 1700  $\text{cm}^{-1}$  are indicative of monosubstituted aromatic rings.<sup>22</sup> Thus, the discrepancies between the bound 7-OTMS and the bulk 7-OTMS are due to residual toluene, which was not present in the reference solution of 7-OTMS shown in Figure 3. The oxidation step indicated in Figure 2c was then performed on the 7-OTMS monolayer. The spectrum of the new surface, given in Figure 4, yields two broad peaks in the range of 1500–1700  $\text{cm}^{-1}$ . The peaks in the region of 1500–1600 and 1700  $\text{cm}^{-1}$  are indicative of carboxylate and carboxylic acid, respectively. The spectrum given in Figure 4 has signatures in both these regions, as was expected for successful oxidation of the vinyl group. The reduction of the peak at 1400  $\text{cm}^{-1}$ , which was due to a vinyl group, also validates oxidation of the vinyl group on 7-octenyltrimethoxysilane. Inspection of this peak, however, suggests that not all of the vinyl groups were oxidized. There

(19) Wasserman, S. R.; Tao, Y. T.; Whitesides, G. M. *Langmuir* **1989**, 5, 1074–1087.

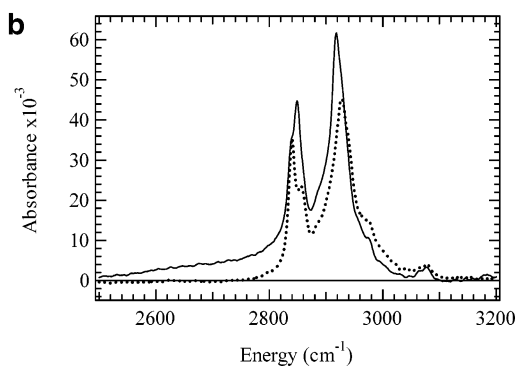
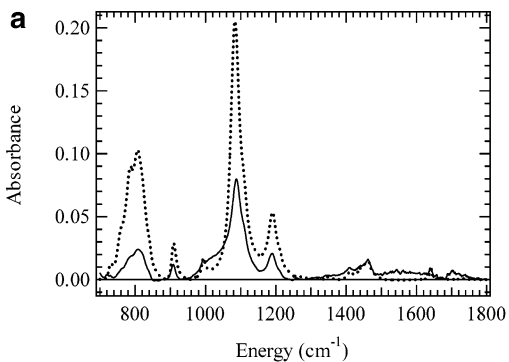
(20) Houchulie, E.; Dobeli, H.; Schacher, A. *J. Chromatogr.* **1987**, 411, 177–184.

(21) Sui, D.; Wilson, J. E. *Protein Expression Purif.* **2002**, 24, 83–89.

(22) Skoog, D. A.; Holler, F. J.; Nieman, T. A. *Principles of Instrumental Analysis*, 5th ed.; Saunders College Publishing/Harcourt Brace College Publishers: Philadelphia, PA, 1998.

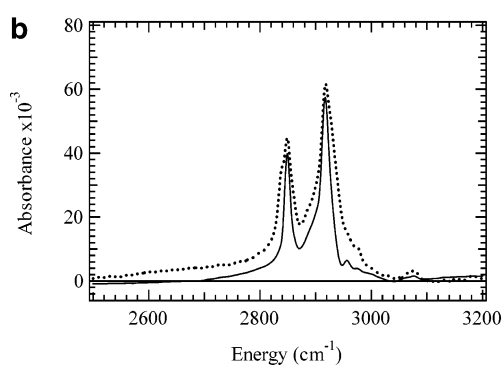
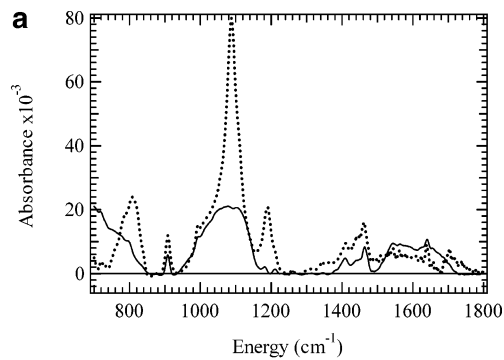


**Figure 2.** Scheme for the attachment of a nitrilotriacetic moiety to the Ge surface: (a) addition of the 7-octenyltrimethoxysilane; (b) attachment of 7-octenyltrimethoxysilane; (c) oxidation of the vinyl group to produce a carboxylic acid; (d) coupling of carbonyldiimidazole (CDI) to the carboxylic acid group; (e) nucleophilic displacement of imidazole with NTA-amine.



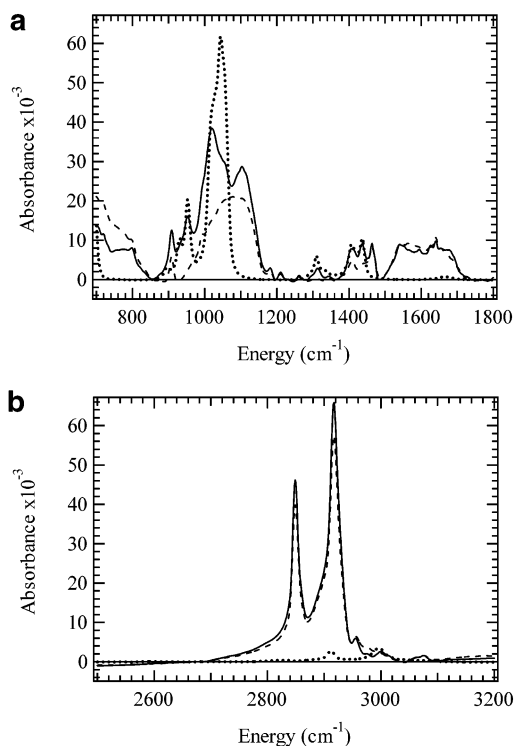
**Figure 3.** Comparison of 7-octenyltrimethoxysilane on the Ge surface (solid line) a reference spectrum of bulk 7-octenyltrimethoxysilane (dotted line): (a) low-frequency region; (b) high-frequency region.

is a competition between oxidation of the vinyl groups and further oxidation of the alkane chains. We determined that an oxidation of 24 h produced the optimum yield of



**Figure 4.** Oxidation of 7-octenyltrimethoxysilane on the Ge surface: (a) low-frequency region; (b) high-frequency region. The carboxylate product is shown as the solid line, and the reference spectrum of 7-octenyltrimethoxysilane on a surface is shown as the dotted line.

functionalized carboxylic acids relative to destructive oxidation of the alkane chain.

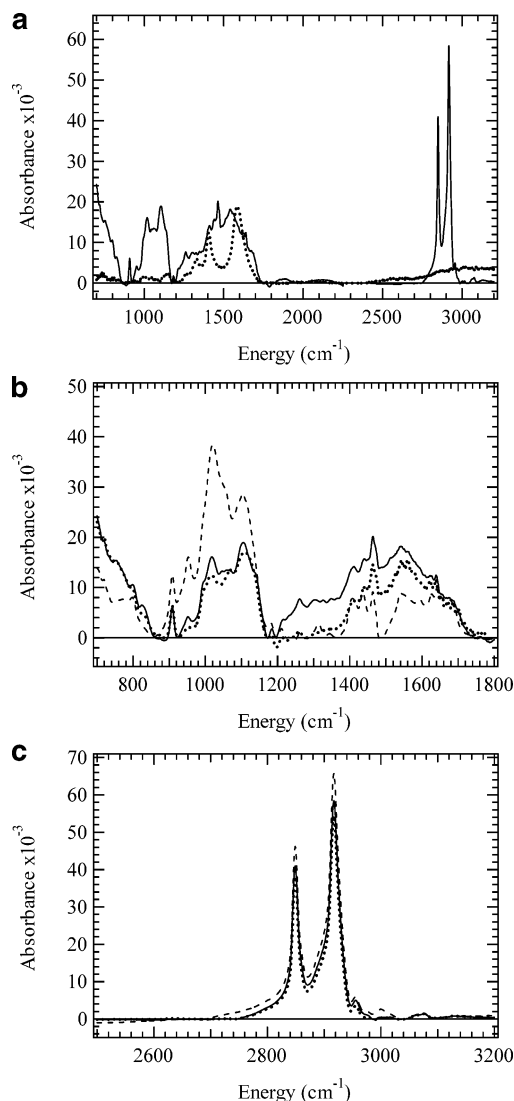


**Figure 5.** The solid spectrum represents carbonyldiimidazole (CDI) on the surface: (a) low-frequency region; (b) high-frequency region. The dotted spectrum represents a scaled reference spectrum of bulk CDI. The dashed spectrum represents the oxidized 7-octenyltrimethoxysilane as shown in Figure 4.

The next step involved the coupling of CDI to the carboxylic acid group produced from the oxidation (refer to Figure 2d).<sup>23</sup> The spectrum of CDI attached to the surface is given in Figure 5 and is compared to the spectrum of CDI in bulk solution. The two spectra are analogous with a strong signature at  $1050\text{ cm}^{-1}$ , which is due to C–N stretching.

The next phase was the attachment of nitrilotriacetic acid (NTA) to the surface given in Figure 2e. The spectrum of this surface was compared to the spectrum of NTA-amine in solution (Figure 6). The spectrum of NTA-amine attached to the surface resembles the sum of the NTA-amine reference spectrum and the CDI surface spectrum in the region of  $1200\text{--}1800\text{ cm}^{-1}$ . The decrease in intensity of the peaks in the region from  $950\text{ to }1200\text{ cm}^{-1}$  is due to the displacement of the imidazole group. The signals in this region are due to C–N and C–C stretching. The reduction in signal is due to the loss of strong C–N stretching expected in an imidazole group. There is not a complete loss of signal in this region since there is medium C–N and C–C stretching in NTA-amine. The NTA-amine surface was washed with  $20\text{ mM NaOH}$  for  $10\text{ min}$ , and then  $\text{NiSO}_4$  was placed in contact with the surface for  $2\text{ h}$ . Once Ni was attached to the surface, it was able to coordinate with two histidine groups. Thus, a  $6\times$  his-tagged protein could bind with three adjacent Ni-NTA groups. The resulting spectrum of the Ni-NTA surface is shown in Figure 6. Since  $\text{NiSO}_4$  does not absorb strongly in the mid-infrared, the spectrum of Ni-NTA on the surface should be similar to NTA-amine on the surface, which was the case as seen in Figure 6.

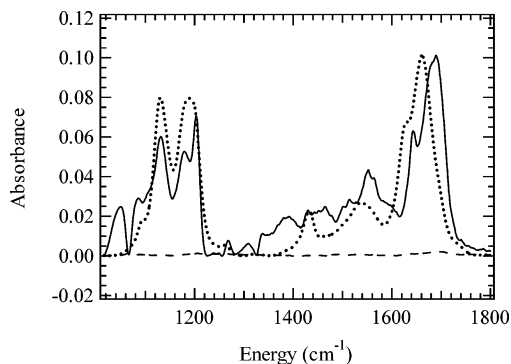
To verify the binding of histidines to the surface-attached Ni-NTA group, a  $6\times$ -his-tagged biotin molecule



**Figure 6.** (a) The solid spectrum represents NTA-amine on the surface, and the dashed spectrum represents a scaled reference spectrum of NTA-amine. (b) Low-frequency region. The solid spectrum represents NTA-amine on the surface and the dotted spectrum represents Ni-NTA. The gray dashed spectrum represents the CDI layer as shown in Figure 5. (c) The same as spectrum b but of the high-frequency region.

was attached to the Ni-NTA surface as shown in Figure 7. The bound his-tagged biotin reference spectrum has peaks indicative of the histidine tag at approximately  $1640$  and  $1630\text{ cm}^{-1}$ , respectively. The band at  $1661\text{ cm}^{-1}$  in the reference his-tagged biotin spectrum was shifted to  $1690\text{ cm}^{-1}$  in the bound his-tagged biotin spectrum. This shift is indicative of a large change in the hydrogen bonding of the biotin molecule. His-tagged biotin reference peaks at  $1130$ ,  $1189$ ,  $1432$ , and  $1538\text{ cm}^{-1}$  matched those in the bound his-tagged biotin spectrum. The remaining peaks in the bound biotin spectrum that differ from those in the reference spectrum are thought to arise from surface–biotin interactions. A  $100\text{ mM}$  rinse with imidazole was used to remove the his-tagged biotin from the surface. As seen in Figure 7, the spectrum of the Ni-NTA surface after the  $100\text{ mM}$  imidazole wash indicated complete removal of the bound his-tagged biotin, thus indicating that the Ni-NTA surface can be used repeatedly. The nearly flat line in the figure is the difference spectrum of the Ni-NTA layer before addition of his-tagged biotin and after the his-tagged biotin is washed away by the  $100\text{ mM}$  imidazole solution.

(23) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: San Diego, CA, 1996.



**Figure 7.** The solid spectrum represents his-tagged biotin attached to the Ni-NTA surface, and the dotted spectrum represents a scaled reference spectrum of his-tagged biotin in bulk solution. The dashed spectrum represents the Ni-NTA surface after washing with 100 mM imidazole. The function of the 100 mM imidazole rinse was to remove bound his-tagged biotin from the Ni-NTA surface.

### Conclusions

Modification of the Ge internal reflectance element was monitored by ATR-FT-IR spectroscopy. The spectra of the silane, CDI, NTA, and Ni-NTA layers were consistent with

reference spectra. Once the NiNTA surface was made, his-tagged biotin was bound and removed from the Ni-NTA surface. The binding biotin was performed multiple times to ensure that the surface was reusable. The use of a biotin label for monitoring successful attachment to NiNTA is a first step toward successful preparation of a biocompatible surface that can be used for the detection of protein binding interactions with surface-attached proteins by ATR-FTIR spectroscopy. A second goal of this research was to determine if the original Ge surface could be restored. Two Ge crystals were modified and had the organic surface removed. After restoration of the Ge crystal, a new organic surface was successfully applied. In conclusion, the method of direct surface modification to a Ge internal reflectance element introduces several applications for the conformational analysis of proteins and protein mixtures as well as having the potential for studying protein-binding interactions.

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