Spectroscopic Evaluation of Protein Affinity Binding at Polymeric Biosensor Films

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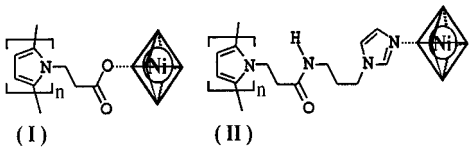
Metal ion affinity chromatography has been applied to the purification of a range of proteins since 1975,[1] finding particular applications in the isolation of genetically modified fusion proteins containing an oligo-histidine sequence.[2] More recently, the reversible coordination of the *his* tag sequence (normally comprising six histidine residues and introduced into the protein at either its N or C terminus) has also been used as a generic immobilization procedure in order to orientate engineered proteins at a metal ion modified interface.[3−8] To date, such affinity immobilizations have involved the use of self-assembling systems,[3−8] of either silanes on glasses,[3] bilayers at the air−water interface,[4−7] or alkanethiols on gold.[8] In all of these cases the headgroup has been modified with a metal chelating agent such as nitrilotriacetic acid (NTA)[3−5,7,8] or iminodiacetic acid (IDA).[6]

We have now further developed metal ion affinity binding using spectroscopic methods to investigate the generic immobilization of engineered fusion proteins within conducting polymer matrixes. In contrast to the multistep synthetic chemistries required for the attachment of chelating groups (e.g., NTA or IDA) to a premodified surface,[3−8] we have examined the specific assembly of the *his*-tagged enzyme, alkaline phosphatase, at Ni^{2+} sites coordinated within a carboxylic acid functionalized polymer. To characterize this interfacial arrangement, we have developed a diagnostic method based upon high-resolution X-ray photoelectron spectroscopy (XPS).

Two functionalized monomers were polymerized and then used to investigate the nature of the nickel ion coordination chemistry, namely, 3-(pyrrol-1-yl)propanoic acid (**I**) and [3-(pyrrol-1-yl)-propanoyl]-1-(3-aminopropyl) imidazole (**II**). Polymer(**I**) was deposited directly by electropolymerization of the appropriate monomer, while poly(**II**) was prepared via the reaction of an intermediatory pentafluorophenol derivatized analogue of poly-(**I**)[9] with 1-(3-aminopropyl)imidazole (Scheme 1). Doping of these films with Ni^{2+} was achieved by immersion of the polymer in an aqueous solution of nickel chloride (100 μM to 500 mM) for an appropriate period of time (e.g., 60 min) followed by rinsing in deionized water. Initially, the nature of the nickel−polymer interaction was studied using reflectance angle infrared spectroscopy (RAIRS) analysis of the poly(**I**) films at various nickel dopant levels, Figure 1. The two peaks of interest are the ν_{asym} (CO_2^-) and ν_{sym} (CO_2^-) bands at 1602 and 1423 cm^{-1}, respectively. It can be seen that there is an increase in the asymmetric band with increasing Ni^{2+} concentration. It is also interesting to note that the $\nu_{(asym-sym)}$ peak separation of 179 cm^{-1} is larger than the corresponding ionic separation (169 cm^{-1}), diagnostic of unidentate binding of Ni^{2+} in a carboxylate ligand system.[10]

Scheme 1



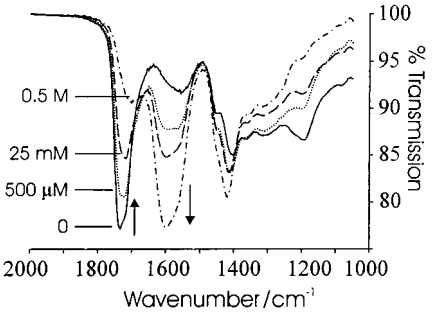


Figure 1. RAIRS spectra of poly(**I**) films doped with varying concentrations of Ni^{2+} in the doping solution. ($\theta = 74°$ on gold coated slides).

The incorporation of nickel ions within the films was confirmed by examining the Ni($2p_{3/2}$) and C(1s) XPS spectra. The films were found to coordinate Ni^{2+} in a concentration-dependent manner, with Figure 2 showing the carboxylate component of the carbon spectra of poly(**I**) at different Ni^{2+}-doping levels and providing evidence for the specific interaction of the nickel ion with the carboxylate groups of the polymer. The shift in the carboxylate component peak to lower binding energies with increasing Ni^{2+} concentration corresponds to the increasing negative charge on the carboxylate groups associated with deprotonation and formation of the anionic species. XPS measurements in the Cl(2p) region show a complete absence of Cl anions within the film, confirming that the counterion for the nickel species is provided by the polymer's deprotonated carboxylate species (i.e., CO_2^- Ni^{2+} complex). Although Ni^{2+}−poly(**I**) is a relatively weak ligand system, the polymer−ion interaction was found to be sufficiently strong to resist the nickel ion being sequestered in 25 mM phosphate buffer solution pH 7.0. The nickel ion could, however, be released in mild acid solution (0.1 M $HClO_4$) or through the introduction of a competitive ligand such as histidine, consistent with observations using traditional Ni^{2+} affinity columns or NTA−SAM systems,[3] and providing the future possibility of being able to regenerate or "pattern" the functionalized surface.

The Ni^{2+}−polymer interaction was further elucidated by collecting the Ni($2p_{3/2}$) XPS signal, with Figure 3a showing a typical spectrum of a Ni^{2+}-doped poly(**I**) film. The main Ni($2p_{3/2}$) signal is located at 856.2 eV with a characteristic satellite peak 4.6 eV higher in energy, a consequence of the interactions between d electrons of the metal center and the specific coordinated ligand.[11] If we consider charge balance and steric restrictions, and that the Ni^{2+} is octahedrally coordinated, then two of the binding sites will be occupied by carboxylate groups, and there will generally be four labile sites (occupied by water molecules) to which a *his* tag sequence on a protein could bind.

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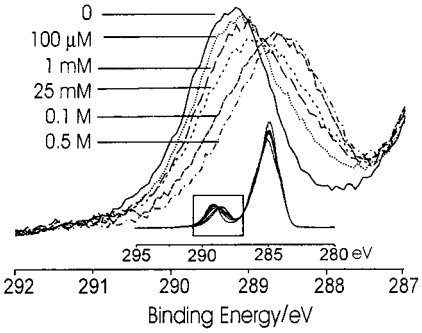

Figure 2. C(1s) XPS spectra of poly(**I**) films doped with increasing Ni^{2+} concentrations in the doping solution. The carboxylate component is found to shift to lower binding energies as the film becomes increasingly populated with Ni^{2+} ion. All XPS spectra are energy corrected against the C(1s) peak at 285.0 eV.

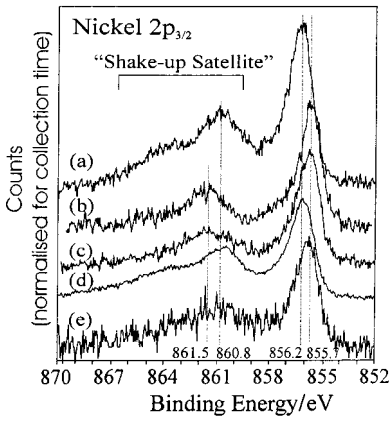

Figure 3. $Ni(2p_{3/2})$ XPS spectra showing (a) Ni doped poly(**I**), (b) Ni doped poly(**II**) and (c) Ni doped poly(**I**) modified with poly(histidine), (d) Ni doped poly(**I**) with non *his* tagged alkaline phosphatase, (e) Ni doped poly(I) with *his* tagged alkaline phosphatase (scale factor ×6). Energy referenced to the C(1s) peak at 285 eV.

To examine the effectiveness of Ni^{2+} chelation by imidazole groups, XPS spectra of nickel-doped poly(**II**) were collected. Figure 3b shows that a similar amount of Ni^{2+} has been incorporated as for poly(**I**), although there is a shift in the position of the $Ni(2p_{3/2})$ peak to a lower binding energy (855.7 eV), as would be expected for Ni^{2+} coordinated to a ligand with a less electronegative center. Significantly, the shake-up satellite for the Ni^{2+}-bound poly(**II**) has moved to a higher binding energy (+5.8 eV from the main $Ni(2p_{3/2})$ line) and has a lower intensity relative to the main $Ni(2p_{3/2})$ line. These spectroscopic features have been found to be reproducible and arise as a consequence of the change in the ligand system surrounding the Ni^{2+}.

To investigate the affinity binding of biomolecules into polymeric systems, poly(histidine) was first immobilized onto nickel-doped poly(**I**) films. The $Ni(2p_{3/2})$ spectrum obtained, Figure 3c provides a signature which is very similar to that of the nickel coordinated in the poly(**II**) system, Figure 3b. The main peak and satellite structure occur at 855.7 and 861.5 eV, respectively, confirming that the peptide is interacting with the polymer-bound nickel ions via its imidazole type ligands.

Enzyme was immobilized in the poly(**I**)−Ni^{2+} films, as would be the case at a biosensor interface, using a solution of either native bacterial alkaline phosphatase (Figure 3d) or the same enzyme but with a hexahistidine tag at the C terminus (Figure 3e). The shift in the $Ni(2p_{3/2})$ peak to a lower binding energy (855.8 eV) is similar to that found for the immobilization of poly(histidine) onto the poly(**I**) film. In addition, the satellite peak is shifted by +5.3 eV from the main $Ni(2p_{3/2})$ peak, and is closer to that found in the nickel−imidazole systems (+5.8 eV) when compared with the unmodified carboxylate poly(**I**) (+4.6 eV). Given that the enzyme (MW ≈ 94 000) is much larger than the *his* tag, (MW ≈ 823), this slight deviation would be expected since not all of the polymer-bound nickel will be capable of binding to the available tag sequences.

The similarity in peak separations and spectral structure between the poly(**II**)−Ni^{2+} and the poly(**I**)−Ni^{2+} in which poly(histidine) or the *his* tag alkaline phosphatase has been immobilized allows us to conclude that the histidine coordination is the primary binding mode (the low signal-to-noise ratio in Figure 3e is a consequence of sampling the ejected photoelectrons through an overlayer of alkaline phosphatase protein).

Throughout the collection of the XPS spectra the line shapes of the C(1s) and Ni ($2p_{3/2}$) spectra were monitored by comparison of the accumulated counts in successive periods of ca. 60 min in order to ensure that there was no degradation of the sample due to X-ray exposure. In contrast to that found for crystalline samples of lysine,[12] it was seen that significant changes in the spectra did not occur within the first three 1-h periods of X-ray exposure.

In addition, a number of controls were carried out. For example it was seen that there is essentially no change in the $Ni(2p_{3/2})$ spectrum when the nickel-doped poly(**I**) films were exposed either to non-*his*-tagged alkaline phosphatase, Figure 3d, or to polylysine. In all cases the immobilization of the peptides/enzymes onto the polymer substrates was corroborated by characteristic C(1s) and N(1s) spectral signatures corresponding to the appropriate amide and C−N binding energies.[13] In the case of the alkaline phosphatase, a weak S(2p) signal could also be observed corresponding to the enzyme's cysteine and methionine residues. By comparison of the magnitude of the C(1s) XPS peaks corresponding to amide linkages in the protein (288.15 eV) and carboxylate of the supporting polymer (289.15 eV) before and after washing the immobilized *his*-tagged protein with pH 3 HCl, it was estimated that a minimum of 50% of protein that was immobilized was bound specifically. Retention of enzymic activity after immobilization was confirmed by means of a *p*-nitrophenyl phosphate spectroscopic end point analysis measured at $\lambda = 405$ nm. The kinetic rate and affinity parameters were calculated as $K_m = 2.5$ mM, $V_{max} = 187$ μmol s^{-1} cm^{-2} at 25 °C, using a Lineweaver−Burke plot.

Finally, the extent of permeation of the nickel ions within the polymer films was also investigated by XPS, by means of adhesive tape to peel the polymer from the electrode surface, thereby revealing the polymer−electrode interface for analysis (i.e., the underside of the film). This approach was effective in demonstrating ion diffusion within the film, with the amounts of Ni^{2+} at the polymer−electrode interface nearly identical to those at the outer (top) polymer−solution interface. The result shows that the film is sufficiently porous to provide potential immobilization sites throughout the film.

In conclusion, we have demonstrated the binding of model peptides and proteins to a conducting polymer interface through the specific interaction with polymer-localized metal ions, using XPS to clarify the nature of the interactions.

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Supporting Information Available: Experimental details regarding materials used and preparation of polymer films, together with the spectroscopic analysis conditions (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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