

Fig. 1. Interaction of two histidine residues with  $\text{Ni}^{2+}$ -NTA.

introducing a short stretch of five or six histidines at defined positions in the protein, creating so-called His-tagged fusion proteins [9]. The His-tag has enough flexibility to bind to both unoccupied sites of the  $\text{Me}^{2+}$ -NTA complex synergistically. The 2:1 histidine: $\text{Me}^{2+}$ -NTA stoichiometry creates a stable immobilization so that the protein can be affinity purified. On the other hand, the stability of a 1:1 histidine: $\text{Me}^{2+}$ -NTA complex is too small for stable complex formation. Therefore, nonspecific binding of proteins without a His-tag is normally not observed. In addition, adsorbed proteins can be easily desorbed by the addition of a suitable chelate such as ethylene diamine tetraacetic acid (EDTA) or a competitor such as imidazole, or by acidification.

$\text{Ni}^{2+}$ -NTA has been used as the basis of protein deposition at gold and graphite surfaces using self-assembled monolayers for applications in both optical and electrochemical immunosensors [10, 11]. Some researchers have tried to directly exploit the targeted interaction of amino acids with conducting polymers such as polyaniline. Both cysteine [12] and histidine [13] residues have been the target as it has been suggested that these amino acids can attack the polyaniline backbone through nucleophilic action [14]. However, it is not clear how much improvement is gained over non-targeted interactions with the polymer using these methods.

Recently, Haddour et al. [15] published a method describing the covalent modification of pyrrole with an NTA ligand and its subsequent incorporation into an electropolymerized film, with copper chelation and attachment of histidine-tagged glucose oxidase. The approach taken in the present work was to investigate the possibility of doping a growing electropolymerized polyaniline/PVS film with NTA to demonstrate whether the NTA would become effectively incorporated into the film, demonstrate whether this film was capable of coordination of  $\text{Ni}^{2+}$  ions and establish if this process would impact negatively on the electrochemical behavior of the conducting polymer film. Immobilization in this way would have several advantages over other techniques. The proteins would be orientated at the electrode surface, potentially allowing full activity of the entire protein population. The proteins would also remain in their native state and not be damaged by covalent interactions. The surface of the electrode could potentially be efficiently regenerated and, it is hoped, that this method would yield greater levels of reproducibility over other immobilization techniques previously applied.

If incorporation of NTA into the PANI/PVS polymer was to be successful, it was essential to ensure that it did not degrade the electrochemical properties of the polymer. To this end, 1 ml of 20% (w/v) NTA was mixed with the aniline/PVS polymerization solution (2% w/v). The numbers of voltammetric cycles performed in PANI/PVS and NTA-PANI/PVS was varied (Fig. 2). It was seen that when polymerization was performed solely in NTA-PANI/PVS (14 cycles), the anodic and cathodic peak currents typical of polyaniline were significantly reduced in magnitude, illustrating a deleterious effect on the growth of the polymer. However, when ten cycles were performed in PANI/PVS and four in NTA-PANI/PVS, the voltammogram was

comparable with 14 cycles in PANI/PVS alone. Thus, by only polymerizing NTA into the upper layers of the polymer, no significant reduction in the redox properties of the polymer was introduced.

Following this, the quantity of NTA used for polymerization was also optimized (Fig. 3). Concentrations of NTA up to 0.6% (w/v) had a positive effect on the anodic peak potential at approx. 300 mV (the oxidation peak corresponding to the conversion of emeraldine to the leucoemeraldine cation radical). Above this concentration, however, peak potentials diminished significantly. Once again, this appears to indicate that high NTA levels interfered with the polymerization of the aniline. Therefore, 0.6% (w/v) NTA-PANI/PVS for four voltammetric cycles following application of ten cycles in PANI/PVS was considered optimal to maximize the level of NTA in the polymer film whilst also maintaining the electrochemical properties of the polymer.

Haddour et al. [15] used a covalent synthetic approach to introduce NTA moieties into the pyrrole monomer. Subsequent cyclic voltammetric studies found a film with good conductive and electrochemical properties. However, the oxidation potential of the pyrrole monomer was increased by at least 100 mV (vs.  $\text{Ag}/\text{Ag}^+$ ), presumably as a consequence of the covalent modification. No negative impact on the electrochemical properties of the polyaniline/PVS film was seen as a consequence of the noncovalent doping of the NTA during electropolymerization. On the contrary, when NTA was introduced into the upper layers of the polymer film, the layer showed enhanced electrochemical properties with higher peak potentials for an equivalent number of voltammetric cycles.

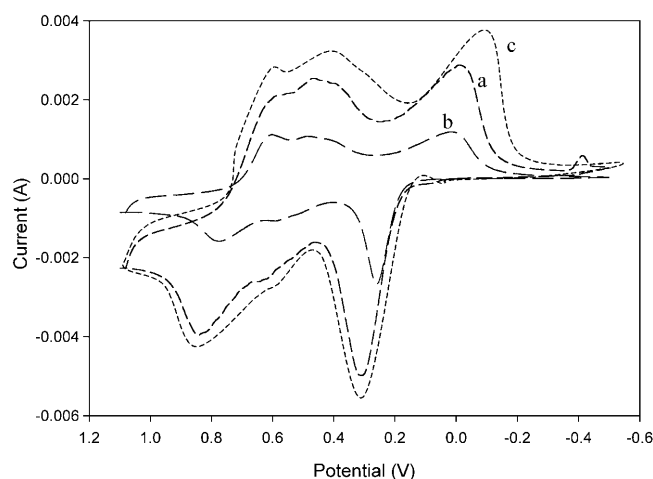


Fig. 2. Polymerization of a) aniline/PVS for 14 voltammetric cycles, b) aniline/PVS with 2% (w/v) NTA for 14 voltammetric cycles and c) aniline/PVS for 10 cycles followed by aniline/PVS with 2% (w/v) NTA for 4 cycles. All cyclic voltammetry was performed in 1 M HCl. Cycling was between  $-500$  and  $1100$  mV (vs.  $\text{Ag}/\text{AgCl}$ ) at  $100$  mV/s. Complete polymerization of the monomer in the presence of NTA resulted in the significantly reduced growth of the polymer. Incorporation of the NTA for the final 4 cycles, however, resulted in comparable polymer films than those produced in the absence of NTA.

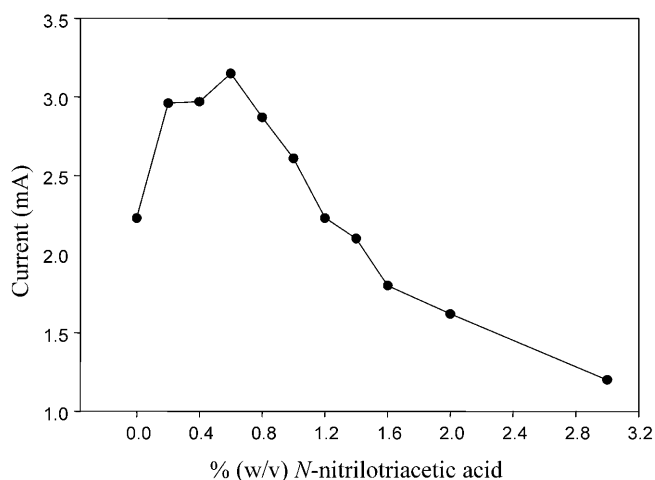


Fig. 3. Effect of NTA concentration on the anodic peak current of the emeraldine/leucoemeraldine cation radical transition. Anodic current peaked at 0.6% (w/v) NTA and decreased above this value.

EDX analysis was performed on electrodes that had been modified with and without NTA and had been incubated in 0.5 M  $\text{NiSO}_4$  solution for 30 min (Fig. 4). In the absence of NTA, only trace quantities of nickel could be detected. However, NTA-modified electrodes showed distinct quantities of nickel at the electrode surface at 7.4 and 8.3 keV, accounting for approximately 10% of the electrode surface coverage. The EDX also showed qualitative changes in the electrode surface, with reductions in chloride content.

The incubation period for nickel deposition was investigated further and analyzed for samples up to 2 h (Fig. 5). It was clear that at 15 min, only 2% of the electrode surface was occupied by nickel. However, this had risen to 10% after 30 min and stabilized at 11% at 1 and 2 h. Thus, it was reasonable to assume that, under the conditions employed, 30 min incubation was adequate to bring about complete binding of the available NTA sites with nickel and this format was used for experiments featuring protein immobilization.

Haddour et al. [15] clearly demonstrated that  $\text{Cu}^{2+}$  ions could become chelated throughout the polymer film. However, it is not clear how accessible these coordination sites would be to a large macromolecule such as a histidine-tagged protein. The performance of these films may be further enhanced by a two stage polymerization with only the NTA-modified component being introduced at the latter stages of the electrochemical synthesis to confine NTA to the upper layers of the polymer and minimize any impact it may have on the polymer properties.

As has been stated, the system of metal chelate affinity chromatography has been found to be an excellent method for the purification of recombinant proteins and many have started to consider this technique to enhance protein immobilization on electrode surfaces, conferring improved orientation, ease of immobilization and improved regeneration. An NTA-based conducting polymer electrode platform could incorporate these distinctive benefits. One

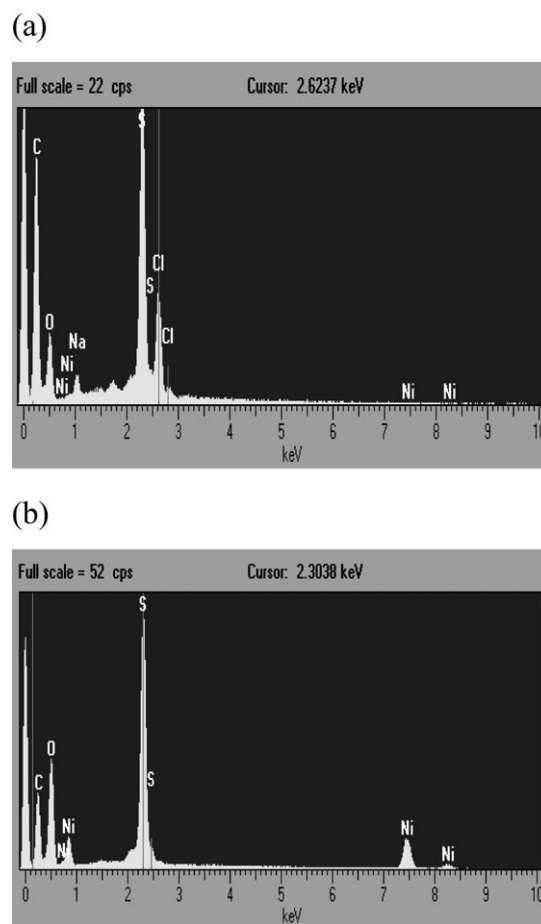


Fig. 4. Energy dispersive X-ray analysis of a) PANI/PVS and b) NTA-PANI/PVS electrodes following incubation for 30 min in 0.5 M  $\text{NiSO}_4$ . Only trace quantities of  $\text{Ni}^{2+}$  could be detected on the PANI/PVS electrode, whereas 10%  $\text{Ni}^{2+}$  coverage was observed in the presence of 0.6% (w/v) NTA.

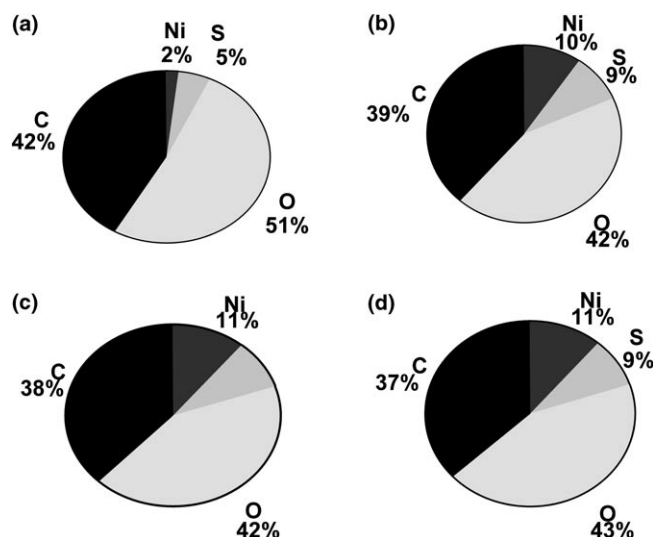


Fig. 5. The effect of incubation time of NTA-PANI/PVS electrodes in 0.5 M  $\text{NiSO}_4$  on the elemental composition as derived by energy dispersive X-ray analysis. The pie charts show that at 15 min (a) only 2%  $\text{Ni}^{2+}$  was present on the electrode. This increased to 10% at 30 min (b), and 11% at 1 h and 2 h (c and d).

significant issue, however, is the requirement for electropolymerization of the polymer film. This is a time-consuming process that can fabricate only small numbers of electrodes. Although this methodology may be applicable to laboratory applications, it would be unsuitable for any mass-production process. In this case, incorporation into alternative strategies such as chemically polymerized films [17] or nanoparticle-based materials [18] might prove a more suitable alternative.

Modification of the conducting polymer PANI/PVS with the metal chelate NTA resulted in a sensor platform with comparable electrochemical characteristics as the unmodified form. The electrode was capable of complexing nickel ions with no reduction in the performance of the polymer film. This platform could be used for further evaluation as an immobilization platform for his-tagged proteins and antibodies.

## Experimental

Aniline (13,293-4) (vacuum distilled and stored frozen under nitrogen), polyvinylsulfonate (PVS, 27,842-4), and nitrilotriacetic acid disodium salt (NTA), were all purchased from Sigma-Aldrich (Poole, Dorset, UK). Ethylenediamine tetraacetic acid disodium salt (EDTA) was purchased from Riedel de Haen (Seelze-Hannover, Germany). Carbon ink (C10903D14) was purchased from Gwent Electronic Materials (Gwent, UK). Silver (Electrode PF-410) and insulation (Electrode 452 SS BLUE) inks were purchased from Acheson (Plymouth, UK). Poly(ethylene) terephthalate substrates (175  $\mu\text{m}$ ) were obtained from HiFi Films Ltd (Dublin, Ireland). All water was ultrapure standard (18 M $\Omega$ /cm).

All electrochemical measurements were carried out either in 1 M HCl or phosphate buffered saline (PBS, 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, 0.1 M KH<sub>2</sub>PO<sub>4</sub>, 0.137 M NaCl, 2.7 mM KCl, pH 6.8).

All electrochemical protocols were performed on a CH Instruments CH1000 potentiostat with CH1000 software (JJ Cambria, Wales), using either cyclic voltammetry or time-based amperometric modes either in a traditional batch cell or a thin-layer flow cell. The thin layer electrochemical flow cell was constructed as previously described [2]. The thin layer cell possessed a platinum wire counter and Ag/AgCl pseudoreference electrode. The cell was 175  $\mu\text{m}$  in depth with an area of 27  $\mu\text{L}$ .

Energy dispersive X-ray (EDX) analysis was carried out with an Oxford Instruments EDX system (Oxford Instruments Analytical Ltd., Bucks., England) with LINK ISIS software and SiLi detector.

Screen-printed electrodes (total size 21 mm  $\times$  14 mm, electrode area 7.07 mm<sup>2</sup>) were fabricated on a DEK 248 (DEK, Weymouth, Dorset, England) screen printing machine as previously described [18]. Each electrode was cleaned by performing a single cyclic voltammetric cycle in 0.2 M H<sub>2</sub>SO<sub>4</sub> between  $-1200$  and  $1500$  mV (vs. Ag/AgCl) at 100 mV/s.

For the polymerization of aniline/polyvinylsulfonate, a mixture of 7.8 mL 1 M HCl, 186  $\mu\text{L}$  aniline and 2 mL PVS was stirred and degassed under nitrogen for 10 min and maintained under a stream of nitrogen. Aniline was polymerized onto the surface of the screen-printed electrodes using cyclic voltammetry. A platinum mesh auxiliary and a Ag/AgCl reference electrode were used. A number of voltammetric cycles were performed between  $-500$  and  $1100$  mV (vs. Ag/AgCl) at 100 mV/s.

For addition of *N*-nitrilotriacetic acid to the PANI/PVS electrode, additional solutions, similar to those described above were prepared with the addition of 20% (w/v) NTA solution. The volumes of 20% (w/v) NTA added to the polymerization solution were between 0 and 1.5 mL (0 to 3% w/v). The polymer-deposited electrodes were then subjected to further rounds of polymerization under the same conditions as already described in Section 2.6.2.

For chelation of Ni<sup>2+</sup> on to the NTA-PANI/PVS electrode, the working electrodes were then washed in ultrapure water and immersed in a solution of 0.5 M NiSO<sub>4</sub>  $\cdot$  6 H<sub>2</sub>O. The electrodes were again washed with ultrapure water. Electrodes were either examined by EDX analysis or used for protein immobilization.

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