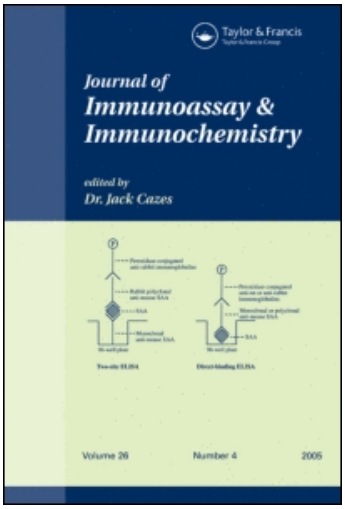

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Investigation of electroplated conducting polymers as antibody receptors in immuno-sensors

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Antibodies raised against the conducting polymer, carbazole as a hapten, react to modulate the polymer's electrochemistry. Using cyclic voltammetry the reaction of the antiserum was discovered to influence the polymer matrix's electrochemistry by an amperometric response. It is suggested that these observation form the basis of a direct sensor for immunoassay.
(**KEY WORDS**: Immunoassay, electrochemistry, conducting polymer, amperometric, sensor, carbazole)

INTRODUCTION

The effective combination of immunochemistry and electrochemistry in an analytical device could provide the basis of direct electrical detection of a wide range of analytes with great sensitivity and specificity. There have been a variety

of attempts to achieve a true immuno-electrode in which binding events are recorded by changes in surface electrochemistry. Solsky and Rechnitz[1-3] developed an antibody responsive membrane electrode, using ionophore-hapten conjugate in a polyvinyl chloride membrane to detect antibody for the hapten. The antibody-responsive membrane was part of a specific test and was dependent on the hapten being connected to a crown-ether, which gives the electrical response when a binding event occurs. The mode of reaction to produce the electrode response was unknown; furthermore the electrode was restricted to the hapten and was therefore not a broadly applicable direct detection system. In the past other electrochemical immuno-assays have relied on complex indirect enzyme methods, where the resultant product of the enzyme immuno-reaction can be measured[4]. Enzymes do have the advantage of increasing the signal measured but they can also have problems with non specific noise and instability.

The prospect that an antibody binding event at an electrode might produce an electrochemical change is one central question which to date has not been addressed. The research, presented in this communication, concentrates on the use of carbazole cross-linked films[5-7] using β-cyclodextrin as a backbone[8]. Carbazole is a heteroaromatic molecule, which is semi-conducting in nature. From the literature on the structure of antigenic epitopes[9] it might be gathered

that a molecule such as carbazole could have a size and shape, which may be immunogenic as a hapten when presented on a carrier protein. Antibodies specific for this hapten, carbazole, have been demonstrated by cyclic voltammetry to have an effect on its electrochemistry[8]. Direct amperometric effects were observed which depend on the concentration of antibody. This observation in which the electrode system would be generally exploitable, opens up considerable prospects for electrochemical configurations of immunoassay.

MATERIALS AND METHODS

Production of antibodies to carbazole.

Polyclonal antibodies against carbazole were made in a rabbit. A preimmunization bleed sample was taken to establish background immunity. The rabbit was injected with carbazole Plant Peptide Derivative (PPD) conjugate with Freund's complete adjuvant at four sites and after one week a post immunization bleed was taken. After one month a second immunization was made with Freund's incomplete adjuvant with the carbazole PPD sample and again injected at four sites. A second post immunization bleed was taken after a one week after the second immunization. Immunization sites were either side of the spine two front two back. Bleeds were taken from the marginal ear vein. Blood was allowed to clot and serum tested for antibody activity by ELISA.

ELISA using BSA N(6-hexanoic)carbazole.

An ELISA (Enzyme Linked Immunosorbent Assay) determined the antibody response for a rabbit to (PPD) N-hexyl carbazole conjugate. Microtitre plates were sensitized with the BSA N(6-hexanoic)carbazole (10μg/ml) made up in sodium carbonate buffer (0.1M, pH 9.6) in PBST (phosphate saline buffer 0.15% tween 20 (Sigma) (200 μl to each well)). This was sensitised at 37 ^{0}C for one hour.

The plate was emptied and washed three times with PBST, removing the PBST at the end of each wash. The plates were blocked with Marvel (1% solution in PBST) (200 μl to each well) and incubated (37^{0}C for one hour). The plates were emptied and washed in PBST three times as before. The serum was added at a range of dilutions to the plates (150μl) and again left to incubate (37^{0}C for one hour). The plates were washed in PBST as before and a 1/1000 dilution of goat anti-rabbit alkaline phosphatase as conjugate (Zymed laboratories inc) in PBST was added to each well (150 μl). The plates were incubated at 37 ^{0}C for one hour and the plates washed in PBST as before.

The enzyme substrate was a solution of paranitrophenolphosphate (Sigma Diagnostics) (one tablet) was made in a buffer solution (5 ml) (diethylamine, pH

9.8, magnesium chloride 50:1). This substrate solution (150 µl) was added to each assay well and the colour allowed to develop over one hour at room temperature. The results were read on a Dynatech model MR7000 plate reader W/L MODE :dual, Test filter : 405 nm, Ref.filter 570 nm.

General cyclic voltammetry experimental.

Cyclic voltammetry was performed on a EG&G model 273A Princeton Applied Research Potentiostat/Galvanostat. (Using Echem and Lotus 1 2 3 to process the data).

Polymers were cast from a monomer stock solution of hexakis[(6)-(2)-(3)-(carbazol-9-yl)hexyl]β-cyclodextrin in dichloromethane (0.1 M tetrabutlyammonium hexaflourophosphate as the electrolyte) onto gold pin electrodes (0.5 mm diameter by 0.5 cm high) using a three electrode arrangement. The polymer electrode was then transferred to a clean electrolyte solution in dichloromethane and scans were repeated until a stable reproducible scan was obtained. The polymer was now stable and was implemented in analysis.

EIA assays for the binding of antibody to electrodes.

In order to demonstrate the antibody binding to the carbazole polymer, electrodes

were cast in dichloromethane, and allowed to dry for ten minutes and dipped into anti-carbazole serum at varying dilutions of serum in PBST (250 μl) and left to incubated for one hour at 37°C. After this time the electrodes were washed in PBST three times and dried on a tissue. The electrodes were dipped into goat anti-rabbit alkaline phosphotase conjugate (1/1000, 250 μl) and incubated for one hour (37°C). The electrodes after this time were washed in PBST as before and dipped into the para nitrophenolphosphate substrate solution (250 μl) as described for the ELISA, and the colour read after one hour.

Electrochemical studies of the effect of anti-carbazole antibody by cyclic voltammetry.

Electrodes were cast in dichloromethane. A final cyclic voltammogram (CV) was made and recorded. These electrodes were air dried for ten minutes. The anti-carbazole serum was diluted to various concentrations to which an electrode was added to each solution (250 μl) for ten minutes. The electrodes were removed and washed in PBST and dried with a tissue. A second cyclic voltammogram was undertaken in dichloromethane which was compared to the original and differences noted.

Molecular modelling of carbazole dimer's electron charge distributions map.

Models were made in Cerius2 (version 3.8 MSI software) on a Silicon

Graphics computer. The model was cleaned and energies minimized before calculating the charge distribution under MOPAC6.

RESULTS

Preliminary investigations of immunization in a rabbit using a PPD N-hexyl carbazole conjugate, demonstrated a good immune response to carbazole (Figure 1) as evidenced by ELISA data. In these ELISA studies the carbazole was conjugated to Bovine Serum Albumin (BSA) in order to provide an alternative carrier totally unrelated to the original immunogen. The pre-immunization serum from the rabbit showed no evidence of non-specific binding of the carbazole hapten BSA N-hexyl carbazole conjugate. One month after immunization a first bleed was taken which showed a serum antibody response to carbazole. A second immunization was given, the second bleed serum showed a further increased antibody response and this serum was used for the electrochemical studies described below. The ELISA values for antiserum dilutions are shown in Figure 1.

It might be expected that these antibodies would bind the carbazole, which is in its monomeric form because that is the nature of the hapten, where the spacer was of a six-carbon length (hexyl) pendant chain. However carbazole forms only dimers[5,6,7] when electroplated at an electrode surface and the question had to be

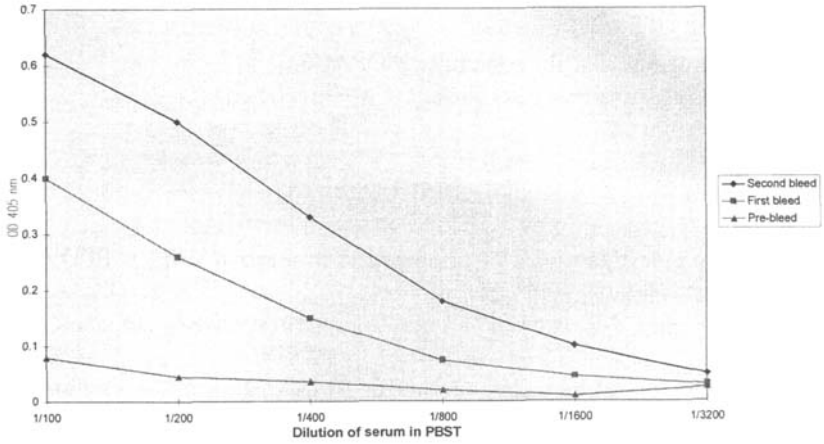

Figure 1. Antibody binding data for rabbit anti-carbazole serum raised by immunization with PPD N-(6 hexanoic) carbazole determined in ELISA using the solid phase antigen BSA N-(6 hexanoic) carbazole.

addressed whether the antibody would react with the dimeric carbazole unit of the polymer structure on the electrode.

In order to test for reactions against the polymer (poly-(hexakis[(6)-(2)-(3)-(carbazol-9-yl)hexyl]β-cyclodextrin)), an EIA (enzyme immuno-assay) format was used in which the polymer film was electrically cast on gold pin electrode (0.5 mm diameter by 0.5 cm high) as a solid phase. The reaction of the rabbit anti-carbazole antibody with the electroplated polymer was followed conventionally by a goat anti-rabbit alkaline phosphatase conjugate. The specific

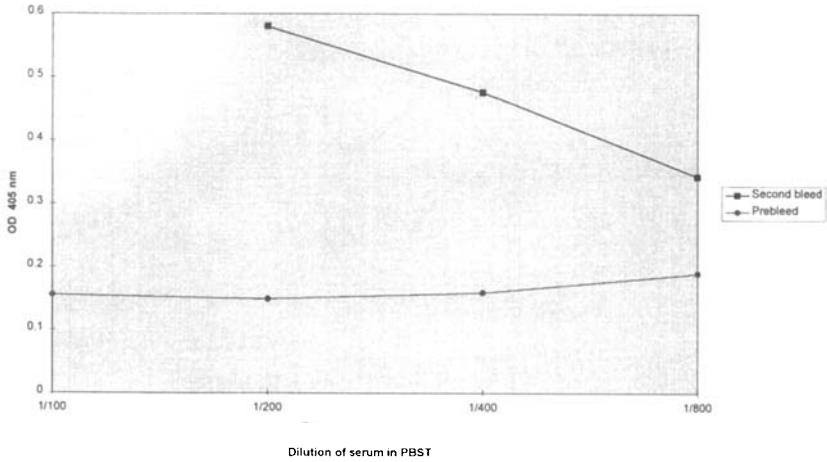

Figure 2. Determination of the binding of anti-carbazole antibody to the dimer unit of hexakis[6-(2)-(3)-(carbazol-9-yl)hexyl] β-cyclodextrin electroplated on gold pin electrodes. Antibody detection is undertaken with an alkaline phosphatase conjugate of a goat anti- rabbit IgG reagent.

antibody raised to the monomer carbazole fully recognised the carbazole unit in its dimeric form electroplated on gold electrodes (Figure 2).

Following these findings electrochemical measurements were made using cyclic voltammetry on the poly-(hexakis[(6)-(2)-(3)-(carbazol-9-yl)hexyl]β-cyclodextrin). The anti-carbazole antiserum effect changed the baseline peak height and shape. In the oxidation phase, it was noted that the second oxidation peak generally registered a higher amperometric reading than the baseline scan (Figure 3). In addition, a shoulder to the second oxidation peak could be clearly

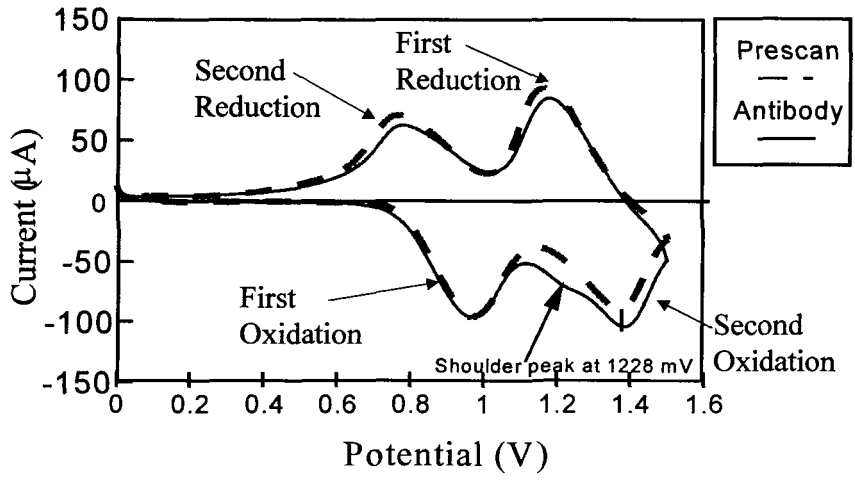

Figure 3. The effect the rabbit anti-carbazole serum on the cyclic voltammogram (scan rate 100 mV/sec) of hexakis[6-(2)-(3)-(carbazol-9-yl)hexyl] β-cyclodextrin polymer on a gold electrode.

seen and continuously appeared at approximately 1228 mV. Prebleed non-immune serum manifested none of these effects.

Tests were conducted with dilutions of anti-carbazole serum in PBS (pH 7.2) on separate coated electrodes to produce individual cyclic voltammetric scans for different antibody concentrations. The result obtained from measuring the cyclic voltammograms shown in Figure 4, suggests a relationship between antiserum concentration and change in peak height for the second oxidation, and first reduction peaks thereby demonstrating a direct immuno-electrochemical response

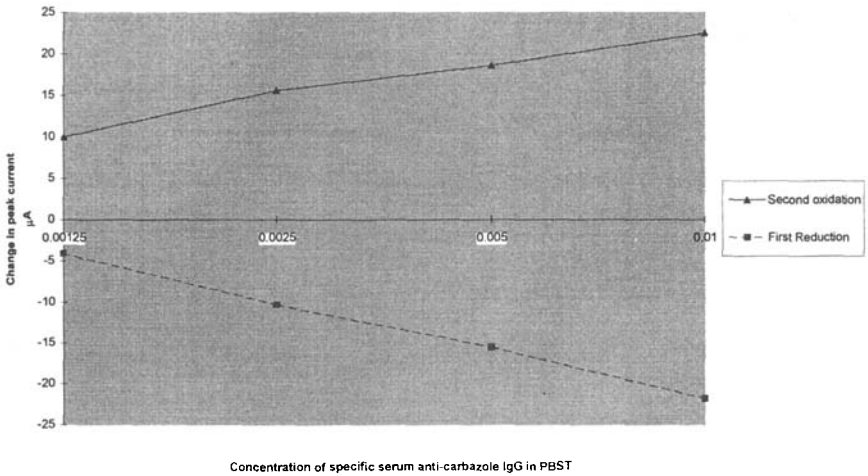

Figure 4. The effect of rabbit anti-carbazole antiserum on electrodes electroplated with hexakis[6-(2)-(3)-(carbazol-9-yl)hexyl] β-cyclodextrin peak current under cyclic voltammetry (PBS) on separate electrodes.

which is dependant on antibody concentration. No such relationship was seen in the other two peaks.

DISCUSSION

The basis for this electrical phenomenon may be explained by understanding how an antibody binds to its hapten via the Fv (the variable region) within the antibody cleft. The specific binding of a ligand by an antibody molecule tends to be relatively hydrophobic. So it can be gathered that a sparingly water-soluble molecule, such as carbazole should tend to form very stable complexes with its antibody. From the known distribution of electron density across the carbazole

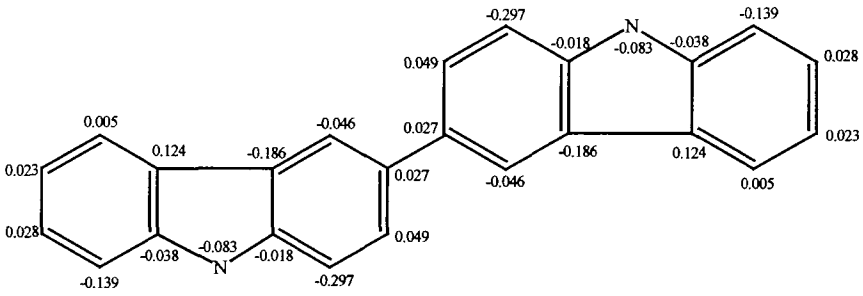

Figure 5. Charge distribution map of the carbazole dimer obtained from the molecular model using MINDO under Cerius 3.8 (MSI software).

molecule (Figure 5), there is a rich potential for establishing noncovalent bonds in the binding cleft of the antibody. It is probable that hydrogen bonding would involve the lone pair of the hetero-atom and that other intermolecular forces, which often involve aromatic groups within the antibody cleft, would be established at sites of differing electron density across the ligand.

Such interactions would be expected to disturb the delocalization of electrons within the ring system and would be reflected by changes in electrochemical redox properties. In addition, it could be expected that the binding of an antibody would produce effects on the flow of counterions into the space around the electroactive carbazole head. Since the incorporation of counterions is necessary for the electrochemical oxidation of every carbazole, then it is to be expected that a binding antibody will interfere by virtue of steric hindrance and charge effects.

The participation of monospecific antibody in modifying the electrochemistry of the electrode, by specifically binding the electrochemical polymer does not in itself provide a signal which is exploitable for a wide range of analytes. However, new prospects have opened with current activities in molecular biology with a capability of producing 'bispecific antibodies'[10]. It is proposed that the present findings provide the basis of an electrochemical signal with one functionality i.e. anti-carbazole. The second functionality of the bispecific antibody is reserved for any analyte in an immunochemical assay. Thus, it is suggested that the observations in this communication can be adapted to immunochemical formats where the immunochemical reaction can occur on a separate solid phase in the form of a competition assay. When a specific antigen is present it will remove the double-headed antibody off the solid phase. This enables free antigen double head to bind to the detection surface where it can be recorded by changes in surface electrochemistry of an electrode system. This will make an assay device that will not require physical separation (i.e. one step) and which would be generic for any analyte. Work is in progress to determine the strength of the amperometric signal in relation to the concentration of specific antibody.

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