Analytical Methods

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Journal:	*Analytical Methods*
Manuscript ID	AY-ART-06-2021-000965.R1
Article Type:	Paper
Date Submitted by the Author:	26-Sep-2021
Complete List of Authors:	McCord, Cynthia; Colorado State University, Chemistry Ozer, Tugba; Yildiz Technical University, Bioengineering Henry, Charles; Colorado State University, Chemistry;

ARTICLE

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Synthesis and Grafting of Diazonium Tosylates for Thermoplastic Electrode Immunosensors

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For electrochemical immunosensors, inexpensive electrodes with fast redox kinetics, and simple stable methods of electrode functionalization are vital. However, many inexpensive and easy to fabricate electrodes suffer from poor redox kinetics, and functionalization can often be difficult and/or unstable. Diazonium tosylates are particularly stable soluble salts that can be useful for electrode functionalization. Recently developed thermoplastic electrodes (TPEs) are inexpensive, moldable, and highly electroactive carbon composite materials. Herein, synthesis and grafting of diazonium tosylate salts were optimized for modification of TPEs and used to develop the first TPE immunosensors. With diazonium tosylates, TPEs were amine functionalized either directly through grafting of p-aminophenyl diazonium salt or indirectly through grafting p-nitrophenyl diazonium salt followed by electrochemical reduction to an amine. Diazonium tosylates were synthesized in situ as a paste in 6 min. Once the reaction paste was spread over the electrodes, near monolayer coverage (1.0 ± 0.2 nmol/cm^2) was achieved for p-nitrophenyl diazonium salt within 5 min. Amine functionalized electrodes were conjugated to C-reactive protein (CRP) antibodies. Antibody-modified TPEs were applied for the sensitive detection of CRP, a biomarker of cardiovascular disease using electrochemical enzyme-linked immunosorbent assays (ELISA). LODs were determined to be 2 ng/mL in buffer, with high selectivity against interfering species for both functionalization methods. The direct p-aminophenyl modification method had the highest sensitivity to CRP and was further tested in spiked serum with an LOD of 10 ng/mL. This low-cost and robust TPE immunosensor platform can be easily adapted for other analytes and multiplexed detection.

Introduction

Electrochemical immunoassays are attractive for bioanalysis due to their fast measurement times (s to min), as well as their excellent sensitivity and selectivity.[1-3] Carbon-based electrodes are often used for immunosensors, as they exhibit wide potential windows compared to metal electrodes, superior biocompatibility, and high chemical stability.[4, 5] Composite carbon electrodes are inexpensive and easy to fabricate and therefore often used in biosensors. Unfortunately, carbon composite electrodes often suffer from slow electrode kinetics and moulding or embossing of these materials is limited. Recently, our group reported thermoplastic electrodes (TPEs) as an alternative to traditional carbon composite electrodes. TPEs are fabricated via a simple method where a dissolved thermoplastic binder is mixed with conductive carbon filler, the material is dried and then heat pressed into a template with the desired shape.[5, 6] TPEs can be easily fabricated and moulded into complex geometries for various sensor and flow device designs.[5-9] The electrochemical

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

behaviour of these materials is similar to traditional commercial electrode materials such as platinum and glassy carbon but far more mouldable and inexpensive, making them excellent materials for electrochemical immunosensors.[5, 6]

Often, the first key step in making an electrochemical immunosensor is modifying the electrode surface. For electrochemical immunoassays, sufficient antibody coverage on the electrode surface is crucial for sensitive detection of analyte of interest.[10, 11] To achieve this, antibodies are adsorbed or coupled to the electrode surface. Adsorption is a simple process; however, coverage can lack the reproducibility, stability, sensitivity, and specificity of coupled antibodies.[10, 12, 13] To couple antibodies to electrodes, electrodes are modified with functional groups including amines, alkynes, and carboxylic acids.[10, 11] Non-covalent affinity-based methods such as pyrene π-π stacking[14-16] and gold-thiol affinity attachment[17-19] are often used, but these methods have their own stability issues, can require lengthy incubations, and often organic solvents are used for modifier dissolution.[20, 21] Many organic solvents are incompatible with common composite binders. Another common functionalization technique is the covalent grafting of diazonium salts.[8] This type of modification can be simple, rapid, versatile, and robust.[12, 22, 23] However, diazonium salts are extremely reactive, thermally unstable, and potentially explosive[24, 25] so synthesis is generally performed around 0°C followed by in situ modification[13, 26-29] or modification is performed in organic solvent with the more stable tetrafluoroborate salts.[8, 30-32]

Diazonium tosylate salts have advantages over other diazonium salts but have not been as widely used or studied. The solubility of tosylate salts is higher than that of the more commonly used tetrafluoroborates or hexafluorophosphates.[33] Synthesis and modification can be performed solvent-free in a water re-action paste.[33-36] Tosylate salts have also proven to be extremely stable, they can be kept dry or in a paste form at room temperature and retain reactivity for months.[33] The source of this stability is hypothesized to be electrostatic interactions causing particularly close contact between the diazonium cation and three tosylate anions as seen with x-ray crystallography.[33] The first paper using diazonium tosylates to modify carbon electrodes was published by Via et al. in 2018. In this report, they used a commercial glassy carbon electrode to grind a water paste consisting of aryl amines, p-toluenesulfonic acid, and sodium nitrite for one-step solvent-free synthesis and spontaneous modification.[36] In this work, we have modified their procedure to be generalizable to a wider range of electrode geometries and optimized the modification for TPEs.

To demonstrate applicability of diazonium tosylate modification for TPE immunoassays, the modification was used for the detection of C-reactive protein (CRP). This biomarker can be monitored in blood serum or saliva and is useful for diagnosis of cardiovascular disease and systemic inflammation.[37, 38] Unlike electrochemical assays, common commercial CRP assays, such as immuno-turbidimetric or immuno-nephelometric methods, can be lengthy and require heavy laboratory equipment.[38] Herein, TPEs were first amine functionalized through two different methods: synthesis of p-nitrophenyl diazonium salt followed by the electrochemical reduction of grafted nitro groups to amines, and direct synthesis of p-aminophenyl diazonium salt. These two diazonium salt modifications were compared in terms of electrode coverage to increase anti-CRP attachment on TPEs for further development of the biosensors. TPE electrode array devices were developed with small-volume electrochemical cells for replicate immunoassay measurements. Here, detection of CRP was performed in a sandwich ELISA format and CRP was quantified through alkaline phosphatase enzymatic turnover of p-aminophenyl phosphate to p-aminophenol. To the best our knowledge, this is the first report directly synthesizing p-aminophenyl diazonium as a tosylate salt and using TPEs as immunosensor platforms.

Experimental

Reagents

BupH™ Phosphate Buffered Saline Packs (PBS), SuperBlock™ (PBS) Blocking Buffer, sulfo-NHS-biotin, sodium nitrite (99.55%), sulfuric acid (H_2SO_4), isopropanol (IPA, HPLC grade), methylene chloride (DCM, Fisher Scientific), and magnesium chloride ($MgCl_2$) were sourced from Thermo Fisher Scientific. p-Toluenesulfonic acid (≥98%), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), potassium ferrocyanide (≥98.5%), potassium ferricyanide (99%), sodium chloride (NaCl, ≥99.5%), and myoglobin from horse heart were sourced from Sigma-Aldrich. P-nitroaniline (99.9%) was sourced from CHEM-IMPEX INT'L INC., and p-phenylenediamine (99%) from Frontier Scientific. Sodium hydroxide (NaOH, 1N), and TRIS-HCl were sourced from J.T. Baker. Bovine serum albumin (BSA, Microbiological grade) was sourced from MP Biomedicals, streptavidin-ALP from MABTECH, and p-aminophenol phosphate (p-APP) from BIOSYNTH. N-hydroxysuccinimide (NHS, ≥98.5%) was sourced from Fluka, and 2-ethanesulfonic acid (MES, 99+%) from Acros Organics. Anti-CRP Goat Polyclonal Antibody and Anti-CRP Mouse Monoclonal Antibody C7 (Biotin) were sourced from Abnova. Human CRP protein (>95% pure) was sourced from Fitzgerald. The fetal bovine serum (FBS, 100%) was sourced from Atlas biological. The buffer concentrations used were 0.1 M sodium phosphate 0.15 M NaCl (pH 7.4) for PBS, 0.1 M MES (pH 6), and 0.1 M tris-HCl 0.1 M NaCl 5 mM $MgCl_2$ (pH 9) for tris-HCl.

Electrochemical Measurements

Electrochemical measurements and pre-treatments were performed with a CHI 660b potentiostat. Cyclic voltammetry (CV) measurements of the modified TPEs were conducted between -0.8 and 0.8 V vs. SCE with a scan rate of 100 mV/s. Nitrophenyl coverage resulting from nitrophenyl diazonium salt modification was calculated as previously described in the literature.[36] For 2.5 mm disk electrodes, experiments were performed in glass vials with a separate 4 mm CE. For TPE array experiments, solutions were pipetted onto the array surface and CEs that were built into the arrays were used. For CV measurements and amperometry electrode treatments, a saturated calomel (SCE) RE was used. The SCE and potentiostat were both sourced from CH Instruments Inc. A homemade Ag/AgCl electrode was used for SWV measurements, and

measurements were performed in 20 µL droplets. Square wave voltammetry measurements were taken from -0.2 V to 0.3 V vs. Ag/AgCl with a 4 mV increment voltage, 25 mV amplitude, and 15 Hz frequency for blocking optimization experiments. SWV parameters such as frequency were optimized and further measurements, were taken from -0.15 to 0.25 V vs. Ag/AgCl with a 2 mV increment voltage, 50 mV amplitude, and 10 Hz frequency. Replicate electrode measurements are shown herein as averaged values with error bars representing the standard deviations.

Electrode Fabrication

For the working (WE) and counter electrodes (CE), Thermomorph™ was used as an inexpensive source of polycaprolactone (PCL). Optix poly(methyl methacrylate) (PMMA) was sourced from Plaskolite, and used in the binder and for the electrode template material. PMMA templates were cut with an Epilog CO_2 laser cutter and designed with the graphic design program CorelDRAW. High Purity Micronized Graphite (16 µm, 99.5-99.99%, Great Lakes Graphite) was used as the conductive carbon source. Thermoplastic electrodes were fabricated by dissolving 1.5 g of plastic (2:1 PCL:PMMA) in ~15 mL of DCM. 3 g of graphite were added to the dissolved plastic, and the material was mixed while drying on a silicon wafer plate. The dried material was removed from the plate with a razor blade and milled in a coffee bean grinder before use. Electrodes were heat pressed into templates at 80–90 °C using a hydraulic heated press (Carver, Inc.) at a pressure of 300-500 psi. Excess electrode material was scraped off and connections were made with silver paint, copper wires, and epoxy purchased from a local store. Initial modification studies were performed on 2.5 mm diameter disk electrodes with separate 4 mm diameter disk CE due to the simplicity of fabricating larger electrodes. Later assays were performed on small-volume electrode arrays consisting of six 1 mm WE and 1.5 mm diameter CE pairs with laser cutter etched ovals to keep solution droplets separate. TPE array devices are shown in Fig. S.6a.

Electrode Pre-Treatment

Prior to modification, electrodes were sanded with 150 grit sandpaper for bulk removal of surface material and any prior modification, then the surfaces were smoothed out via polishing with 600 grit sandpaper. After each polish, the electrodes were sonicated for one min in Milli-Q water to remove loosely packed material. Cathodic pre-treatments were performed at -1.5 V vs. SCE for 30 seconds in 0.1 M H_2SO_4. Anodic pre-treatments were performed at +1.5 V vs. SCE for 30 seconds in 0.1 M NaOH. Electrodes were stored in Milli-Q water when not in use and allowed to air dry prior to modification.

Safety

No violent reactions were observed for these syntheses, however diazonium salts can be highly reactive. Working in small quantities and performing synthesis on smooth surfaces is recommended.[25,36] P-toluene sulfonic acid acts as a stabilizing agent for the diazonium salts so it is vital to mix in excess prior to adding the sodium nitrite reactant.[36] Toxic substances and nitrogen oxides are produced in this reaction, so PPE and working in a hood are necessary.[36]

Diazonium salt synthesis and modification

The diazonium synthesis reactions and modification are shown in Scheme 1. Reaction pastes were synthesized by grinding the aryl amine and p-toluene sulfonic acid in an open glass vial using a glass stir rod. After 1 min passed and a homogenous paste was observed, sodium nitrite was added, and the paste was mixed vigorously for another 5 min. For early optimization of the synthesis and modification, p-nitrophenyl diazonium salt was synthesized using 0.2 mmol p-nitroaniline, 0.6 mmol p-toluenesulfonic acid, 75 µL water, and 0.5 mmol sodium nitrite. For p-aminophenyl diazonium salt synthesis, p-phenylene diamine can be added in excess of sodium nitrite to produce primarily monodiazonium salts to modify electrodes with aminophenyl groups.[39] p-Aminophenyl diazonium salt was synthesized using 0.3 mmol p-phenylene diamine, 0.6 mmol p-toluenesulfonic acid, 200 µL water, and 0.2 mmol sodium nitrite. The batch quantities were tripled for TPE array modifications, thus, up to three TPE arrays could be modified using a single batch of diazonium paste. The amounts of water to add for each paste type were optimized by adding water in 25 µL aliquots until an easily spreadable paste was achieved.

Following synthesis, the diazonium salt reaction pastes were spread over the TPE surfaces and left in petri dishes containing wet paper towels to prevent the paste from drying out. After 5 min, the paste was rinsed off with 0.1 M sulfuric acid, then the electrodes were washed thoroughly with Milli-Q water. To remove unreacted paste components with poor water-solubility, the electrodes were sonicated in isopropanol, then rinsed thoroughly with Milli-Q water again.

The amounts of water to add for each paste type were determined by adding water in 25 µL aliquots until an easily spreadable paste is achieved. Electrode pre-treatment, sonication time, and paste incubation time were optimized using the p-nitrophenyl diazonium salt modification and nitrophenyl coverage was determined with CV. Coverage stability was also tested by looking at nitrophenyl coverage with CV. For the CRP ELISA experiments, aryl amines were pre-ground with a mortar and pestle to achieve a more uniform paste and more consistent voltammetry (S.1).

Blocking Optimization

Different blockers of nonspecific adsorption were incubated on the diazonium modified TPE surfaces. Blocking optimization was performed on reduced p-nitrophenyl and p-aminophenyl diazonium salt modified electrodes. Nitrophenyl surface groups were electrochemically reduced at -1V vs. SCE for 30s in 0.1 M KNO3. Diazonium modified electrodes were incubated with 1% (w/v) BSA in PBS, SuperBlock™, and 1% (w/v) BSA diluted in SuperBlock™ for one hour. 1:100 dilutions of streptavidin-ALP solutions were prepared in Tris-HCl and incubated on the electrode surfaces for 30 min. Electrodes were incubated in 4 mM p-APP substrate in tris-HCl for 20 min. Nonspecific enzymatic turnover of p-AP was measured following the 20 min incubation period with SWV. Three Tris-HCl washes were performed be-tween each step. 20 µL

aliquots were used for all modification, wash, and detection steps. All modification steps for this experiment and further assays were performed in humidified petri dishes to prevent evaporation.

Streptavidin-ALP biotin assay as proof of concept

To provide a proof-of-concept for the use of diazonium tosylate modified TPEs for immunosensors, biotin was conjugated to TPEs and used to capture streptavidin-ALP as a model system, shown in Scheme 2a. Biotin-streptavidin-ALP assays were performed on reduced p-nitrophenyl, p-aminophenyl and reduced p-aminophenyl diazonium salt modified electrodes. Nitrophenyl and aminophenyl modified electrodes were electrochemically reduced at -1V vs. SCE for 30s in 0.1 M KNO_3. Electrodes were incubated with 1 mM sulfo-NHS-biotin in PBS for one hour. 1% (w/v) BSA in Superblock was incubated on the electrodes for 1 h to block nonspecific binding. 1:100 dilutions of streptavidin-ALP solutions were prepared in Tris-HCl and incubated on the electrode surfaces for 30 min. Background signal was tested with SWV in Tris-HCl prior to 20 min incubation in 4 mM p-APP substrate in tris-HCL. Enzymatic turnover of p-AP was measured following the 20-min incubation period with SWV. Three tris-HCl washes were performed between each step. 20 µL aliquots were used for all modification, wash, and detection steps. Relative streptavidin-biotin binding was quantified by background subtracting the Tris-HCl blank measurements from the measurements taken after 20 min in p-APP and integrating the resulting p-AP peaks to determine peak area.

CRP ELISA

ELISA assays were performed on reduced nitrophenyl, and reduced p-aminophenyl diazonium salt modified electrodes as shown in Scheme 2b. Nitrophenyl and aminophenyl modified electrodes were electrochemically reduced at -1V vs. SCE for 30s in 0.1 M KNO_3. Modified TPEs were incubated with 4 mM NHS in PBS for one hour. Anti-CRP/EDC (25 µg/mL; 4 mM) solutions were prepared in MES buffer and incubated on the electrode surfaces for one hour. 1% (w/v) BSA in SuperBlock™ was incubated on the electrodes for one hour to block nonspecific binding. CRP samples (0.1, 1, 10, 100, 1000, 10000 ng/mL in PBS) and myoglobin controls (1000 ng/mL in PBS) were incubated for 30 min. Biotinylated anti-CRP (5 µg/mL in PBS) was incubated for 30 min. A 1:1000 dilution of streptavidin-alkaline phosphatase was made in Tris-HCl and incubated on the electrode surfaces for 30 min. Background signal was tested with SWV in Tris-HCl prior to 20 min incubation in 4 mM p-APP substrate in Tris-HCl. Enzymatic turnover of p-AP was measured following the 20 min incubation period with SWV. Three washes were performed between each step. For the early steps, washes were performed in PBS. Following biotinylated anti-CRP modification, washes were performed in Tris-HCl. 20 µL aliquots were used for all modification, wash, and detection steps. The setup for ELISA experiments is shown in Fig. S.6b.

Assay response was quantified by subtracting background signal of the Tris-HCl as a blank solution from the SWV signal obtained after 20 min in p-APP (an example of raw and background subtracted data is shown in Fig. S.7). The final p-AP peaks were used to determine peak area and generate CRP calibration curves for the reduced nitrophenyl and reduced aminophenyl modified electrodes shown in Fig. 4. The calibration curves in Fig. 4 were fitted with four-parameter logistic regressions using MATLAB (S.8).

Serum Samples

Electrodes were modified with anti-CRP and blocker as described in the previous section. FBS serum was used as a surrogate for human serum. The serum sample was diluted and spiked with CRP to final concentrations of 0, 1, 10, 100, and 1000 ng/mL CRP in 100-fold diluted serum. Diluted serum samples were incubated for 30 min on the modified electrode surfaces and further washes and modification steps were performed as described in the previous section.

Results and Discussion

Electrode Pre-Treatment Optimization

Electrode pre-treatment optimization included: (i) sanding alone, (ii) sanding and anodic treatment, and (iii) sanding and cathodic treatment. Following pre-treatment electrodes were modified and nitrophenyl coverage was calculated for each pre-treatment (Fig. 1a). Pre-treatment voltammetry is shown in Fig. S.2. and quantitative coverage data is presented in Fig. 1a. It was observed that anodic treatment of the electrodes resulted in inconsistent nitrophenyl coverage, high background current, and poorly resolved peaks. Sanded and cathodically treated electrodes had more consistent nitrophenyl coverage compared to oxidized electrodes. Since cathodic treatment of the electrodes exhibited higher coverage and sharper peaks than sanding pre-treatment, cathodic pre-treatment was used for further experiments.

Sonication Time

To investigate the effect of the duration of sonication on nitrophenyl coverage, the electrodes were sonicated in IPA for durations of zero to 20 min. The nitrophenyl coverage of the electrodes was calculated and presented in Fig. 1b. It was observed that nitrophenyl coverage was much higher without the sonication step compared to coverage after sonication in IPA. For the modified electrode CVs without sonication, there was a double peak for nitro reduction, indicating a large amount of adsorbed p-nitroaniline and nitrophenyl diazonium salt. After 5 min sonication in IPA, there was a large decrease in coverage to an expected level for a nitrophenyl monolayer (0.5-0.8 nmol/cm^2).[36] This suggests the removal of loosely adsorbed paste material.[36] After 10 min the nitrophenyl coverage stabilized and coverage variability between electrodes was at its lowest, so 10 min sonication in IPA was used for further experiments.

Modification Time

The impact of incubation time and humidity condition of modification was evaluated, and the data is presented in Fig. 1c. To increase the humidity, electrodes were placed in petri dish containing wet paper towels. Increasing the reaction time or

humidity did not have a statistically significant impact on the nitrophenyl coverage. However, performing the reaction in a humidified petri dish kept the paste from drying out making it easier to remove the reaction paste through rinsing following modification. Where-as the nitrophenyl coverage for 5 min incubation of reaction paste was 0.5 ± 0.2 nmol/cm^2, it was 0.6 ± 0.1 nmol/cm^2 for 15 min under humidified conditions. Since most to all the modification occurs within the first 5 min of incubation of reaction paste on the electrode surface, a 5 min modification in a humidified petri dish was used for all further experiments.

Coverage Stability

Stability tests were performed with the electrodes modified with the optimized diazonium procedure and kept in Milli-Q water in a covered beaker for up to a week. The nitrophenyl coverage was determined using CV. The obtained data for this experiment is shown in Fig. 1d. As can be seen in Fig. 1d, coverage did not decrease with time; the replicates tested the day of modification had 0.3 ± 0.1 nmol/cm^2 coverage and the replicates tested a week later had 0.4 ± 0.1 nmol/cm^2 coverage. This shows that this nitrophenyl modification on TPEs is stable for at least one week.

Geometry and nitrite impact on nitrophenyl coverage

Nitrophenyl coverage of the electrodes was further investigated by employing CV using 1 mm diameter electrode arrays modified with the optimized procedure in the presence and absence of sodium nitrite. As can be seen in Fig. 2a from the substantial increase in nitrophenyl reduction peak area when sodium nitrite is used, this reaction is responsible for the majority of the nitrophenyl coverage on the electrode surface. The average nitrophenyl coverage calculated for the 2.5 mm diameter disk electrodes in Fig.1c was compared to the average coverage calculated from the 1 mm diameter array electrodes to investigate the impact of electrode geometry on coverage. The average coverage/geometric area on 1 mm electrode arrays (1.0 ± 0.2 nmol/cm^2) was two-fold higher than 2.5 mm disks (0.5 ± 0.2 nmol/cm^2). Further investigation into the coverage and capacitance of arrays vs. disks (S.3) shows that the capacitance/geometric area for the 1 mm arrays is 2.6 times higher than that of the 2.5 mm disks: 490 ± 50 µF/cm^2 for arrays, and 190 ± 70 µF/cm^2 for single recipe disks. Higher capacitance suggests higher real surface area/geometric surface area. Therefore, we hypothesize this coverage disparity is due to higher surface roughness on the array electrodes introduced during the electrode fabrication and modification steps. SEM images of pre-treated but unmodified, and aminophenyl and nitrophenyl modified TPE arrays are shown in Fig. S.5 and little difference is seen in surface morphology of the three samples.

Direct modification with p-aminophenyl diazonium salt

Aminophenyl monodiazonium salts can be synthesized directly by limiting the amount of sodium nitrite to prevent didiazonium salt formation.[39] Lyskawa et al. demonstrated that when 1 molar equivalence of sodium nitrite to p-phenylenediamine is used 90% of the product is the monodiazonium and 10% of the product is the didiazonium salt.[39] When 1 molar equivalence was used for the diazonium tosylate reaction paste, the thickness and color of the paste varied significantly between batches. As small amounts were used, this inconsistency was likely due to variability in whether the nitrite or aryl amine was the limiting reactant. High background current was also seen for a wide potential range from several unanticipated peaks in the CVs shown in Fig. S.4. To reduce paste variability, 0.75 molar equivalence was tried instead, and the paste colour became more consistent and there were fewer background peaks in the CVs, shown in S.4. The CVs of the optimized aminophenyl modification (Fig. 2b) with and without sodium nitrite also show that the reaction with sodium nitrite is responsible for the majority of the electroactive surface groups.

Blocking Optimization

To prevent signal from nonspecific adsorption, different blockers were evaluated. Peak currents for p-AP were used to quantify relative nonspecific adsorption and data is shown in Fig. 3a. When SuperBlock™ alone was used, the highest amounts of non-specific adsorption were seen for both diazo modification strategies, 0.23 ± 0.03 µA and 0.24 ± 0.02 µA p-AP peak current for reduced p-nitrophenyl and p-aminophenyl diazonium salt modified electrodes, respectively. BSA showed less nonspecific adsorption on average but higher variability in blocking on reduced p-nitrophenyl electrodes, but much higher blocking for p-aminophenyl modification than SuperBlock™, 0.15 ± 0.08 µA and 0.02 ± 0.01 µA for reduced p-nitrophenyl and p-aminophenyl diazonium salt modified electrodes, respectively. BSA diluted in SuperBlock™ was the best blocker for aminophenyl modification and was not statistically different from BSA for reduced p-nitrophenyl modification 0.039 ± 0.004 µA and 0.04 ± 0.03 µA for reduced p-nitrophenyl and p-aminophenyl diazonium salt modified electrodes, respectively. Therefore, BSA in Superblock™ was used for all subsequent experiments.

Streptavidin-ALP Biotin Assay

All amine functionalization strategies showed promising signal response on biotinylated electrodes and low response on unbiotinylated controls. Data for this experiment is shown in Fig. 3b-d. Reduced p-nitrophenyl-modified electrodes had the lowest signal, but the sharpest and best resolved peaks. Aminophenyl-modified electrodes, without further reduction, show similar response to reduced nitrophenyl modified electrodes. However, aminophenyl-modified electrodes have broader response peaks and lower reproducibility. When aminophenyl-modified electrodes are reduced electrochemically prior to further modification, the signal is significantly higher and much more reproducible, although the peaks are still broader than reduced nitrophenyl-modified electrodes. These results, along with the results of blocking optimization, suggest denser surface coverage is achieved from direct aminophenyl-modification. Improvement of the signal following reduction agrees with our hypothesis that oxidized amine forms exist on the surface following direct aminophenyl modification.

CRP ELISA Calibration in Buffer

For the CRP ELISA assays in buffer, both methods showed similar dynamic ranges from 1-1000 ng/mL. The ELISA results indicate that the assay is useful for monitoring CRP in serum at 100-fold dilutions. Healthy CRP levels in blood are <1 µg/mL, but levels can rise 10^3-fold in patients with cardiovascular risk.[40] The LODs for both methods were 2 ng/mL CRP and were calculated from the myoglobin blanks (blank+3SD$_{blank}$), as the presence of other protein is more representative of a real system than buffer blanks. The

myoglobin blank pAP peak areas for each modification strategy (19 ± 2 nVA for nitrophenyl; 44 ± 1 nVA for aminophenyl modification) were not statistically different from pAP peak areas from buffer blanks (20 ± 2 nVA for nitrophenyl; 44 ± 4 nVA for aminophenyl modification) showing that the assay is specific to CRP. Although the LODs were the same: 2 ng/mL, the reduced aminophenyl modification strategy showed more sensitive response to increasing CRP concentration in the dynamic range and was therefore used for the assay in serum.

CRP ELISA in Spiked Serum Samples

The LOD in serum was five times higher than the LOD in myoglobin at 10 ng/mL due to an increase in nonspecific signal and variability in the 0 ng/mL serum blank. This may be attributed to some degree of cross-reactivity of the CRP antibodies and bovine CRP present in FBS. The pAP peak area data for spiked 100-fold diluted serum showed no loss in sensitivity relative to that of buffer. The 4-PL fit coefficients determined in serum fell within the 95% confidence interval of the fit coefficients in buffer (S.8). The serum results were not significantly different from the buffer results for the concentrations tested, apart from 1 ng/mL concentration which is below the assay LOD.

Conclusions

In this study, diazonium tosylates were used as a novel immunoassay functionalization method to develop the first TPE immunosensors. Nitrophenyl and aminophenyl diazonium tosylates were in-situ synthesized in water reaction pastes from aryl amines under ambient conditions and were used to successfully modify TPEs with amine functional groups. This new immunosensor functionalization method was rapid (5 min monolayer coverage), versatile, and stable for at least a week. Small-volume TPE arrays were developed, functionalized with diazonium tosylates, conjugated to antibodies and used to perform selective CRP ELISAs in PBS buffer, with clinically relevant detection limits. These TPE biosensors also demonstrated no loss in sensitivity when the assay was performed in serum, which is promising for future utility for cardiovascular disease. Further optimization would be required for determination in saliva where healthy CRP levels average around 289 pg/mL and cardio-vascular disease patients average just below the LOD at 1680 pg/mL.[37] However, with further assay optimization and volume reduction, determination in saliva could likely be achieved. In addition, this sensing platform can be easily adapted for simultaneous detection of various biomarkers with the use of modified small-volume TPE-arrays for POC diagnostics at reduced costs.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was supported through funding from the National Science Foundation (CHEM1710222) and the National Institutes of Health (R21EB030349).

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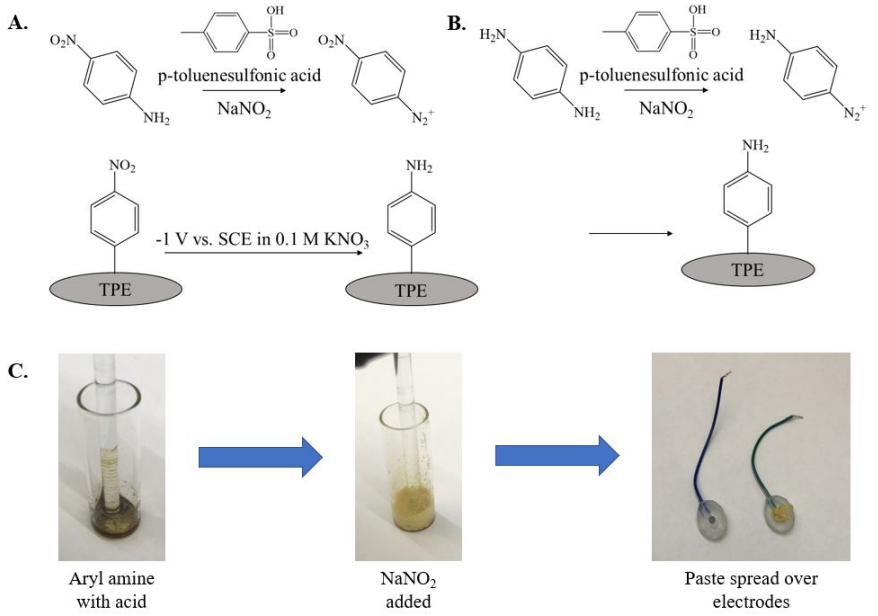
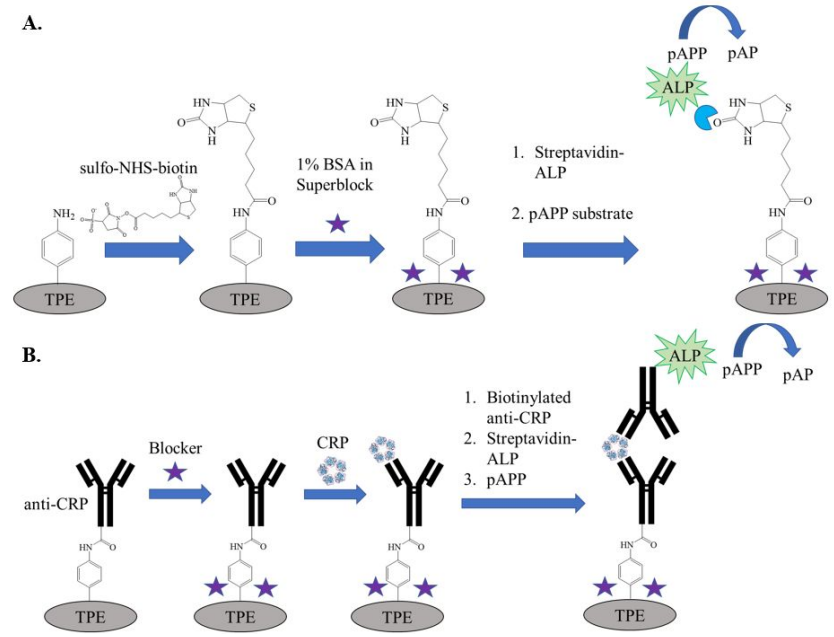
Scheme 1. Diazonium salt synthesis and modification schematics. A) p-nitrophenyl diazonium salt modification and subsequent reduction of nitro groups to amines, B) p-aminophenyl diazonium salt modification, and C) reaction and modification images with p-nitrophenyl diazonium salt shown.



Scheme 2. Assay modification schematics. A) Streptavidin-ALP biotin proof of concept assay. B) CRP ELISA assay.



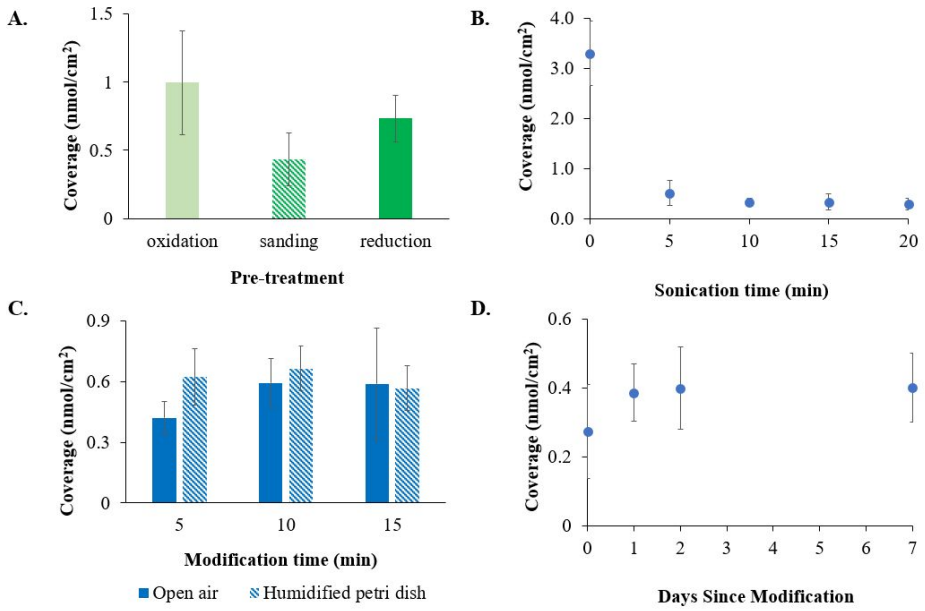


Fig. 1. P-nitroaniline modification optimization and stability testing. A) Pretreatment optimization (n=4), B) sonication time in IPA following modification (n=4), C) modification time and conditions: open air (5 and 10 min n=5; 15 min n=7) and humidified petri dish (5 min n=9; 10 and 15 min n=5), and D) Coverage stability.

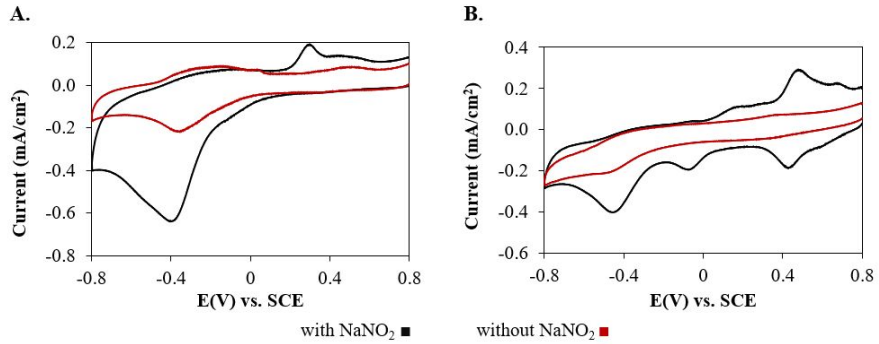


Fig. 2. Averaged cyclic voltammetry of modified 1 mm diameter TPEs in N_2 degassed 0.1 M H_2SO_4 v=100 mV/s, modified with and without $NaNO_2$. A) p-nitrophenyl diazonium salt modification (with $NaNO_2$ n=16; without $NaNO_2$ n=6), B) p-aminophenyl diazonium salt modification (with $NaNO_2$ n=6; without $NaNO_2$ n=6).

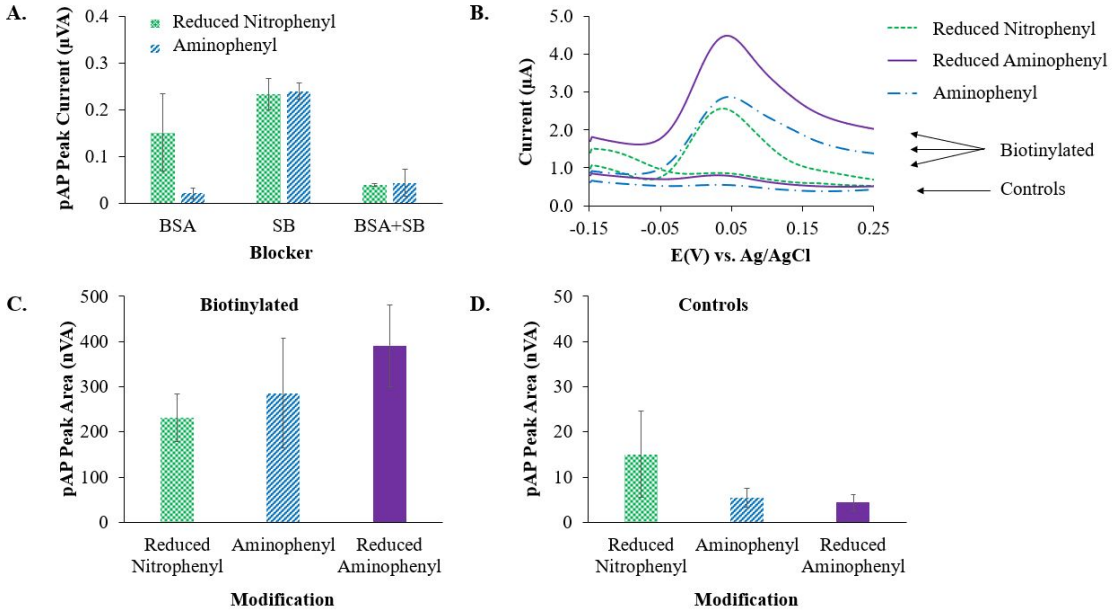


Fig. 3. Streptavidin-ALP assay data for reduced nitrophenyl, direct aminophenyl, and reduced aminophenyl modified electrodes from ALP generated p-AP SWV. A) Quantitative blocking optimization peak current data (BSA n=6; SB and BSA+SB n=3), B) averaged voltammograms for biotinylated TPEs and unbiotinylated controls (biotinylated n=6; controls n=3), C) quantitative peak area data for biotinylated electrodes, and D) quantitative peak area data for unbiotinylated controls.

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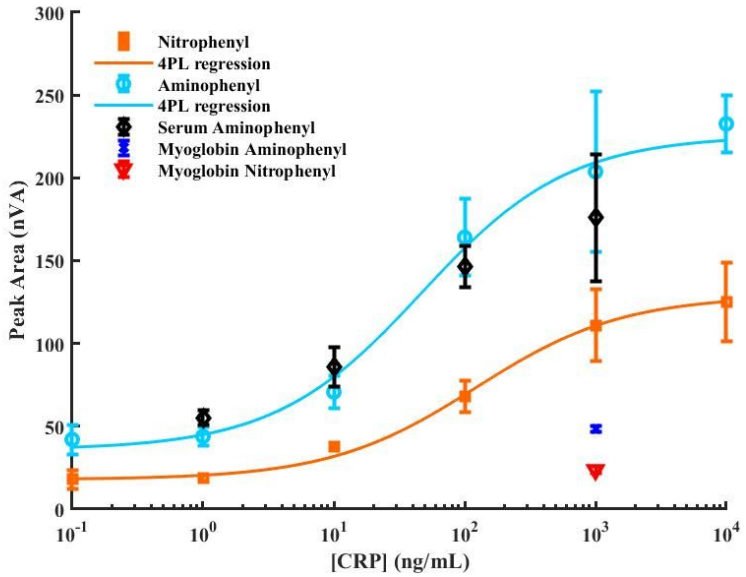

Fig. 4. CRP ELISA assay p-AP peak area data for reduced nitrophenyl (Nitrophenyl) modified electrodes and reduced aminophenyl (Aminophenyl) modified electrodes for various concentration of CRP in PBS buffer pH=7.4 (0.1-10^4 ng/mL; n=4 for all but PPD 10^3 ng/mL [n=3]), CRP spiked 100x diluted serum (1-10^3 ng/mL; n=3), and 10^3 ng/mL myoglobin (n=3).