Cheap and Sustainable Biosensor Fabrication by Enzyme Immobilization in Commercial Polyacrylic Acid/Carbon Nanotube Films

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ABSTRACT: Novel glucose biosensors were constructed by loading glucose oxidase (GOX) into the nanopores of homogenous carbon nanotube (CNT) films on the surface of Pt disk electrodes and trapping the enzyme by subsequent deposition of polyacrylic acid (PAA), forming PAA/GOx-CNT-modified Pt disks. In amperometric biosensing with anodic hydrogen peroxide (H2O2) detection at a potential of +600 mV, increasing electrolyte glucose concentrations produced instantaneous steps in the H2O2 oxidation current. Glucose biosensor amperometry was feasible down to 10 μM, with a sensitivity of about 34 μA mM⁻¹ cm⁻² and linear current response up to 5 mM. The biosensors reliably determined glucose concentrations in human serum and a beverage. Successful trials with PAA/GOx-CNT-modified screen-printed Pt electrode disks demonstrated the potential of this means of enzyme fixation in biosensor mass fabrication, which offers a unique combination of cheap availability of the two matrix constituents and sensor layer formation through simple drop-and-dry steps. PAA/GOx-CNT/Pt biosensors are green and user-friendly bioanalytical tools that do not need large budgets, special skills, or laboratory amenities for their production. Any user, from industrial, university, or school laboratories, even if inexperienced in biosensor construction, can prepare functional biosensors with GOx, as in these proof-of-principle studies, or with other redox enzymes, for clinical, environmental, pharmaceutical, or food sample analysis.

INTRODUCTION

Modern electrochemical enzyme biosensing combines the potentials of catalytic proteins with the qualities of modern potentiostats and high-tech sensor design. Evolution has produced protein biocatalysts of high efficiency and specificity that transform their substrates into products required in the body, while progress in electronic hardware and software development has resulted in the availability of computerized equipment such as small-bench footprint or hand-held amplifiers (potentiostats). Sub-nanoampere current acquisition, device portability, and wireless connection to electrochemical sensors and electrode array multiplexing are all now feasible, and methodology development in electrochemistry and nanomaterial science has created reliable procedures and advanced electrodes for the electroanalysis of redox-active analytes, even in complex sample matrices with signal disturbance by interferents. The methodology offers outstanding performance in analyte detection and is used extensively in clinical, environmental, pharmaceutical, forensic, and food sample analysis and in the testing of disease biomarkers, allergens, pollutants, drugs, nutrients, and toxic contaminants.

Enzymes are dynamic structures of folded amino acid chains that tend to lose biocatalytic function through structural distortion or denaturation when placed in extreme environments. An important consideration during the manufacture of enzyme biosensors is thus the gentle immobilization of the analyte-specific protein on the surface of the selected amperometric detector, usually a noble metal or carbon disk electrode and nowadays often designed as an integral part of mass-fabricated screen-printed electrode platforms. Existing strategies for functional enzyme fixation involve adsorption, covalent bonding, cross-linking and entrapment in or behind semipermeable polymeric electrode coatings, or combinations of these approaches. By far, the most frequently used immobilization technique is to embed proteins in thin films of synthetic or natural macromolecules, with polymeric aniline, thiophene or pyrrole, and biopolymer chitosan being representative examples. Polyacrylic acid (PAA), the immobilizing matrix component used in this study, is a large-scale
commercial commodity in industries dealing with water and wastewater, detergents and cleaners, paints and coatings, inks, oil and gas pipelines, pharmaceuticals, and personal care. Early PAA application in sensor fabrication exploited the carboxylic acid groups on films of radio frequency plasma polymerized acrylic acids for covalent attachment of chemical electrode surface modifiers.14 Probably, the first cases of PAA use as a biosensor component were the carbodiimide-assisted covalent bonding of glucose oxidase (GOx) to PAA15, or polyethylene/PAA16 membranes that were prepared and used for aqueous glucose measurements via amperometric hydrogen peroxide15 or Clark-type oxygen16 detection. In 2002 Kurzawa, Hengstenberg, and Schulmann reported that an industrial PAA formulation, specially prepared as an anodic electrodeposition paint (EDP) for the protection from corrosion of, for instance, the insides of food cans17,18 and also exploited for carbon–fiber micro- and nano electrode fabrication,19–21 electrochemical scanning tunneling microscopy,22–24 and scanning electrochemical microscopy tip25 preparation, worked very well, through electrochemically induced precipitation, in establishing enzyme-entrapping PAA thin films on the surface of common millimeter-diameter disk electrodes and even of individual members of electrochemical microelectrode array platforms.26 A unique feature of the proposed methodology was spatial focusing of PAA/enzyme co-electrodeposition on the microscopic structures, which is not possible in manual procedures. Further tests with a library of PAA/EDP variants from combinatorial synthesis proved that the substrate response characteristics of resulting PAA/EDP-based glucose biosensors were related to the degree of hydrophobicity and the swelling properties of the PAA polymer matrix.27,28 Other examples of successful PAA-based enzyme biosensors, mostly using glucose oxidase (GOx) as enzyme model, involved polyacrylamide/PAA,29 polyaniline/polyacrylonitrile/PAA,30 polyaniline/PAA,31 polytetrafluoroethylene/PAA,32 silica/ferrocene-tagged PAA,33 graphite oxide-polyethyleneimine/PAA,34 and composites or thin films of PAA hydrogels35 as electrode coatings for biocatalyst immobilization. Existing biosensor design involving PAA involves chemical synthesis steps to make the polymer and functional additives and thus requires appropriate skills and facilities. For those seeking more practicable and reproducible enzyme biosensing, and with the desire to follow the principles of Green (Analytical) Chemistry, we offer here a simplified, sustainable alternative PAA-based technology for enzyme immobilization on sensor surfaces that needs only commercially available chemicals and uses just simple drop-and-dry procedures to create the layers that capture the biocatalyst on the electrode surface with excellent protection against loss. With the GOx/PAA couple as an example, the details of the new biosensor fabrication are provided, with their relevant analytical figures of merit and the results of analytical performance tests on model and real samples. Moreover, the suitability for biosensor mass fabrication was proved by successful proof-of-principle trials aimed at the modification of commercial screen-printed platinum (Pt) electrodes.

**EXPERIMENTAL SECTION**

**Materials.** Salts for electrolyte (buffer), GOx (from Aspergillus Niger, #G1741), and PAA (average M, ~250,000, 35 wt % in H2O, #416002) for GOx/PAA biosensor fabrication were Sigma-Aldrich Corporation reagents acquired from S.M. Chemical Supplies Co., Ltd. (Bangkok, Thailand). Carboxylated single-walled carbon nanotube (CNTs) with 1.0–3.0 at % carboxylic acid entities were from Carbon Solutions, Inc. (Riverside, CA), and the GOx substrate β-D-(+)-glucose was purchased in an anhydrous form from Italmar (Thailand) Co., Ltd. (Bangkok, Thailand). Ultrapure de-ionized (DI) water was used for buffer and stock solution preparations. The biosensor storage solution and electrolyte for all glucose biosensor tests was 0.1 M sodium phosphate buffer (Na-PB), pH 7.0.

**Biosensor Fabrication.** Precursors were commercial 3 mm diameter Pt disk electrodes with firm PEEK (poly-ether-etherketone) polymer insulation (Metrohm Siam Co., Ltd., Thailand). Before use, the Pt electrode disks were polished on an electrode-polishing pad that was soaked with a suspension of 0.4 μm alumina powder in water. A thin adherent film of CNTs was then formed on the Pt disks through a simple drop-and-dry step with 5 μL of a freshly ultrasonicated and thus homogenous 5 mg mL−1 suspension of the nanomaterial in DI water.36 Next, the nano-porous CNT electrode deposit was loaded with GOx in a second drop-and-dry step with 5 μL of a 20 mg mL−1 solution of the enzyme in 0.1 M Na-PB. A final drop-and-dry step with 5 μL of a 10,000X dilution of the viscous commercial PAA suspension (0.5 mg mL−1) formed the polymeric topcoat that protected against loss of GOx by leakage. Completed biosensors were kept for 30 min in stirred Na-PB to remove loosely attached components of the immobilization matrix. Commercial 3 mm diameter screen-printed Pt disk electrodes (Pt-SPEs #50, Metrohm Siam Co., Ltd., Thailand) were also tested with the modification procedure. It is worth mentioning here that a drop-and-dry step with 5 μL of a more dilute suspension of PAA (0.1 mg/mL) failed to prevent GOx escape from the sensor surface (Figure S1). Concentrations higher than 0.5 mg mL−1 were not tested as thicker PAA coatings were expected to adversely affect the sensitivity and response time of resulting biosensors, because of slower diffusion of substrate through the polymer topcoat to the entrapped GOx underneath.

**Biosensor Amperometry.** A Palm sense-4 potentiostat (PalmSens BV, The Netherlands) connected to a three-electrode electrochemical cell was used for the amperometric biosensor tests. A fritted Ag/AgCl/3 M KCl assembly and a Pt wire served as reference electrode (RE) and counter electrode (CE), while the working electrode (WE) was the enzyme-modified Pt disk electrode of standard or SPE design. Data acquisition was controlled by the PalmSens-4 software PSTrac5.8, and individual measurements detected the glucose response of the PAA/GOx-CNT/Pt biosensors as anodic hydrogen peroxide (H2O2) oxidation currents through an adjustment of the WE potential to +0.6 V versus RE, this potential being chosen from experience gained in previous glucose biosensing studies.36–40

**Glucose Detection in Blood Serum and a Beverage.** The quality of PAA/GOx-CNT/Pt biosensors for the quantification of glucose in real samples was first evaluated through their use in standard addition mode testing of blood serum. Serum samples were prepared by centrifugation of a clotted whole-blood sample donated by one of the investigators in this study. Blood samples from other individuals were not involved, and formal authorization was therefore not required. A reference glucose concentration in the tested serum was assessed with a commercial Accu-Chek Active [Roche Diagnostics (Thailand) Ltd., Thailand] blood glucose meter, as used by diabetic patients for their normal blood tests. The mean of three Accu-Chek measurements provided the reference value for the data from the trials with PAA/GOx-CNT/Pt biosensors.
A second type of real sample was a carbonated soft drink (Pepsi, Suntory Pepsi Beverage Co., Ltd., 16 g sugar per 200 mL bottle). Before analysis, beverage samples were centrifuged at 10,000 rpm for 5 min and the supernatant was sieved through a 0.45 μm cellulose acetate filter. The reference value for the obtained Pepsi glucose concentration was equivalent data from the determination by HPLC with a refractive index (RI) detector, which is a common method for the quantitative analysis of dissolved sugar.

**RESULTS AND DISCUSSION**

Previously proposed electrochemical PAA-based enzyme biosensors showed reliable and stable analytical performance, indicating that hydrophilic PAA sensor modifications are compatible with enzymic catalysis by, for instance, GOx, which performs well as an analyte converter and signaling unit over satisfactory periods of operation and storage. A technical drawback, however, is that the available options involve complex procedures in fabrication of their functional matrix components. This discourages new users and is a barrier to interested applicants lacking the practical expertise and the laboratory settings for chemical synthesis. The skill- and synthesis-free alternative biosensor fabrication with PAA-based enzyme immobilization described here is a sequence of three drop-and-dry steps with stock solutions of commercially available, low-cost materials (Figure 1).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** PAA/GOx-CNT/Pt glucose biosensor fabrication procedure as a simple sequence of three drop-and-dry steps with solutions or suspensions of commercial stock materials.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Results of PAA/GOx-CNT/Pt glucose biosensor amperometry. (A) Original current trace from a calibration trial, with aliquots of glucose solution added successively to stirred test buffer. (B) Magnified view of the first step of the i/t trace in (A). (C) Calibration plot of data from the amperometric recording in (A) including the results of linear regression analysis for the first section up to 5 mM glucose. (D) Illustration of the practical limit of detection of PAA/GOx-CNT/Pt glucose biosensors. Electrolyte for all trials was 0.1 M Na-PB, and amperometry was at 25 °C, with an anodic H2O2 detection potential of + 0.6 V vs RE.
produced H₂O₂. Figure 2A is a typical amperometric recording operated at +0.6 V versus RE for anodic detection of enzymically amperometric calibration trials with the modified Pt disks, on day 1 (black dots), day 3 (blue squares), day 7 (red triangles), and day 14 (green triangles) after preparation, with storage in refrigerated 0.1 M Na-PB between individual assessments. (B) Zoomed view of the linear range for glucose quantification with PAA/GOx-CNT/Pt biosensors, with a standard deviation of ±10.9% for glucose sensitivity of 32.6 μA mM⁻¹ cm⁻², with a standard deviation of ±10.9% for glucose sensitivity of 32.6 μA mM⁻¹ cm⁻².

CNT deposit offered a nano-porous functional layer for GOx penetration and capture. The current purchase price for the minimum order of 1 g of the commercial CNTs is $280 US, so 40,000 fabrication repeats are possible from a single CNT order, giving a cost of just 0.7 cent for each biosensor item.

The second drop-and-dry step loaded GOx, the glucose detector, through the nanopores into the three-dimensional CNT matrix, while the third and final step locked the CNT/GOx composite in place with randomly arranged PAA polymer strings that kept protein molecules trapped within the graphitic pockets. Commercial PAA was a viscous solution of the polymer with a purchase price of about $200 US per 250 mL, which would, at the applied PAA load on the microgram scale, provide half a million CNT/GOx-primed Pt disks with protection against enzyme leakage (cost: 0.04 cent per biosensor). Extreme simplicity (just pipetting microliter volumes of stock solutions of commercial reagents and waiting for solvent evaporation) and cheapness (a cost of less than 1 cent per biosensor item) are obvious advantages of the immobilization scheme. Its usefulness was verified in the following set of performance tests with the bioanalytical sensor tools.

The linearity and sensitivity of glucose analysis with PAA/GOx-CNT/Pt biosensors were explored through the usual amperometric calibration trials with the modified Pt disks operated at +0.6 V versus RE for anodic detection of enzymically produced H₂O₂. Figure 2A is a typical amperometric recording acquired during multiple supplementations of trial buffer with small volumes of glucose stock solution. Analytically relevant features of the trace are distinct steps in the H₂O₂ current response to elevations of the glucose concentration in the test solution, with just 6 and 15 s needed to reach 90% and 100% of the final steady-state signal, respectively (Figure 2B). Figure 2C is a calibration plot computed from the data in Figure 2A. At higher glucose concentrations in trial buffer, the immobilized GOx approached saturation with substrate, so the signal current reached a plateau. Before signal saturation, the response linearity extended with an R² value of 0.996 up to 5 mM of dissolved glucose, and the sensitivity (curve slope), normalized to the geometric surface area of the modified Pt disk electrode, was 34.1 μA mM⁻¹ cm⁻².

A set of final test runs determined the practical detection limit and showed that increases in glucose concentration in the measuring buffer as small as 10 μM produced measurable steps in the biosensor current (Figure 2D). The feasible working range for glucose quantifications with PAA/GOx-CNT/Pt biosensors is thus 0.01–5 mM. Screen-printed Pt disks with a drop-dried PAA/GOx-CNT modification performed equally well for glucose detection (Figure S1), and the proposed approach is thus suitable for mass fabrication of biosensors.

For eight similarly prepared PAA/GOx-CNT/Pt biosensors, the linear range extended reproducibly to 5 mM, with a mean value and standard deviation for glucose sensitivity of 32.6 μA mM⁻¹ cm⁻² and ±10.9%, respectively (Figure 3A,B), indicating satisfactory reproducibility in fabrication. Measurement repeatability and storage stability of PAA/GOx-CNT/Pt biosensors were verified through the amperometric testing of the same sensor on the morning after its completion and then again after various times of storage in refrigerated Na-PB. The calibration plots and their zoomed linear sections for days 1, 3, 7, and 14 are shown in Figure 3C,D. The relevant curves are almost perfectly superimposable, indicating that the linear range of the proposed electrochemical glucose detection stayed constant throughout the 2 weeks of sensor storage and individual sensitivities were close to the mean value of 30.14 μA mM⁻¹ cm⁻², with a standard deviation of ±10.9%.
deviation of just ±3.3%. This confirmed that the applied polymer PAA surface deposit was biocompatible and successfully hindered loss of active GOx, which ensured both response stability and storage feasibility.

Control experiments were carried out with GOx-CNT/Pt biosensors that lacked the enzyme escape protection with a PAA glaze. Amperometric sensor testing revealed that about 75% of glucose response was lost during 30 min of storage in Na-phosphate buffer in the absence of PAA; longer storage did not further reduce the current response to 1 mM glucose addition (Figure S3). Apparently, about three-quarters of the GOx in the CNT matrix was only loosely entrapped and able to escape by diffusion into the bulk solution, while escape of the remainder was prevented and some glucose oxidation near sensor surface continued, with measurable H2O2 generation.

Before analysis of real blood serum samples and a beverage, the PAA/GOx-CNT/Pt biosensors were used for the determination of glucose in Na phosphate buffers of known content. This first analytical task was conducted with three similarly prepared biosensors as triplicate replications of assays in standard addition mode of quantification. Figure S4 shows, as a representative example, one of the nine amperometric sensor recordings from this trial, while Table S1 is a summary of the trial outcome. Individually, biosensor copies 1, 2, and 3 reached percentage model sample recoveries of 99.3 ± 0.8, 101.3 ± 1.4, and 104.1 ± 0.6, while their average recovery performance was 101.6 ± 2.2%.

The consistent ability of the three tested biosensors to measure the glucose content of model samples accurately was a first proof of the usefulness of PAA/GOx-CNT/Pt biosensors for glucose analysis, and it confirmed again the reproducibility of their fabrication and reliable biocatalytic response. The excellent analytical performance with Na-PB model samples was also achieved when operating the PAA/GOx-CNT/Pt biosensors for glucose quantifications in blood serum from a non-diabetic donor and in the carbonated beverage Pepsi. Original amperometric recordings and the resulting standard addition plots for analyte concentration determination are available in Figures 4A,C and 4B,D for the serum and Pepsi samples, respectively.

Scaled against reference values from parallel analysis with a commercial blood glucose meter (serum, 5.97 mM) and high-pressure liquid chromatography with refractive index detector (Pepsi, 243.51 mM), the glucose concentrations and percent recovery rates for the serum and Pepsi glucose samples were 5.8 ± 0.1 and 240.3 ± 16.0 mM and 97.5 ± 2.2 and 98.7 ± 6.6, respectively (see also Table S2). Two remarks on the results of the serum and beverage testing are relevant here. First, endogenous ascorbate and urate did not interfere with the blood glucose quantification even though both organic anions are oxidizable at the applied anodic H2O2 detection potential of +600 mV versus RE. In trial buffer at pH 7.0 the carboxylic acid groups on the PAA strings of the biosensor surface modification are predominantly in a deprotonated and therefore negatively charged form. The associated electrostatic repulsion that anionic serum components experience near interfacial anionic PAA was obviously strong enough to suppress ascorbate and/or urate contributions to signal generation, just as interference elimination is feasible through application of semipermeable membranes, for example, as a negatively charged perfluorinated Naion topcoat.42,43 Easily formed PAA deposits are a cheap modification for enzyme electrodes and offer protection both from enzyme leakage and from signal interference. Second, like Pepsi beverages from elsewhere in the world, the tested Thai Pepsi from Suntory Pepsi Beverage Co., Ltd., Bangkok, used a blend of fructose and glucose for sweetening. For concerned
consumers, the total sugar content of 16 g per 200 mL of Suntory Pepsi is available on the bottle label. The measured glucose content of the tested Suntory Pepsi sample was 240.3 ± 16.0 mM or 8.7 ± 0.6 g/200 mL, and thus, some 51–58% of the sugar in this version of Pepsi was identified as glucose by the GOx-assisted detection with PAA/GOx-CNT/Pt biosensors. Apparently, Thai Pepsi is produced with slightly more glucose and less fructose than its equivalents in Europe and Australia and in the USA, where the glucose fractions are usually about 50 and 40%, respectively.44

■ CONCLUSIONS

We report the construction of efficient amperometric glucose biosensors, with GOx as the target-specific biocatalyst immobilized onto Pt disk WEs by entrapment in the nanopores of an adherent homogenous CNT sensor surface film; once loaded, escape of GOx was prevented by superimposition of a layer of polymeric PAA strings, which effectively sealed the pore exits. The current responses of PAA/GOx-CNT/Pt biosensors were fast (a few seconds), proportional to glucose concentration of up to 5 mM and quantifiable down to 10 μM glucose. A tabulated comparison of the analytical figures of merit of the PAA/GOx-CNT/Pt glucose biosensors of this study with those of published biosensors with PAA as an immobilization matrix component is shown in Table S3. Reliably correct quantification of the glucose content of blood serum and a carbonated beverage verified the suitability of the probe design for real sample analysis. Moreover, the successful modification of screen-printed Pt electrode disks offers the possibility of mass fabrication of biosensors. Realization of the proposed PAA/CNT-based biosensor architecture requires only cheap, commercially available materials and simple drop-and-dry procedures. This conforms to the principles of Green (Analytical) Chemistry, as it avoids the synthesis of sensor components, with its associated chemical use and waste generation. The simplicity of the PAA/GOx-CNT/Pt biosensor preparation procedure is an important asset for potential applicants across the science and health disciplines as it facilitates tool reproduction without special skills or laboratory amenities. Anyone with access to cheaply available CNT, PAA, and GOx can prepare the bioanalytical tool, whether an electro- or analytical chemist experienced in sensor fabrication or a member of a high school or undergraduate class. We also suggest the readily feasible replacement of GOx in the biosensors, used here as a proof of principle, by other redox enzymes so as to adapt the electrochemical probes to the analysis of other analytes. Our own application targets are exploitations of the sustainable PAA/CNT immobilization matrix for the development of wearable enzyme-based biosensors for minimally invasive substrate detection in sweat, saliva, and tears. Future work will explore the replacement of Pt with graphitic carbon, as a cheaper disk electrode material, to further optimize the cost-effectiveness of the biosensors.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00925.

Calibration plots for PAA/GOx-CNT/Pt-SPEs with different loadings of PAA, calibration data for a PAA/GOx-CNT-modified Pt disk on an SPE platform; results from a GOx leak test of CNT-GOx/Pt biosensors with no PAA glaze; amperometric recordings, standard addition plots, and a tabulated summary from an application of PAA/GOx-CNT/Pt biosensor to the quantification of the glucose content of Na phosphate buffer solutions; amperometric recordings, standard addition plots, and a tabulated summary from quantification of the glucose content of blood serum and Pepsi beverage; and tabulated comparison of the analytical figures of merit of the PAA/GOx-CNT/Pt glucose biosensors of this study with those of published biosensors with PAA as an immobilization matrix component (PDF)

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Author Contributions

K.S. and S.T. carried out the measurements. A.S. guided the laboratory work, was responsible for the study conception and funding acquisition, and produced the first manuscript draft. All three authors contributed to data analysis, graphic preparation, and manuscript revision and all gave approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CNT, carbon nanotubes; GOx, glucose oxidase; PAA, poly(acrylic acid); WE, working electrode; CE, counter electrode;
RE, reference electrode; SPE, screen-printed electrode; Na-PB, sodium phosphate buffer

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