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Combined covalent and noncovalent carboxylation of carbon nanotubes for sensitivity enhancement of clinical immunosensors†

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Abstract

We report here for the first time with quantitative details that the combination of pi–pi stacking of pyrenecarboxylic acid with chemically carboxylated multiwalled carbon nanotubes (MWNT-COOH) offers superior sensitivity compared to MWNT-COOH alone for serum insulin measurements and that this combination is broadly applicable for biosensors, drug delivery, and catalytic systems.

Graphical Abstract

The conductive large surface area with easy functionalization of carbon nanotubes has allowed the development of highly sensitive electrochemical immunosensors and electrodes featuring chemical or enzymatic catalysts yielding high electrocatalytic currents.^{1–8} In particular, conventional covalent carboxylation (*e.g.*, by acid treatment) and recently

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developed noncovalent carboxylation of carbon nanotubes have remarkable significance in the areas of electronics, biosensors, engineering, materials science, fuel cells, and renewable energy.^{9–15} New functionalization strategies to obtain a fundamental understanding of carbon nanostructures in enzyme biosensors and electrocatalysis are still evolving.^{16–18}

In this study, we π – π stacked 1-pyrenebutyric acid (Py-COOH) with MWNT-COOH on gold screen printed electrodes (AuSPEs) for covalent immobilization of monoclonal insulin antibody (Ab_{surface}) and detection of serum insulin. With the goals of minimizing the serum matrix effect and amplifying the current signal, we captured insulin present in serum using horseradish peroxidase (HRP)-labeled second insulin antibody (detection antibody) attached to magnetite (Fe_3O_4) nanoparticles. To our knowledge, this is the first report showing the effect of combining covalent and noncovalent carboxylations of MWNTs for developing highly sensitive serum insulin amperometric immunosensors. Applicability of this methodology to other enzyme-catalyzed reactions for sensing and electrocatalysis adds immense significance to the findings in this report.

Scheme 1 shows the first amperometric insulin immunosensor designed in this study for measuring insulin levels in human serum. The detailed experimental procedure for the construction of the immunosensor under optimized conditions is provided in the ESI.[†]

In brief, an AuSPE was dry coated with MWNT-COOH. Py-COOH was then π – π stacked onto the basal plane sidewalls of the MWNT-COOH surface.^{1,19,20} Following this, the surface -COOH groups were activated by treatment with a freshly prepared solution of 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC, 0.4 M) and *N*-hydroxysuccinimide (NHS, 0.1 M) mixture in deionized water to convert them into *N*-succinimidyl esters for covalent attachment with the lysine residues of Ab_{surface} (amine-carboxylic acid coupling chemistry).²⁰ The surface was blocked with bovine serum albumin (2% BSA) to minimize nonspecific binding.²¹ The sensor was incubated with serum insulin conjugates prepared by capturing insulin from serum using poly(acrylic acid)-functionalized magnetite nanoparticles (MNPs) that are covalently attached with HRP-labeled rabbit polyclonal anti-insulin antibody (Ab_{HRP}). The resulting serum insulin conjugate is denoted as MNP– Ab_{HRP} –insulin.

Amperometric signals were monitored for the binding of MNP– Ab_{HRP} –insulin conjugates onto Ab_{surface} at an applied constant potential of -0.1 V vs. a pseudo Ag reference electrode. All procedures were completed off-line except the detection step. For detection, a solution containing a 1 mM hydroquinone (HQ) mediator and 0.5 mM H_2O_2 in phosphate buffered saline (PBS), pH 7.4 was delivered onto the sensor surface *via* a syringe pump at a rate of $200 \mu\text{L min}^{-1}$. The HRP-label (present in the MNP– Ab_{HRP} –insulin conjugate) catalyzed oxidation of HQ to quinone in the presence of H_2O_2 was monitored as current signals, which are proportional to the concentration of bound serum insulin on the AuSPE sensor surface (Scheme 1). Similar to the above immunosensor design of MWNT-COOH/Py-COOH/ Ab_{surface} /BSA on AuSPEs, we designed a sensor without the Py-COOH linkers (*i.e.*, MWNT-COOH/ Ab_{surface} /BSA on AuSPEs) for serum insulin measurements using the above

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described MNP–Ab_{HRP}–insulin conjugation strategy. The MNP–Ab_{HRP} strategy offers the following advantages: isolation of insulin from complex serum by simple use of a small magnet, improved antibody–antigen binding selectivity, increased signals from large antibody–insulin complexes carrying MNPs (1 mg contains $\sim 1.8 \times 10^{12}$ particles, Chemicell Inc.), and minimized interferences of signals from the free serum matrix.^{22,23}

Fig. 1 shows the experimental setup of the immunosensor for serum insulin detection. Once the immunosensor electrode was connected to a 3-electrode configuration in a microfluidic channel, a constant -0.1 V potential was applied. A syringe pump was used to deliver 1 mM HQ and 0.5 mM H₂O₂ containing PBS solution over the serum insulin-bound sensor surface. The corresponding amperometric signals were recorded on a computer connected to a potentiostat.

The scanning electron microscopy (SEM) image of bare AuSPEs (Fig. 2A) shows the characteristic roughness that would be expected for the purchased rough gold surface of SPEs manufactured by curing gold ink over a ceramic substrate at low temperature (DropSens Inc.). Dry-coating of MWNT-COOH onto AuSPEs resulted in cylindrical tubular structures of the nanotubes (Fig. 2B). Further modifications of the electrode with pi–pi stacked Py-COOH, covalently attached Ab_{surface}, and BSA blocking, respectively (details under Experimental, ESI[†]), changed the tubular structures to become thicker and denser (Fig. 2C, D, and E). Following the selective binding of the MNP–Ab_{HRP}–insulin conjugate onto Ab_{surface}, the presence of the nanoparticles on the sensor surface was observed (Fig. 2F).

Fig. S1 (ESI[†]) shows the stepwise Fourier transform infrared spectroscopy characterization of the designed serum insulin immunosensor. MWNT-COOH coated electrodes displayed a C=O stretching at 1730 cm^{-1} and an O–H stretching at 3380 cm^{-1} (Fig. S1A, ESI[†]). The characteristic C–H wagging vibration of Py-COOH is observed in Fig. S1B (ESI[†]) at 787 and 840 cm^{-1} .²⁴ With immobilization of proteins (*i.e.*, Ab_{surface} and BSA), amide-I C=O stretching and amide-II N–H bending peaks were observed at 1640 and 1550 cm^{-1} , respectively, along with an N–H stretching at $\sim 3500\text{ cm}^{-1}$.²⁵ Binding of serum insulin from the MNP–Ab_{HRP}–insulin conjugate onto the surface Ab of MWNT-COOH/Py-COOH/Ab_{surface}/BSA AuSPEs increased the intensities of amide-I and amide-II signals along with the C=O peak due to the formation of a sandwich immunoassembly featuring serum insulin molecules sandwiched between the Ab_{surface} and Ab_{HRP} (Fig. S1C–E, ESI[†]).

Fig. S2 (ESI[†]) illustrates the stepwise Raman characterization of the immunosensor. Fig. S2A (ESI[†]) shows the characteristic D and G bands of MWNT-COOH that shifted to a lower frequency after pi–pi stacking with Py-COOH molecules.²⁶ The D and G bands shifted from 1354 and 1593 cm^{-1} to 1345 and 1588 cm^{-1} , respectively. This was attributed to the partial electron transfer from Py-COOH to MWNT-COOH and a relatively weaker sp² C–C bond strength in the hybrid complex than on nanotubes.²⁷ This confirmed that pi–pi stacking of Py-COOH molecules on the MWNT-COOH electrode occurred. Fig. S2B (ESI[†]) shows the Raman spectra for every modification on the electrode surface. All spectra exhibited carbon related D, G, and 2D modes at approximately 1350 , 1600 , and 2900 cm^{-1} , respectively. After binding of the MNP–Ab_{HRP}–insulin conjugate (150 pM serum insulin treated) onto the

surface insulin anti-body, a weak phonon band for the Fe_3O_4 core of MNPs was observed at $\sim 150\text{ cm}^{-1}$. Together, the spectral and microscopic studies confirmed the applicability of the designed immunosensor for serum insulin measurements.

Fig. 3A and B show the amperometric current signals for various serum insulin concentrations detected by the designed immunosensors with and without Py-COOH linkers on MWNT-COOH coated AuSPEs, respectively. The amperometric trace for serum insulin of a 20-times diluted type 2 diabetes patient's serum in PBS (that corresponds to 5% serum) is also shown in Fig. 3A. From the calibration plot of measured currents with insulin concentration (Fig. 4a), we determined the patient serum insulin concentration to be $343 \pm 32\text{ pM}$, which agreed with the estimation using the standard enzyme-linked immunosorbent assay (commercial serum insulin kit, Mercodia Inc., Sweden) method.²³

Fig. 4(a and b) compares the current responses for different serum insulin concentrations measured by the two immunosensor strategies. The slopes of the linear relationship plots between current and concentration correspond to sensitivity. From the slopes, we determined that AuSPEs fabricated with the pi-pi stacked Py-COOH molecules on MWNT-COOH showed nearly 3-times greater sensitivity compared to those without the Py-COOH molecules (Fig. 4). The linear range of the designed immunosensor with Py-COOH stacking was from 2 to 125 pM and for the sensor without the Py-COOH the linear range was from 5 to 125 pM serum insulin (Fig. 3). The decrease in current signals at higher concentrations could have likely arisen from the surface saturation of the electrode with the MNP- Ab_{HRP} -insulin conjugate and the associated resistance for the communication of the electron transfer mediator with the electrode. Nevertheless, the linear range of the sensor is sufficient to differentiate type 1 ($<50\text{ pM}$) from type 2 ($>70\text{ pM}$) diabetes serum insulin levels under fasting conditions. More importantly, the sensitivity enhancement accomplished in this work by the combination of MWNT-COOH with Py-COOH is also significant for diagnostic biosensors that measures elevated or diminished levels of disease biomarkers over normal conditions.

The limit of detection (LOD)²⁸ for serum insulin was estimated to be 1.35 pM for the Py-COOH stacked MWNT-COOH immunosensor electrodes and the LOD was 3.22 pM in the case of only the MWNT-COOH modified immunosensor electrodes with no Py-COOH stacking. In addition, we observed better insulin affinity for $\text{Ab}_{\text{surface}}$ on the MWNT-COOH/Py-COOH modified AuSPE sensor (calculated apparent electrochemical Michaelis-Menten constant,²⁹ $K_{\text{M}}^{\text{app}}$, was 168 pM) than the MWNT-COOH only modified AuSPE sensor ($K_{\text{M}}^{\text{app}}$ 270 pM) (Fig. S3 (ESI[†]) for Line-weaver-Burk plots). This can be attributed to the greater number of available carboxyl groups provided by the combination of Py-COOH and MWNT-COOH for enhanced $\text{Ab}_{\text{surface}}$ attachment and in turn to more $\text{Ab}_{\text{surface}}$ binding sites for the binding of MNP- Ab_{HRP} -insulin conjugates with stronger affinity. We experimentally verified this presumption by quantitation of surface immobilized -COOH groups and $\text{Ab}_{\text{surface}}$ as detailed below.

Electrochemical quantitation of the surface -COOH groups in the two types of immunosensors revealed $3.7 (\pm 0.7) \times 10^{-10}\text{ mol cm}^{-2}$ of -COOH in MWNT-COOH/Py-COOH modified AuSPEs compared to $0.8 (\pm 0.1) \times 10^{-10}\text{ mol cm}^{-2}$ of -COOH in the

MWNT-COOH only modified AuSPEs. Quantitation of carboxyl groups was done by converting them to acyl chlorides followed by derivatization with 4-nitrophenol *via* an ester linkage.³⁰ The voltammetric reduction of the aryl nitro-labels on the surface allows for the quantitation of carboxyl groups (see Fig. S4 (ESI[†]) for cyclic voltammograms).³⁰

Thus, the higher number of –COOH groups available in the combined MWNT-COOH/Py-COOH modification (4.6-fold) could offer greater covalent attachment of Ab_{surface} molecules than the MWNT-COOH modification alone on AuSPEs, which would result in enhanced serum insulin detection sensitivity. We quantitated Ab_{surface} using the NanoOrange Protein Quantitation Kit (Thermo Fisher Scientific). The amount of Ab_{surface} on the MWNT-COOH/Py-COOH modified AuSPEs was estimated to be 8.7 ± 2.5 pmol cm⁻², whereas it was 2.9 ± 0.4 pmol cm⁻² in the case of only MWNT-COOH modified AuSPEs. This quantitation confirmed that the combination of covalent and noncovalent carboxylations of MWNT allowed a greater amount of immobilized Ab_{surface} (about 3-fold) compared to MWNT-COOH only modified AuSPEs.

In summary, we demonstrated for the first time the significance of combining covalent and noncovalent functionalization of carbon nanotubes for sensitive immunosensing applications. We applied this surface chemistry to design a novel amperometric immunosensor to measure serum insulin concentrations with direct type 1 and type 2 diabetes relevant pM detection levels. The results presented suggest that functionalized carbon nanostructure based sensors, drug delivery, and catalytic systems can benefit by additional incorporation of noncovalent pyrenyl or other similar noncovalent strategies to offer improved performance. The presented surface design methodology is not limited to electrochemical detection because this carbon nanostructure functionalization strategy can also be utilized for optical and spectral based detection methods and for any enzyme immobilized electrocatalytic applications. Similarly, the detection is not limited to serum insulin alone; any other biomolecules and small molecules can be detected in principle by utilizing the demonstrated combination of covalent and noncovalent functionalization of carbon nanotubes and other related carbon nanomaterials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Experimental setup. The top view of the microfluidics channel is shown on the left.

Fig. 2. SEM images of (A) bare AuSPE, (B) AuSPE/MWNT-COOH, (C) AuSPE/MWNT-COOH/Py-COOH, (D) AuSPE/MWNT-COOH/Py-COOH/Ab_{surface}, (E) AuSPE/MWNT-COOH/Py-COOH/ Ab_{surface}/BSA, and (F) AuSPE/MWNT-COOH/Py-COOH/ Ab_{surface}/BSA/MNP–Ab_{HRP}–insulin (the presence of surface bound nanoparticle conjugates can be seen in the image).

Fig. 3. Immunosenor current signals with increasing serum insulin concentration in 5% serum captured onto MNP-Ab_{HRP} conjugates and then bound with surface immobilized insulin-antibody on (A) with and (B) without Py-COOH linkers on MWNT-COOH coated AuSPEs. Experimental conditions: -0.1 V vs. pseudo-Ag reference, 1 mM HQ, 0.5 mM H₂O₂, flow rate 200 μ L min⁻¹, room temperature (23 °C), $N=4$ replicates.

Fig. 4. Current responses for immunosensor electrodes fabricated (a) with and (b) without Py-COOH linkers on MWNT-COOH coated AuSPEs for serum insulin detection ($N=4$ replicates).

Scheme 1.

Schematic of the designed serum insulin immunosensor.