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Ultrasensitive Label Free Electrical Detection of Insulin in Neat Blood Serum

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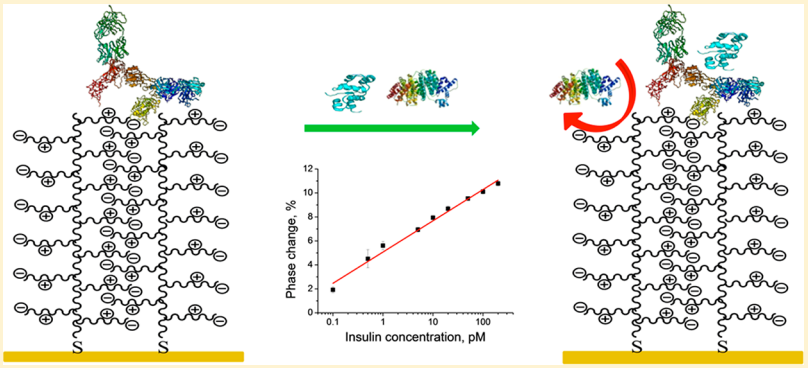
Supporting Information



ABSTRACT: Electrical assays potentially offer a highly sensitive, cheap, portable, automated, and multiplexed means of protein biomarker detection, characteristics with an ability to underpin both disease stratification and the development of point of care diagnostics. Most conveniently applied in a reagent free manner, all sensitive assays such as these suffer, however, from profound problems when applied in complex fluids such as blood serum. We report herein, the development, and clinical application, of a highly sensitive and selective electrical insulin biosensor based on a chemisorbed zwittorionic polymer support and a novel reagentless sensing technique based on phase monitoring electrochemical impedance spectroscopy. The polymer adlayer is exceptionally effective in both reducing background response and maintaining receptive antibody binding efficacy, while the non-Faradaic analysis avoids potential interference from background electro-active molecules. Applied to the detection of even a low molecular weight protein (here, insulin), a linear range from 0.1 to 200 pM and an unprecedented femtomolar detection limit are possible in undiluted blood serum.

The sensitive and selective detection of protein biomarkers plays a central role in the diagnosis of infection and a multitude of disease states as well as assessments of general physiological trauma. It will undoubtedly underpin future advances in improved disease stratification, early diagnosis, and assessment of treatment efficacy.[1,2] The extrapolation of current methodologies to a higher throughput, "point of care" format has, however, been severely hampered by the slow, cumbersome, and somewhat error prone standard laboratory methods of protein assaying, such as those based on immunoassay, mass spectroscopy, or turbidimetric analysis. Of the range of methods available, those which are label free bring advantages associated with decreased cost and sample preparation time and eliminate the potentially perturbative effects of chemical labeling.[3−5] However, label-free assays are normally considered to be less sensitive/less amplified than labeled equivalents and are prone to potentially profoundly detrimental nonspecific signals. Coupled with the knowledge that a significant number of biomarkers are present in complex biological fluids at extremely low levels (picomolar or even femtomolar),[6−8] this means that a convenient and label-free detection in real samples (such as blood serum) remains exceptionally demanding.

Electroanalytical methods potentially offer a highly sensitive, cheap, portable, automated, and multiplexed means of reporting protein binding at suitably prepared interfaces.[9,10] Though additionally potent when underpinning label free methods, such assays inherently suffer from the effects of nonspecific signal (in addition to potentially faradaic background if electroactive materials are present) when seeking to detect specific targets in complex fluids such as serum. In order to overcome these barriers and bring sensitive label free electrical biosensors into complex media, we have combined herein the use of a surface assembled zwittorionic polymer support and a non-Faradaic

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electrochemical impedance spectroscopy (EIS) modality of analysis.

It is known that zwitterionic polymers, such as poly(carboxybetaine methacrylate) (PCBMA), constitute excellent antifouling materials.[11−15] They have not, to date, been assembled on transducing interfaces in a manner conducive to highly sensitive electroanalytical sensing (where layer chemical stability and thickness are important). EIS methods of analysis are associated with the application of a sinusoidal voltage (imposed at a fixed mean voltage) to an electrode with the measurement of associated current (and, from this, associated impedance and capacitance, etc.). A modified metal electrode immersed in electrolyte will have associated a "double layer". Any binding events at this surface will perturb the double layer and the associated assayed impedance/capacitance characteristics. This methodology has been applied, in a variety of formats (predominantly faradaic), to the detection of proteins, nucleic acids, and small molecules, in aqueous solution, typically with nanomolar to piocomolar detection limits.[16] In order to minimize the possible detrimental impact (on either receptor layer viability or background signal) we have sought here to use non-Faradaic EIS, where only a low potential is applied to the receptive electrode and no redox probe is added to solution. In contrast to prior analytical impedance methods (where the tranducing parameters are interfacial impedance, resistance, or capacitance), we use here the phase offset between the input voltage and output current as the sensing signal. This is an electrode surface area independent signal that directly (and very sensitively) reflects change in interfacial capacitance (that is, any physical change that impacts the electrode interface capacitor will likewise generate a detectable change in phase, recorded).[17,18] To the best of our knowledge, there exists no precedence for the application of this methodology in a reagentless format or to the detection of a protein biomarker in complex fluid. The combination of surface assembled and chemisorbed PCBMA polymer and non-Faradaic EIS underpins an ability to assay a protein target with negligible nonspecific response or electroactive interference and an unprecedented sensitivity. To introduce and underline the utility of this methodology, which can be powerfully applied to the detection of any biomarker for which there is a receptor, we have sought here to apply it to a particularly demanding problem, the assaying of (low molecular weight) insulin in undiluted blood serum. Insulin levels are potent reporters of carbohydrate and glucose metabolism and, as such, are powerful markers of diabetes and related diseases (diseases which are rapidly accelerating in occurrence).[19,20] The biosensor is fabricated through the immobilization of antitarget antibody onto a PCBMA interface chemisorbed and photopolymerized *in situ* on a gold electrode surface. Strikingly, the interfaces respond in a highly selective manner to the target, with striking levels of sensitivity, and are totally interference-free even in undiluted blood serum.

EXPERIMENTAL SECTION

Materials and Apparatus. Electrochemical experiments were performed on an Autolab Potentiostat 12 equipped with an FRA2 module (Metrohm Autolab B.V.). A conventional three-electrode system with gold disk working electrode (1.6 mm diameter, BASi), a platinum wire counter, and a silver/silver chloride (Ag/AgCl, filled with 1.0 M KCl) reference electrode (CH Instruments) was used, together with a glass electrochemical cell with a working volume of 5 mL (accepting 0.5−5 mL of analytical solution; typically a 2−3 mL solution was used for measurements in buffer and 1 mL or less for serum sample analyses). For the analysis of some patient samples, microelectrode arrays (6 screen printed gold electrodes with a diameter of 200 μm DropSens) (Oviedo, Spain) were used.

Human insulin, human blood serum, bovine serum albumin (BSA), cysteamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and *N*-hydroxysuccinimde (NHS) were purchased from Sigma Aldrich. Patient serum samples were collected by Clinical Biochemistry, John Radcliffe Hospital, Oxford, U.K. Monoclonal anti-insulin antibody (mouse IgG1 isotype) was purchased from Santa Cruz Biotechnology, Inc. Phosphate buffered saline (PBS, 10 mM, pH 7.4) was prepared by dissolving PBS tablets (Sigma Aldrich) in ultrapure water and filtering using a 0.22 μm membrane filter. All other chemicals were of analytical grade. Ultrapure water (18.2 MΩ/cm) was obtained from an Ultrapure (Elga Water Flex3) system and used throughout.

Carboxybetaine methacrylate (CBMA) monomer was synthesized according to a previous report.[21] Briefly, 10 mL of 12 mM β-propiolactone in anhydrous acetone was added dropwise to 50 mL of 10 mM 2-(dimethylamino)ethyl methacrylate in anhydrous acetone. The mixture was stirred under nitrogen protection at about 15 °C for 5 h, and the white precipitate then washed with 50 mL of anhydrous acetone and 100 mL of anhydrous ether before being dried with a vacuum desiccator and stored at 4 °C.

Residual serum samples post insulin analysis using the chemiluminescent immunoassay, Centaur XP analyzer, (Siemens Diagnostics, Frimley, U.K.) were used as reference materials for comparison to the developed sensor. Samples were anonymized and covered a range of concentrations observed in clinical practice.

Surface Modification. Gold electrodes were polished sequentially with 3.0, 1.0, and 0.1 μm diamond spray (Kemet International Ltd.) and then ultrasonically washed in ultrapure water (5 min) prior to immersion in freshly prepared piranha solution (concentrated H_2SO_4/30% H_2O_2, v/v 3:1. Caution: treat with extreme care!) for 10 min. Finally, the electrodes were electrochemically pretreated according to a previous report[22] with some modification; cyclic voltammetry scans were conducted in 0.5 M KOH over the potential range from −0.35 V to −1.35 V at a scan rate of 2 V/s, until curves were stable. Following this treatment, scans were carried out in 0.5 M H_2SO_4 over the potential range from −0.35 to 1.5 V (4 V/s) until a stable sharp reduction peak is obtained.

The pretreated gold electrodes were dried in a flow of nitrogen gas and immersed in a solution of 5 mM cysteamine in ethanol for 12 h at room temperature to form an amine-terminated monolayer. The electrodes were then rinsed with ethanol, then water and dried in a flow of nitrogen gas, prior to incubation in a solution containing 0.4 M EDC, 0.1 M NHS, and 0.5 mM CBMA for 3 h, so as to attach CBMA monomers to the electrode surfaces. In total, 17.2 mg of CBMA, 0.2 mg of ethylene glycol dimethacrylate, and 1.17 mg of 2-hydroxy-2-methylpropiophenone were dissolved in 0.1 mL of water in preparation of a photopolymerization solution.[14] The CBMA modified electrodes were then drop-coated with 1.0 μL of this prior to illumination under 254 nm light for 30 min (to initiate polymerization across the surface). After washing and soaking in PBS (10 mM, pH 7.4) for 24 h to equilibrate the zwitterionic

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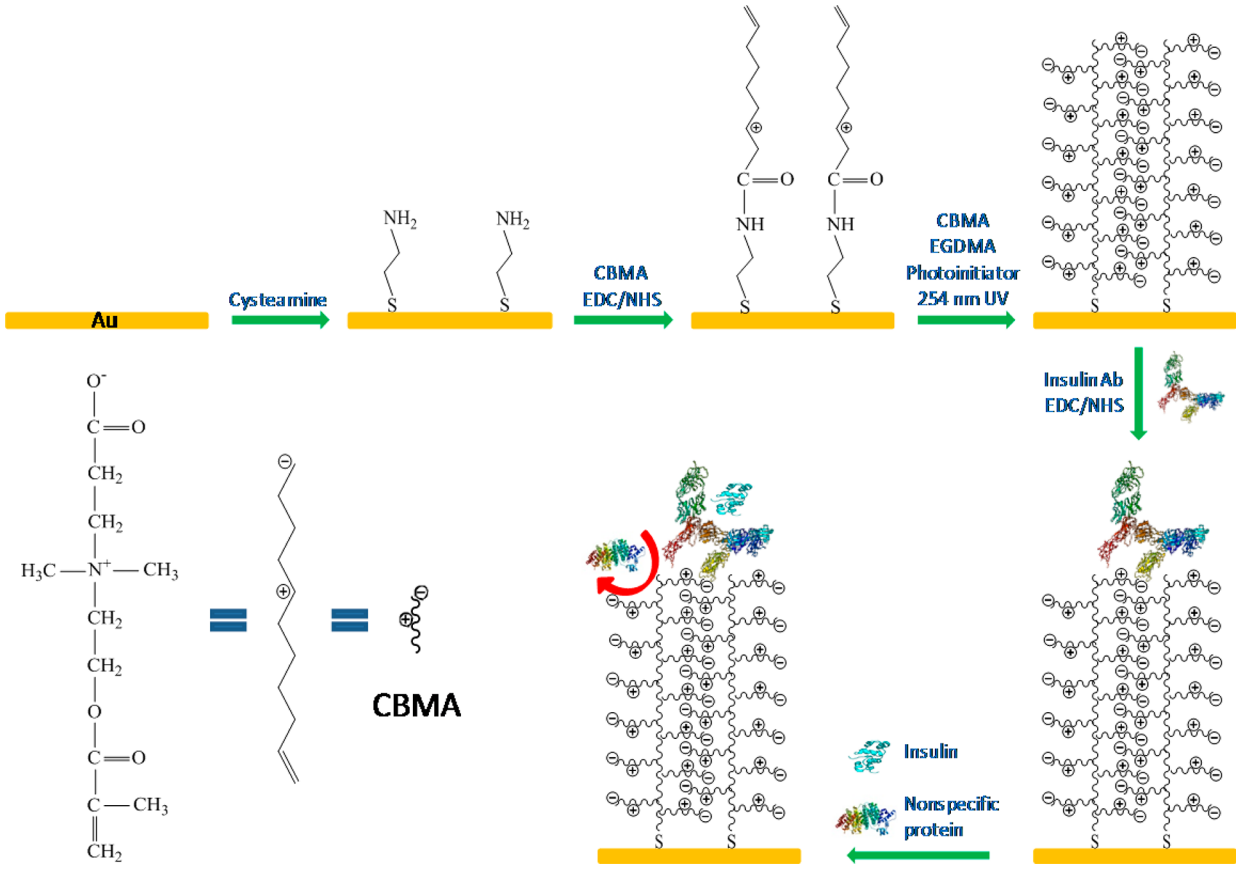

Figure 1. Schematic illustration of the insulin receptive interface fabrication through the immobilization of antibody on a photopolymerized and covalently bound PCBMA layer on gold.

polymer, the PCBMA modified electrodes were then incubated in 0.4 M EDC and 0.1 M NHS for 40 min, followed by incubation in a PBS solution containing 1.0 μM insulin antibody for 3 h at room temperature. In order to deactivate the unreacted activated carboxyl groups on the electrode surface, the modified electrodes were finally soaked in 100 μM BSA for 3 h, then rinsed extensively with PBS prior to analysis. Preliminary attempts to regenerate and reuse the sensing interfaces using 0.2 M Gly−HCl buffer, pH 2.0 containing 1% DMSO for 5 min have not, as yet, been adequately reproducible, quite possibly because of detrimental interactions between DMSO and the supporting hydrogel.

Electrochemical Impedance Spectroscopy. Non-Faradaic EIS measurements were conducted in PBS (10 mM, pH 7.4) with a frequency range of 0.1 to 100 Hz or 100 kHz, a waveform magnitude of 5 mV, with a potential of 0.0 V applied to minimize any spurious background signal or contributing field effects. EIS curves were initially displayed in the format of Bode plots, with phase plots subsequently used for analysis at optimized frequencies (phase was found to be a significantly more sensitive function than impedance). For the detection of insulin in PBS, the prepared receptive interfaces were incubated in 10 mM pH 7.4 PBS containing specific concentrations of insulin at room temperature for 20 min and EIS responses recorded after washing with PBS. To evaluate biosensor selectivity, BSA and different dilutions of sera (1−100% v/v in 10 mM pH 7.4 PBS) were tested similarly. Assays in undiluted serum were carried out by receptive surface incubation in 50 μL for 20 min, prior to PBS rinsing and EIS analysis in pure PBS. All EIS measurements were carried out at room temperature (20 ± 1 °C).

RESULTS AND DISCUSSION

Figure 1 summarizes the sensor surface fabrication steps. A cysteamine adlayer modified gold electrode was covalently modified with carboxyl containing CBMA monomers with the assistance of EDC and NHS. The linked monomers were then photopolymerized with solution phase monomers to form a chemisorbed zwitterionic PCBMA polymer interface. This formed interface, characterizable by EIS, shows excellent stability over 1 month (Figure S1 in the Supporting Information). [The hydrogel and extreme hydrophilic nature of these films preclude any meaningful SEM, TEM, spectroscopic, contact angle, or ellipsometric characterization]. Finally, anti-insulin antibodies were amide coupled to the abundant carboxyl groups of PCBMA, forming an antifouling receptive interface selective for insulin.

Non-Faradaic EIS was used to monitor the binding of target insulin at such interfaces initially in PBS (10 mM, pH 7.4). Target binding leads to a change in complex electrical impedance and, specifically, the phase offset between input voltage and output current (Figure S2 in the Supporting Information). The latter, independent of electrode surface area, is monitored by standard impedance methods. As shown in Figure 2A, significant phase change is detectable in the low frequency (0.1−10 Hz) regime (note: phase change is related to the double layer capacitance of the electrode interface and is notably more sensitive to binding than the associated change in impedance (Figure 2B)). During these analyses, the sampling

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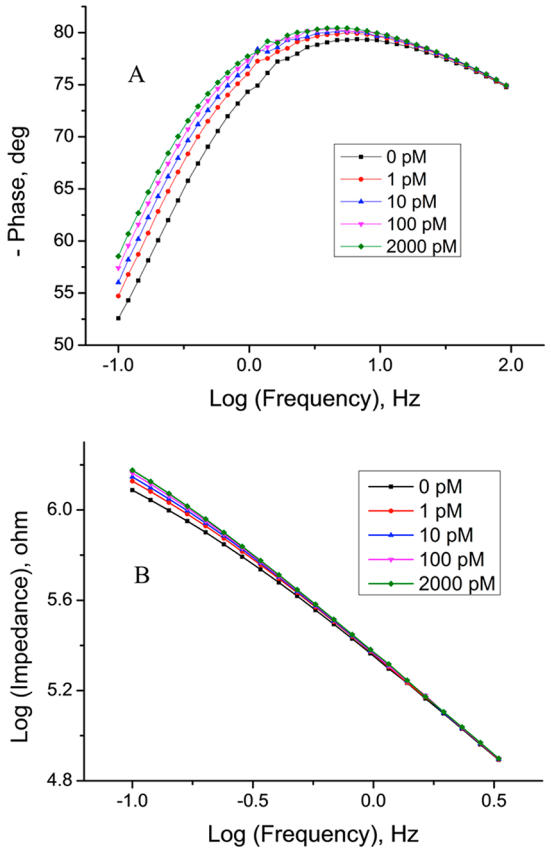

Figure 2. (A) Phase curves, depicting the detected phase offset between input and output signals as a function of applied frequency and (B) impedance curves of non-Faradaic EIS Bode plots of a typical sensory surface. Both plots are depicted as a function of exposure to different concentrations of insulin spiked into 10 mM pH 7.4 PBS. Note that standard impedance analyses (B) are only adequately sensitive even at low frequencies where assay time is prohibitively long.

frequency can be optimized to that which is most responsive (see Figure S3 in the Supporting Information), here 0.2 Hz, prior to calibrating sensor response against insulin spiked into buffered aqueous solution (Figure 3).

As shown in Figure 3, these assays are exceedingly sensitive, responding to target at sub-femtomolar levels before saturating at levels in excess of 500 pM. In fitting the data to a Langmuir expression,[23,24] the dissociation constant K_D of this insulin antibody immobilized interface is calculated to be 3.76 ± 0.37 pM, toward the lower end of prior K_D assessments of this monoclonal antibody immobilized on different chemistries.[25−27] This is a highly reproducible observation that we ascribe to being reflective of the properties of the supporting zwitteronic polymer. Though antibody surface immobilization is often at the detriment of their binding affinity,[28,29] the superhydrophilic nature of this polymer has very recently been shown to facilitate an enhanced protein binding affinity by virtue of water withdrawal from hydrophobic regions of the protein surface. Regions that play a significant role in mediating protein−protein interactions.[30,31] It appears, then, that the hydrogel interfaces generated herein provide a highly effective means of supporting antibody binding efficacy.

A more detailed analysis of the sensor response to insulin reveals that, prior to saturation, the non-Faradaic EIS phase of the sensor surface changes linearly with the logarithmic value of insulin concentration across a 0.1−200 pM range, with a limit

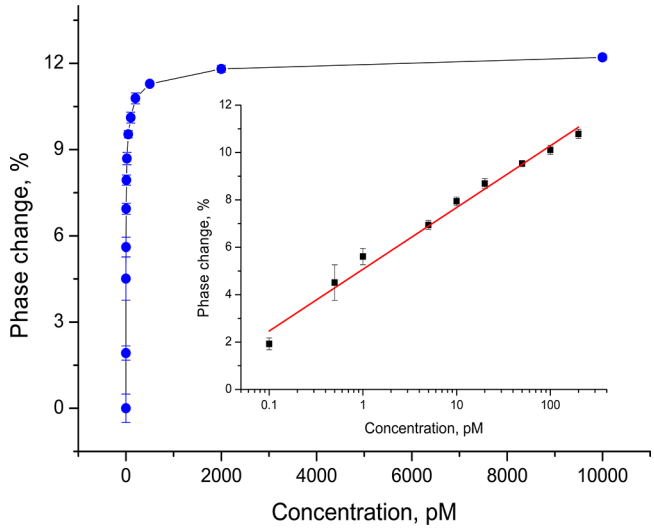

Figure 3. Phase change of the biosensor after incubation with controlled concentrations of insulin in PBS (10 mM, pH 7.4). Inset shows the corresponding calibration curve (R = 0.994). Error bars represent the standard deviations across three repeated measurements.

of detection (LOD) of 42.6 fM. To the best of our knowledge, this LOD is substantially lower than that of any other prior insulin sensor and is also unprecedented in the label free detection of any biomarker by EIS methods.[32−34] As clinically relevant insulin levels lie in the low picomolar range,[35] these interfaces and their analyses comfortably fulfill the requirement of detection across the entire clinically relevant range, an ability we ascribe to the combination of a chemically stable, antifouling, and biocompatible supporting interface, with a highly sensitive and novel impedance based assessment of target binding. The current sensor-to-sensor (sensitivity) variation is within 10% across different Au electrodes and less than 5% if the same electrode is replenished and reused. [It is important to note that this variance is excluded from presented clinical data since all electrodes were internally calibrated against standard insulin solutions prior to use.]

The direct and sensitive detection of disease biomarkers in the presence of a large excess of nonspecific protein, though central to convenient (facile and/or point of care) application as noted above, exceedingly demanding, particularly for low molecular weight targets (insulin 5.8 kDa) present at subpicomolar levels with an unamplified label free approach. The selective response of these interfaces was initially analyzed and observed to be strikingly good; the response to 10 pM target is 60-fold that to BSA at a concentration that is 100 000 times higher (Figure 4). In the absence of immobilized antibody, it is also important to note that sensor response is negligible (phase change less than 1%) even at high levels (1 nM) insulin.

The application of such selective and quantitative sensing in neat blood serum is, however, more demanding still and commonly the failing point of otherwise effective sensory interfaces (which can accordingly only be operated in highly dilute serum).[33,36] The response of these anti-insulin PCBMA interfaces to increasing volume equivalents of blood serum is summarized in Figure 4 where it is evident that the superhydrophilic and nonfouling character[13] of the supporting hydrogel is such that phase response, even in neat serum, is <3% of the interfacial response to 10 pM insulin. Further assays of insulin spiked into different serum solutions were carried out

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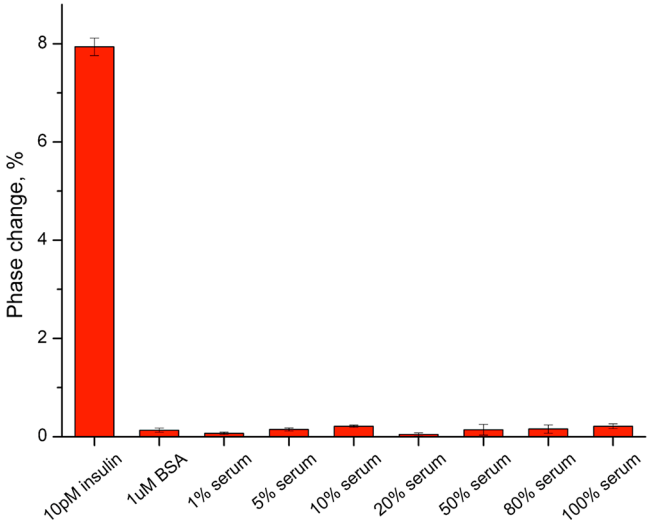

Figure 4. Sensor phase response (with the native receptive interface) on exposure to its specific insulin target (far left), micromolar BSA in PBS. In order to calibrate the levels of nonspecific adsorption interference within different dilutions of blood serum (in 10 mM PBS, pH 7.4), the interfaces, after insulin quantification in pure PBS, were presaturated in 10 nM insulin, prior to analysis in sera (so as to exclude contributions from the specific binding of insulin naturally present in serum and report solely the degree of nonspecific signal).

and revealed quantification to consistently be within 2% of that in pure PBS (see the Supporting Information Figure S4. [On the basis of our own assessments, the insulin levels in this commercial serum are <50 pM. This means that the amount of insulin actually present in a 50 pM sample in 10% serum is <55 pM, increasing to <100 pM in neat serum. As shown in the Figure 3 inset, the sensor response change between 50 pM and <100 pM is <5% broadly in line with the results of Figure S4 in the Supporting Information]. The application of these interfaces to the quantification of insulin in a cohort of patient samples (spanning a broad range of concentrations) is summarized in Figure 5, where it is evident that the assays are well behaved and compare well (most results being within 5% of each other) with comparative quantification of the same samples by a standard chemiluminescence assay. A more

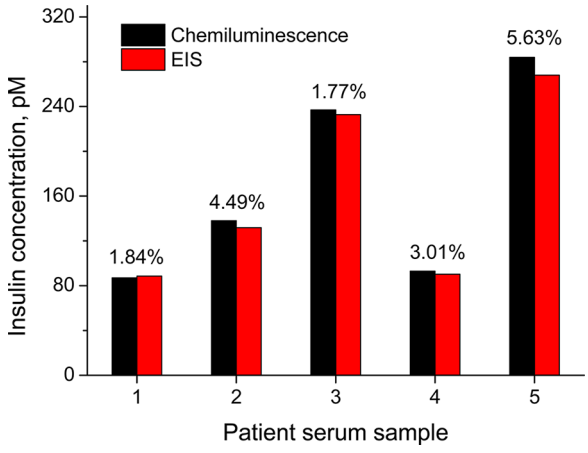

Figure 5. Representative comparative real patient serum sample assays with the non-Faradaic EIS method (macro disc electrodes; each sample analyzed in triplicate) and a clinically approved chemiluminescent immunoassay. The % differences are shown.

detailed Bland Altman analysis across patient samples assessed at both macro gold electrodes and microelectrode arrays is shown in Figure S5 in the Supporting Information, where the quantification differences between EIS and chemiluminescence are largely within 10% across the entire concentration range. This is highly encouraging as current levels of agreement between commonly used methods in clinical use can show up to 200% variation in results through differences in assay sensitivity and specificity.[37] The higher disagreement with one specimen at very low insulin concentration may reflect the superior selectivity of the interfaces employed in the EIS method. An ability to assay low molecular weight protein targets at picomolar levels in undiluted serum is one we believe to be unprecedented by this electroanalytical method.

CONCLUSIONS

We present herein a uniquely sensitive and nonfouling electrical biosensor capable of calibrating an important and low molecular weight protein marker across its entire clinically relevant range. In combining the excellent antifouling and antibody supporting characteristics of a chemisorbed zwitterionic polymer layer and a new and highly sensitive reagentless phase analysis, the interfaces exhibit only minimal response to neat blood serum but respond with high sensitivity to insulin. In comparison to those utilized in the faradaic EIS detection of insulin on a PEGylated surface,[27] the interfaces used herein exhibit both substantially better antifouling characteristics (100% serum vs 50% serum) and higher sensitivity (LOD 42.6 fM vs 1.24 pM). The assays are reagentless, fast, readily expanded to the detection of other proteins individually or in a multiplexed format, and perform well with real patient sample analysis. We believe such abilities add more to the armory available to the development of convenient point of care diagnostics.

ASSOCIATED CONTENT

Supporting Information

Additional supporting figures as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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