Biosensors and Bioelectronics 24 (2009) 1949–1954

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Rapid detection of anti-chromatin autoantibodies in human serum using a portable electrochemical biosensor

Konstantin N. Konstantinov[a], Ravil A. Sitdikov[b], Gabriel P. Lopez[b], Plamen Atanassov[b], Robert L. Rubin[c,*]

[a] Dept. of Internal Medicine, University of New Mexico Health Sciences Center, United States
[b] Dept. of Chemical & Nuclear Engineering, University of New Mexico, United States
[c] Dept. of Molecular Genetics and Microbiology, MSC08-4660, University of New Mexico Health Sciences Center, 1 University of New Mexico, Albuquerque, NM 87131, United States

ARTICLE INFO

Article history:
Received 18 July 2008
Received in revised form
26 September 2008
Accepted 29 September 2008
Available online 17 October 2008

Keywords:
Autoantibody immunoassay
Electrochemical sensor
Point-of-care biosensor

ABSTRACT

The current system employed electrochemical sensor technology for the measurement of autoantibodies in human sera. Anti-chromatin antibodies are an early and sensitive indicator of systemic lupus erythematosus (SLE) and are typically quantified using enzyme-linked immunosorbent assays (ELISA). Electrochemical detection compared favorably with ELISA using a sample of 30 SLE sera ($r = 0.9$), and non-specific binding by normal serum immunoglobulin was undetectable. The electrochemical sensor assay required <20 min processing time and utilized a hand-held apparatus with a disposable electrode. These results demonstrate the applicability of this technology to the rapid measurement of a clinically relevant analyte with an apparatus of potential simplicity and low cost.

1. Introduction

Laboratory measurement and monitoring of biomarkers are mandatory components in the management of clinical disease. Numerous technical approaches and methodologies have been devised to measure antibodies in clinically relevant samples. This can be a challenging undertaking because the specific antibody of interest is generally present in serum in a vast excess of irrelevant immunoglobulin with essentially the same composition and chemical properties. While solid phase assays have generally come to dominate the immunoassay world, most of these involve a substantial time for the read-out using expensive, non-portable equipment that requires considerable technical effort to operate, interpret, and report. Consequently, there is a need for instrumentation that can be produced inexpensively and operated easily at a point-of-care clinic or home environment, with portable biosensors becoming one of the fastest growing technological developments (Melanson, 2007).

Biosensors based on electrochemical reactions have emerged as a highly promising technique for the measurement of clinically relevant analytes (Privett et al., 2008). They are ideally suited for clinical applications due to their high sensitivity and selectivity, portable field-based size, rapid response time and suitability for mass fabrication at low cost (Wilkins and Sitdikov, 2006). Despite their potential advantages over laboratory-based analytical techniques, numerous issues remain to be addressed before portable biosensors for antibodies are ready for routine patient use (Wang et al., 2008). While biosensors generally show excellent characteristics for synthetic or pristine laboratory samples, they are often not sufficiently robust for real-life samples. Limitations of portable biosensors include operational stability of the biological receptor and/or the physical transducer, poor reproducibility between sensors and reduced specificity in complex matrices, resulting in high background signals. Frequently, therefore, the main obstacles are encountered once the sensor is used outside the laboratory and applied to in situ sample monitoring conditions (Andreescu and Sadik, 2004).

Rheumatic diseases are common and confront society with serious medical, social and financial burdens imposed by their chronic and debilitating nature (Davidson and Diamond, 2001). Each autoimmune rheumatic disease is associated with a particular set of autoantibody markers, which are used to define disease, predict flares, or monitor efficacy of therapy (Lernmark, 2001). Typically, these tests are performed in centralized clinical laboratories where expensive equipment can be consolidated and quality control of assays sustained. Nevertheless, methods for detection

* Corresponding author. Tel.: +1 505 272 4640; fax: +1 505 272 9550.
E-mail address: rlrubin@salud.unm.edu (R.L. Rubin).

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doi:10.1016/j.bios.2008.09.032

of autoantibodies continue to be expensive, protracted, and labor-intensive, and preserving the identity of assay results when testing multiple samples requires constant vigilance. Consequently, this field of laboratory testing may be especially amenable to biosensor applications.

Anti-chromatin (-nucleosome) autoantibodies are a sensitive indicator of systemic lupus erythematosus and related diseases (Rahman and Isenberg, 2008; Rubin and Fritzler, 2007). We applied a simple, rapid electrochemical-based biosensor to the measurement of anti-chromatin antibodies and validated it in part by comparison to a well-accepted standardized assay. The new assay not only overcomes practical deficiencies in current autoimmune serologic tests, but also should be readily adapted to other antigen or antibody targets of clinical value.

2. Experimental

2.1. Human serum samples

Sera from patients diagnosed with systemic lupus erythematosus (SLE) based on accepted criteria (Tan et al., 1982; Hochberg, 1997) have been previously described (Burlingame et al., 1994). Samples were stored at −20 °C. Normal human sera (NHS) were obtained from blood bank and laboratory personnel and stored and used in the same way as the SLE sera.

2.2. Preparation of immobilized antigen on membranes

Chromatin was purified from calf thymus, stripped of histone H1 (Lutter, 1978) and stored in 50% glycerol at −20 °C. Its concentration (of the DNA component) was determined by absorbance at 260 mμ based on $E = 25$ for 1 mg/ml. Protein/DNA ratio = 1.27 by BCA assay (see below). On the day of use chromatin was diluted to 20 μg/ml (in DNA, 25 μg/ml histone protein) in phosphate-buffered saline ("PBS") (0.01 M Na phosphate, 0.14 M NaCl, +0.01% thimerosal (Sigma), pH 7.2) at 5 °C. Unmodified hydrophobic polyvinylidene fluoride membranes of 0.45 μm pore size ("Immobilon-P PVDF membranes", Millipore Corp., Bellerica, MA or "BioTrace PVDF membranes", Pall Life Sciences Corp., Port Washington, NY) were cut to 1.5 cm × 7.6 cm, wetted in methanol, hydrated and immersed in 10–20 ml chromatin solution. After overnight incubation at 5 °C, the membrane was transferred to a solution of 5% non-fat dry milk (Kroger Corp., Cincinnati, OH) in PBS for 1–3 h, followed by 1% Tween-20 (polyoxyethylene sorbitan monolaurate; Sigma) in PBS for 0.5–1.0 h and finally rinsed several times in 0.05% Tween-20 in PBS ("PBS-tween"). Casein (Gallard-Schlesinger Inc., Plainview, NY) at 1% in PBS can be substituted for non-fat milk as the non-specific blocking agent with similar results.

2.3. Protein determination

The amount of chromatin protein bound to the PVDF membrane and the ELISA plate well was determined by the bicinchoninic acid colorometric assay ("BCA", Pierce Biotechnology Inc.). Replicate 260 mm^2 pieces of PVDF membrane were adsorbed with chromatin and washed under the standardized conditions described above except they were not post-coated with blocking solution. Membranes were immersed in 2.0 ml of the BCA reagent mix and 0.1 ml saline. Protein bound to the ELISA wells was determined in a similar way except 0.2 ml of the BCA reagent and 10 μl saline were added to each well, and the contents of five wells were pooled for spectrophotometric determination. The 30-min incubation was performed at 37 °C per manufacturer's recommendation. Standard curves were generated either in test tubes or microtiter plate wells using calf thymus histone (Worthington Chemical Co.), whose concentration was determined by absorbance at 230 mμ based on $E = 4.2$ for a 1 mg/ml solution (Chung et al., 1978). Background color development from membranes or plate wells to which no chromatin was added was subtracted from the appropriate samples.

2.4. Immunosensor design

A chromatin-coated membrane was placed over a plastic strip on which 8, 3-electrode sensors were screen printed at a center-to-center distance of 0.9 cm (AndCare, Alderon, Durham NC). A lucite block with 8, 6 mm diameter holes aligned to the electrode strip was placed above the membrane so that up to 0.3 ml solution could be introduced onto a 6-mm diameter circle of exposed membrane. Below the electrode strip was place another lucite block with eight 6 mm diameter aligned cavities, which was sealed with a plastic gasket at its interface with the electrode strip, and works as a waste reservoir. A 1-mm diameter perpendicular hole interconnecting the eight lower sample holes exited the lower chamber (waste reservoir) through a stainless-steel hollow rod outlet to which a vacuum can be applied. The entire assembly, which measured 76 mm × 35 mm × 20 mm, was held together with a clamp and is further described in Section 3.

2.5. Amperometric immunoassay

The test sera were diluted 1:50 in "serum diluent" consisting of 1 mg/ml gelatin (Baker Chem. Co., Phillipsburg, NJ), 0.75 mg/ml bovine gamma globulin (Calbiochem/EMD) + 1 mg/ml bovine serum albumin (Cohn fraction V; Sigma) + 0.05% Tween-20 + 0.01% thimerosal in PBS. The serum diluent was also supplemented with 1 mg/ml non-fat dry milk or casein to block binding of antibodies to milk proteins observed in some sera. Diluted sera were ultrafiltered through 0.45 μm pore size membranes. After loading each of the eight wells of the assembly with 200 μl diluted serum, a weak vacuum was applied to the lower chamber using a peristaltic pump (403 U/VM4, W-M Alitea AB, Stockholm, Sweden) set at 15 RPM to draw the samples through the membrane over the course of ∼1 min. A volume of 0.2 ml PBS-Tween was added to each well, and drawn through by vacuum. This was repeated for a total of three wash cycles. Then 0.2 ml peroxidase-conjugated goat anti-human IgG (Caltag, San Francisco, CA) diluted 1:1000 in serum diluent was added to each well and drawn through the membrane by vacuum. Wells were then washed 3× with PBS-Tween. To all the wells was added simultaneously 0.1 ml peroxidase substrate solution hydrogen peroxide (H_2O_2) + 3,3′,5,5′-tetramethylbenzidine ("TMB liquid substrate solution for ELISA", Sigma) at room temperature, and ∼10% of the solution was immediately drawn through the membrane. The electrode terminals were connected through a cable to an amperometric reader (ANDCare 800 8-well electrochemical strip reader, Alderon, Durham NC)), whose parameter settings and output were monitored by interfacing with a laptop computer using the manufacturer's software. Readings were generally made at 2 min intervals, preceded by a 10-s vacuum pulse to pull ∼10% of the substrate solution through the membrane. The potential between the working and reference/counter electrodes was set at −100 mV, and 5 ms voltage pulses were sequentially and repeatedly applied to each working electrode. Using this intermittent pulse amperometry over the course of 4 s at each electrically addressed well position, 20 readings of current were measured during the last microseconds of each pulse, and these were saved and averaged to produce the recorded signal at the selected timepoint for each sample. The electrical current maximum was typically set at 100 μA, the lowest sensitivity setting of the ANDCare Reader. Total

processing time was typically less than 20 min from the time of sample addition to the last reading.

2.6. Determination of anti-chromatin activity by ELISA

Anti-chromatin antibody quantification was performed by ELISA as previously described (Burlingame and Rubin, 1990, 2002). Briefly, Immulon 2HB microtiter plates (Dynex Laboratories Inc., Alexandria, VA) were coated with H1-stripped chromatin at 5.0 μg/ml in PBS overnight. Wells were blocked with 1% gelatin for 1 h. After rinsing with PBS-Tween, 200 μl serum diluted 1:200 in "serum diluent" (described above) were added in duplicate and incubated for 2 h with agitation at room temperature. Plates were washed with PBS-Tween and wells incubated with agitation for 1.5 h with peroxidase-conjugated goat anti-human IgG diluted 1:1000 in serum diluent. After washing with PBS-Tween, colored product was developed during 1 h using 0.005% H_2O_2 + 1 mg/ml 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as the secondary substrate in McIlvaine's buffer, pH 4.6. Optical densities (OD) at 410 mμ were measured using a Thermo Labsystems (Fisher) reader, and values beyond the range of direct measurement at 1 h were extrapolated from OD at earlier timepoints as described (Burlingame and Rubin, 1990) so that antibody activity of all samples was expressed as OD at 1 h.

2.7. Assay procedure and analysis

The ELISA and amperometric immunoassays were performed with the same reagents where possible and within a 2-month period. Each amperometric immunoassay included a positive control sample and a negative control with only secondary antibody as well as several normal sera. Amperometric readouts were corrected for secondary antibody background binding by subtraction using the signal produced by the negative control at each timepoint, which was typically <1 μA. Variability in the electrode response between assays due largely to repetitive use necessitated normalizing the readouts for samples run in different assays. This was done by using the average readout of the 13 individual runs of the same positive control to calculate a correction factor, the ratio of this value to the individual run value, and multiplying this number by the value obtained for the test sample determined in the same run. In some experiments chromatin-coated strips were blocked with casein and submerged in PBS-Tween for 0, 8, 15 and 22 days at 5 °C prior to assay with various SLE and NHS; in this case background subtraction but no inter-run normalization was performed.

3. Results

3.1. Multichannel flow-through immunosensor design

A flow-through immunoassay procedure was applied to an electrochemical detection system and adapted for measuring anti-chromatin autoantibodies in serum from human blood. The biosensor consists of a polymeric membrane with immobilized chromatin on which the antigen-antibody interaction occurs. These immune complexes are detected with a reagent antibody conjugated to peroxidase, and enzyme catalyzed product formation is detected in real-time by a flow-through 3-electrode transducer. The product sensing electrode set can be fabricated as a free-standing single channel device or arranged in an array of several (in this case eight) channels machined from a single plastic block. The schematic of the disposable flow-through electrochemical immunosensor in single well format is shown in Fig. 1.

The array of eight 3-electrode sensors consists of screen printed electrode assemblies of carbon working, carbon counter,

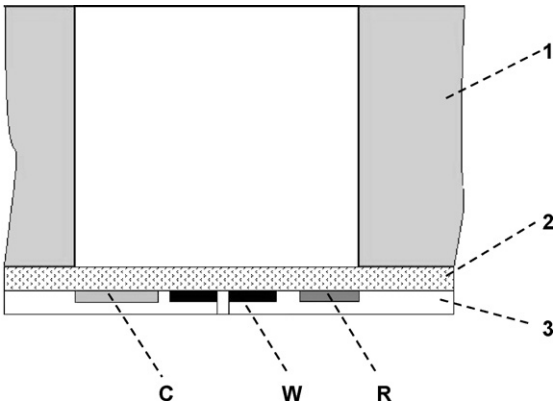

Fig. 1. Schematic of disposable flow-through electrochemical immunoassay detection unit, single well format: (1) lucite manifold, (2) immuno-selective membrane with immobilized H1-stripped chromatin, (3) screen-printed electrode strip with references (R), working (W) with hole in the center and counter (C) electrodes.

and silver-chloride reference inks and are situated perpendicular to the direction of liquid flow through the well. This design minimizes sweeping action due to lateral fluid flow across the surface of the working electrode and allows enzymatic reaction products to form adjacent to the electrode. The flow-through design also substantially enhances antigen–antibody binding by reducing the thickness of the fluid film, which in turn reduces diffusional constraints to immuno-interactions. This improves the rates of bimolecular interactions between immobilized chromatin, the analyte serum antibodies and the enzyme-conjugated detecting antibodies. Together with the use of intermittent pulse amperometry to affect electrochemical transduction, the apparatus is designed to enhance the magnitude of the signal output.

3.2. Electrochemical detection of anti-chromatin antibodies

Diluted serum samples and subsequently the peroxidase-conjugate secondary antibody were introduced manually into an eight-well manifold device as shown in Fig. 2. Immediately after their addition, the fluid was drawn by negative pressure over the course of about 1 min through the porous, chromatin-coated membrane. The amount of peroxidase-labeled antibody bound to the

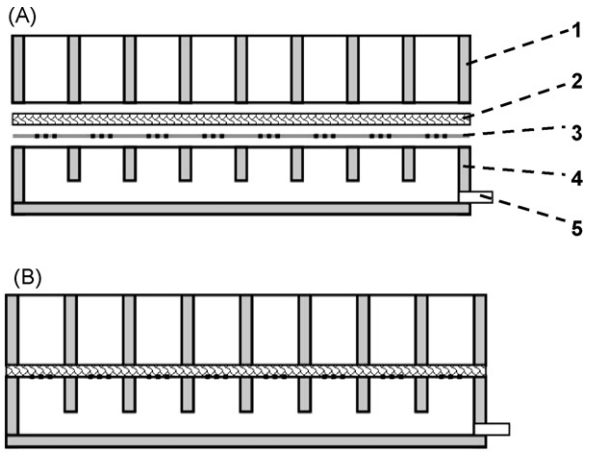

Fig. 2. Eight-channel flow-through immunosensor assembly. (A) Eight-hole plastic body (1), chromatin-coated immuno-selective membrane (2), eight-sensor electrode array strip (3) sealed under the membrane, waste reservoir (4) and outlet (5). (B) The assembled eight-channel immunosensor.

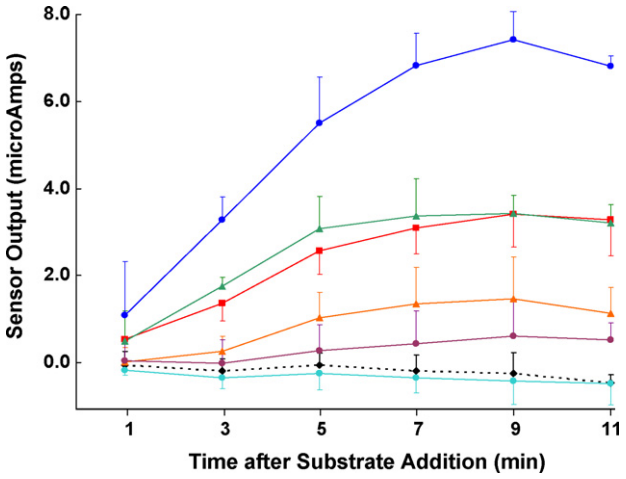

Fig. 3. Kinetics of anti-chromatin antibody response. Six sera from patients with SLE and one normal serum (dashed line) were applied to the anti-chromatin biosensor, and the signal output was measured over time. Shown are the mean ± R.S.D. of four independent determinations by two investigators. Amperometric readouts were minimally corrected for secondary antibody background binding as described in Section 2.7, resulting in slightly negative values at some timepoints for 1 SLE and the normal serum. Interassay normalization was not performed in this study.

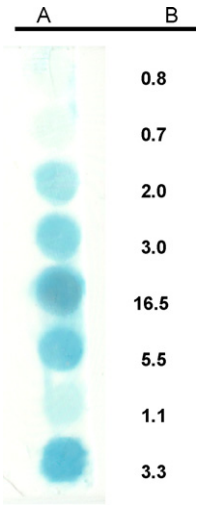

Fig. 4. Visual record of reactivity. Six SLE sera were tested in the biosensor and signal output in microamperes was recorded at 7 min (B). TMB product accumulation on the chromatin-coated membrane is shown (A). The top and next position used serum diluent and a normal human serum, respectively, in the first stage of the assay.

membrane was manifested after addition of H_2O_2 and TMB, the primary and secondary substrates, respectively. In order to ensure contact with the electrode of the oxidized TMB product that accumulated within and above the membrane, a brief vacuum pulse was applied just before each reading. Upon applying intermittent millisecond pulses of electrical potential, a current is generated due to the electro-reduction of TMB^+ to TMB. Current signals are measured during the last microseconds of each pulse.

Fig. 3 shows average results of signals generated from four assays of six SLE samples with various levels of anti-chromatin antibodies. A rapid rise in current occurred during the first few minutes, after which relatively stable readings were obtained. Presumably, product development over the electrodes initially occurred at maximum catalytic rate, but periodic draw-down of the substrate/product solution prevented further product accumulation, resulting in an approximate steady-state concentration of product in contact with the electrode. Decreased rate of catalysis over time may also result from substrate depletion and possibly from inhibition of peroxidase by H_2O_2 (Hernandez-Ruiz et al., 2001) or the TMB diimine oxidation product. Blue-colored product representing the charge-transfer complex of the parent diamine and the diimine oxidation product (Josephy et al., 1982) also accumulated transiently on the membrane, and the amount of deposited product approximately correlated with the amperometric readout of the sample (Fig. 4).

3.3. Comparison with ELISA

The signals produced with sera from 30 SLE patients using the electrochemical sensor were compared to those produced by ELISA, the standard and widely used method for measuring anti-chromatin antibodies. Fig. 5 compares the results of the 7-min electrical readout of the sensor with the 1 h optical density value generated in the ELISA using the same samples and detecting antibody. It should be noted that while the two assays both employed a peroxidase-based amplification systems, the sensor measured the current associated with the electrochemical reduction of the oxidized TMB product, while the ELISA simply measured the optical density of the oxidized ABTS product. The assays showed a high degree of correlation ($r = 0.89$); similar results were seen at the 3 and 5 min readings ($r = 0.87$ and 0.87, respectively). The slope of the line remained essentially unchanged between 3 and 7 min, indicating that the fluidics achieved maximum sensitivity within 3 min after addition of the substrate.

3.4. Normal serum binding in the electrochemical sensor

Although the data in Fig. 5 indicate that there was little in the way of background binding in the electrochemical sensor assay, normal sera were also tested in this system. As shown in Fig. 6 using an expanded scale, normal sera showed no net IgG binding in this assay. Some normal sera had negative values because they displayed lower background binding than the secondary antibody alone, which was subtracted from the values shown in the figures. Apparently, unknown components of some normal sera inhibit the marginal but detectable binding of the secondary antibody. Overall, normal serum binding was not a problem in this electrochemical sensor system.

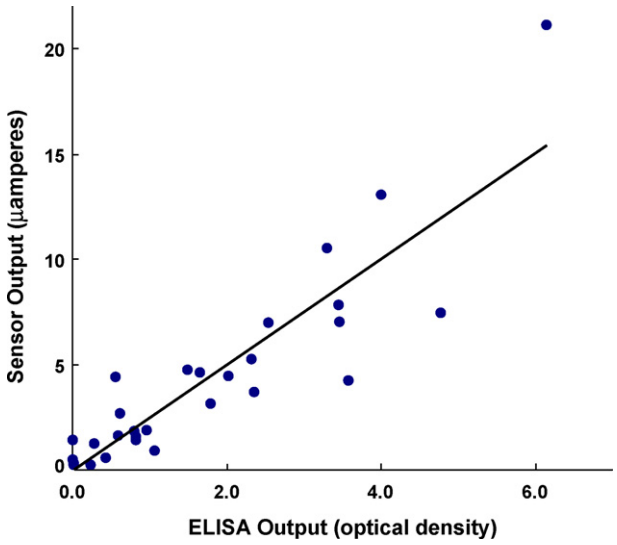

Fig. 5. Relationship between output with the biosensor vs. ELISA with a panel of SLE sera.

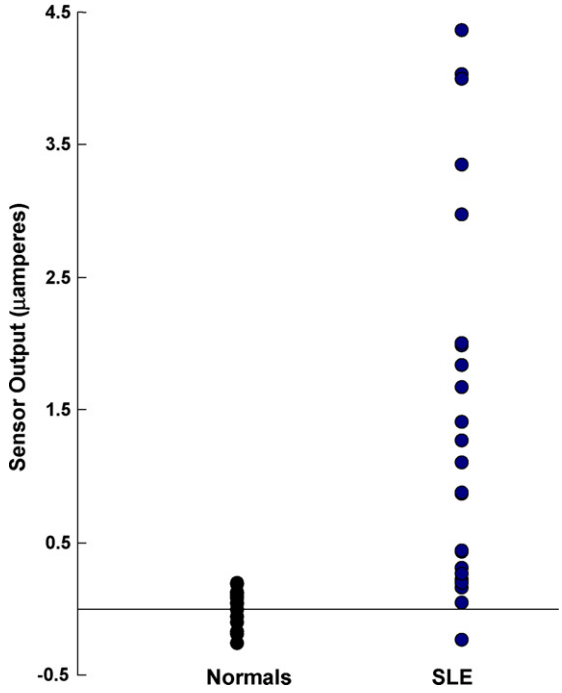

Fig. 6. Reactivity of normal human sera. Sera from normal people were applied to the biosensor and the signal output was measured at 7 min. Also shown for comparison is the reactivity of SLE sera with low and moderate anti-chromatin activity.

3.5. Quantity of immobilized antigen

We also determined the amount of protein bound to the PVDF membrane and the polystyrene microtiter plate well. The area of the ~7-mm diameter membrane exposed to the analytes contained 2.6 ± 0.26 μg (R.S.D.) protein while the ELISA plate had 0.29 μg/well when coated at 5 μg/ml (1 μg/well) and 0.35 ± 0.17 μg/well (R.S.D.) when coated at 20 μg/ml. This corresponds to 69 μg/mm^2 and up to 2.2 μg/mm^2 immobilized chromatin on the membrane and the polystyrene, respectively.

3.6. Stability of antigen-coated membrane

The effect of short-term storage of the antigen-coated membrane was investigated. As shown in Table 1, storage of chromatin-coated membranes up to 3 weeks had no effect on their antigenicity when compared to membranes prepared the day of use. This suggests that long-term storage of protein-coated membranes will be feasible.

Table 1
Anti-chromatin antibody reactivity of membranes stored for 22 days vs. used the same day of preparation.

Sample	Antibody response in μA after storage (days)[a]	
	0	22
SLE1	4.35 ± 1.61	4.05 ± 1.32
SLE2	4.70 ± 1.71	6.31 ± 0.55
NHS	0.28 ± 0.11	0.15 ± 0.12

[a] Shown are 5 min readouts (±R.S.D.) of duplicate determinations using two SLE sera with moderate anti-chromatin activity and one normal serum. Similar results were obtained at 7 min and with membranes stored for 8 and 15 days.

4. Discussion

4.1. Electrochemical sensor vs. ELISA

The present adaptation of an amperometric sensor allowed quantitative detection of a specific IgG autoantibody in human serum with negligible binding of IgG from normal serum. The strong correlation between this method and a standard ELISA for measuring anti-chromatin antibodies indicates that both assays had comparable sensitivity, specificity, dynamic range and discriminating ability. However, the electrochemical sensor system was much faster than the ELISA method and employed instrumentation that could readily be fabricated into a portable, hand-held device.

Differences in design and signal generator account for the higher sensitivity of the electrochemical sensor over the ELISA method. Because polyvinylidene fluoride membrane used as the immunoreactive solid phase in the electrochemical sensor has a microporous interior and consists of a wettable hydrophobic polymer, it has a high protein binding capacity (Starita-Geribaldi and Sudaka, 1990). Approximately 30× as much chromatin was bound to the PVDF membrane compared to polystyrene based on surface area, resulting in ~8-fold more exposed antigen, despite the antigen-coated region of the sensor occupying only one-fourth the surface area of the microtiter plate well. Together with the lower serum dilution and the use of fluidics to drive antibody into the antigen-coated solid phase, the rates of bimolecular antigen–antibody interactions were increased, resulting in an assay time of 15–20 min for the electrochemical sensor compared to 4–5 h for the ELISA. Also, the use of intermittent pulse amperometry produces substantially greater electrical signals than differential pulse or direct current amperometry due to less depletion of the analyte during the period of measurement (Wojciechowski et al., 1999).

4.2. Electrochemical sensor: outstanding problems and prospects

Minimizing molecular interactions other than those intended to detect is a critical characteristic of an immunoassay, largely determining its positive/negative discriminatory capacity. In measuring specific antibodies in non-pristine or complex fluids, binding of irrelevant immunoglobulin to hydrophobic surfaces has been a major obstacle in the development of biosensors (Veetil and Ye, 2007). By using appropriate agents to block non-specific interactions, no background binding of non-immune IgG was detected in the current system. We did not determine absolute lower-limit of detection or maximum sensitivity of the system because our goal was limited to comparison to an existing assay in a clinically relevant setting. However, it is likely that antigen detection using a capture antibody on the solid phase could achieve substantially higher sensitivity than most current methodologies because of the high protein-binding capacity of the solid phase and the combined amplifying power of the secondary antibody, enzyme substrate and electronics.

Several technical hurdles will need to be overcome to make this system widely usable as a point-of-care detector or in a remote setting. Because the electrochemical sensor involved drawing samples through a membrane that can act as a microporous filter, all the samples were pre-ultrafiltered to preclude possible problems with protein aggregates that occasionally develop upon storage and freeze/thaw cycles of serum samples. While pre-filtering should not be necessary with fresh samples or samples thawed only once, the flow-through requirement of the apparatus makes attention to this detail more important than in the ELISA method. Also, controlled fluidics will need to be incorporated into the apparatus to drive reagents and washing solutions over the electrodes to replace the external vacuum pump in the current configuration.

A secondary antibody with conjugated enzyme to amplify the signal and generate the oxidized analyte undergoing electrochemical reduction is necessary. These cumbersome steps may be minimized by engineering an auto-fluidics device, similar to a blood glucose meter (Andreescu and Sadik, 2004). Finally, while the stability of the antigen-coated membrane as well as other components of this system appears to be excellent, shelf-life issues will need to be examined in more detail.

4.3. Importance of autoantibody measurement and alternative methods

Detection of serum autoantibodies is standard practice for the diagnosis and classification of autoimmune rheumatic diseases (Sheldon, 2004) and has been increasingly used for detection of cancer (Tan and Zhang, 2008; Lu et al., 2008), neurological disorders (Zifman and Amital, 2008; Buckley and Vincent, 2005) and occupational exposure (Cooper et al., 2006). Several novel platforms have been recently adapted to measurement of autoantibodies including surface plasmon resonance imaging (Metzger et al., 2007; Buhl et al., 2007; Lokate et al., 2007; Kurowska et al., 2006), quartz crystal microbalance analysis (Drouvalakis et al., 2008) and electrical impedence spectroscopy (Balkenhohl and Lisdat, 2007). While in some cases approaching comparable sensitivity and specificity to existing methodologies, initial cost and complexity of operation suggest that these technologies would be more appropriate for a centralized clinical laboratory where multiplex analyses would be of value.

For small clinics or remote clinical settings, disposable, amperometric-based biosensors have emerged as diagnostic devices that combine point-of-care screening analysis with accurate, low cost systems and with minimal operator involvement (Ribone et al., 2006). Recent applications of this class of sensor include determination of tumor markers (Wu et al., 2007), liver disease markers (Song et al., 2007) and purified IgG antibody to West Nile virus (Ionescu et al., 2007). We believe the current system is the first report of the successful adaptation of a potentially portable electrochemical biosensor for quantitative measurement of antibodies in human serum.

5. Conclusions

The current system applied principles of electrochemical signal transduction and microfluidics to a small biosensor for the measurement antibodies to chromatin, an important autoantibody in the diagnosis and management of patients with systemic lupus erythematosus. The entire immunoassay required only 20 min processing time but produced signals of comparable magnitude to and which were highly correlative with a standard immunoassay. With the application of disposable screen-printed electrodes and further miniaturization, this device has the potential to make practical the measurement of antibodies in near real-time and in remote clinical settings where centralized diagnostic laboratories are unavailable.

Acknowledgment

This work was supported in part by a grant from the National Science Foundation to G.P.L.

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