A membrane-based displacement flow immunoassay

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Received 5 January 1998; accepted 18 March 1998

Abstract

The use of a membrane-based continuous flow displacement immunoassay for detection of nanomolar quantities of explosives is demonstrated, and the kinetics of this system are characterized through experimentation. Antibodies of 2,4,6-trinitrotoluene (TNT) are immobilized onto a porous membrane with surface reactive sites designed to facilitate the covalent binding of the antibody. After saturating the immobilized antibody binding sites with labeled antigen, target analyte is introduced in flow, and the displacement reactions are monitored using a fluorometer. The displaced labeled antigen detected is proportional to the concentration of the analyte introduced to the antibody-labeled antigen complex. Multiple assays were performed at flow rates of 2.0, 1.0, 0.50, and 0.25 mL/min using membranes saturated with varying TNT antibody concentrations. The signal intensity (i.e. the concentration of displaced labeled antigen) was independent of antibody concentration at 1.0 mL/min, but proportional to antibody concentration at 0.25 mL/min. Our data suggests that the lower flow rate created a longer interaction time between the injected analyte and the antibody-labeled antigen complex, resulting in greater displacement of the labeled antigen and higher signal intensities than seen at higher flow rates. © 1998 Elsevier Science S.A. All rights reserved.

Keywords: Explosives detection; Displacement immunoassay; Membrane; Biosensor; Immobilized IgG

1. Introduction

Immobilized biomolecules are emerging as popular analytical tools given their reusability and sensitivity. This has led to the development of a wide variety of immunoassay detection systems in numerous fields including drug testing (Parsons et al., 1993; Holt et al., 1995), medical diagnostics (Bluestein et al., 1990; Apple et al., 1995) and environmental monitoring (Van Emon and Lopez-Avila, 1992; Shriver-Lake et al., 1995). Traditional immunoassays, whether direct binding, sandwich, or competitive assay formats, measure the binding of antigen to antibody and are dependent upon the association kinetics of the antibody–antigen reaction (Rabbany et al., 1994a). However, the displacement or "flow" immunoassay (Ligler et al., 1993) stands apart from standard immunoassays that rely on the association rates of antibody to antigen. The displacement immunoassay measures the dissociation of labeled antigen from immobilized antibody in the presence of unlabeled antigen under flow conditions.

Continuous flow immunoassays possess many inherent properties which provide advantages over other detection systems. Contamination is minimal; the entire flow system and reactor are enclosed with the exception of the small port used to introduce the sample. Moreover, the numerous discrete washing steps included in traditional immunoassays are eliminated. Lastly, in contrast to their counterparts, assays conducted under continuous flow conditions normally possess rapid response times. In the aforementioned displacement immunoassay, the antibody is immobilized onto a solid support such as microporous beads which are placed under flow conditions. In Whelan et al. (1993), displaced fluorescent antigen was detected in concentrations ranging from 20 to 1200 ng/mL with a response time of 5 minutes. The overall sensitivity of the system is dependent upon the dissociation constant of the antibody (Rabbany et al., 1994b).

For bead-based displacement immunoassays, the rate at which the labeled antigen dissociated from the anti-
body in the presence of unlabeled antigen increased with decreasing flow rate because the analyte was provided more time to interact with the immobilized complex (Wemhoff et al., 1992; Rabbany et al., 1995). These experiments suggested that immobilization of an antibody and labeled antigen on a porous membrane may not provide a suitable matrix for the displacement assay because this geometry would not allow sufficient time, under flow conditions, for the unlabeled antigen to interact effectively with the complex to displace the labeled antigen.

Nevertheless, there are numerous advantages in using a membrane instead of a bead matrix. These advantages include facility and reproducibility of immobilizing the antibody, simpler insertion of the membranes into the reaction chamber, and more reproducible mass transfer characteristics for the displacement reaction. The beads are difficult to load into the column in exact quantities, and the quantity of beads in the column has a direct effect on the magnitude of the signal generated. A smaller number of beads reduces the probability that an unlabeled antigen molecule will encounter and displace a labeled antigen from the antibody, thereby reducing the signal intensity. Flow stream tunneling and the packing of the matrix in bead-based columns reduce the surface area over which the injected analyte can interact with the immobilized complex and, thus, the signal intensity generated. A membrane-based system solves these problems because it possesses a stable two-dimensional geometry with negligible height. In addition, the flow apparatus can be miniaturized for easier storage and reduced cost if a membrane replaces the beads as the matrix support for antibody immobilization.

Similar displacement assays have been performed on a membrane in a dipstick format for detection of drugs of abuse (Kidwell, 1994), and low molecular weight environmental pollutants (Behnke et al., 1996) in single-use non-quantitative assays. In comparison, the membrane-based displacement assay described herein provides quantitative information and is sufficient for testing more than 40 positive samples. In this study, the performance of a membrane-based flow immunoassay is examined and compared with the bead-based assay. This is accomplished by introducing various analyte concentrations of the explosive trinitrotoluene (TNT) into the flow system and monitoring the fluorescence of the eluant. The effects of flow rate and antibody concentration on the performance of the membrane-based detection system are characterized. The Environmental Protection Agency (EPA) has proposed 2 ng/mL TNT as the maximum limit for the lifetime health advisory level for drinking water (Jenkins et al., 1994).

2. Materials and methods

2.1. Antibody purification and immobilization

Monoclonal antibodies 11B3 (mouse IgG1) with a specificity for 2,4,6-trinitrotoluene (TNT) were generated by immunizing pristane-primed mice with 2,4,6-trinitrobenzene TNB-OVA. The IgG fraction was isolated using protein G (Whelan et al., 1993).

Porous Immunodyne ABC membranes (Pall Corp., Port Washington, NY) of 0.45 μm pore size were cut into circular disks having a diameter of 6.0 mm and height of 1.0 μm. The membrane consisted of a nylon mesh which was modified to incorporate unspecified surface reactive sites that facilitated the covalent binding of proteins, water, and other compounds containing nucleophilic groups. The membranes were incubated for three hours in 50 μL of TNT antibody solutions ranging in concentrations from 0.35 to 2.8 mg/mL. After immobilizing the antibody, the membranes were placed into 100 μL of a 5 mg/mL Hammarsten casein solution containing 0.01% Triton X-100 for one hour in order to block any remaining binding sites on the membrane.

For immobilization on porous beads, protocols as described previously were followed (Bart et al., 1996). Briefly, 0.3 mg/mL of anti-TNT antibody was covalently attached to 100 μL of polyacrylamide beads (Empath AB1, Pierce, Rockford, IL). The beads had an average diameter of 60 μm and contained an azlactone group capable of forming a stable bond with any primary amine group of the antibody (i.e. lysine groups).

2.2. Labeled antigen saturation

To prepare for saturation with labeled antigen, the membranes were washed three times with 100 μL of phosphate buffer solution (PBS) containing 0.01% Triton X-100, in order to remove nonspecifically-bound proteins. The membranes, or a 100 μL aliquot of the beads, were then dispensed into a small disposable flow-through column containing a frit at its base (Isolab, Inc., Akron, OH), with a 100 μL head volume. The membrane was secured in place by using a barrel of a 1 mL plastic syringe, cut to the proper height. The available antibody binding sites were saturated with 50 μL of a 30 μM fluorophore-labeled antigen (CY5-TNB) solution and incubated at room temperature overnight. The particular sulfoindocyanine dye used for the analyte labeling was used because of its superior physical properties, such as solubility, high quantum yield, and spectral characteristics (Bart et al., 1996).

2.3. Membrane incorporation into the flow system

The membrane was tested in the laboratory flow system format consisting of a Rabbit-Plus peristaltic pump...
(Rainin Instruments, Emeryville, CA) and a fluorescence detector (Jasco Model 821-FP, Easton, MD) equipped with a 12 μL flow cell. A 10 mM PBS solution containing 2.5% ethanol, to ensure solubility of the TNT and a surfactant, 0.01% Tween-20, was pumped through the flow system in order to remove nonspecifically-bound fluorophore-labeled antigen and to increase the speed of the displacement reaction.

The experiments were run at flow rates ranging from 0.25 to 2.0 mL/min, while the assay eluant was monitored at an excitation of 635 nm and an emission of 661 nm. When the background fluorescence stabilized, 100 μL samples of analyte diluted with the buffer solution were introduced into the flow using a Rheodyne five-way valve sample injector. Analyte injections, which ranged from 5 to 1200 ng/mL, were made in triplicate. A 1000 ng/mL concentration of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) was used as negative control, since TNT and RDX are the two most commonly used explosives and are often mixed together to form military munitions. A Hewlett Packard integrator (Palo Alto, CA) was used to record and quantify the displaced labeled antigen.

3. Results

3.1. Dose-response curves for membrane and bead assay

Standard curves are illustrated for a membrane prepared using 8.75 nmol/mL TNT antibody and for a bead-based assay, conducted earlier using 5.0 nmol/mL TNT antibody (Fig. 1). These two curves display the signal reproducibility between the triplicate injections of various analyte concentrations over membranes and beads subjected to the same flow conditions. The signal intensities between the two standard curves for analyte concentrations ranging from 5 to 600 ng/mL are similar.

3.2. Repetitive displacement studies

To examine the reproducibility of signal intensity, a repetitive displacement assay was conducted using a single membrane at varying flow rates. The membrane was incubated in an antibody solution of 8.75 nmol/mL prior to use and the flow rate was varied from 2.0 to 0.25 mL/min. The signal intensities measured for six repetitive 100 μL injections of a 1200 ng/mL analyte solution are shown in Fig. 2. An increase in signal intensity is shown to correspond to a decrease in flow rate. Moreover, the statistical significance of the increase in signal intensity with a decrease in the flow rate is supported by the paired t-test. At the 0.25 mL/min flow rate, the fluorescence signal began to decrease with each additional injection, suggesting depletion of the bound labeled antigen on the membrane.

3.3. Effect of antibody density

The effect of antibody concentration on signal intensity is depicted in Fig. 3. Fig. 3A shows the data obtained at flow rate of 0.25 mL/min for three membranes that had been prepared using antibody solutions of 2.2, 4.4, and 17.5 nmol/mL, respectively. The signal intensity was observed to increase proportionally with the antibody concentration at each particular analyte injection. In contrast, at a higher flow rate of 1.0 mL/min (Fig. 3B), the signal intensity failed to show the same direct relationship with antibody concentration, due to the decrease in the time available for displacement. The detection limit remained in the low nanomolar range for both flow rates and all antibody concentrations.

3.4. Membrane lifetime

The ability to use the membrane-based immunoassay for multiple assays is illustrated in Fig. 4. Using a single membrane for 35 consecutive 100 μL injections, reproducible signals were obtained for samples ranging in concentration from 5 to 1200 ng/mL at a flow rate of
Fig. 2. Repetitive displacement assay using an analyte concentration of 1200 ng/mL injected into the same membrane column at flow rates of 2.0, 1.0, 0.5, 0.25 mL/min. A paired t-test determined the statistical significance for the signal intensities obtained at each flow rate.

1.0 mL/min. These results indicate the reaction is reproducible and that the membranes can be used for repeated analysis. In situations where many of the samples passed through the membrane contain no more than a very low concentration of the antigen of interest, we have run more than 50 samples on a single membrane.

4. Discussion

An understanding of the displacement characteristics of the membrane-based immunoassay is critical to the development of a highly sensitive and consistent detection system. We compared the performance of the membrane-based assay with that of the bead-based assay and observed similar relationships between signal intensity and concentration. As mentioned earlier, numerous advantages can be cited in favor of using a membrane as the support matrix for the displacement reaction. The inability to pack the reaction chamber with porous beads uniformly and precisely causes channeling (i.e. creation of an inhomogeneous flow rate) and hence, reduces the reproducibility and sensitivity of the signal. Re-association between the displaced labeled antigen and the immobilized antibody can occur in a bead immunoassay as a result of the matrix’s height, which reduces the displacement signal. In contrast to the bead-based assay’s columnar geometry, the membrane’s disc geometry minimizes re-association of the displaced labeled analyte. In addition to ease of assembly, membrane immunoassays enable repetitive assays to be performed without loss of sensitivity. The membrane-based immunoassay can continually achieve a sensitivity of approximately 5 ng/mL using a 100 μL sample of injected TNT with an assay time of 2–4 minutes, until the CY5-TNB becomes depleted significantly.

To further characterize the performance of the displacement immunoassay on a membrane, two factors, flow rate and antibody concentration, were investigated. When the flow rates were reduced from 2.0 to 0.25 mL/min, higher signal intensities resulted, presumably due to an increase in the interaction time between the injected analyte and the antibody-labeled antigen complex. This confirms our preliminary studies on membranes (Rabbany et al., 1996) and parallels earlier experiments that demonstrated a similar flow rate dependency in both the bead-based displacement immunoassay (Wemhoff et al., 1992) and a membrane-based sandwich immunoassay. Our experiments also indicated that antibody concentration had a profound effect on signal...
Fig. 4. Reusability of a membrane assay with increasing concentrations of analyte. This figure illustrates that highly reproducible signal intensities were obtained for 35 injections of monotonically increasing concentrations of analyte.

intensity, but only at lower flow rates. At higher flow rates, such as 1 mL/min, the generated signal remained independent of the antibody concentration. The lower residence time for the injected analyte associated with the higher flow rate explains this phenomenon. Under such conditions, the amount of antigen which has an opportunity to interact with membrane-bound antibody may be limiting, as opposed to the amount of antibody available for interaction.

Because immunoassay times and sensitivities will vary with assay format, they should be selected to meet user requirements. For example, this anti-TNT monoclonal antibody has been used in ELISA’s (Whelan et al., 1993), competitive fiber-optic biosensors (Shriver-Lake et al., 1995), displacement microcapillary assays (Narang et al., 1997), bead-based assays (Rabbany et al., 1997), and the membrane displacement assays reported here. The ELISA’s required multiple incubation/reagent steps and 2–3 h to complete. Recent displacement assays using microcapillaries as the solid substrate have shown two to three orders of magnitude increase in sensitivity over the bead-based assay, but had run times of 15 minutes compared to 1 minute (Narang et al., 1997). Results from the membrane assay showed a marked improvement in reproducibility over the previously-reported bead-based methods. Also, while the membranes had sensitivity comparable to the bead columns, they provided significant advantages in terms of preparation, insertion into the immunosensor, stability in flow, and reproducibility. Moreover, the data suggest that the membranes can be reused more often than bead columns with comparable amounts of antibody–antigen complex immobilized, even when all the samples added are positive. It may be that the two-dimensional geometry of the membrane reduces the potential for rebinding of dissociated labeled antigen to immobilized antibody, partially offsetting the reduced interaction time between the initial displacement of labeled antigen and unlabeled antigen.

Additional experiments are currently underway to elucidate concerns pertaining to assay reproducibility, sensitivity, and detection limit. For realistic application in the detection of low molecular weight molecules, the potential nonspecific interaction of the membrane with samples containing mixture of analytes must be considered. In this vein, the expansion of the membrane-based immunoassay to include other antigens and to perform simultaneous multianalyte tests should be attainable. Variations can be made in the flow rate, antibody concentration and column size to optimize the assay for both sensitivity and functionality.

In conclusion, a flow immunosensor has been implemented for repetitive displacement of explosives using a single disposable membrane. Despite the very short contact time, the target analyte can be detected in the low nanomolar range. Thus, an immunosensor incorporating the membrane-based assay has proven to be rapid, reusable, and reagentless while providing a quantitative assessment of antigen concentration.

Acknowledgements

This work was supported by the Strategic Environmental Research and Development Program (SERDP) in addition to the Environmental Security Technology Certification Program (ESTCP) and by a grant to Hofstra University from the Naval Research Laboratory. The views expressed are those of the authors and do not represent those of the US Navy or the Department of Defense.

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