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Short communication

Rapid antibody screening by membrane chromatographic immunoassay technique

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Abstract

This paper describes a novel membrane chromatographic immunoassay technique suitable for rapid screening of antibodies in serum samples. This technique could potentially be utilized for antibody screening in situations where screening for exposure to one of several possible antigens is required. A synthetic microporous membrane is first selectively loaded with antibodies from the test serum sample by hydrophobic interaction. The in situ affinity membrane thus formed is sequentially pulsed with the antigens corresponding to the antibodies being screened. From the antigen chromatogram peak profile thus obtained, inferences about the antibodies present in the serum sample can then easily be made. This technique in addition to being rapid and direct is conceptually simple, and does not use any expensive media or reagents. It would potentially be useful for rapid diagnosis of infections as well as for rapid assessment of conditions such as envenomation or exposure to toxic substances.
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1. Introduction

The use of membranes for immunological assays is widespread, primarily on account of the high analyte-binding surface area available. Membranes are mainly used as support matrices for immobilizing immunological analytes at specific locations as in Western transfer immunoassay [1] or in dot blot immunoassays [2]. Membranes could also be used in a manner similar to thin layer chromatography where the analytes are allowed to be transported in a transverse direction within the membrane using a solvent moving by capillary action or by forced flow [3,4]. Yet another way in which membranes are utilized in immunoassays is as porous convective flow-through media [5–7]. In all of these different approaches, the membrane provides a solid surface on which antigen–antibody recognition and binding is localized, thus facilitating observation and quantification.

Conditions such as microbial infections, envenomation and exposure to toxic substances lead to immunological responses in the host if the intruding agent is antigenic in nature. This usually results in the formation of antibodies towards these antigens. Therefore, an indirect method by which these conditions can be diagnosed is by looking for the specific antibodies in the host system, e.g. streptococcal infection can be diagnosed from the presence of anti-streptococcal antibodies in the serum. Most immunoassays for detecting antibodies towards intruding agents look for one specific antibody at a time. Moreover, conventional immunoassays are notoriously slow and involve multiple steps and use of multiple reagents. Therefore, in situations where it is required to screen for one of several possible intruding agents, systematic diagnosis using such conventional techniques could be time consuming. Ideally, a rapid single test which could look for several possibilities at the same time would be desirable. In a recent paper the author has described a membrane chromatographic immunoassay method for the rapid detection and quantitative analysis of a specific serum antibody [7]. Membrane chromatography relies on convective transport of analytes to their binding and recognition sites on the membrane surface and is hence fast. The immunoassay technique described in the current paper is suitable for rapid serum antibody screening which could potentially be utilized in the situation described in the previous paragraph, i.e. where screening for exposure to one of several possible antigens is required.

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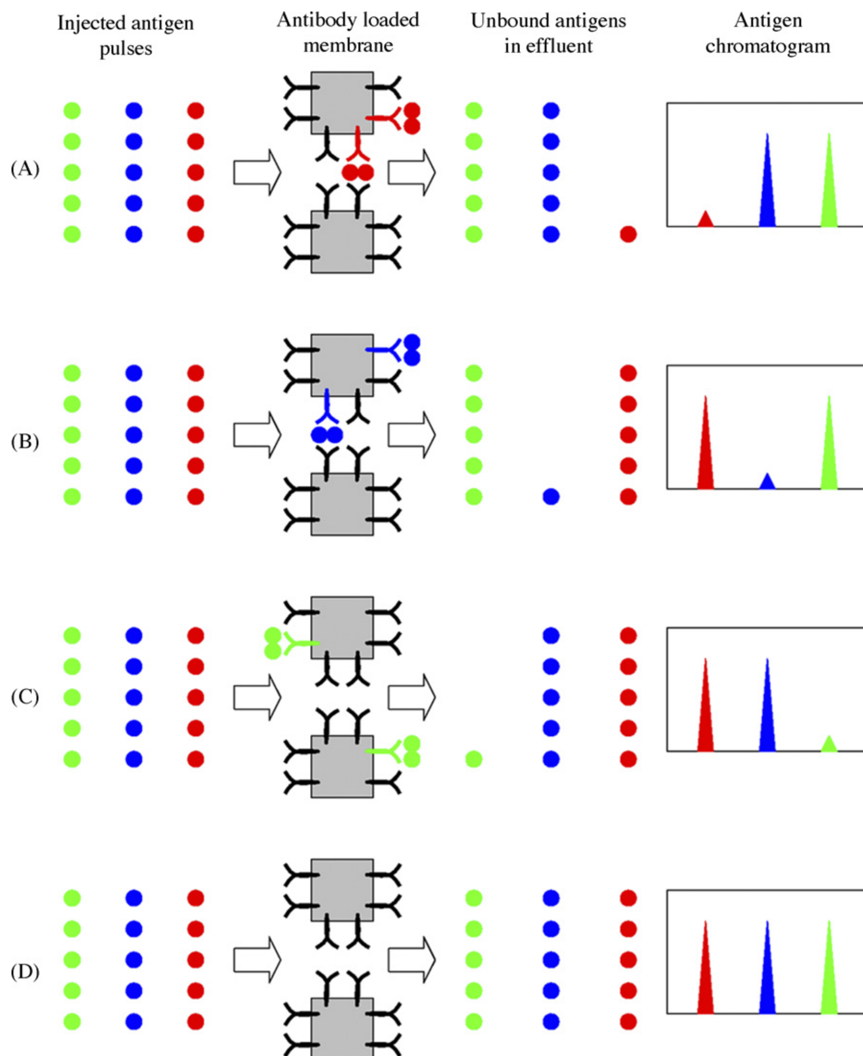

Fig. 1. Scheme for antibody screening using the membrane chromatographic immunoassay technique (three antibodies and their corresponding antigens are like color coded, i.e. red, blue and green; all other serum antibodies are represented by the black antibody molecules).

The working principle of the immunoassay technique is shown in Fig. 1. The antibody molecules present in a test serum sample are first reversibly immobilized on a microporous membrane by pulsing it. This antibody binding which is mediated by hydrophobic interactions in presence of a high anti-chaotropic salt concentration is selective in nature, i.e. only antibody molecules bind to the membrane. The unbound non-antibody proteins present in the serum sample are then washed out of the membrane in order to prevent non-specific protein–protein interactions. The in situ affinity membrane thus formed by antibody coating on the synthetic membrane is then sequentially pulsed with all the antigens, the antibodies corresponding to which are being screened. If the serum sample were free from the antibodies being screened, the antigens pulses would pass through the membrane unhindered and show up as chromatographic peaks using appropriate flow-through antigen detectors placed downstream to the membrane (see Fig. 1D). From this, a control reference antigen chromatographic profile is obtained. If the antibody corresponding to an antigen were present in the serum sample being tested, the peak corresponding to that particular antigen would be attenuated in the chromatogram thus obtained relative to the control reference antigen chromatographic profile (see Fig. 1A–C where the antigen–antibody pair are color coded). Based on this, information about a possible infection or envenomation or indeed exposure to a toxic substance could be rapidly inferred. Proof of concept of this novel immunoassay method is provided by screening for antibodies in rabbit serum against three protein antigens: human hemoglobin, human serum albumin and ovalbumin.

2. Experimental

2.1. Material

Human serum albumin (200 g/l) solution was kindly donated by the Scottish National Blood Transfusion Services UK. Chicken egg albumin or ovalbumin (catalogue no. A-2512), human hemoglobin (catalogue no. H-7397), anti-human serum albumin (whole antiserum raised in rabbit, catalogue no. A-3293), anti-chicken egg albumin (whole antiserum raised in

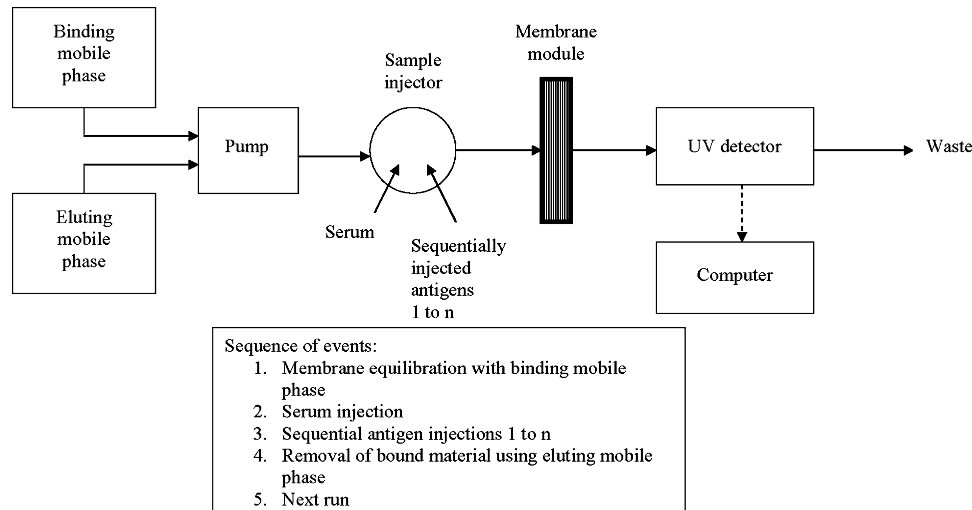

Fig. 2. Experimental set-up and methodology for the immunoassay technique.

rabbit, catalogue no. C-6534), anti-human hemoglobin (whole antiserum raised in rabbit, catalogue no. H-4890), standard rabbit serum (catalogue no. R9133), sodium phosphate (mono- and di-basic) and ammonium sulphate were purchased from Sigma. PVDF microfiltration membrane (hydrophilic PVDF, 0.22 μm pore size, GVWP) was kindly donated by Millipore. All sample solutions used in the immunoassays were prepared using ultrapure water (18.2 MΩ-cm) obtained from a NANOpure Diamond (Barnstead) water purification unit. 20 mM sodium phosphate buffer (pH 7.0) was used as the eluting buffer while 1.5 M ammonium sulphate solution prepared using the eluting mobile phase was used as the binding buffer. All buffers were microfiltered and degassed prior to use.

2.2. Experimental set-up

An AKTAprime liquid chromatography system (Amersham Biosciences) was used for carrying out the immunoassays as shown in Fig. 2. A membrane module within which stacks of disc membrane could be housed was designed and fabricated in-house. A stack of five membrane discs each having effective diameter of 18.5 mm was housed within this module. A 2 ml sample loop was used for loading the serum samples on to the membrane while the antigens were pulsed using a 100 μl sample loop. The UV absorbance (at 280 nm) of the effluent stream from the membrane module along with the system pressure were continuously recorded and logged into a computer using PrimeView (Amersham Biosciences).

2.3. Experimental methods

The sequence of events in the immunoassay experiments is outlined in Fig. 2. All experiments were carried out at ambient temperature in the laboratory, i.e. 24 °C. The three antigens selected for this study were human hemoglobin, human serum albumin and ovalbumin, all of which could be detected at 280 nm wavelength using a flow through UV detector. Previous experiments had shown that satisfactory rabbit antibody loading on the microporous PVDF membrane took place in the presence of 1.5 M ammonium sulphate concentration. Therefore, all serum and antigen samples used in the immunoassay contained ammonium sulphate at this concentration. All membrane chromatographic experiments were carried out at 2 ml/min flow rate.

The serum samples for antibody screening were prepared by mixing 0.1 ml of rabbit serum with 1.4 ml of 20 mM sodium phosphate buffer (pH 7.0) and 1.5 ml of 3 M ammonium sulphate solution also prepared using the same buffer. These samples were then centrifuged at ambient temperature for 20 min at 10,000 rpm (9.3 rcf) in a micro centrifuge (Eppendorf, 5415D). In each immunoassay experiment 2 ml of centrifuged serum sample was injected into the membrane module followed by washing out of unbound non-antibody proteins using the binding buffer. Once the UV absorbance reached the baseline and stabilized indicating that all unbound proteins had been removed from the membrane module, the antigen solutions were sequentially pulsed through the membrane module followed by their detection using the flow through UV detector. The antigen solutions used were 0.05 mg/ml each of human hemoglobin, human serum albumin and ovalbumin all prepared using the binding buffer and 100 μl of each antigen was injected. At the end of each experiment, the material bound on the membrane was completely removed by switching over to the eluting buffer, thus making the membrane ready for the next experiment. Each immunoassay took approximately 6 min to complete.

3. Results and discussion

Prior to carrying out the immunoassay experiments simple chromatographic adsorption tests demonstrated that none of the three antigens bound to the PVDF membrane in presence of 1.5 M ammonium sulphate concentration at 0.05 mg/ml protein concentrations in each case. For this immunoassay to work, it is critically important that the antigens do not bind on the native membrane. Another key requirement is that the antigen–antibody binding on the membrane should deplete enough antigen from the injected pulse for there to be a notice-

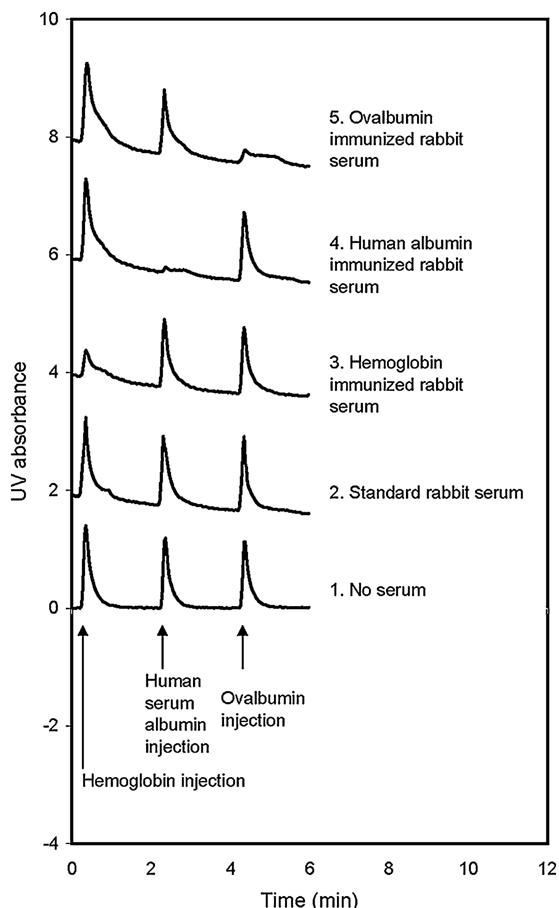

Fig. 3. Antigen profiles obtained from multi-immunoassay experiments (three antibodies were screened in this study).

able effect on the antigen peak. Loading less amounts of antigen would guarantee this but the amount of antigen injected should also be significantly higher than its detection limit to ensure reliable results. Extensive initial trial-and-error experiments were carried out to identify the operating serum and antigen concentrations for the immunoassay technique. Membrane fouling is a major problem in membrane based techniques. Extensive fouling could potentially reduce the reliability and reproducibility of the immunoassay technique. Fouling is influenced by the analyte concentration and type as well as by the permeate flux, i.e. the rate at which the analyte solution is pushed through the membrane. Initial experiments were also carried out to identify testing conditions (i.e. analyte concentration and flow rate) at which fouling would be acceptably low.

Fig. 3 shows a summary of the antigen chromatograms obtained from five immunoassay experiments. In experiment 1, the three antigens were sequentially pulsed through a native membrane stack, i.e. not loaded with any antibody, the purpose of this being to obtain a standard antigen profile. As can be seen in the figure, this resulted in three peaks of roughly similar heights. In experiment 2, the membrane was first loaded with a standard rabbit serum sample, i.e. not containing antibodies toward any of the antigens being tested. By pulsing the three antigens sequentially through the membrane the control reference antigen profile was obtained. By comparing the standard antigen profile with the control reference it is evident that the amount of non-specific antigen binding on the standard rabbit serum antibodies was negligible. The only difference resulting from the presence of the loaded antibodies was the introduction of a slight baseline drift in the antigen chromatogram. In all subsequent experiments carried out using antibody loaded membranes, this type of baseline drift was observed. However, the magnitude of drift was not significant enough to cause problems in data interpretation.

Experiment 3 was carried out by loading the membrane with a serum sample obtained from a rabbit immunized with human hemoglobin. The antigen profile thus obtained (see Fig. 3) clearly shows that the hemoglobin peak was significantly smaller than those in the standard antigen profile and in the control reference antigen profile while the human albumin and ovalbumin peaks were largely unchanged. This indicates that the hemoglobin bound to the anti-hemoglobin antibody molecules present on the membrane while the two other antigens, i.e. human serum albumin and ovalbumin passed through unhindered. Experiment 4 was carried out by loading the membrane with a serum sample obtained from a rabbit immunized with human serum albumin. The antigen profile obtained in this experiment (see Fig. 3) clearly shows that the human serum albumin peak almost disappeared indicating that this protein was very effectively captured by the anti-human albumin antibody molecules that had been loaded on the membrane prior to antigen injection. The hemoglobin and ovalbumin peaks appeared normally in the antigen profile indicating that the serum sample did not contain antibodies towards these proteins. Experiment 5 was carried out by loading the membrane with a serum sample obtained from a rabbit immunized with ovalbumin. The antigen profile thus obtained (see Fig. 3) shows that the ovalbumin peak was significantly attenuated when compared to that in the control reference profile. However, the hemoglobin and human serum albumin peaks appeared normally in the antigen profile indicating that the serum sample did not contain antibodies towards these proteins. These results quite clearly demonstrate that the immunoassay technique could indeed be used for rapid antibody screening. Repeat experiments carried out using the same membrane as well as with fresh membranes showed similar profiles. For a particular antigen its peak was found to be within plus or minus 5% when its corresponding antibody was absent in the serum sample being tested. When its corresponding antibody was present, there was a run-to-run variation of plus or minus 15% in terms of the area of the attenuated antigen peak. This was due to the fact that small peak areas were being compared. In the current work, proof-of-concept of the technique is provided by screening for antibodies towards three protein antigens. Quite clearly there was negligible interference between the different antigen–antibody interactions involved in the screening tests. Therefore, in theory many more antibodies could potentially have been screened using this immunoassay technique.

In the current study, the antigens being proteins a flow-through UV absorbance detector satisfactorily served as the antigen detector. Other properties of molecules such as intrinsic fluorescence and visible light absorption could also be used to measure antigen concentrations where applicable. The use of labeled

antigens (e.g. fluoro-, chromo- or radio labeled) could potentially significantly increase the sensitivity of this immunoassay. Using the current UV absorbance based approach the antigen detection limit was in the range of 0.25–0.5 μg protein.

Most membrane immunoassays are based on non-specific protein binding on membranes which could lead to interference due to non-specific protein–protein interactions. Moreover, reliance on non-specific binding results in low antibody or antigen loading on the membrane due to competition for binding sites. In most immunoassays, the antigen–antibody recognition usually takes place at physiological conditions which also favour non-specific protein–protein electrostatic interactions. Several selective pressures were utilized in the current immunoassay to increase accuracy and reduce interferences. First of all, the selective binding of antibody molecules from the serum samples at high ammonium sulphate concentration ensured that non-specific protein–protein interactions of the antigens with non-antibody proteins were avoided. The use of a selective antibody binding mechanism also increased the amount of antibody loaded. Conducting the antigen–antibody recognition at a high salt concentration ensured that non-specific antigen binding due to electrostatic interactions was also minimized. Another advantage of using hydrophobic interaction for antibody immobilization on the membrane is that the binding is not sensitive to the presence of most other substances as long as the ammonium sulphate concentration is maintained above the threshold needed for binding. Yet another advantage of using hydrophobic interactions for antibody immobilization is that the protein binding does not take place due to specific ligand–molecule interactions and hence the Fab sites on the antibody molecules are expected to be more accessible for antigen binding than in ion-exchange and affinity based antibody immobilization. A major requirement with immunological assays is that the assay conditions do interfere with antigen–antibody binding. There is enough evidence to suggest that hydrophobic interactions are crucial for antigen–antibody binding [8–10]. Srinivasan and Ruckenstein [11] have suggested that van der Waals forces which also play an important role in antigen–antibody binding would increase in the presence for anti-chaotropic salts such as ammonium sulphate. Hence, the high ammonium sulphate concentration at which the immunoassay is carried out rather than interfering with the immunological recognition and binding has a benign effect on such interactions.

4. Conclusion

A membrane chromatographic immunoassay method suitable for rapid antibody screening is discussed in this paper. This technique is direct and does not require the use of expensive reagents and media. Proof of concept of this technique is provided by screening for rabbit antibodies against three protein antigens. The experimental results clearly show that there is negligible interference in the recognition and binding of the different antigen–antibody pairs. The use of hydrophobic interactions for antibody immobilization ensures high and selective loading on the membrane thereby eliminating non-specific interactions between antigens and non-antibody serum proteins. Carrying out the antigen–antibody binding in the presence of high salt concentration also eliminates non-specific electrostatic interactions. This immunoassay technique could potentially be utilized for antibody screening in situations where screening for exposure to one of several possible antigens is required.

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