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New immunoassay technique using antibody immobilized on a membrane and a flow cuvette as reaction vessel

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Immunoenzymatic detection systems have been developed using human IgG as a model antigen. A membrane with covalently immobilized specific antibodies was placed into a specially constructed ultranarrow flow cuvette and solutions containing the antigen and antibody-peroxidase conjugate were then successively passed through the flow capillary cell. After washing, the membrane was placed into the substrate solution and the intensity of developed colour on the membrane was recorded visually or by a reflection spectrophotometer. The lower detection limit was about 5×10^{-11} M and the overall analysis time was 10 min. Photoimmobilization was used to immobilize the antibody and thereby permitting control of the protein surface concentration on the membrane as well as the dimensions and shape of the activated region.

Key words: Enzyme immunoassay; Dot analysis; IgG; Flow injection analysis; Peroxidase

Introduction

Many of the widely used solid-state enzyme immunoassays (EIA) are based on immobilization of antibodies on membrane supports followed by formation of specific immunocomplexes with the antigen to be analyzed on the matrix surface and the subsequent detection of these complexes by enzyme-labelled antibody (Tijssen, 1985; Dzantiyev et al., 1987). The major disadvantages of these techniques are their multiple-step design and the fact that they are time-consuming (Osipov et al., 1989). Immobilization on the support as a necessary condition of the heterogeneous EIA procedures facilitates the elimination of non-bound components. However, it also leads to diffusion limitations related to the necessity for antigen molecules and antibody-enzyme conjugate molecules to migrate toward the matrix surface for immunobinding. The result is a fairly time-consuming assay. One way to solve this problem is to shorten the diffusion distance by using a narrow flow cuvette for the immunochemical reactions. In this article we describe an approach for conducting the membrane EIA in a narrow flow cell which considerably reduces the overall time of analysis.

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Abbreviations: EIA, enzyme immunoassays; HRP, horseradish peroxidase; DABA, diethylacetal of *p*-azidobenzaldehyde; KS-49, regenerated cellulose on nylon net; DAB, 3,3′-diaminobenzidine tetrahydrochloride; PBS, phosphate-buffered saline; PBST, PBS, containing 0.05% Tween 20.

Materials and methods

Horseradish peroxidase (HRP), specific activity 830 IU/mg, RZ $= A_{403\ nm}/A_{280\ nm} = 3.0$, was purchased from NPO Biolar (Latvia). Sodium periodate was obtained from Merck. Diethylacetal of *p*-azidobenzaldehyde (DABA) was originally synthesized by A.A. Cshegolev at Chemical Enzymology Department of Moscow State University. The antisera to human IgG and HRP were obtained by immunizing rabbits with proteins. The following membrane matrices were used: chromatography paper FN-11 (Filtrak, Germany), regenerated cellulose on nylon net (KS-49, Polymersynthesis, Vladimir, Russian Federation). 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 3,3′-diaminobenzidine tetrahydrochloride (DAB) were purchased from Sigma. The colour on the strips was quantified using the Shimadzu dual wavelength flying-spot scanner CS-9000.

Preparation of IgG-enzyme conjugate

The antiserum to human IgG was raised in rabbits and antibodies were obtained from the serum by fractionation with ammonium sulphate followed by passage through a DEAE-Toyoperl 650 M (Toyo, Soda, Japan) column. Rabbit IgG-peroxidase conjugate was obtained using the periodate method (Nakane and Kawaoi, 1974). The conjugate was purified on a Toyopearl HW 55 column (90 × 3 cm id.).

Immobilization of immunoglobulins on the membrane supports

For sensitization, the matrices were placed in a benzene solution of diethylacetal of *p*-azidobenzaldehyde (0.45 mg/ml), then dried in air at room temperature in the dark. The deuterium lamp of a Shimadzu dual wavelength flying-spot scanner CS-9000 was used for illumination (model DS-350U, 30 W) (the matrices were placed on the scanning stage of the CS-9000). Then the matrices were washed with ethyl alcohol and treated with 0.1 M HCl for 15–30 min.

DABA-activated matrices were placed in immunoglobulin solution (1×10^{-6}–1×10^{-8} M) in 0.01 M phosphate buffer (PBS) (pH 7.4; 0.14 M NaCl). The matrices were incubated for 40 min at room temperature or for 12–14 h at 4°C. Then the supports were treated with $NaBH_4$ solution in PBS (1 mg/ml) for 15 min at room temperature. Noncovalently bound proteins were desorbed by washing the membrane in PBS with 0.05% Tween 20 (PBST).

The construction of the narrow capillary membrane cell

The specially constructed flow cuvette consisted of two plates which could be bolted together tightly. There was a small flow channel with a depth of 0.5 mm between two spacers placed between the plates. A piece of membrane (KS-49) with immobilized specific antibodies was placed in the channel and the cell hermetically sealed by a rubber gasket around the flow channel. The surface dimensions of the working space in the cuvette were approximately 10 × 2 × 0.5 mm.

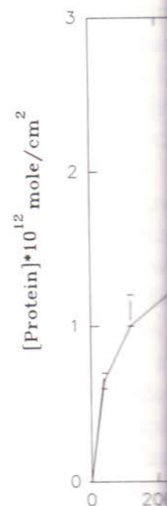
Enzyme immunoassay in the narrow capillary membrane cell

A matrix strip was placed in the flow capillary cell and the following solutions were pumped successively through the cuvette: 10^{-11}–10^{-9} M human IgG solution in PBST; 10^{-8}– 5×10^{-9} M specific antibody-horseradish peroxidase conjugate solution in PBST; and the washing buffer (PBST). The pumping rate was also varied. After pumping, matrices were taken out and developed in the standard detection solution of 0.5 mg/ml DAB, 3×10^{-3} M H_2O_2 and 0.02% $CoCl_2$ in PBS. The intensity of staining was then measured using a Shimadzu CS-9000 instrument.

Results and discussion

Immobilization of immunoglobulins on the photoactivated matrix

Different porous supports with photoimmobilized antibodies were used to analyze biologically active compounds. A two-step immobilization procedure was chosen as a method for immobilization of immunoglobulins, to control the quantity of immobilized protein and also to create a number of variably activated zones on the matrix (Kazanskaya et al., 1989).



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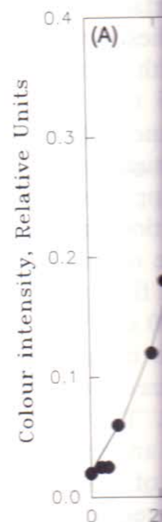

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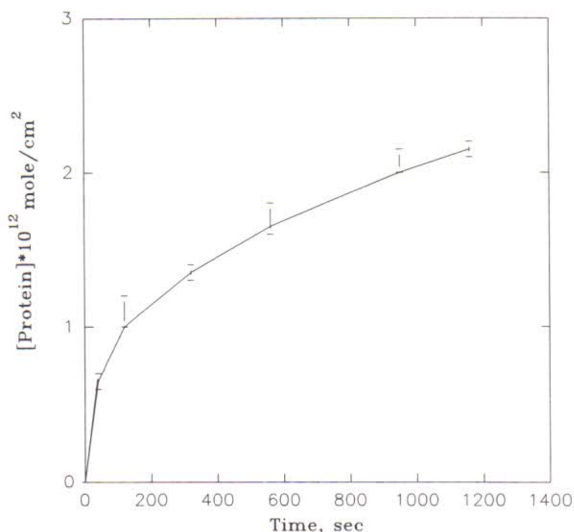
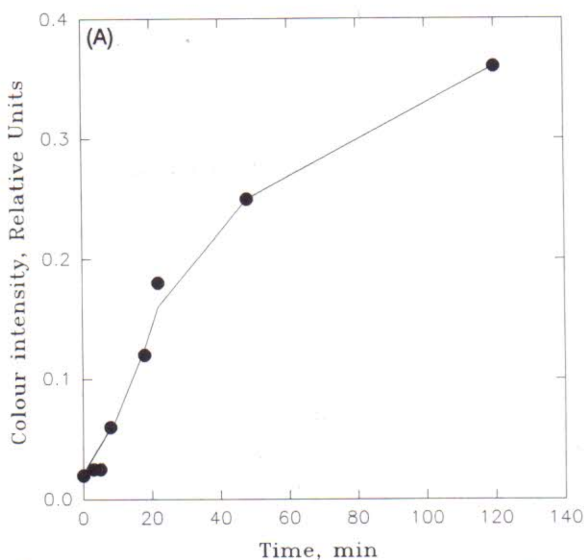


Fig. 1. Surface protein (conjugate IgG-HRP) concentration on an FN-11 matrix versus illumination time. Impregnated DABA matrices were activated by UV light from a deuterium lamp of CS-9000 with a wavelength of 300 nm.

Of the various compounds proposed as photoactive reagents for protein photoimmobilization on solid supports (Kazanskaya et al., 1989), the best signal/background ratio was obtained for PABA. This compound is water-insoluble and it is, therefore, necessary to use only water-free organic solutions. This feature strongly restricts a choice of matrix which can be used as a support for antibody immobilization in organic solvents since many are degraded. The PABA absorbance range is from 200 to 320 nm with a maximum at about 250 nm; the effective photolysis constant is 5.8×10^{-3} s^{-1} (Kazanskaya et al., 1989). Being UV-light sensitive photoreagent molecules localized on the surface of the matrix are transformed into nitrene derivatives which react covalently with the matrix base extremely efficiently.

The covalent immobilization of protein on the activated matrix is then accomplished by formation of the Schiff's bases between amino groups of the protein molecule and aldehyde groups formed on the surface of matrix. Any UV light source can be applied for the photoactivation by PABA. Fig. 1 shows the apparent surface concentration of covalently bound IgG-HRP conjugate on the FN-11 matrix versus D2 lamp illumination time. The quantity of covalently bound protein on the matrix was determined by subtraction of non-



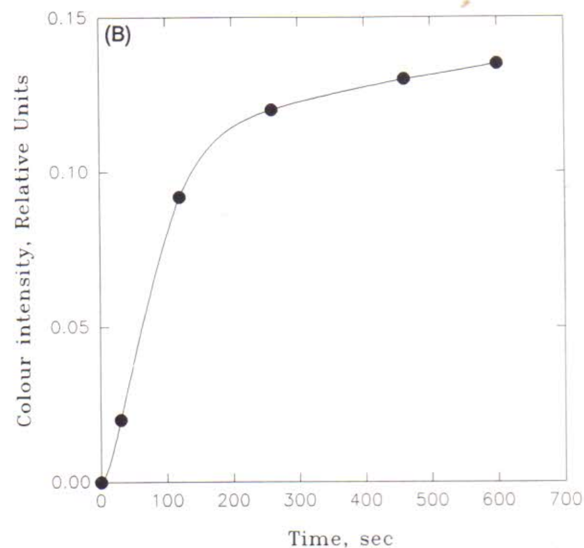


Fig. 2. *a*: kinetics of the interaction between antigen (HRP) and photoimmobilized antibodies. The reaction was carried out under nonflow standard conditions. The initial concentration of antigen was 3×10^{-10} M and the time for colour development was 3 min. A substrate solution containing 0.5 mg/ml DAB, 3×10^{-3} M H_2O_2 and 0.02% $CoCl_2$ in PBS was used for development. *b*: kinetics of the interaction between antigen (HRP) and photoimmobilized antibodies. The solution of HRP was passed through the narrow flow cell. The flow rate was 0.35 ml/min. The initial concentration of antigen was 3×10^{-10} M. The time for colour development was 3 min and a substrate solution of DAB was used for development.

covalently bound protein from its total quantity on the support (the conjugate IgG peroxidase was used). The increase in immunoglobulin density on the matrix is linear until 4–5 min of illumination with this kind of light source have elapsed. The matrix is saturated with protein after 30–35 min of illumination. The maximum apparent surface concentration of the immobilized antibody on the matrix was 2×10^{-12} mol/cm^2. This value is comparable to the value characterizing the quantity of antibodies adsorbed on the walls of polystyrene microplates for enzyme immunoassays (Cantarero et al., 1980).

Enzyme immunoassay using a flow cell

We have examined experimentally our supposition that decreasing the diffusion distance to the acceptor molecule immobilized on the solid support shortens the time needed to reach the necessary complex concentration on the solid phase and thus shortens the assay time. Rabbit anti-horseradish peroxidase (HRP) antibodies were photoimmobilized on the porous supports

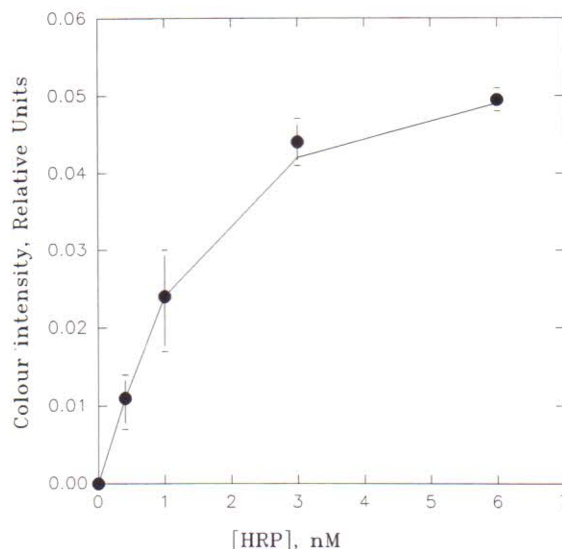


Fig. 4. Standard curves of horseradish peroxidase as antigen. Solution of HRP was passed through the flow cell containing the immunosupport with photoimmobilized anti-HRP rabbit antibodies on a KS-49 matrix. The reaction time was 80 s and the time for colour development was 1 min. A substrate solution of DAB was used for development.

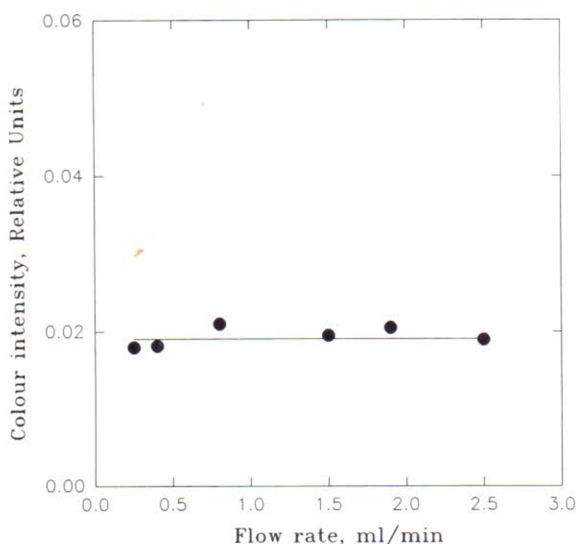


Fig. 3. The staining intensity of a membrane site versus the solution flow rate (in volume units). Antigen (HRP) solution was passed through the flow cell containing the immunosupport with anti-HRP rabbit antibodies photoimmobilized on a KS-49 matrix. The reaction time was 300 s and the initial concentration of antigen was 3×10^{-10} M. The time for colour development was 3 min and the substrate solution of DAB was used for development.

(see materials and methods section). The antigen solution was leaked along the surface of the membrane with immobilized antibodies placed in a collapsible hermetic flow cell with a narrow groove (about 2 mm wide and 0.5 mm deep). Taking the thickness of the membrane used into account (about 0.1 mm), the maximum flow dimensions of the cross section were approximately 2×0.4 mm. The antigen concentration in solution was kept constant and the time of reaction (the time for which antigen solution flowed over the matrix) was varied from 10 to 600 s.

The binding between the antigen in solution and the antibodies photoimmobilized on the membrane were studied kinetically. The membrane was placed in a solution of antigen and after incubation for a fixed period of time, the membrane was washed and incubated in substrate solution. Fig. 2a shows the influence of the reaction time on the final signal when the reaction is carried out under standard conditions without mixing. The antigen concentration in solution was 3×10^{-10} M. Analyzing the dependence in Fig. 2a, one can conclude that the

matrix staining provided by a definite concentration of immunochemical complex reaches a maximum in 90–120 min under these conditions. This conclusion is in agreement with the results of previous investigations of antigen-antibody interactions on other supports (polystyrene microplates, cuvettes, etc.) (Tijssen, 1985). This very fact necessitates carrying out the solid-phase EIA in 1.5–4 h, while under the same conditions, the antigen-antibody reaction in homogeneous solution reaches equilibrium within a few minutes (Arefyev et al., 1990).

It is evident from Fig. 2*b* that the use of the narrow (capillary) flow cuvette as the environment for the immunochemical reaction permits the time to achieve equilibrium to be reduced to a few minutes. In this case, the time of the development of the maximum signal is about 200–300 s.

It was important to determine the effect of the flow rate at a constant reaction time on the extent of the reaction between soluble antigen and immobilized antibodies. The intensity of staining the membrane site versus the solution flow rate (in volume units) at a reaction time of 300 s is presented in Fig. 3. The flow rate was varied from 0.35 to 2.5 ml/min. Since the volume of sample passing through the flow cuvette was kept constant, the linear flow rate was proportional to the volume flow rate. The analysis of the dependence does not suppose any correlation between the experimental flow rates and the surface protein concentration of the immune complex. It should be noted, however, that the seven-fold increase in volume of solution passed through the system corresponds to a change in rate from 0.35 to 2.5 ml/min. Consequently, the lowering of the flow rate in this range does not change the absolute quantity of the antigen on the membrane surface, but increases the bound portion of the substance passing through the flow cuvette. Hence, in proportion to the surface concentration of immune complex, signals can be obtained with smaller amounts of solution, or

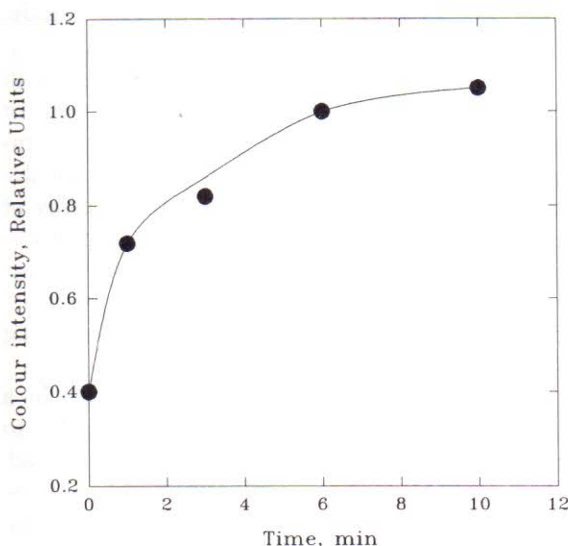


Fig. 5. Kinetics of the interaction between antigen (human IgG) and rabbit antibodies photoimmobilized on a KS-49 matrix. The solution of antigen was passed through the flow cell in which the immunosupport was placed. The flow rate was 0.35 ml/min. The initial concentration of antigen was 1.5×10^{-10} M. The time of the second reaction in anti-human IgG rabbit antibody-HRP conjugate solution was 5 min. The time for colour development was 3 min and a substrate solution of DAB was used for development.

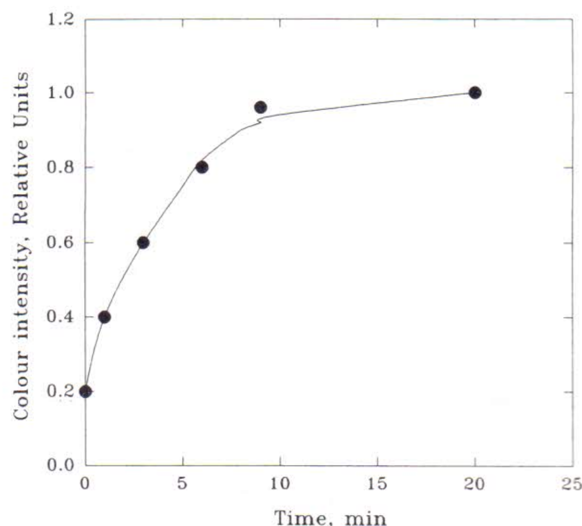


Fig. 6. Kinetics of the interaction between conjugate anti-human IgG rabbit antibodies with HRP and immobilization on an immunosupport antigen (human IgG). The solution of conjugate was passed through the flow cell in which the immunosupport was placed. The flow rate was 0.35 ml/min. The initial concentration of IgG-HRP was 5×10^{-9} M and the time for the first reaction was 5 min. The time for colour development was 3 min and a substrate solution of DAB was used for development.

after a shorter contact time between the antigen and immobilized antibodies.

The above conclusions were then used to elaborate a test system for the model antigen–HRP and the calibration curve obtained is shown in Fig. 4. The antigen solution volumes pumped by a peristaltic pump were chosen in such a way that the time taken to pass the solution through the cell was approximately 70–80 s and the anti-HRP rabbit antibodies were photoimmobilized on a KS-49 matrix.

The total analysis time, including the time to wash out excess enzyme-labelled antibodies and stain in substrate solution was only 3–4 min.

This system was also used to quantitatively assay human IgG. Goat anti-human IgG antibodies were photoimmobilized on the regenerated cellulose membrane. This immunosupport was then placed in a flow capillary cell and treated successively with human IgG standards, washing buffer, the solution of HRP-labelled rabbit anti-human IgG antibodies and then again washing buffer.

The kinetics of human IgG binding to antibodies immobilized on the porous support and of the binding of anti-human IgG-HRP conjugate at various protein concentrations (Figs. 5 and 6) suggest that the maximum signal has already developed after 3–6 min. It was reported previously, that the time to reach the 'plateau' in a conventional ELISA is approximately 60–120 min (Dzantiyev et al., 1987). Shortening the time of immunochemical contact (as in kinetic immunoassays) inevitably leads to a significant decrease in the antigen detection limit (Arefyev et al., 1990). However, in the immuno-testing procedure presented, the lower detection limit is practically the same as in the steady-state immunoassay, the time of analysis being shortened more than ten times.

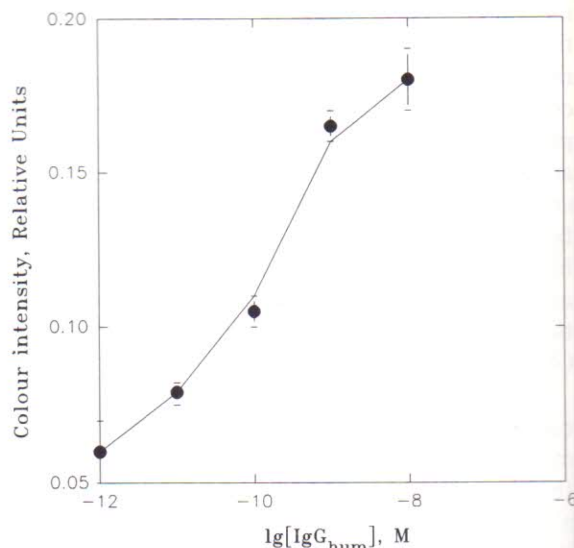


Fig. 8. Standard curves for human IgG. The time requested for the first reaction was 4 min and the time for second reaction was 2 min. The initial concentration of IgG-HRP was 1×10^{-8} M and the time for colour development was 3 min. A substrate solution of DAB was used for development.

In the analysis, the conjugate was used in a great excess (1×10^{-8}–5×10^{-9} M) in order to measure all of the immune complexes formed. The conjugate solution volumes pumped were chosen to provide a contact time of 1.5–3 min and for the automated introduction of components into the system, a flow-injection analyzer was used (Fig. 7). Aliquots of human IgG and the solutions of anti-IgG antibody–HRP conjugate were injected using a two-valve microinjector into two phosphate buffer flows. The additional loop in the scheme provided a 1.5 min delay in conjugate flow in relation to the sample flow. The

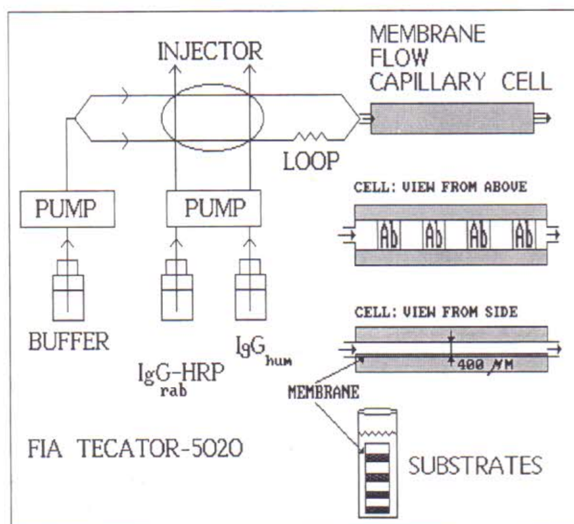


Fig. 7. FIA manifold for flow membrane enzyme immunoassay for human IgG. See text for futher details.

overall cycle time, the time of enzymatic reaction and the total analysis time were about 5–6, 1–2 and 10 min, respectively. It follows from the calibration curve for the matrix staining intensity versus human IgG concentration (Fig. 8) that the lower detection limit of the method is 5×10^{-11} M.

It should be noted that the application of a porous membrane matrix as a solid support allows one to carry out fast semiquantative analysis using only a flow capillary cell and peristaltic pump without any specialized equipment.

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