Covalent immobilization of proteins onto photoactivated polystyrene microtiter plates for enzyme-linked immunosorbent assay procedures

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Method Article

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Abstract

Enhancement of the speed and sensitivity of an ELISA technique was achieved by doing it on a polystyrene microtiter plate preactivated by a simple photochemical reaction. Immobilization of Epicoccum nigrum antigen or goat anti-rabbit IgG onto the photoactivated polystyrene microtiter plates was found to occur in only 45 min with higher binding than that obtained through adsorption during the same period onto the untreated surface. Nearly 1.5–2-folds higher readings were obtained when the ELISA was carried out with the solid phase prepared on the photoactivated surface rather than on the untreated surface. Moreover, solid phases prepared on the activated surface could detect IgE \(E. nigrum antibody) even at 1/50 \(v/v) dilutions, whereas a solid phase prepared on the untreated surface failed to do so. Around three times higher ELISA values were obtained in the activated plate than the untreated plate when IgE was diluted to 1/5 \(v/v). Such photoactivated surface could be of great importance in diagnostic tests involving the ELISA technique particularly to confirm false negative cases.

Introduction

Conventional ELISA procedure carried out by immobilizing antigen or antibody on a polystyrene plate through adsorption is time consuming or inconsistent due to detachment of biomolecules during washings. On the other hand, covalent binding is more sensitive, minimizes nonspecific binding and rapid. \(1), and later \(2), introduced an active functional group to an inert polystyrene surface by nitrating the aromatic ring of the polystyrene followed by the reduction of the nitro group to an amino group. Alternatively, a functional group can be introduced onto a polystyrene surface by a grafting \(3) or gaseous plasma technique \(4). However, all the approaches are tedious and time consuming multistep procedures. Earlier, we have shown the activation of polystyrene surface by a rapid and simple method using 1-fluoro-2-nitro-4-azidobenzene \(FNAB) in a photochemical reaction \(6). Here, we report a rapid and simple method for a double-antibody sandwich \(DAS) ELISA and a direct-antigen coating \(DAC) ELISA by covalently immobilizing antigen or antibody onto the photoactivated polystyrene surface \(7).

Reagents

• 1-fluoro-2-nitro-4-azidobenzene \(FNAB, IUPAC nomenclature: 4-azido-1-fluoro-2-nitrobenzene) can be purchased from Apollo scientific ltd, UK \(cat no. 248-878-6). Alternatively, it can be made from 4-Fluoro-3-nitroaniline by a simple diazotization reaction as described earlier \(6). CAUTION. FNAB is explosive and should be handled with care, especially when using large quantities. FNAB can be stored safely in dark in a loosely capped bottle at 4°C in a refrigerator. • Polystyrene microtiter plates, \(Greiner Labortechnik, Germany). • Anti-rabbit IgG, \(Sigma Aldrich, cat. no. I3382) CAUTION Store all reagents at 2-8ºC. If slight turbidity occurs upon prolonged storage clarify the solution by centrifugation before use. • Rabbit IgG, \(Sigma Aldrich, cat. no. I4508). • Anti-rabbit IgG-alkaline phosphatase conjugate, \(Sigma Aldrich, cat. no. R3115). • Human IgE ELISA Quantitation Set \(Cat. no. E80-108, Bethyl laboratories, USA). CAUTION Store all reagents at 2-8ºC. Do not freeze reagents. All reagents must be kept at room temperature \(20-25C) before use. Components supplied in the kit are: Affinity purified Goat anti-

Human IgE Coating Antibody A80-108A, 1 ml at 1 mg/ml Human IgE Calibrator, RC80-108-6, 1.0 ml. HRP Conjugated Goat anti-Human IgE Detection Antibody A80-108P, 0.1 ml at 1 mg/ml. • Epicoccum nigrum was prepared according to published procedure. • Bovine serum albumin \(BSA), Sigma \(USA). BSA solution should be prepared in PBS. CRITICAL: BSA solution should be filtered prior to use to avoid microbial contamination. • p-nitrophenyl phosphate \(Sigma Aldrich, USA cat. no. P7998). Store in cool place. Recommended storage temperature: -20 °C keep container tightly closed in a dry place. CAUTION: Avoid contact with skin and eyes. Avoid formation of dust and aerosols. • Phosphate buffered saline \(PBS) was prepared by mixing 0.85% NaCl to 0.01 M phosphate buffer \(pH 7.2). To make 0.01 M phosphate buffer add 1.217 g Na2HPO4, 0.379 g NaH2PO4. Add distilled water to make 1 l solution. • Washing buffer was made by adding 0.1% Tween 20 to PBS. • Substrate dye buffer - It is prepared by adding 1 mg of p-nitrophenyl phosphate to 1ml of 50 mM bicarbonate buffer, pH 9.6 containing 0.02% MgCl2. It was used for colour development. • Stop Solution -2 M NaOH.

Equipment

• Refrigerator \(Godrej , India) • UV Stratalinker 2400, Stratagene, USA • Polystyrene microtiter plates \(Greiner Labortechnik, Germany) • ELISA reader \(Biorad iMark™ Microplate Reader, USA).

Procedure

1. Activation of polystyrene surface The wells of a polystyrene microtiter plate were activated by FNAB under UV light for 15 min at a wavelength of 365 nm as described earlier \(6). 2. Detection of analyte In activated and untreated polystyrene microtiter plates, analyte \(rabbit IgG, human IgE, Epicoccum nigrum) was detected by immobilizing capture molecule \(anti-rabbit IgG, anti-human IgE, Epicoccum nigrum antigen) onto the activated and untreated polystyrene microtiter plates followed by blocking , binding of analyte and secondary antibody-conjugate. The plates were washed by washing buffer after each step. Color development was carried out by adding substrate dye buffer. After 30 min, 50 µl of 2 M NaOH were added to stop the colour development. Absorbance was recorded at 405 nm in an ELISA reader.

Timing

1. Activation of polystyrene surface –approx. 25 minutes a) Coating of FNAB to polystyrene surface 5-10 minutes b) UV light exposure- 15 minutes c) Washing and drying - 5 minutes 2. Detection of Analyte - 8 h 15 minutes a. Immobilization of capture molecule \(0.5 µg/100µl carbonate–bicarbonate buffer, pH 9.6 per well) onto the photoactivated surface-45 minutes at 37 oC. b. Blocking \(200µl/well of 2% BSA was used as a blocking agent) -1 h at 37o C c. Incubation of analyte \(0.5 µg/100µl phosphate buffer saline , pH 7.2 per well)- 3h at 37 oC. d. Incubation time of secondary antibody \(100µl of a 1/6000 dilution in PBS per well) - enzyme conjugate - 3h at 37 oC e. Incubation time of substrate dye buffer \(200µl) - 30 minutes.

Anticipated Results

• A two fold increase in ELISA values was obtained when goat anti-rabbit IgG was immobilized over 45 min on the photoactivated surface compared with the untreated surface . The photoactivated plate gave more than a 2.5-fold increase in ELISA values compared to the untreated plate when 125 ng/ml of goat anti-rabbit IgG was used for immobilization. As shown in fig. 1 and fig.2, goat anti-rabbit IgG immobilized on the photoactivated plate was found to detect as little as 62 ng/ml of rabbit IgG. The solid phases prepared by immobilizing capture biomolecule onto the photoactivated microtiter plates for 45 min gave better ELISA value than did the untreated plate. Moreover, solid phases prepared on the activated surface could detect even at a 1/50 \(v/v) dilution whereas solid phases prepared on the untreated surface failed to do so. • ELISA values which were about three folds higher were obtained with the activated plate compared to the untreated plate when analyte \(Epicoccum nigrum antibody) was diluted to 1/5 \(v/v) \ (Fig. 3). As the solid phase prepared on the photoactivated surface can detect lower concentration of antibodies, it would be ideal for disease diagnosis, particularly to confirm false negative cases. • Unlike the activation of polystyrene microtiter plates by conventional time consuming multistep methods, the photoactivation method using FNAB is a single-step rapid procedure and can be carried out by relatively unskilled personnel. Because of the ease by which the photoactivated surface is prepared, ELISA applications could be a potentially powerful alternative to the simple passive adsorption technique. In brief, solid phases prepared on the photoactivated polystyrene surface permit more efficient ELISA procedures in terms of rapidity, sensitivity and reproducibility than do the untreated surface and make possible a range of diagnostic tests. • Amount of reagents used for ELISA is not final and can be changed according to the need. In fact it requires less amount of reagents than used for conventional ELISA procedure.

References

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Figures

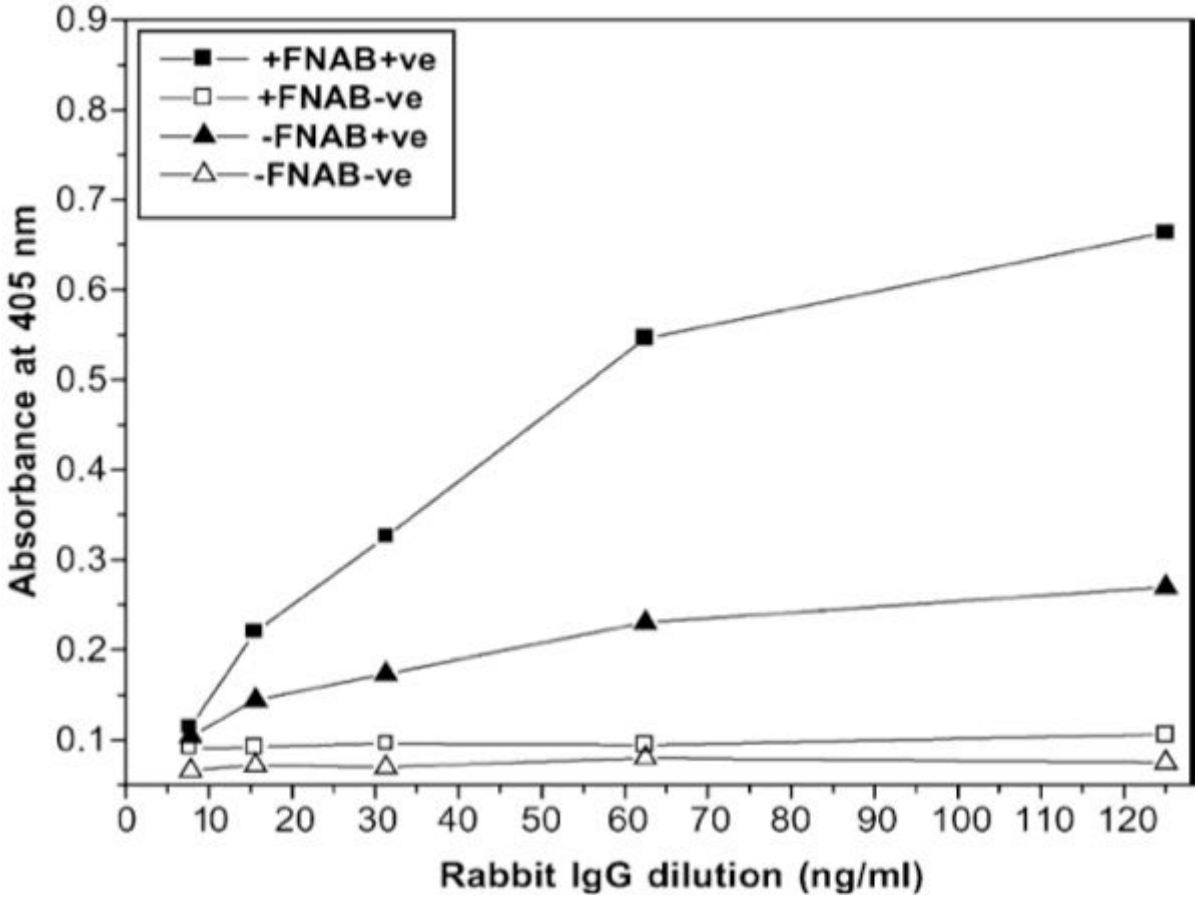

Figure 1

figure 2 Response curves for serial dilutions of rabbit IgG added to immobilized goat anti-rabbit IgG on activated and untreated microtiter plates. Response curves for serial dilutions of rabbit IgG added to immobilized goat anti-rabbit IgG on activated and untreated microtiter plates. The open symbols represent normal goat sera as negative control. A double dilution series (250, 125, 62.5, 31.25, 15.62 and 7.8 ng/ml) of rabbit IgG was made and each dilution was added to a well with immobilised goat anti-rabbit IgG.

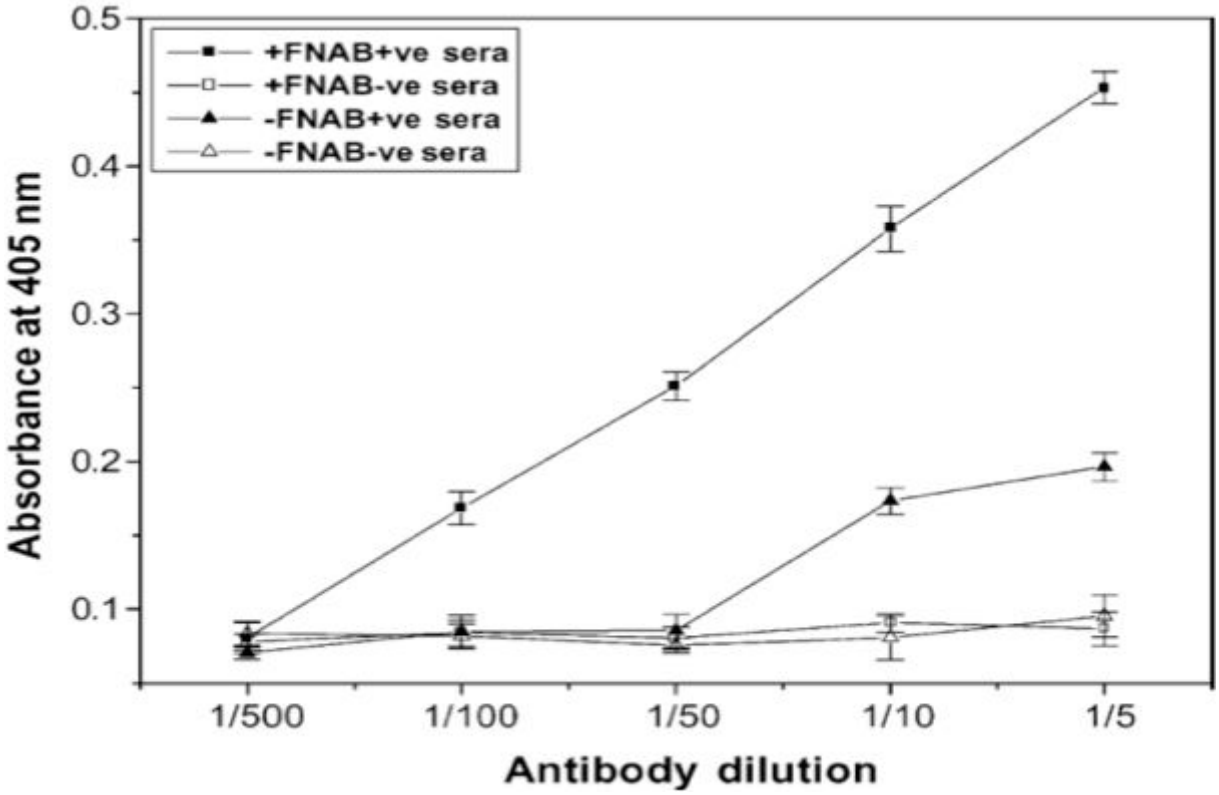

Figure 2

figure 3 Sensitivity studies of antigen immobilized to the photoactivated and untreated wells using different dilutions (1:500, 1:100, 1:50 and 1:5 v/v) of antibody Sensitivity studies of antigen immobilized to the photoactivated and untreated wells using different dilutions (1:500, 1:100, 1:50 and 1:5 v/v) of antibody. Solid and open squares represent activated wells with positive and negative sera from patients, respectively. Solid and open triangles represent untreated wells with positive and negative sera from patients, respectively. The error bars represent the standard errors of the means.

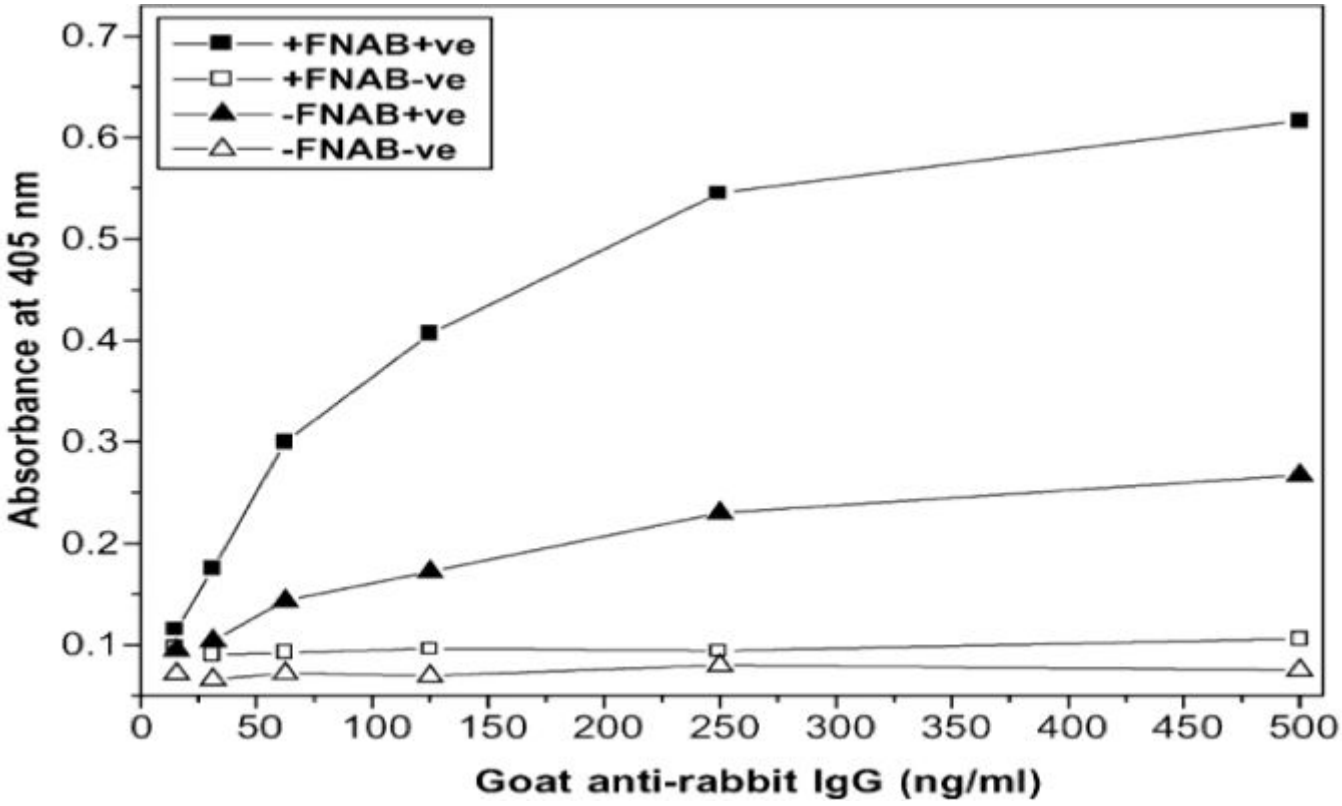

Figure 3

figure1 Immobilization of goat anti-rabbit IgG on activated and untreated microtiter plates at different concentrations. Immobilization of goat anti-rabbit IgG on activated and untreated microtiter plates at different concentrations. The open symbols represent normal goat sera as negative control.