

Image-based ELISA on an activated polypropylene microtest plate—A spectrophotometer-free low cost assay technique



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ABSTRACT

In this communication, we report ELISA technique on an activated polypropylene microtest plate (APP_μTP) as an illustrative example of a low cost diagnostic assay. Activated test zone in APP_μTP binds a capture biomolecule through covalent linkage thereby, eliminating non-specific binding often prevalent in absorption based techniques. Efficacy of APP_μTP is demonstrated by detecting human immunoglobulin G (IgG), human immunoglobulin E (IgE) and *Aspergillus fumigatus* antibody in patient's sera. Detection is done by taking the image of the assay solution by a desktop scanner and analyzing the color of the image. Human IgE quantification by color saturation in the image-based assay shows excellent correlation with absorbance-based assay (Pearson correlation coefficient, $r=0.992$). Significance of the relationship is seen from its p value which is $4.087e-11$. Performance of APP_μTP is also checked with respect to microtiter plate and paper-based ELISA. APP_μTP can quantify an analyte as precisely as in microtiter plate with insignificant non-specific binding, a necessary prerequisite for ELISA assay. In contrast, paper-ELISA shows high non-specific binding in control sera (false positive). Finally, we have carried out ELISA steps on APP_μTP by ultrasound waves on a sonicator bath and the results show that even in 8 min, it can convincingly differentiate a test sample from a control sample. In short, spectrophotometer-free image-based miniaturized ELISA on APP_μTP is precise, reliable, rapid, and sensitive and could be a good substitute for conventional immunoassay procedures widely used in clinical and research laboratories.

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1. Introduction

The enzyme-linked immunosorbent assay (ELISA) technique is the backbone of the diagnostic assay for detection of numerous diseases involving antigens and antibodies (Engvall and Perlman, 1971; Crowther, 2001). Conventionally, antigen or antibody is quantified spectrophotometrically by measuring the absorbance of the assay solution in an ELISA plate reader. Disadvantages of conventional ELISA, normally performed in a 96-well microtiter or ELISA plate are: (i) ELISA plate itself is costly, (ii) ELISA reader is a bulky and an expensive instrument which is not easily available in resource-poor areas, (iii) requires large volumes of costly reagents and analyte and (iv) requires long incubation time. Therefore, for low-resource settings it is necessary to have a device or an assay system that is cheap, specific, sensitive and most importantly, does not require costly instrument. Recently, spectrophotometer-free image-based assay systems are gaining importance due to their low cost and easy operation. Perez et al. carried out image-based analysis for clinical diagnosis by taking the image of a microtiter plate after assay through a mobile phone and quantified the image

of each well using RGB color space (Baro de la Fuente et al., 2006; Perez et al., 2008; Garcia et al., 2009). Wang et al. used cell phone to facilitate microchip enzyme-linked immunosorbent assay (ELISA) based detection of human epididymis protein 4, a biomarker for ovarian cancer in urine. They have used a non-lithographic technique to fabricate microchips made of polymethyl-methacrylate (PMMA) and polystyrene (Wang et al., 2011). However, higher background color in control samples in all these cases was obtained, thereby limiting the detection at lower concentrations. Another group of scientists (Carrilho et al., 2009; Carrilho and Whitesides, 2009; Cheng et al., 2010; Li et al., 2010; Martinez et al., 2007, 2008a, 2008b, 2010a, 2010b; Sia et al., 2004; Vella et al., 2012) modified paper for image-based biochemical assays; nevertheless, sensitivity of assay on paper involving ELISA is much lower than that obtained by ELISA experiments in a typical microtiter plate (Cheng et al., 2010). Tian et al. (2011) have made test zones on a transparency film by sticking cellulose powder on it and used this for ELISA. Cellulose-based assay systems often show non-specific interaction between antibody-conjugate and the cellulose fiber leading to high negative control thus, affecting the specificity as well as sensitivity of the assay.

In the present work, we report a novel approach to minimize non-specific binding by performing ELISA on an activated polypropylene microtest plate (APP_μTP). PP_μTP is made from PP sheet

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where test zones are made in the form of small cavities. In APP μ TP, inert and hydrophobic test zones are photochemically activated so that it can immobilize a capture biomolecule through a covalent linkage. This prevents non-specific binding often prevalent in absorption based techniques. The other promising features of this assay are: (i) it is image-based; hence no need of spectrophotometer, (ii) miniaturized; hence requires less amount of reagents and analyte, (iii) rapid and (iv) sensitive. A Schematic representation of image-based ELISA on APP μ TP is shown in Fig. 1.

2. Materials and methods

2.1. Materials and instrumentation

Anti-human IgG, human IgG, anti-human IgG-HRP conjugate, bovine serum albumin (BSA) and o-phenylenediamine dihydrochloride (OPD) were purchased from Sigma (USA). Polystyrene microtiter plates (Greiner Labortechnik, Germany) were activated by FNAB as described by Nahar et al. (2001). *Aspergillus fumigatus* antibody was obtained from the sera of patients ($n=7$) suffering from allergic broncho pulmonary aspergillosis (ABPA) and used as positive test sera. Pooled sera samples from healthy volunteers were used as a negative control. Patients were from the V.P. Chest Institute outpatient center in Delhi, India. Informed consent was obtained from the patients and healthy individuals. When human IgG was used as a test sample (positive sera), rabbit IgG was taken as a negative control. All buffer solutions were freshly prepared in triple DW before use. Phosphate buffered saline (PBS) was prepared by mixing 0.85% NaCl to 0.01 M phosphate buffer (pH 7.2). Washing buffer was prepared by adding 0.1% Tween 20 to PBS. Substrate dye buffer was prepared by adding 6 mg OPD and 8 μ l H₂O₂ in 6 ml of citrate buffer (pH 5.2, 0.2 M). Experiments were performed in triplicates.

2.2. Activation of PP μ TP

PP μ TP was made from locally purchased white polypropylene sheet of 0.8 mm thickness. The sheet was cut into a strip having dimension of 9 cm in length and 3 cm in width. On the strip, array of test zones (small cavities) were made by mildly hammering or pressing with a blunt end iron rod. Each cavity was separated by 0.5 cm. Cavity of the PP μ TP had an average diameter of 3.5 mm and a depth of around 1 mm. All the dimensions were measured by an electronic caliper. We also checked the uniformity of cavities by measuring their volumes using distilled water.

Test zone of a PP μ TP was activated by introducing an active functional group onto it by a photolinker, 1-fluoro-2-nitro-4-azidobenzene (FNAB) according to the published procedure (Naqvi et al., 2002). Briefly, FNAB solution (0.0625 mg/2.5 μ l in CH₃OH/cavity) was poured into cavities of a PP μ TP plate. After evaporation of the solvent, the plate was exposed to UV light at a wavelength of 365 nm for 20 min in an UV Stratalinker 2400, Stratagene, USA, washed with methanol and dried.

2.3. Conventional ELISA procedure

Conventional ELISA was carried out on an activated polystyrene microtiter ELISA plate by immobilizing anti-human IgE or anti-human IgG (1 μ g/100 μ l) at 4 °C overnight (11 h), blocking by 2% BSA in PBS (200 μ l) in 1 h at 37 °C, antibody (100 μ l) binding in 3 h at 37 °C and antibody–enzyme conjugate (100 μ l) binding in 3 h at 37 °C. After each step, the wells were washed thoroughly with washing buffer. Color development was carried out by adding 100 μ l of substrate-dye buffer in each well followed by 20 μ l of 5% H₂SO₄ to stop color development. Absorbance was recorded at 490 nm in an ELISA reader (Biorad iMark™ Microplate Reader, USA).

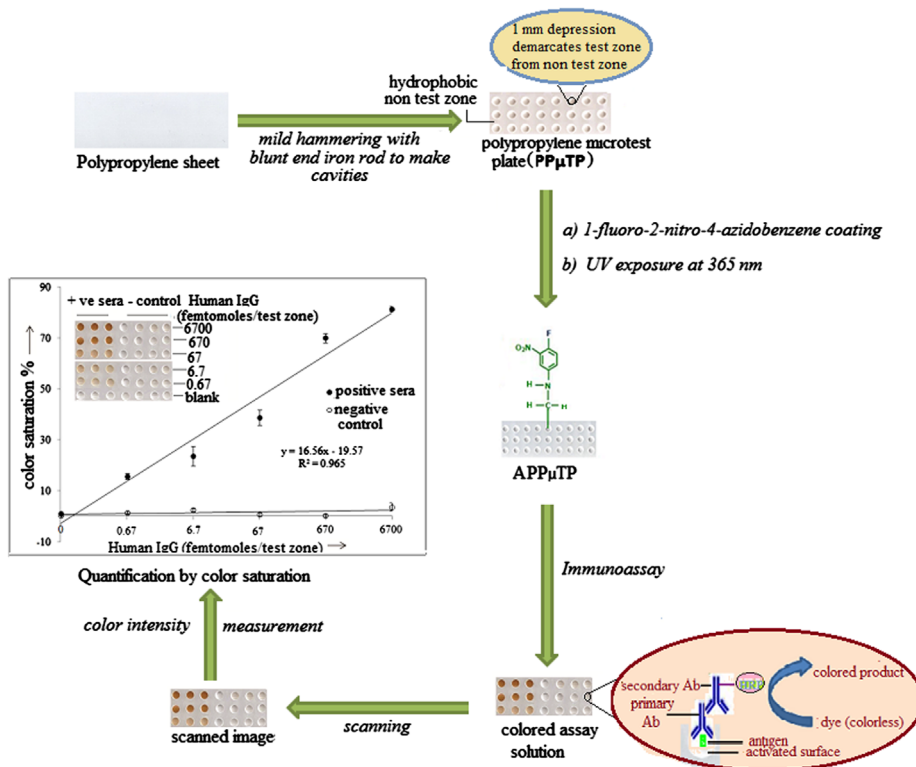


Fig. 1. Schematic representation of image-based ELISA on APP μ TP. Test zones were activated by FNAB for covalent immobilization of a capture molecule. After immunoassay on APP μ TP, color assay solution was scanned and the intensity of color was analyzed as color saturation percentage.

2.4. ELISA on APP μ TP

ELISA on an APP μ TP was carried out with the same reagents as in conventional procedure, except that the volume of each reagent used in the test zone was 10 μ l. Also, instead of conventional thermal incubation, ELISA steps on APP μ TP were carried out by ultrasound waves in 40 min according to published procedure (Sharma and Nahar, 2009). Briefly, for each ELISA step APP μ TP was floated for 10 min on a sonicator bath operating at a frequency of 40 kHz and an output power of 120 W with a starting temperature of 37 °C. Color was developed by adding 8 μ l of substrate-dye buffer and then stopped by 2 μ l of 5% H₂SO₄. The APP μ TP was then scanned on a desktop scanner (HP photo smart C6388) by placing the plate upside down to get the image. From Adobe Photoshop the mean value of each R, G and B of the scanned image was obtained which was then converted to HSB (Hue, Saturation, and Brightness) using freely available “Macbeth color calculator” software. Image was then quantified as saturation percentage. We have termed “saturation” as “color-saturation” to make it clear as well as to differentiate it from conventional chemical understanding of saturation.

2.5. Preparation of standard curves by spectrophotometric and image-based methods for human IgE quantification

Different concentrations of human IgE were detected in a microtiter ELISA plate using Human IgE ELISA Quantitation Set (Cat. no. E80-108, Bethyl laboratories, USA) following manufacturer's protocol. Absorbance was recorded at 490 nm in an ELISA reader and a standard curve was made.

For image-based quantification, color solution from microtiter plate was transferred to a PP μ TP plate and scanned to get the image. Color saturation percentage was then calculated for each concentration of human IgE and a standard curve was made.

Standard curve was also made by detecting different concentrations of human IgE on APP μ TP according to Section 2.4.

2.6. Detection of human IgG on APP μ TP

Different amounts of human IgG (0.67 nanomoles to 67 femtomoles) were detected by performing ELISA on APP μ TP as described in Section 2.4.

2.7. Comparison of ELISA performed on APP μ TP, microtiter ELISA plate and paper

Detection of human IgG was carried out by ultrasound waves in 40 min on APP μ TP, paper disc and microtiter plate by performing ELISA using anti human IgG and human IgG at a concentration of 1 μ g/ml, blocking with 2% BSA and anti human IgG conjugate of 1:2000 dilution. Amount of reagents for microtiter plate were as described in Section 2.3 and for paper disc and APP μ TP as described in Section 2.4. All the assays were done by calculating color saturation; for microtiter plate, image-based assay was done by transferring the assay solution to a new PP μ TP.

2.8. Rapid ELISA on APP μ TP

In a time dependent study, ELISAs on APP μ TPs were carried out as described in Section 2.4 in 8, 16, 24, 32 and 40 min respectively by ultrasound waves. For comparison, ELISA was also performed on APP μ TP in 18 h by thermal incubation as in conventional procedure.

2.9. Detection of *A. fumigatus* antibody in patients' sera

Detection of *A. fumigatus* antibody was carried out by ultrasound waves in 40 min on a APP μ TP and an activated microtiter ELISA plate using *A. fumigatus* antigen (1 μ g/ml) as capture biomolecule, patient's serum (1:100 dilution) as a test sample, pooled sera sample from healthy volunteer as a negative control and anti-human IgG–HRP conjugate (1:2000) as a detecting agent. Amount of reagents for microtiter plate and APP μ TP were taken as described in Sections 2.3 and 2.4 respectively. After assay, the colored solutions from microtiter plate were transferred to a new PP μ TP. Both the plates were scanned and the results were quantified as color saturation.

3. Results and discussion

3.1. Choice of APP μ TP as a miniaturized assay device

PP μ TP is made up of polypropylene sheet which is hydrophobic, inert and does not bind biomolecule through absorption; hence PP μ TP is not suitable for assays where biomolecule immobilization is required. Therefore, for such assay, there is a need to activate the test zone for biomolecule binding. The test zones are activated using a photolinker, FNAB as described earlier (Naqvi et al., 2002; Nahar et al., 2001). FNAB dissolved in methanol, is loaded into the cavities of PP μ TP. To exclude reaction of methanol with the highly reactive nitrene, the solvent was evaporated from the cavities prior to exposure to UV light. When the FNAB coated plate is exposed to UV light, it generates highly reactive nitrene, which binds to the test zones of polypropylene surface through nitrene insertion mechanism whereas the fluoro group of the photolinker remains intact. This fluoro group results in activation of the surface and allows binding through the amino groups of a incoming protein molecule without any catalyst or reagent. Thus, in APP μ TP only a desired biomolecule is immobilized in the test zone by covalent bonding. Also, in case of paper as a matrix, one has to make a substantial portion of the paper hydrophobic by chemical modification to avoid cross contamination through spreading of the analyte. On the other hand, activated cavities for test zones in APP μ TP demarcate them from hydrophobic non-test zones which are above the level of test zones.

3.2. Comparison of standard curves prepared by spectrophotometric and image-based methods for human IgE quantification

For spectrophotometric quantification of human IgE we have prepared standard curve by performing ELISA in the wells of an activated microtiter plate using Human IgE ELISA Quantitation Set. For image-based quantification, color solution from microtiter plate is transferred to a PP μ TP plate and scanned on a desktop scanner by placing the plate upside down. Due to surface tension property, the liquid remains in the cavities. Although digital camera or even a mobile phone camera can be used to capture the image, we preferred desktop scanner as it gives reproducible results. Nowadays many printers are available with an integrated scanner and is not very costly. On the other hand, image from camera varies from person to person and time to time depending on the intensity of light, distance etc. Scanned image was then quantified as saturation (color saturation) percentage. Saturation is a color term commonly used by digital imaging experts and is measured as percentage value. Color saturation is obtained from freely available digital imaging software according to Section 2.4. ELISA results by color saturation method shows excellent correlation with absorbance (Pearson correlation coefficient, $r=0.992$) as calculated by R software. The p value was $4.087e-11$ which

indicates the significance of a relationship (Fig. 2a). This clearly shows that image analysis by the color saturation method is as good as the absorbance based method and can safely substitute spectrophotometer by a cheap scanner. Efficacy of APP μ TP is also checked by making a standard curve for quantification of human IgE by performing ELISA on it. The results expressed as color saturation percentage show a strong correlation with increasing concentration of human IgE (Fig. 2b). This is particularly more important as it requires 10 times less reagent than microtiter plate but with similar efficiency.

3.3. Dose dependent detection of human IgG on APP μ TP

One of the main criteria of an ELISA assay is that it should not give false positive result. Most frequently, false positive result occurs due to improper blocking, insufficient washing or absorption of secondary antibody-HRP conjugate (reporter molecule) directly to the support. We have checked the efficacy of the APP μ TP by detecting human IgG in a dose dependent manner. To find out if there is any false positive result, we have taken rabbit IgG as a negative control. As seen from Fig. 3a, there is no non-specific binding in the negative control sera. This is expected as APP μ TP does not bind through absorption. APP μ TP is also sensitive and can detect 0.67 femtomoles (10 pg) of human IgG in a test zone. It also shows that color saturation is directly proportional to the amount of human IgG.

3.4. Efficacy of APP μ TP with respect to microtiter and paper ELISA

Recently paper is emerging as a low cost device for image based assay. Therefore, we have checked performance of APP μ TP with

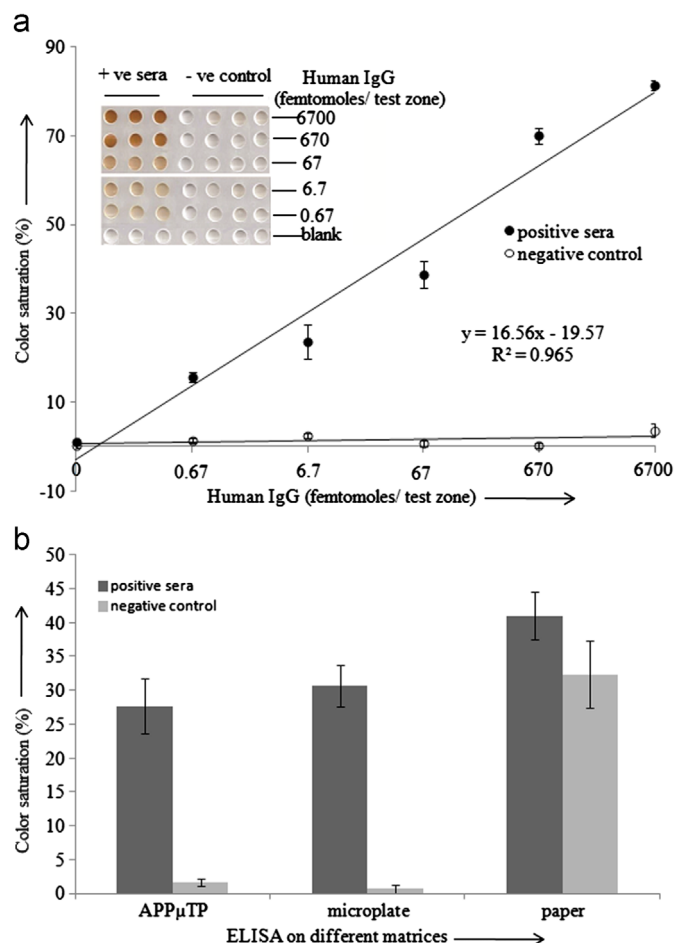


Fig. 3. (a) Dose dependent detection of human IgG on APP μ TP and (b) comparative study of ELISA performed on APP μ TP, microtiter ELISA plate and paper. Color saturation was obtained from the scanned images of the resultant assay solutions.

respect to microtiter ELISA plate and Whatman paper for the detection of human IgG. The results show that human IgG detection on APP μ TP is akin to that of microtiter based assay with no non-specific binding. In contrast, high non-specific binding in control sera is observed in the paper ELISA leading to false positive result (Fig. 3b). This is in agreement with the reported literature (Cheng et al., 2010). From this study it is evident that APP μ TP is much better option as a low cost assay device than either paper or microtiter plate.

3.5. Rapid ELISA on APP μ TP

One of the important parameters of low cost ELISA is that it should be rapid. Earlier, we demonstrated the use of ultrasound waves, an alternate source of energy, in reducing the ELISA timings for antibody detection to 40 min from 18 h-conventional ELISA (Sharma and Nahar, 2009). The method involves optimum use of ultrasonic waves, generated in a laboratory sonication bath, for rapid ELISA on a microtiter plate. Herein, we try to find out whether ELISA timings can be reduced by ultrasound waves on APP μ TP. For this, we have performed each step of ELISA on APP μ TP by ultra sound waves in a sonicator bath in 2, 4, 6, 8 and 10 min corresponding to total ELISA incubation time of 8, 16, 24, 32, and 40 min respectively. The results were further compared with 18 h-conventional ELISA performed on APP μ TP. For better comparison, all the results are expressed in color saturation percentage. As expected, there is negligible non-specific binding on APP μ TP ELISA, either performed by long conventional

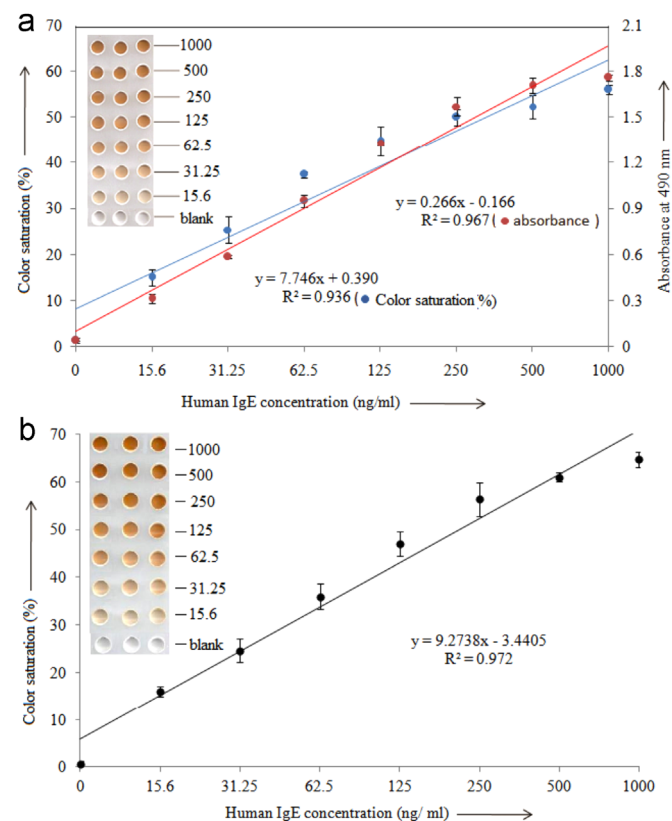


Fig. 2. Standard curves for human IgE quantification: (a) by performing ELISA on an activated microtiter plate by absorbance (●) based method and its comparison with color saturation (●) obtained from color image (inset) of PP μ TP filled with colored assay solution from microtiter plate and (b) by performing ELISA on APP μ TP.

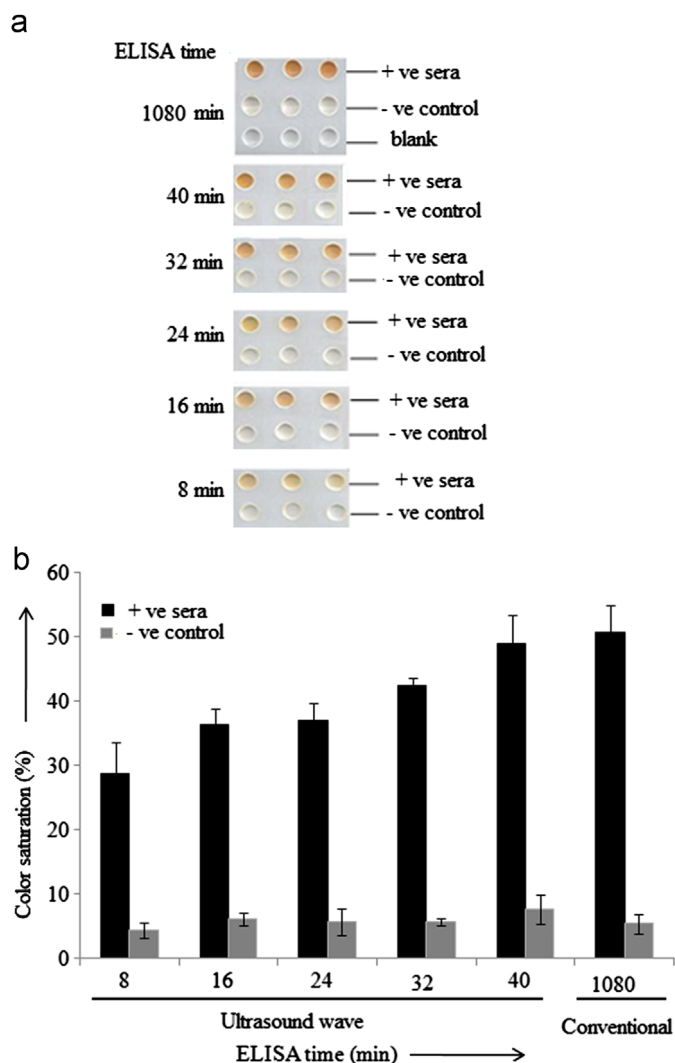


Fig. 4. Detection of human IgG by rapid ELISA on APP μ TP by ultrasound waves in different times. Detection of human IgG by conventional ELISA was carried out in 18 h. (a) Scanned images of experiments and (b) quantification of resultant colored assay solutions in terms of color saturation.

incubation or rapid ultrasonic waves. Although, 40 min-ultrasound ELISA is comparable with 18-h ELISA, the analyte on APP μ TP can be detected even in 8 min. That means if first two steps are prefabricated, ELISA can be done in 4 min only (Fig. 4). Being one of the fastest ELISA in the current scenario, the 4 min-ELISA can also be used as a point of care (POC) diagnosis. Although mechanism of rapid ELISA by ultrasound waves is not fully understood, it is likely that some localized hot spots are generated during sonication that accelerate covalent and non-covalent binding of biomolecules during the ELISA process.

3.6. Detection of *A. fumigatus* antibodies in patients' sera on APP μ TP

Efficacy of APP μ TP was further validated by detecting *A. fumigatus* antibodies in patients' sera and comparing it with microtiter plate based ELISA. As already seen, color saturation is akin to that of absorbance; therefore we express the results as color saturation. Detection of antibodies on APP μ TP strongly correlates to each absorbance-based assay. Most importantly, there is negligible color saturation values for negative controls on both the plates suggesting that APP μ TP could be an excellent substitute of microtiter-based assay (Fig. 5).

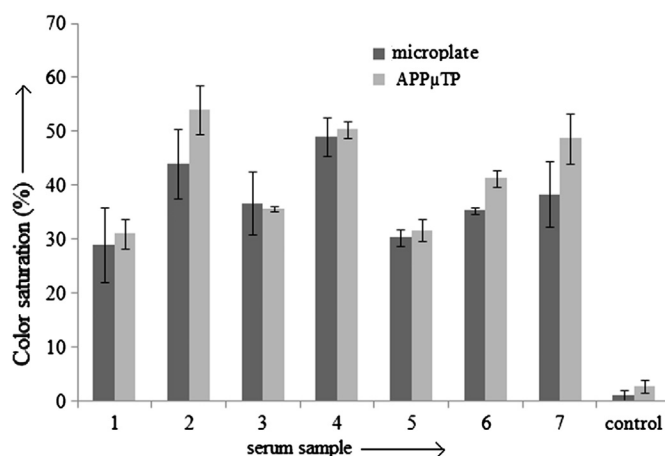


Fig. 5. Detection of *Aspergillus fumigatus* antibodies in patients' sera on microtiter ELISA plate and APP μ TP by ultrasound waves in 40 min. Pooled patients' sera from healthy individuals are taken as a negative control. Results were expressed as color saturation percentage.

4. Conclusions

False assay result is detrimental to any biochemical assay, more importantly if the assay is for diagnosis. Image-based diagnostic assay is a new area and is mostly carried out on paper. Often ELISA on paper gives high non-specific binding leading to false positive result. In this report, we have responded to this problem by performing image-based ELISA on APP μ TP. Hydrophobic and inert test zone of PP μ TP is photochemically activated to make it enable to immobilize a capture biomolecule through a covalent linkage. APP μ TP is validated by detecting human IgG with insignificant non-specific binding, a necessary prerequisite for ELISA assay. Another important aspect of ELISA is the quantification of analyte. Detection of human IgE, human IgG and *A. fumigatus* antibodies in patients' sera are as precisely quantified on APP μ TP as in conventional ELISA procedure. Efficacy of APP μ TP is also checked with respect to microtiter plate and paper-based ELISA. ELISA on APP μ TP is akin to that of conventional ELISA; in contrast, paper based-ELISA shows high non specific binding in control sera. Also, we have carried out ELISA on APP μ TP by ultrasound waves and even in 8 min it can convincingly differentiate test sample from control sample. In conclusion, spectrophotometer-free ELISA on APP μ TP could be an excellent alternative either to conventional or paper-based ELISA.

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