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Paper-Based ELISA: A Novel Diagnostic Approach for Monitoring Aqueous Humour VEGF Level in Ocular Diseases

Yu-Ting Tsao, Wei-Hsuan Sung, Hung-Chi Chen, Min-Yen Hsu and Chao-Min Cheng

Abstract

We commonly diagnose ocular diseases via both morphological changes and symptoms. It is necessary to develop biochemically based assays for early or follow-up diagnosis of these diseases with a focus on robustness and ease of handling. To lay out a prospective path toward this goal, we describe and propose the use of ultrahigh sensitive paper-based ELISA (p-ELISA), which uses a treated piece of filter paper to monitor the activity of ocular diseases (i.e., detecting the vascular endothelial growth factor (VEGF) concentration in aqueous humour for proliferative diabetic retinopathy or age-related macular degeneration diagnosis). The advantages of p-ELISA include the following: (1) the capacity to directly measure biomarker concentrations in aqueous humour using only a tiny sample volume (as little as 2 μL); (2) significantly increased sensitivity compared to conventional ELISA (fg/mL levels); and (3) inexpensive materials and a short operation duration. P-ELISA is a novel point-of-care diagnostic tool with the significant potential to advance ophthalmological treatment guidelines by facilitating early detection and routinely monitoring therapeutic response.

Keywords: paper-based ELISA, VEGF, diagnosis, ocular disease, ophthalmology, aqueous humour

1. Introduction

Prevention, diagnosis and treatment are the core principles of modern medical care. Two of these principles, prevention and treatment, are inextricably linked to the third, precise diagnosis. Suitable early diagnostic criteria must be established on properly understanding the pathophysiology of a specific disease and how it impacts the human body. Precise diagnosis naturally leads to precise prevention and treatment. By developing current biotechnologically relevant technologies, medical practitioners will have increasingly adequate tools for precise diagnoses and suitable monitoring of treatment methodologies. Most ophthalmologists, however, still diagnose ocular diseases based primarily on structural and functional changes. In this chapter, we will discuss biochemically based diagnostic tools for ophthalmology. The introduction consists of two parts: (1) an introduction to current

biochemically based diagnostic tools and the clinical applications of ELISA and (2) a discussion on the importance of VEGF monitoring for several eye diseases. Following this, we will present an overview and details on paper-based ELISA (p-ELISA), a rapid, cost-effective, easy-to-handle, sensitive, and robust method for monitoring aqueous humour VEGF level in ocular diseases.

1.1 ELISA in clinical applications

Biochemically based diagnostic tools have been widely used for a variety of specific clinical disease diagnoses, prognoses, and the evaluation of treatment efficacy. We have used such tools to measure specific tumor biomarkers as part of a multiple cancer screening tool (e.g., PSA screening for the prostate cancer), and to monitor CRP level to evaluate patient inflammatory state [1]. The focus of such biochemically based diagnostic tools is quantification or qualification of a target substance in body fluids to assist medical practitioners with precise disease diagnosis or treatment status [2]. To distinguish specific substances from body fluids, researchers have used multiple strategies including, but not limited to, enzyme-catalyzed reactions [3], antibody/antigen interactions, spectrophotometry [4], and electrophoresis [5]. Enzyme-catalyzed reactions and antibody/antigen interactions are two of the most widely applied technologies among biochemically based diagnostic tools. In the enzyme-catalyzed reactions model, the specific binding capabilities of enzymes facilitate the measurement of enzyme catalytic activities as a means of quantifying or qualifying a target substrate [6]. Raabo and Therkildsen first applied this strategy to measure blood sugar using glucose oxidase (GOD) [3]. In the antibody/antigen interactions model, quantitative capacity is provided by the immunological affinity between an antibody and a specific substrate or antigen, one of which is immobilized in the solid phase while the other is distributed in the liquid phase. This method is widely used in clinical biochemistry (e.g., detection of human chorionic gonadotropin in a pregnancy test [7]). Such methods are linked to the development of the enzyme-linked immunosorbent assay (ELISA), first described by Engvall and Perlmann, who combined the specificity of antibody/antigen interactions with signal amplification via high-turnover catalytic enzymes to provide both high sensitivity and specificity [8]. ELISA is traditionally performed in a 96-well plastic plate. Current formats of ELISA typically include the direct type, indirect type, sandwich type, and competitive type, with procedural details for each described in the chapter references [9]. The clinical importance of ELISA is the capacity to detect trace amounts of a particular peptide or protein (also known as antigen or antibody in an ELISA kit) in a rapid and executable way. ELISA is widely used as a screening or diagnostic tool. It is used for infectious disease screening, as in the detection of human immunodeficiency virus antigen (HIV-Ag) for the diagnosis of acquired immune deficiency syndrome (AIDS) [10]. It is employed for toxicology as in the detection of organophosphate in wheat end products [11]. And it is used for allergen screening in the food industry [12]. Conventional ELISA requires volumes of approximately 200 μL for test completion. Unfortunately, fluid sources such as aqueous humour are limited, which rules out conventional ELISA methodology for ocular disease detection. A modified ELISA, p-ELISA for instance, that could use minute sample volumes would be an invaluable tool in such cases.

1.2 Importance of monitoring VEGF levels within the human eye

Measuring the concentrations of specific circulating substrates (e.g., vascular endothelial growth factor (VEGF)) in aqueous humour or vitreous humour can provide ophthalmologists with a relevant tool for evaluating ocular diseases. Elevation

of the VEGF plays a critical role in the pathological angiogenesis of age-related macular degeneration (AMD) and diabetic retinopathy (DR) [13], the two leading causes of blindness in developed countries. AMD is a degenerative disease characterized by loss of central vision. Clinically, we can classify AMD into neovascular and non-neovascular types. DR is one of the major complications of type 1 and type 2 diabetes mellitus. Over 30 million people suffer from vision-threatening DR worldwide [14]. Overexpression of VEGF in neovascular AMD and DR leads to neovascularization and vascular leakage, which eventually results in retinal thickening and edema [13, 15]. Both of the diseases mentioned above are diagnosed by symptomatic and structural examination using an ophthalmoscope, fluorescein angiography (FA), or optical coherence tomography (OCT). Both can be treated with intravitreal injection of VEGF inhibitors (for neovascular AMD and DR). Injection of anti-VEGF agents has become one of the most effective treatments for neovascular AMD and DR [16, 17]. Pegaptanib, ranibizumab, bevacizumab and aflibercept are the currently available anti-VEGF agents. Although anti-VEGF therapy has saved many patients from visual loss, the cost of anti-VEGF therapy is relatively high and requires lifelong treatment (e.g., in neovascular AMD, the cost was about €22,818–37,926 per year for bevacizumab, ranibizumab, and aflibercept therapy [18]). Unfortunately, the efficacy of anti-VEGF therapy varies from person to person, which provokes controversy over treatment time schedules. Individualized therapy is frequently advocated but limited by deficient tools to precisely monitor the progression of the pathological angiogenesis in eyes. It is commonly known that elevation of VEGF appears before clinically detectable structural or functional changes [19]. Clearly then, easy detection of VEGF levels within the human eye can decidedly assist early diagnosis, severity assessment (as a quantitative tool), and drug-efficacy evaluation.

Both the blood-ocular barrier and insufficient quantities of aqueous humour have limited the possibilities for biochemically based diagnostic tools for ocular diseases. In most cases, an ophthalmologist can only diagnose and follow up on ocular diseases via examination of morphological changes of the eyes and symptoms analysis. Unfortunately, anatomical changes and functional visual loss are largely, though not completely, irreversible. Therefore, it is necessary to develop an early detection technique to diagnose and monitor ocular diseases before visual acuity loss, and such a technique must be capable of using only small sample amounts. We believe that p-ELISA is just such a tool. It can be used to monitor VEGF levels with minute sample amounts and is capable of ultrahigh sensitivity and specificity.

2. How do we apply p-ELISA to monitor VEGF levels in ocular diseases?

Although the vitreous humour occupies the majority of the space in the human eye (about 4 mL in an adult eye) and contacts directly to the retina, it is difficult to extract the vitreous humour as a diagnostic sample for retina evaluation for two reasons: (1) the vitreous humour is stagnant and not actively replenished and (2) the risk of vitreous hemorrhage and retinal detachment when extracting vitreous humour. Several studies have reported that cytokine level in aqueous humour is highly correlated with vitreous level and severity of AMD and DR [20, 21]. For this reason, extracting aqueous humour is a safer source of material for monitoring VEGF level. Aqueous humour is produced from the ciliary process at an average rate of 2.4 $\mu\text{L}/\text{min}$ [22] and fills the anterior and the posterior chambers. The total volume of an adult's aqueous humour is about 250 μL . The maximum amount of aqueous humour that can be extracted from the anterior chamber at one time before potential chamber collapse is approximately 200 μL .

The conventional ELISA plate requires relatively large sample volumes per assay (50–200 μL) compared to p-ELISA. Both the tolerance and repeatability of each test are restricted by sample volume, so small volumes are obviously problematic. While sample volume can be magnified via dilution, sensitivity and specificity are consequently diminished [23]. Moreover, complicated incubating and blocking steps in conventional ELISA are time-consuming and tedious and lead to a decrease in sensitivity and specificity. Conventional ELISA requires modification to more practical sample volumes on the scale of microliters, and sensitivity and specificity must be preserved or amplified.

P-ELISA requires only a piece of filter paper, and can effectively be used with well volumes as low as 3 μL . The high surface-to-volume ratio of cellulose fibers in paper greatly reduces reaction time so that a diagnostic procedure can be completed within a single hour (compared to approximately 4 h for conventional ELISA). P-ELISA also relies on methodology that is different from the conventional ELISA sandwich model: it uses an indirect model. Instead of using Avastin, the more traditional monoclonal antibody, p-ELISA uses HRP-conjugated Avastin. The simplified protocol and high affinity between antigen/antibody gives rise to ultrahigh sensitivity and specificity (fg/mL levels). Furthermore, recording and analyzing p-ELISA results can be completed by using a commercial handheld cellphone camera and image-processing software. In this way, expensive ELISA plate readers are replaced by inexpensive and common technology that allows small-scale laboratories to perform ELISA analysis in an easy-to-handle and cost-effective manner.

2.1 Production of the paper plate

P-ELISA was first introduced in 2010 by Chao-Min Cheng [24] who used a 96-microzone paper plate instead of a traditional plastic well to perform ELISA. His first 96-microzone paper plate was made using filter paper processed by photolithography to generate hydrophobic barriers between each test zone. He found that three-dimensional (3D) structures of the cellulose fiber network in the test zones boosted the reaction rate and reduced the required, effective sample volume to only 3 μL . Photolithography allowed mass plate production but required expensive equipment and a complicated production process. A less costly and simpler procedure was subsequently found: inkjet printing [25]. Of the various inkjet printing methods, wax printing and screen printing were found to be the two best, and now most common, strategies to produce 96-microzone paper plates. Wax printing uses a commercial wax printer to create hydrophobic patterns on filter paper, and screen printing uses commercially available polymer solutions [26, 27]. Both of these two methods are flexible, inexpensive, and easy to use.

Wax printing is an easy to execute and effective process for forming test zones on paper. The nature of the paper substrate allows for the creation of hydrophilic test zones, and the wax, printed in circles, makes defined hydrophobic barriers. The result is a sheet of paper with small, defined wells arrayed in a format that mimics that of a 96-well plate. This 96-well-plate format was first designed on a computer using commercial software (white areas as the hydrophilic test zones and black areas as the hydrophobic barriers), and then printed on Whatman qualitative paper with a commercial wax printer (Xerox Phaser 8560DN). The wax-printed paper was then placed on a hotplate (135°C) to melt the printed wax enough to allow it to penetrate all the way from the printed side through to the opposite side of the paper [28]. Careful attention was paid to this melting process to avoid heating for too long, which would result in the wax spreading out too far from its originally printed shape. Typically this process took from 10 to 20 min, but visual observation always took precedence over total time if necessary. Alternatively to using a hotplate, an

oven could be used for this process, in which case the melting process was shorter in length. Test zone creation was visually verified by observing whether or not the black wax appeared on the back side of the paper or by placing a drop of water on the paper to test the integrity of the printed hydrophobic area. While the format of the paper plate test zone was identical to that of a traditional, plastic 96-well plate for conventional ELISA (circles of 5 mm in diameter), each well or test zone required only 3 μ L of solution to fill (see **Figure 1**). Although a smaller test zone would require less sample volume, we chose the conventional 96-well plate format to facilitate familiarity and a relationship to standard protocols used in conventional laboratories.

2.2 How to perform ELISA on the paper plate

To simplify the ELISA protocol, we used an indirect ELISA model instead of the conventional sandwich ELISA model. Our protocol comprised 5 steps as follows: (1) antigen immobilization within test zones; (2) blocking the test zones with buffer; (3) adding enzyme conjugated detection antibodies; (4) washing away unbound antibodies; and (5) adding substrates for a color-producing enzymatic reaction. Antigen immobilization was completed via application and physical adsorption, a process based on the non-specific interaction between the antigens and the paper fibers [29]. This step requires approximately 3 μ L of solution and 10 min of drying under ambient conditions. Blocking the test zones with the blocking buffer prevented nonspecific antibody binding. This step also required approximately 3 μ L of solution and 10 min of drying. Enzyme-conjugated detection antibodies were added to conjugate with the immobilized antigen. Incubation period was dependent on experimental design. Washing away the unbound antibodies was completed with a piece of blotting

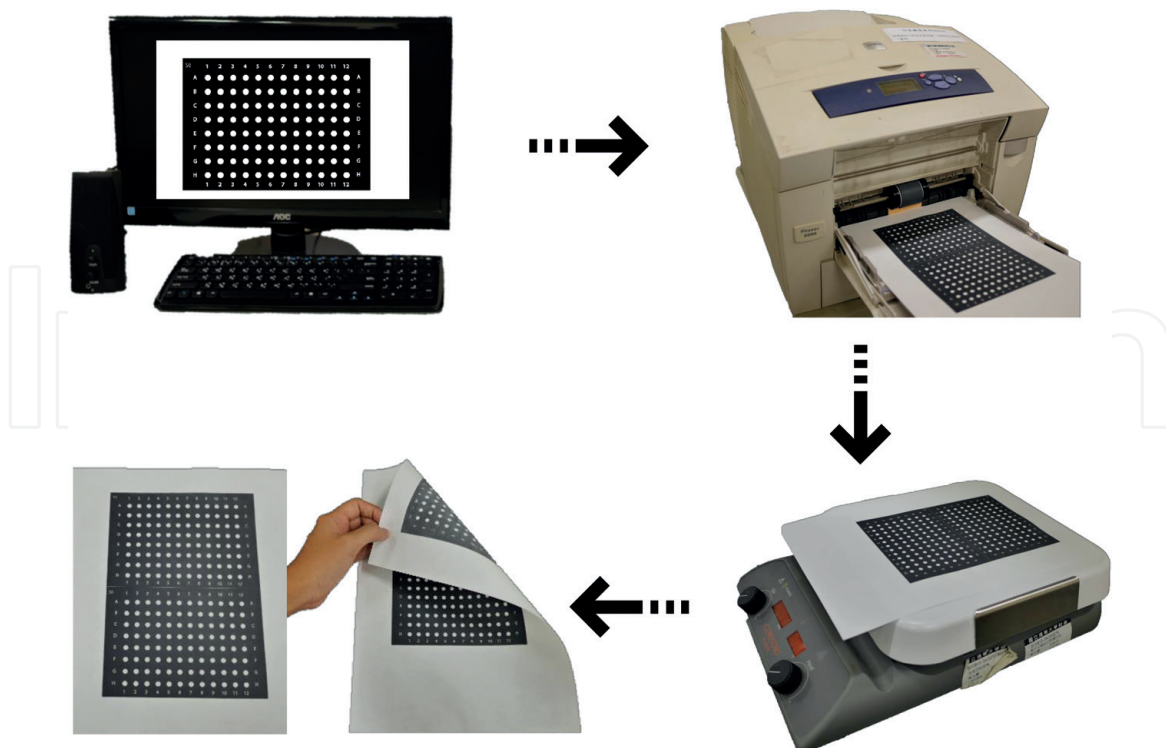


Figure 1. The schematic of the paper plate production. The pattern of the paper plate was first designed using commercial software on a computer, and the pattern was printed onto Whatman qualitative paper using a wax printer. In the next step, the printed black wax was heated to melt it through the filter paper using a hot plate with the temperature set at approximately 135°C. This created defined hydrophobic barriers surround hydrophilic test zones. The pattern of the test zones should be clear on both side of the filter paper in the finished product.

paper placed beneath the paper plate to absorb the washing buffer, or the paper plate was shaken in a basin of washing buffer. The final step, adding substrates to initiate a color-producing enzymatic reaction with the conjugate enzyme, was performed immediately after the washing step. Total processing time for this colorimetric reaction varied from case to case, and some conditions, such as humidity and ambient temperature, affected evaporation rate, so controlling such variables is important for reducing experimental error. Following chemical processing, colorimetric results can be conveniently recorded using a desktop scanner or a commercial cellphone camera and then analyzed via software using a linear red-green-blue (RGB) system. Although a desktop scanner is the most commonly used analytical device for paper-based diagnostic tools due to its high sensitivity, the cellphone camera and application model has gained significant popularity due to its portability, convenience, and cost efficiency. An image interpretation application (e.g., Petgeia) can be used to analyze the colorimetric reaction. Moreover, several recent papers have shown that cellphone camera analytical results are comparable to those from a desktop scanner [30, 31]. Combining the convenience of using a simple paper substrate with a lightweight cellphone camera and application dramatically increases the practicality of such an approach for effective, real-time, point-of-care (POC) diagnostics that can significantly impact health outcomes for a number of people, especially those in resource-poor environments.

2.3 Protocol from aqueous humour extraction to aqueous VEGF level detection

We can now more easily relate the p-ELISA process to our real-life protocol for detecting VEGF levels in aqueous humour. The aqueous humour extraction was performed under a normal and proper process. All the patients were examined by the slit lamp first to evaluate the condition of anterior chamber, and the anterior chamber paracentesis was then performed by the following step: the patient was placed in a supine position and all the procedure was done under the microscope. Local anesthesia was given by dripping 0.5% proparacaine hydrochloride two to three times with an interval of 5 min. The surgical field was spread by the lid speculum and the eyeball was fixed at the limbus opposite to the paracentesis site. First ocular surface was disinfected by diluted beta-iodine solution for 5 min. Then, a 27 gauge needle was inserted through the paralimbal cornea carefully with the tip overlying and parallel to the iris. The bevel of the needle should be placed forward and carefully avoid hurting the lens. A volume of about 50–100 μL aqueous humour was extracted and put into storage for further analysis. 50–100 μL aqueous humour would be sufficient for detecting the VEGF level by repeating 20 wells in p-ELISA model, which required only 2 μL samples for analysis in each well. After sampling, prophylactic antibiotic drop and ointment were given. The whole procedure took less than 10 min to complete. For detecting the VEGF levels in aqueous humour, first the VEGF concentration calibration curve was produced by adding varying VEGF concentrations from a commercially available VEGF kit into a row of test zones. These concentrations ranged from 10^{-14} g/mL to 10^{-6} g/mL ($n = 8$) (**Figure 2**) [32]. The calibration curve was calculated using the Hill equation and the coefficient of determination (R^2) was found to be 0.9938. The resulting color intensity of different VEGF concentrations has an approximately linear relationship without considering the blank value [33]. When testing aqueous humour, we reduced the requisite sample volume from 3 to 2 μL due to the limited volumes available. After adding 2 μL of patient aqueous humour into several test zones, we allowed the sample to dry for 10 min. We then added 2 μL 1% BSA blocking buffer into the test zones and waited another 10 min for drying. In the next step, 5 μL of 0.8 mg/mL horseradish peroxidase (HRP)-conjugated Avastin was added as the

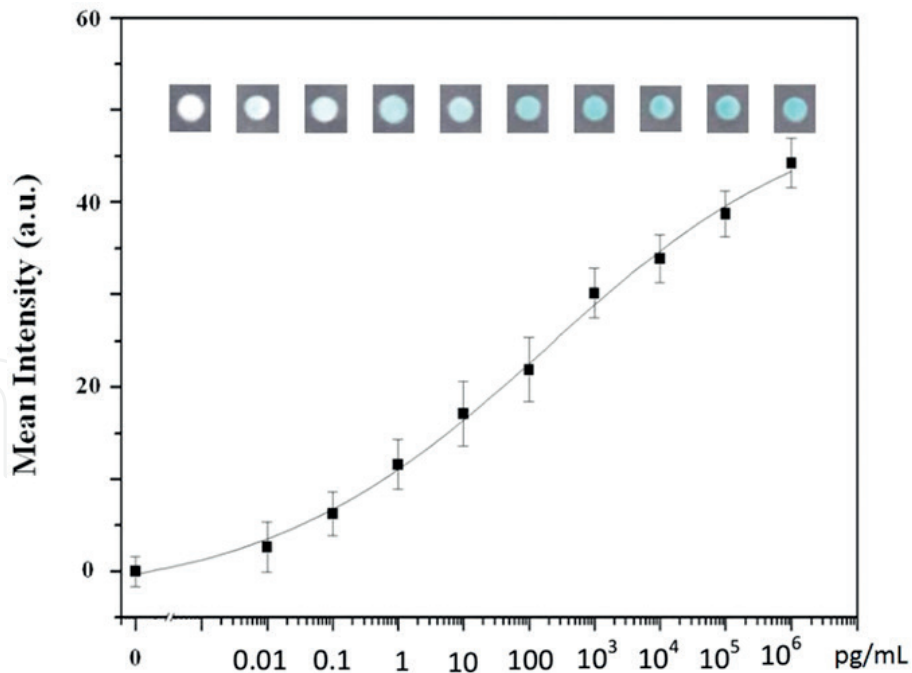


Figure 2.

The calibration curve of the commercially provided VEGF (kit form) using p-ELISA [34]. Eight different concentrations of commercial VEGF ranging from 10^{-14} g/mL to 10^{-6} g/mL were used. The calibration curve was calculated using the Hill equation, and the coefficient of determination (R^2) is 0.9938.

antibody, and the plates were incubated for 10 min to allow for conjugation with the immobilized VEGF. We then added 2.5 μ L of streptavidin to enhance the colorimetric signal. After washing away the excess antibody with washing buffer, a 2 μ L solution of 3,3',5,5'-tetramethylbenzidine (TMB) + H_2O_2 was added into the test zones. This facilitated the enzyme substrate-driven colorimetric reaction with the HRP-conjugated Avistin. Resulting colorimetric output signals were dynamically recorded using a cellphone camera (from HTC Inc., Taiwan). For comparison, colorimetric results were also scanned using a commercial desktop scanner (EPSON; No.:GT-10000+). All images taken by these two optical recording approaches were then analyzed with image processing software (Adobe, Photoshop CS5), in order to compare the differences from each approach. This process was used to refine the more convenient cellphone-based recording system [33]. All procedures are performed in a laminar flow hood to prevent environmental bias and took less than 1 h to complete. The total equipment required in this protocol included several filter papers, a commercial wax printer, an oven, a pipetman, a commercial cellphone, and a desktop scanner, each of which are accessible and cost-effective. In addition to these efficiencies, the requirement of tiny sample volumes contributed to a reduction in overall cost for each test compared to conventional techniques.

The sensitivity of our p-ELISA to detect VEGF in a buffer system was ~ 33.7 fg/mL, which was outstanding for protein-detection technology and 150 times more sensitive than conventional ELISA (~ 5 pg/mL). The ultrahigh sensitivity of this new assay was attributed to several advantageous protocol features. First, we modified and prepared the antibody from human recombinant VEGF-A antibody to HRP-conjugated Avastin, and Avastin was the therapeutic antibody for VEGF, which carried high specificity toward VEGF. This therapeutic-based monoclonal antibody helped streamline the protocol with upgraded specificity. The HRP-conjugated Avastin was made following the protocol provided in an EasyLink HRP Conjugation Kit as described in detail in our reference information [33]. Secondly, we deviated from the conventional sandwich ELISA protocol to use an indirect ELISA protocol, which simplified the complicated blocking and washing steps. Lastly, we replaced

the alkaline phosphate protocol for our colorimetric response with HRP (colorless to blue, instead of purple), allowing us to obtain the stronger output signals. The comparison between p-ELISA and conventional ELISA protocol for detecting aqueous humour VEGF levels is demonstrated in **Table 1**.

2.4 Clinical application of p-ELISA to detect VEGF levels in ophthalmological patients

In our previous study, we quantified aqueous humour VEGF levels in patients with several ocular diseases using p-ELISA. The mean VEGF levels detected in 13 senile cataract patients was 14.4 ± 8.5 pg/mL (mean \pm SD), in 14 patients with proliferative DR (PDR), it was 740.1 ± 267.7 pg/mL, in 17 patients with AMD, it was 383 ± 155.5 pg/mL, and in 10 patients with retinal vein occlusion (RVO), it was 219.4 ± 92.1 pg/mL respectively [33]. In one study using conventional ELISA, Jian-Ping Tong reported that the mean VEGF levels from 10 senile cataract patients was 108.3 ± 72.3 (mean \pm SD) pg/mL, while in 12 patients with choroidal neovascularization due to AMD it was 668.9 ± 340.0 pg/mL [20]. Similarly using conventional ELISA, Hideharu Funatsu reported mean VEGF levels of 26 patients with PDR as 376.5 ± 187.8 [21], and H Noma reported mean VEGF levels from 24 patients with branch RVO as 299.1 pg/mL [34]. The pathogenesis of senile cataracts is rooted in degenerative effects on lens structure, which is different from the pathological angiogenesis of PDR, AMD, and RVO. Therefore, patients with senile cataract would be a good control group for our experiment. As we can see from the results mentioned above, both the p-ELISA and conventional ELISA showed considerable disparity in VEGF levels between patients with senile cataract and patients with PDR, AMD, or RVO (the levels of VEGF were low in the senile cataract group and high in PDR, AMD, or RVO groups). The aqueous VEGF levels measured by

	P-ELISA for VEGF		Conventional ELISA for VEGF	
Equipment	Paper plate Desktop scanner Smartphone camera		Plastic plate Plate readout	
Antigen/primary antibody	VEGF/HRP-conjugated avastin		VEGF/human recombinant VEGF-A antibody	
Secondary antibody	None		HRP conjugate	
Cost for equipment	100 USD		20,000 USD	
Dilution	No		Yes	
Detection sensitivity	0.03 pg		18.75 pg/mL	
Detection range	0.01–100,000 pg/mL		31.25–2000 pg/mL	
Reagent/duration	Volume (μL)	Time (mins)	Volume (μL)	Time (mins)
(1) Immobilize VEGF	2	7	70	120
(2) Blocking	2	7	100	30
(3) Antibody	75	20	30	60
(4) Colorimetric reaction (add TMB+ H ₂ O ₂)	2	10	100	3
Total per zone	13.5	44	300	213
Total sample volume require per test	40 (repeat 20 wells)		9600 (total 96 wells)	

Table 1.
A comparison between conventional ELISA and p-ELISA in the detection of VEGF levels.

p-ELISA were comparable to those from conventional ELISA, supporting the idea that pathological angiogenesis can be adequately diagnosed using p-ELISA. Further case studies with greater sample numbers are necessary to confirm this.

In another one of our previous studies, we used p-ELISA to detect aqueous humour VEGF levels before and after intravitreal injection (IVI) of anti-VEGF antibodies.

The results showed that the mean VEGF levels in 16 patients with neovascular AMD, myopic neovascularization, or polypoidal choroidal vasculopathy were 545.71 ± 810.29 pg/mL (mean \pm SD) before IVI of anti-VEGF antibodies. After IVI of anti-VEGF antibodies, the mean VEGF levels became 0.072–0.131 pg/mL (N = 15) within 5 weeks and 163.06 ± 367.06 pg/mL (N = 15) after 5 weeks. We also evaluated the efficacy of the ranibizumab and bevacizumab by detecting VEGF levels via p-ELISA and found that 50% of patients (6/12) that took ranibizumab demonstrated earlier VEGF elevation within 49 days compared to 11.11% (2/18) in patients that took bevacizumab ($p = 0.0342$) [32]. The minimal sample volume requirement and ultrahigh sensitivity of p-ELISA allowed us to monitor VEGF levels closely. Using this approach, ophthalmologists could prescribe personalized VEGF inhibitor treatment schedules for their patients. In addition, the delicate data output of p-ELISA could also assist clinicians and pharmaceutical companies to evaluate the effects of anti-VEGF antibodies. In summary, using p-ELISA to monitor aqueous VEGF level can be a useful tool to assist diagnosis of several ocular diseases, evaluate treatment efficacy of anti-VEGF treatment, and promote the development of new drugs.

Although the IVI of anti-VEGF antibodies has become one of the most powerful treatment strategies for patients with pathological angiogenesis in eye diseases, there are still many limitations to the clinical application of VEGF inhibitors:

(1) drug efficacy varies from person to person; (2) the adverse effects of IVI of VEGF antibiotics include post-injection endophthalmitis [35, 36], uveitis, rhegmatogenous retinal detachment, vitreous hemorrhage [36, 37], and the risk for sustained intraocular pressure elevation [36, 38]; and (3) the potential of systemic adverse events such as an increase in bleeding tendency [36]. Therefore, a good procedure for monitoring VEGF levels within the human eye would be beneficial to patients who receive VEGF inhibition therapy. In patients with good response to anti-VEGF therapies, a clinical ophthalmologist can decrease the frequency of VEGF inhibitor injections based on scheduled VEGF level monitoring by p-ELISA. Decreasing the frequency of IVI of VEGF antibiotics not only saves unnecessary medical expenses for patients, it spares the patients from the risk of therapeutic complications. For those patients with poor response to anti-VEGF therapies, early detection of elevated VEGF via ultrasensitive p-ELISA can alert a clinical ophthalmologist to increase treatment frequency or change the therapeutic approach. Because elevations in VEGF level appear earlier than morphological changes or visual acuity changes, monitoring VEGF levels can provide helpful early detection of disease and further prevent undesirable vision loss in those afflicted [39] (**Figure 3**). In addition to being ultrasensitive, p-ELISA for VEGF level detection takes only a short time (<1 h) compared to conventional ELISA (4–5 h). In a conventional outpatient clinical setting, aqueous humour extraction for VEGF level detection requires an initial visit, time-consuming testing, and a follow-up visit to discuss possible treatment options. With p-ELISA, a patient could be examined and receive results within an hour, hastening and increasing the efficacy of possible treatment (schematically described in **Figure 4**). Using p-ELISA for diagnosis as well as follow-up monitoring provides a more effective and less costly means of caring for patients without sophisticated laboratories and expensive ELISA readers. It offers a truly viable POC device that can be used in rural areas, in the developing world, and in emergency or resource-poor environments in ways that may revolutionize existing ocular disease diagnosis and treatment.

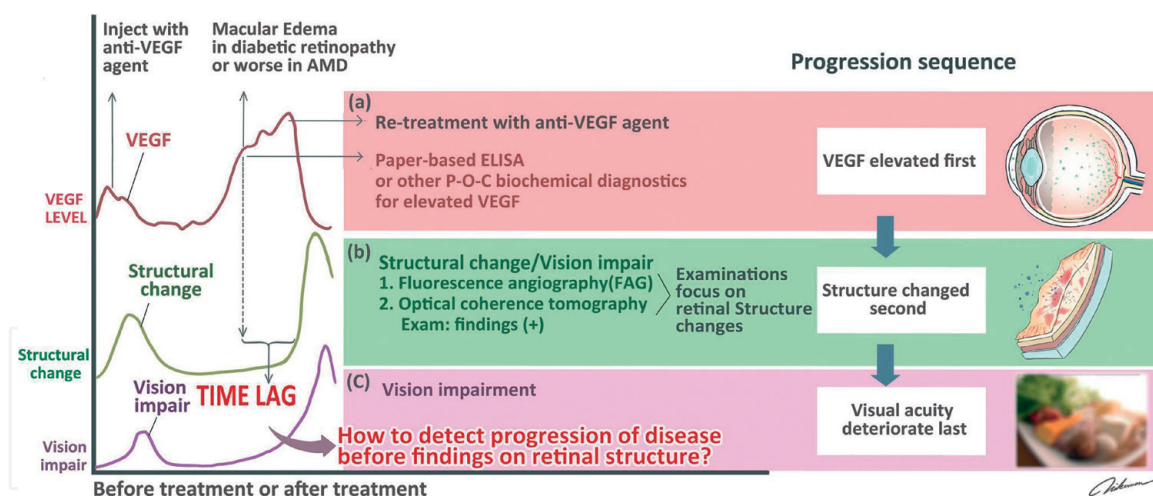


Figure 3.

The correlation between elevated VEGF, structural change, and vision impairment in patients receiving anti-VEGF therapy [39]. The Y coordinates represent VEGF levels, the structural changes, and vision impairment. The X coordinates represent the time sequence after the injection of anti-VEGF agent. Once the effects of the anti-VEGF agent began to wear off, VEGF levels initially elevated, leading to pathological angiogenesis, retinal structural changes, and vision impairment. Using an ultra-sensitive device such as p-ELISA to detect elevated VEGF levels would reduce the likelihood of structural changes and vision impairment. (a) P-ELISA or other POC diagnostics can detect elevated VEGF levels in the first disease progression sequence. (b) The structural changes could be noticed by FAG or OCT after a time lag of approximately 16 days following initial VEGF elevation. (c) Vision impairment would follow soon after the structural changes occurred.

2.5 The future prospects of paper-based ELISA

P-ELISA, first developed by the Whitesides Research Group at Harvard University to detect IgG and HIV antigen in serum, has become a useful diagnostic tool in many medical fields including the diagnosis of infectious disease (e.g., HIV, *E. coli*, and dengue fever [24, 28, 40]), the detection of autoimmune antibodies (e.g., anti-noncollagenous 16A (NC16A) in patients with bullous pemphigoid [41]), potential tumor marker assessment [42] and human cognitive performance determination (e.g., detecting specific neuropeptides such as neuropeptide Y following traumatic brain injuries and post-traumatic stress disorder [43]). We first used p-ELISA in the ophthalmological field to detect VEGF levels in aqueous humour and had good results in both disease diagnosis and treatment followed-up. P-ELISA is uniquely posed to meet the needs of eye disease diagnosis because it requires only tiny sample amounts to provide ultrahigh sensitive results. It is foreseeable that p-ELISA will take its place among commonly practiced approaches for diagnosing and treating ocular diseases. There are, in fact a great many detectable cytokines and growth factors in aqueous humour besides VEGF including tumor necrosis factor (TNF)- α , interleukin (IL)-2, -4, -5, -6, -8, -10, serum amyloid A (SAA), and migration inhibitory factor (MIF), and p-ELISA may ultimately be useful for detecting each of them. The elevation or depression of each of these markers is associated with ocular disease states [44–47]. It is worth noting that could the capacity to analyze a host of biomarkers with only tiny amounts (2 μ L) of aqueous humour could lead to a comprehensive optical health examination that goes beyond and is more impactful than existing morphological and functional examinations.

As bright as the future is for this novel diagnostic technology, there are still existing limitations. Aqueous humour sampling is invasive, and low volume extraction does not eliminate risks of infection, hyphema, or lens capsule rupture. A less invasive strategy for evaluating ocular diseases would be ideal. We do know that tears carry a number of cytokines that are highly correlated with corneal and conjunctival disease. Elevation of IL-6, IL-8, and VEGF appears

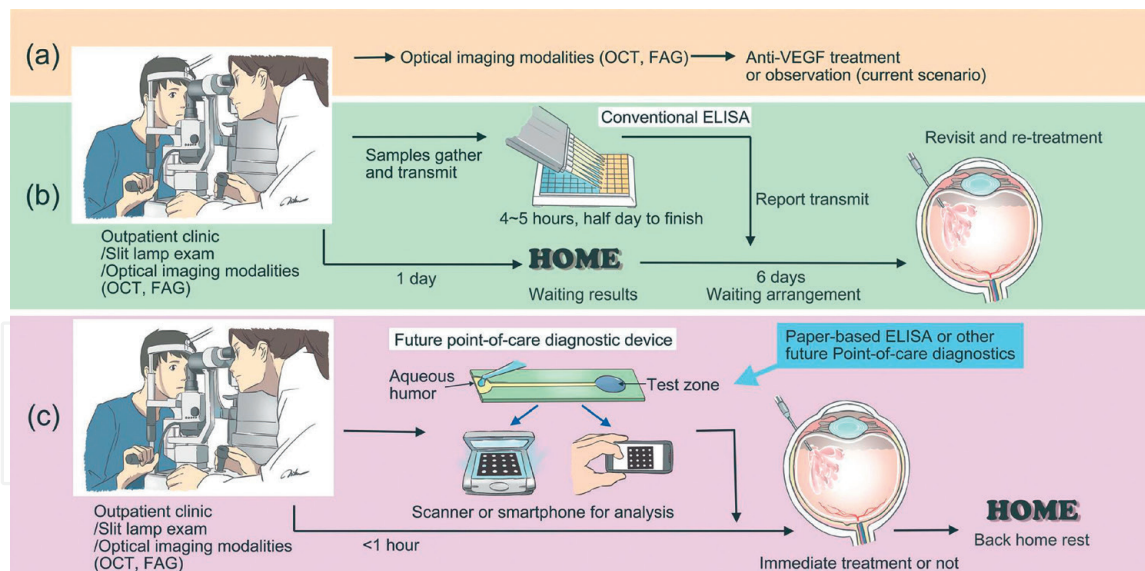


Figure 4. The 3 different outpatient clinic scenarios for the follow-up examination of the patients receiving anti-VEGF treatment [39]. (a) Optical imaging modalities: the patient receives the optical imaging test only to determine whether to receive anti-VEGF treatment or observation. (b) Optical imaging + conventional ELISA VEGF level test: the patient receives the optical imaging test and extraction of aqueous humour occurs at the first visit. Because the conventional ELISA requires 4–5 hours to finish, the patient must arrange another appointment with the doctor to see the reports and decide whether to receive the anti-VEGF treatment. The next visit is usually one week later. This modality provides more information but has the risk of delaying treatment. (c) Optical imaging + p-ELISA VEGF level test: the patient receives the aqueous humour extraction, sees the testing reports, and decides whether to receive the treatments immediately within one visit. The short testing time of p-ELISA (<1 hr) allows the patient to obtain timely and proper treatment.

in patients with corneal neovascularization [48]. Elevation of inflammatory cytokines (IL-1 β , IL-6, INF- γ , and TNF- α) indicates inflammation from ocular surface diseases [49, 50]. Detection of the trace amounts of biomarkers in tears by using ultra-sensitive p-ELISA is a direction for further investigation. P-ELISA has greatly simplified the complicated steps of conventional ELISA and eliminated the need for expensive equipment, but the process still requires professional medical technologists and simple laboratories for analyzing results, at least until cellphone camera applications become more developed. The properties of the p-ELISA as a lab-on-paper device make it a promising future POC device. Further modifying p-ELISA devices from an ophthalmologist-friendly diagnostic tool to a patient-friendly POC device remains a challenge. Development of an in situ device for ophthalmological analysis would provide considerable healthcare impact in developing countries with limited resources and alleviate the currently unbalanced ophthalmologist to patient ratio. This paper-based diagnostic tool is easy to handle, fast, sensitive, and requires only small samples and simple equipment to quantify specific biomarkers. P-ELISA has the potential to provide impactful benefit in the evaluation of ocular diseases and improve ocular health in real world.

3. Conclusions

In this chapter, we described how paper-based ELISA (p-ELISA) was robust, user-friendly, and required only small sample amounts (e.g., 2 μ L of aqueous humour to measure VEGF) to return results that demonstrated very high sensitivity (~33.7 fg/mL). Detection of aqueous humour VEGF level via p-ELISA provides not only early diagnosis of ocular diseases with pathological angiogenesis (e.g., PDR,

AMD, and RVO), but assists in outlining accurate disease treatment. In regards to its effect on ocular disease diagnosis and treatment, p-ELISA can provide precise and quantitative biochemical data prior to the onset of morphological and functional changes. An early diagnosis could lead to early treatments and save patients from undesirable visual loss. As an aid to treatment monitoring, p-ELISA can record the efficacy of anti-VEGF therapy based on continuous follow-up measurement of VEGF level in aqueous humour. In this way, clinical ophthalmologists could provide ideal and individualized treatment schedules for different patients. Furthermore, this cost-effective and portable diagnostic device could provide far reaching and effective ocular health care for underserved populations especially in developing countries. P-ELISA is a simple diagnostic device that may well widen our views on eyes.

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Conflict of interest

We declare that there is no conflict of interest.

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