INVESTIGATION OF POLYVINYLIDENE FLUORIDE FOR ENZYME-LINKED IMMUNOSORBENT ASSAY APPLICATIONS USING C-REACTIVE PROTEIN

By

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Abstract

For over 40 years, the sandwich Enzyme-Linked Immunosorbent Assay (ELISA) method, conducted in an insulated laboratory setting, has been a stepping stone in quantifying amounts of various proteins in the human body. Sandwich ELISA works by immobilizing and activating antibodies when the protein of interest is present. In particular, substrates depending on protein specificity are required to carry out sandwich ELISA.

Polyvinylidene fluoride (PVDF) can be acted as a sandwich ELISA platform for C-Reactive Protein (CRP) immunoassays outside the laboratory setting. Due to PVDF's superior mechanical properties, PVDF was able to act as a stable substrate for immobilizing anti-CRP antibodies in the CRP immunoassay. To validate if PVDF can be used as a reliable ELISA platform, several dilution experiments involving horseradish peroxidase (HRP) and tetramethylbenzidine (TMB) reagent were conducted. When the concentration of CRP is elevated beyond the threshold amount 0.0643 mg/L, the PVDF membrane strip would turn into a light blue color. However, below normal CRP concentrations (between 0.00005 mg/L and 0.0643 mg/L) the PVDF membrane strip would turn a noticeably dark blue color as a comparison. This color change shows the presence of antibody binding, releasing HRP from the HRP-conjugated anti-CRP antibodies to bind to the TMB reagent. Therefore, PVDF should be a stable candidate for ELISA applications at the qualitative level.

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Relevant Abbreviations

AIDD: Autoimmune Disease Detector

CRP: C-reactive protein

ELISA: Enzyme-Linked Immunosorbent Assay

TMB: Tetramethylbenzidine

PVDF: Polyvinylidene Fluoride

HRP: Horseradish peroxidase

Introduction

The purpose of this study was to find an affordable and non-invasive method for detecting autoimmune diseases. Specifically, the aim was to find a stable platform to host a CRP immunoassay by sandwich ELISA without requiring an insulated laboratory setting. The senior design project associated with this study, AIDD (Autoimmune Disease Detector) utilizes ELISA by using conjugated anti-CRP antibodies with HRP and CRP to present a signal when a certain amount of CRP is applied to a PVDF membrane test strip.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA has been widely used as a detection method for common immunological concepts such as quantification of proteins since its conception in 1971 (Jonsson, 2016). ELISA, however, has limited accessibility as it is primarily conducted in a laboratory. Immunoassays in paper form (P-ELISA) typically use nitrocellulose or filter paper as their reaction substrate for autoimmune antibody detection (Hsu, et al., 2014). P-ELISA methods typically involve preparation of well-plates using wax printing, which is often very invasive, and not typically accessible for a quick and simple diagnosis of the protein of interest. Our senior design project focused on detecting the amount of CRP in human saliva to deduce whether the individual has early onset of any sort of autoimmune disease, shown as elevated levels of CRP concentration. Nitrocellulose paper and filter paper do not provide an effective binding substrate for anti-CRP antibodies. Therefore, anti-CRP and CRP immunoassays need a different substrate.

Polyvinylidene Fluoride (PVDF)

Many lateral flow immunoassays require the use of many different pad layers, including a sample pad, conjugated release pad, a membrane of some sort, a backing card, and an absorbent pad (Koczula & Gallotta, 2016). Often, creation of lateral flow immunoassays requires strenuous effort, as nitrocellulose and filter paper often do not have optimal binding properties, especially in the case of CRP. We have discovered that PVDF strips can provide a simple substrate for P-ELISA using anti-CRP and CRP.

C-Reactive Protein (CRP)

CRP is a pentamer with 5 calcium ion groups used for ligand binding. High concentrations of CRP have been associated with chronic inflammatory processes (Salazar, et al., 2014), including autoimmune diseases, such as Rheumatoid Arthritis and Type II Diabetes Mellitus. From a biophysical perspective, CRP can be dissociated into a monomeric form (Salazar, et al., 2014), which is not as stable as the pentameric form, but produces significant binding when various receptors are presented, such as anti-CRP. The anti-CRP antibody binds with CRP at the calcium ion bonding site (Jonsson, 2016) and upon binding, the antibody releases any factors associated with the antibody. For instance, the binding of HRP to anti-CRP will be unbound when CRP is attached to anti-CRP due to calcium ion binding. Once this occurs, sandwich ELISA is completed, and the immunoassay will give present a specific readable signal due to an attached dye, such as TMB reagent which was used in this study.

Research

Validation of Colorimetric Assay with PVDF

Due to PVDF's superior mechanical strength, insolubility, and abrasion resistance (Polyvinylidene Fluoride, n.d.), PVDF is often used in several engineering applications. Its insolubility arises from its alternating carbon difluoride and methylene groups, providing polarity to PVDF. Due to PVDF's resistance to harsh chemical and thermal conditions, PVDF can be served as an efficient structural polymer assay and immunoassay substrate (Polyvinylidene Fluoride, n.d.).

PVDF can also serve as a biopolymer for colorimetric assays (Kolusheva, Kafri, Katz, & Jelinek, 2001). Visible color transitions are present for colorimetric immunoassays as soon as the displayed epitope is recognized by its specific antibody in aqueous solution and with the appropriate dye, specifically CRP with anti-CRP in this study (Kolusheva, Kafri, Katz, & Jelinek, 2001). This can only occur if the membrane used has a hydrophobic N-terminus for the proteins to become immobilized and let ELISA occur. Even more so, according to Zhao, et al., colorimetric assays involving such a setup only requires immobilization of antibodies to the specific membrane followed by the relevant reagent, whereas typical ELISA procedures take up to several hours. PVDF provides the structural support for such an assay, in which antibodies initially become immobilized onto the PVDF membrane so that N-terminus sequencing can occur (Zhao, et al., 2012) for a successful proposed colorimetric readout.

Incorporation of salivary CRP according to ELISA and its application to PVDF

AIDD was initially created to provide a non-invasive basis for detecting general autoimmune diseases according to the amount of CRP in a saliva sample. The maximum threshold

for detecting early onset autoimmune disease is 0.0643 mg/L of CRP and the minimum threshold is 0.00005 mg/L of CRP (Desai & Mathews, 2014). CRP in saliva was used for testing. CRP has become to be known as a marker for systemic inflammation and amounts higher than 0.0643 mg/L indicate presence of inflammatory conditions and conditions associated with obesity such as Type II Diabetes Mellitus (Desai & Mathews, 2014). Immunoassays such as ELISA have been reported to be compatible with CRP and associated antibodies (Desai & Mathews, 2014), which is why AIDD utilizes salivary CRP as the protein of interest with PVDF membrane acting as the test strip.

Design Methodology

Reagents Used:

Item	Company	Concentration	Catalog No.
Affinity Purified Goat anti-Human CRP Antibody	Immunology Consultants Laboratory, Inc.	2 µg/mL	GCRP-80A
HRP Conjugated Goat anti-Human CRP Antibody	Immunology Consultants Laboratory, Inc.	0.1 µg/mL	GCRP-80P
C-Reactive Protein (CRP)*	Lee Biosolutions	2.0 mg/mL	140-11A
1-Step™ Ultra TMB-ELISA Substrate Solution (TMB reagent)	ThermoFischer Scientific	Unit Size: 250 mL	34028
10x Tris/Glycine/SDS Buffer**	Bio-Rad Laboratories, Inc.	Unit Size: 1.0 L	161-0732

*C-Reactive Protein used in this study is equivalent in properties to CRP found in human saliva

**Tris buffer was donated by Hong Xu from UCR's Department of Bioengineering.

Obtaining PVDF

1 in. x 3 in. PVDF strips were donated by Dr. Meera Nair from UCR's School of Medicine's Division of Biomedical Sciences. The donated PVDF strips were packaged using dual layer blue paper to protect the hydrophobic barrier on the PVDF strips.

Initial testing for color change using anti-CRP, HRP-conjugated anti-CRP, and CRP

Considering that PVDF is a suitable membrane to host an ELISA for CRP and HRP-conjugated anti-CRP, initial testing was not conducted on the PVDF membrane to conserve the donated supply. To ensure a significant color change, two Eppendorf tubes were prepared. The first tube contained a vortexed mixture of 0.1 µL of 2 µg/mL anti-CRP and 0.1 µL of 0.1 µg/mL

HRP-conjugated anti-CRP. The other Eppendorf tube mixture contained the same amount of anti-CRP and HRP-conjugated anti-CRP in addition with 0.1 µL of CRP.

Immobilizing HRP-conjugated anti-CRP antibodies on PVDF strips

A mixture of 0.775 mL of 10x Tris/Glycine/SDS buffer (Tris buffer) with deionized water was created to ensure a 5:95 dilution for significant antibody binding with PVDF. 2 µL of the diluted Tris mixture was extracted and coated onto a small portion of one 1 in. x. 3 in. PVDF strip where the CRP sample would be placed. The PVDF strip was then lyophilized in a vacuum chamber for 15 minutes. Following lyophilization, 0.1 µL of HRP-conjugated anti-CRP antibodies were loaded onto the portion of lyophilized Tris buffer. The PVDF strip was then lyophilized in the vacuum chamber for 20 minutes. The loaded PVDF strip at this stage is considered the final product design.

Ensuring colorimetric change on the PVDF membrane

2 mg/mL CRP was diluted to make a dilute concentration of 0.1286 mg/L concentration of CRP. 0.1286 mg/L of CRP was created to make sure that the concentration went above the threshold of 0.0643 mg/L (Desai & Mathews, 2014) to ensure noticeable antibody binding. 0.1 µL of the diluted CRP was added to the lyophilized portion of the PVDF. The strip was lyophilized for 10 minutes in a vacuum chamber. 5 µL of TMB reagent was then added onto the HRP-conjugated anti-CRP and CRP lyophilized region.

Schematic of final proposed ELISA on PVDF

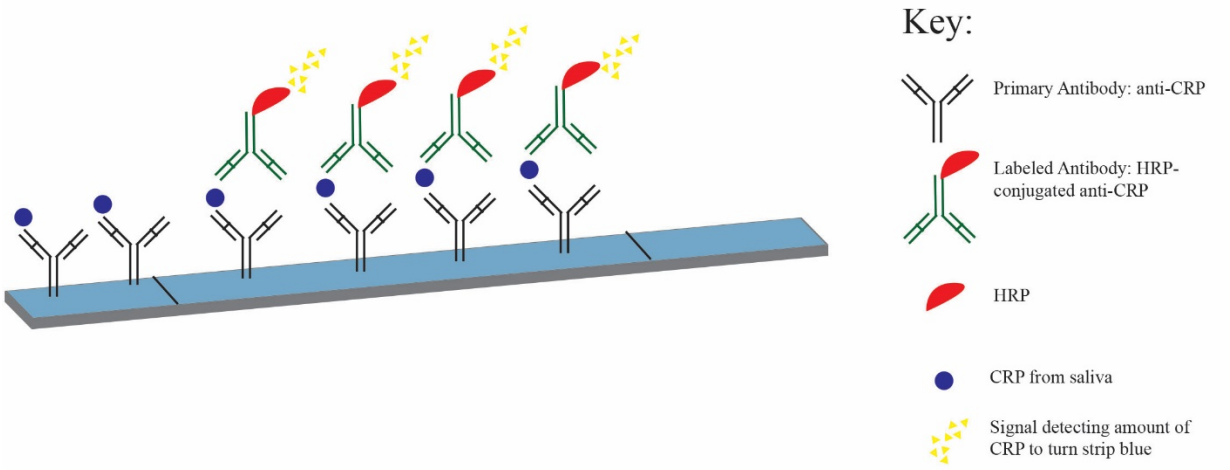


Figure 1. ELISA schematic after the PVDF membrane is lyophilized with Tris buffer along with anti-CRP, HRP-conjugated anti-CRP, CRP, and added TMB reagent. The blue bar represents a 1 in. x 3 in. PVDF membrane strip. This schematic is not drawn to scale. This schematic was generated using Adobe Illustrator.

PVDF color gradient after added TMB

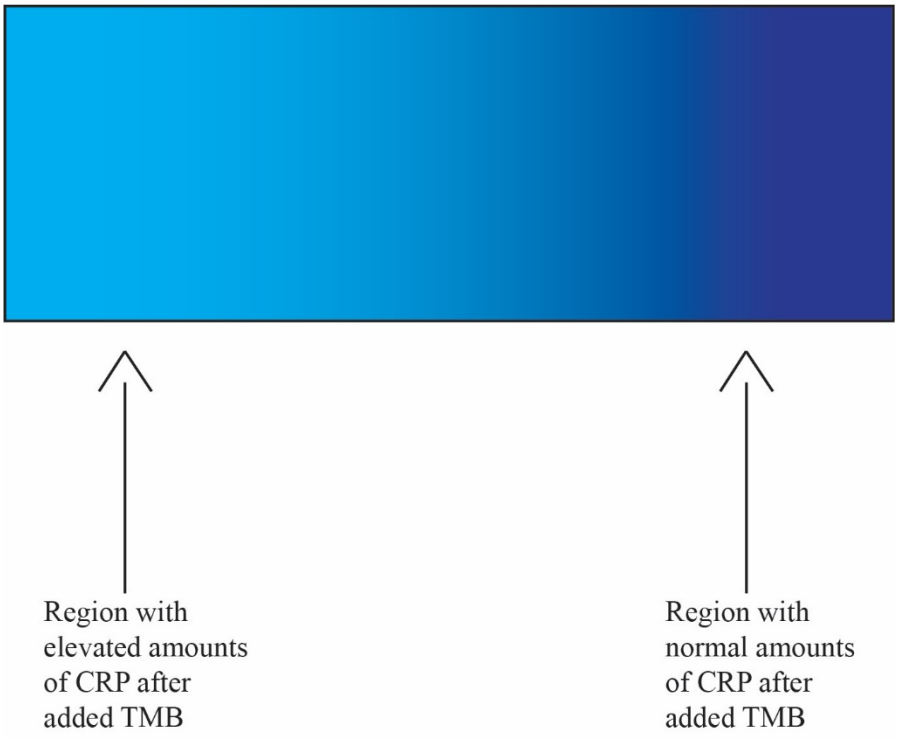


Figure 2. Blue color gradient after TMB is added to the PVDF test strip. The left side of the model indicates a light blue color region, signifying elevated amounts of CRP more than or near 0.0643 mg/L, the maximum concentration of CRP designated for a healthy individual (Desai & Mathews, 2014). The right side of the model indicates a dark blue color region, signifying normal amounts of CRP less than 0.0643 mg/L.

Results

Initial testing of color change

This portion of the study was conducted collectively by all students in the AIDD Senior Design Group.

No color change was exhibited in the initial Eppendorf tubes containing solely anti-CRP, HRP conjugated anti-CRP, and CRP. The solution was colorless. The tubes containing TMB solution buffer in conjunction with HRP conjugated anti-CRP antibodies showed a blue color change, as shown in Figure 3. Figures 3A and 3B show Eppendorf tubes that contain the mixture with only anti-CRP, HRP-conjugated anti-CRP, and TMB reagent. Figures 3C and 3D show Eppendorf tubes containing the mixture with anti-CRP, HRP-conjugated anti-CRP, TMB reagent, and added CRP in amounts higher than 0.0643 mg/L to show ensured antibody binding.

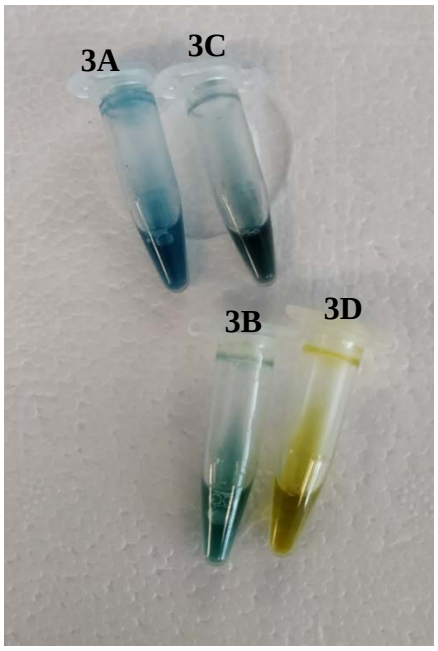


Figure 3. Initial testing results without the use of PVDF. All Eppendorf tubes contain dilute amounts of anti-CRP, HRP-conjugated anti-CRP, and TMB. **3A** and **3B** contain CRP, appearing bluer in color. **3C** and **3D** do not contain CRP, appearing lighter in color.

Immobilizing HRP-conjugated anti-CRP and anti-CRP on PVDF

A prototype of PVDF loaded with diluted Tris buffer, HRP-conjugated anti-CRP, and anti-CRP was created and appeared colorless after lyophilization. Although colorless, the region where all 3 solutions were added had small linear streaks. This prototype is shown in Figure 4.

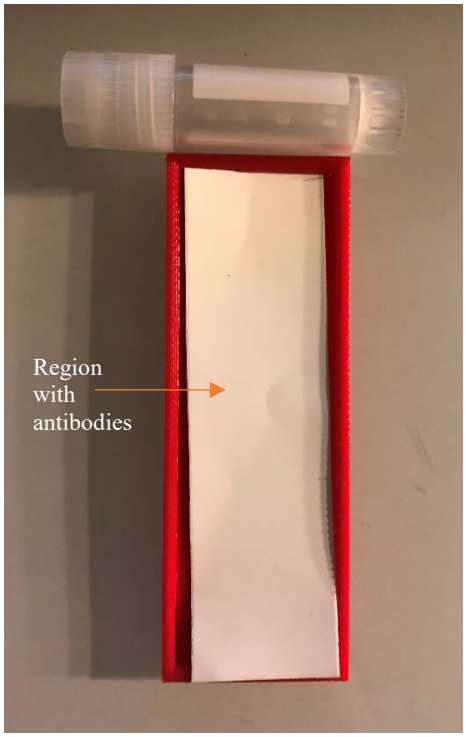
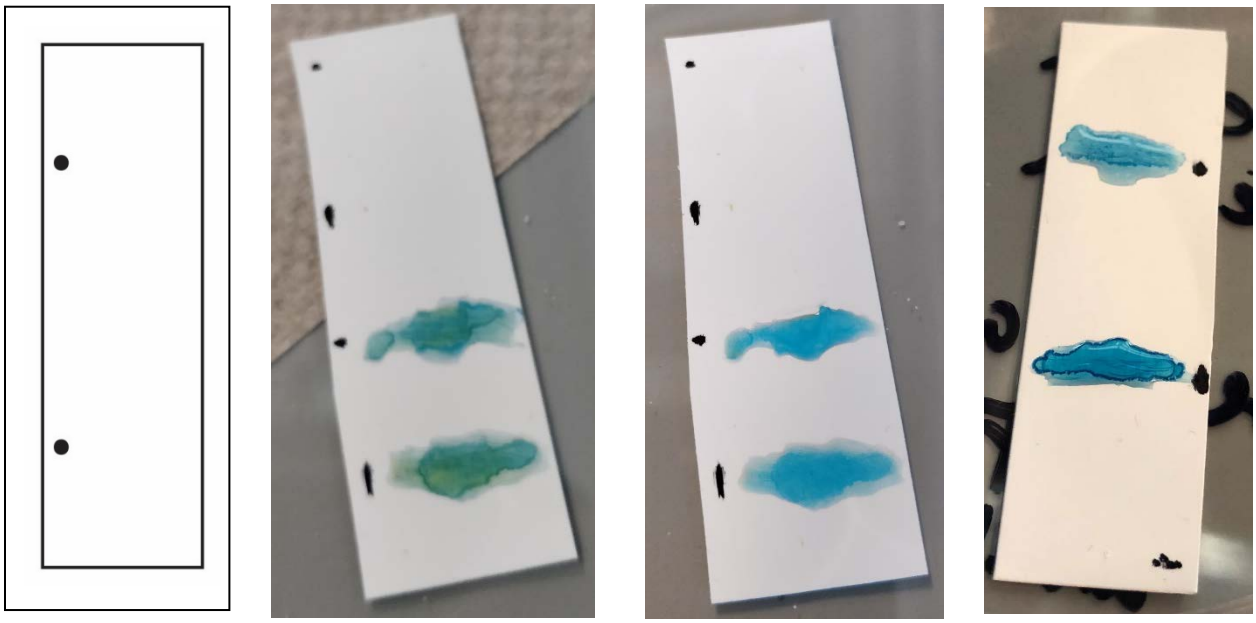


Figure 4. The final prototype of AIDD. This apparatus contains the loaded PVDF membrane containing Tris buffer, anti-CRP, and HRP-conjugated anti-CRP in one linear region. The capsule at the top is empty but once AIDD is packaged for production, the capsule will contain 50 µL of TMB.

Ensuring colorimetric change on PVDF

It was found that after insertion of diluted amounts of CRP followed by TMB reagent, a visibly blue color was shown at the ELISA region. The following strips were compared in this scenario—one with no CRP added and one with CRP added, as shown in Figure 5. Figure 5A shows the schematic for this part of the experiment, where the region to the left of the top black dot would have CRP added, while the region to the left of the bottom black dot would have no CRP added. Figure 5B shows the PVDF strip with diluted concentrations of the antibody mix in Tris buffer 5:45 mL dilution (5 mL of Tris buffer with 45 mL of deionized water) , with the top strain showing added CRP and the bottom strain showing no CRP. Figure 5C shows the PVDF strip with more diluted concentrations of the antibody mix in Tris buffer 5:95 mL dilution (5 mL of Tris buffer with 95 mL of deionized water), again with the top streak containing added CRP and the bottom streak containing no CRP. Figure 5D shows the loaded PVDF strip with a CRP concentration of 0.1286 mg/L in the top strain and the bottom strain containing no CRP.



5A **5B** **5C** **5D**

Figure 5 (A-D). Figure **5A** represents the proposed schematic for comparing two regions of antibody and CRP binding. The region to the left of the top black dot signifies the region where Tris buffer, anti-CRP, HRP-conjugated anti-CRP, CRP, and TMB are added and lyophilized, while the region to the left of the bottom black dot contains all the same materials without CRP. These regions correspond to the regions in 5B-5D. Figure **5B** includes a 5:45 µL dilution of Tris buffer containing all materials. Figure **5C** shows regions of a 5:95 dilution of Tris buffer containing all materials. Figure **5D** shows regions of extreme amounts of CRP found in human saliva, to a threshold CRP concentration of 0.1286 mg/L.

Discussion

According to Figure 3, TMB only gives rise to a blue color change when it binds to free HRP. The condition to which HRP existing as a free enzyme occurs when CRP is present in the immunoassay. The two left-most Eppendorf tubes in Figure 3 showed blue colors compared to the tubes on the right, which is in line with the expected color change with included CRP. Due to the tendency for HRP to be released from anti-CRP once the antibody bind to the calcium ion units on CRP, TMB was able to bind with HRP to produce a visually light blue color.

Although the loaded PVDF as shown in Figure 4 looks like a normal PVDF strip, the lyophilized region with Tris buffer, HRP-conjugated anti-CRP, and anti-CRP is embedded in the strip. 50 μL of TMB will be packaged with AIDD for adequate coating to ensure a visual colorimetric output.

Figure 5 used two regions on the PVDF membrane strips to represent color changes due to the intention to conserve the limited supply of PVDF. It was noted that according to Figures 5B, 5C, and 5D, the color with each ELISA region, with increasing dilution factors from Figure 5B to Figure 5C, changed according to the gradient present in Figure 2. The color gradient change was visually noticeable in Figure 5D, which shows extreme amounts (around double the amount of CRP considered normal—0.0643 mg/L) being lighter in color compared to the region below it with no CRP added.

Due to PVDF's rigid structural properties, anti-CRP antibodies were able to be immoblized after lyophilization. Due to the distinct color change generated on the PVDF strip, PVDF was able to act as a Sandwich ELISA platform for HRP-conjugated anti-CRP binding. In this sense, PVDF acts as a suitable platform for quantifying amounts of CRP in human saliva by use of a colorimetric immunoassay.

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