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Study of Pneumococcal Surface Protein, PspA, Incorporated in Poly(Vinyl Alcohol) Hydrogel Membranes

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Abstract

This study investigates poly(vinyl alcohol) (PVA) membranes as controlled release micro-matrices, which can be useful in therapeutic applications for optimizing the administration of drugs. Currently, the use of hydrogels is limited by protein size. This study investigates the delivery of PspA, a large protein of approximately 38 kD. Pneumococcal surface protein A (PspA) has been shown to provide protective immunity against pneumococcal infection and is considered as a pneumococcal vaccine. The protein release experiments demonstrated that from an initial pH 7.4, approximately 60% of PspA diffuse into a neutral environment with an initial burst and a declining rate reaching equilibrium. The results indicate that the protein was successfully incorporated and released from the membrane over time. The hydrogel and protein interaction is temporary, and the membrane system is ideal for protein drug delivery. The data confirm that the protein did not aggregate and was active after release. The protein release is promising and a step forward to develop microneedles to facilitate high molecular weight protein delivery as well as vaccine delivery.

Keywords

Hydrogel, Recombinant PspA, Drug Delivery, Vaccine, Poly(Vinyl Alcohol) (PVA) Membrane, Streptococcus Pneumoniae

1. Introduction

Despite research and advances in the use of therapeutic proteins, delivery ve-

hicles have limited their use [1] [2]. Therapeutic protein delivery has been explored via hydrogel membrane systems to avoid degradation and restrictive gastrointestinal absorption, intravenous [1] [2] [3]. The use of membranes will potentially decrease the use of needles for vaccines and, consequently, increasing patient comfort and compliance. Controlled drug delivery has progressed over the years and is improving with the use of different types of hydrogel membranes. Understanding polymers, protein release mechanisms, and molecular interactions are necessary for efficient design and fabrication [1] [3]. Desired protein drug release kinetics requires an understanding of the proposed mechanism and delivery material [4] [5]. *Streptococcus pneumoniae* (pneumococcus) is a gram-positive, alpha-hemolytic bacterium with 91 serotypes. *S. pneumoniae* is known for causing human diseases such as osteomyelitis, keratitis, septic arthritis, endocarditis, peritonitis, cellulitis, brain abscesses, and otitis media. It is a primary cause of morbidity and mortality; therefore, a vaccine has been administered since the 1970s [6]. Studies have shown a reduction in the prevalence of the disease as a result of the various vaccines [7]. This study investigates the interaction, release rates, and diffusion efficiency of different proteins from a hydrogel membrane.

A surface protein of *S. pneumoniae*, PspA (Pneumococcal Surface Protein A), elicits protection against pneumococci. PspA contains four distinctive domains, which include the N-terminal domain, alpha-helical domain, a proline-rich domain, and a choline-binding [8]. The alpha-helical domain contains protection-eliciting epitopes. It has a high degree of serological variability with a size ranging from 67 to 99 kDa, however, this study uses a truncated version (the alpha helix), 38 kDa [9]. The truncated PspA has been proven to elicit immunity [10]. PspA is highly electrostatic with an elongated rod-like shaped, coiled-coil structure [9], and the functional N-terminal end is electronegative. Regarding PspA virulence, studies have shown that the presence of PspA protects pneumococci from clearance in the blood of infected mice [11] [12]. The evidence demonstrated that PspA incorporated into a poly(ethylene oxide) matrix inhibits complement activation through the interference with the deposition of C3b; this inhibition can occur in classical and alternative pathways [6] [11]. Studies on PspA reported that it is a promising vaccine candidate [6] [11] [13].

Poly(vinyl alcohol) (PVA) cross-linked with glutaraldehyde (GA), a prolonged-release micrometrics, is useful for drug delivery [14] [15]. PVA swells and absorbs water to create a swelling-controlled delivery system, and various studies are being conducted worldwide for the application of hydrogels as controlled released drug delivery systems [14] [16] [17]. We are studying the PVA membrane for its use and ability to deliver a large protein into the desired environment [14] [18]. Furthermore, the use of PVA increases the bioavailability of drugs [17] and decreases drug loss due to the increase in viscosity [19] [20] [21]. PVA membranes are non-biodegradable, and the retained structure allows the space for drug release [22] [23] [24]. A saturated solution of the drug is en-

trapped within the PVA membrane structure, and the drug diffuses across the hydrogel matrix when the surface of the PVA hydrogel encounters hydrophilic solution [19] [22] [25] [26]. Therefore, the PVA crystalline-like structure can be modified to control the diffusion rate of the drug via the cross-linking ratio [23] [27] [28].

With the isolation of a truncated fragment of PspA from *E. coli*, this study investigates the release of PspA from the open-mesh of hydrogel membranes cross-linked with glutaraldehyde. Many characteristics play a part in this study, including testing protein release and diffusion at pH 7.4 (which represents any biological environment), calculating the Equilibrium Solution Constant, and determining protein presence in the gel after incorporation using ATR-FTIR. This study also investigated the release mechanism of PspA and how a sufficient amount of protein is released from a membrane over time. With the characterization of the PspA filled thin film hydrogel membrane, it provides the necessary information to develop a drug delivery system. This system represents a proof of concept for a large protein. The mechanism of hydrogel formation allows modifications that control protein entrapment and release.

2. Materials and Methods

2.1. Materials

The hydrogels used for this study were prepared using poly(vinyl alcohol) PVA (98% hydrolyzed, the molecular weight of 70,000 - 100,000 g/mol^{-1}), acetic acid (AA), and glutaraldehyde (GA) (50% aqueous solution). All materials for the formation of membranes were purchased from Sigma-Aldrich chemical company (St. Louis, MO, USA). Nitrogen gas was purchased from Airgas, USA. Ampicillin was also purchased from Sigma-Aldrich chemical company. Sodium phosphate and Luria broth were purchased from Fisher Scientific. All chemicals were used without further purification and were of analytical grade. The alpha-helical domain of PspA was purified from the *E. coli* clone. Anti-6x His tag antibody was purchased from Abcam, USA. Anti-PspA mouse monoclonal antibody XiR278 (IgG) was gifted from Dr. Larry McDaniel at the University of Mississippi Medical Center. Secondary antibody Alkaline Phosphatase-conjugated AffinPure Goat Anti-Mouse IgG (H + L) was purchased from Jackson ImmunoResearch Laboratories, USA.

2.2. Growth and Purification of Pneumococcal Surface Protein A (PspA)

PspA is highly expressed in *E. coli*. 5 ml of *E. coli* overnight growth was inserted into a flask containing 1 liter of Luria Broth (LB) and 75 μg/ml of ampicillin. The starter culture was then incubated in a shaker for 2.5 hours with an RPM of 200 at 37˚C. Cells were grown to an OD600 of 0.6 - 0.9 at 37˚C, and 1 mM IPTG was added for the expression of PspA. Cells were allowed to continue growing for 4 hours after induction. Cells were harvested and sonicated. The lysate was sub-

jected to purification using the Fast Protein Liquid Chromatography (FPLC) system from ÄKTA Amersham Pharmacia was used to purify PspA. Recombinant PspA was designed with His-tag on the C-terminal to facilitate the purification steps using IMAC nickel loaded HiTrap IMAC FF column (Ni-NTA). Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot was used to confirm the isolation, purity, and activity of PspA.

2.3. Preparation of PspA-Loaded Poly(Vinyl) Alcohol (PVA) Hydrogels

The hydrogels were made by dissolving 5 g of high molecular weight PVA in 40 ml of boiling deionized water, allow the solution to cool to room temperature. Mix 250 µl of PspA (final concentration, 0.12 mg/ml or 0.7 mg/ml) and 960 µl of PVA solution with 108.75 µl of 10% (v/v) sulfuric acid (catalyst), 36.25 µl of 10% (v/v) acetic acid (buffer), 36.25 µl of 50% (v/v) methanol (quencher) and 108.75 µl of 1% (v/v) Glutaraldehyde (cross-linker). The solution was poured on a glass plate to form a membrane. The membrane was cross-linked overnight at room temperature inside a nitrogen gas chamber to control humidity.

$$\left(0.12 \text{ or } 0.7 \text{ mg/ml PSA}\right)\left(1.5 \text{ ml PVA}\right) = \left(C_O\right)\left(250 \text{ ul PspA}\right)$$

C_O = Concnetration of PspA stock.

Equation (1) Equation for Constitutional formula of PspA, Poly(vinyl alcohol).

2.4. Diffusion Cell Experiments (DC)

Hydrogel membranes were placed between two adjacent diffusion cells to perform drug diffusion experiments. One chamber was vacant, and the other contained 5 mL of 20 mmol sodium phosphate buffer solution exposing only one face of the membrane to a concentration gradient. The solution is stirred continuously using a stirring rod and maintained at 37˚C by circulating heated water through the jacketed diffusion cells. A UV spectrophotometer is used to calculate the concentration of protein diffused into the buffer solution. Samples are taken periodically over 48 hours, and absorbance is recorded at 277 nm for PspA. The protein concentration was calculated using Beer's Law, A = ε*l*c.

2.5. Equilibrium Solution Content (ESC) of Protein-Loaded PVA Hydrogel

The equilibrium solution content was calculated to determine the characterization of the porous structure of the hydrogel for the drug delivery system of PspA. This measurement provides the relative measure of swelling to approximate the mesh size of the hydrogel. The ESC was determined by placing the hydrogel in buffer solutions ranging at pH 7.4 for this study. ESC, swelling ratio, and mesh size were calculated using Equations (2)-(4) [29].

$$\text{ESC} = \left(\frac{W_{wet} - W_{dry}}{W_{wet}}\right) \times 100\%$$

Equation (2) Equation for ESC.

$$\text{Swelling ratio} = \frac{W_{wet}}{W_{dry}}$$

Equation (3) Equation for the Swelling ratio.

W_{wet} = membrane wet weight, W_{dry} = membrane dry weight.

$$\xi_S = l_c \left(\frac{M_c}{M_r} \right)^{\frac{1}{2}} C_n^{\frac{1}{2}} v_2^{\frac{1}{3}}$$

Equation (4) Equation for Mesh size.

C_n is the rigidity coefficient, M_c is the number of average molecular weight between junction, M_r is the molar mass of repeating units of PVA, l_c is the C-C bond length (0.154 nm), v_2 is the swollen polymer volume fraction after equilibrium.

2.6. Application of Mass Transport Equations

Higuchi's Equation can be applied because the experimental system follows the same basic principles from the original derivation. Higuchi's equation assumes a pseudo-steady-state in which the initial drug concentration is at least ten times greater in magnitude than the drug solubility [30].

$$k_H = \frac{M_t}{\sqrt{t}}, \quad D = \frac{\pi}{t} \left(\frac{M_t}{2M_0 A} \right)^2$$

Equation (5) Higuchi K Constant and Equation (6) Diffusion Coefficient.

M_t = the mass [mg] diffused at time t, $\sqrt{t}$ = square root of time [seconds];

M_t = the mass [mg] diffused at time t;

M_0 = initial mass [mg] diffused at time 0, t = time [seconds];

A = the exposed surface area of hydrogel.

2.7. Protein Release Study (PR)

PspA hydrogel membranes were submerged in 5 mL of 20 mmol sodium phosphate buffer of pH 7.4. The tubes are incubated at 37˚C with agitation. UV spectrophotometer was used to measure PspA concentration by recording absorbance at 277 nm. The aliquot was returned to the sample. SDS-PAGE and native gel electrophoresis were used to confirm PspA diffusion from the PVA membranes. Western blot was used to validate that the released protein was active.

2.8. Fourier Transform Infrared Spectroscopy (FTIR)

Membranes with and without protein were dried at 90˚C, and ATR-FTIR (IR-Affinity) was used to analyze the surface of the membrane. This technique was used to determine functional groups, such as hydroxyl groups present within the cross-linked membrane matrix, and peaks associated with the presence of protein.

2.9. Human Corneal Epithelial Cells

Human Corneal Epithelial Cells (HCEC) were purchased from Invitrogen and maintained in Keratinocyte media supplemented with 0.2% v/v Bovine pituitary extract (BPE), 1 μg/mL Recombinant human insulin-like growth factor-I, 0.18 μg/mL Hydrocortisone, 5 μg/mL Bovine transferrin and 0.2 ng/ml Human epidermal growth factor. HCEC were grown to confluency and were introduced to the hydrogel with or without the protein (PspA), for 24 hours in separate experiments. The supernatant was collected to use in both Cytokine Arrays and Enzyme-linked immunosorbent assay (ELISA). The assay's protocol was performed according to the manufacturer's guidelines.

2.10. Cytokine Assays

Supernatants from Human Corneal Epithelial Cells (HCEC) exposed to growth media, and PspA hydrogel was collected 18 - 20 hours following exposure. Homogenates were analyzed with human cytokine antibody array 7 (Ray Biotech) according to the manufacturer's specifications. Briefly, antibody array membranes 7 were placed into eight-well trays and blocked with 2 ml of blocking buffer for 30 minutes. Blocking buffer was decanted, and membranes were incubated at 25˚C for 2 hours with l ml of supernatants. Samples were decanted, and membranes were washed three times with 1x wash buffer I followed by 2x wash buffer II at 25˚C with shaking. Diluted biotin-conjugated antibodies (1 ml) were added to each membrane and allowed to incubate at 4˚C overnight. Membranes were again washed with 1x wash buffers I and II prior to being incubated at 25˚C for 2 h with diluted horseradish peroxidase-conjugated streptavidin. Following a final wash, the membranes were incubated with detection buffers C and D. The chemiluminescent signal was then detected by exposure to film, and intensity was determined by densitometry. Signal intensities were quantitated using the Gel Doc system (BioRad), and the increased levels of cytokines from the exposure were compared.

2.11. ELISA

HCEC exposed supernatants were analyzed with specific ELISA Kits Interleukin-6 (IL6), Interleukin-8 (IL8), Interferon gamma (IFN-γ), Tumor necrosis factor alpha (TNF-α), Interleukin-12 (IL-12) P40, Interleukin-12 (IL-12) P70, Interleukin-16 (IL-16) and GRO-α (Ray Biotech). The homogenates were incubated in the ELISA plate at 4˚C overnight, which was followed by wash according to the wash solutions in the kit. Diluted biotin-conjugated antibodies specific to the cytokine being evaluated was added to each well and allowed to incubate at 4˚C overnight. Wells were washed and incubated at 25˚C for 45 min with diluted horseradish peroxidase-conjugated streptavidin. The wells were washed, and 100 μl of TMB One-Step Substrate Reagent was added to each well and allowed to incubate at room temperature for 30 min. The reaction was stopped solution and read at 450 nm immediately with the Biotech plate reader at 450

nm for ELISA analysis.

Statistical Analysis: Multi-way ANOVA and post-hoc Tukey HSD tests were performed using GraphPad Prism 8 software. Significance was defined by $\alpha = 0.05$.

All experiments were carried out at the University of Arkansas except the Cytokine Assays and ELISAs.

3. Results and Discussion

3.1. Recombinant PspA Purification

E. coli BL21 cloned with PspA plasmid lysate was purified using Ni-NTA. The SDS-page stained with Coomassie Brilliant Blue shows a strong band at 38kD for 32.25 mM and 62.5 mM fractions (**Figure 1(a)**). The 32.25 mM imidazole fraction (lanes 4 - 6) shows some impurities while the 62.5 mM imidazole fractions (lanes 7 - 9) show a pure 38 kD band, which represents recombinant PspA. Western blot result confirmed the 38 kD fraction has a His-tag protein signal related to the recombinant PspA (**Figure 1(b)**). Purified PspA from 62.5 mM imidazole fraction was used for future experiments and application.

3.2. Evaluation of PspA Protein Diffusion

The mechanism of drug release occurs initially only close to the surface of the hydrogel film. The drug (in this study protein is equivalent to the drug) is in high concentration within the membrane; therefore, as it diffuses out and the interstitial void left behind is quickly filled with drug transported from further behind the membrane's surface [30]. In this scenario, the dissolution of drug molecules into the newly available region is constant [30] [31]. At the diffusion front where the hydrogel meets the buffer solution, a pseudo-steady state is assumed for the system. This assumption can be made because of the initial large quantity of drug in the medium, and the "perfect sink" scenario is achieved via constant agitation [30]. The assumption also neglects any swelling and dissolving of the membrane. As expected, the concentration gradient is the driving force of mass transport in the medium.

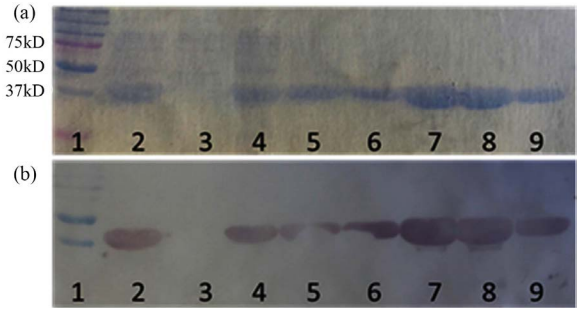


Figure 1. Coomassie Brilliant Blue staining and Western blot using Anti-6x His-tag antibody of a riveting IMAC fraction of recombinant PspA. For both methods; a. CBB and b. WB. Lanes 1—molecular weight marker; 2—lysate; 3—flow through; 4 - 6—fraction 9 to 11 respectively; 7 - 9—fraction 18 to 20, respectively.

The results of the PspA diffusion cell experiments are presented in **Figure 2**. At the neutral pH of 7.4, the maximum diffusion experienced was ~60%. The "initial burst effect" is experienced, and the diffusion profile demonstrates a constant release until equilibrium is reached (**Figure 2(a)**) [32] [33]. After 12 hours, the rate appears to decline. The calculated Diffusion coefficient [cm^2/s] in **Figure 2(b)**, correlates with the release percentage data. The rate is initially faster and plateaus around 18 hours in the experiment.

3.3. Swelling Behavior of Protein-Loaded PVA Hydrogel

The equilibrium solution content was calculated to characterize the porous structure of the hydrogel for the drug delivery system of PspA. The results show that swelling of 1% GA membranes is close and above 80% after 24 hours of swelling and above 90% after 48 hours of swelling (**Figure 3(a)**). The equilibrium solution results from release experiments illustrate a similar ESC value between 0.12 mg/ml drug-loaded membranes and its respective controls (**Figure 3(a)**). However, the data shows that at the higher concentration, 0.7 mg/ml, PspA membranes at 24 h have a 12.5% larger ESC compared to 24 h control ($p \leq 0.0001$). The ESC value for 0.7 mg/ml has less than 1% increase at 48 hours, suggesting that 0.7 mg/ml membranes are near equilibrium at 24 hours, while 0.12 mg/ml membranes may take over 48 hours to do the same. At 0.12 mg/ml concentration, the protein molecules are more dispersed, making it more difficult for collisions between molecules to happen, therefore slowing down the diffusion out of the membrane. The results demonstrate that protein concentration affects the swelling and mesh size of the hydrogel [32] [34]. The higher protein concentration produces a greater concentration gradient that leads to a rapid increase in swelling and pore size (Å) until equilibrium is reached [24].

There were no significant changes in swelling properties between 0.12 mg/ml PspA membranes with corresponding controls at 24 and 48 hours (**Figure 1(b)**). Both membranes nearly double in the swelling ratio at 48 hours. However, at 24 hours, the 0.7 mg/ml PspA membrane had three times the swelling ratio of its respective control ($p \leq 0.0001$). While the control membrane had a significant increase in swelling with time ($p = 0.0029$) [24] [35], the 0.7 mg/ml PspA membrane did not. The results indicate that the 0.7 mg/ml hydrogel was already swollen to near its maximum capacity with the more significant protein concentration, while the control and 0.12 mg/ml PspA membranes expanded with the influx of buffer at 48 hours.

An evaluation of the hydrogel pore structure shows that mesh size increases with the protein concentration of the hydrogel (**Figure 3(c)**). The mesh size for 0.12 mg/ml PspA membranes relative to its controls is not significantly different, signifying that 0.12 mg/ml is such a low concentration that the gel mesh size is virtually unaltered [23] [34]. However, increasing the concentration to 0.7 mg/ml of PspA causes the mesh size to rise by 335 Å relative to 24 hr control ($p \leq 0.0001$). While the control and 0.12 mg/ml membrane continued to increase in mesh size at 48 hours, the 0.7 mg/ml membrane only showed a neglectable

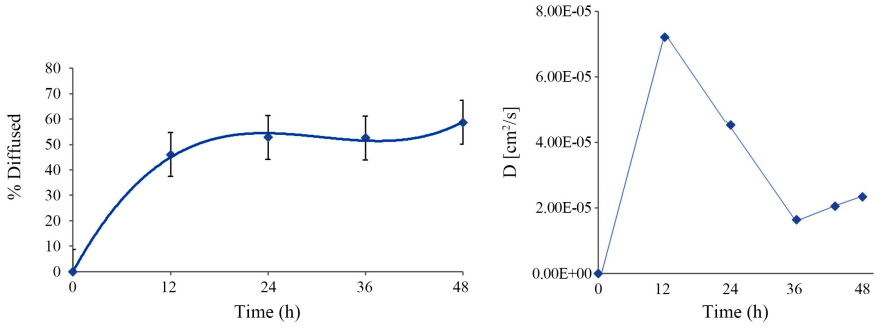


Figure 2. (a) The percentage of PspA released in 5 mL Sodium Phosphate, pH 7.4 (b) Calculated Diffusion Coefficient [cm²/s].

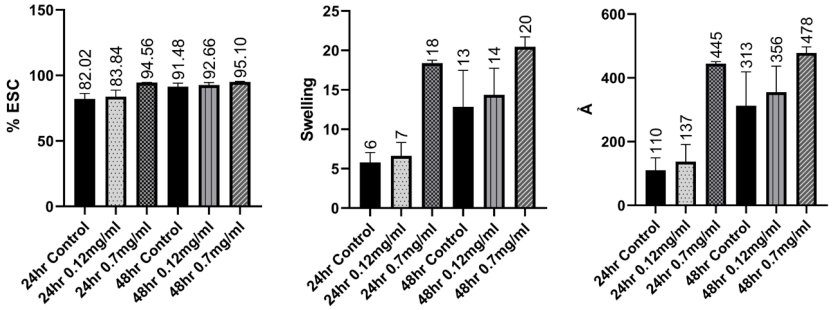


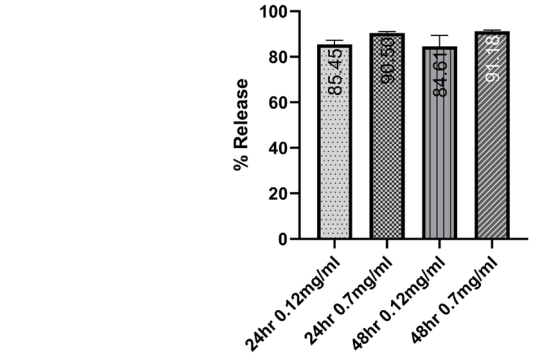
Figure 3. (a) Equilibrium Solution Content of protein-loaded PVA hydrogel at 0.12 mg/ml and 0.7 mg/ml PspA concentrations. (b) Swelling ratio of hydrogels from 0.12 mg/ml and 0.7 mg/ml PspA membranes at 24-hours and 48-hours. (c) Mesh size in Å of hydrogels from 0.12 mg/ml and 0.7 mg/ml PspA membranes at 24-hours and 48-hours.

increase. The higher protein concentration causes the mesh to be so stretched that as protein is released and buffer diffuses in, the size remains nearly the same. At lower protein concentration, however, the membrane pores are not fully stretched, so as the buffer moves into the hydrogel, the pore increase in size, and the membrane swells.

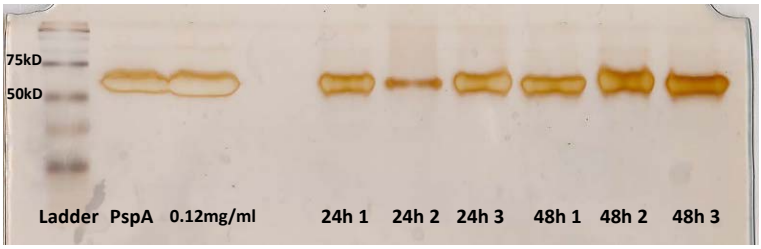
Two different methods were used to confirm protein release. The UV-vis result shows no significant changes in the amount of protein released from 24 hours to 48 hours for 0.7 mg/ml membranes and 0.12 mg/ml membranes, indicating that all the protein within the hydrogels is released at 24 hours. The 24-hour membranes had 5% less release than 48-hour most likely due to the increased space between the molecules, and the random motion of proteins in and out of the membrane, which explains the larger standard deviation for the 0.12 mg/ml membranes (**Figure 4(a)**). Native gel confirmed that encapsulating the protein within the hydrogel does not lead to protein aggregation and that the protein integrity is not affected after release (**Figure 4(b)**).

Recombinant PspA was designed with 6x His-tag in C terminus to facilitate purification of the protein using Ni-NTA. Also, 6x His-tag was used in this experiment as a standard to estimate the relative activity of PspA after release from the hydrogel. After release, the 6x His-tag PspA molecules from the hydrogel can

be detected using western blot with anti-6x His-tag antibody while the anti-PspA antibody had been used to identify the active PspA after release [10]. **Figure 5** validates the release of PspA at 0.12 mg/ml concentration from the hydrogel and confirms its activity due to its strong interaction with Anti-PspA (IgG). These results confirm the released PspA is completely active, and the hydrogel system facilitates a high molecular weight protein delivery. Thus, the system is suitable for vaccine delivery.



(a)



(b)

Figure 4. (a) Protein release calculated from UV-Vis concentration; (b) The native gel of 0.12 mg/ml membrane release after 24 hours and 48 hours.

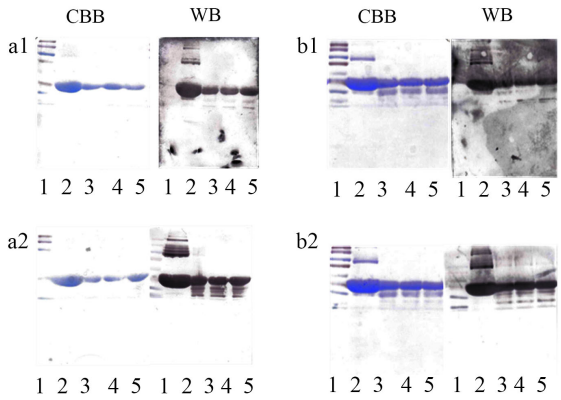


Figure 5. 1. Coomassie Brilliant Blue (CBB) staining and Western blot (WB) of PspA released from hydrogel using Anti-6x His-tag antibody. 2. Coomassie Brilliant Blue staining and Western blot of PspA released from hydrogel using Anti-PspA antibody. a1 & a2. Release after 24 hours. Lane 1—molecular weight marker: 2—0.7 mg/ml PspA; 3 - 5—PspA release. b1 & b2. Release after 48 hours. Lane 1—molecular weight marker; 2—0.7 mg/ml PspA; 3—2 PspA released.

3.4. ATR-FTIR Spectroscopy

The interaction of cross-linkage was examined by Attenuated Total Reflection-Fourier Transform InfraRed (ATR-FTIR) spectroscopy. The resulting spectra show all major peaks (3300, 2940, 1731, 1141, and 1087) related to hydroxyl and acetate groups, which are indicative of a PVA hydrogel cross-linked with glutaraldehyde. Aldehyde peaks at 1735 (C=O stretch), 2850 (C-H stretch, and 1650 (OH def) cm^{-1} with intermolecular hydrogel bonding of water are also visible. The spectrum showed bands at 1140 (anti-sym) and 872 (sym) cm^{-1} indicative of the C-O-C-O-C stretch vibrations from acetal functional groups, which can represent an Acetal Ring Group or Ether Linkage Formation Structure resulting from binary functionalization. The PVA hydrogel with PspA incorporated displayed new vibration bands at 1648 and 1542 cm^{-1} in comparison to PVA hydrogel without protein [15] [16]; these bands represent the Amide I and Amide II similar to what others have seen when protein is present [36] [37]. The readings confirmed the incorporation of protein, seen in **Figure 6**. The appearance of these bands was decreased after release experiments, thus, indicating the release of PspA.

3.5. Cytokines Expression and ELISA

As proof of the safety of the hydrogel system, cytokine antibody array analysis was utilized to investigate host immune factors that are involved in the ocular environment when the hydrogel is present and the release of the PspA protein. The corneal homogenates collected at post-exposure were analyzed using human inflammatory cytokine arrays. Chemiluminescent was used to detect signal intensities, and the signals were highlighted to show areas that had increased signals (**Figure 7**). The cytokine difference relative to the media and PspA hydrogel was determined and listed in **Table 1**. Of 60 cytokines assayed, there was a slight increase in the presence of GRO alpha and IL-8 in both conditions. The results demonstrated no contrasts in the cytokine expression profiles in the corneal homogenates collected from exposure to media alone and PspA hydrogel.

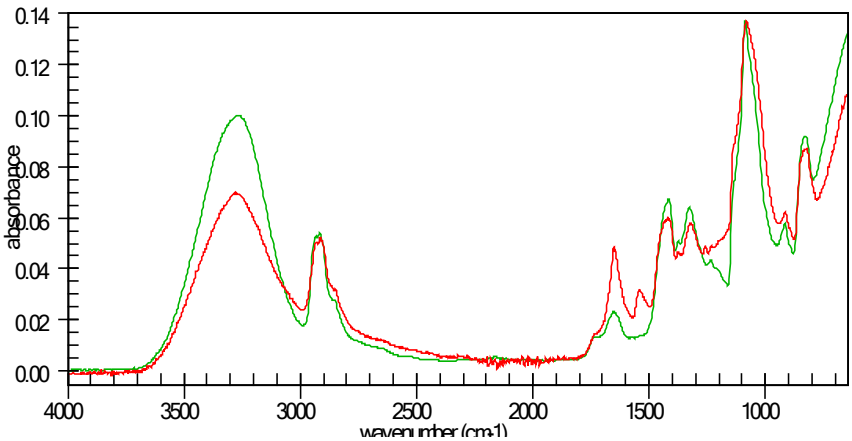
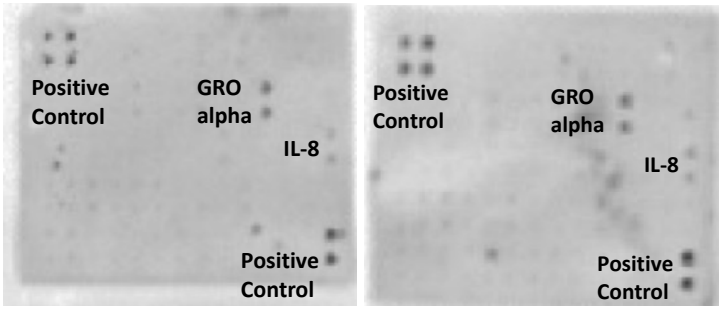


Figure 6. ATR FTIR, spectra of 1% GA PVA hydrogel membrane (green), and 1% GA PVA hydrogel membrane with the protein, PspA, incorporated (red).



(a) (b)

Figure 7. Film images of cytokine arrays. A total of 60 cytokines were analyzed, (a) Representative of media 18 hours post-infection (b) Representative of PspA hydrogel 18 hours post-infection.

Table 1. Cytokine profiles of Human Corneal Epithelial Cells Exposure to Hydrogel Drug Delivery System with PspA.

Sample	IL-8[1]	Gro-alpha[1]
Control	0.167 ± 0.0718	0.048 ± 0.0033
PspA	0.153 ± 0.0956	0.045 ± 0.0033
Hydrogel System	0.151 ± 0.0590	0.046 ± 0.00042

[1]Numbers are expressed as Mean ± Standard Error of the Mean (SEM).

To further evaluate the introduction of the hydrogel delivery system in the ocular environment, the innate immune response was assayed using cytokine antibody arrays. The results from the cytokine arrays were identical in cytokine expression. In these experiments, we identified GRO alpha and IL-8 were slightly upregulated in both media and PspA hydrogel. The immune response was further investigated utilizing specific ELISA assays of cytokines present during infection. It was noted that there was not an increase in the presence of the proteins, which is indicative of a normal immune response based on the data from the control samples.

4. Conclusion

With the isolation of a truncated fragment of PspA from *E. coli*, this study investigates the release of PspA from the open-mesh of hydrogel membranes. With the characterization of the PspA filled thin-film hydrogel membrane, it provides the necessary information to develop a modern biotechnology technique for the drug delivery system of high molecular weight proteins. This technique expands the current use of hydrogels and its application for vaccine delivery. The system represents a proof of concept and applies to proteins of similar size. The mechanism of hydrogel formation allows modifications that control protein entrapment and release. In conclusion, time and protein concentration affect the hydrogel pore size and drug release. Altogether, the data indicate that membranes with low protein concentration behave similarly to control membranes. Pore swelling and mesh size increase with time, with larger pores having higher

protein release. Most of the protein within the hydrogel gets released within 24 hours, with membranes with 0.7 mg/ml concentration having the most release. The results from this study give insight into the minimum presence of cytokines, which are indicators of the innate immune response. Based on these findings, the presence of the hydrogel will not trigger an immune response that could potentially hinder the action of the drug therapy. Based on this study, we have provided evidence of an efficient hydrogel membrane system for protein delivery. With the characterization of PspA into a thin film hydrogel, it provides the necessary information of PspA transient entrapment within the membrane. This study provides the groundwork for applications of this hydrogel membrane delivery system in various infection models.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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