A NEW PROTOCOL IN PROTEIN IMMOBILIZATION USING PYR-BH3 FOR LEUKOCYTE REPROGRAMMING

Leonid Mirson
McGowan Institute for Regenerative Medicine: Medical Devices Lab

INTRODUCTION

One or the largest health problems in the United States of America is that of severe infection leading to sepsis or septic shock. In Intensive Care Units throughout the country, sepsis is the number two leading cause of death [1]. Though the discovery of antibiotics has gone a long way to treading sepsis in patients, overall prognosis is still poor; furthermore, there has been no new effective treatment developed in the last thirty years. New protocols and drugs have been discovered for symptomatic treatment such as controlling blood pressure, heart rate, and breathing, but no treatment has been successful at treating the underlying cause [2].

Sepsis is a disease process that is more complex than the body’s regular response to infection. Sepsis, in many cases, is due to the body’s over-reaction to an infection leading to release of numerous cytokine proteins and elevating white blood cell count [2]. Patterns of large cytokine protein release, specifically the Interleukin proteins, have been associated with increased mortality and poorer prognosis [3]. It is now widely believed that excessive activation of neutrophil chemotaxis due to increased cytokine production may cause neutrophils to aggregate and damage healthy tissue through oxidative processes or by forming clots [1].

Therefore, we aim to reduce neutrophil chemotaxis and response to the cytokines that the body produces when sepsis occurs in order to prevent damage to healthy tissues. We hope to achieve our goals through use of a concept known as cell surface receptor down-regulation. According to Sabroe et al., it has been demonstrated that binding of Interleukin-8 to the neutrophil causes the Interleukin-8 receptor to internalize inside of the cell and deactivate for a period of time [4]. By utilizing the idea of receptor down-regulation, we plan on attaching, or covalently immobilizing, cytokine proteins onto polymethylpentene fibers. A septic patient’s blood will then be run through fibers and recirculated back to the patient, with the goal that the neutrophils in the blood will be deactivated and unable to significantly respond to strong cytokine signaling in the body.

Our project’s primary focus at this stage is to improve our protein immobilization procedure. Though there are many variables and reagents that can be altered, one of the most important chemical reagents that we use to bind protein to the fibers is sodium cyanborohydride (NaCNBH3). It is our reducing agent and is also the largest source of our problems. NaCNBH3 is an extremely hydroscopic powder, and therefore absorbs water very quickly. It has a very short shelf life and after few uses becomes impossible to measure accurately. This leads to difficulty in reproducing results due to high variability in the amount of protein immobilized. Furthermore, NaCNBH3 is extremely dangerous to use due to the formation of cyanide gas if it comes into contact with a significant amount of water or acid [5]. Due to the combination of these factors, it has been decided that we seek to find a new reducing agent to be used in our experiment. The reducing agent that was decided upon is 5-ethyl-2-methyl pyridine borane complex, referred to as Pyr-BH3 henceforth.

OBJECTIVE

Our goal is to create a protocol that successfully immobilizes the same amount of protein onto fibers using the Pyr-BH3 as compared to the NaCNBH3, and definitively quantify the amount of protein immobilized onto the fibers with both reducing agents.

SUCCESS CRITERIA

The project will be deemed a success if all the goals are met. Specifically, quantification of protein immobilized with the Pyr-BH3 reducing agent reveals the same amount of protein or greater to that immobilized with the NaCNBH3 reducing agent. There will also be a negative control in place to eliminate contamination as a possible reason for a high reading. Therefore, success can only be determined if the protein concentration from both reducing agents is significantly higher than that of the negative control. Finally, the results of the protein quantification must be consistent over a number of trials and sample sizes.

METHOD

Pyr-BH3 was decided upon as the new reducing agent of choice for a number of reasons. After conducting a literature search, it was determined that Pyr-BH3 is cost-effective, has similar reducing capabilities to NaCNBH3, is safe to handle, comes in liquid form (which is significantly easier to measure), has a long shelf life, and has a low rate of hydrolysis [6]. The hydrolysis is very important because much of the immobilization process takes place in water and is one of the limiting factors that eliminated many other potential reducing agents.

The protein chosen to test immobilization capabilities was BSA, or Bovine Serum Albumin. It was chosen because unlike cytokine protein, it is cheap and readily available. The starting testing protocol chosen for the Pyr-BH3 was identical to that of the NaCNBH3 protocol, save for the reagent difference.

The protocol takes roughly 6 hours to run and is fairly complex with many steps. Essentially it begins by putting a set of fibers into the plasma amination machine located in our lab. This machine vaporizes amine group monomers and attaches them directly to the fibers. The duration, quantity, and temperature settings of the plasma machine were not altered because it was previously determined that the amount of amine
groups placed on the fibers fully saturated them with the current recipe. Following this, a series of washes took place in phosphate buffered solution at a specific pH in order to remove any contamination. The next steps of the process included attaching a layer of gluteraldehyde to the amine groups, followed by attaching cross-linking chitosan to the gluteraldehyde, attaching BSA protein to the chitosan layer, and finally adding the proper amount of reducing agent. All the layers of compounds help to attach a maximum amount of protein to the fibers. The reducing agent is a critical step that follows the protein attachment – it stabilizes the attachment and makes a stronger bond to the fibers, thus completing the immobilization.

The amount of protein on the fibers was then quantified by use of the microBCA assay. Each fiber was cut into 15 pieces which served as samples, and each sample was placed into a well plate with the microBCA working solution. The amount of protein on the fiber is directly proportional to the color change that would result after incubation in the solution, and this color change was read with a spectrophotometer and analyzed to determine quantitatively how much protein was on the fiber.

After each immobilization and assay, one variable was changed at a time until a successful protocol was created. Variables changed include duration of steps, number of steps, concentration of protein, gluteraldehyde, and chitosan, temperature of solutions, and pH of solutions.

RESULTS

The results of the most recent, and most successful, protocol are displayed in figure 1. The protein immobilized was measured as protein mass per surface area of fiber in units of micrograms/cm². The protein immobilized with the Pyr-BH₃ and NaCNBH₃ was measured as 4.1 and 0.45 micrograms/cm², respectively. The negative control, which was just a plain, washed fiber, had a protein content measured at 0.50 micrograms/cm². Each group consisted of two fibers, for a total of 30 samples per group. The Pyr-BH₃, despite the large variability indicated by the standard deviation bars, immobilized a far larger amount of protein compared to the NaCNBH₃ with p<0.01. However, it is clear that the NaCNBH₃ and the negative control (plain aminated fibers) are nearly identical.

DISCUSSION

Most of the success criteria were met. Spectroscopy revealed a significantly increased protein density on the fiber which used the Pyr-BH₃ reducing agent to immobilize compared to the fiber which used the NaCNBH₃ reducing agent to immobilize. Furthermore, the Pyr-BH₃ fibers revealed significantly more protein attached to them as compared to the negative control plain fibers. However, the quantity of protein immobilized to the fibers using the NaCNBH₃ reducing agent was less than that of the negative control. In the past we have seen an extremely large variability in the protein immobilized with the NaCNBH₃, most likely due to hydroscopic nature of the substance leading to a shorter shelf life than anticipated. Further trials are needed to definitively demonstrate that the amount of protein immobilized with the Pyr-BH₃ is indeed greater than the amount of protein immobilized with the NaCNBH₃.

ACKNOWLEDGMENTS

Principle Investigator: Dr. Federspiel
Mentor: Alex Malkin

REFERENCES

Figure 1. The figure shows a comparison between the protein content found on each fiber with NaCNBH$_3$, Pyr-BH$_3$, and the negative control (aminated fibers)