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Development of Assay Platforms for Monitoring Human C-Reactive Protein

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Abstract

It is imperative to have rapid diagnostic assays available that provide healthcare workers with a rapid yes/no or semi-quantitative response to a biomarker of interest. Agglutination assays use the clustering of antibodies in the presence of an antigen to semi-quantitatively monitor specific protein levels. Our research details the development of a latex agglutination test (LAT) for the detection of C-reactive protein (CRP), an important marker of inflammation in various disease states, at physiologically-relevant levels. Blue latex beads were covalently coated with Protein A for oriented antibody immobilization, then functionalized with anti-CRP antibody at various concentrations. We determined the optimal concentrations of Protein A and anti-CRP and will carry out an assessment of particle size effects on visualization and detectable CRP levels. Further experiments will improve this test's sensitivity and develop this into a platform assay for other biomarkers. For quantitative measurements in a laboratory environment, we also have developed a sandwich ELISA assay for CRP using anti-CRP conjugated with alkaline phosphatase. These platforms will establish and optimize the immunoassays, then we will use novel nanoparticles such as fluorescent liposomes and europium chelate particles to provide greater sensitivity and ease of use versus traditional latex beads or enzymes used for signal amplification.

Introduction

- Elevated serum C-Reactive Protein (CRP) levels in humans is a biomarker of inflammation and is used to assess cardiovascular risk. (1,2)
- CRP acts as a risk factor for peripheral vascular diseases, stroke, or cardiovascular disease at levels of approximately 1.34 µg/mL to 6.45 µg/mL, whereas normal levels in healthy patients are X. (3)
- Latex agglutination tests use the clustering of antibodies in contact with an antigen to monitor specific protein levels in a sample using biomolecule coated latex beads.
- Sandwich ELISA assays measure the antigen (CRP) bound between two layers of antibodies (anti-CRP). This minimizes the need for sample purification before analysis and enhances the sensitivity compared to direct or indirect ELISA assays(4)

We aim to develop a latex agglutination assay using anti-CRP coated microparticles and a sandwich ELISA assay for CRP using anti-CRP conjugated with alkaline phosphatase to quantify human CRP at disease-relevant concentrations.

Methods

Latex Agglutination Test using anti-CRP Coated Microparticles

- 1 um carboxylated polymer particles were coated with Protein A at 0.25x-2.0x the concentration needed for a monolayer around the bead with EDC.
- Protein A coated beads were conjugated to an anti-CRP antibody at concentrations of 1.25, 2.5, 5.0, and 10 µg/mL, then washed.
- 10 uL of 0.5% conjugated beads and 10 uL of 1.5 µg/mL CRP were mixed and viewed under a microscope or by eye for 15 minutes.

Sandwich Assay using anti-CRP conjugated with alkaline phosphatase

- Anti-CRP antibody was coated overnight at a concentration of 2.5 µg/mL.
- The plate was washed and then blocked for 2 hours, then washed again.
- CRP was added in a serial dilution down the plate starting at 100 ng/mL for 1 hour.
- The plate was washed and alkaline phosphatase conjugated anti-CRP was added at a 1:1000, 1:2000, 1:4000, and 1:8000 dilution in triplicate columns for 1 hour.
- The plate was washed and fluorescein diphosphate substrate was added prior to reading.

Results

- Binding latex beads with Protein A allowed for the better orientation of anti-CRP leading to more effective binding and reduced antibody concentration. (Fig 1)
- The assay was able to detect 1.5 µg/mL CRP levels by eye and under a microscope with 2x Protein A with 10 µg/mL anti-CRP antibody proving to be the most effective. (Fig 2)
- Latex beads that were functionalized to anti-CRP without being coated with protein A were not capable of detecting CRP at relevant levels.

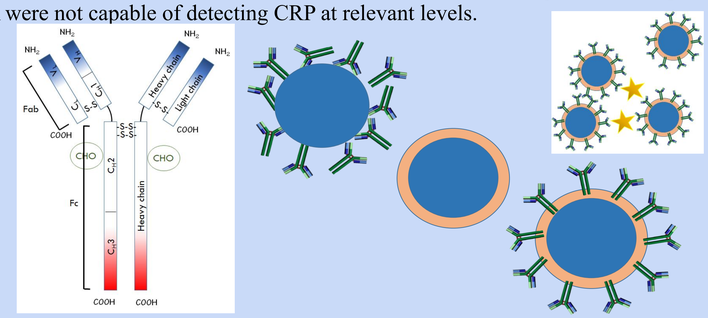

Fig 1. Latex beads conjugated to Protein A to allow for oriented immobilization of anti-CRP

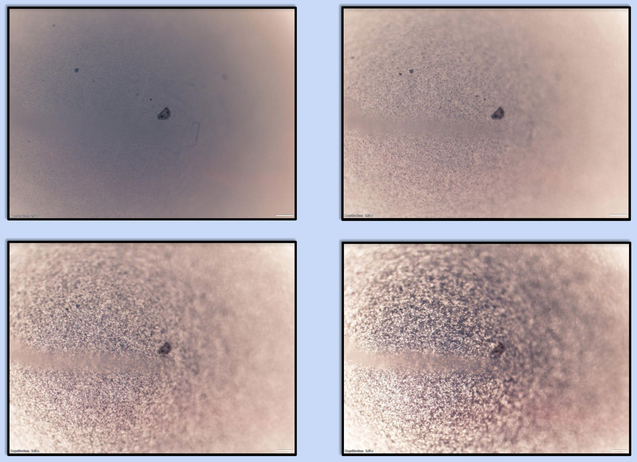

Fig 2. Agglutination Timeline of 1x protein A, 5 µg/mL anti-CRP conjugated beads with 2 µg/mL CRP over 15 minutes with photos taken every 5 minutes.

- As seen in the sandwich ELISA, the fluoresence intensity is proportional to the concentration of CRP in the 1.5-100 ng/mL range. (Fig. 3 and Fig. 4).

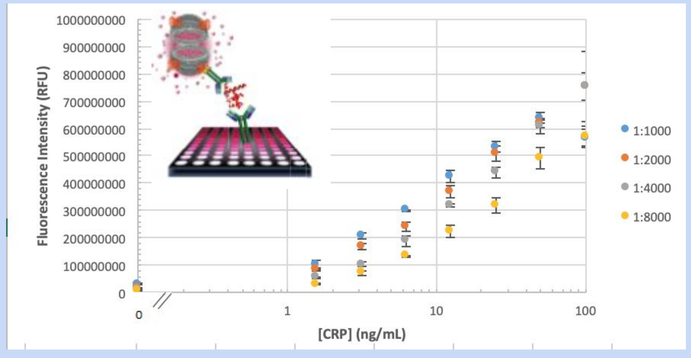

Fig. 3. Sandwich ELISA performance with varying dilutions of alkaline phosphatase conjugated anti-CRP.

Results

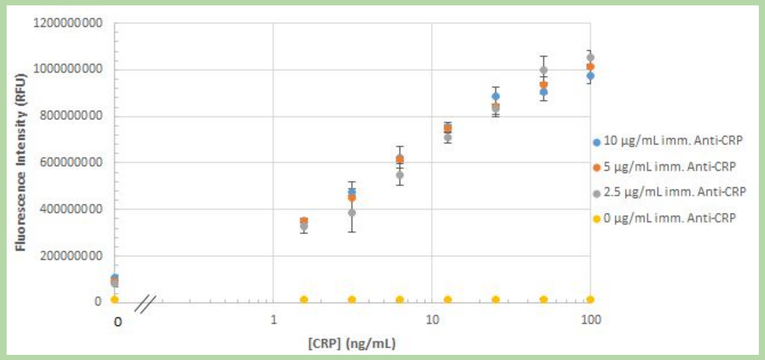

Fig 4. Sandwich ELISA performance with varying immobilized anti-CRP concentrations

Discussion

- Our previous work was unable to develop an agglutination assay using liposomes, potentially because the liposomes were too small for visible detection or electrostatically charged.
- This latex agglutination test is able to detect elevated levels of CRP that are indicative of inflammation and cardiovascular risk.
- Additional agglutination assays could be developed for testing other valuable biomarkers.
- Further concentrations of Protein A and anti-CRP antibody as well as different particle sizes may improve this test's sensitivity.
- The development of this CRP Sandwich ELISA assay is the first step in developing an assay using europium chelate particles for signal amplification.
- In future work, we will use Protein A to immobilize the antibodies on the europium bead surface by their Fc fragments, thus providing ideal orientation of the antigen binding regions.

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