

## MAGNETIC IMMUNOASSAY FOR RAPID ASSESSMENT OF ACUTE MYOCARDIAL INFARCTION (AMI)

Y. Haik<sup>1</sup>, M. Cordovez<sup>1</sup>, C.-J. Chen<sup>1</sup> & J. Chatterjee<sup>1</sup>

<sup>1</sup> *Biomagnetic Engineering Lab, FAMU-FSU College of Engineering, Tallahassee, FL 32310*

**INTRODUCTION:** Cardiac disease, especially acute myocardial infarction (AMI) affects a growing number of people in the United States and other parts of the world. A complication of current diagnosis procedures is that a number of ambulatory AMI patients are discharged with negative findings only to have recurrent and often more serious complications at home. Of the more than 5 million individuals with acute chest pains admitted to emergency rooms in the U.S., about 3 million are admitted to an intensive care unit or telemetry ward because traditional methods of diagnosis are not sensitive enough. More importantly, 2-8% of patients with chest pains who are discharged from emergency departments develop acute myocardial infarctions resulting in adverse events and malpractice actions [1]. The development of a simplified cost-effective and accurate procedure to diagnose AMI would greatly aid medical care providers, especially those in emergency rooms where the majority of AMI patients are initially evaluated. Additionally, the use of improved myocardial injury markers would improve detection in patients with minor or silent MI. Furthermore, the ability to perform such diagnostic tests at the point of care (POC) allows for the timely evaluation of patients within the emergency room or even in an ambulance. Care can then be delivered immediately, based on the results of these assays.

Our study presents a magnetic immunoassay that was developed to simultaneously test for two markers of AMI (Myoglobin and Fatty Acid Binding Protein).

Myoglobin (MYO), a single polypeptide chain (17 kDa), and Fatty Acid-Binding Protein (FABP, 15 kDa) are two small cardiac proteins that show elevated serum levels soon after the infarction, significantly increasing their concentration within 2 hours and peaking after 4-6 hours. The levels of myoglobin and FABP in plasma rise from about 32 µg/L and 3 µg/L to over 200 µg/L and 100 µg/L, respectively, often within less than 5 hours after an AMI [1]. Recent studies have shown that the combined measurement of Myoglobin and FABP in plasma allows for discrimination between myocardial and skeletal muscle injury with the ratios for these markers differing between heart

(MYO/FABP ratio 4:5) and skeletal muscle (MYO/FABP ratio 20:70, depending on muscle type) [2].

Regardless of the cardiac marker, measurement of marker concentrations has relied on various types of immunoassays. The most common methods are radioimmunoassay, latex agglutination, and two-site immunoassay.

Current immunological methods have the dual drawbacks of often being time consuming, with determination ranges of 3-4 hours, and requiring special equipment, which limits their usefulness in emergencies. Today, with increased emphasis on cost-effective decision-making and rapid treatment, hospitals are in need of the rapid and efficient determination of an AMI for patients admitted to the emergency department (ED) room with acute chest pain.

The use of magnetic particles in immunological assays has grown considerably, as the particles' magnetic properties permit their easy separation and/or concentration in large volumes, allowing for faster assays and in some cases improved sensitivity over currently available commercial methods. We chose a standard solid-phase ELISA (Enzyme Linked Immunoassay), consisting of the formation of a complex or "sandwich" by attaching two different antibodies to different epitopes on the same target antigen or protein. One antibody was attached to a solid surface (superparamagnetic microsphere), and the other to a small chemical enzyme (Alkaline Phosphates). The first antibody, attached to a surface, is used for the separation of the antigen from the background, while the second antibody, labeled with a enzyme, reacts with an introduced chemical reagent to give a relative indication of the concentration of the antigen (the extent of this reaction is proportional to the concentration of the antigen).

**METHODS:** Albumin superparamagnetic particles with a magnetic core made of  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> (maghemite) were prepared [3]. The magnetic particles were prepared in-house with diameters ranging from 6-10 nm. These magnetic particles were then coated with albumin. Figure 1 shows albumin coated magnetic microspheres. Protein coupling efficiency was measured for the composite particles. Avidin was used as a model ligand due to its strong bond-

forming ability with various ligands used in immunoassays. It was found that only 30% of the calculated amount of avidin required for monolayer formation on particles was used to coat the particles and the remaining portion was not adsorbed. Measurements with a Superconducting Quantum Interface Device (SQUID) showed that the particles were superparamagnetic. They can thus be easily separated from a solution with a small magnet and immediately re-dispersed into the liquid phase, without clumping, by removal of the magnet.

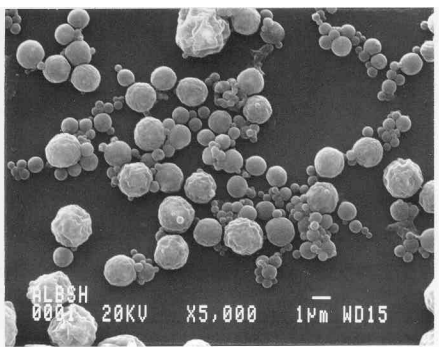


Fig. 1: SEM of albumin microspheres.

The immunoassay developed follows a standard solid-phase ELISA, consisting of the formation of a complex or “sandwich” by attaching two different antibodies to different epitopes. The main difference to the standard method is the use of superparamagnetic microspheres for the separation and concentration of the antibodies-antigen complex from the background. The antibodies conjugate with the myoglobin protein forming the microsphere-myoglobin-enzyme complex. Using an external magnet, the superparamagnetic properties of the microspheres allow for the repeated washing of the sample, separating the complex from all background media. After the complex has been isolated, a chemical reagent is introduced that reacts with the enzyme label in the complex to give a relative measurement of myoglobin concentration in a sample.

Biotin serves as the bridging link between the microspheres and the antibodies and the Alkaline Phosphatase (AP) and the antibodies. The antibodies used are two complementary clones of monoclonal mouse anti-human cardiac myoglobin (mouse isotope IgG1: 908 and 4E2) chosen because their epitopes are placed widely apart on the myoglobin protein. They were obtained from Research Diagnostics Inc. delivered in 1 mg antibody/ml PBS, pH 7.4 containing 0.1% sodium azide as preservative.

Both antibodies were coupled to two different labels: the avidin coupled superparamagnetic

microspheres and the streptavidin coupled alkaline phosphatase enzyme marker. Coupling was achieved by biotinylating the antibodies and incubating them with the avidin and streptavidin coupled labels.

Calf intestinal Alkaline Phosphatase (MW 140kD), an enzyme that hydrolyzes pNPP (para-nitrophenolphosphate MW 371) to produce a yellow substrate, was used. The reaction is easily measured with a Spectrophotometer at 405nm and can be stopped at any time with the addition of 2M NaOH, which makes it possible to store results for later comparisons and verifications. While measurements using calf intestinal Alkaline Phosphatase (AP) can be affected by the presence of endogenous alkaline phosphates activity, the markers used in the immunoassay can be easily substituted.

Antibodies were biotinylated using a Sulfo-NHS-LC-Biotinylation Kit from Pierce Chemicals. The long chain (LC) arm biotin, containing a 22 Å spacer arm between the reacted primary amine and the biotin moiety, was used to minimize the possible effects of steric hindrance when conjugating the Biotin to the much larger paramagnetic microspheres and to increase the sensitivity. After the antibodies were biotinylated and the microspheres coupled with avidin, the procedure for conjugating the antibodies to their respective labels was carried out.

Human Serum Albumin (HSA) was added to the antibody-label conjugates to block all other possible binding sites for biotin and/or avidin in both the microsphere-antibody conjugate and on the Alkaline Phosphatase-Antibody conjugate. The addition of HSA (a major soluble protein in human blood) can also partially mimic the conditions in the whole blood samples for clinic use. We found HSA did not interfere with our AMI-marker measurements. Since there is a high concentration of HSA in human blood, we expect that no additional blocking agents are needed in our AMI assay system in clinic use [4].

**RESULTS:** Experiments to establish the myoglobin calibration curve based on the absorbance levels for the reaction of pNPP to AP at 405 nm were conducted. Myoglobin (obtained from Sigma) concentration samples of (0.0 µg/ml, 0.05 µg/ml, 0.10 µg/ml, 0.15 µg/ml, and 0.20 µg/ml) were incubated with labeled antibodies test amounts previously incubated with HSA solution using 4 ml cuvettes and 9 ml washes. Incubated the materials for 5 minutes before applying the magnetic immunoassay.

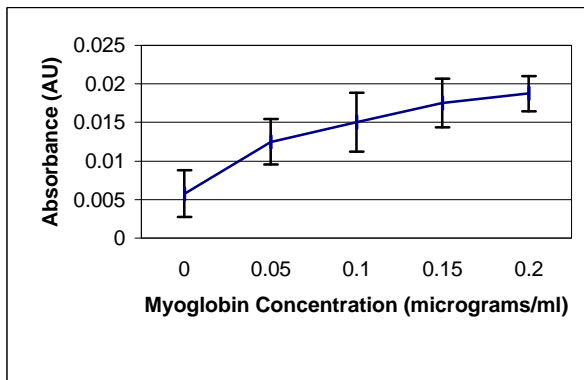


Fig. 2: Calibration curve for myoglobin based on the reaction of pNPP to AP following coupling with labeled antibodies and magnetic separation using the magnetic immunoassay.

Figure 2 shows the calibration curve for myoglobin with error bars representing the standard deviation for each data set. Each data point represents the average absorbance of 4 separate experiments. The center point concentration of 0.10  $\mu\text{g/ml}$  represents a discriminator value for AMI, a myoglobin concentration used by previous researchers as indicative of AMI, and values both above and below that value were used in the curve to demonstrate the validity of the magnetic immunoassay in the concentration ranges of most likely use. The validation of the magnetic immunoassay is supported by the data of figure 2 allowed us to modify the magnetic immunoassay to target other cardiac proteins to support the assumption that the magnetic immunoassay used could be modified to a variety of proteins.

Using the same magnetic immunoassay methodology and simply modifying the antibody labels with the appropriate anti-FABP antibodies, samples for 4 different concentrations of FABP were used to create a concentration curve based on the absorbance levels for the reaction of pNPP to AP. Figure 3 shows the values for absorption for different concentrations of FABP based on the pNPP-AP reaction. Notice in Figure 3 the significant difference between the reading with no

FABP present and all the others, but little difference between the samples containing FABP. A possible reason for this was the low concentration of superparamagnetic microspheres, which caused the saturation of antibody binding.

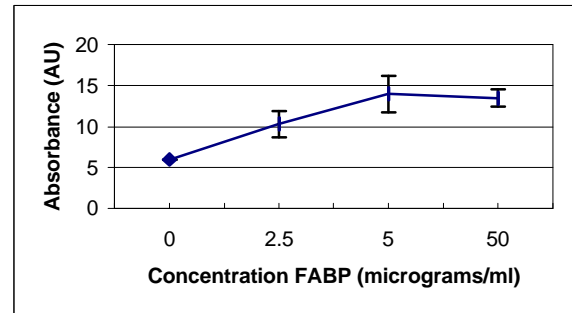


Fig. 3: Calibration curve for FABP.

A laboratory scale magnetic immunoassay was used to prototype a self-enclosed POC device for the measurement of diagnostic proteins. Figure 4 shows a schematic of translating the steps used in the immunoassay into a workable device. The device consists of a mechanism to introduce the sample into the testing chamber (a syringe is currently used). Mixing is obtained by manually shaking the sample. A set of magnets is being used to attract the coupled magnetic microspheres to the AMI marker and the Alkaline Phosphatase. Cyclic washing is used to decant the unwanted solution while maintaining the magnets to capture the microspheres. ParaNitrophenolphosphate is then added to the solution to react with AP for 1 min, followed by stopping the process with 2 M NaOH and measuring the AMI marker concentration in a spectrophotometer. The process is currently being automated.

Fluorescence labels were additionally used instead of the chemical reagent because (a) fluorescence could be adequately measured using at 499 nm in any standard spectrophotometer as a function of light absorption, and (b) using fluorescein as the quantifying label simplifies the process, as there is

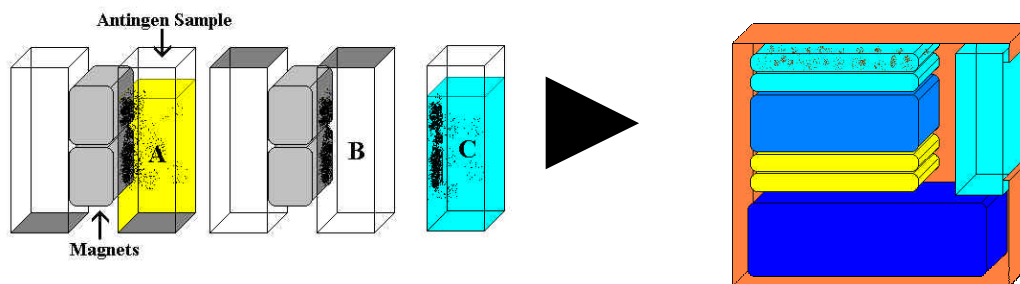


Fig. 4: Translating the constitutive steps of the magnetic immunoassay into a self-enclosed POC device.

no separate reaction step needed as in the case of most chemical enzyme tags. Fluorescein SMCC-BSA was used as the antibody label for quantifications.

A simple calibration curve for a single series of 7 different concentrations ranging from 0 to 0.063  $\mu\text{g/ml}$  was performed. This test was done to assess the ability of absorbance measurements of fluorescein by the equipment, and to evaluate sample handling and initial testing procedures. Figure 5 shows the calibration curve for fluorescein.

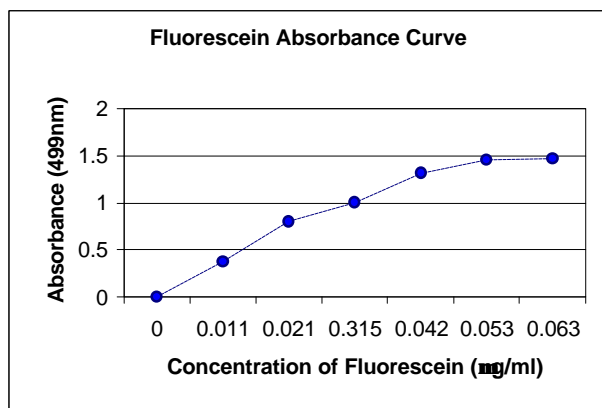


Fig. 5: Calibration curve for fluorescein SMCC-BSA.

Measurements were found to not be stable. The absorbance values for absorption increased with time, making it necessary for all the measurements to be conducted after exactly the same time intervals. Measurements could thus not be reread at a later time point.

Using Alkaline Phosphatase has proven adequate to predict AMI markers concentration.

**DISCUSSION & CONCLUSIONS:** According to the American College of Cardiology/American Heart Association guidelines for the management of patients with AMI, a proper goal for an emergency department AMI protocol would be a targeted clinical examination and a door-to-needle (as in treatment for AMI) time that is less than 30 minutes. Current immunological methods have the dual drawbacks of often being time consuming, requiring determination ranges of 3-4 hours, and requiring special equipment, which limits their usefulness in emergencies. This study presents the development of a magnetic immunoassay that has been used for detecting for two cardiac markers simultaneously. The time needed to present a result for both markers is less than 3 minutes. The magnetic immunoassay uses a standard solid-phase ELISA that consists of the formation of a complex or “sandwich” by attaching two different antibodies

to different epitopes on the same target antigen or protein. One antibody is attached to a nanomagnetic particle, and the other is attached to a chemical enzyme (AP). Current work includes transforming the developed immunoassay into a miniaturized point of care device.

**REFERENCES:** <sup>1</sup> M. Hudson, R. Christenson, L. Newby et al. (1999) *Clin Chim Acta* **284**:223-237. <sup>2</sup> J. Monique, K. Wodzig, M. Simoons, et al (1999) *Cardiovascular Res.* **44**:315-324. <sup>3</sup> J. Chatterjee, Y. Haik and C-J. Chen (2001), *Coll. Poly.Sci.*, **279**:1073-1081. <sup>4</sup> Cordovez, M. (2001) “Point of Care Diagnosis of Acute Myocardial Infarction Using Magnetic Immunoassays,” Master Thesis, FSU.

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