Rapid Isolation of AMI Markers using Nanomagnetic Particles

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ABSTRACT

Due to the increasing number of AMI patients accompanied with a difficulty in diagnosing the anomaly, a major push to develop innovative approaches to tackle the recognition of myocardial infarctions has been noticed. Most of these advances utilize immunoassays to decrease the time of acute myocardial infarction (AMI) detection, granting the patient a better chance of survival. This article presents a technique for rapidly isolating cardiac markers from a sample solution. The paper also presents techniques to correlate the concentrations of AMI markers with detectable labels.

Keywords: AMI, Nanomagnetics, Diagnostics, Detection.

1 INTRODUCTION

Coronary artery disease (CAD) is the leading cause of morbidity and mortality in the world. The emergency department evaluation of patients with potential acute coronary syndromes has traditionally included initial cardiac marker testing for suspected Acute Myocardial Infarction (AMI) [1]. Because of lack of sensitivity in current measuring methods for AMI half million people in America are mistakenly discharged from ER to have AMI later at home [2]. Currently electrocardiographs (ECG) are used in concert with blood serum protein elevations to diagnose the infarction. It has been shown that the diagnostic sensitivity of an ECG is approximately 50% in determining myocardial damage [3]. The number of individuals who have been discharged from emergency departments provides further evidence of the need for a more efficient method of AMI detection. It has been indicated that approximately 25% of the patients sent home with an acute myocardial infarction had ST elevations that were misjudged or overlooked by the physician [4]. Also, an estimated 80% of the patients admitted to the coronary care unit for suspected acute myocardial infarction are discharged without having this diagnosis confirmed [5, 6]. More importantly, 2-8% of the 2 million individuals that were not admitted developed AMIs resulting in added injury to the individual and malpractice actions taken against the institution [7].

Today, with increased emphasis in cost-effective decision-making and rapid treatment, hospitals are in need for the rapid and efficient determination of AMI for patients admitted to the emergency room with acute chest pain. Point of care or "near-patient" testing allows diagnostic assays to be performed at the site of patient care delivery in locations such as the emergency department or the intensive care unit where treatment decisions are made and care is delivered based on the results of these assays immediately [8]. Several markers result from the cardiac tissue injury. Myoglobin, Creatine Kinase, Fatty Acid-Binding Protein (FACB), Troponin, and Glycogen Phosphorylase (AP) are released into the blood stream immediately after the cardiac injury. Recent studies have shown the ability of Myoglobin and Fatty Acid-Binding Protein (FABP) as biochemical markers for the early diagnosis of AMI, especially in the first few hours after the onset of the AMI event [9]. Although common, conventional antibody-antigen interaction immunoassays evaluation techniques require laboratory setting to be performed and are not available at the point of care.

An ideal marker that could be used to detect myocardial injury would possess a number of characteristics. It must have high protein concentrations within the myocardium, while being present in low concentrations in non-cardiac tissue; it also should rapidly be released after cardiac injury, persists in plasma, and allows the development of accurate and rapid assays [10]. Among other cardiac markers, myoglobin is one of the ideal serum cardiac markers that can be used to test the presented assay and fulfills the aforementioned measurement criteria. Myoglobin is a protein which consists of a single polypeptide chain that composed of 153 amino acids and has a molecular mass of 17.8 KD. In the instance of an AMI twice the normal concentration of myoglobin is found within 2 hours and the level peaks within 4 hours.

Presently the use of magnetic particles in immunoassays is growing; due in part to their ability to

facilitate the separation of the targeted compounds and their higher sensitivity in harvesting the required compound.

This study uses magnetic immunoassay technology to detect the minute concentration of Myoglobin in blood. It utilizes a standards solid-phase enzyme linked immunosorbent assay (ELISA), the sandwich forms by attaching two different antibodies to different epitopes on the same target antigen which in our case is Myoglobin. One antibody is attached to a solid surface of the magnetic microsphere whereas the other is labeled with Alkaline Phosphatase (AP) enzyme and attached to the specific epitope on the Myoglobin. The first antibody is used for the separation of Myoglobin from the blood sample whereas, the AP labeled antibody is used to measure the relative concentration of Myoglobin in blood stream. (Figure.1).

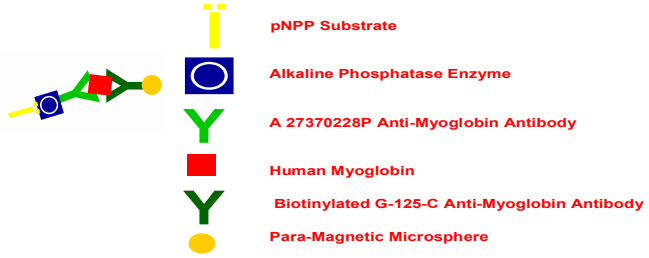

Figure 1. Schematic representation of a two-site immunoassay

Attaching AP enzyme to the end of the anti-myoglobin antibody will further facilitate the detection of AMI. Once the AP substrate is added, the reaction will occur and a bright yellow color will result. This yellow color can then be detected by any photodetector device at 405 nm wavelength.

2 MATERIALS AND METHODS

Materials:

All magnetic Microspheres (MMs) used in this study were produced using protocols that were established by the Biomagnetic Engineering Laboratory [13, 14]. The MMs are coated with albumin and coupled with avidin to conjugate the anti-myoglobin antibody. The antibodies used in this research are two complementary clones of anti-human cardiac myoglobin IgG. The primary antibodies (G-125-C) are polyclonal clone of antibodies produced in goat whereas the secondary antibodies (A27370228P) are monoclonal ones produced in Murine Hybridoma cells. These clones were chosen because their epitopes are placed widely apart on the myoglobin protein. Both antibody clones were purchased from Biospacific, Inc. The G-125-C antibodies were biotinylated using the EZ-link Sulfo-NHS-LC biotinylation kit obtained from Pierce chemicals. Pierce offers two biotinylation kits: long chain arm and standards biotin kits. In this study, long chain arm biotin containing a 22 Armstrong spacer arm between the reacted primary amine and the biotin moiety was used in order to minimize the possible effects of steric hindrance when conjugating the biotin to the much larger MMs and to increase the sensitivity. Alkaline Phosphatase enzyme was linked to anti-myoglobin A27370228P antibodies using a special labeling kit obtained from Pierce chemicals.

Coupling Anti- Myoglobin Antibody clone G-125-C to Avidin Coated Microspheres:

To form the first part of the conjugate, the calculated avidin concentration on the mms was used to estimate the needed antibody concentration. 1ml of (0.1mg/ml) biotinylated G-125-C antibody solution was combined with 7.6 ml of (0.01g/ml) avidin coupled microsphere solution. These figures were obtained by using HABA method to determine the minimum number of biotin groups on both antibody clones. Using HABA method, a minimum of 15 biotin groups/ antibody gives 10 nmoles of avidin needed to couple with 0.1 mg of G-125-C antibodies. Each 0.076 g of avidin coupled microsphere solution has 10 nmoles avidin or 7.6 ml of solution at 0.01 g/ml. The final solution of G-125-C antibodies and avidin coupled microspheres were incubated while mixing at 37°C for 1 hour.

Coupling Anti-Myoglobin A27370228P with Alkaline Phosphatase Enzyme:

The EZ-Link® Maleimide Activated Alkaline Phosphatase Kit obtained from Pierce chemicals was used to label the antibody with Alkaline Phosphatase (AP) enzyme. The kit provides all the reagents necessary to prepare quality conjugates of (AP) with antibodies and other proteins. The (AP) supplied in the kit is pre-activated to exact specifications to ensure both efficient conjugation to sulfhydryl (-SH) groups and retention of phosphatase activity.

Experimental Procedure:

At the beginning of the experiment, both antibodies were saturated with Human Serum Albumin (HSA) to ensure blocking all other possible binding sites for biotin and/or avidin in both the microsphere-antibody conjugate and on the AP-antibody conjugate.

The G-125-C antibodies-MMs conjugate was prepared by adding 1 ml of biotinylated G-125-C antibodies to 7.6 ml of (0.01 mg/ml) of MMs and incubated together for 1 hr at 37°C while mixing. Afterward, the whole ELISA complex was formed by adding the both conjugates; G-125-C antibodies-MMs complex and AP labeled antibodies to 2ml of (100 µg/l) Myoglobin in a 4 ml cuvette and incubated for 1 hr at 37°C while mixing [17, 18, 19].

After the incubated time, a 1.5 tesla magnet was applied to the side of the cuvette to isolate the desired conjugate (MMs –G-125-C-Myoglobin-AP labeled A27370228P) from the solution. The isolated conjugate was then washed several times by Tris Buffered Saline (TBS) while the magnet applied.

Finally, 50 µL of p-nitrophenyl phosphate (pNPP) solution was added to the washed conjugate, mixed gently and allowed to react for 5 minutes before the a spectrophotometric measurement was taken at 405 nm.

3 RESULTS

The determination of peripheral Alkaline Phosphatase (AP) enzyme attached to the end of the whole conjugate was the key to indirectly determine the concentration of Myoglobin that was taken up by the conjugate. The colorless pNPP substrate solution was incubated at RT before it was added to the ELISA conjugate and incubated for 2-3 minutes at RT. The chemical reaction that occurred between the (AP) enzyme and its substrate resulted in a bright yellow color which can be detected spectrophotometrically at A_{405}. Since different serial concentrations of Myoglobin ranged from (0 µg/L – 700 µg/L) were used in forming the conjugate, the resulted different color intensities were each single concentration correlated to the amount of Myoglobin that was used in the conjugate. Figure 2 shows the absorbance at different concentrations of Myoglobins. The same technique was used to detect for troponin (another cardiac marker) however with another label (HRP). Figure 3 shows the results obtained for troponin.

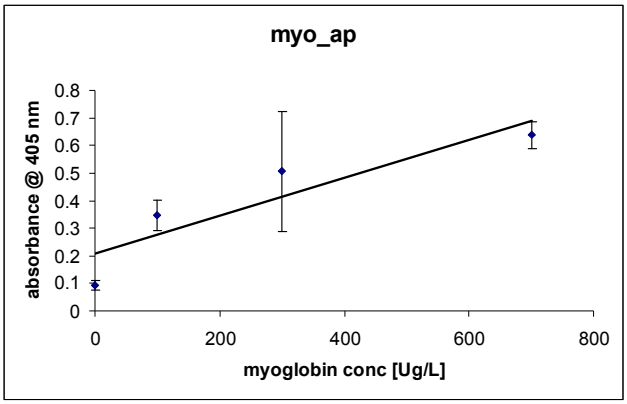

Figure 2: Absorbance at different Myoglobin concentrations.

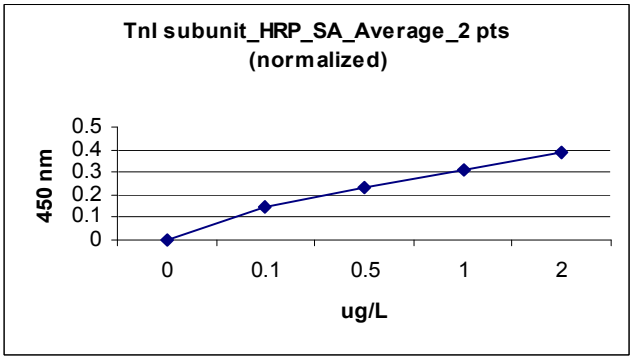

Figure 3: Troponin detection

It should be emphasized that the background effects have been minimized by using a series of HSA saturation procedure to minimize the effect of secondary conjugations.

4 DISCUSSION

Early and quick diagnosis of acute myocardial infarction (AMI) has recently captured researchers' attention. Many approaches have been developed to tackle the problem where most of them utilize immunoassay techniques for fast detection and direction of admitted patients in emergency department (ED). Time is a critical factor that plays a role in diagnosing the severity of patients' conditions as well as in directing patients correctly. This study presents the feasibility of the use of AP photodetection and determination techniques of patients' blood samples to help workers in ED to make the right decision in judging their admitted patients.

In developing a diagnostic technique for AMI, many analytical parameters have to be considered. Repeatability, sensitivity, and accuracy are the most important parameters that any diagnostic technique should have [20]. We consider, for AP measurement, for example, the technique to be repeatable if the relative standard deviation of many readings is smaller than 3%. In our case, all readings of the three experimental trials fell within that range. Both figures 2 and 3 show linear behavior for the absorbance at the different concentrations of cardiac markers that were tested. The protocols of using AP and HRP are very close in the application. A comparison between the two labels on Myoglobin was tested. Figure 4 a and b show the results obtained

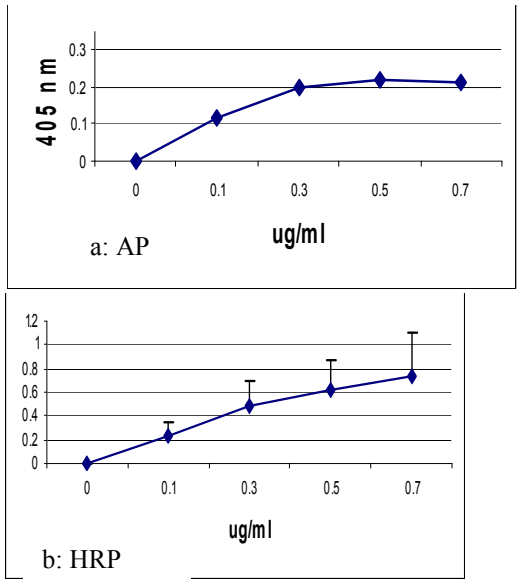

Figure 4 Comparison between AP and HRP behavior as markers on Myoglobin

The figure shows that HRP as a label scled linearly better than that of AP particularly at high concentrations of Myoglobin.

The AP saturates at higher concentrations while HRP continues to scale linearly. Additionally the HRP shows more sensitivity than that of AP.

Another important parameter for the user is evidently the accuracy of the measurement. Accuracy of immunological methods, like the one used in this study, is dependent upon the fidelity of the antibody-antigen binding. The literature indicates that the ability of specific antibodies to bind antigens is well documented [21]. In this study, both antibody categories G-125-C and A27370228P specifically attach to different epitopes on the surface of cardiac markers. The accepted value of such a measurement must be obtained since the first measurement [20]. However, a very small number of users in ED are not expected to spend time to obtain a mean value from a set of different results. Therefore, the magnetic immunoassay approach is sensitive enough to help ED workers make their decisions.

Speed is the most important parameter that this study accomplished. The total time that is needed to complete the test was approximately 60 minutes. Assuming that both terminals of the conjugate were prepared, the time is needed only to react cardiac markers to both conjugates' terminals. However, the application of this assay will depend on user's requirements where the need for rapid results may or may not be balanced by other considerations.

In conclusion, the Magnetic ELISA that this study presents is an innovative Myoglobin detection method that involves avidin-biotin interaction. The literature shows that ELISA with Avidin-Biotin reaction has better assay sensitivity than the ELISA using the direct antibody absorption format [22]. This technique was sensitive enough in measuring a Myoglobin concentration of 50 µg/L which is lesser than the AMI detection limit.

REFERENCES

[1] Hedén, Bo, Öhlin, Hans, Rittner, Ralf, & Edenbrandt, Lars (1997). *Circulation*, 96:1798-1802.

[2] Michael P. Hudson, Robert H.Christenson,L. Kristin Newby, Andrew L. Kaplan, E. Magnus Ohman. Clinica Chimica Acta 1999; 284:223-237.

[3] Gibler W, Lewis L, Erb R, et al. *Ann Emerg Med.* 1990;19:1359-1366.

[4] McCarthy BD, Beshansky JR, D'Agostino RB, Secker HP. *Ann Emerg Med* 1993; 22: 579-582.

[5] Puleo RP, Meyer D, Wathen C, Tawa CB, Wheeler S, Hamburg RJ, Ali N, Obermueller SD, Triana JF, Zimmerman JL, Perryman B, Roberts R. (1994). *N Engl J Med,* 331:561-566.

[6] Roberts R, Kleiman NS. (1994). *Circulation,* 89 (2), 872-881.

[7] Hudson, Michael., Christenson, Robert., Kristin Newby, L., Andrew, Kaplan & Magnus Ohman, E. (1999). *Clinica Chimica Acta, 284(2), 223-237.*

[8] De Winter, Robbert; Koster, Rudolph; Sturk, Augueste; & Sanders, Gerard. *Circulation*, 92 (12), 3401-3407.

[9] Adams J, Abendschein D, Jaffe A. (1993). *Circulation,* 88 (2),750-763.

[10] Adams, Jesse E. (1999). Clinica Chimica Acta 284 (2), 127–134.

[11] Bennett, Betsy & Ala, Mobile. (n.d.). Available online at: http://www.sma.org/smj/97july3.htm.

[12] Vote, David; Owen, Doar; Moon, Richard & Toffaletti, John. (2001). *Clinica Chimica Acta*. 305 (1-2), 81-87.

[13] Haik Y, Pai V and Chen C. J. J. Mag. Magn. Mat. (1999),194: 254-261.

[14] Chatterjee, J., Haik, Y., & Chen, C. (2003), J. Mag. Magn. Mat. 257,113–118.

[15] Duncan, R.J.S., *et al.* (1983). *Anal. Biochem.* 132, 68-73.

[16] Suter M, Butler J.E and Peterman, J.H. (1989). *Molecular Immunology*; 26:221-230.

[17] Hedges JR, Young GP, Henkel GF,(1992). *Ann Emerg Med,*21, 1445-1450.

[18] Marcello, Galvani; Donatella, Ferrini; Roberto, Puggioni; Saverio, Ruggeri; & Filippo, Ottani. (1998). *International Journal of Cardiology.* 65 ((Suppl.1), S17-S22.

[19] Mauro Panteghini. (1998). *Clinica Chimica Acta*, 272(1, 6), 23-31.

[20] Boujtita, M, Boitard, M, & El Murr, N. (1999). *Biosensors & Bioelectronics* 14, 545-553.

[21]Shelton, D., Van Kessel, J., and Wachtel, M. (2003). *Journal of Microbiological Methods*, 55, 717-725.

[22] Hao Yu, H. Ahmad , & Vasta, G. (1998). *Analytical Biochemistry*, 261(1), 1-7.