Troponins as Smart Biosensors for Point-of-care Detection of Acute Myocardial Infarction

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ABSTRACT

Troponin molecules and their T, C and I subtypes have shown significance in early point-of-care assessment of acute myocardial infarction. Other slowly released proteins are Creatine kinase MB, Fatty acid-binding Protein and myoglobin. Conventional antibody-antigen immunoassays do measure these markers and need laboratory setting. So, these are not suitable for point of care. The magnetic particles in immunoassay enhance the utility of immunoassay due to their ability to facilitate the separation of the targeted compounds and their high sensitivity of analyte. The present report presents a innovative idea to explore the possibility of: 1. troponin subtypes by MALDI mass spectrometry technique; 2. developing sandwich magnetic immunoassay using solid-phase Enzyme linked Immunosorbent Assay (ELISA) based on formation of "sandwich" complex of two different antibodies at two binding sites on same target troponin antigen. One antibody was attached with magnetic nanoparticles at on binding site and other antibody with fluorescent marker with glucose at other binding site. The concentration of troponinT in the blood sample was correlated with proportional concentration of glucose measured by glucometer. The application of nanotechnology in troponin analysis minimizes the artifacts of troponin analysis in AMI. Using site-specific agent such as a magnetic nanosphere is novel choice to estimate 1 pico gm/L. In addition, the magnetic nanoparticles enhance the analyte detectibility, sensitivity to analyte with optimal reaction speed. Furthermore, the nanospheres can be localized with use of external magnet. The technique provides simple point of care method. Furthermore, we proposed a simple technique of use of proteomics and magnetic nanoparticles to isolate and quantify AMI marker troponin in the blood sample wih use of glucometer. The MALDI mass spectrometry technique has potential of exploring more AMI sensitive Troponin or other proteins. The proposed magnetic immunoassay of point-of-care method shows potential as rapid reliable cost-effective efficient as cardiac protection device. The technique is simple, user-friendly and cost-effective. The nanotechnology based glucometer may provide portable analyzer for quicker point-of-care testing at minimum cost.

Key words: *troponin, AMI, magnetic immunoassay, Glucose*

1 INTRODUCTION

Troponin has different subtypes T, I and possible other subtype C and significantly leached out from the damaged cardiac walls soon after myocardial infarction. Other slowly released proteins are Creatine kinase MB, Fatty acid-binding protein and myoglobin. Conventional antibody-antigen immunoassays do measure these markers and need laboratory setting. So, these are not suitable for point of care and needs troponin subtype specificity. The magnetic immunoassay enhances its utility due to ability to separate the targeted troponins [1]. We got success on: 1. exploring troponin subtypes by MALDI mass spectrometry technique; 2. developing solid-phase Enzyme linked Immunosorbent Assay (ELISA) based on formation of "sandwich" complex of two different antibodies at two binding sites on same target troponin antigen. One antibody was attached with magnetic nanoparticles at on binding site and other antibody with fluorescent marker with glucose at other binding site. The concentration of troponin T in the blood sample is proportionate with glucose measured by glucometer- a simple point of care method. The point of care method has potential as rapid reliable cost-effective efficient as cardiac protection device. The MALDI mass spectrometry technique has potential of exploring more AMI sensitive Troponin or other protein.

The AMI marker troponin in the blood sample using glucometer is simple, user-friendly and cost-effective commercial miniature device. The use of proteomics in blood sample troponin analysis (proteome profile) further explores the other proteins involved in leaching process and enhances the accuracy and sensitivity of troponin specific markers. Paramagnetic particles have been extensively used for imaging and separation of proteins to capture AMI proteins. The assumptions were: 1. Magnetic immunoassay by antitroponin-troponin T/C/I subtypes predicts AMI using alkaline phosphatase reaction; 2. Measurement of electrical component by miniature device characterizes the colorimetric glucose concentration proportional to troponinT concentration; 2. MALDI-MS TOF spectrometry reveals the troponin protein subtypes and peptides active in AMI.

2 TROPONINS AND SUBTYPES BY MALDI

TroponinT and other subtypes C/I markers determine the AMI soon after infarction and present as binary cTnI-cTnC or ternary cTnI-cTnC-cTnT complex with.scanty information of actual amino acid residues. The major player troponinT (TpT) is a single peptide chain (17kDa) as a small cardiac protein. The three different troponin specific proteases used for enzymatic digestion increase the total protein aminoacid sequence coverage by mass spectrometry of three cardiac troponin subunits. Combined amino acid sequence coverages for cardiac troponin I, T, and C (cTnI, cTnT, cTnC) from AMI tissue is 54%, 48%, and 40%, respectively. A major drawback of routine troponin assay is the lack of standardization and long assay time while point-of-care assay needs quick and simple method. Other difficulty is nonspecific antibodies used against different regions on the cTnI molecule and calibration of technique. These problems lead to the necessity of AMI specific troponin subunit or other specific protein(s) search by matrix assisted laser desorption time-of-flight (MALDI TOF) mass spectrometry. Using this troponin information, it becomes easy to develop simple point-of-care glucodetection based magnetic immunoassay of cardiac troponin(s).

3 TROPONINS IN AMI AND POINT-OF-CARE TEST

TroponinT and other subtypes C/I markers show elevated serum levels soon after the infarction. The levels of plasma troponin rise about 15 ug/L. Still it is believed that TpT is not specific to cardiac injury. The immunoassays have emerged as potential quantitative methods to measure troponin in tissues based on specific binding of troponin antigen with homologous antibodies. The commonly used methods for cardiac markers are radioimmunoassay, latex agglutination and two-site immunoassay. However, these methods are time consuming 3-4 hours and not feasible in emergency rooms of hospitals. Point-of-care miniature devices allow diagnostic assays at the site of patient care delivery in emergency departments or intensive care unit.

4 MALDI TOF-MASS SPECTROMETRY

AMI left ventricle cardiac peptide mixtures after enzyme digestion can be analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (AXIMA-CFRTMplus; Applied Biosystems with cyano-4-hydroxycinnamic acid, HABA and sinnapinic acid used as an energy-absorbing matrix). External calibration was performed with peptide standard mixtures from peptide Mass Standards Kit (BioRad Biosystems). Protein identification by Kompact software was carried out using the Shimdzu Biotech peak-Fit and other search engine (Protein Prospector; available online at http://prospector.ucsf.edu) with the following criteria: minimum matching peptides, 3; number of missed cleavages,1; fixed modification, Met oxidation, Ser and Thr phosphorylation; variable peptide mass tolerance, SwissProt database. The troponin protein amino acid sequence as a percentage is based on its amino acid sequence from the fragments observed by mass spectrometry. This amino acid sequence does not reflect the absolute amount of the identified troponin protein. The whole isolated troponin protein complex after enzymatic digestion results peptide mixture. The peptides can be analyzed by MALDI-TOF mass spectrometry. In our troponin experiment, Peak-fit software confirmed the presence of all three troponin subunits. Depending on the protease used for protein digestion, different peptide sequences were obtained for the respective troponin subunits (Table 1). All proteases yielded peptide mixtures but essentially all would not allow the identification of the various troponin subunits by mass spectrometry (Table 1). Combining amino acid sequences from all enzyme digests, total protein sequences for cTnI, cTnT, and cTnC were 55%, 48%, and 42%, respectively (Fig. 2). The similar experiments were possibly with the anti-cTnI pAb P1, anti-cTnT mAb JLT-12, anti-cTnT pAb P1, and anti-cTnC mAb 1A2 troponin anitibodies extracted of all three cardiac troponin subunits. Earlier these subnits were confirmed by Western blotting and mass spectrometry; the protein sequence coverages, however, varied [5]. The mAb 8I-7 showed the greatest protein sequence coverage for all subunits. It was selected for the subsequent experiments on human serum total protein sequence coverages of 33%, 11%, and 28%, respectively[5]. For AMI patient, mainly cTnIcTnT or cTnI-cTnC-cTnT complexes are expected as shown in Figure 1.

Table 1. Total amino acid sequence by MALDI of cardiac troponins enzyme digests of human myocardial tissue.

	Trypsin	Asp-N	Lys-C	Total
TnC	**None**	**44 (aa22–160)**	**None**	**42 (aa 26–160)**
TnI	**24 (27–201)**	**36 (104–209)**	**35 (41–206)**	**55 (25–210)**
TnT	**19 (125–290)**	**None**	**30 (75–290)**	**48 (75–290)**

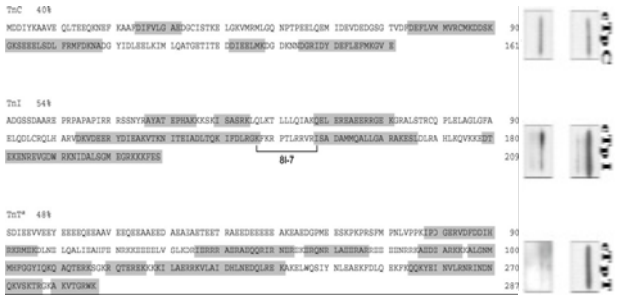


Figure 1: MALDI-TOF MS of cardiac troponins isolated from AMI myocardial tissue suggested amino acid patterns(left) for 3 distinct proteins(right)

5 MAGNETIC IMMUNOASSAY

The use of coated magnetic particles for cell and protein separation has been established at our lab [2]. Our laboratory has developed magnetic particles made of iron oxide in the range of 6 nm to 30 nm. These particles exhibit superparamagnetic behavior. The surface properties of these magnetic particles can be manipulated by coating them with protein, polymer and silica. In addition, a large number of functional groups of amines, carboxylic acid, hydroxyl, apoxy, amide, aldehyde, ketone, chloromethyl, sulphate, hydrazide provide variety of linkage for attachment on spheres' surface. Our sandwich magnetic immunoassay uses solid-phase enzyme linked immunoassay (ELISA) using sandwich by attaching two different antibodies to different epitopes on the same cardiac wall troponin target antigen. One troponin antibody attached to a superparamagnetic solid surface is used to separate the troponin antigen (cardiac marker) from the blood sample, while the second antibody is attached to glucose molecules. The immune complex is used to measure the relative concentration of cardiac troponin marker in blood stream by measuring attached glucose molecules at the end of the anti-cardiac marker antibody to detect AMI by miniature glucometer. The Figure 1 shows schematics of the magnetic immunoassay proposed for troponin detection and concentration measurement in AMI patient blood sample. Using magnetic nanospheres estimated limit was reported from our lab up to 1 pico gm/L [2,3]. The nanospheres eliminate laborious steps of diluting reactants and enhance analyte detectibility, sensitivity to analyte with optimal reaction speed. Furthermore, the nanospheres can be easily localized with use of external magnet. The research team at Center for Nanomagnetics and Biotechnology has developed and established a magnetic immunoassay using two different labels (a) enzymatic label and (b) glucose lebels.

6 SYNTHESIS OF NANOMAGNETIC PARTICLES AND BIO-APPLICATIONS

Encapsulated superparamagnetic particles or nanospheres separate different biological components from noncoagulated blood samples e.g. albumin or avidin coated magnetic microspheres and polystyrene coated magnetic microsheres attached to a red blood cell. Avidin ligand is choice due to its strong bond-forming ability in immunoassays. It had been found that only 30% of calculated amount of avidin required for the monolayer formation on particles was used to coat the particles and the remaining portion unabsorbed. Magnetization of these particles by superconducting Quantum Interface Device (SQUID) showed the characteristics superparamagnetic behavior of the composite particles. These spheres can be easily separated from a solution with a small magnet.

Figure 2:Sketch of immune complex for magnetic immunoassay.

7 MAGNETIC IMMUNOASSAYS FOR POINT-OF-CARE DIAGNOSIS OF ACUTE MYOCARDIAL INFARCTION (AMI)

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 (binding sites) on the same target antigen or protein was used. One antibody was attached to a solid surface (superparamagnetic microspheres), and the other was attached with enzyme. The first antibody attachéd to the surface was used for separation of the antigen from background, while the second antibody, labeled with an enzyme, reacts with substrate to give a relative indication of the concentration of antigen. In immunoassay, human troponin as antigens and monoclonal anti-troponin antibody targeting uses a superparamagnetic microsphere to separate antibody-antigen complex. In two-site immunoassay, two monoclonal antibodies for troponin target different epitopes get lebeled with an enzyme label or superparamagentic microsphere. The antibodies conjugate with troponin protein forming the microsphere-troponin-enzyme complex. Using external magnet, the suparamagnetic microsphere separate the complex from all background media in the same sample container. In the isolated sample, a chemical reagent is introduced to react with enzyme label in the complex to give a relative measurement of troponin concentration in a sample.

8 ANTIBODY BIOTINYLATION

Biotin serves as the bridging link between the nanospheres with antibodies and the enzyme. The biotin-avidin system for conjugation of labels to antibodies offers convenience, availability and the economic use. In our lab, we chose antibodies as two complimentary clones of monoclonal mouse antihuman cardiac Myoglobin (mouse isotop IgG1:908 and 4E2) because of their epitopes were placed widely apart on the myoglobin protein[1]. Both antibodies were coupled to two different labels. The avidin was coupled superparamagentic microspheres and the Streptavidin coupled enzyme marker by biotinylating the antibodies and incubating them with the Avidin and Streptavidin coupled labels. Strepatavidin is a terameric protein with four binding sites that is similar to Avidin in its molecular structure and has a similar binding capacity. ($Ka = 10^{15}/M$). The alkaline Phosphatase (MW 140 kD), is convenient and suitable for POC device. The antibody

biotinylation was done using Sulfo-NHS-LC-biotinylation kit (Pierce Chemicals). Pierce offered two biotinylated kits: Biotin with long chain arm (22 A spacer arm between primary amine and biotin moiety) and standard Biotin[1]. The long chain arm biotin couples with much larger paramagnetic microspheres and enhances sensitivity. After biotinylation of antibodies and microspheres coupled with avidin, the conjugation of antibody was carried out with respective labels. Other important step was the addition of human serum albumin (HSA) 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.

9 TESTING OF THE NANOPARTICLES IN GLUCODETECTION DEVICE

The magnetic particles isolate cardiac markers from whole blood. After labeling these cardiac markers with glucose molecule, allows a glucometer to measure the concentration of the Troponin cardiac marker in the sample by glucose oxidase-peroxidase system. This reaction generates a current proportional to the amount of glucose present and translates by the meter to the digital glucose conversion. The proposed device has validity, realiability, quick and less time consuming, specimen accessibility, simple technique, high efficiency, disposable and low cost. Terminal glucose is measured by one of three methods: electrical, colorimetric measurements using the following reaction.

Glucose + O2 → Gluconic acid + H_2O_2

H_2O_2 + reduced chromogen(uncolored) → H_2O + Oxidized chromogen(colored)

Glucose assay methods: Glucose Oxidase-peroxidase assay

Electrical:measures electrical resistance on strips (suitable)

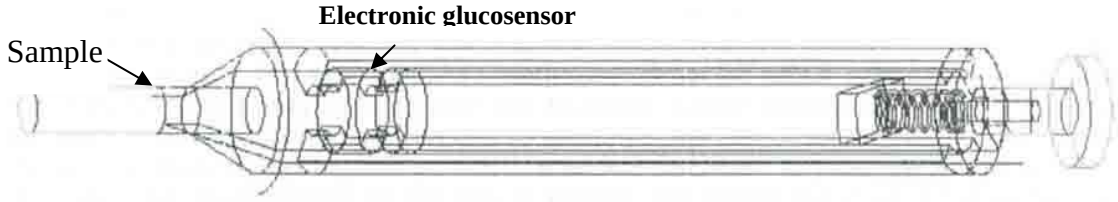
Colorimetric method: measures color intensity by spectrophotometer as shown in Figure 3.

Infra red glucose biosensor: real-time glucose monitoring(as most suitable in miniature devices)

10 CONCLUSION

Detection and measurement of Troponin subtypes in AMI is established by immunoassays but less sensitive in early detection. Troponin rapid and early AMI sensitive point-of-care assay is challenge. MALDI provides proteome fingerprint for early AMI sensitive proteins as guide to develop monoclonal troponin antibody based immunoassay utilizable for point-of-care miniature device.

Figure 2: A possible point-of-care glucometer based miniature pen-like device for Troponin measurement.



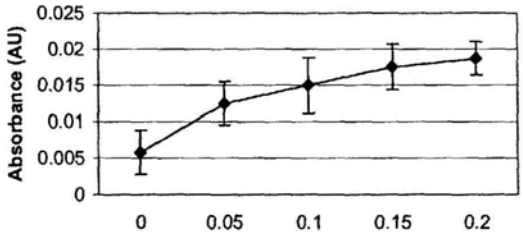


Figure 3: The relationship of antitroponin specific immune complex linked with glucose absorbance(y axis) vs troponin T calculated concentration(x axis) is shown.

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