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(54) **SYSTEM AND ASSAY FOR DETECTION OF
CARDIAC MARKERS FOR ASSESSING
ACUTE MYOCARDIAL INFARCTION**

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(57) **ABSTRACT**

Assay systems and methods are provided for detecting a target antigen in a physiological fluid (e.g., blood, serum, or urine). The method includes linking via a first antibody a magnetic microparticle to the target antigen in the physiological fluid; linking via a second antibody a glucose molecule to the target antigen; utilizing a magnetic field to separate the magnetic microparticle-linked antigen from the physiological fluid to form a test sample; and detecting the glucose in the test sample to determine the concentration of target antigen in the physiological fluid. The target antigen can be a protein or marker resulting from cardiac tissue injury, which can be used to assess acute myocardial infarction. An exemplar target antigen is myoglobin. The glucose detection preferably is one that can be done rapidly, e.g., with a conventional glucometer, and may include measuring the electrical resistance, color, or pH of the test sample.



-  **Glucose molecule**
-  **Biotinylated Con-A Lectin**
-  **Streptavidin**
-  **Biotinylated 4E2 antibody**
-  **Myoglobin**
-  **Biotinylated 908 Antibody**
-  **Para-Magnetic Microsphere**

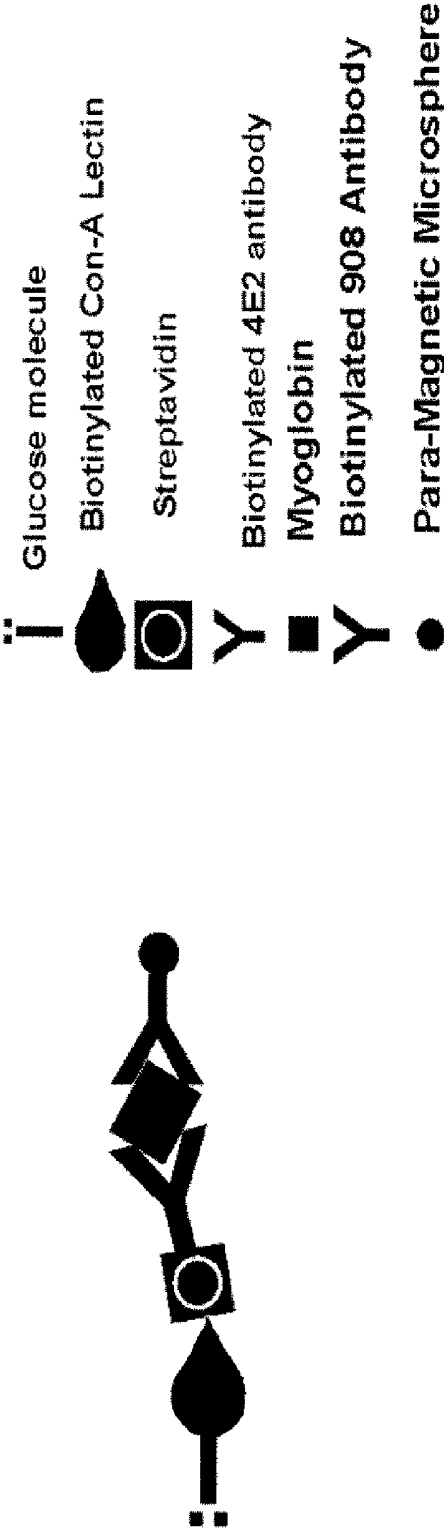


FIG. 1

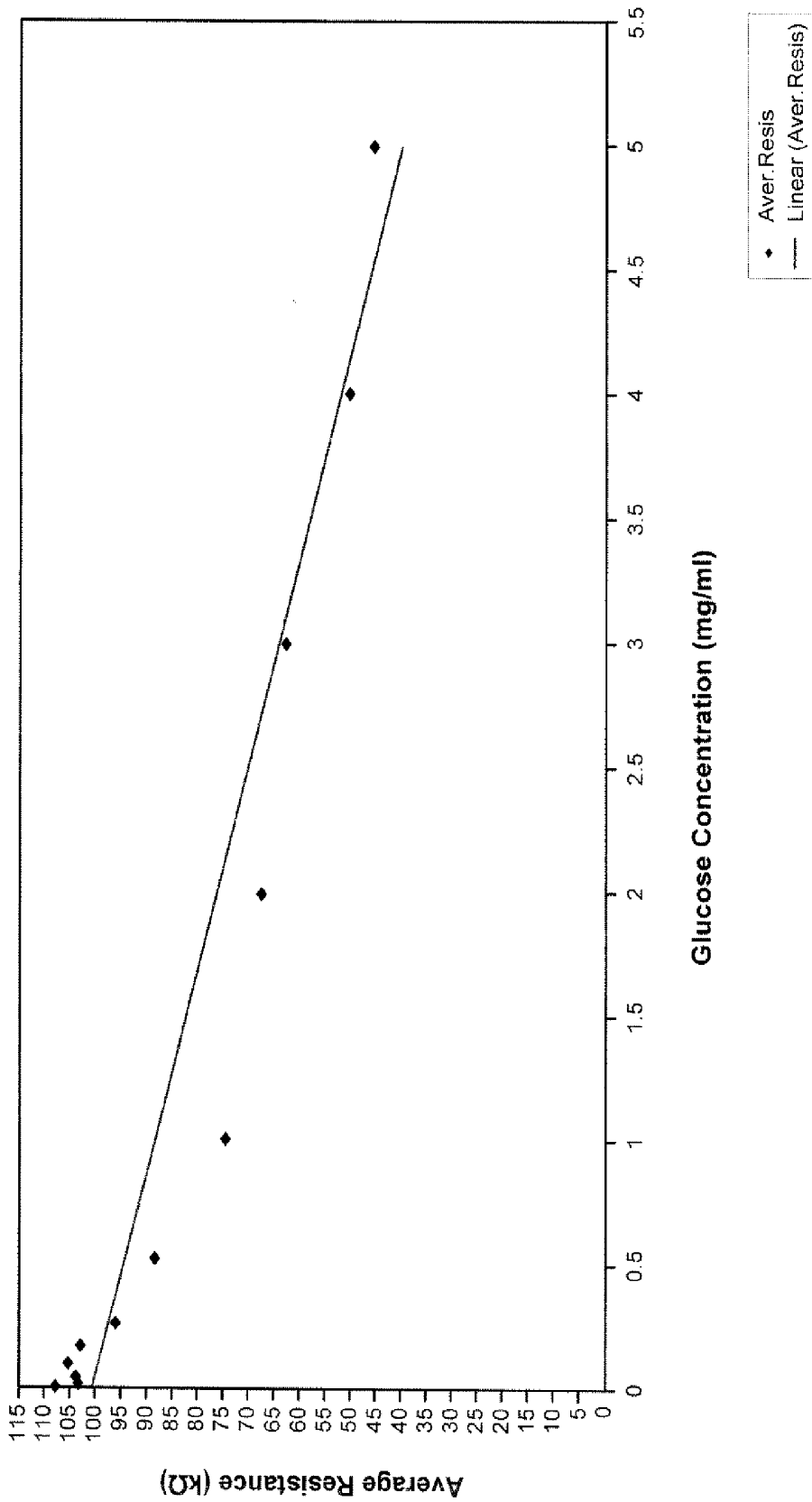


FIG. 2

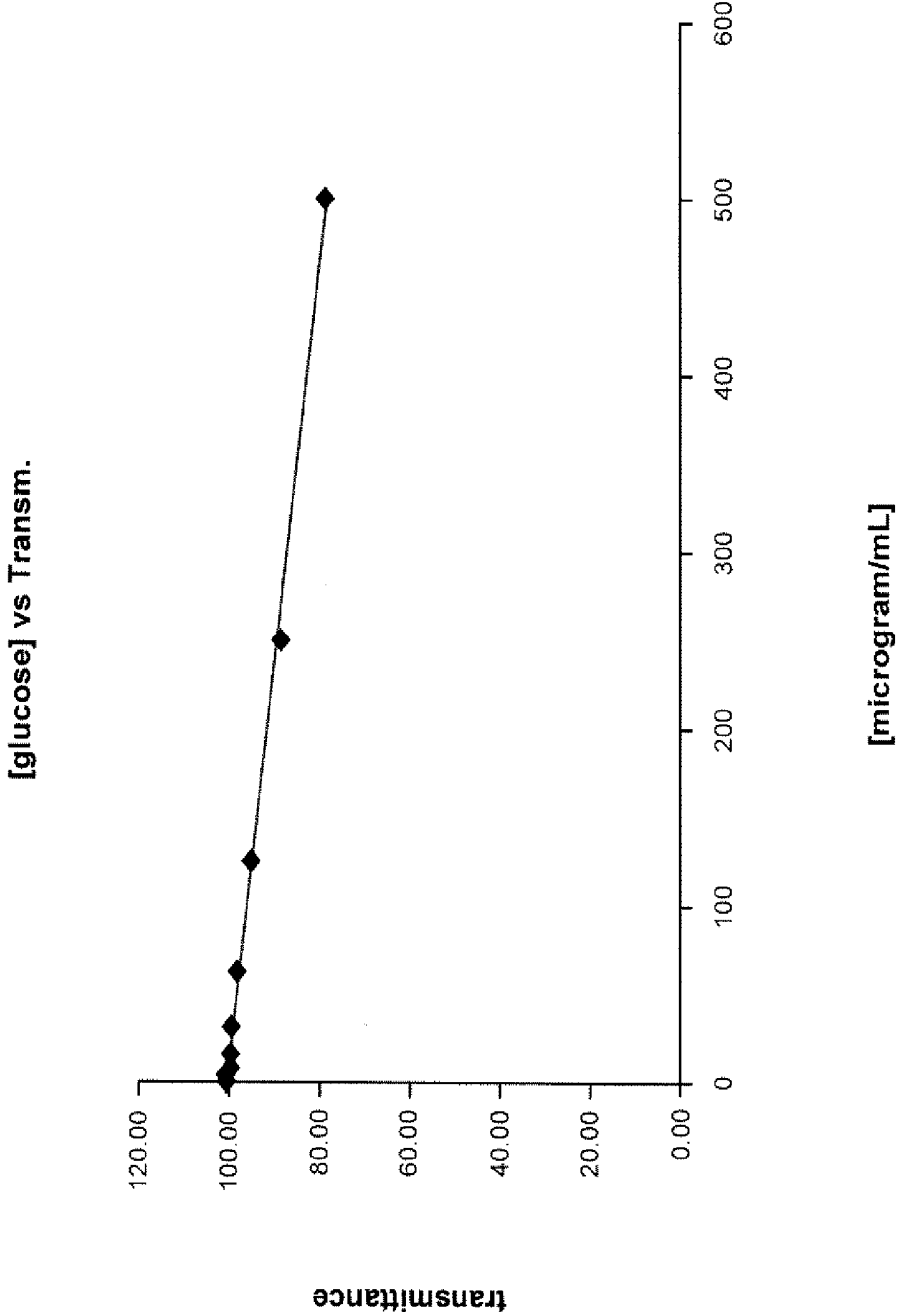


FIG. 3

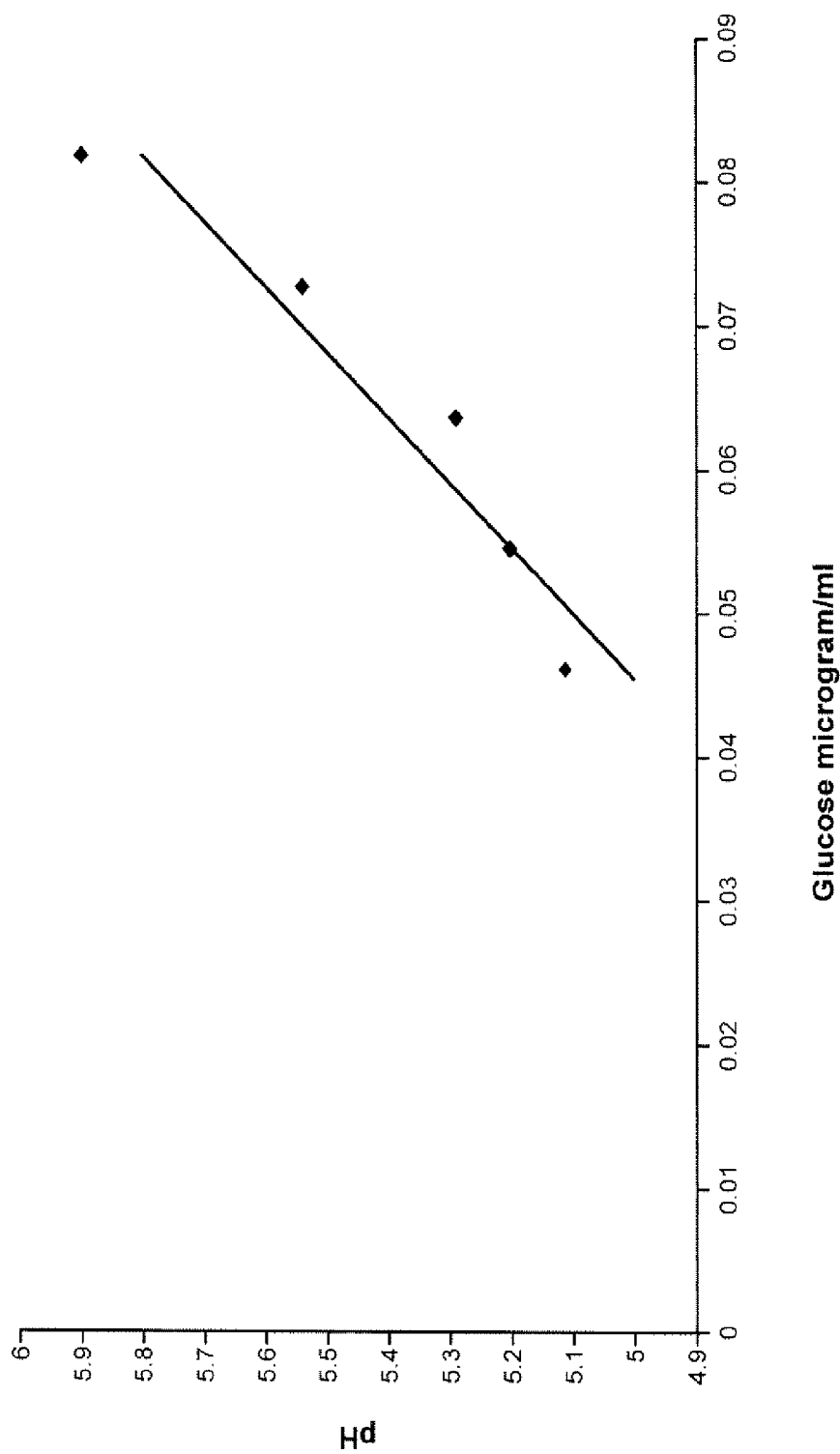


FIG. 4

**SYSTEM AND ASSAY FOR DETECTION OF
CARDIAC MARKERS FOR ASSESSING
ACUTE MYOCARDIAL INFARCTION**

CROSS-REFERENCE TO RELATED
APPLICATIONS

[0001] This application is a divisional of U.S. patent application Ser. No. 11/204,463, filed Aug. 16, 2005, which claims benefit of U.S. Provisional Application No. 60/602,420, filed Aug. 18, 2004. The applications are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

[0002] This invention relates generally to diagnostic immunoassays for medical applications, and more particularly to methods for rapidly determining whether a person has suffered an acute myocardial infarction.

[0003] Acute Myocardial Infarction (AMI), commonly referred to as a heart attack, is a disorder in which a region of the heart muscle is damaged due to an inadequate supply of oxygen thereto. For instance, a clot in a coronary artery that blocks the supply of oxygen to the particular region of the heart and results in death or damage to the cells in this region can cause an AMI. Typically, the AMI occurs as a result of a coronary artery that has been narrowed due to the effects of arteriosclerosis. The damaged tissue causes a permanent loss of contraction of this portion of the heart muscle.

[0004] There is a great desire for early determination as to whether AMI is occurring for patients that present themselves at an emergency room of a hospital. A significant number of patients complain from chest pain and/or other symptoms concomitant with AMI (Heden et al., *Circulation*, 96: 1798-802 (1997)). This early determination allows hospital personnel to determine which patients can be sent home and which patients have suffered an AMI. For patients who have suffered an AMI, thrombolytic therapy should be started as soon as possible after the AMI for maximum benefit. Unfortunately, a high percentage of patients who present with chest pains are admitted to the intensive care unit or telemetry ward of the hospital. In addition, a small percentage of patients that are sent home after the clinicians determined such patients did not suffer an AMI, shortly thereafter are admitted to the hospital as having suffered an AMI. These problems are due to traditional diagnostic regimens lacking sufficient sensitivity in a large portion of the cases presented.

[0005] 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 (Gibler et al., *Ann Emerg Med* 19:1359-66 (1990)). 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 (McCarthy et al., *Ann Emerg Med* 22:579-82 (1993)). In addition, an estimated 80% of the patients admitted to the coronary care unit for suspected acute myocardial infarction are discharged without having this diagnosis confirmed (Puleo et al., *N Engl J Med*, 331:561-66 (1994); Roberts & Kleiman, *Circulation*, 89(2):872-81 (1994)). 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 (Hudson et al., *Clinica Chimica Acta*, 284(2):223-37 (1999)).

[0006] 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 AMI detection, granting the patient a better chance of survival (Hudson et al., *Clinica Chimica Acta* 284:223-37 (1999)).

[0007] The release of specific cardiac proteins from the injured cardiac tissue into the patient's bloodstream has become an important parameter for the diagnosis of AMI. A proper protein marker to use in such an assay is one that is present in measurable concentrations in the early AMI onset stage as well as having high clinical sensitivity and specificity (De Winter et al., *Circulation*, 92(12):3401-07. Several proteins or markers can result from the cardiac tissue injury. Myoglobin, creatine kinase, fatty acid-binding protein (FACB), cardiac specific troponins, and glycogen phosphorylase (AP) are released into the blood stream immediately after the cardiac injury. The measurement of these markers has become an increasingly important for the diagnosis and sizing of AMI. Depending on the differences in their concentration in the blood stream, these markers are used both for diagnosis, by detecting the presence of increase concentration of these proteins in patients who have AMI signs, and sizing of an AMI, by taking serial measurements of these markers to estimate the extent of damage (Adams et al., *Circulation*, 88(2):750-63 (1993)).

[0008] 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 noncardiac tissue. It also should be rapidly released after cardiac injury, persists in plasma, and allow the development of accurate and rapid assays (Adams, *Clinica Chimica Acta* 284(2): 127-34 (1999)).

[0009] The CK-MB isoenzyme (86 kDa) is one of the most common markers used for the evaluation of patients with suspected AMI (Achar et al., *Am. Fam. Physician* 72(1):119-26 (2005)). Damaged myocardial tissue releases CK-MB in a characteristic fashion following the injury, allowing clinicians to often determine the timing and extent of an AMI, with the concentration level of CK-MB normally being 3 U/L and peaking at over 25 U/L 16-20 hours after infarction. Unfortunately, CK-MB is insufficiently sensitive during the early phases of an AMI, with an increase in concentration also present during massive skeletal muscle damage. The late peak of CK-MB concentration has led to its primary use in confirming an AMI at 24 hours post-injury (Id.; Cubrilo-Turek et al. *Acta Med Croatica* 58(5):381-8 (2004); Gibler et al., *Ann Emerg Med* 19:1359-66 (1990)). Recently, the use of the two CK-MB subforms (CK-MB 1 and CK-MB 2) has been proposed for a more accurate diagnosis of early AMI. CK-MB 2 is found in myocardial tissue, and upon tissue destruction is released into the blood as the more negatively charged subform, CK-MB 1. A level of CK-MB >1 U/L and a ratio of CK-MB 2 to CK-MB 1 >1.5 has been reported to have a better sensitivity within the first six hours post-AMI compared to CK-MB alone (Penttila et al., *Clin Biochem* 35(8):647-53 (2002); Lin et al., *Clin Chem* 50(2):333-38 (2004)). Troponin is a protein also found in the skeletal and cardiac muscle that

has become a standard marker for the diagnosis of AMI (Achar et al., *Am Fam Physician* 72(1):119-26 (2005); Berroeta et al., *Ann Fr Anesth Reanim*, (2005)). Used in conjunction with CK-MB, troponin can detect not only minor myocellular necrosis but also can be used as an indicator for patients at risk for ACS [Penttila et al., *Clin Biochem* 35(8): 647-53 (2002); Lin et al., *Clin Chem* 50(2):333-38 (2004); Berroeta et al., *Ann Fr Anesth Reanim*, 2005)). Troponin entails three forms (C, I, and T) which along with actin and myosin regulate muscle contraction. While troponin C is identical in cardiac and skeletal muscles, troponins I and T are site specific. Therefore, a rise in cardiac specific troponin I and T is suggestive for myocardial damage. Both forms rise within the first 3-4 h of the injury, peak generally within 24 h, and remain elevated for 10-14 days, therefore qualifying as late markers for a recent AMI in patients who do not receive an immediate medical assistance. Although troponin I and T have similar sensitivity and specificity for the detection of myocardial infarction, troponin T may be elevated under other pathological conditions such as renal failure or polymyositis. Recently, the use of troponin I over the T isoform in the diagnosis of ACS and AMI has been reported (e.g., Eisenman et al., *Singapore Med. J* 46(7):325-27 (2005)). Moreover, troponin I alone has been shown to be a better marker for risk stratification in patients with chest pain than when combined with CK-MB and myoglobin Qggers et al., *Am Heart J*. 148(4):574-81 (2004); Eggers et al., *Coron Artery Dis* 16(5): 315-19 (2005)). Recent studies have shown that FABP and myoglobin can be used to detect a myocardial injury sooner after an infarction than CK-MB or troponin T (Achar et al., *Am Fam Physician* 72(1):119-26 (2005); Ishii et al., *Clin Chem* (2005); Chen et al., *J. Huazhong Univ Sci Technolog Med Sci* 24(5):449-51, 459 (2004); Sallach et al., *Am J. Cardiol* 94(7):864-67 (2004)). Myoglobin (17 kDa) and FABP (15 kDa) are two small cardiac proteins that show elevated serum levels soon after the infarction, significantly increasing their concentration within 2 hours after infarction and peaking 4-6 hours after an infarction. The concentrations of myoglobin and FABP in plasma rise from about 32 µg/L and 3 µg/L respectively to over 200 µg/L for myoglobin and 100 µg/L for FABP often under 5 hours after an AMI (Van Nieuwenhoven et al., *Circulation* 2848-54 (1995); Wodzig et al., *Eur. J Clin Chem Clin Biochem* 71:135-40 (1997)). Myoglobin is a heme protein that participates in the oxygen transport in the muscle, hence any severe destruction of striated muscle may result in significant elevations of its plasma concentration. Although myoglobin has a low cardiac specificity, it has a high sensitivity for myocardial injury that makes it a useful marker for AMI when used in parallel with other cardiac markers. Recent studies show that the combined measurement of myoglobin and FABP in plasma allows the 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). Alternately, combined measurements of myoglobin concentrations with skeletal muscle specific markers by supplementary tests such as CK-MB, cardiac specific troponins, or carbonic anhydrase III can also be used to differentiate between myocardial and skeletal muscle damage. A potential drawback is that both markers, FABP and myoglobin, unlike other cardiac markers, are eliminated from plasma by renal clearance, which means that measurements could be inaccurate in patients with chronic renal failure. In these cases, cardiac markers concentrations

should be interpreted in conjunction with the individual estimated renal clearance rates of the patients. Although both markers lack cardiac sensitivity, myoglobin has been used more often as an early AMI marker. Despite recent studies that indicate cardiac FABP may provide information superior to other markers in the early onset of ACS (Ishii et al., *Clin Chem* (2005); Chen et al., *J. Huazhong Univ Sci Technolog Med Sci* 24(5):449-51, 459 (2004)), its use in the diagnosis of myocardial injury is not unanimously accepted.

[0010] Development of a cost effective, simple and efficient assay determining whether an AMI has taken place would greatly assist health care providers in timely and accurately diagnosing AMI suspected patients. It would therefore be desirable to provide improved methods and assay compositions for AMI detection. It would also be desirable to provide better methods and assays for measuring myoglobin, as well as other assayable antigens.

SUMMARY OF THE INVENTION

[0011] Assay systems and methods are provided for detecting an target antigen in a physiological fluid.

[0012] In one aspect, the method comprises linking via a first antibody a magnetic microparticle to the target antigen in the physiological fluid; linking via a second antibody a glucose molecule to the target antigen; utilizing a magnetic field to separate the magnetic microparticle and linked target antigen from at least a portion of other components in the physiological fluid, thereby forming a test sample; and detecting the glucose in the test sample to determine the concentration of target antigen in the physiological fluid.

[0013] In a preferred embodiment, the target antigen comprises a protein or marker resulting from cardiac tissue injury, which can be used in the assessment of acute myocardial infarction. One such target antigen is myoglobin. Others include creatine kinase, fatty acid-binding protein, troponins, and glycogen phosphorylase. Representative examples of physiological fluids that may be sampled and assayed include blood, serum, and urine.

[0014] The glucose detection preferably is one that can be done rapidly and includes measuring the electrical resistance, color, or pH of the test sample. In one embodiment, the glucose detection comprise measuring light transmittance through the test sample. In another embodiment, the glucose detection comprises oxidation of the glucose to gluconic acid at a platinum electrode.

[0015] In one embodiment, the first and second antibodies comprise complementary clones of monoclonal anti-human cardiac myoglobin antibody. In one example, the second antibody comprises a monoclonal mouse anti-human cardiac myoglobin Mouse isotope IgG:908, and the first antibody comprises a monoclonal mouse anti-human cardiac myoglobin Mouse isotope JgG:4E2.

[0016] In one embodiment, the second antibody is linked to a streptavidin. In one embodiment, the streptavidin is linked to a Con-A lectin which is linked to the glucose molecule. In a specific example, the second antibody is a biotinylated anti-myoglobin antibody and the anti-myoglobin antibody is linked to streptavidin which is linked to a biotinylated Con-A lectin which is linked to the glucose molecule.

[0017] In a preferred embodiment, the magnetic microparticles comprise microspheres coated with a polymer or protein. For example, the encapsulating material can comprise albumin, avidin, or a combination thereof.

[0018] In one particular assay for detecting an AMI-indicating antigen in a physiological sample, the method includes the steps of combining the physiological sample with magnetic microparticles linked to a first biotinylated antibody, wherein the first antibody binds to a first site on the AMI-indicating antigen present in the sample; applying a magnetic field to the physiological sample to separate the linked myoglobin from the blood sample to form a test sample comprising the magnetic particle-linked AMI-indicating antigen; combining the test sample with a second biotinylated antibody, wherein the second antibody binds to a second site on the AMI-indicating antigen, and wherein the second antibody is linked to a glucose molecule; and detecting the glucose in the test sample to determine the concentration of the AMI-indicating antigen, such as myoglobin, in the physiological sample, such as blood, serum, or urine.

[0019] In another aspect, an immunoassay system is provided, which includes a first antibody linkable to an assayable protein antigen present in a physiological fluid; a magnetic microparticle linked to the first antibody; a second antibody linkable to the assayable protein antigen; and a glucose molecule linked to the second antibody.

[0020] In one particular embodiment, an immunoassay kit is provided for detecting myoglobin or another AMI-indicating antigen, which includes a first part that comprises magnetic microparticles linked to antibodies selected to be linkable to a first epitope of the myoglobin or other AMI-indicating antigen; and a second part that comprises second antibodies selected to be linkable to a second epitope of the myoglobin, wherein the second antibody is linked to a lectin that is linked or linkable to a glucose molecule or enzyme tag.

[0021] In another aspect, a method is provided for detecting a target antigen in a physiological fluid which comprises the steps of linking via a first antibody a magnetic microparticle to the target antigen in the physiological fluid; utilizing a magnetic field to separate the magnetic microparticles with the target antigen linked thereto from at least a portion of other components in the physiological fluid, thereby forming a test sample; linking via a second antibody an enzymatic tag or glucose molecule to the target antigen; and detecting, using a calorimetric method, the linked enzymatic tag or glucose molecule in the test sample to determine the concentration of target antigen in the physiological fluid.

BRIEF DESCRIPTION OF THE FIGURES

[0022] FIG. 1 is a schematic representation of one embodiment of the magnetic immunoassay described herein for detection of myoglobin.

[0023] FIG. 2 is a graph of glucose concentration versus average electrical resistance in one test example.

[0024] FIG. 3 is a graph of glucose concentration versus light transmittance in one test example.

[0025] FIG. 4 is a graph of glucose concentration versus pH in one test example.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0026] Improved assays and methods have been developed using magnetic immunoassay technology to separate the target of interest from a fluid sample and detect minute concentrations of an assayable target antigen. In a preferred appli-

cation, they are used to assess myocardial injury by indirect detection of myoglobin or another protein or marker resulting from cardiac tissue injury.

[0027] In a preferred embodiment, the assay utilizes a magnetic solid-phase enzyme linked immunoassay (magnetic ELISA), wherein the sandwich forms by attaching two different antibodies to different epitopes on cardiac markers (i.e., an AMI-indicating antigen, such as myoglobin). One antibody is attached to a solid surface of the magnetic microsphere, and the other is attached to a glucose molecule. The first antibody is used for the separation of the AMI-indicating antigen from the physiological fluid sample whereas, the second antibody, attached to some glucose molecules, is used to measure the relative concentration of the AMI-indicating antigen in the physiological fluid. Attaching glucose molecules at the end of the anti-myoglobin antibody will further facilitate the detection of AMI because it is easily measured by a conventional glucometer. FIG. 1 shows a schematic representation of one embodiment of the magnetic immunoassay.

[0028] In other embodiments, the magnetic assay technique is adapted to separate and detect other antigens (for other uses besides AMI testing) to which two different antibodies can be attached (i.e., linked, bound) where one antibody is attached to a solid surface of a magnetic microparticle, and the other antibody is attached to a glucose molecule. Essentially any antigen (e.g., soluble protein) can be separated and detected using the appropriate antibodies linked to the magnetic microparticles and glucose molecules. In yet another embodiment, the glucose can be substituted with another sugar (e.g., mannose, fructose, galactose) that can be readily detected.

[0029] As used herein, the term “antibody” is intended to include immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site that specifically binds (immunoreacts with) an antigen, such as Fab and F(ab')₂ fragments. As used herein, the term “antibody” includes polyclonal and monoclonal antibodies, and variants such as single-chain (recombinant) antibodies, “humanized” chimeric antibodies, and immunologically active fragments of antibodies. For the purposes of this invention, a “chimeric” monoclonal antibody is a murine monoclonal antibody comprising constant region fragments (Fc) from a different animal. For the purposes of this invention, a “humanized” monoclonal antibody is a murine monoclonal antibody in which human protein sequences have been substituted for all the murine protein sequences except for the murine complementarity determining regions (CDR) of both the light and heavy chains. Standard techniques for the generation and isolation of antibodies are well-known and commonly employed by those of skill in the art. A number of standard techniques are described in Kohler & Milstein, *Nature* 256:495-97 (1975); Kozbor et al., *Immunol Today* 4:72 (1983); Cole et al., *Monoclonal Antibodies and Cancer Therapy*, pp. 77-96 (Alan R. Liss, Inc., 1985); Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses* (Plenum Publishing Corp., New York, N.Y. 1980); Lemer, *Yale J. Biol. Med.*, 54:387-402 (1981); Geftter et al., *Somatic Cell Genet.*, 3:231-36 (1977); and Galfre et al., *Nature* 266:55052 (1977).

[0030] Complementary clones of an antibody are useful in the detection of the assayable antigen. As used herein, “complementary clones” of an antibody means antibodies that bind non-overlapping epitopes on the same antigen.

[0031] As used herein, the terms “comprise,” “comprising,” “include,” and “including” are intended to be open, non-limiting terms, unless the contrary is expressly indicated.

Rapid Detection Method and Assay

[0032] In one aspect, an assay method is used to detect minute blood concentrations of myoglobin or another protein (e.g., creatine kinase, a fatty acid-binding protein, a glycogen phosphorylase, a troponin) indicative of AMI. In one embodiment, the assay method includes linking a first antibody linkable to an AMI-indicating antigen in a physiological sample; linking a magnetic microparticle to the first antibody; linking a second antibody to the AMI-indicating antigen; linking a glucose molecule to the second antibody; utilizing a magnetic field to separate the magnetic particle and linked AMI-indicating antigen from a portion of other components in the physiological sample, thereby forming a test sample; and detecting the glucose in the physiological sample to determine the concentration of the AMI-indicating antigen (or at least to determine the relative amount of the AMI-indicating antigen as compared to a standard or normal level of AMI-indicating antigen in the physiological fluid). In one embodiment, the glucose molecule is linked to the second antibody and the AMI-indicating antigen via a biotinylated lectin and streptavidin link. In one embodiment, the biotinylated lectin comprises a Con-A lectin. For one embodiment where myoglobin is the AMI-indicating antigen, the first antibody comprises a biotinylated 908 antibody and the second antibody comprises a biotinylated 4E2 antibody. (There also are other epitopes that can be used.) The two antibodies are bound to different epitopes on the human myoglobin or other target antigen. In another embodiment, it is possible to have a direct link between avidin or streptavidin and the glucose molecule. Con A also can be used. In one embodiment, the magnetic particles comprise microspheres coated with albumin, avidin, or a combination thereof.

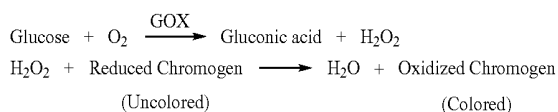
[0033] In another aspect, the immunoassay for detecting myoglobin or another antigen can be provided in a kit form, similar to ELISA kits known in the art. In one example, the kit includes a first part which comprises magnetic microparticles bound to antibodies selected to be linkable to an antigen of interest; and a second part which comprises a second antibody-streptavidin-lectin, wherein the lectin is selected to be linkable to glucose and the second antibody is selected to be linkable to the antigen of interest. In a preferred embodiment, the kit includes a first part which comprises magnetic microparticles linked to antibodies selected to be linkable to a first epitope of human myoglobin; and a second part which comprises a second antibody selected to be linkable to a second epitope of human myoglobin, wherein the second antibody is linked to a lectin, which is linked to a glucose molecule.

[0034] In one embodiment of the technique, the assay method is applied to detect myoglobin or another AMI-indicating protein present in a physiological fluid (e.g., blood, serum, or urine) taken from a patient presenting a possible acute myocardial infarction. In a preferred embodiment, the physiological fluid is blood. In a preferred embodiment, a method is provided for detecting myoglobin in a blood sample which comprises: combining the blood sample with magnetic microparticles linked to a first biotinylated antibody, said first antibody binding to a first site on myoglobin present in the blood sample; combining the blood sample with a second biotinylated antibody, said second antibody binding to a second site on the myoglobin present in the blood

sample, wherein said second antibody is linked to a glucose molecule; using a magnetic field and a separation process to separate the linked myoglobin from the blood sample to form a test sample comprising the myoglobin; and then detecting the glucose in the test sample, wherein the glucose detection can be correlated to the concentration of myoglobin in the blood sample.

[0035] The magnetic separation step can be conducted using techniques known in the art, for example, as taught in U.S. Pat. No. 6,132,607, which is incorporated herein by reference. In one embodiment, a separation chamber is used which comprises a plurality of magnets and a plurality of channels having an inlet and an outlet. The magnets are arranged on one side of the channels and are in contact with one another to create a magnetic field. The magnetic particle containing-fluid is introduced into the inlet of the channels of the separation chamber, and the magnetic field of the magnets captures the microparticle-attached-components within the channels and the remaining mixture exits the outlet of the channels of the separation chamber.

[0036] Glucose concentration can be measured by any of a variety of known techniques. For example, the technique can be any one of three general methods: electrical, calorimetric, or acidity measurement. In a typical glucose testing system, the following glucose reaction is utilized:



[0037] In one embodiment, the glucose is measured electrically, based on the first part of the reaction. (See, e.g., Bennett, Blood Glucose Determination: Point of Care Testing (Available online at: <http://www.sma.org/smj/97july3.htm>)). In one example, reagent strips are provided having a test area impregnated with enzyme glucose dehydrogenase and two metal strips that serve as electrodes. The enzymatic action on glucose liberates an electron, which reacts with a mediator present in the strip. The monitor in which the strip is placed applies a voltage across the electrodes, causing the mediator to be reconverted to its oxidized form. This reaction generates a current proportional to the amount of glucose linked with the conjugate and is translated by the meter to a numeric glucose concentration.

[0038] Conventional glucose measurement instruments typically have a reporting range of 20 mg/dL to 600 mg/dL. Concentrations outside that range will not be detectable in such conventional glucometers. In the case of AMI, the normal concentration of myoglobin in blood varies from 32 µg/L to over than 200 µg/L, which means that the small glucose concentration in samples tested with the presently described assay methods will not be detectable by conventional glucometers. Accordingly, an alternative way to detect glucose concentration may be to measure the electrical current generated in a glucose test strip using two electrodes connected with a voltmeter, where the electrodes of the glucose test strip transfer electrical current generated from the reaction between glucose oxidase (GOX) enzyme and its substrate, glucose, from the reaction site to the glucometer. In that way, measuring the electrical current or resistance in those elec-

trodes can be used to detect the concentration of glucose in a sample. The greater the current, the greater the concentration of the attached glucose.

[0039] In a calorimetric approach, glucose reacts with its enzyme glucose oxidase (GOX) to form gluconic acid and hydrogen peroxide (H₂O₂). The hydrogen peroxide then reacts with a chromogen to form a pink-colored compound, which can be monitored by reflectance photometry (Vöte et al., *Clinica Chimica Acta*. 305(1-2):81-87 (2001)).

[0040] In a third approach to glucose detection, a conventional pH meter is used to detect the acidity of a sample solution, where the acidity, in the form of gluconic acid, results from the reaction between glucose and its enzyme. The acidity of gluconic acid then can be detected by a conventional pH meter, whereby a more acidic test solution indicates that more glucose is attached to the conjugate.

[0041] Those skilled in the art can readily compare the glucose concentration to a standard or target value for a particular antigen, in order to obtain useful information. In a preferred embodiment, a doctor, paramedic, or emergency room worker can take a small blood sample from a patient who is presenting a possible AMI, test the sample with the magnetic immunoassay described herein to test the patient's myoglobin level.

[0042] For diabetics suffering from AMI, a reference glucose concentration in the blood stream can be used to adjust for the glucose concentration used to measure the levels of AMI-indicating antigen in the blood.

[0043] In another aspect of the immunoassay, an enzyme tag is used instead of the glucose. For example, an enzymatic reaction such as one involving alkaline phosphatase can be used to generate a change in color which can be used to detect for the concentration of cardiac markers in a physiological fluid. That is, the enzyme tag produces a color change when reacted, so that a calorimetric technique can be used to detect or measure the pathogen in the same way that colorimetric-based glucometer would be used with a glucose-induced color change.

[0044] The magnetic microparticles facilitate separation and detection of the target antigen. As used herein, the term "magnetic microparticles" includes magnetic, paramagnetic, superparamagnetic ferromagnetic and ferrimagnetic materials. The microparticles may comprise iron, nickel, cobalt, gadolinium, manganese and/or their alloys. In one embodiment, the microparticles comprise an iron oxide, such as magnetite, or more preferably, maghemite ($\lambda\text{Fe}_2\text{O}_3$). In other embodiments, the magnetic nanoparticles comprise an alloy or a mixture of elemental materials.

[0045] The microparticles preferably are in the form of microspheres. In preferred embodiments, the microspheres have a diameter of 500 nm or less.

[0046] In a preferred embodiment, the microspheres have a magnetic core, preferably of a superparamagnetic material, with a size of about 30 nm or less, which is encapsulated with a polymer or protein. In one embodiment, the magnetic particles comprise microspheres coated with albumin, avidin, or a combination thereof. In various embodiments, the magnetic particles may be encapsulated as described in U.S. Application Publication No. 2004/0065969 to Chatterjee, et al. and U.S. Application Publication No. 2004/0146529 to Chen, et al. The disclosures of these publications are expressly incorporated by reference herein.

[0047] The methods and compositions can be further understood with the following non-limiting examples.

Example 1

Magnetic Immunoassay for Myoglobin

Materials Preparation

[0048] Magnetic microspheres (MMS) were produced using protocols that were established by Haik, et al., *J Magnetism & Magnetic Materials* 194:254-61 (1999); Chatterjee et al., *J Magnetism & Magnetic Materials* 257:113-18 (2003). The MMS were coated with albumin and coupled with avidin to conjugate the anti-myoglobin antibody. The synthesis of the magnetic microspheres was accomplished according to a published protocol (Chatterjee et al., *J Magnetism & Magnetic Materials* 257:113-18 (2003)) in three major steps: coprecipitation of ferrous chloride and ferric chloride by sodium hydroxide, material heating and sonication, and material peptization with nitric acid. Both ferrous and ferric chloride were dissolved in a 1:2 molar ratio in distilled water. The solution then was mixed immediately with a highly concentrated sodium hydroxide, stirred continuously, heated to 90° C., and sonicated to produce ultra fine magnetic particles, which then were peptized by nitric acid. Finally, the microspheres were coated with albumin and avidin to make them attachable to the biotinylated antibodies.

[0049] The antibodies used were two complementary clones of monoclonal mouse anti-human cardiac myoglobin (Mouse isotope IgG: 908 and 4E2). These clones were chosen because their epitopes are placed widely apart on the myoglobin protein. Both antibody clones were purchased from Research Diagnostics, Inc.

[0050] Both antibodies were biotinylated using an EZ-link Sulfo-NHS-LC biotinylation kit obtained from Pierce Chemicals (Rockford, Ill., USA). Long chain arm biotin containing a 22 Armstrong spacer arm between the reacted primary amine and the biotin moiety was selected to minimize the possible effects of steric hindrance when conjugating the biotin to the much larger MMS and to increase the sensitivity.

[0051] Streptavidin (SA) is a 60,000 Dalton protein composed of four identical subunits, each of which has a binding site for biotin. Isolated from the microorganism *Streptomyces avidinii*, this protein resembles egg white avidin in its biotin binding properties. Its affinity for biotin is several orders of magnitude lower than egg white avidin; it lacks carbohydrate, and has a lower isoelectric point than avidin. Streptavidin generally requires a longer spacer arm between the biotin residues and the protein or probe to which it is attached and it is less stable than avidin. The SA was obtained from Vector Laboratories (Burlingame, Calif.).

[0052] Con A is one of the most widely used and well-characterized lectins. It has broad applicability primarily because it recognizes a commonly occurring sugar structure, α -D-mannose and α -D-Glucose through its four saccharide binding sites. At neutral and alkaline pH, Con A exists as a tetramer of four identical subunits of approximately 26,000 Daltons each. Below pH 5.6, Con A dissociates into active dimers of 52,000 Daltons. The biotinylated Con A lectins also were obtained from Vector Laboratories.

[0053] Coupling Anti-Myoglobin Antibody Clone 908 to Avidin Coupled Microspheres:

[0054] One milliliter of (0.1 mg/ml) 908 biotinylated antibody solution was combined with 7.6 ml of (0.01 g/ml) avidin coupled microsphere solution. Using the HABA method to

determine the minimum number of biotin groups on both antibody clones, a minimum of 15 biotin groups/antibody gives 10 nmoles of avidin needed to couple with 0.1 mg of 908 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 908 antibodies and avidin-coupled microspheres were incubated while mixing at 30° C. for 30 minutes.

[0055] Coupling Anti-Myoglobin 4E2 with Glucose Molecule

[0056] To reach the optimal performance between the reactants, concentrations of 5 µg/ml of biotinylated anti-myoglobin 4E2, 1 µg/ml of Con-A, 20 µg/ml of SA, and 5 mg/ml of glucose were prepared according to the recommendation of Suter et al., *Molecular Immunology* 26:221-30 (1989). Con-A and SA were added to attach each other in an eppendorf tube in a volume of 200 µl of each and incubated at 30° C. for 30 minutes while mixing. Then, 200 µl of biotinylated 4E2 antibodies were added to the previous solution and incubated for an additional 30 minutes at 30° C. while mixing to bind with the SA. Next, 200 µl of (5 mg/ml) glucose was added to the mixture and incubated for another 30 minutes at 30° C. while mixing to be adhered to the Con-A lectin surface.

[0057] Determination of Samples' Sizes:

[0058] Normal myoglobin concentration in the blood stream of a human patient ranges from 32 µg/L to over 200 µg/L (Hedges et al, *Ann Emerg Med*, 21:1445-50 (1992); Marcello et al., *Int'l J. Cardiology* 65(Suppl.1):S17-S22 (1998); Mauro Panteghini, *Clinica Chimica Acta* 272(1,6):23-31 (1998)). A discriminator value of 100 µg/L myoglobin was chosen as indicative of AMT (Quaro Panteghini, *Clinica Chimica Acta* 272(1,6):23-31 (1998)). It was determined that if a discriminating concentration of 100 µg/L of myoglobin containing 5.55×10^{-3} nmoles antibody, then 0.833 µg of antibodies are needed to bind to all possible epitopes. Using 2.4 excess, which is 2.4 times the needed amount of antibody, a test amount of 2 µg of antibody needed/myoglobin sample of 100 µg/L. The amounts used were 170 µL microsphere labeled 908 antibody solution (0.0116 µg/µL) and 200 µL of (5 µg/ml) 4E2 antibody solution.

[0059] An excess amount of antibody solution was used to assure those concentrations larger than the discriminator value could be detected. Any concentration smaller than the discriminator value can be measured as the excess labeled is washed away during the immunoassay.

Experimental Procedure and Results:

[0060] After preparing the necessary components in their appropriate concentrations—(1 µg/ml) Con-A, (20 µg/ml) SA, (5 µg/ml) biotinylated 4E2 antibody, (0.0116 µg/µL) biotinylated 908 antibody, (100 µg/ml) myoglobin, and (5 mg/ml) glucose solution—an exact volume amount of 200 µl of Con-A and SA were mixed together in an eppendorf tube and incubated for 30 minutes at 30° C. while mixing in order to link them together. Then, the same volume amount of biotinylated 4E2 antibody was added to the tube and incubated under the same conditions. Finally, a glucose solution of the same amount was added to the mixture and treated in the same way to obtain the whole conjugate of (4E2 antibody-SA-Con A complex).

[0061] The second part of the ELISA conjugate, the 908 antibodies-MMS, was prepared by adding 1 ml of biotinylated 908 antibodies to 7.6 ml of (0.01 mg/ml) of MMS and incubated together for 30 minutes at 30° C. while mixing.

Then, 200 µl of (100 µg/ml) myoglobin was added to the 908 antibodies-SPM complex and incubated under the same conditions.

[0062] Next, the two parts were added together and incubated in the same way. Then, two 1.5 Tesla magnets were applied to the side of the tube to isolate the reactants (MMS-908-Myo-4E2-SA-Con A-glucose) from other unneeded solution components. The isolated reactants were then washed several times with Tris Buffered Saline (TBS) while the magnets were applied.

[0063] Glucose tests were then performed to measure the amount of glucose taken up by the conjugate. Electrical, calorimetric, and acidity glucose determination approaches were used. In the electrical approach, a sample volume of 5 µL of each myoglobin concentration was applied to a regular strip of an electrical glucometer (Therasense Freestyle, Ultra One Touch model) to measure the electrical resistance generated due to the reaction between glucose oxidase (GOX) enzyme and its substrate, where glucose was measured using two electrodes connected to a voltmeter (type Fluke 23). The readings showed that the electrical resistant generated in strips is inversely related to the different glucose concentration; the highest concentration had the lowest resistant (FIG. 2). In the calorimetric assay, a volume of 600 µL of glucose oxidase reagent solution was warmed for 5 minutes in a water bath under 37° C. and then a 50 µL of sample solution of each concentration of myoglobin was added to the preheated reagent solution and incubated for 10 minutes in a water bath under 37° C. The samples then were measured using a spectrophotometer, and the resulted solution had different intensities of pink color depicting the amount of glucose in each sample (see FIG. 3). In the acidity approach, a sample of 500 µL of glucose oxidase enzyme was added to a sample of 1 ml of each myoglobin concentration and allowed to react for 5 minutes to form gluconic acid solution, and then a pH meter was used to measure the acidity of each sample. Readings showed a proportional relationship between each glucose concentration and its pH level. The higher glucose concentration had the higher acidity level (see FIG. 4).

[0064] Publications cited herein and the materials for which they are cited are specifically incorporated by reference. Modifications and variations of the methods and devices described herein will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the appended claims.

I claim:

1. An immunoassay system comprising:
 - a first antibody linkable to an assayable protein antigen present in a physiological fluid;
 - a magnetic microparticle linked to the first antibody;
 - a second antibody linkable to the assayable protein antigen; and
 - a glucose molecule linked to the second antibody.
2. The immunoassay system of claim 1, wherein the assayable protein antigen comprises myoglobin.
3. The immunoassay system of claim 1, wherein the assayable protein antigen is selected from creatine kinase, fatty acid-binding protein, troponins, and glycogen phosphorylase.
4. The immunoassay system of claim 1, wherein the second antibody is linked to a streptavidin.

5. The immunoassay system of claim 4, wherein the streptavidin is linked to a lectin which is linked to the glucose molecule.

6. The immunoassay system of claim 1, wherein the second antibody is a biotinylated anti-myoglobin antibody and the anti-myoglobin antibody is linked to streptavidin which is linked to a biotinylated Con-A lectin which is linked to the glucose molecule.

7. The immunoassay system of claim 1, wherein the first and second antibodies comprise complementary clones of monoclonal anti-human cardiac myoglobin antibody.

8. The immunoassay system of claim 7, wherein the second antibody comprises a monoclonal mouse anti-human cardiac myoglobin Mouse isotope IgG:908.

9. The immunoassay system of claim 7, wherein the first antibody comprises a monoclonal mouse anti-human cardiac myoglobin Mouse isotope IgG:4E2.

10. The immunoassay system of claim 1, wherein the magnetic microparticle comprises a microsphere coated with a polymer or a protein.

11. The immunoassay system of claim 10, wherein the coating comprises albumin, avidin, or a combination thereof.

12. An immunoassay kit for detecting myoglobin comprising:

a first part which comprises magnetic microparticles linked to first antibodies selected to be linkable to a first epitope of myoglobin; and

a second part which comprises second antibodies selected to be linkable to a second epitope of the myoglobin, wherein the second antibodies are linked to lectin that is linked or linkable to glucose molecules or enzyme tags.

13. The immunoassay kit of claim 12, wherein the second antibodies are linked to streptavidin.

14. The immunoassay kit of claim 12, wherein the streptavidin is linked to the lectin which is linked to the glucose molecules.

15. The immunoassay kit of claim 12, wherein the second antibodies are biotinylated anti-myoglobin antibodies and the anti-myoglobin antibodies are linked to streptavidin which is linked to biotinylated Con-A lectin which is linked to the glucose molecules.

16. The immunoassay kit of claim 12, wherein the first and second antibodies comprise complementary clones of monoclonal anti-human cardiac myoglobin antibody.

17. The immunoassay kit of claim 12, wherein the second antibodies comprises a monoclonal mouse anti-human cardiac myoglobin Mouse isotope IgG:908.

18. The immunoassay kit of claim 12, wherein the first antibodies comprises a monoclonal mouse anti-human cardiac myoglobin Mouse isotope IgG:4E2.

19. The immunoassay kit of claim 12, wherein the micro-particles are microspheres which comprise a core of a super-paramagnetic material coated with a protein or a polymer.

20. The immunoassay kit of claim 12, wherein the coating comprises albumin, avidin, or a combination thereof.

21. An immunoassay system comprising:

a first antibody linkable to an assayable Acute Myocardial Infarction-indicating antigen;

a magnetic microparticle linked to the first antibody,

a second antibody linkable to the assayable Acute Myocardial Infarction-indicating antigen; and

a sugar molecule linked to the second antibody.

22. The immunoassay system of claim 21, wherein the sugar molecule comprises mannose, fructose, or galactose.

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专利名称(译)	用于检测用于评估急性心肌梗塞的心脏标志物的系统和测定法		
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摘要(译)

提供了用于检测生理流体(例如血液,血清或尿液)中的靶抗原的测定系统和方法。该方法包括通过第一抗体将磁性微粒与生理液体中的靶抗原连接;通过第二抗体将葡萄糖分子与靶抗原连接;利用磁场将磁性微粒连接的抗原与生理液分离,形成试验样品;检测试样中的葡萄糖,测定生理液中的目标抗原浓度。靶抗原可以由心脏组织损伤引起的蛋白质或标记物,其可以用于评估急性心肌梗塞。示例性靶抗原是肌红蛋白。葡萄糖检测优选是可以快速进行的,例如使用常规血糖仪,并且可以包括测量测试样品的电阻,颜色或pH。

