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(54) Title: METHOD AND APPARATUS FOR DETERMINATION OF FATTY ACID MARKERS USING ELECTRICAL IMPEDANCE MEASUREMENT

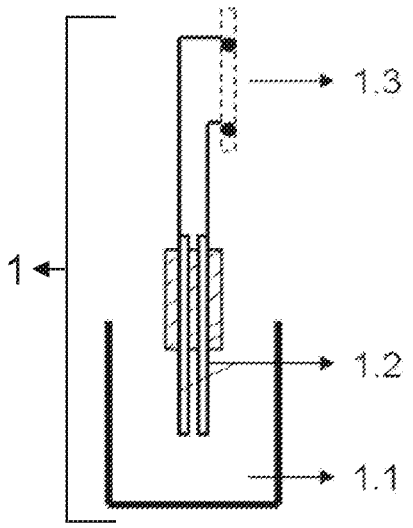


FIGURE 1

(57) Abstract: The invention comprises a method and apparatus for determining unbound (free) fatty acids and/or fatty acid binding capacity of albumin in case of acute organ/tissue ischemia, or in acute/chronic specific medical conditions associated with modified albumin by fatty acids, and diagnosing medical conditions associated with increased free fatty acids and/or reduced fatty acid binding capacity of albumin in samples, using electrical impedance measurement. The method includes specific electrolysis reaction, so-called anodic decarboxylation reaction or Kolbe's electrolysis, which takes place on the anode surface. The reaction occurs between two fatty acid anions carried out by the specific electrical current or potential. The method eventually includes condensation of two fatty acid anions in one long hydrocarbon molecule on the anode surface. Accumulation of many hydrocarbon chains creates an insulating layer on the anode surface; this in turn increases the electrical impedance between the electrodes. The impedance change reflects the level of free fatty acids and/or the fatty acid binding capacity of albumin that is increased or decreased in the ischemia and/or specific medical conditions. The invention also comprises an apparatus consisting of an "instrument" and "single-use kit".

DESCRIPTION

METHOD AND APPARATUS FOR DETERMINATION OF FATTY ACID MARKERS USING ELECTRICAL IMPEDANCE MEASUREMENT

TECHNICAL FIELD

This invention is related to the method and apparatus ensuring diagnosis of acute or chronic organ/tissue ischemia or specific medical conditions associated with altered fatty acid metabolism by detecting free fatty acids and/or fatty acid binding capacity of albumin in serum, plasma, or blood sample of a patient. More specifically, the described method and apparatus are useful for diagnosis as well as prognosis and follow-up of acute cardiac ischemia.

PREVIOUS TECHNIQUE

Untreated myocardial ischemia can result in an acute myocardial infarction, which can be diagnosed by testing for physiological markers of necrosis. Well-known markers include creatine kinase, troponin, and myoglobin that are released from cells following cell death, usually hours later after beginning of the chest pain. However, there is an emerging need for early and specific markers that would be positive immediately after (within minutes) the beginning of ischemia (before cell death), to allow treatment of the disease prior to irreversible damage.

Albumin is the most abundant plasma protein, the main determinant of colloid osmotic pressure. Among its most important functions are fatty acid transport (at least 7 binding sites present on albumin), drug binding and transport, modulation of endothelial permeability, metal chelating property, and free radical scavenging.

Albumin has been involved and/or affected in many diseases. N-terminal modification of the albumin which decreases the metal (such as cobalt, nickel, copper) binding capacity of the molecule immediately after (within minutes) the cardiac insult (so-called ischemia modified albumin), has previously been reported and proposed as a biomarker for myocardial ischemia [patent no: US 5,227,307 (Bar-Or et al.), no: US 5,290,519 (Bar-Or et al.), Bar-Or D, et al. A

novel assay for cobalt-albumin binding and its potential as a marker for myocardial ischemia-a preliminary report. J Emerg Med 2000: 19; 311–315]. The patent application publication no: US 2005/0021235 (Bar-Or et al.) disclose the usage of this ischemia modified albumin (IMA) for myocardial ischemia and have led to a clinical assay for albumin cobalt binding (ACB test). The test uses colorimetric methods to determine the amount of unbound metal ions present in the sample; this indirectly indicates albumin cobalt binding capacity (this capacity reduces in acute ischemia). Detection of reduced cobalt binding capacity of albumin by monitoring the amount of unbound metal in the sample is also issue for some other patents and patent applications such as no: US 7063782 (Wayment et al), application publication no: US 2009/0294304 (Hyland et al.), and application publication no: US 2010/0291611 (Bolbot et al.); they use, however, electrochemical detection of unbound transition metal ions in the sample. Indeed, we now know that albumin has at least 4 different cobalt binding sites: N-terminal, Site A, Site B, and Cys-34 (Bal W, et al. Binding of transition metal ions to albumin: Sites, affinities and rates. Biochimica Biophysica Acta 2013; 1830: 5444-5455). The albumin cobalt binding assay has approved for more than a decade by the FDA, but it has never gained expected clinical achievement.

Human serum albumin has at least seven binding sites with varying affinities for medium and long chain fatty acids (Petitpas, I et al. Crystal structures of human serum albumin complexed with monounsaturated and polyunsaturated fatty acids, J. Mol. Biol. 2001; 314: 955–960). Under normal physiologic condition, 0.1-2 molecules of fatty acids are bound to each albumin molecule, but the molar ratio of fatty acid/albumin can reach up to 6, during fasting or extreme exercise or in patients with liver and cardiovascular diseases (e.g., acute myocardial ischemia) (Brodersen, R., et al. Multiple fatty acid binding to albumin in human blood plasma. Eur. J. Biochem. 1990; 189: 343–349., Wolfe, R. et al. Role of triglyceride-fatty acid cycle in controlling fat metabolism in humans during and after exercise. Am. J. Physiol. 1990; 258: E382–E389). The numbering of these seven sites (FA sites 1-7) in the crystal structure of albumin is arbitrary and not based on affinity for fatty acid

molecules. Although the binding pockets seem well adapted to accommodate fatty acid molecule, they are not specific for fatty acids, thus they are also capable of binding other ligand molecules. For example, drug binding site I (Sudlow site I) (representative ligands are warfarin and phenylbutazone) which is located on subdomain IIA of the albumin, overlaps with FA7 (fatty acid binding site no.7). Similarly, drug binding site II (Sodlow site II) (representative ligands are diazepam and propofol) which is located on subdomain IIIA of the albumin, overlaps with FA3 and FA4. Elsewhere on the albumin, FA1 also acts as the primary site for binding hemin, while FA5 is a secondary site for propofol and throxine. FA6 is a secondary site for ibuprofen and diflunisal (Ghuman, J et al. Structural basis of the drug-binding specificity of human serum albumin. J. Mol. Biol. 2005; 353: 38–52, Krenzel ES, et al. Correspondance of fatty acid and drug binding sites on human serum albumin: A two-dimensional nuclear magnetic resonance study. Biochemistry 2013; 52: 1559-1567). Among the seven binding sites, extensive studies have identified FA2, FA4, and FA5 as the highest affinity sites for fatty acids on the albumin, while the remaining sites are lower affinity for fatty acids (Simard JR, et al. Location of high and low affinity fatty acid binding sites on human serum albumin revealed by NMR drug-competition analysis. J Mol Biol 2006; 361: 336-351). Human albumin ligands sharing the same binding site or having separated but close binding sites with different affinity may compete with each other, one may inhibit binding of other one, or even one may dissociate other one from the albumin surface. For example, in case of occupied albumin by fatty acids, the addition of only one equivalent of hemin is sufficient to quantitatively replace the fatty acid bound to FA1 (it is one of the low affinity fatty acid binding site and only single available binding site for hemin) (Junk MJN, et al. Characterization of the solution structure of human serum albumin loaded with a metal porphyrin and fatty acids. Biophysical J 2011; 100: 2293-2301).

Although the albumin cobalt binding test was approved by the FDA in 2003 as a new method for ruling out myocardial ischemia among patients coming to the emergency department, it has recently been questioned because of its low specificity (Kim, JS et al. Ischemia-Modified Albumin: Is it a reliable

diagnostic and prognostic marker for myocardial ischemia in real clinical practice? Cardiology 2010, 116, 123–129, Oh BJ et al. Insignificant role of the N-terminal cobalt binding site of albumin in the assessment of acute coronary syndrome: Discrepancy between the albumin cobalt binding assay and N-terminal targeted immunoassay. Biomarkers 2012; 17: 394-401). Bhagavan NV et al. were first demonstrated plausible relationship between increase in FFA levels and similar increase in IMA (lower cobalt binding capacity) in the sera of patients with acute myocardial infarction (Bhagavan NV, et al. Utility of serum fatty acid concentrations as a marker for acute myocardial infarction and their potential role in the formation of ischemia modified albumin: A pilot study. Clin Chemistry 2009; 55: 1588-1590). Similar concern has also been raised in the patent application publication no: US 2011/0045520 (Bhagavan et al.). Another recent study has further demonstrated allosteric inhibition of cobalt binding to albumin by fatty acids (Lu J, et al. Allosteric inhibition of cobalt binding to albumin by fatty acids: Implications for the detection of myocardial ischemia. J Med Chem 2012; 55: 4425-4430). Actually it is not surprising to see the parallelism between IMA and albumin occupied (bound) by fatty acids, since all cobalt binding sites excluding the N-terminal share common places with the fatty acid binding sites on albumin. Jalan R et al have recently demonstrated strong correlation between fatty acid bound to FA1 and/or FA2 of albumin, and reduced cobalt binding capacity of albumin (Jalan R et al. Alterations in the functional capacity of albumin in patients with decompensated cirrhosis is associated with increased mortality. Hepatology 2009; 50: 555-564). They have also speculated the possible overlap between cobalt binding sites and FA1 / FA2 sites of albumin (Actually Site A, Site B, and Cys34 binding sites of cobalt, and FA 1/FA 2 share common place on albumin surface). So it seems reasonable to say that, reduced cobalt binding to albumin which is termed IMA is actually due to occupation of some (Site A, Site B, or Cyt-34) metal binding regions by the bound fatty acids. According to the contemporary science, N-terminal site of albumin never binds fatty acids (Petitpas, I et al. Crystal structures of human serum albumin complexed with monounsaturated and polyunsaturated fatty acids, J. Mol. Biol. 2001; 314: 955–960, Krenzel ES, et al.

Correspondance of fatty acid and drug binding sites on human serum albumin: A two-dimensional nuclear magnetic resonance study. Biochemistry 2013; 52: 1559-1567, Simard JR, et al. Location of high and low affinity fatty acid binding sites on human serum albumin revealed by NMR drug-competition analysis. J Mol Biol 2006; 361: 336-351, Fujiwara S et al. Faty acid binding to serum albumin: Molecular simulation approaches. Biochim Biophysic Acta 2013; 1830: 5427-5434). This data is well suited with the study by Oh BJ et al that, reduced cobalt binding to albumin which is detected by albumin cobalt binding test is not related to the albumin's N-terminal modification (contrary to Bar-Or's original description in 2000) (Oh BJ et al. Insignificant role of the N-terminal cobalt binding site of albumin in the assessment of acute coronary syndrome: Discrepancy between the albumin cobalt binding assay and N-terminal targeted immunoassay. Biomarkers 2012; 17: 394-401).

Human albumin ligands sharing the same binding site or having separate but closed binding sites with different affinity may compete with each other. This competition may result in binding inhibition or even blocking of a ligand, or partial or complete displacement of already bound ligand in the presence of other ligand (Rudman D, et al. Effect of free fatty acids on binding of drugs by bovine serum albumin, by human serum albumin and by rabbit serum. Pharmacol Exp Ther 1971; 272: 176-261). Fujiwara S et al have recently summarized the list of ligands that share common binding site with fatty acids (Fujiwara S et al. Faty acid binding to serum albumin: Molecular simulation approaches. Biochim Biophysic Acta 2013; 1830: 5427-5434). Fatty acid binding affinity to and displacement from albumin are also affected by the albumin unfolding (protein denaturation). It is well-known entity of the relevant science that, human albumin loses its 3-domain alpha-helix configuration and even undergoes fragmentation while the impact of denaturizing agent increases. Recently Anand U et al. have reviewed the effect of various denaturants on the albumin with a special emphasis on protein unfolding and refolding dynamics (Anand U et al. Binding, unfolding and refolding dynamics of serum albumin. Biochimica Biophysica Acta 2013; 1830; 5394-5404). Urea and heating are two well-known examples of albumin denaturant agents. (Tayyab S

et al. Use of domain specific ligands to study urea-induced unfolding of bovine serum albumin. Biochem Biophysic Res Com 2000; 277; 83-88; Rezaei-Tavirani M et al. Conformational study of human serum albumin in predenaturation temperatures by differential scanning calorimetry, circular dichroism and UV spectroscopy. J Biochem Mol Biol 2006; 39: 530-526). Protein (e.g., albumin) unfolding and refolding is well-known and promising technology which is a subject of several patents. For example, patent application publication no: US 2011/0059181 (Hu et al.) disclosed a methods for hydrophobic drug delivery comprising first producing unfolding albumin which expose its hydrophobic core, then incorporating the drug into the unfolded albumin, and finally obtaining water-soluble drug-albumin complex after allowing refolding of the albumin.

Human serum contains a mixture of at least 6 fatty acids; these are (in approximate percentage) oleic acid (38%), palmitic acid (25%), linoleic acid (22%), stearic acid (10%), arachidonic acid (3%), and linolenic acid (2%). Myocardial ischemia causes a hyperadrenergic state which results in breakdown of tissue and plasma phospolipids and triglycerides with resultant increased plasma concentration of free fatty acids (FFA) within minutes after beginning of chest pain. Nearly all blood fatty acids are tightly bound to albumin and transported throughout the body while a very small percentage is present in unbound form (so-called unbound or free fatty acid) (Richieri GV, Kleinfeld AM. Unbound free fatty acid levels in human serum. J Lipid Res. 1995;36: 229-240). In particular, patients with acutely ischemic myocardium exhibit a rapid increase in serum FFA levels that can exceed normal average values by as much as 3-fold (Opie LH. Metabolism of free fatty acids, glucose and catecholamines in acute myocardial infarction. Am J Cardiol 1975;36:938-953). The patent no: US 6,750,030 (Kleinfeld et al.) and patent application publication no: US 2011/0045520 (Bhagavan et al.) disclosed usage of FFA level as a biochemical marker of acute myocardial ischemia, since they found 2-fold and 5-fold increased FFA levels in their patients' blood respectively although their measurement methods are different. The patent application publication no: US

2008/0305550 (Huff et al.) also disclosed a method of determining the ischemic event by determining the amount of fatty acid that is complexed to albumin.

Elevation of circulating ischemia modified albumin concentration is not specific for myocardium; theoretically it can be generated during any organ/tissue ischemia and in a series of non ischemic acute or chronic disease or medical conditions (Gaze DC. Biomarkers of cardiac ischemia. INTECH 2013; Morozov MI. Ischemia modified albumin as a marker of myocardium and skeletal muscle damage. Int J Cardiovasc Res 2014: 3; 1-5). There must be a series of diseases or medical conditions where albumin loses partially its cobalt or fatty acid binding capacity, or blood level of fatty acid and/or modified albumin increase. For example, fatty liver which reflects some primary and secondary chronic liver diseases, characterized by increased blood fatty acid levels. Amirtharaj GJ, et al have recently demonstrated increased bound (to albumin) fatty acid level and decreased cobalt binding capacity of albumin in patients' blood sample with fatty liver (Amirtharaj GJ, et al. Fatty acids influence binding of cobalt to serum albumin in patients with fatty liver. Biochimica Biophysica Acta 2008; 1782: 349-354). They also have showed causative relationship between bound (to albumin) fatty acids and decreased cobalt binding to albumin. Jalan R et al have showed reduced cobalt binding to albumin in acute liver failure patients (Jalan R et al. Alterations in the functional capacity of albumin in patients with decompensated cirrhosis is associated with increased mortality. Hepatology 2009; 50: 555-564).

Increase in fatty acid level after intravenous administration of heparin has been known for many years. Floating heparin molecule (one of the most negatively charged molecule in the blood) interacts with the positively charged lipoprotein lipase enzyme which is embedded in the glycocalyx layer of endothelium in stable conditions and displace it into the circulation. Increased lipoprotein lipase activity rapidly converts triglycerides into fatty acids with resultant immediate (within 15-30 minutes after single dose) up to 2-3 fold increase of free fatty acids (this increase nearly identical to increase of fatty acid level observed in acute myocardial ischemia) (DeSmet R, et al. Heparin-induced release of protein-bound solutes during hemodialysis is an in vitro

artifact. Clin Chem 2001; 47: 901-909; Nelson PG. Effect of heparin on serum free fatty acids, plasma catecholamines, and the incidence of arrhytmias following acute MI. Brit Med J 1970; 3: 735737; Rogers WJ. Effect of heparin-induced free fatty acid elevation on myocardial oxygen consumption in man. Am J Cardiol 1977; 40: 365-372; Brunner MP. Effect of heparin administration on metabolomic profiles in samples obtained during cardiac catheterization. Circ Cardiovasc Genet 2011; 4: 695-700).

The Kolbe's electrolysis or anodic decarboxylation reaction is among the oldest reaction described in electrochemistry which has been known since the mid of the 19th century. It has originally been described in transformation of fatty acids into saturated hydrocarbons to be used as fuels. The fundamental nature of the Kolbe's reaction is clear: a discharge of the carboxylic acid anions with subsequent decarboxylation and "fusion" of the radicals to form a hydrocarbon chain, as depicted below:

$$R1COO^- + R2COO^- \rightarrow R1\text{-}R2 + 2CO2 + 2e^-$$

At present, the term Kolbe's electrolysis is understood to imply electrocondensation at the anode not only of fatty acids but also of their various derivatives, such as the half-esters of dibasic acids. Saturated fatty acids with straight-chains of varying lengths have been subjected to the Kolbe's electrolytic condensation reaction and primary synthesis products in satisfactory yields have been obtained in all cases with the exception of formic acid. Unsaturated fatty acids having one or more double bound with carbon chain of varying lengths have also documented to undergo electrolytic condensation reaction. The reaction efficiency is low, however, in fatty acids having double bond on α (2) and β (3) positions. Side reaction can also occur during the formation of the desired primary compound especially when the synthesis is carried out in an aqueous medium with the presence of certain anions or at low current densities. The example of by-product is the formation of the alcohol containing one carbon atom less than the original carboxylic acid. In Kolbe's reaction water is usually used as solvent, but some other solvents such as alcohols and acetic acids may also be used. Since first description, this reaction has been a subject of several patents in the field of various hydrocarbon

production from waste materials, renewable natural raw materials, or biomass, such as patents no: US 3,992,268 (Antos G), no: WO 2007095215 (Bradin D), patent application publication no: US 2013/0203953 (Pereira et al.), patent application publication no: US 2014/0353163 (Kuhri et al.). The patent application publication no: US 2008/0161418 (Dierker M) disclosed a method by using Kolbe's electrolysis for the production of branched hydrocarbons from the fatty acids to use in emollients and cosmetics.

The Kolbe's electrolysis is mainly determined by the reaction conditions, like anodic current or potential, anode materials, temperature, and electrolyte composition. Some suitable anode materials that have been reported before are platinum, iridium, gold, nickel, graphite. In general, increase of current density favors the hydrocarbon synthesis. At specific current densities, the so-called critical potential of about 2.1-2.8 V is reached on the anode above which Kolbe's condensation proceeds expectantly. The current density per electrode surface area of 250 mA/cm2, therefore, is recommended for efficient yield of end product, although the current density as low as 1 mA/cm2 (0.01 mA/mm2) sufficiently effective to drive the Kolbe's electrolysis. It is assumed that at the critical potential the carboxylate anion forms a layer at the anode surface, whereby the solvent is desorbed (leading to generation of primary hydrocarbon product), whilst below the critical potential the solvent is preferentially oxidized (leading to generation of by-products) (Schafer HJ. Recent contributions of Kolbe electrolysis to organic synthesis. Topics in Current Chemistry. 1990; 152: 91-151). To maintain low voltage level, the electrodes are arranged so as to leave 0.1-2 mm gap between the electrodes. As a rule, the yield of end product falls with increasing temperature. Foreign anions in the electrolyte solutions inhibit the principal anodic reaction and their presence leads to the formation of by-products. The presences of certain metallic cations even in very small amounts strongly affects or even completely suppress the course of the Kolbe's electrolysis, possibly due to formation of oxide layers at the anode. These cations include Pb+2, Mn+2, Ca+2, Cu+2, Co+2, Fe+2, and are especially effective at low current densities. The pH is another determinant of the reaction; the principle reaction occurs at neutral or weakly acidic pH levels.

Finally, the composition of electrolyte solution also determine the efficiency of the primary (Kolbe's) reaction; increased concentration of carboxylic acid anions leads to higher yields of the principal condensation product, the extent of side reactions increases as the concentration is lowered.

In the electrolytic condensation of fatty acids, the anode generally becomes coated with a layer of insoluble polymers (hydrocarbon chains) which are formed during the reaction and inhibit it, especially when the acids contain several double bonds and long chain (Svadkovskaya GE, et al. Electrolytic condensation of carboxylic acids. Russian Chemical Reviews, 1960; 29: 161-180, Schafer HJ. Electrochemical conversion of fatty acids. Eur J Lipid Sci Technol 2012; 114: 2-9). By using polaromicrotribometry, the formation of this hydrophobic layer on anode has been detected at the critical potential where Kolbe's reaction starts. It has been suggested by the author that this film is responsible for controlling (slowing and even stopping) the kinetics of Kolbe's reaction. (Bruno F, et al. Polaromicrotribometric study of a hydrophobic polymer film on platinum electrode during Kolbe electrosynthesis in aqueous media. Electrochimica Acta 1972; 17: 1161-1170). Attempts to increase the efficiency of Kolbe's electrolysis by preventing undesirable deposits on the anode are disclosed in patent no: US 3,787,299 (Beck et al.) (flowing electrolyte between the electrodes was recommended to sweep over depositions), and no: US 3,756,928 (Eisele et al.) (intermittently stopped electrolysis and short-circuited electrodes in current-off periods was recommended to decrease depositions). It is the main subject of this invention that is undesirable (now, we desire it anymore) deposition on the anode during the course of the electrolysis. These deposits create an insulating hydrocarbon layer on the anode surface; this in turn increases the electrical impedance between the electrodes (this normally slows down the reaction and inhibits synthesis of hydrocarbon), thus allows detection of fatty acid content in the sample by measuring impedance change.

BRIEF DESCRIPTION OF THE INVENTION

In contemporary clinical practice, there are no point-of-care (POC) or on-side tests which is suitable for diagnosis of acute medical conditions associated

with ischemia in early period (within minutes), such as myocardial ischemia. An ideal test would be a small simple device providing quick and accurate results that can be used at the bedside. The aim of this invention is to provide such a diagnostic test.

It is an object of the present invention to provide a diagnostic test that detects a change in biochemical composition of the patient's sample, wherein this change relates to fatty acid composition or fatty acid binding capacity of albumin.

It is another object of the present invention to provide a diagnostic test that has at least one pair of electrode (anode and cathode), wherein the specific electrolysis reaction known as Kolbe's electrolysis or anodic decarboxylation reaction takes place on the anode surface under specific electrical current and/or potential.

Another object is to provide a diagnostic test that allows Kolbe's electrolysis reaction on the anode surface, wherein two fatty acid anions lose their polarity and condense into one long chain of hydrocarbon molecule which subsequently accumulates on the anode surface.

It is another object of the present invention to provide a diagnostic test that drive decarboxylation reaction of fatty acids on the anode surface, wherein accumulated hydrocarbon molecules make an insulating layer on the anode surface which gradually increases impedance between electrodes.

Additionally, it is a diagnostic test that determines changes in impedance measurements relative to time in sample(s) from ischemic patient or patient with specific medical conditions, wherein patients have higher level of free fatty acids and/or lower level of fatty acid binding capacity of albumin, when compared to normal individuals and/or patient's another sample.

It is another object of the present invention to provide a diagnostic test that uses data processing techniques to identify electrochemical output data for quantifying the difference between patient and normal individuals and/or the difference between patient's at least two samples. It is an apparatus for assaying a patient's condition at the patient's side.

MEANINGS OF FIGURES

Figure 1. Single-Use Kit, General Appearance

Figure 2. Instrument, General Appearance

Figure 3. Single-Use Kid and Instrument, After Engagement

The correspondence of the part numbers indicated in the figures are given below.

1. Single-use Kit
   - 1.1. Specific Focus
   - 1.2. Electrodes
   - 1.3. Connectors
2. Instrument
   - 2.1. Socket
   - 2.2. Port
   - 2.3. Potentiostat / Amperostat
   - 2.4. Microprocessor
   - 2.5. Screen

DETAILED DESCRIPTION OF INVENTION

A free (unbound) fatty acid levels and/or fatty acid binding capacity of occupied (by fatty acids)/modified (by any blood-born ligands) albumin in a patient may be used to diagnose or monitor acute ischemia or specific medical conditions. The method and apparatus described here can be used as an indicator of presence, severity, activity, and staging of acute ischemia or specific medical conditions during screening, at the initial diagnosis, in the treatment period and follow-up.

In one aspect, acute ischemia is acute myocardial ischemia in which a few fold (up to 6) increased bound fatty acid/albumin ratio (normal physiologic ratio is 0.1-2) and up to 2-3 fold increased free (unbound) fatty acid level are observed in patient's blood sample immediately after chest pain. Scientists being aware of albumin-ligand interactions have nowadays accepted the fact that fatty acid binding sites (albumin has at least 7 sites) and up to three of four

cobalt binding sites (except for N-terminal site) share same binding places in part on the albumin; therefore increased fatty acids (either bound or unbound fatty acids) might compete the cobalt ions or even displace already bound cobalt ions during cobalt binding test in acute myocardial ischemia. Contrary to original description of ischemia modified albumin which has been postulated to occur after N-terminal modification of the molecule (by Bar-Or D. in 2000), along with the recent supportive clinical and laboratory studies clearly described in the Background of the Invention section, the inventor has reasonably concluded that ischemia modified albumin may be actually an albumin fully occupied by fatty acids (up to 6-7 molecule of fatty acid per one albumin; indeed it possesses at least 7 fatty acid binding sites, three of which are high affinity). Determination of albumin-fatty acid complex (albumin occupied by fatty acids) to diagnose specific medical condition has been subject of the patent application publication no: US 2008/0305550 (Huff et al.). Determination of fatty acid level has been subject of some other patents, like patent no: US 6,750,030 (Kleinfeld et al.), and patent applications, like publication no: US 2011/0045520 (Bhagavan et al.) and US 2002/0160537 (Kleinfeld A); none of which have disclosed the method described here.

In another aspect, acute ischemia is arterial or venous ischemia in acute clinical settings affecting various human organ, tissues or body parts which is characterized by altered unbound or bound (to albumin) blood fatty acid levels and/or reduced fatty acid binding capacity of albumin; including, but not limited to, pulmonary embolism, preeclampsia, sepsis, stroke, carbon monoxide poisoning, deep vein thrombosis, lower and upper limb ischemia, intestinal ischemia, testicular torsion, trauma related ischemia, placental ischemia and insufficiency in pregnancy, hypothermia, iatrogenic ischemia (i.e., organ harvesting for transplantation, ischemic preconditioning, remote ischemic preconditioning), post operative ischemia, exercise induced ischemia, ischemia with thrombolytic/fibrinolytic therapy, ischemia with thrombectomy, ischemia with reperfusion, ischemia with percutaneous transluminal angioplasty (PTA) and stenting.

In another aspect, specific medical conditions are a series of acute or chronic, congenital or acquired diseases, disorders or non-physiologic conditions which are characterized by primarily or secondarily altered fatty acid metabolism accompanied by increased/altered unbound or bound (to albumin) blood fatty acid levels or reduced fatty acid binding capacity of albumin; including, but not limited to, fatty liver, steatohepatitis, acute and chronic liver failure, cirrhosis, jaundice, acute or chronic hepatitis, renal failure, adiposity, obesity, metabolic syndrome, pancreatitis, polycystic ovary syndrome, mitochondrial fatty acid oxidation disorders, any medical conditions associated with mitochondrial dysfunctions, diabetes with increased insulin resistance, cardiovascular disease, peripheral arterial disease, atherosclerosis, hypertension, any medical conditions with increased oxidative stress.

In another aspect, specific medical conditions are a series of chronic diseases, disorders or non-physiologic conditions affecting various human organs, body parts, tissues, specific cell or cell groups, cell organelles, membranes, proteinaceous molecules, receptors, enzymes, hormones, immunoglobulins, DNA, RNA, chemicals, or electrolytes which are characterized by modification of albumin with altered albumin-fatty acid binding capacity; including, but not limited to, diabetes mellitus and other medical conditions associated with oxidative stress such as sepsis, renal diseases, liver diseases, cardiovascular diseases, neurodegenerative diseases, rheumatologic diseases, premalignant and malignant diseases, and aging. In diabetes mellitus, the slow non-enzymatic reaction, termed glycation (glycosylation), affects the albumin molecule with attachment of glucose or other carbohydrate compounds such as galactose and fructose to the free amine groups of albumin. Decreased fatty acid binding capacity of glycated albumin has been demonstrated (Yamazaki E, et al. Kinetics of fatty acid binding ability of glycated human serum albumin. J Biosci 2005; 30: 475-481) and recently re-established (Blache D, et al. Glycated albumin with loss of fatty acid binding capacity contributes to enhance arachidonate oxygenation and platelet hyperactivity: relevance in patients with type 2 diabetes. Diabetes 2015; 64: 960-972). Glycated albumin may be used as an alternative marker for glycemic control. It may also be

suggested using as a marker in diabetes complication, including nephropathy, retinopathy, neuropathy, gestational diabetes, and Alzheimer's disease. Structural and/or functional modification of albumin in acute or chronic specific medical conditions is not only limited to glycation (glycosylation) of albumin, but also includes other posttranslational changes such as oxidation, citrullination, cysteinilation, homocysteinilation, glutathionilation, and phosphorylation of the protein during various medical conditions; all these posttranslational changes may reduce fatty acid binding capacity of albumin . The present invention discloses a method and apparatus to determine bound (to albumin)/ unbound fatty acid levels and magnitude of fatty acid binding capacity of albumin in a patient's sample by using specific electrolysis reaction (Kolbe's electrolysis) to screen, diagnose, stage, follow-up of acute ischemia or specific medical conditions. Patent applications like publication no: US 2008/0305550 (Huff et al.) and publication no: US 2014/0147874 (Cotter et al.) have disclosed detection of modified albumin in various medical conditions; however, none of which have disclosed the method described here.

In a preferred embodiment, the method comprise: providing a sample from patient; determination of fatty acid level in a sample by using impedance change measurement carried out by a specific electrolysis reaction; and defining whether measurement is indicative of acute ischemia or specific medical conditions by comparing the results of normal individuals. The specific electrolysis reaction is the Kolbe's electrolysis or anodic decarboxylation reaction which takes place on the anode (embedded in the patient's sample) under specific electrical current density and voltage. Under Kolbe's electrolysis, fatty acids lose their carboxylate anions, remaining radicals condense and generate long hydrocarbon chain, then a hydrocarbon deposits start to accumulate on the anode surface, and finally many hydrocarbon chains coat the anode surface and gradually increase the impedance between the electrodes. The magnitude and/or changing pattern of measured impedance (either instantaneous or time dependent) reflects the amount of fatty acids in the sample. This is also indicative of the fatty acid binding capacity of sample's albumin (this is probably identical to the ischemia modified albumin). Either the

free fatty acid level or fatty acid binding capacity of albumin can be used to determine the occurrence, nonoccurrence, or current activity level of an acute ischemia or specific medical conditions.

In another preferred embodiment, the method comprises: providing a sample from patient; determination of fatty acid level in a sample by using impedance measurement carried out by a specific electrolysis reaction. The method also includes adding fatty acid which is soluble in the sample at the starting, before the starting or after the starting of electrolysis (and impedance measurement), thus creating a competition milieu in the sample for fatty acid anions migrating towards either the albumin molecules or the surface of anode electrode -both are strong attractor for fatty acid anions-, and finally defining whether measurement is indicative of acute ischemia or specific medical conditions by comparing the results obtained from normal individuals and/or said patient's another sample without mixing extra fatty acids. The specific electrolysis reaction is the Kolbe's electrolysis or anodic decarboxylation reaction which takes place on the anode (embedded in the patient's sample) under specific electrical current density and voltage. Under Kolbe's electrolysis, fatty acids lose their carboxylate anions, remaining radicals condense and generate long hydrocarbon chain, then hydrocarbon deposits start to accumulate on the anode surface, and finally many hydrocarbon chains coat the anode surface and gradually increase the impedance between the electrodes. If the sample's albumin is occupied by fatty acid molecules/ ligands, or modified by any sample's molecules in acute ischemia or specific medical conditions, nearly all added fatty acid molecules are attracted by the anode, and thus Kolbe's reaction results in intense formation of isolating hydrocarbon layer, and rapid and high level impedance measures. If the sample's albumin possesses physiologic level (0.1-2 fatty acid per albumin) bound fatty acid molecules (normal albumin), a part of added fatty acid molecules is attracted by and bound to albumin molecules, while only the remaining part goes to anode, thus weaker Kolbe's reaction results in slower and low level impedance measure. The magnitude and/or changing pattern of measured impedance (either instantaneous or time dependent) reflects representatively the amount of fatty

acids in the sample. This is also representatively indicative of the fatty acid binding capacity of sample's albumin (this is probably identical to the ischemia modified albumin). Either the free fatty acid level or fatty acid binding capacity of albumin can be used to determine the occurrence, nonoccurrence, or current activity level of an acute ischemia or specific medical conditions.

In another preferred embodiment, the method also includes adding (Kolbe's) reaction inhibitors in the sample at the beginning or before starting electrolysis (and impedance measurement), thus extinguishing anodic decarboxylation reaction, then comparing the results with normal individuals and/or subject's another sample without inhibitor, to determine whether the measurement is indicative of acute ischemia and/or specific medical conditions. If the sample's albumin possesses physiologic level (0.1-2 fatty acid per albumin) bound fatty acid molecules (normal albumin), the inhibited part of the reaction will be in small amount, thus resultant inhibited reaction creates only small amount of drop in impedance measure. If the sample's albumin is occupied by fatty acid molecules/ ligands, or modified by any sample's molecules, with resultant high level free fatty acid level and/or reduced albumin-fatty acid binding capacity (acute ischemia or specific medical conditions), the inhibited part of the reaction will be in relatively large quantity. This inhibited reaction creates large amount of drop in impedance measure. The magnitude and/or changing pattern of measured impedance (either instantaneous or time dependent) reflects representatively the amount of free fatty acids. This is also representatively indicative of the fatty acid binding capacity of sample's albumin (this is probably identical to the ischemia modified albumin). Either the free fatty acid level or fatty acid binding capacity of albumin can be used to determine the occurrence, nonoccurrence, or current activity level of an acute ischemia or specific medical conditions.

In another preferred embodiment, the method comprise: initially creation of specific physical or pharmacological challenge in patient's body and/or circulatory system which increases fatty acid and/or ischemia modified albumin level in blood; then providing a sample from patient; determination of fatty acid level in a sample by using impedance measurement carried out by a specific

electrolysis reaction; and defining whether measurement is indicative of acute ischemia or specific medical conditions by comparing the results of normal individuals and/or the subject's another sample obtained before applaying challenge. Physical challenges include, but not limited to, cold immersion/exposure of body or body part(s), exercise of body or body part(s), provocative intermittent pressure cuff application on extremities, having a meal. Pharmacological challenges include, but not limited to, parenteral administration of heparins, heparinoids, and cathecolamines. The specific electrolysis reaction is the Kolbe's electrolysis or anodic decarboxylation reaction which takes place on the anode (embedded in the patient's sample) under specific electrical current density and voltage. Under Kolbe's electrolysis, fatty acids lose their carboxylate anions, remaining radicals condense and generate long hydrocarbon chain, then hydrocarbon deposits start to accumulate on the anode surface, and finally many hydrocarbon chains coat the anode and gradually increase the impedance between the electrodes. The magnitude of measured impedance (either instantaneous or time dependent) reflects representatively the amount of fatty acids in the sample. This is also representatively indicative of the fatty acid binding capacity of sample's albumin (this is probably identical to the ischemia modified albumin). Either the free fatty acid level or fatty acid binding capacity of albumin can be used to determine the occurrence, nonoccurrence, or current activity level of an acute ischemia or specific medical conditions.

In another preferred embodiment, the method comprise: providing a sample from patient; determination of fatty acid level in a sample by using impedance measurement carried out by a specific electrolysis reaction. The method also includes adding an albumin ligand(s) in the sample which share(s) same or even common binding sites with fatty acids just before starting electrolysis (and impedance measurement) to displace already bound fatty acid, then providing migration of released fatty acid towards the surface of anode electrode which is strong attractor for fatty acid anions, and defining whether measurement is indicative of acute ischemia or specific medical conditions by comparing the results of normal individuals and/or subject's another sample

obtained before adding albumin ligand(s). The specific electrolysis reaction is the Kolbe's electrolysis or anodic decarboxylation reaction which takes place on the anode (embedded in the patient's sample) under specific electrical current density and voltage. Under Kolbe's electrolysis, fatty acids lose their carboxylate anions, remaining radicals condense and generate long hydrocarbon chain, then hydrocarbon deposits start to accumulate on the anode surface, and finally many hydrocarbon chains coat the anode and gradually increase the impedance between the electrodes. If the sample's albumin is full of (occupied by) fatty acid molecules (like ischemia modified albumin) as in acute ischemia or specific medical conditions, added ligands replace many fatty acid molecules, released fatty acids are attracted by the anode, and thus Kolbe's reaction results in formation of isolating hydrocarbon layer and corresponding impedance measures. If the sample's albumin is lack of fatty acids at most of the fatty acid binding regions as in normal physiologic condition (when excluding 3 high affinity binding sites, albumin has at least 4 low affinity binding sites which are most likely lack of fatty acids since it possesses 0.1-2 fatty acids per molecule in physiologic condition), added ligands specific for known low affinity fatty acid binding sites bind readily to albumin without any fatty acid displacement. This in turn results in weak Kolbe's reaction with corresponding low level impedance measure. The magnitude of measured impedance (either instantaneous or time dependent) reflects representatively the amount of fatty acids in the sample. This is also representatively indicative of the fatty acid binding capacity of sample's albumin (this is probably identical to the ischemia modified albumin). Either the free fatty acid level or fatty acid binding capacity of albumin can be used to determine the occurrence, nonoccurrence, or current activity level of an acute ischemia or specific medical conditions.

In another preferred embodiment, the method comprise: providing a sample from patient; determination of fatty acid level in a sample by using impedance measurement carried out by a specific electrolysis reaction. The method further includes applying chemical, physical, mechanic, magnetic, electromagnetic, electrostatic, photic, sonic, thermal, manometric influence

alone or in combination in the sample that result in un-folding of albumin protein structure leading to release of already bound fatty acids starting before or during electrolysis (and impedance measurement), then released fatty acid anions migrate towards the surface of anode electrode which is strong attractor for fatty acid anions, and defining whether measurement is indicative of acute ischemia or specific medical conditions by comparing the results of normal individuals and/or subject's another sample with normal (non-unfolding or non-denatureted) albumin . The specific electrolysis reaction is the Kolbe's electrolysis or anodic decarboxylation reaction which takes place on the anode (embedded in the patient's sample) under specific electrical current density and voltage. Under Kolbe's electrolysis, fatty acids lose their carboxylate anions, remaining radicals condense and generate long hydrocarbon chain, then hydrocarbon deposits start to accumulate on the anode surface, and finally many hydrocarbon chains coat the anode surface and gradually increase the impedance between the electrodes. If the sample's albumin is full of (occupied by) fatty acid molecule (i.e., ischemia modified albumin) as in acute ischemia or specific medical conditions, after un-folding, at least some of already bound fatty acids release from the albumin, released fatty acids are attracted by the anode, and thus Kolbe's reaction results in formation of isolating hydrocarbon layer and corresponding impedance measures. If the sample's albumin carries only 0.1-2 fatty acid molecules as in normal physiologic condition, un-folding of the protein releases less or even no fatty acids. This in turn results in weak or no Kolbe's reaction with corresponding low level impedance measure. The magnitude of measured impedance (either instantaneous or time dependent) reflects representatively the amount of fatty acids in the sample. This is also representatively indicative of the fatty acid binding capacity of sample's albumin (this is probably identical to the ischemia modified albumin). Either the free fatty acid level or fatty acid binding capacity of albumin can be used to determine the occurrence, nonoccurrence, or current activity level of an acute ischemia or specific medical conditions.

In another preferred embodiment, the method comprises applying two or more above-mentioned methods at the same time, consecutively, or intermittently. It also includes providing two or more samples from patient; representative determination of fatty acid levels and/or fatty acid binding capacity of albumin in samples by using impedance measurement carried out by Kolbe's electrolysis; and defining whether the data is indicative of acute ischemia or specific medical conditions by comparing the result with one another, each other and/or normal individuals.

The method disclosed herein can be used alone, or in conjunction with other diagnostic tests to improve the accuracy and specificity of the diagnosis. These include commonly used myocardial injury biomarkers like cTnI, cTnT, myoglobin, CK-MB. The method can also be used for screening purpose, to identify individuals who appear to be "at risk" for further testing by this or other means.

The subject sample may be selected, for example, from the group consisting of blood plasma, serum, urine, or other body fluids containing albumin and/or fatty acids. Preferably, the sample is blood. To improve accuracy, two or more samples may be tested concomitantly, consecutively, or intermittently.

The assay can be used, for example, to evaluate patients presenting to an emergency room, or for ongoing care within a hospital, clinic, polyclinic, laboratory setting, or in a medical practitioner's office or in emergency transit (e.g., ambulance), during home visit by medical staff, during or following surgery or therapeutic treatment (e.g., angioplasty, thrombolysis), or even in any places where above-mentioned opportunities and/or personnels are not available and non-medical persons are authorized to perform test, especially in emergency conditions (e.g., rural area, trans-continental flights). The assay has the advantage that it can be easily and reproducibly obtained from individuals residing anywhere since it necessitates low operating energy from standard batteries, is easy to perform, and has simple rapid reading.

The assay comprises an apparatus consisting of an instrument and single-use kit. The single-use kit has a specific locus (1.1) capable of holding or confining sample where electrolysis takes place, two electrodes (1.2) in the locus, and a connection part (1.3) of the electrodes to the instrument. The instrument has a specific socket (2.1) suitable for the single-use kit, specific port (2.2) suitable for the electrodes' connection part which allows the electrical connection of the electrodes to instrument, potentiostat/ amperostat (2.3) , microprocessor (2.4), and display screen (2.5).

The single-use kit includes specially designed structure like, but not limited to, cartridge, cuvette, plate, or strip; all have specific loci (1.1) capable of holding or confining sample which is in particular produced of blood compatible material such as, but not limited to, polystyrene, polymethyl methacrylate, or polyethylene . The specific locus (1.1) is a form of reservoir in cartridge and cuvette, a shallow pit in plate or strip, or flat area in plate or strip provided that it can hold sample for some time owing to its porous, fibered, or hydrophilic structure. The reservoir type kit may accept sample in a volume of 0.05-5 ml, preferentially 0.1-2 ml, and more preferentially 0.1-1 ml. The pit type kit may accept sample in a volume of 1-10 droplets, preferentially 1-5 droplets, more preferentially 1-2 droplets. The flat type kit may accept sample in a volume of 1-5 droplets, preferentially 1-2 droplets.

A typical single-use reservoir type kit may include apparatus to stir the sample during reading, for example magnetically or electromagnetically activated stirring bars. All three types (reservoir, pit, and flat) may be heated to 36-37 °C during reading.

After adding sample in the locus (1.1) of kid, the electrolysis is started without considerable amount of delay or immediately, since electrodes (1.2) immersed in the sample may react with other sample (e.g., blood) contents, which would impede impedance measurement.

After starting of the assay, the initiation impedance is assigned either a value of zero ohms of resistance or actual resistance in ohms. Immediately after starting, the Kolbe's electrolysis reaction began; this in turn results in accumulation of hydrocarbon layer on the anode. This accumulation of

hydrocarbon adds electrical resistance to the circuit. The changes in resistance which is generally read continuously for at least 3 minutes, up to 10 minutes, preferentially 5-6 minutes are measured and quantified in ohms. Results of impedance measurement are quantified as follows: 1. Ohm at a given time in the test, 2. Slope, or rate of the reaction, in ohms change per minute, 3. Maximum extent of impedance, in ohms, 4. Plotting a graph (real time or after completion of the measurement) showing impedance changes against time and subsequent calculation of area under curve, in ohm x minutes or arbitrary units. The increase in impedance is directly proportional to the quantity of free fatty acid in the sample. In one embodiment, these fatty acids are individual's original unbound fatty acids. In other embodiment, they are individual's original bound fatty acids initially which are displaced later on after starting the test. In another embodiment, they are externally added fatty acids while assaying the sample. In further embodiment, they are double or triple mixture of original, displaced or added fatty acids.

In one embodiment, the single-use kit (any types) may contain known amount of albumin ligands, fatty acids (or their esters or alcohols), reaction inhibitors or combination of them within the locus (1.1) (they are placed here during the process of manufacturing, or upon reaching the kit to end-users). Upon adding sample in the locus (1.1), the ligand and/or fatty acid dissolve within the sample and interact with sample's albumin. In another embodiment, upon adding sample in the locus (1.1), the reaction inhibitor dissolves within the sample and interacts with electrodes (1.2) and subsequently inhibits the reaction. In another embodiment, known amount of albumin ligands, fatty acids, reaction inhibitors or combination of them are added into the sample already placed in the locus (1.1); upon mixing, the ligand and/or fatty acid interact with sample's albumin and/or electrodes (1.2), reaction inhibitors interact with electrodes (1.2). Example of albumin ligands are heme and biluribin, but all albumin ligands capable of replacing fatty acids on the albumin which are known to those having skill in the science can be selected. Example of fatty acids are oleic acid, palmitic acid, linoleic acid, stearic acid, arachidonic acid, linolenic acid, but all medium and long chain fatty acids, saturated or mono or

poly unsaturated fatty acids, single or branched fatty acids, fatty acids or carboxylic acids carrying substituent (for example ester, acyamino, acyloxy, nitrilo, halo, aryl, alkyl, aralkyl, hydroxyl groups or heterocyclic groups) in the α (2) and β (3) positions or in a more remote position from the carboxylic group, their esters or alcohols, or other fatty acids/carboxylic acids capable of binding to albumin and/or driving Kolbe's electrolysis which are known to those having skill in the science, can be selected. The reaction inhibitors include, but not limited to, salt of divalent cations, like Pb+2, Mn+2, Ca+2, Cu+2, Co+2, Fe+2 ; but all other substances capable of inhibiting Kolbe's reaction which are known to those having skill in the science can be selected.

The single-use kit has two electrodes (1.2), one cathode and one anode, within the locus (1.1) capable of holding or confining sample. Examples of electrode material are platinum, iridium, rhenium, palladium, indium, rubidium, gold, nickel, graphite, silver, copper, combination of them, or their alloys, but all other material or their combination capable of driving Kolbe's electrolysis reaction known to those having skill in the science can be selected. The material selected for the electrodes should provide a low electrical resistance. Since Kolbe's reaction results in gradual increase of electrical impedance between the electrodes, electrodes of a lower inherent resistance will create a stronger resistance change. Materials with a low electrical resistance include, but not limited to, copper, silver, gold, or their alloys. When contacted to patient's sample (i.e., blood), the electrodes must also have a low oxidation tendency, since oxidation on the electrodes may impede impedance measurement related to the Kolbe's reaction. Examples of materials resistant to oxidation include, but not limited to, platinum, gold, and silver. In one embodiment, the electrodes are made from the materials having low electrical and high oxidation resistance. In another embodiment, the electrodes are made from the materials having low electrical resistance; they, however, are coated another material having high oxidation resistance, like, but not limited to, copper coated with silver.

The electrodes may have curved (e.g., cylindrical wire) or flat surface. The surface area of the electrode may be 0.01-100 mm2, distance between electrodes may be 0.1-5 mm.

Examples:

The invention is further described by the examples, which are solely for the purpose of illustrating specific embodiments of this invention, and are not to be construed as limiting the scope of the invention in any way.

We purchased commercially available 1ml plastic cuvette, and a copper wire covered by silver at a diameter of 0.3mm. We assembled these materials to create hand-made cartridge that hold 0.6ml sample volume while two parallel wire electrodes separated by 1mm were fully embedded in the sample with effective surface of approximately 3.3mm2 (4mm in length) in each. The electrodes were connected to potentiostat which keeps electrical potential stable during testing. All impedance readings assuming starting from 0 (zero) kilo-ohms were plotted against time (6 minutes) and area under curve (AUC) measures were calculated.

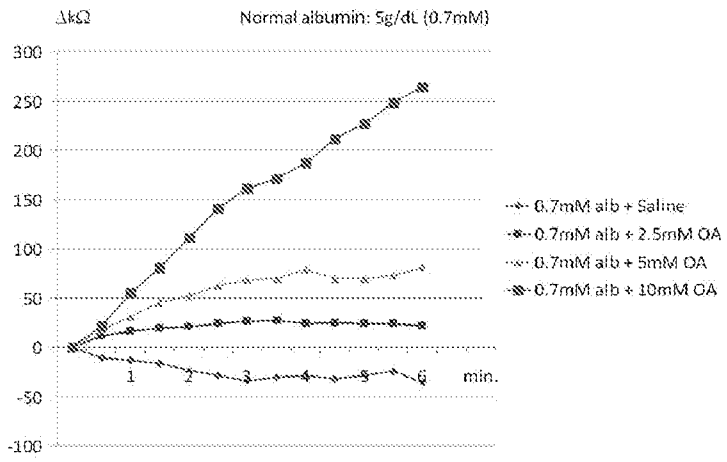
We purchased sodium oleate (sodium salt of oleic acid (OA)- one of the most abundant fatty acid found in human blood) from Sigma Aldrich. We prepared three different sodium oleate solutions at a concentration of 2.5mM, 5mM, and 10mM by mixing lyophilized sodium oleate powder with serum physiologic.

In human blood samples, normal level of albumin is 3.5-5.5 g/dL. We prepared human albumin solution at a concentration of 5 g/dL (0.7 mM) and 10 g/dL (1.4mM) by diluting de-fatted 20% human albumin purchased from Sigma Aldrich; thus we had “normal” albumin solution at a concentration similar and twice to human blood. The “normal” albumin solution tested here represents hypothetical subject without blood fatty acids.

Since one albumin molecule has at least 7 fatty acid binding sites, we mixed same volume of 1.4mM albumin and 10mM (1.4x7 □ 10) sodium oleate solution by 10-minute gentle swinging. Fatty acids are high-affinity ligands for albumin, the binding process is usually completed within a minute after mixing.

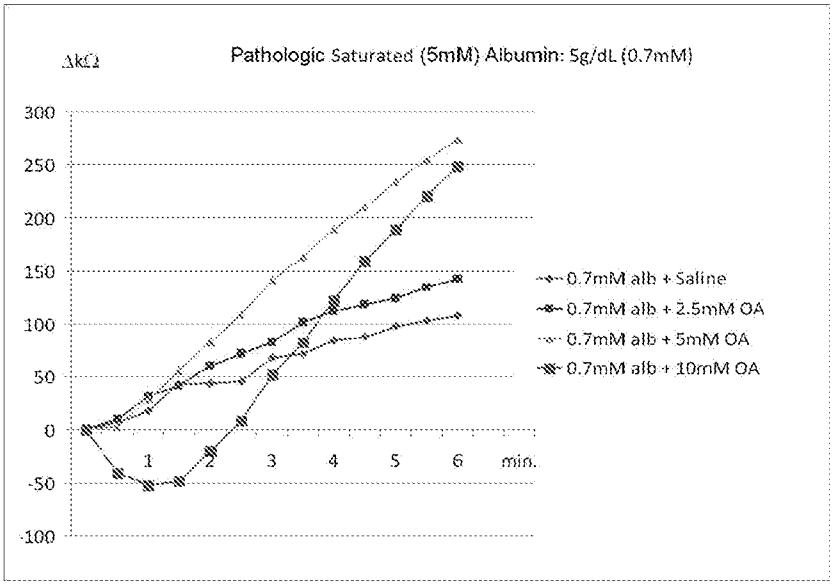
Thus, we finally got so-called “saturated” albumin solution, in which we had seven OA molecules per one albumin molecule in the sample. Due to 1:1 dilution effect, the final saturated albumin concentration was 0.7mM (with 5mM OA) that is similar to human blood. By using same method, we also prepared 0.7mM albumin solution saturated with 1.25mM OA (1.75 OA molecules per one albumin molecule). The former saturated albumin solution represents subjects having acute ischemia or specific medical conditions with elevated blood free fatty acid levels; this is called “pathologic saturated” albumin solution. The latter saturated albumin solution represents physiologic normal subjects with normal blood fatty acid levels, since in physiologic conditions about 0.1-2 albumin binding sites are occupied by fatty acids circulating in the blood; this is called “physiologic saturated” albumin solution.

All measurements were performed by using hand-made cartridge embedded in warm water bath at a temperature of 36 °C. We prepared test solution by mixing 0.3ml albumin (normal or saturated) solution and 0.3ml complementary solution (sodium oleate or serum physiologic), thus total final test solution was always 0.6ml. Immediately after mixing, we started measurement.

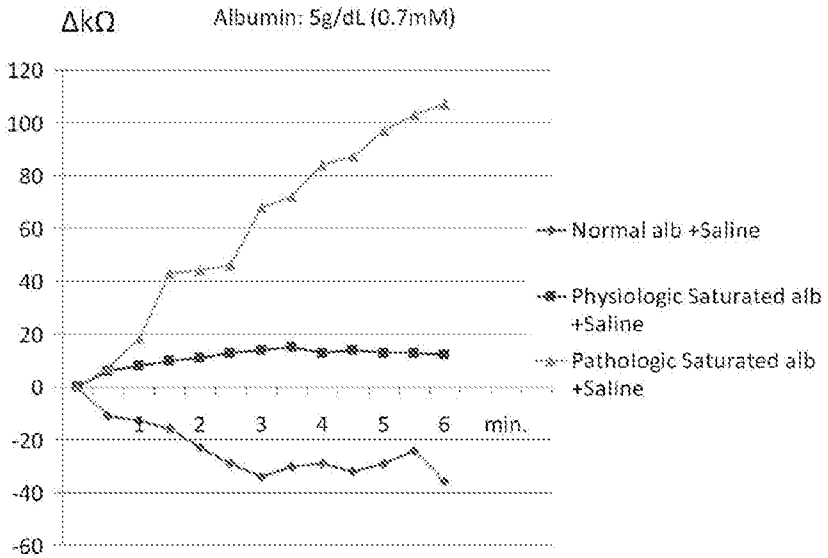


Example 1: Graphical representation of impedance changes against time among four different scenarios of “normal” albumin (hypothetical subject without blood fatty acids). Note the decrease in impedance during testing in the first scenario in which no oleic acid (OA) is added. The impedance gradually

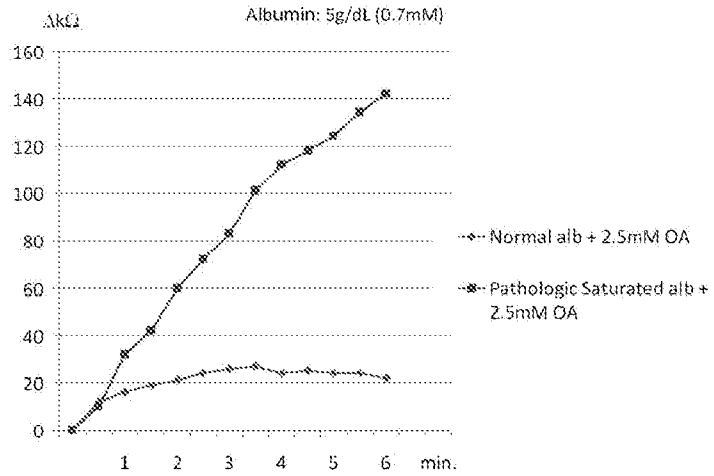
increases in other scenarios in which OA is added in the sample; the higher the added OA concentration, the more upward movement of impedance graphs.



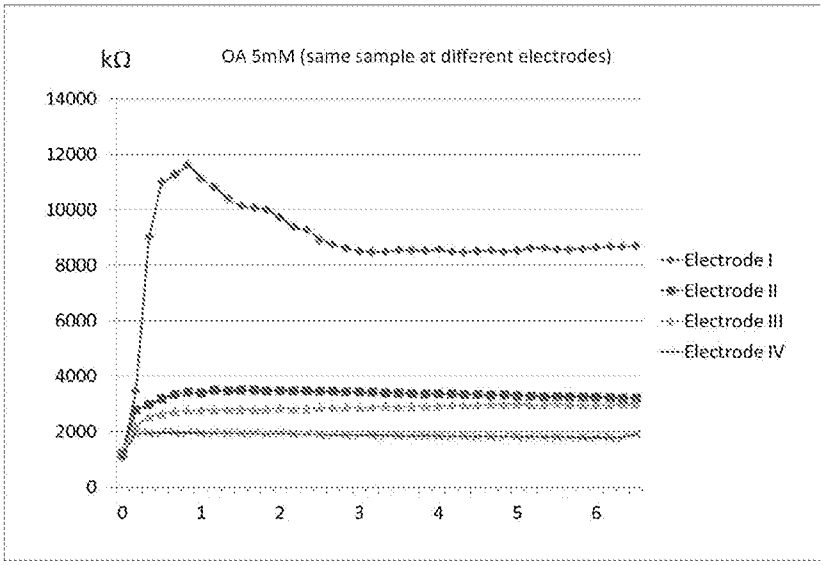
Example 2: Graphical representation of impedance changes against time among four different scenarios of "pathologic saturated" albumin. Note the increase in impedance during testing in all four scenarios. In the last scenario, the impedance decreases initially then increases. This temporary decrease in impedance is probably related to abundance of OA in the sample.



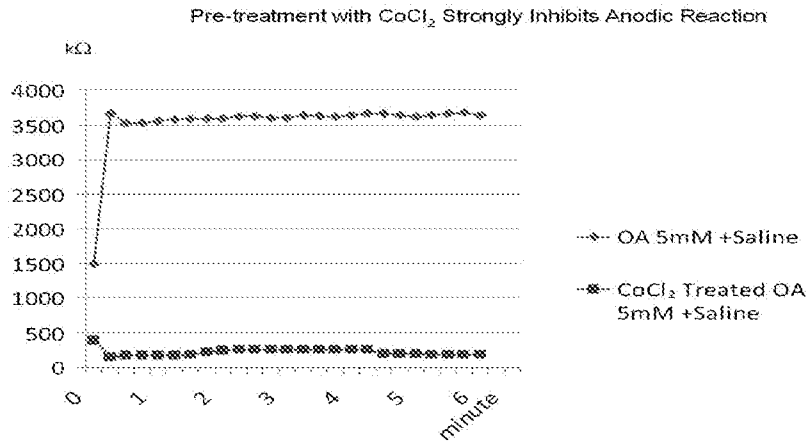
Example 3: Graphical representation of impedance changes against time among three different scenarios; normal albumin representing hypothetical subject, physiologic saturated albumin representing normal subject, and pathologic saturated albumin representing acute ischemia or specific medical conditions, preferentially acute myocardial ischemia. Note the marked difference among three graphs. There are no OA molecules in the first scenario's sample; its impedance decreases during test. In the second scenario –physiologic saturated albumin- which represents normal subject (there are 1.75 OA molecules per one albumin molecule), only slight increase in impedance is seen during test. This means that there is small amount of free (unbound) OA molecule in the sample. Also note the marked impedance increase in the third scenario -pathologic saturated albumin- which represents acute ischemia or specific medical conditions (there are 7 OA molecules per one albumin molecule). This means that there is considerable amount of free (unbound) OA molecule in the sample. This might be due to the fact that, although the number of fatty acid binding sites of albumin and the number of OA molecules are roughly equalized in the "pathologic saturated" albumin solution, some OA molecules probably never bind to albumin's fatty acid binding sites, or some other already bound to albumin's low affinity sites may be dissociated due to intermolecular dynamics during testing. Additionally, consumption (and condensation) on the anode surface and concomitant decrease in concentration of OA in the sample may further favor (speed up) dissociation of OA from the albumin, thus promote anodic reaction and increase impedance eventually. AUC of pathologic saturated albumin (representative ischemic subject) is approximately 5 times much higher than physiologic saturated albumin (representative normal subject).



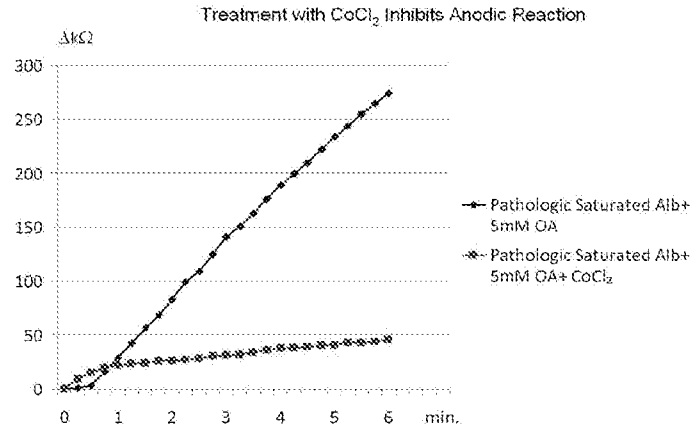
Example 4: Graphical representation of impedance changes against time among two different scenarios; normal albumin representing hypothetical subject and pathologic saturated albumin representing acute ischemia or specific medical conditions, preferentially acute myocardial ischemia. Albumin molecule has three high-affinity (FA2, FA4, FA5), one medium-affinity (FA7), and three low-affinity (FA1, FA3, FA6) fatty acid binding sites (at least total seven binding sites). In the first scenario representing hypothetical subjects, after adding 2.5mM OA (meaning 3.5 molecules OA per one albumin molecule), some OA molecules are attracted by the albumin, while some others are attracted by the anode. This competition results in occurrence of relatively low-level anodic reaction with resultant low-level impedance increase. In the second scenario –pathologic saturated albumin- representing subjects with acute ischemia, since all (or most of) fatty acid binding sites of albumin have already been occupied by OA molecules, after adding 2.5mM OA, all (or nearly all) added OA molecules stay unbound in the sample. All free OA molecules coming from either added OA group or dissociated (from albumin) OA group, eventually are attracted by the anode during test, since there is no competition. This, in turn, results in occurrence of relatively high-level anodic reaction with resultant high-level impedance increase. Note the marked difference between two scenarios below. AUC of representative ischemic subject is approximately 3.7 times much higher than representative hypothetical subject.



Example 5: Graphical representation of impedance changes against time in a sample containing OA (sodium oleate) at a concentration of 5mM. We recorded impedance changes against time during 6 minutes in the same sample (without adding anything) by using four identical but different electrode pairs. Note the rapid upward elevation to reach maximum impedance level for each electrode within 30-45 seconds. Also note the higher the number of electrodes, the lower the level of maximum impedance in each consecutive electrode. This is due to consumption effect of anodic decarboxylation (Kolbe's) reaction; each anode electrode condenses (consumes) a part of carboxylic acid (sodium oleate in this example) in the sample to create a layer of hydrocarbone, thus concentration of OA in the sample decreases gradually, starting from the Electrode I to Electrode IV. This, in turn, results in stepwise decrease in maximum impedance; highest in Electrode I, and lowest in Electrode IV, strongly indicating that the observed impedance changes is related to Kolbe's reaction.



Example 6: Graphical representation of impedance changes against time among two different scenarios. The first is the mixture of 0.3ml 5mM OA (sodium oleate) and 0.3ml saline with final OA concentration of 2.5mM. In the second scenario, we prepared 0.3ml $CoCl_2$ solution in saline at a concentration of 0.02mM. Then we mixed 0.3ml 0.02mM $CoCl_2$ and 0.3ml 5mM OA with final OA concentration of 2.5mM and final $CoCl_2$ concentration of 0.01mM. Note the marked impedance drop in the second scenario; this means that $CoCl_2$ markedly inhibits reaction occurred in this example. Actually, the presences of certain metallic cations (like Co+2) even in very small amounts strongly affects or even completely suppress the course of the Kolbe's electrolysis, as described previously in this text. So, this example further proves the occurrence of Kolbe's electrolysis reaction in our settings.



Example 7: Graphical representation of impedance changes against time among two different scenarios. The first is the mixture of 0.3ml 0.7mM albumin saturated with 5mM OA (sodium oleate) (pathologic saturated albumin) and 0.3ml 5mM OA. In the second scenario, we added $CoCl_2$ in the solution described in the first scenario with a final $CoCl_2$ concentration of 0.01mM. Note the marked impedance drop in the second scenario; this means that $CoCl_2$ markedly inhibits reaction occurred in this example. This example additionally proves the occurrence of Kolbe's electrolysis reaction in our settings.

CLAIMS

1. A test for screening, diagnosing, and follow-up of ischemia or specific medical condition characterized by being electrochemical.
2. The electrochemical test of claim 1 characterized by being an apparatus for defining fatty acid marker by using electrical impedance, while the anodic decarboxylation (Kolbe's electrolysis) reaction takes place.
3. The fatty acid marker determination apparatus of claim 2 characterized by comprising an instrument (2) and single-use kit (1).
4. The instrument (2) of claim 3 characterized by comprising a specific socket suitable for the single-use kit (2.1), specific port suitable for the electrodes' connection part which allows the electrical connection of the electrodes to instrument (2.2), potentiostat/amperostat (2.3), microprocessor (2.4), and display screen (2.5).
5. The single-use kit (1) of claim 3 characterized by comprising a specific locus capable of holding or confining sample where Kolbe's electrolysis takes place (1.1), at least two electrodes in the locus (1.2), and a connection part of the electrodes to the instrument (1.3).
6. A method for defining fatty acid marker by measuring electrical impedance characterized with the steps;
   - Adding patient sample in the specific area on the kit,
   - Starting electrolysis reaction thus allowing condensation of fatty acid anions over the anode to generate hydrocarbon chains,
   - Increasing of impedance gradually while accumulation of hydrocarbon layer over the anode,
   - Reading impedance between electrode and recording impedance change ($\Delta\Omega$) against time,
   - Obtaining numeric and/or graphical data in terms of instantaneous impedance change, rate of impedance change, maximum level of impedance change, and area under curve (AUC) on the impedance change against time graphic,

- Comparing and interpreting numeric and/or graphical data with normal individual.

7. A method for defining fatty acid marker by measuring electrical impedance characterized with the steps;

- Adding patient sample in the specific area on the kit,
- Adding predetermined amount of fatty acid anions in the specific area on the kit,
- Starting electrolysis reaction thus allowing condensation of fatty acid anions over the anode to generate hydrocarbon chains,
- Increasing of impedance gradually while accumulation of hydrocarbon layer over the anode,
- Reading impedance between electrode and recording impedance change ($\Delta\Omega$) against time,
- Obtaining numeric and/or graphical data in terms of instantaneous impedance change, rate of impedance change, maximum level of impedance change, and area under curve (AUC) on the impedance change against time graphic,
- Comparing and interpreting numeric and/or graphical data with normal individual and/or patient's another sample without adding fatty acid anions.

8. It is a method for defining fatty acid marker by measuring electrical impedance characterized with the steps;

- Adding patient sample in the specific area on the kit,
- Adding an inhibitor of Kolbe reaction in the specific area on the kit,
- Starting electrolysis reaction thus allowing condensation of fatty acid anions over the anode to generate hydrocarbon chains,
- Increasing of impedance gradually while accumulation of hydrocarbon layer over the anode,
- Reading impedance between electrode and recording impedance change ($\Delta\Omega$) against time,
- Obtaining numeric and/or graphical data in terms of instantaneous impedance change, rate of impedance change, maximum level of

impedance change, and area under curve (AUC) on the impedance change against time graphic,

- Comparing and interpreting numeric and/or graphical data with normal individual and/or patient's another sample without adding inhibitor of Kolbe reaction.

9. It is a method for defining fatty acid marker by measuring electrical impedance characterized with the steps;

- Applying physical and/or pharmacological challenge to patient,
- Adding patient sample in the specific area on the kit,
- Starting electrolysis reaction thus allowing condensation of fatty acid anions over the anode to generate hydrocarbon chains,
- Increasing of impedance gradually while accumulation of hydrocarbon layer over the anode,
- Reading impedance between electrode and recording impedance change ($\Delta\Omega$) against time,
- Obtaining numeric and/or graphical data in terms of instantaneous impedance change, rate of impedance change, maximum level of impedance change, and area under curve (AUC) on the impedance change against time graphic,
- Comparing and interpreting numeric and/or graphical data with normal individual and/or patient's another sample obtained before applying physical and/or pharmacological challenge.

10. It is a method for defining fatty acid marker by measuring electrical impedance characterized with the steps;

- Adding patient sample in the specific area on the kit,
- Adding predetermined amount of ligands in the specific area on the kit, which can release already bound fatty acids from the albumin,
- Starting electrolysis reaction thus allowing condensation of fatty acid anions over the anode to generate hydrocarbon chains,
- Increasing of impedance gradually while accumulation of hydrocarbon layer over the anode,

- Reading impedance between electrode and recording impedance change ($\Delta\Omega$) against time,
- Obtaining numeric and/or graphical data in terms of instantaneous impedance change, rate of impedance change, maximum level of impedance change, and area under curve (AUC) on the impedance change against time graphic,
- Comparing and interpreting numeric and/or graphical data with normal individual and/or patient's another sample without adding ligands capable of releasing fatty acid from the albumin.

11. It is a method for defining fatty acid marker by measuring electrical impedance characterized with the steps;

- Applying external effects over patient sample to obtain denaturation of albumins,
- Adding effected patient sample in the specific area on the kit,
- Starting electrolysis reaction thus allowing condensation of fatty acid anions over the anode to generate hydrocarbon chains,
- Increasing of impedance gradually while accumulation of hydrocarbon layer over the anode,
- Reading impedance between electrode and recording impedance change ($\Delta\Omega$) against time,
- Obtaining numeric and/or graphical data in terms of instantaneous impedance change, rate of impedance change, maximum level of impedance change, and area under curve (AUC) on the impedance change against time graphic,
- Comparing and interpreting numeric and/or graphical data with normal individual and/or patient's another sample without applying external effect (without denaturation).

12. It is a method for defining fatty acid marker by measuring electrical impedance characterized with the steps;

- Obtaining at least two samples from the patient,
- Performing at least two processes selected from claim 6-11, at the same time, consecutively, or intermittently,

- Comparing and interpreting numeric and/or graphical data with one another and/or each other and/or normal individuals.

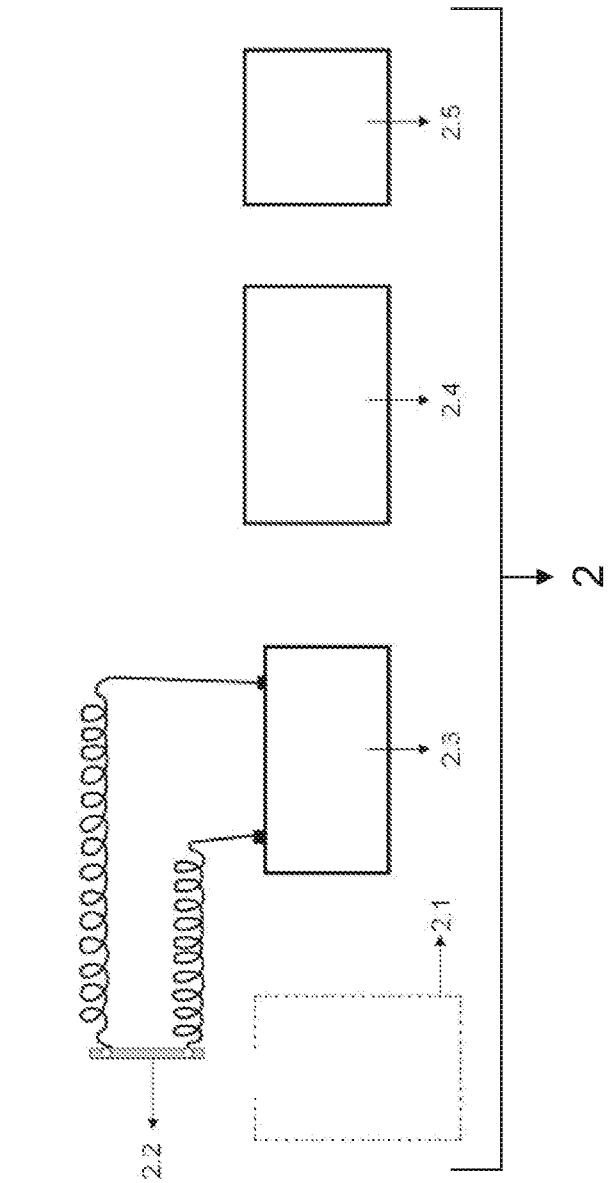


FIGURE 2

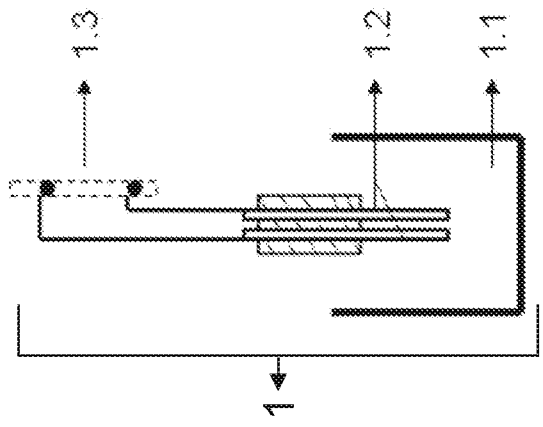


FIGURE 1

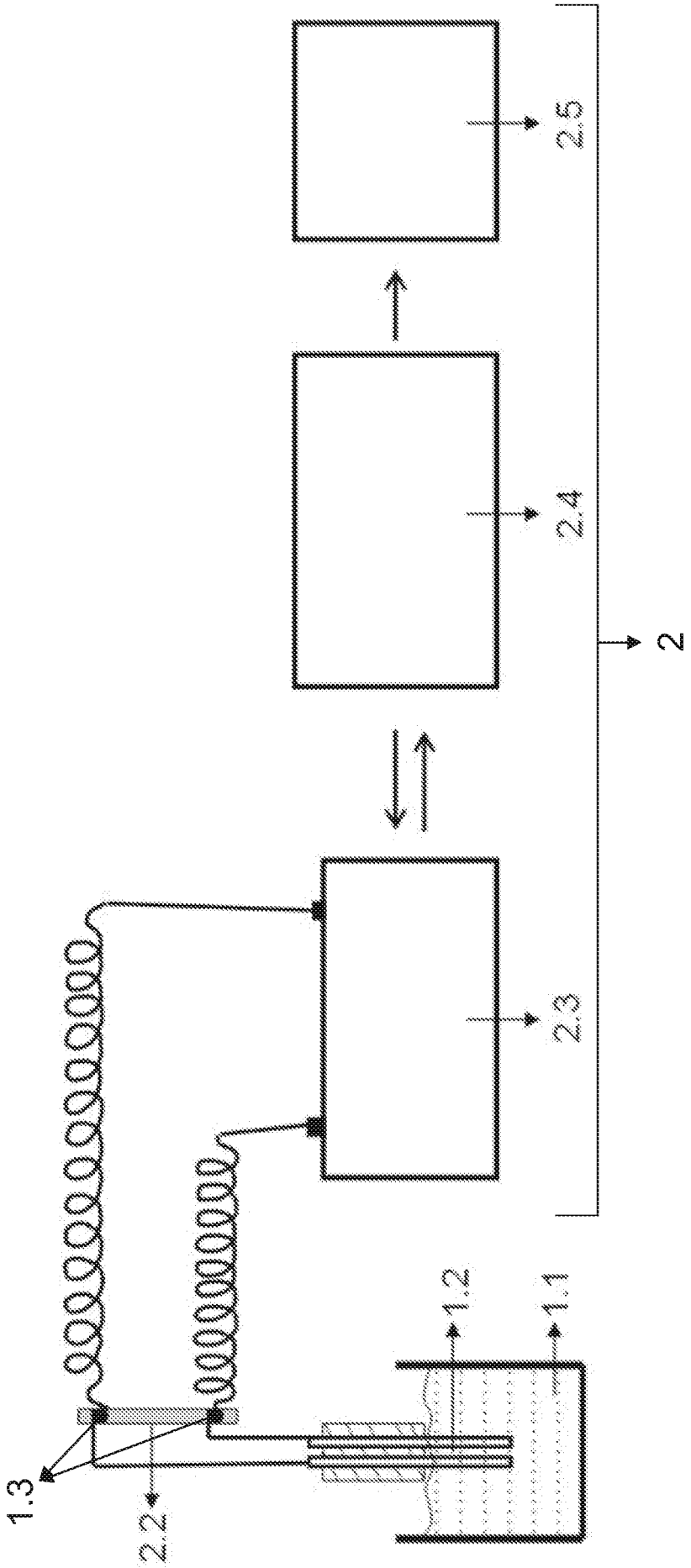


FIGURE 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/TR2016/050515

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/92 G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KIN FONG LEI ET AL: "Real-Time Electrical Impedimetric Monitoring of Blood Coagulation Process under Temperature and Hematocrit Variations Conducted in a Microfluidic Chip", PLOS ONE, vol. 8, no. 10, 7 October 2013 (2013-10-07), page e76243, XP055088672, DOI: 10.1371/journal.pone.0076243 abstract, page 2, right-hand column, 2nd paragraph, figures 1-3 and 6	1-5

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 March 2017	10/03/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lindberg, Pia

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	US 2011/045520 A1 (BHAGAVAN NADHIPURAM V [US] ET AL) 24 February 2011 (2011-02-24) cited in the application abstract, paragraph [0007]-[0009], [0014], [0029], [0030], [0045]-[0048].	6-12
A	US 2008/305550 A1 (HUFF HOLLIE [US] ET AL) 11 December 2008 (2008-12-11) cited in the application abstract, paragraphs [0011], [0012], [0049], [0060]-[0064].	6-12
A	Gv Richieri ET AL: "Unbound free fatty acid levels in human serum", Journal of Lipid Research, 1 February 1995 (1995-02-01), pages 229-240, XP055350211, UNITED STATES Retrieved from the Internet: URL:http://www.jlr.org/content/36/2/229.full.pdf#page=1&view=FitH [retrieved on 2017-02-28] abstract	6-12
A	US 4 369 250 A (GINDLER E MELVIN) 18 January 1983 (1983-01-18) claims 1-4	6-12
A	VIJAY KUMAR ROY ET AL: "Plasma Free Fatty Acid Concentrations as a Marker for Acute Myocardial Infarction", JOURNAL OF CLINICAL AND DIAGNOSTIC RESEARCH, vol. 7, 1 January 2013 (2013-01-01), pages 2432-2434, XP055350222, ISSN: 2249-782X, DOI: 10.7860/JCDR/2013/7682.3566 abstract	6-12

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	& David L Trout ET AL: "Titration of free fatty acids of plasma: a study of current methods and a new modificati,on*", Journal of lipid research, 1 January 1960 (1960-01-01), pages 199-202, XP055350230, Retrieved from the Internet: URL:http://www.jlr.org/content/1/3/199.full.pdf#page=1&view=FitH [retrieved on 2017-02-28] the whole document -----	6-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/TR2016/050515

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