METHOD FOR EXOSOMAL BIOMARKER DETECTION BY ELECTRIC FIELD-INDUCED RELEASE AND MEASUREMENT

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ABSTRACT

The molecules harbored in exosomes play important roles in biological science. A highly desirable goal for exosome research is the rapid, simple, simultaneous tracking and quantification of exosome harbored molecules. Disclosed herein are methods and devices for inducing the release and measurement of biomolecules harbored in exosomes. The disclosed method, Electric Field Induced Release and Measurement (EFIRM) technique, uses an electrical field to simultaneously disrupt exosomes to release the contents and measure the harbored exosomal RNA/proteins. The exosome vesicle contents can be released within minutes. This provides a potential on-site method for the detection of exosome-harbored biomolecules.

Specification includes a Sequence Listing.
FIGURE 1A – FIGURE 1B

Exosome extraction

Fluorescently labeled antibody conjugates

Exosome detection with electric and magnetic field

Protein

RNA

Magnetic field

Exosome extraction

Electrochemical sensor

Fluorescently labeled detector probe

Biotin labeled capture probe

HRP-Antibody conjugates (anti-GFP/anti-fluorescein)
FIGURE 2A – FIGURE 2D
FIGURE 3A – FIGURE 3C

a) Exosome → GAPDH mRNA Release

b) [Images of microscopic views of exosomes]

Exosome Incubation time at room temperature (minutes)

- w/ E-field
- w/o E-field

- w/ Triton
- w/o Triton
**FIGURE 4A – FIGURE 4D**

(a) CD63-GFP EFIRM
- Relative GFP
- Sample dilution factor

(b) Relative GAPDH
- Sample dilution in log scale

(c) Human CD63-GFP
- Relative GFP
- Human sample: Mouse sample

(d) Human GAPDH
- Relative GAPDH
- Human sample: Mouse sample
FIGURE 5

Relative CD63-GFP in saliva

CD63-GFP

Saline

Relative CD63-GFP in serum
METHOD FOR EXOSOMAL BIOMARKER DETECTION BY ELECTRIC FIELD-INDUCED RELEASE AND MEASUREMENT

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application is a Continuation of U.S. application Ser. No. 14/119,843 filed Jun. 20, 2014, now U.S. Pat. No. 9,932,635, which is a 371 National Phase of PCT Application No. PCT/US2012/039471 filed May 24, 2012, which claims priority benefit of U.S. Provisional Application No. 61/489,634 filed May 24, 2011, the disclosures of each are incorporated by reference their entireties.

STATEMENT OF GOVERNMENT RIGHTS

[0002] This invention was made with Government support under Grant No. DE017790 awarded by the National Institutes of Health. The Government has certain rights in the invention.

REFERENCE TO A “SEQUENCE LISTING,” A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED ON A COMPACT DISK.

[0003] The sequence listing contained in the file named “008074-5043-US_ST125”, created on Apr. 2, 2018 and having a size of 4 kilobytes, has been submitted electronically herewith via EFS-Web, and the contents of the txt file are hereby incorporated by reference in their entirety.

BACKGROUND


[0006] A method for releasing the exosome encapsulated constituents for analysis is to apply lysis reagents. The lysis reaction time typically varies from several minutes to several hours and can compromise the integrity of the biomoles. Therefore, for translational and clinical applications, a need exists for rapid exosome-specific extraction and highly sensitive, specific detection of exosome-associated bio-molecules. These and other needs are addressed by the methods and devices disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] FIG. 1A-FIG. 1B depicts a schematic representation of the Electric Field Induced Release and Measurement (EFIRM) system. a) Anti-hCD63 antibodies were conjugated to magnetic beads, the beads were mixed with exosomes, and the mixture was transferred to the electrochemical sensor array for exosome extraction. b) A magnetic force was applied to collect the exosome-magnetic bead complexes onto the electrodes in the sensor array. An electric field was applied to induce release and then to measure both RNA and protein molecules. The magnetic beads were approximately 1-2 μm in diameter, as measured by transmission electron microscopy.

[0008] FIG. 2A-FIG. 2D depicts transmission electron microscopy (TEM) images of magnetic bead based exosome extraction. a) Exosomes (arrows) were extracted from human saliva with anti-hCD63-conjugated magnetic beads; b) Magnified view of human exosomes (arrows) on a bead; c) Mouse saliva after extraction with anti-hCD63-conjugated magnetic beads. No exosome is apparent. d) Human saliva after extraction with magnetic beads coated only with streptavidin (negative control).

[0009] FIG. 3A-FIG. 3C depicts a comparison between electric field (E-field) and Tritton X-100 lysis for releasing exosomal GAPDH mRNA from human saliva. Control groups were not treated with the E-field or Tritton X-100, but were incubated for the same time period. a) Schematic illustration of an exosome disrupted with E-field and RNA released. b) TEM images before (left) and after (right) E-field (top) or Tritton X-100 (bottom) treatment. (i and iii) Exosomes (arrows) attached to anti-hCD63 magnetic beads; (ii and iv) Exosomes were disrupted after treating with (ii) a cyclic square wave (csw) E-field for 200 s, or (iv) with Tritton X 100 for 20 min. The background is the lacy support film for TEM. c) GAPDH mRNA was measured by E Fir M afer csw E-field (top) or Tritton X-100 (bottom) treatment. Measurements of released RNA (filled bars) are presented as the ratio between the mRNA signal and the blank control (casein PBS buffer with no exosomes). Positive controls (without w/o) electrical field treatment in the release step, open bars were undegraded mRNA measured in untreated exosome samples; therefore, these mRNAs were not exposed to saliva enzymes.
FIG. 4A-FIG. 4D depicts Western blot and qPCR measurements of hCD63-GFP protein and GAPDH mRNA in exosomes shed from H460 cells using E-field. a) Titration of the GFP moiety of hCD63-GFP by EFIRM and Western blotting. b) Titration of GAPDH mRNA by EFIRM and qPCR. Data in a) and b) are fitted by a linear model. The PCR readout is presented on a log scale. The human specificity of EFIRM measurements was tested by detecting human exosomal (c) CD63-GFP protein and (d) GAPDH mRNA in mixtures of endosomes from human lung cancer H460 cells and interfering exosomes from mouse Lewis lung carcinoma LL2/LLC1 cells. Both the GAPDH mRNA probe and the anti-GFP antibody were human-specific. Therefore, this assay tested whether mouse targets could interfere with detection of human targets.

FIG. 5 depicts the correlation between human CD63-GFP levels in saliva and serum measured with EFIRM technology. Samples are from mice that had been injected with a human lung cancer cell line that expressed CD63-GFP (solid squares) or with saline alone (cross and circles). Each point represents the ratio of electrochemical current readings from serum (X-axis) and saliva (Y-axis) relative to the blank control reading. Relative values close to 1 indicate low hCD63-GFP levels, because there was a small difference between the signal and the blank control (casein PBS). The linear regression is indicated with the dashed line (R=0.77).

SUMMARY OF THE INVENTION

The rapid degradation of released bio-molecules implicates a need for detection technologies that can make accurate assessments within seconds to minutes. For example, mRNA levels were reduced to less than 40% within 1 minute of release from macromolecules into saliva. Park N J, Li Y, Yu T W, Brinkman B M N, Wong D T (2006) Clin. Chem. 52: 988-994. Stabilization reagents are therefore necessary for accurate exosome biomarker detection. The combination of exosome releasing agents and stabilization reagents renders the process complicated and may introduce interference.

An electric field, particularly one with a non-uniform profile, can stimulate vesicle deformation in biological samples, and it can direct the flow of the released bio-molecules. Wei F, Qu P, Zhai I, Chen C, Wang H, Zhao X S (2006) Langmuir 22:6280-6285; Wei F, Liao W, Xu Z, Yang Y, Hong D T, Ho C M (2008) Small 5:1784-1790. An electric field can cause redistribution or polarization of lipid vesicular structures that protect bio-molecules. The non-uniform electrical field will either rupture the membrane or disrupt the tertiary structure of the exosomal lipid bilayer, which causes temporary pore formation; in both cases, the harbored bio-molecules can be released.

Accordingly, disclosed herein are methods and devices for directly detecting bio-molecules associated with the exosome by electrical field-induced release and measurement.

In one aspect, the present disclosure provides a method for detecting a biomarker present in an exosome, the method comprising: providing a biological fluid comprising exosomes; extracting exosomes from the biological fluid to form an enriched exosome composition; contacting the enriched exosome composition with a detection reagent that specifically binds to the biomarker; applying an electrical field to the enriched exosome composition in the presence of the detection reagent; detecting an interaction between the biomarker and the detection reagent.

In one aspect, the present disclosure provides a method for detecting a biomarker present in an exosome, the method comprising: providing a biological fluid comprising exosomes; contacting the biological fluid with an exosome extraction reagent to form a complex between the exosome and the exosome extraction reagent; the exosome extraction reagent comprising a first affinity moiety bound to a magnetic bead, wherein the first affinity binding moiety specifically binds to a surface-exposed exosome marker; separating the complex formed between the exosome and the exosome extraction reagent from the biological fluid; contacting a solid phase with the complex formed between the exosome and the exosome extraction reagent, the solid phase comprising a first contact region under the influence of a magnetic field, a second contact region, and a detection reagent immobilized at the second contact region, the detection reagent comprising a second affinity moiety that specifically binds to the biomarker; immobilizing the complex formed between the exosome and the exosome extraction reagent at the first contact region; applying an electrical field to the immobilized complex formed between the exosome and the exosome extraction reagent at the first contact region; and detecting an interaction between the biomarker and the detection reagent.

In some embodiments of the methods described above, the biomarker is a protein.

In some embodiments of the methods described above, the biomarker is a nucleic acid. In a specific embodiment, the nucleic acid is an mRNA.

In some embodiments of the methods described above, the biological fluid is selected from the group consisting of saliva, whole blood, blood plasma, blood serum, amniotic fluid, bile, colostrum, breast milk, cerebrospinal fluid (CSF), lymph, gastric acid, nasal mucus, pleural fluid, semen, tears, and urine. In a specific embodiment, the biological fluid is saliva. In another specific embodiment, the biological fluid is whole blood, blood plasma, or blood serum.

In some embodiments of the methods described above, the step of extracting exosomes from the biological fluid comprises: contacting the biological fluid with an exosome extraction reagent that specifically binds to a surface-exposed exosome marker to form a complex between the exosome and the exosome extraction reagent; and separating the complex formed between the exosome and the exosome extraction reagent from the biological fluid.

In some embodiments of the methods described above, the surface-exposed exosome marker is CD63.

In some embodiments of the methods described above, the exosome extraction reagent comprises an antibody.

In some embodiments of the methods described above, the exosome extraction reagent comprises an exosome-specific lectin.

In some embodiments of the methods described above, the exosome extraction reagent comprises a bead attached to a first affinity moiety that specifically binds to a surface-exposed exosome marker. In a specific embodiment, the bead is a magnetic bead.

In some embodiments of the methods described above, the detection reagent comprises a second affinity...
moiety immobilized to a solid phase, wherein the second affinity moiety specifically binds to the biomarker.

[0026] In some embodiments of the methods described above, the second affinity moiety comprises an antibody.

[0027] In some embodiments of the methods described above, the second affinity moiety comprises a nucleic acid.

[0028] In some embodiments of the methods described above, the solid phase comprises an electrochemical sensor. Non-limiting examples of electrochemical sensors are described in U.S. Patent Application Publication No. 2010/030706, the content of which is hereby incorporated herein by reference in its entirety for all purposes.

[0029] In some embodiments of the methods described above, the absolute value of the maximum voltage of the electric field does not exceed 1 volt (V).

[0030] In some embodiments of the methods described above, the absolute value of the maximum voltage of the electric field does not exceed 500 millivolt (mV).

[0031] In yet other embodiments of the methods described above, the absolute value of the maximum voltage of the electric field does not exceed 3.0 V, 2.5 V, 2.0 V, 1.5 V, 1.4 V, 1.3 V, 1.2 V, 1.1 V, 1.0 V, 0.9 V, 0.8 V, 0.7 V, 0.6 V, 0.5 V, 0.4 V, 0.3 V, or 0.2 V.

[0032] In some embodiments of the methods described above, the electric field is applied for less than 300 seconds.

[0033] In some embodiments of the methods described above, the electric field is applied for 200±30 seconds.

[0034] In yet other embodiments of the methods described above, the electric field is applied for 60±30 seconds, 90±30 seconds, 120±30 seconds, 150±30 seconds, 180±30 seconds, 210±30 seconds, 240±30 seconds, 270±30 seconds, 300±30 seconds, 330±30 seconds, 360±30 seconds, 390±30 seconds, 420±30 seconds, 450±30 seconds, 480±30 seconds, 510±30 seconds, 540±30 seconds, 570±30 seconds, 600±30 seconds, or more seconds.

[0035] In some embodiments of the methods described above, the electric field is a non-uniform electric field.

[0036] In some embodiments of the methods described above, the non-uniform electric field is a cyclic square wave electrical field (csw-E-field).

[0037] In some embodiments of the methods described above, the csw-E-field comprises 15 cycles of ~300±50 mV for 9±2 seconds followed by 200±50 mV for 1±0.5 second.

[0038] In some embodiments of the methods described above, the csw-E-field comprises 20 cycles of ~300 mV for 9 seconds followed by 200 mV for 1 second.

[0039] In one aspect, the present disclosure provides a method for analyzing exosome biomolecules, the method comprising: providing an exosome sample; and applying an electrical field to the exosome sample, such that the exosome no longer excludes the extra-membrane environment; and thereby analyzing exosome biomolecules.

[0040] In some embodiments of the methods described above, the method further comprises applying a magnetic force prior to applying the electric field.

[0041] In some embodiments of the methods described above, the electric field is a cyclic square wave.

[0042] In some embodiments of the methods described above, the electric field is less than 1 volt.

[0043] In some embodiments of the methods described above, the electric field is less than 500 milli-Volts.

[0044] In some embodiments of the methods described above, the electric field is applied for 200 seconds or less.

[0045] In some embodiments of the methods described above, the electric field is a non-uniform electric field.

[0046] In some embodiments of the methods described above, the exosomes are isolated from saliva.

[0047] In some embodiments of the methods described above, the saliva is human saliva.

[0048] In some embodiments of the methods described above, the exosome is bound by an antibody.

[0049] In some embodiments of the methods described above, the antibody is attached to a magnetic bead.

[0050] In some embodiments of the methods described above, the biomolecules are a polypeptide and/or a nucleic acid.

[0051] In some embodiments of the methods described above, the nucleic acid is a ribonucleic acid.

[0052] In some embodiments of the methods described above, the polypeptide is an integral exosomial membrane protein.

[0053] In one aspect, the present disclosure provides a method for releasing an encapsulated biomolecule comprising applying an electrical field to an encapsulated biomolecule, wherein the biomolecule is encapsulated in an encapsulating structure and wherein the electrical field is sufficient to cause the encapsulating structure to degrade sufficiently so as to release the encapsulated biomolecule and wherein the electrical field is a cyclic square wave.

DETAILED DESCRIPTION

[0054] Biological assay systems are important in biotechnology and medical diagnostics. A useful biological agent detection method is using DNA or RNA detection through a polymerase chain reaction (PCR), real-time polymerase chain reaction (RT-PCR), or reverse transcription polymerase chain reaction. Alternatively, assaying for the presence of a particular protein can also be used to detect or study biological samples of interest. Before a biological sample can be analyzed using PCR or protein detection methods, it must first be prepared for analysis. A challenge in biological sample preparation is lysis.

[0055] Lysis methods can be generally divided into reagent-assisted or reagent-less lysis methods. The former uses lytic agents such as detergent to lyse cells. However, these lytic reagents can interfere following analysis process and the system can be overly complicated due to lytic agent injection and sample rinsing step. Therefore, reagentless lysis methods have been developed with various approaches.


[0057] Mechanical lysis uses a crushing or grinding action to cleave cell membranes or a sharp object to pierce the cell membrane. For example, glass beads can be centrifuged along with the sample at a high angular velocity, cleaving cells as they grind past each other. Some centrifuge systems also rock the sample using three dimensional movements to
increase the efficiency of lysis. Another method of mechanical lysis is to pressure the cell sample and flow it past a piercing object at a high velocity. However, in mechanical lysis methods, the size of the sharp structures should be decreased as the sample size decreases. Moreover, the fabrication process can be challenging, and costly. [0058] The thermal lysis methods are using high temperature (~94°C) to lyse the cell. Heating cells to a high temperature causes cellular protein denaturation and cell membrane breakdown. This method is not ideal because the harsh process of thermal lysis may destroy the target to be detected.

[0059] Electrical lysis methods are usually based on the irreversible electroporation of the lipid bilayer of the target particles. Electrical lysis methods do not possess the drawbacks inherent in mechanical and thermal lysis methods.

[0060] While electrical lysis has been applied to mammalian cells, bacteria and viruses (Park, K., Akin, D., Bashir, R. (2007) Biomed Microdevices 9:877-883), it was unknown whether electrical lysis could be applied to exosomes and for exosome analysis before the instant disclosure.

[0061] Accordingly, in some embodiments, a method of electrical lysis of an exosome and collection of at least a portion of contents thereof is provided. The method entailing applying an alternating electrical current to an exosome such that the exosome is lyzed and collecting at least a portion of the contents thereof.

[0062] In order for the present disclosure to be more readily understood, certain terms and phrases are defined below as well as throughout the specification.

[0063] Definitions

[0064] All references cited herein are incorporated by reference in their entirety as though fully set forth. Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton et al., Dictionary of Microbiology and Molecular Biology 3rd ed., J. Wiley & Sons (New York, N.Y. 2001); March, Advanced Organic Chemistry Reactions, Mechanisms and Structure 5th ed., J. Wiley & Sons (New York, N.Y. 2001); and Sambrook and Russell, Molecular Cloning: A Laboratory Manual 3rd ed., Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y. 2001), provide one skilled in the art with a general guide to many terms used in the present disclosure. As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in accordance with manufacturer defined protocols and/or parameters unless otherwise noted.

[0065] As used herein, the terms “exosome” and “luminal vesicle” are used interchangeably to refer to a small membrane vesicle having a diameter between about 40-100 nm in diameter present in biological fluids. Exosomes are released by various cell types (e.g., tumor cells, neurons, B- and T-lymphocytes, and intestinal epithelial cells) into the extracellular environment, e.g., various biological fluids. Briefly, this occurs when late endosomes (multivesicular bodies) fuse with the plasma membrane and are released from the cell.

[0066] “Exosome contents” or “contents of the exosome” refer to the polynucleotides, polypeptides, hormones and organic and inorganic molecules found within the lipid membrane of the exosome. Exosomes are described below, in the section entitled “Exosome.” Examples of polynucleotides found within the exosome include, without limitation, DNA and RNA as describe above.

[0067] As used herein, the term “enriched exosome sample” refers to a sample containing exosomes isolated or extracted from a biological sample or fluid. Methods for extracting exosomes from biological fluids are well known in the art and include, without limitation, centrifugation, differential centrifugation, ultracentrifugation, precipitation, and affinity capture.

[0068] As used herein, the terms “biomarker present in an exosome” or “biomarker” are used interchangeably to refer to a molecule attached to the surface, embedded within, or encapsulated within an exosome present in a biological fluid. In certain embodiments, the biomarker is a polypeptide (e.g., a protein or peptide), nucleic acid (e.g., DNA, RNA, mRNA, tRNA, or rRNA), lipid, or carbohydrate moiety. In certain embodiments, the biomarker is present at a higher or lower concentration in an exosome present in an individual with a particular disease or condition, as compared to the level of the biomarker in an exosome present in an individual who does not have the particular disease or condition. Biomarkers generally refer to a gene, mRNA, protein that is present in a biological sample, which is useful for the diagnosis of a disease, for providing a prognosis, or for preferential targeting of a pharmacological agent to an affected cell or tissue.

[0069] As used herein, the term “affinity moiety” refers to a binding molecule (e.g., antibody, aptamer, peptide, or nucleic acid) that specifically binds to a particular target molecule (e.g., a biomarker present in an exosome or a marker present on the surface of an exosome).

[0070] As used herein, the term “exosome extraction reagent” refers to an agent comprising an affinity moiety (e.g., an antibody, aptamer, peptide, or nucleic acid) that specifically binds to a marker present in the surface of an exosome (e.g., a polypeptide, nucleic acid, lipid, or carbohydrate moiety). Exosome extraction reagents may further include, for example, a solid phase onto which the affinity reagent is immobilized (for example: a bead such as a magnetic bead, a chip, a strip, or a surface such as a well in a microtitre plate). In one embodiment, the marker present in the surface of an exosome is CD63. Other, non-limiting, examples of markers found on the surface of exosomes are described in Théry C. et al. (Nat Rev Immunol. 2002 Aug;2(8):569-79), the content of which is hereby incorporated herein by reference in its entirety for all purposes.

[0071] As used herein, the term “detection reagent” refers to an agent comprising an affinity moiety (e.g., an antibody, aptamer, peptide, or nucleic acid) that specifically binds to a biomarker present in an exosome (e.g., a polypeptide, nucleic acid, lipid, or carbohydrate moiety). Detection reagents may further include, for example, a detectable moiety (e.g., a radioisotope, a fluorescent label, a magnetic label, an enzyme, or a chemical moiety such as biotin or digoxigenin). The detectable moiety can be detected directly, or indirectly, by the use of a labeled specific binding partner of the detectable moiety. Alternatively, the specific binding partner of the detectable moiety can be coupled to an enzymatic system that produces a detectable product. In one embodiment, the biomarker present in an exosome is CD9, CD63, CD81, CD82, or a combination therein. Other, non-limiting, examples of biomarkers present in an exosome are described in Théry C. et al. (Nat Rev Immunol. 2002
As used herein, the term “specifically binds” refers to a molecule that binds to a particular target molecule (e.g., a biomarker present in an exosome or a marker present on the surface of an exosome) with at least 2-fold greater affinity, as compared to a non-targeted molecule. In certain embodiments, a molecule specifically binds with at least 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, 1000-fold, 5000-fold, or greater affinity, as compared to a non-targeted molecule.

The term “nucleic acid” or “polynucleotide” refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogous of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides.

The term “gene” means the segment of DNA involved in producing a polypeptide chain. It may include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

The term “isolated,” when applied to a nucleic acid or protein, denotes that the nucleic acid or protein is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein that is the predominant species present in a preparation is substantially purified. In particular, an isolated gene is separated from open reading frames that flank the gene and encode a protein other than the gene of interest. The term “purified” denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure. An isolated nucleic acid can be a component of an expression vector.

Purity is determined by any art-recognized method of analysis (e.g., band intensity on a stained gel, polyacrylamide gel electrophoresis, HPLC, mass-spectroscopy, or a similar means).

“Lysis” or to “lyse” refers to disrupting the integrity of a closed lipid membrane such that the contents of the closed lipid membrane are accessible and/or the contents are released into the extra-membrane environment. Using a cell as an example, lysis refers to causing the breaking of the cellular membrane such that the contents of the cell are in direct contact with the extracellular milieu. A “lysis” is a liquid or solid collection of materials following a lysis procedure.

As used herein, the term “AC current” refers to an alternating electrical current that flows in one direction, reverses and flows in the opposite direction. As used herein, the term “DC current” refers to a direct electrical current that flows in one direction only. As used herein, the term “inverter” refers to an electrical device that converts DC current to AC current.

A “nonuniform electric field” refers to an electric field created by an electrical potential difference between two electrodes. The nonuniform electric field includes at least some electric field lines that are more locally concentrated at one electrode relative to the other electrode, e.g., more concentrated at the dispensing end relative to the second electrode or a grounded target surface. In some embodiments, the second electrode can be one or more ring electrodes, plate electrodes, and/or grounded target surfaces.

The term “square wave” is used herein as it is understood in the art, namely, a wave that is essentially in the form of an abrupt rise in value from a zero level followed by a period maintained at some maximum value followed by an abrupt decrease in value to the zero level. When plotting value against time, variations in the value produces a wave form made essentially of vertical and horizontal lines. Departures from absolutely vertical and horizontal lines through all portions of the wave are acceptable as long as the wave form of the electric field has an essentially square or rectangular form as understood by those skilled in the art.

As used herein, the terms “biological fluid” and “biological sample” are used interchangeably and refer to an extracellular sample from a patient. In certain embodiments, the biological sample is a biological fluid, while in other embodiments, the biological sample may be a non-fluid extracellular sample. Non-limiting examples of biological samples that can be used in the methods described herein include saliva, whole blood, blood plasma, blood serum, amniotic fluid, bile, colostrum, breast milk, cerebrospinal fluid (CSF), lymph, gastric acid, nasal mucus, pleural fluid, semen, tears, and urine.

As used herein, the term “subject” refers to any animal (e.g., a mammal), including, but not limited to, humans, non-human primates, rodents, and the like, which is to be the recipient of a particular treatment. Typically, the terms “subject” and “patient” are used interchangeably herein in reference to a human subject.

The term “differentially expressed” or “differentially regulated” refers generally to a protein or nucleic acid that is overexpressed (upregulated) or underexpressed (downregulated) in one biological sample compared to at least one other sample from a subject.

The terms “overexpress,” “overexpression,” “upexpress,” “upregulate,” or “upregulated” interchangeably refer to a biomarker that is present at a detectably greater level in a biological sample in comparison to biological sample from a control subject. Overexpression can be 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more in comparison to a sample from a control subject. In certain instances, overexpression is 1-fold, 2-fold, 3-fold, 4-fold, 5, 6, 7, 8, 9, 10, or 15-fold or more higher levels of transcription and/or translation in comparison to a sample from a control subject.

The terms “underexpress,” “underexpression,” “underexpressed,” “downregulate,” or “downregulated” interchangeably refer to a biomarker that is present at a detectably lower level in a biological sample in comparison to a biological sample from a control subject. In certain instances, underexpression is 1-fold, 2-fold, 3-fold, 4-fold or more lower levels of transcription and/or translation in comparison to a control. Overexpression and underexpression can be detected using conventional techniques for detecting mRNA (e.g., RT-PCR, PCR, hybridization), proteins (e.g., ELISA, immunohistochemical techniques, mass...
spectroscopy, Luminex® xMAP technology), or microbes (e.g., microbial nucleic acid profiling).

Biological assay systems are important in biotechnology and medical diagnostics. A useful biological agent detection method is using DNA or RNA detection through a polymerase chain reaction (PCR), real-time polymerase chain reaction (RT-PCR), or reverse-transcriptase polymerase chain reaction. Alternatively, assaying for the presence of a particular protein can also be used to detect or study biological samples of interest. Before biological sample can be analyzed using PCR or protein detection methods, it must first be prepared for analysis. A challenge in biological sample preparation is lysis.

Lysis methods can be generally divided into reagent-assisted or reagent-less lysis methods. The former uses lytic agents such as detergent to lyse cells. However, these lytic reagents can interfere following analysis process and the system can be overly complicated due to lytic agent injection and sample rinsing step. Therefore, reagentless lysis methods have been developed with various approaches.

In some embodiments, a method of electrical lysis of an exosome and collection of at least a portion of contents thereof is provided. The method entailing applying, an alternating electrical current to an exosome such that the exosome is lysed and collecting at least a portion of the contents thereof.

Electrical Lysis

The application of electricity has been used to achieve lysis of viruses, prokaryotic and eukaryotic cells. However, whether electricity could be used to effectively lyse exosomes, and the conditions of such use, was unknown prior to the instant disclosure. Electrical lysis of cells has been demonstrated as a modification of various electroporation techniques.


Exosome

Exosomes are small vesicles that are released into the extracellular environment from a variety of different cells such as but not limited to, cells that originate from, or are derived from, the ectoderm, endoderm, or mesoderm including any such cells that have undergone genetic, environmental, and/or any other variations or alterations (e.g. Tumor cells or cells with genetic mutations). An exosome is typically created intracellularly when a segment of the cell membrane spontaneously invaginates and is ultimately exocytosed (see for example, Keller et al., Immunol. Lett. 107 (2): 102-8 (2006)).

Exosomes can have, but not be limited to, a diameter of greater than about 10, 20, or 30 nm. They can have a diameter of about 30-1000 nm, about 30-800 nm, about 30-200 nm, or about 30-100 nm. In some embodiments, the exosomes can have, but not be limited to, a diameter of less than about 10,000 nm, 1000 nm, 800 nm, 500 nm, 200 nm, 100 nm or 50 nm. As used throughout, the term “about,” when referring to a value or to an amount is meant to encompass variations in some embodiments±10% from the specified amount, as such variations are appropriate.

Exosomes may also be referred to as microvesicles, nanovesicles, vesicles, deorosmes, bleb, blebbly, prostatesomes, microparticles, intraluminal vesicles, endosomal-like vesicles or exocytosed vehicles. As used herein, exosomes can also include any shed membrane bound particle that is derived from either the plasma membrane or an internal membrane. Exosomes can also include cell-derived structures bounded by a lipid bilayer membrane arising from both herniated evagination (blebbing) separation and sealing of portions of the plasma membrane or from the export of any intracellular membrane-bound vesicular structure containing various membrane-associated proteins of tumor origin, including surface-bound molecules derived from the
host circulation that bind selectively to the tumor-derived proteins together with molecules contained in the exosome lumen, including but not limited to tumor-derived microRNAs or intracellular proteins. Blebs and blebbing are further described in Charras et al., Nature Reviews Molecular and Cell Biology, Vol. 9, No. 11, p. 730-736 (2008). Exosomes can also include membrane fragments.

Circulating tumor-derived exosomes (CTEs) as referenced herein are exosomes that are shed into circulation or bodily fluids from tumor cells. CTEs, as with cell-of-origin specific exosomes, typically have unique biomarkers that permit their isolation from bodily fluids in a highly specific manner.

Exosomes can be directly assayed from the biological samples, such that the level of exosomes is determined or the one or more biomarkers of the exosomes are determined without prior isolation, purification, or concentration of the exosomes. Alternatively, exosomes may be isolated, purified, or concentrated from a sample prior to analysis.

In some embodiments, an exosome may be purified or concentrated prior to analysis. Analysis of an exosome can include quantitatively the amount one or more exosome populations of a biological sample. For example, a heterogeneous population of exosomes can be quantitated, or a homogeneous population of exosomes, such as a population of exosomes with a particular biomarker profile, or a specific bio-signature, or derived from a particular cell type (cell-of-origin specific exosomes) can be isolated from a heterogeneous population of exosomes and quantitated. Analysis of an exosome can also include detecting, quantitatively or qualitatively, a particular biomarker profile or a bio-signature, of an exosome, as described below.

An exosome can be stored and archived, such as in a bio-fluid bank and retrieved for analysis as necessary. An exosome may also be isolated from a biological sample that has been previously harvested and stored from a living or deceased subject. In addition, an exosome may be isolated from a biological sample which has been collected as described in King et al., Breast Cancer Res 7(5): 198-204 (2005). An exosome may be isolated from an archived or stored sample. Alternatively, an exosome may be isolated from a biological sample and analyzed without storing or archiving of the sample. Furthermore, a third party may obtain or store the biological sample, or obtain or store the exosomes for analysis.

An enriched population of exosomes can be obtained from a biological sample. For example, exosomes may be concentrated or isolated from a biological sample using size exclusion chromatography, density gradient centrifugation, differential centrifugation, immunomembrane ultrafiltration, immunoabsorbent capture, affinity purification, microfluidic separation, or combinations thereof.

Size exclusion chromatography, such as gel permeation columns, centrifugation or density gradient centrifugation, and filtration methods can be used. For example, exosomes can be isolated by differential centrifugation, anion exchange and/or gel permeation chromatography (for example, as described in U.S. Pat. Nos. 6,899,863 and 6,812,023), sucrose density gradients, organelle electrophoresis (for example, as described in U.S. Pat. No. 7,198,923), magnetic activated cell sorting (MACS), or with a nanomembrane ultrafiltration concentrator. Various combinations of isolation or concentration methods can be used.

Highly abundant proteins, such as albumin and immunoglobulin, may hinder isolation of exosomes from a biological sample. For example, exosomes may be isolated from a biological sample using a system that utilizes multiple antibodies that are specific to the most abundant proteins found in blood. Such a system can remove up to several proteins at once, thus uncovering the lower abundance species such as cell-of-origin specific exosomes.

This type of system can be used for isolation of exosomes from biological samples such as blood, cerebrospinal fluid, urine and/or saliva. The isolation of exosomes from a biological sample may also be enhanced by high abundant protein removal methods as described in Chrony et al. J. Proteome Res 2004; 3:1120-1127. In another embodiment, the isolation of exosomes from a biological sample may also be enhanced by removing serum proteins using glycopeptide capture as described in Zhang et al, Mol Cell Proteomics 2005; 4:144-155. In addition, exosomes from a biological sample such as urine may be isolated by differential centrifugation followed by contact with antibodies directed to cytoplasmic or anti-cytoplasmic epitopes as described in Pisitkun et al., Proc Natl Acad Sci USA, 2004; 101:13368-13373.

EXAMPLES

Example 1

Affinity Capture of Exosomes by Immuo-Magnetic Beads

Effective exosome extraction requires efficiency and specificity. The technology described herein integrated exosome extraction enhanced with electrical field and magnetic bead techniques combined with simultaneous biomolecule release and detection. The detection was performed with amperometric electrochemical sensor technology(21) based on an array of 16 bare gold electrode chips (Gene Fluidics, USA). The entire procedure is illustrated in FIG. 1.

Magnetic beads are an efficient tool for exosome extraction, because they facilitate exosome monitoring and detection of attached biomarkers. In this study, anti-human CD63 antibody (hCD63) attached to magnetic beads was used to capture exosomes that expressed membrane-bound CD63(14) (FIG. 1).

To accomplish this, streptavidin-coated magnetic beads (Invitrogen, USA) were mixed with biotinylated anti-hCD63 (Aneell, USA) on a mixer for 30 min at room temperature. Then, 10 mL of H460 cell medium, serum, human saliva and murine saliva were incubated with the anti-hCD63-conjugated magnetic beads in casein-PBS (Invitrogen, USA). Murine saliva was used to demonstrate the specificity of the magnetic bead-based exosome extraction, which lacks the hCD63 protein. Samples were mixed for 2 h at room temperature to form exosome-magnetic bead complexes. Next, exosome-magnetic bead complexes were attracted onto the electrochemical sensor by applying an array of magnets. The unattached species were removed by washing.

Transmission electron microscopy (TEM) was used to examine exosomes after extraction with anti-hCD63 antibody-conjugated magnetic beads (FIG. 2a-b). For TEM measurements samples were loaded onto carbon-coated grids. Due to interference between the magnetic beads and the electric field of the electron microscope, specific grids
Capture probe:  
5'-Biotin-AGGTCCACCACTGACACGTTG

Detector probe:  
5'-SCGATGGGGACACGGAAGGCC-Fluorescein-3'

[0110] The TEM images showed exosomes located on the surface of the magnetic beads. The sizes of the extracted particles ranged from 70-100 nm, consistent with the size distribution of typical exosomes (10, 14, 22) (FIG. 1b). The exosome extraction efficiency was approximately 85%, determined by comparing the levels of hCD63 found in extracted exosomes to the levels found in exosome-depleted human saliva. The TEM results showed no mouse-saliva exosomes attached to the beads (FIG. 1c). Furthermore, when beads were coated with streptavidin only, no exosomes were captured (FIG. 1d).

Example 2
Release of Harbored mRNA from Human Saliva
Exosomes via Cyclic Square Wave (CSW) Electrical Field

[0111] Triton™ X-100 has been shown to disrupt the integrity of salivary exosomes, but this leads to endogenous RNA degradation by RNases in the saliva matrix (18). Therefore, a rapid release process and immediate detection are necessary to effectively measure exosomal mRNA. A cyclic square wave electric field (csw E-field) was assessed to determine whether the csw E-field would facilitate the release and detection of RNA from human saliva exosomes (FIG. 3a). For comparison, the release efficiency was measured against Triton™ X-100 based lysis carried out on the same human saliva exosomes, captured by the same magnetic beads, and performed in parallel with the csw E-field assay.

[0112] Glycerinaldehyde 3-phosphate dehydrogenase (GAPDH) or GPDH mRNA is present in most exosomes. Therefore, measurement of GAPDH mRNA was utilized for comparing csw E-field to Triton™ X-100 based lysis.

[0113] For GAPDH mRNA detection, the electrodes were coated with a surface matrix of conducting polymer pyrrole. Then, the surface of the electrode was pre-coated with oligonucleotide capture probes with sequences specific for human GAPDH and a biotin label at the 5' end. The immobilization of the capture probe was carried out by applying a csw E-field for 20 cycles of 9 s at -300 mV followed by 1 s at +200 mV (200 s total) (21). After the exosome extraction step, the exosome-magnetic bead complex was collected onto the capture probe-coated electrode with an array of magnets placed underneath the electrochemical sensor. When loading the sample onto the electrode, 10 nM of detector probe (with a fluorescein-labeled 3' end) was mixed in with the exosome-magnetic bead complexes. The csw E-field was then applied to release the harbored GAPDH mRNA from the exosomes. The csw E-field was 20 cycles of 9 s at -300 mV followed by 1 s at +200 mV (200 s total).

[0114] In parallel, samples from the same batches were lysed with 0.5% Triton™ X-100 (Sigma, USA) for 20 min at room temperature to compare release and protection efficiencies. The released mRNA hybridized with the oligonucleotide capture and detector probes. Then, we added 150 unit/ml of anti-fluorescein antibody conjugated to horseradish peroxidase (HRP: 1:1000 dilution; Roche, USA). Finally, the 3,3',5,5'-tetramethylbenzidine (TMB) substrate for horseradish peroxidase was loaded, and an amperometric signal was read out.

[0115] Exosome-magnetic bead complexes were examined by TEM before and after treatment with the csw E-field or Triton™ X-100 (FIG. 3b).

[0116] After both treatments, the exosomes on the beads disappeared. The levels of GAPDH mRNAs were measured at different time points by csw E-field after the application of electrical field or Triton™ X-100 (FIG. 3c). The results showed that the initially high GAPDH mRNA levels decreased gradually over the incubation time for both csw E-field and Triton™ X-100 disrupted samples. In contrast, the untreated exosomes (positive controls) maintained constant GAPDH mRNA levels during the experiment. These data demonstrate that both csw E-field and Triton™ X-100 detergent released the GAPDH mRNA from human saliva exosomes. With exposure to the saliva matrix, the signals dropped to less than 40% within one min. After 20 min, the readouts decreased down to the background level. The kinetics of the GAPDH mRNA signal decay demonstrates that the exosome protected and stabilized endogenous mRNA. Without the exosome protection, the GAPDH mRNA level rapidly decayed.

Example 3
Development of CSW-Field Technology to Simultaneously Detect Exosomal Surface Proteins and Harbored mRNA

[0117] H460 cells were used to evaluate EFIRM technology for simultaneous measurements of an exosomal membrane protein (hCD63-GFP) and an exosomal mRNA (GAPDH). Purified hCD63-GFP exosomes were isolated by ultracentrifugation from medium conditioned by H460 cells. Exosomes were then incubated with magnetic beads coated with anti-hCD63. When the csw E-field was applied, both the GAPDH mRNA-DNA probe hybridization and the exosome-anti-GFP antibody binding were measured within minutes on the same detection chip.

[0118] The sensitivity of EFIRM for detecting exosomal GAPDH mRNA and hCD63-GFP protein was determined by benchmarking the results against conventional methods of Western blotting for detecting the hCD63-GFP protein and nested q-PCR for detecting the GAPDH RNA.

[0119] For the hCD63-GFP protein detection, there was no need to disrupt the endosome, because the protein was membrane-bound and the GFP portion was accessible to the medium. Therefore, the rabbit anti-GFP antibody conjugated to HRP (Invitrogen, USA) was mixed with the exosome-
hCD63-magnetic bead complex in solution for 1 h at room temperature. After washing, the mixture was collected on the electrodes with the applied magnetic field. Then, the TMB substrate was added, and amperometric measurements were carried out. GFP protein levels are expressed relative to the negative control. This was calculated as the ratio between the sample and the blank casein PBS buffer.

Western blotting and EFRIM showed similar protein sensitivities at the same dilutions (Fig. 4a). The electrochemical level of GAPDH mRNA by EFRIM and the CT value on a log 10 scale from q-rtPCR also showed a similar trend, but different concentrations (Fig. 4b). These results demonstrated that the sensitivity of EFRIM was comparable to that of conventional methods for detecting both mRNA and GFP protein.

The specificity of EFRIM technology for detecting human protein (hCD63-GFP) and mRNA (hGAPDH mRNA) endosomal targets in the presence of interfering mouse exosomes. The detection probes were based on human-specific sequences for the GAPDH mRNA and the CD63 protein. We performed the EFRIM measurements of exosomal hCD63-GFP protein and GAPDH mRNA on mixtures of human and mouse exosomes at different volume ratios. The corresponding electrochemical signals are presented in Figs. 4c and 4d. As the ratio between human and mouse exosomes, the pure human exosomes (ratio=1) showed higher signal intensities than the pure mouse exosomes (ratio=0). Even when the human exosomes were highly outnumbered by mouse exosomes (human exosome: mouse exosome=0:2), the signal remained above 2 standard deviations from the pure mouse exosome sample. This result showed that the EFRIM technology was highly specific in the detection of both human GAPDH mRNA and hCD63-GFP protein.

Example 4

EFRIM Technology for Detecting Exosomal hCD63-GFP in Body Fluids

EFRIM successfully detected exosomal surface protein and harbored mRNA simultaneously, whether EFRIM technology could be applied to an in vivo system was therefore tested. Nude mice were implanted with the human lung cancer cell line H460, which expressed hCD63-GFP.

Male athymic BALB/c nude mice were obtained from Charles River (USA) and weighed 20-22 g at the beginning of the experiments. The mice were housed in sterilized, filter-topped cages and maintained under sterile conditions. Each mouse received an injection of $1 \times 10^6$ H460 cells that expressed hCD63-GFP, or 100 µl (n=11) saline in the left chest cavity. Briefly, the mice were anesthetized with 1-3% isoflurane in oxygen with a precision vaporizer. The mice were then placed in a right lateral, decubitus position and a suspension of H460 cells or saline was injected (100 µl) slowly into the left intercostal space at the dorsal mid-axillary line, just below the inferior border of the scapula with a 30-ga needle attached to a 1-cc syringe. The needle was advanced approximately 5 mm through the chest wall into the pleural space. After injection, the needle was retracted, and mice were turned to the left lateral decubitus position for recovery. After 20 days, mice reached a moribound condition, and serum and saliva was collected before euthanization.

EFRIM was then to measure hCD63-GFP positive exosomes in saliva and serum. FIG. 5 shows the correlation between exosomal hCD63-GFP levels in saliva and serum. All 20 samples had measurable hCD63-GFP levels by EFRIM in both serum and saliva. Data points close to the line (slope=1) indicate that the serum and saliva had similar protein concentrations; data points below the line indicate a higher signal in serum than in saliva, and vice versa. Therefore, the human CD63-GFP-exosome levels from serum and saliva correlated well (R=0.73) in the 20 mouse samples (both serum and saliva were measured for each mouse). Furthermore, the mice injected with hCD63-GFP and those injected with saline (control group) showed significant differences in the hCD63-GFP positive exosomes in both serum and saliva. The low relative values (1 was the same as the blank control) measured in the saline group suggested that the saline injection did not generate high GFP signals in either serum or saliva. The detection required only 10 µl of raw sample in an on-site measurement. The time from the raw sample loading to detection was approximately 3 h.

REFERENCES


33. All references cited herein are hereby incorporated by reference herein in their entirety and for all purposes to the same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

34. Many modifications and variations of this application can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the application is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which the claims are entitled.
A method for detecting a biomarker present in an exosome, the method comprising:
(a) providing a biological fluid comprising exosomes;
(b) contacting the biological fluid with an exosome extraction reagent to form a complex between the exosome and the exosome extraction reagent, the exosome extraction reagent comprising a first affinity moiety bound to a magnetic bead, wherein the first affinity binding moiety specifically binds to a surface-exposed exosome marker;
(c) separating the complex formed between the exosome and the exosome extraction reagent from the biological fluid;
(d) contacting a solid phase with the complex formed between the exosome and the exosome extraction reagent, the solid phase comprising a first contact region under the influence of a magnetic field, a second contact region, and a detection reagent immobilized at the second contact region, the detection reagent comprising a second affinity moiety that specifically binds to the biomarker, wherein the biomarker is selected from a protein, a nucleic acid and an mRNA;
(e) immobilizing the complex formed between the exosome and the exosome extraction reagent at the first contact region;
(f) applying an electrical field to the immobilized complex formed between the exosome and the exosome extraction reagent at the first contact region; and
(e) detecting an interaction between the biomarker and the detection reagent.

The method according to claim 26, wherein the biological fluid is saliva.

The method of claim 30, wherein the biological fluid is whole blood, blood plasma, or blood serum.

The method according to claim 26, wherein the surface-exposed exosome marker is CD63.

The method according to claim 26, wherein the exosome extraction reagent comprises an antibody.

The method according to claim 26, wherein the exosome extraction reagent comprises an exosome-specific lectin.

The method according to claim 26, wherein the second affinity moiety comprises an antibody.

The method according to claim 26, wherein the second affinity moiety comprises a nucleic acid.

The method according to claim 26, wherein the solid phase comprises an electrochemical sensor.

The method according to claim 26, wherein the absolute value of the maximum voltage of the electric field does not exceed 1 volt.

The method of claim 39, wherein the absolute value of the maximum voltage of the electric field does not exceed 500 millivolt (mV).

The method according to claim 26, wherein the electric field is applied for less than 300 seconds.

The method of claim 41, wherein the electric field is applied for 200±30 seconds.

The method according to claim 26, wherein the electric field is a non-uniform electric field.

The method of claim 43, wherein the non-uniform electric field is a cyclic square wave electrical field (csw E-field).

The method of claim 44, wherein the csw E-field comprises 15 cycles of −300±50 mV for 9±2 seconds followed by 200±50 mV for 1±0.5 second.

The method of claim 45, wherein the csw E-field comprises 20 cycles of −300 mV for 9 seconds followed by 200 mV for 1 second. 1.

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