Video Article

Detection of Exosomal Biomarker by Electric Field-induced Release and Measurement (EFIRM)

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Abstract

Exosomes are microvesicular structures that play a mediating role in intercellular communication. It is of interest to study the internal cargo of exosomes to determine if they carry disease discriminatory biomarkers. For performing exosomal analysis, it is necessary to develop a method for extracting and analyzing exosomes from target biofluids without damaging the internal content.

Electric field-induced release and measurement (EFIRM) is a method for specifically extracting exosomes from biofluids, unloading their cargo, and testing their internal RNA/protein content. Using an anti-human CD63 specific antibody magnetic microparticle, exosomes are first precipitated from biofluids. Following extraction, low-voltage electric cyclic square waves (CSW) are applied to disrupt the vesicular membrane and cause cargo unloading. The content of the exosome is hybridized to DNA primers or antibodies immobilized on an electrode surface for quantification of molecular content.

The EFIRM method is advantageous for extraction of exosomes and unloading cargo for analysis without lysis buffer. This method is capable of performing specific detection of both RNA and protein biomarker targets in the exosome. EFIRM extracts exosomes specifically based on their surface markers as opposed to size-based techniques.

Transmission electron microscopy (TEM) and assay demonstrate the functionality of the method for exosome capture and analysis. The EFIRM method was applied to exosomal analysis of 9 mice injected with human lung cancer H640 cells (a cell line transfected to express the exosome marker human CD63-GFP) in order to test their exosome profile against 11 mice receiving saline controls. Elevated levels of exosomal biomarkers (reference gene GAPDH and protein surface marker human CD63-GFP) were found for the H640 injected mice in both serum and saliva samples. Furthermore, saliva and serum samples were demonstrated to have linearity (R = 0.79). These results are suggestive for the viability of salivary exosome biomarkers for detection of distal diseases.

Video Link

The video component of this article can be found at https://www.jove.com/video/52439/

Introduction

Exosome research is an emerging field of investigation that examines lipid microvesicles that carry RNA[1], DNA[2], and protein[3] cargo. Previous investigations of exosome biology have led to identification of exosomes in biofluids such as blood[4], urine[5], breast milk[6], and saliva[7]. Studies have demonstrated that exosomes play a role in different cellular pathways, remotely meditating communication between different systems of the body[8]. Because of the role exosomes play in intercellular communication, it is hypothesized that they may package biomolecule targets (protein, RNA, and DNA) correlated with disease states. *In vitro*[3] and animal model[9] studies appear to corroborate this hypothesis. In investigating exosomal content for biomarker discovery, it is necessary to develop a methodology for selective exosome isolation from biofluids, induced expulsion of cargo from exosomes, and quantification of exosome biomolecules. In the extent of this work, exosomes will be defined as a structure having a diameter of approximately 70-100 nm and possessing surface marker CD63.

Researchers typically first purify exosomes by ultracentrifugation[10] and then process exosomal content through the usage of lysis buffer kits. Usage of lysis buffer methods requires incubation times ranging from minutes to hours. This process may potentially harm exosome cargo and lead to sample degradation. For example, salivary exosome RNA released via lysis buffer into the surrounding extracellular environment possesses a half-life of under 1 min, making measurement of exosomal RNA post-lysis buffer a particularly difficult task without the addition of stabilization reagents[11]. The compounded effect of adding various reagents for lysis and stabilization may introduce agents that complicate and interfere with the analysis of exosomal content. An alternative approach may be helpful for rapidly unloading exosomal content and safely preserving the cargo for characterization.

In this work, we propose the usage of a non-uniform electric field for the release of exosomal content. Electric-fields have been known to carry the ability to polarize and disrupt the lipid bilayer that forms cell membranes. Our experimental work explores usage of non-uniform cyclic square waves (CSW) for disrupting the microvesicle structure of exosomes and releasing carried cargo. This method uses voltages in the several hundred millivolt range, meaning that most biomolecules will not be disrupted. We demonstrate that the usage of a cyclic-square wave is able to actuate release of salivary exosome mRNA content into the surrounding fluidic environment. This release of exosomal content is seamlessly integrated with an electrode system that can be used to quantify the biomarker expression levels[12,13]. This proposed method allows for rapid, sensitive, and lysis buffer free analysis of exosome content.

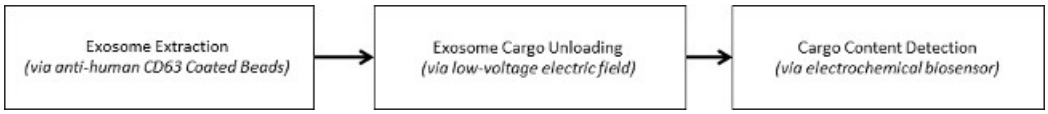

Figure 1. Overview of EFIRM Workflow. The EFIRM method is broadly divided into the three major phases that are necessary for purifying and analyzing exosomes.

This CSW based exosomal content release and analysis method is used in conjunction with CD63-specific magnetic microbeads for exosome isolation. These CD63-affinity beads allow for the selective isolation of exosomes from salivary samples (and other biofluids). Following incubation and extraction of exosomes using the magnetized beads, the beads are migrated to the electrochemical sensor system for the CSW based content release and analysis portion of the experiment. **Figure 1** gives an overview of the workflow of the EFIRM method.

Protocol

1. Magnetic Bead-based Exosome Extraction

1. Pipette a well-mixed solution of 5 µl of Streptavidin-Coated magnetic microparticles into 495 µl of phosphate buffered saline (PBS) buffer in a microcentrifuge tube to resuspend the beads. Wash and resuspend the beads with 500 µl of PBS three times using a magnetic rack. The rack is an array of magnets on the side of a housing unit that can hold the sample microcentrifuge tubes.
    1. For each wash, first let the tubes sit on the rack for 1 min, and then use a pipette tip to carefully remove the supernatant buffer without disturbing the beads.
    2. Place the tubes on a regular rack without magnets at the side. Add 500 µl of PBS into the tubes, and use the pipette to mix the solution and beads together. Then put the tubes back on the magnetic rack to again separate the beads from the solution.
    3. Perform this removal of buffer via magnetization and resuspension in PBS a total of three times. This performs an initial wash of the magnetic particles.

2. Resuspend the beads into 490 µl of PBS buffer, with the tube placed on the non-magnetized portion of the magnetic rack. Pipet 5 µl of biotinylated mouse anti-human CD63 antibody at 1.0 mg/ml stock concentration into the mixture of beads. Use the pipette to mix the beads and antibody in solution.
3. Place the microcentrifuge tubes with bead and biotinylated antibody mixture on a sample rotator. Set the rotator parameters for the sample rotator for reciprocal rotation at 90° tilting for 5 sec and vibrating at 5° for 1 sec. Rotate the sample-bead mixture tubes at these parameters for 30 min at RT.
4. Remove unbound antibody after conjugation.
    1. Following 30 min of rotation at RT, place the tubes back in the magnetic rack for 5 min.
    2. Perform three washes of beads by removing the liquid phase using a micropipette and wash with 500 µl of PBS. After the triple wash, resuspend the beads in 490 µl of casein-PBS and place on the unmagnetized portion of the rack.

5. Exosome extraction using antibody-coated beads.
    1. Label each tube with targeted sample ID. Pipette a 10 µl sample of serum or saliva into the microcentrifuge tube. Use the pipette to mix the sample and magnetic beads by pipetting several times.
    2. Place the tubes with sample and anti-human CD63 antibody beads on rotator and rotate for 2 hr at RT. Use the same rotator parameters as described in step 1.2.
    3. Following 2 hr of sample rotating, perform a triple wash by magnetizing to separate beads from solution, removing liquid phase with micropipette, and resuspending beads in 500 µl of Tris-HCl buffer. The resultant beads are now bound to the exosomes and are ready for the electrical field release and measurement.

2. Electric Field Induced Released and Measurement of Exosomal Content

1. Initial Precoating of Electrode with GADPH Primer
    1. Apply a plastic well to an electrode array to prevent cross-contamination of the individual electrodes. For this experiment, use a 16-sensor electrode array with each unit electrode in the array consisting of a working, counter, and reference electrode made of bare gold.
    2. Prepare a stock mixture of 100 nM DNA probe, 0.3 M KCl, and 10 mM pyrrole by pipetting stock reagents into a tube with ultrapure distilled water. Mix thoroughly by vortexing.
    NOTE: For this study, the DNA probe selected corresponds to the GAPDH reference gene, which is known to exist within exosomes. The probe sequence used is: 5'-Biotin-AGGTCCACCACTGACACGTTG-3'. Use this mixture on all the electrodes.
    3. Pipet 60 µl of the monomer-DNA probe mixture onto the surface of each gold electrode. Examine the electrodes to ensure that there is adequate coverage of the working, counter, and reference electrodes by the liquid mixture.

4. Electropolymerize monomer-probe mixture to create a conducting polymer layer on the electrode surface by applying a cyclic square wave (CSW) electric field profile to the electrode surface. This electric field consists of applying +350 mV for 9 sec and immediately switching to +950 mV for 1 sec. Apply this cyclic square wave profile to the electrode for 10 cycles, for a total of 100 sec of applied electric field.
5. Rinse sensor surface 3 times with distilled water and dry with nitrogen gas to remove liquid from the surface of the electrode. Ensure that liquid is properly removed from the electrode.

2. Exosome Cargo Unloading
    1. Load 5 µl of 1 µM of a detector probe into 495 µl of the bead-exosome complex mixture and use a pipette to mix.
    NOTE: The detector probe is a DNA primer sequence conjugated to a fluorescein molecule at the 3' end. The detector probe sequence used for this study corresponds to the GAPDH mRNA found within exosomes. The sequence of the fluorescein conjugated detector probe is: 5'-GCAGTGGGGACACGGAAGGCC-Fluorescein-3'.
    2. Pipette 60 µl of the probe and bead-exosome complex mixture onto a gold electrode surface with a magnet array underneath. This magnet array consists of sixteen 2.54 mm diameter neodymium magnets aligned to correspond to the working electrodes of the sensor. **Figure 2A** illustrates the placement of the magnets and bead-exosome solution.
    3. Once sample is loaded on the electrode surface, apply 20 cycles of the CSW electric field with 9 sec at -300 mV and 1 sec at +200 mV (200 sec total). The exosomal cargo that is released will hybridize to the primers on the surface of the electrode. If surface markers of the exosome are the subject of investigation, skip this portion of the experiment. **Figure 2B** illustrates this process.
    4. Wash-off the unbound analytes on the electrode surface by triple rinsing the electrode surface with distilled water. Dry the electrode with nitrogen gas.

3. Reporter Antibody and Readout
    1. Add 60 µl of 150 unit/ml anti-fluorescein antibody conjugated to horseradish peroxidase (HRP in 1:1,000 dilution) diluted in Casein/PBS.
    2. Use an electric-field driven conjugation to complex anti-fluorescein HRP to the probe sandwich. Apply -200 mV for 1 sec and +500 mV for 1 sec for 5 cycles to the electrode surface. **Figure 2A** shows the capture and detector probe complexes for both a protein and nucleic acid system.
    3. Triple wash sensor surface using distilled water and dry with nitrogen gas.
    4. Following the wash-off of unbound excess anti-fluorescein antibody, add 60 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate. Load this substrate on to each sensor surface using a multichannel pipet.
    5. Perform amperometric readout of the current by measuring the electrode current at -200 mV for 60 sec using an electrochemical potentiostat capable of simultaneous measurement of 16 channels. **Figure 2C** is an example of current profile during readout.

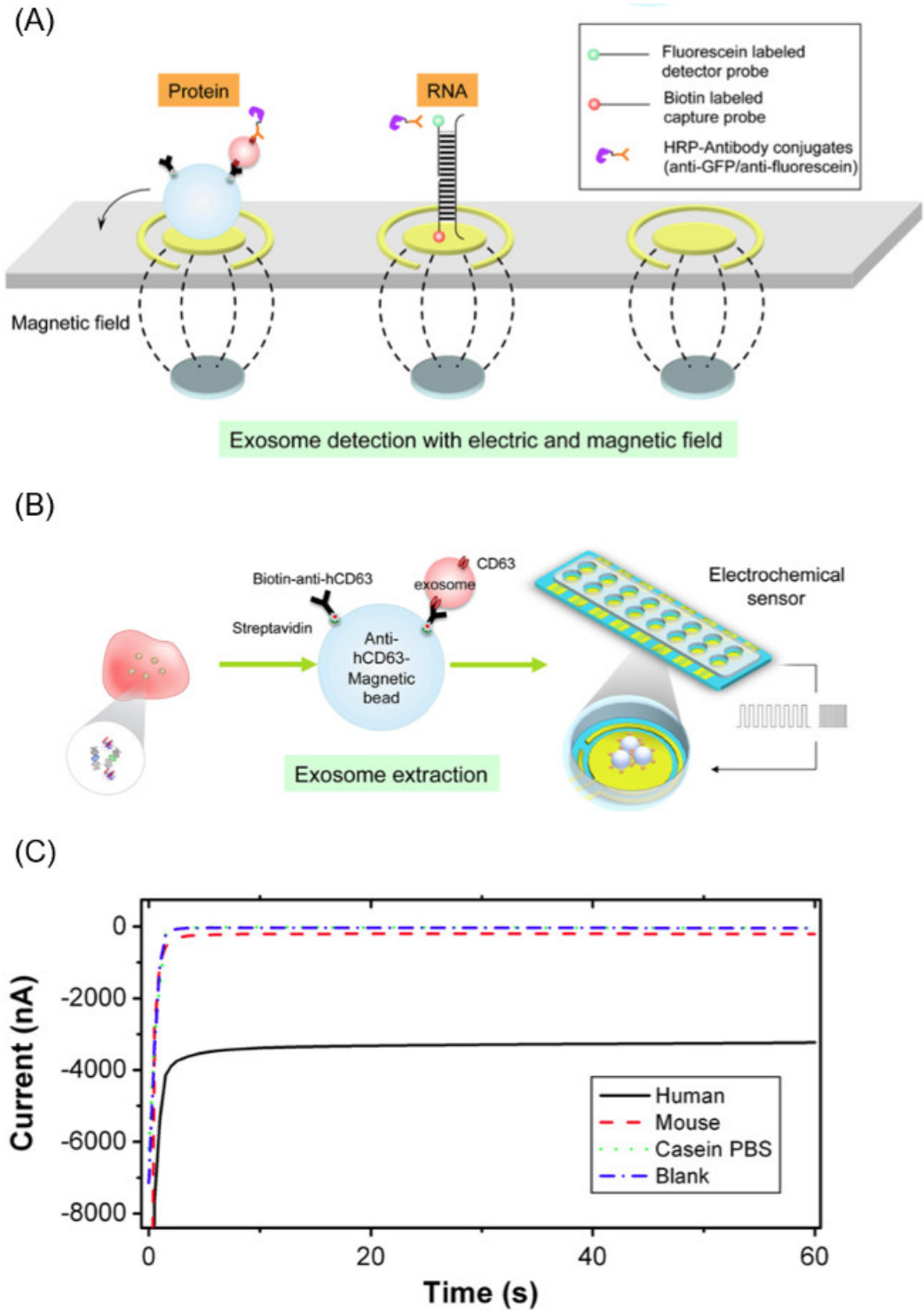

Figure 2. Components of EFIRM Method. (**A**) Method of extracting exosomes from biofluid using anti-human CD63 coated magnetic microparticles and then unloading exosome cargo using cyclic square waves applied to the particle-exosome complex. (**B**) Scheme of electrode biosensor used for detecting RNA/DNA/protein targets from the released exosome. (**C**) Representative example of amperometric readout from the EFIRM methodology, where larger current magnitude corresponds to higher levels of a biomolecule. This figure is from Wei *et al.*[14] Please click here to view a larger version of this figure.

Representative Results

Validation of Exosome Capture of Beads Using TEM

Isolation of exosomes from saliva using anti-human CD63 magnetic beads was validated following extraction protocol by using transmission electron microscopy (TEM) images. TEM shows magnetic beads with 70-100 nm granules immediately adjacent (see **Figure 3A**, and **3B**), consistent with the known profile of exosomes. No 70-100 nm granules were observed for the magnetic beads in saliva that did not have anti-human CD63 antibodies conjugated to them previously (see **Figure 3C** and **3D**).

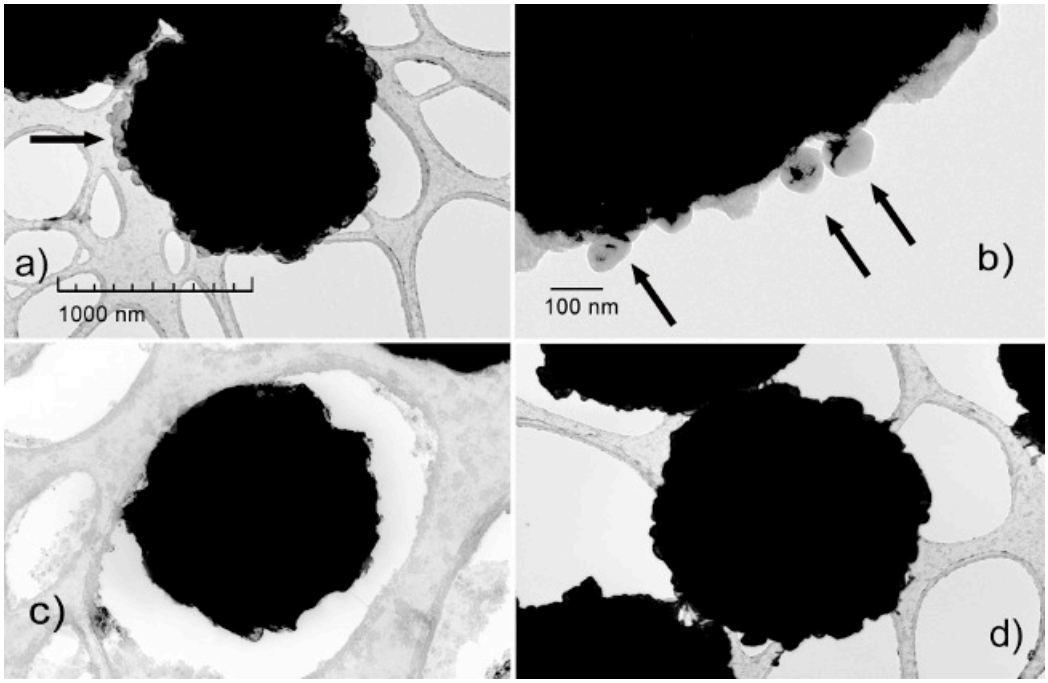

Figure 3. TEM images of magnetic microparticle. (**A**) Image of anti-human CD63 magnetic nanoparticles with small granules of 70-100 nm adjacent. (**B**) Enlarged view of magnetic nanoparticle and observed granules. (**C**) Image of magnetic beads with no antibody conjugated. (**D**) Magnified view of magnetic beads with no antibody conjugated. This figure is from Wei *et al.*[14] Please click here to view a larger version of this figure.

Study of Release of Exosomal Content Using CSW Electric Field

TEM images of anti-human CD63 beads show 70-100 nm granules adjacent to magnetic beads (see **Figure 3A**). Following the capture of the exosomes using the magnetic beads, samples are processed with two different methods in parallel. The first method used was Triton-X 100 detergent to lyse exosomes and the second method was the cyclic square wave (CSW) electric-field lysis buffer free method discussed in this work (details of Triton-X 100 method are described by Wei e*t al.*[14]). These processed exosome-bead complexes were analyzed by TEM and a mRNA reference gene was quantified using the electrochemical method described in this work. The reference gene used in this study was GAPDH, which is present in most exosomes.

TEM images show that in both the Triton-X 100 and CSW electric field treated samples, there was a noted absence of the exosome structures adhered to the magnetic beads after both the CSW and Triton-X 100 treatment methods (see **Figure 4B-ii** and **Figure 4B-iv**). The measurement of the GAPDH expression levels in both the Triton-X 100 and CSW electric field treated samples possessed a similar profile: namely that following the different lysing protocols, there was a decrease in measured GAPDH levels as time progressed, indicating an exposure of exosomal mRNA to the extravesicular environment of the saliva (see **Figure 4C**).

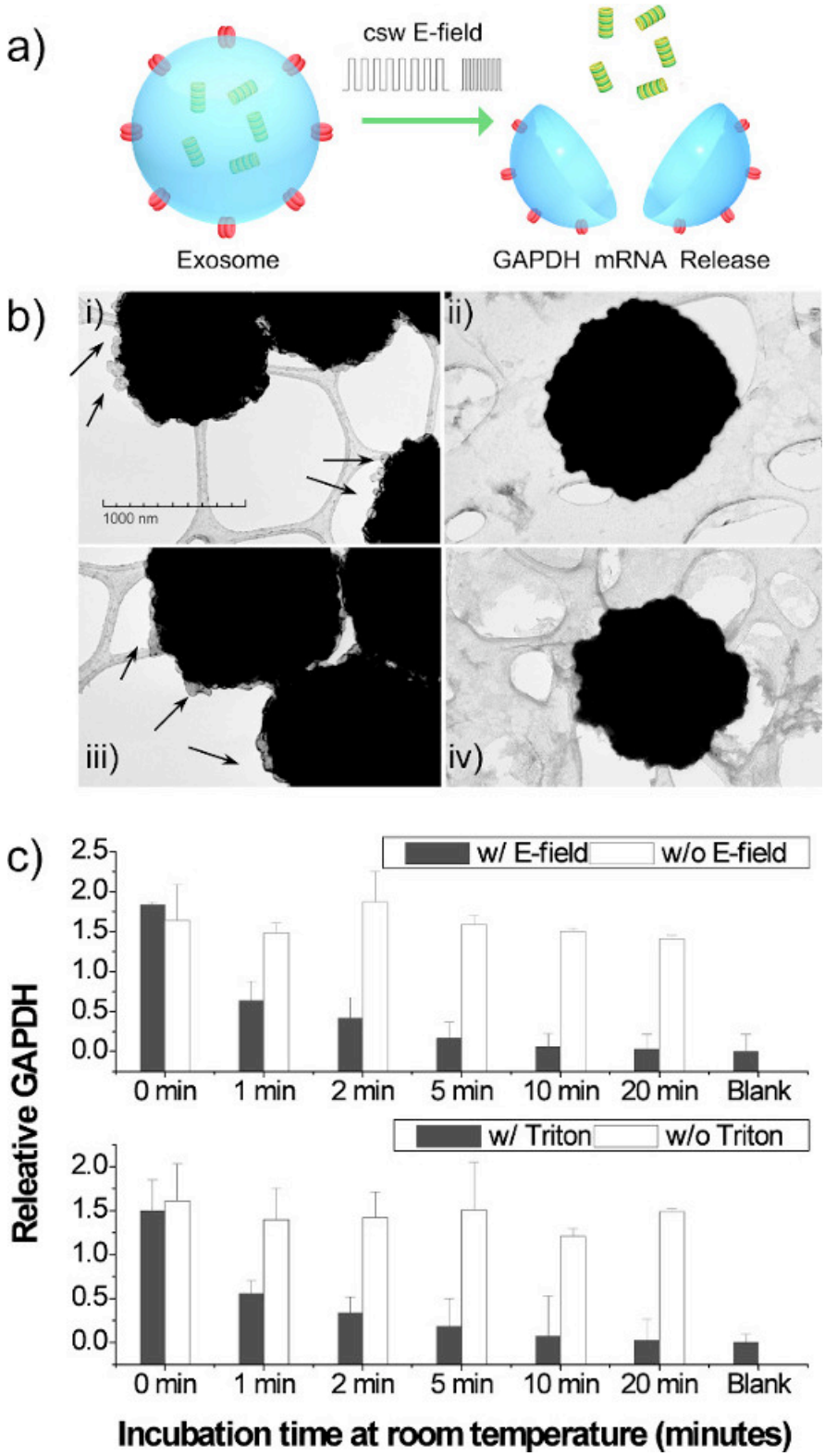

Figure 4. Results relating to Cyclic Square Wave Exosome Cargo Release. (**A**) Diagram of electric CSW release method. (**B**) TEM images of exosomes: (i) Before CSW release, (ii) After CSW method, (iii) Before lysis buffer method, (iv) After lysis buffer method. Background of TEM images is lacey support. (**C**) Quantification of exosome GAPDH mRNA levels when lysis buffer and electric field CSW is applied. This figure is from Wei *et al.*[14] Please click here to view a larger version of this figure.

Examining Performance of EFIRM for detection of surface proteins and harbored mRNA

Evaluation of EFIRM and comparison to traditional methods was conducted. EFIRM was compared to quantification of protein using western blot (for human CD63-GFP, a surface marker for exosomes) and qPCR for the quantification of RNA (using GAPDH as an mRNA target). Because the human CDP63-GFP was on the surface of the exosome, CSW was not applied to unload exosome cargo. The results of the protein and RNA tests are respectively shown in **Figure 5A** and **5B**.

Specificity tests were also conducted for EFIRM by mixing the human exosomes with exosomes from mouse samples. These specificity tests involved different ratio mixtures of mouse to human samples. Results of the experimentation demonstrate that when mouse exosomal content was five times greater than human content, human exosomal proteins and mRNA could still be identified and quantified (see **Figure 5C** and **5D** for protein and RNA, respectively).

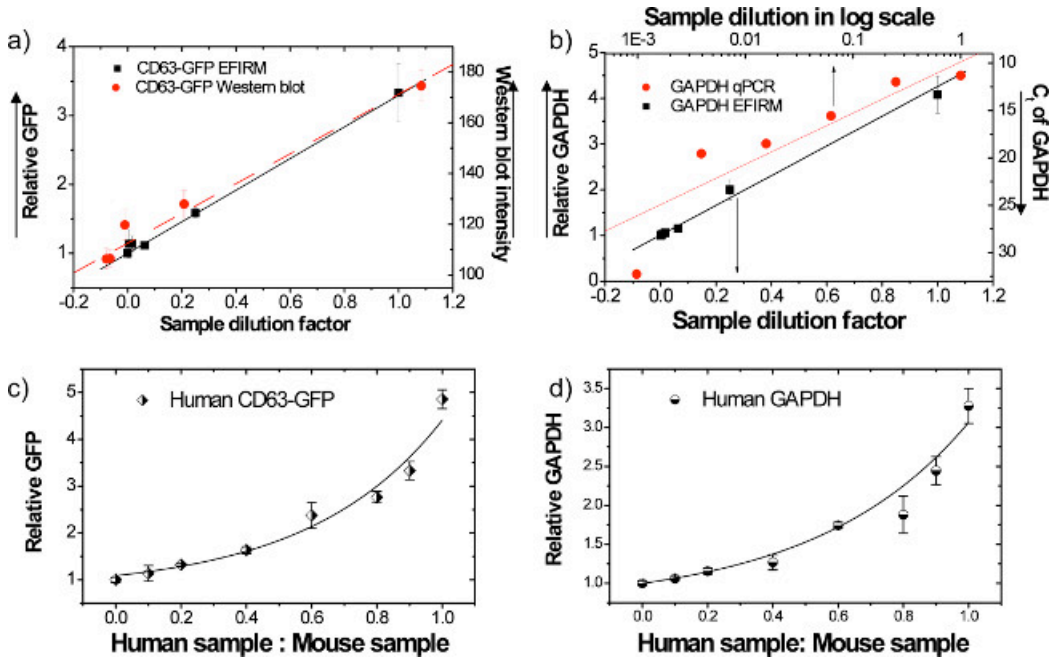

Figure 5. EFIRM Performance Evaluation for mRNA and protein levels using dilute samples of exosomes. (**A**) Comparison of EFIRM and western blot on GFP moiety. (**B**) Comparison of EFIRM and qPCR for GAPDH mRNA. (**C**) Relative detected GFP signal levels for human exosomes diluted in different amounts of mouse exosome. (**D**) Relative detected signal for GAPDH mRNA for human exosomes diluted in different amounts of mouse exosome. This figure is from Wei *et al.*[14] Please click here to view a larger version of this figure.

Exosomal Content Quantified From Mouse Saliva

In order to determine the viability of detecting exosomal content in salivary samples, a nude mouse model was used. This nude mouse model received an interpleural injection of 1 x 10^6 human lung cancer cell H460 (a cell line which was transfected to also express the exosome marker human CD63-GFP) or saline. There were 11 mice that received a negative control of 100 µl of saline, and 9 mice received the cell injection. Following 20 days, serum and saliva samples were collected from the two groups of mice.

Usage of the EFIRM method allowed quantification of the relative levels of human CD63-GFP protein in the mice serum and saliva. A comparison of the relative levels between the two serum and saliva of the mice demonstrated linear correlation between the two biofluids (R = 0.79). Additionally, significant difference was observed in the human CD63-GFP levels for the H460 injected mice against the saline injected mice. This suggests that exosomes from distal tumor cells are trafficked into the vasculature and saliva. The results comparing serum and saliva are shown in **Figure 6**.

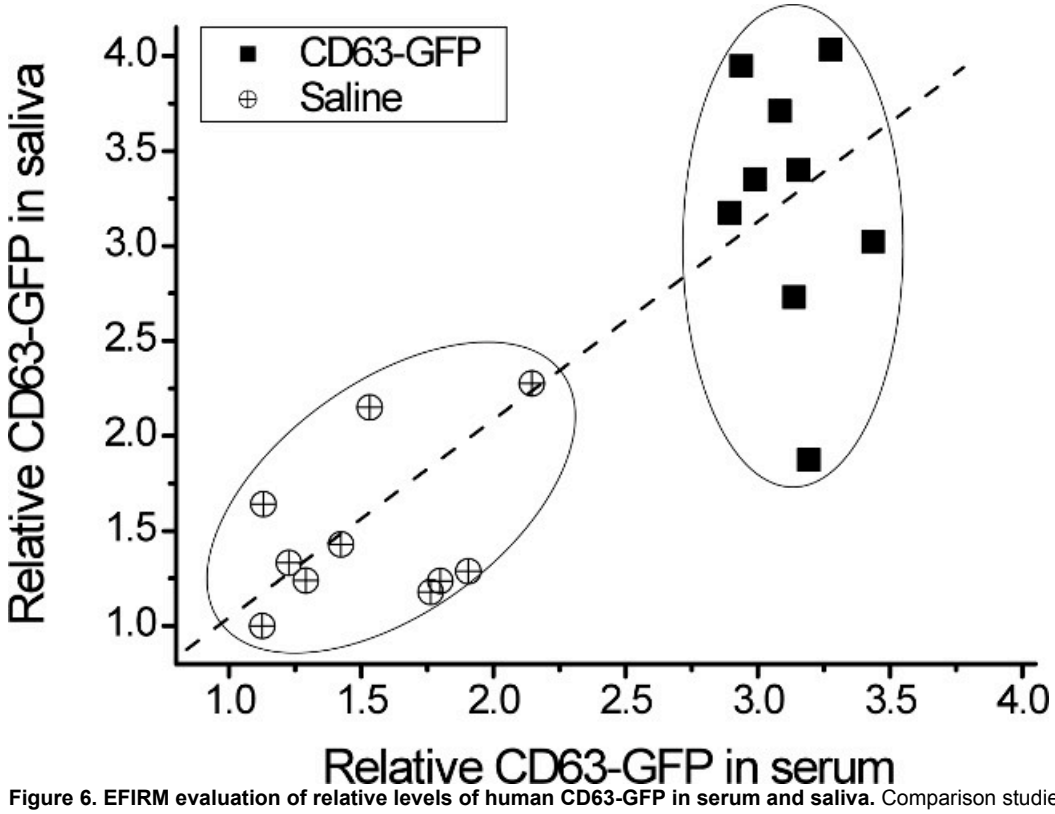

Figure 6. EFIRM evaluation of relative levels of human CD63-GFP in serum and saliva. Comparison studies between the relative levels of human CDP63-GFP from mice injected with human lung cancer cells. Linearity exists between serum and saliva samples for human CD63-GFP. Levels of human CD63-GFP are elevated in mice injected with cells (compared to saline injected mice). This figure is from Wei *et al.*[14]

Discussion

As the results indicate, anti-human CD63 coated magnetic nanoparticles are able to specifically capture small particles that have a size ranging from 70-100 nm. This captured particle is consistent with the previously observed profile of exosomes. Furthermore, the usage of the low-voltage CSW following the capture of the particles is shown to remove them from the bead surface and cause DNA degradation profiles similar to that of a traditional lysis buffer based method for cargo release. This data indicates that the workflow of exosomal cargo release can be simplified through the application of a cyclic square wave for the disruption of the exosome lipid membrane. This simplified method allows for rapid cargo release without the need for lysing buffers that may adversely affect exosome cargo, and as a result the EFIRM method appears to be a preferable method for exosomal content studies compared to the traditional methods of ultracentrifugation and usage of lysis buffers. The sensitivity, specificity, and ease of the EFIRM method makes it ideal for extracting exosomes from a biofluid and rapidly testing internal content for both nucleic acids and protein targets.

The EFIRM methodology was applied to the study of a nude mouse model that was injected with H460 human lung cancer cell. The H460 human lung cancer cells were additionally transfected with human CD63-GFP so that exosome surface markers CD63 would have a fluorescent property. Following a period of 20 days, mouse serum and saliva samples were collected, and the EFIRM methodology was used to analyze their exosomal content. This study demonstrated linear correlations between the serum and saliva samples (R = 0.79). The implications of these results are twofold: First, the ability to capture exosomes from biofluids suggests that distal cancer cells are able to transmit exosomes to other segments of the body, most notably in this study the oral cavity. Second, the linearity observed between the serum and saliva samples suggests that saliva can be a credible biofluid for the capture of exosomes that are produced in a diseased state of the body (*i.e.*, cancer) and analysis of their molecular content.

The method described and the subsequent results show that the method is appropriate for extracting exosomes from biofluids based on their surface markers (in the scope of this work, CD 63 is the surface marker selected). For future projects, it is of merit to examine whether targeting additional surface markers exosomes (such as CD 9) or combining the EFIRM method with other techniques will improve the extraction and testing of exosome content.

Exosome biology is a promising area of study for translational medicine, and the following study's data suggests that salivary exosomal analysis can be a venue for identifying disease discriminatory biomarkers. Future investigation seems appropriate in determining whether distal disease biomarkers can be used for non-invasive diagnostics. EFIRM as a method may help pave the way to more effective analysis of such targets within exosomes.

Disclosures

David Wong is co-founder of RNAmeTRIX Inc., a molecular diagnostic company. PeriRx LLC sublicensed intellectual properties pertaining to molecular diagnostics from RNAmeTRIX. David Wong is a consultant to PeriRx.

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