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# Electric Field-Induced Disruption and Releasing Viable Content from Extracellular Vesicles (EVs)

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## Abstract

In order to more fully elucidate the biogenesis of exosomes and understand the role of exosomes in disease processes, it is necessary to develop methods to capture exosomes and induce the unloading of viable cargo. Traditionally, ultracentrifugation followed by chemical based exosome lysis techniques is used to isolate these extracellular vesicles and release their internal content. Here we describe a novel technique for capturing and releasing exosomal content through magnetic bead-based exosome extraction coupled with electric field induced release and measurement (EFIRM). The usage of low-voltage electric fields allows the exosome to be lysed without chemical treatment, and surface immobilized probes can allow for the rapid capture of the content of lysed exosomes. EFIRM as an integrated exosome lysing and analysis system offers great potential for the investigation of exosomes in clinical and basic science contexts.

### Keywords

Exosomes; Extracellular vesicles; Electric field induced release and measurement (EFIRM); Biomarkers; Diagnostics

## 1. Introduction

Exosomes are 30–100 nanometer microvesicular structures that have been found to be associated with the function of intercellular communication between different organ systems of the body(1). As Zhang et al (2) have noted, the transfer of oncogenic proteins and of nucleic acids via exosomes modulates the activity of recipient cells contribute to roles in tumor growth, progression, metastasis, and drug resistance(3). Consequently, it is of particular interest to develop methods to precisely unload the internal cargo of exosomes and precisely analyze their internal molecular content(4). The techniques for unloading the exosomal cargo involve usage of chemical agents for lysing exosome samples or usage of the electric field induced release and measurement (EFIRM) technique. EFIRM is a lysis-buffer free technique for rapidly unloading exosome cargo and analyzing their internal content through the application of a low-voltage cyclic square wave (See Figure 2A) (5).

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There have been previous demonstrations of the use of the EFIRM technique for the analysis of mRNA targets from murine models of pancreatic and lung cancer ((6),(7),(8)). During lysis buffer method incubation times, unloaded content released from exosome samples degrade over time. Wei. et al (5) used transmission electron microscopy to visualize the effect of both the chemical lysis buffer and the electric field based lysis method on CD63 coated exosomes, demonstrating that both methods worked identically (see Figure 2B). The degradation profiles of exosomal mRNA was also demonstrated to be the same in both the chemical lysis and EFIRM lysis methods (see Figure 2C). One advantage of the EFIRM technique to note is that EFIRM integrates the exosome lysis procedure together with the high-sensitivity detection (and capture) of molecular content from the exosome (see Figure 1C); this feature can allow for reducing the potential of content degradation when exosome cargo is exposed to the extravesicular milieu. This article details the methods utilized in our work for the performance of exosome cargo unloading using both chemical-based and EFIRM-based techniques. Since EFIRM is able to immediately capture the lysed content from exosomes, this feature will also be included in the protocol.

Successful exosomal content analysis includes and requires both an initial extraction of exosomes from a biofluid proceeding the lysis process(9). There are two primary exosome extraction techniques that will be described: the first to be discussed is the ultracentrifugation method, which allows for sized-based purification of exosomes with minimally contaminated pellets of exosomes. The second approach to be discussed is a magnetic bead-based exosome precipitation technique, which utilizes surface specific marker antibodies (such as anti-CD63 antibody) to capture exosomes. This bead-based approach allows for exosome extraction with a lower sample volume and can be performed quickly, but does not allow for fractionation of microvesicles by size. EFIRM and chemical lysis techniques have both been used with both exosome extraction methods. The various extraction and unloading techniques that can be used in conjunction with EFIRM must be weighed by the individual researcher based on the study goals.

## 2. Materials

### 2.1 Ultracentrifugation-based exosome extraction

1. Culture medium specific to cells of interest
2. Atmosphere of 95% air and 5% $CO_2$
3. Phosphate-buffered saline (PBS)
4. 50 mL polypropylene centrifuge tubes
5. Counter-top centrifuge
6. 100 mL polycarbonate tubes
7. 500 mL disposable filter unit (0.22 μm)

### 2.2 Radioimmunoprecipitation assay (RIPA) buffer-based Exosome Cargo Unloading

1. RIPA lysis buffer

a. 50 mM Tris-HCl (pH 7–8)
b. 150 mM NaCl
c. 0.1% SDS
d. 0.5% Sodium deoxycholate
e. 1% Triton X-100

2. Protease and phosphatase inhibitor cocktail tablet
3. 100 mL polycarbonate tube

### 2.3 Magnetic Bead-based Exosome Extraction

1. Streptavidin-coated magnetic microparticles
2. Phosphate Buffered Saline (PBS)
3. Magnetic bead rack
4. Microcentrifuge tube
5. 1.0 mg/mL biotinylated mouse anti-human CD63 antibody (or other exosomal surface marker designated appropriate for study)
6. Microcentrifuge tube rotator rack
7. Casein-PBS
8. Biofluid of interest (e.g. serum, saliva, cell culture media)
9. 1M Tris-HCl

### 2.4 Electric Field Induced Release and Measurement of Exosomal Content (EFIRM)

1. Gold 3-electrode system (working, counter, and reference electrodes)
2. Monomer DNA probe mixture
   a. 100 nM DNA probe
   b. 0.3 M KCl
   c. 10 mM pyrrole
   d. Ultrapure Water
3. Microcentrifuge tube
4. Electrochemical potentiostat
5. Distilled water
6. Nitrogen gas
7. Detector probe (hybridizes to the DNA probe (*see* Note 6)
8. Magnet array of neodymium magnets
9. 150 unit/mL anti-fluorescein horseradish peroxidase 1:1000 dilution (HRP)

10. Casein-PBS

11. 3,3', 5,5'-tetramethylbenzidine (TMB) substrate solution

## 3. Methods

Carry out all procedures at room temperature unless otherwise specified.

### 3.1 Ultracentrifugation-based Exosome Extraction from Cell Culture

1. Grow cells of interest in flasks with culture medium. Place in an incubator at 37°C and at an atmosphere of 95% air and 5% $CO_2$ until the cells reach 55% to 75% confluency. The time period of this step depends on cell-type.

2. Remove the culture medium, wash with phosphate-buffered saline (PBS), and finally replace medium with a similar volume of exosome-free medium. Return the cells to the incubator (*see* Note 1).

3. Collect the conditioned media with a pipette. Transfer the filtered media to 50 mL polypropylene centrifuge tubes. Centrifuge cells at 300 x g for 10 minutes and collect the conditioned medium supernatant with a pipette into new 50 mL polypropylene centrifuge tubes (*see* Note 2).

4. Centrifuge the collected media at 2000 x g for 20 minutes. Pipette the supernatant and transfer to 100 mL polycarbonate tubes (maximum volume 68mL) for use with a countertop centrifuge (*see* Note 2).

5. Mark one side of each tube with a marker (do not use anything permanent), orient the tube in the rotor with the mark facing up, and centrifuge the medium at 16,500 x g for 20 minutes. Transfer the supernatant to a 500 mL disposable filter unit (0.22 μm). Transfer filtered media to new 100 mL polycarbonate tubes (*see* Note 3).

6. Before centrifugation, mark the approximate location on the tubes of where the pellet would be. Then, centrifuge at 120,000 x g for 70 minutes and then remove the supernatant completely by aspiration. The product of this step will be exosomes (*see* Note 4).

7. Allow the pellet to dry in the tube, then immediately re-suspend the pellet in each tube in 1 mL of ice-cold PBS, using a micropipette. Pool the re-suspended pellets from all the tubes containing materials from the same cells in a single ultra-centrifuge tube, and then add PBS to fill the tube completely (*see* Note 4).

8. Centrifuge at 100,000 x g again for 70 minutes, and then remove the supernatant as completely as possible by aspiration. Proceed with caution as to not lose pellet.

9. Exosomes

   To re-suspend the pellet (i.e., exosomes), gently add a small volume (~160 μL/68 mL of starting volume) of PBS and allow the pellet to sit in the PBS for ~15–30 minutes at room temperature. Time will depend on size of pellet. Afterwards,

gently swirl the PBS over the pellet until it fully dissolves. Aliquot your re-suspended exosomes into smaller volumes ~50 μL and store at -80°C for long-term storage (up to 1 year) and for downstream applications. Avoid repeated freezing and thawing.

### 3.2 Magnetic Bead-based Exosome Extraction

1. Pipette a well-mixed solution of 5μL of Steptavidin-coated magnetic microparticles into 495 μL of PBS buffer in a microcentrifuge tube to resuspend the beads using a magnetic rack to hold multiple tubes. Wash and resuspend the beads by letting the tubes sit on the rack for 1 min, then using 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 mix the solution with the beads using the pipette. Then put the tubes back on the magnetic rack to again separate the beads from the solution. Perform this removal of buffer for a total of three times.
3. Move the tubes to the non-magnetized portion of the magnetic rack and resuspend the beads into 490 μL of PBS buffer. Pipette 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.
4. After placing the microcentrifuge tubes with the mixture on a sample rotator, set the parameters for the sample rotator for reciprocal rotation at 90° tilting for 5 seconds and vibrating at 5° for 1 second. Rotate the sample-bead mixture tubes for 30 minutes at RT.
5. After 30 minutes of rotation at RT, place the tubes back in the magnetic rack for 5 minutes. 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.
6. Label each tube with targeted sample ID. After pipetting a 10 μL sample of serum or saliva into the microcentrifuge tube, use the pipette to mix the sample and beads.
7. Place the tubes with sample and anti-human CD63 antibody beads on rotator and rotate for 2 hours at RT, using the same rotator parameters as described in step 4.
8. Perform a triple wash by magnetizing to separate the 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.

### 3.3 Radioimmunoprecipitation assay (RIPA) buffer-based Exosome Cargo Unloading

1. Dissolve exosome pellet in RIPA lysis buffer (~160μL/68mL of starting volume for cell culture based exosome extract) containing protease and phosphatase inhibitors (1 inhibitor tablet each/10 mL of RIPA). Pipette thoroughly, followed

by vortex. To further lyse exosomes, sonicate the sample in a water bath 3X for 5 minutes at room temperature with vortex in between each sonication step. Centrifuge sample at 13,000 x g for 5 minutes at room temperature and transfer supernatant to a 100 mL polycarbonate tube. Store at -80°C for long-term storage.

## 3.4 Electric Field Induced Release and Measurement of Exosomal Content (EFIRM)

### 3.4.1 Precoating of Electrode with a DNA Probe

1. For standard electric field-induced release and measurement (EFIRM) experiments, a gold 3-electrode system consisting of working, counter, and reference electrodes was used as the base for the cargo unloading procedure.
2. In a microcentrifuge tube, prepare a stock mixture that will consist of 100 nM DNA probe, 0.3 M KCl, and 10 mM pyrrole in Ultrapure Water in a microcentrifuge tube. Vortex the mixture gently to ensure an adequate mixture of the different components of the solution (*see* Note 5).
3. Pipette 60 μL of the prepared monomer DNA probe mixture and ensure the monomer mixture provides adequate coverage of the individual working, counter, and reference electrodes. Inspect the seals and dividers of the electrodes used to ensure that there is no cross contamination between different electrodes.
4. Apply a cyclic square wave (CSW) electric field profile to each electrode surface to initiate the formation of a conducting polymer surface coated with capture probes. The polymerization process is initiated by first applying a +350 mV potential for 9 seconds and then immediately changing the potential to +950 mV for 1 second. This CSW profile is applied to each electrode for a total of 10 cycles, which means in total the application of the electric field will be 100 seconds of applied electric field.
5. After applying the electric field profile for 100 seconds, rinse each electrode surface 3 times with distilled water and dry with nitrogen gas. This procedure is performed to ensure that unbound monomeric units from the mixture are not remaining on the electrode and will not interfere with the cargo unloading phase.

### 3.4.2 Exosome Cargo Unloading

1. Pipette 5 μL of 1 μM of detector probe into 495 μL of the exosome extract mixture. Mix the detector probe solution with the exosome extract by repeatedly pipetting. Pipette 60 μL of the new mixture into each well. Under each electrode, there should be a magnet array that consists of neodymium magnets aligned to each corresponding electrode sensor. (*see* Note 6 and Figure 1B)
2. After adding the mixture to each well, inspect to ensure that mixture completely covers each electrode. Tap the side of the plate to ensure an even distribution of liquid. Apply 20 cycles of electric field across the electrode, which each cycle consisting of 9 seconds at -300 mV potential and 1 second at +200 mV potential

(for a total of 200 seconds). During this process, the exosomal content will be released and hybridized onto the surface probe.

3. Wash off the unbound contents in the mixture by rinsing each electrode surface 3 times with distilled water. Dry the electrodes with nitrogen gas.

### 3.4.3 Reporter Antibody and Readout

1. Pipette 60 μL of 150 unit/mL anti-fluorescein antibody conjugated to horseradish peroxidase (HRP 1:1000 dilution) into a tube. Add Casein/(PBS) to dilute the mixture.
2. Apply an electric-field to conjugate the complex anti-fluorescein HRP to the probe surface. Apply a-200 mV potential for 1 second and +500 mV potential for 1 second for 5 cycles (total 10 seconds).
3. Wash the electrode surface by rinsing 3 times with distilled water. Following wash, dry with nitrogen gas.
4. Pipette 60 μL of 3,3', 5,5'-tetramethylbenzidine (TMB) substrate solution onto the surface of each well. Measure the electrode current at -200 mV for 60 seconds to perform amperometric readout.

## Notes

1. Cells that grow fast may be overconfluent and start dying after 48 hours. In this case, take the conditioned medium after 24 hours. Cells that are very sensitive may not like growing in exosome-free medium or in FBS-free medium. In this case, take the supernatant after 24 hours.
2. Ensure that none of the pellet is collected or contaminates the supernatant. Use a pipette and leave behind 2mL of media.
3. Ensure that none of the pellet is collected or contaminates the supernatant. There will probably not be a visible pellet at this step. When removing the supernatant with the pipette, hold the tube at an angle, so that the pellet is always covered with supernatant, and stop removing supernatant when half a centimeter of liquid is still covering the pellet.
4. For this high-speed centrifugation, all tubes should be at least three-quarters full. Use PBS to fill tubes. Centrifugation time is calculated to allow a full hour at 100,000 × g, i.e., ~10 min to reach 100,000 × g plus 1hr at the final speed. A longer time (up to 3hrs) will not damage the exosomes.
5. The probe utilized should be designed to be complementary with the sequence that you desire to capture from the exosome. The GAPDH probe, for example, consists of the sequence 5′-Biotin-AGGTCCACCACTGACACGTTG. If one seeks to only lyse the exosome without capturing the sequence, one can skip to the cargo unloading step of this section.

6. In the case of detecting GAPDH as a reference, the detector probe 5′-GCAGTGGGGACACGGAAGGCC-Fluorescein-3′ was used. The inclusion of a detector probe can be neglected if one seeks to only lyse the exosome without performing detection using the electrode.

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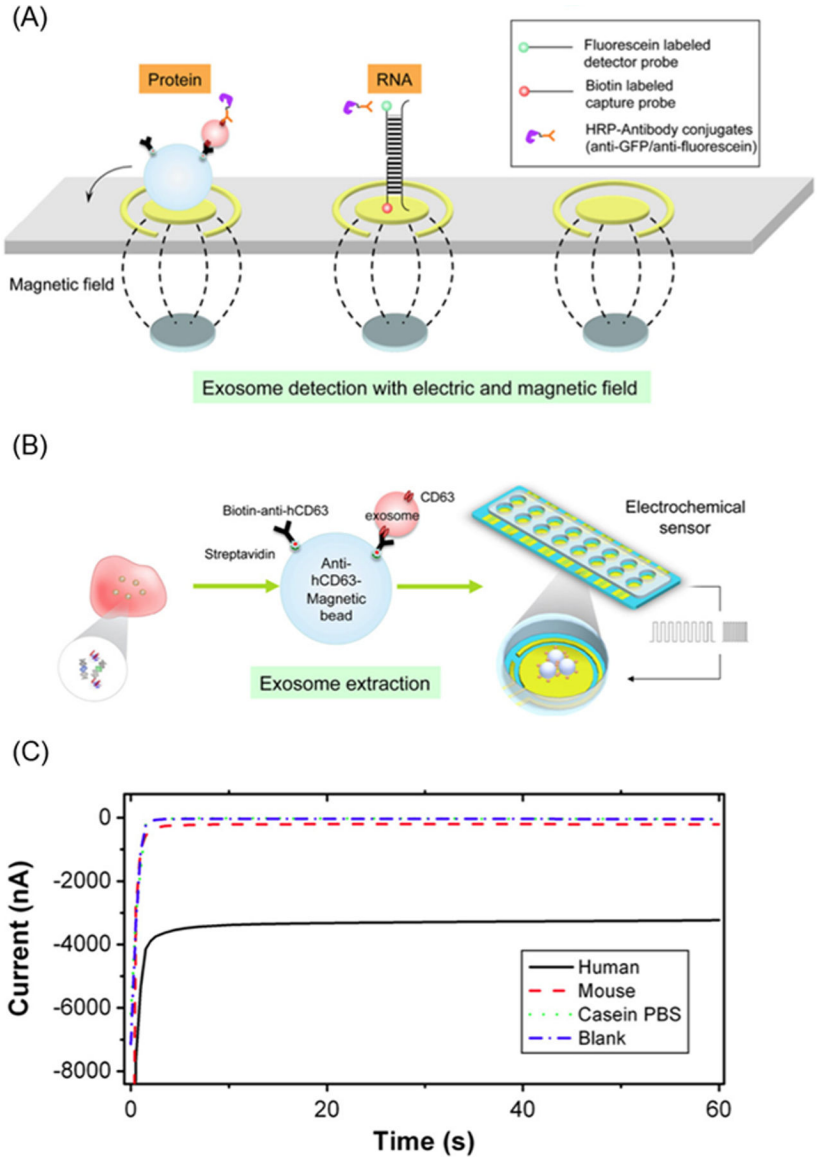


**Figure 1: Components of EFIRM Method.**

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. Figure is from Wei *et al* (reproduced with permission)

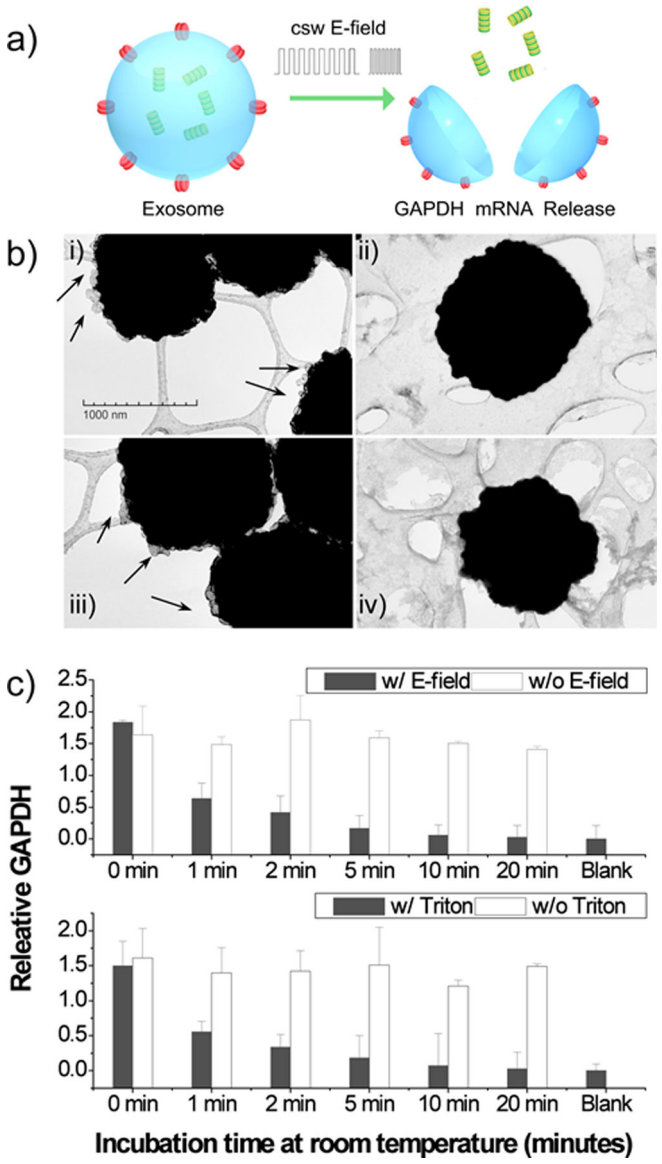


**Figure 2: Results relating to Cyclic Square.**

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.* (reproduced with permission)