Correlating Live Cell Viability with Membrane Permeability Disruption Induced by Trivalent Chromium

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Supporting Information

ABSTRACT: Cr(III) is often regarded as a trace essential micronutrient that can be found in many dietary supplements due to its participation in blood glucose regulation. However, increased levels of exposure have been linked to adverse health effects in living organisms. Herein, scanning electrochemical microscopy (SECM) was used to detect variation in membrane permeability of single cells (T24) resulting from exposure to a trivalent Cr-salt, CrCl₃. By employing electrochemical mediators, ferrocenemethanol (FcMeOH) and ferrocenecarboxylic acid (FcCOO⁻), initially semipermeable and impermeable, respectively, complementary information was obtained. Three-dimensional COMSOL finite element analysis simulations were successfully used to quantify the permeability coefficients of each mediator by matching experimental and simulated results. Depending on the concentration of Cr(III) administered, three regions of membrane response were detected. Following exposure to low concentrations (up to 500 μM Cr(III)), their permeability coefficients were comparable to that of control cells, 80 μm/s for FcMeOH and 0 μm/s for FcCOO⁻. This was confirmed for both mediators. As the incubation concentrations were increased, the ability of FcMeOH to permeate the membrane decreased to a minimum of 17 μm/s at 7500 μM Cr(III), while FcCOO⁻ remained impermeable. At the highest examined concentrations, both mediators were found to demonstrate increased membrane permeability. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability studies were also conducted on Cr(III)-treated T24 cells to correlate the SECM findings with the toxicity effects of the metal. The viability experiments revealed a similar concentration-dependent trend to the SECM cell membrane permeability study.

1. INTRODUCTION

Many heavy metal ions, such as cadmium and arsenic, have toxic properties, leading to detrimental effects in living organisms.¹ By contrast, metals such as zinc, iron, and calcium, take part in biological systems and are required for healthy growth and development of an organism. These trace essential heavy metals are necessary in small quantities but become toxic at higher concentrations.² The toxicity of metals in the body can also be dependent on the metal oxidation state. For example, Cr(III) is regarded as an essential micronutrient that is often found in many dietary supplements to promote cellular homeostasis.³⁻⁵ This is due to its involvement with low-molecular-weight chromium-binding-substance that maintains the active conformation of the insulin receptor, important for blood glucose regulation. However, high concentrations of Cr(III) exposure can lead to toxicity.⁶⁻⁸ On the other hand, Cr(VI) is known to induce oxidative stress, cytotoxicity, and carcinogenicity, regardless of its concentration.⁶,⁷,⁹⁻¹⁵ Elevated levels of Cr(III) have been associated with heightened production of reactive oxygen species (ROS).¹,⁵,¹³,¹⁶⁻¹⁹ In some cases, Cr(III) has been shown to lead to higher ROS levels than the toxic Cr(VI) oxidation state.¹¹ The mechanism of Cr(III) toxicity is believed to involve not only elevated levels of ROS, but also the direct interaction of Cr(III) with DNA. The resultant DNA adducts lead to genomic instability.⁶ However, Cr(III) does not easily cross the cell membrane and is commonly brought into the cell by active means such as pinocytosis, reducing its toxic effects.¹⁶⁻²¹

Cr is known to bioaccumulate primarily in the kidneys, liver, and lungs of mammals, potentially leading to adverse health effects in these tissues as concentrations increase.¹³,¹²⁻²⁴ Monitoring exposure to Cr, commonly through urine content, has determined a substantial half-life of ~10 years in the body.²²,²³ Due to its ability to bioaccumulate in the urinary tract and its potential to cause cellular damage, our current study focuses on T24 cells and human urinary bladder carcinoma.

In molecular biochemistry and biology, there are many research tools. For instance, ROS and reactive nitrogen species (RNS) signaling and redox reactions can be investigated via fluorescence spectroscopy and electrochemistry.²⁵⁻²⁹ How-
ever, many techniques focus on bulk analysis of cell samples. This provides an excellent indication of population traits; single cell techniques are needed to examine sample heterogeneity. Single live-cell studies are challenging. Since bioanalytical tools such as spectroscopy, atomic force microscopy, and flow cytometry can provide information on a series of individual cells, such as ROS release, events that are linked to a specific area of a live cell are tough to assess. Neither can these tools analyze chemical activity on an individual spot of interest over a cell membrane.

Scanning electrochemical microscopy (SECM) is a viable method of studying biological samples while leaving their homeostasis unaltered and has been successfully employed in numerous cellular studies. SECM provides a method of single cell characterization and can be utilized for location specific analysis of the cell membrane, limited only by the diameter of the electrode. This technique has proven useful for a range of investigations involving reaction kinetics, surface and interface processes, microstructure fabrication, cellular imaging, membrane transport, multidrug resistance, nerve cell signaling, cellular ROS and reactive nitrogen species (RNS), metabolic interactions, and cellular redox processes. Furthermore, SECM can be used for the rapid quantification of single live cell topography and surface reactivity.

These dynamic cellular processes can be interpreted from variations in the faradic current at the ultramicroelectrode (UME) as it approaches the cell from above. This type of analysis is commonly performed in feedback mode, with the addition of a nontoxic electrochemical mediator. At small probe-to-cell distances, the membrane permeability can affect the tip current. In proximity to the membrane, the availability of the mediator from the bulk solution to the UME becomes hindered. This hindered diffusion causes a decrease in current termed as negative feedback. However, if the mediator is permeable to the cellular membrane, it may cross the membrane and reach the UME tip. This has an effect of weakening the negative feedback that would be observed when compared to an impermeable membrane of the same geometry. The resultant data are commonly reported as probe approach curves (PACs), displaying the normalized current as a function of normalized distance to the membrane. Since it is known that a correlation between membrane permeability and cellular viability/activity exists, here, SECM was used to explore the membrane responses of T24 cell exposure to trivalent CrCl3. Operating in the depth scan mode, the UME is scanned in a two-dimensional (2D) \( x-z \) plane above the cell, generating a 2D current map consisting of hundreds of individual PACs. Then, three-dimensional (3D) COMSOL finite element analysis simulations were utilized to generate theoretical depth scan images. Through the comparison of theoretical and experimental data, permeability coefficients were extracted. Very interestingly, membrane permeability on a specific spot of interest can be determined very straightforward, and membrane permeability mapping above an area over a live cell can be obtained easily.

2. RESULTS AND DISCUSSION

2.1. Off-Axis Characterization of Cell Membrane Permeability. SECM depth scanning moves the UME in the \( x-z \) plane of solution over a single live cell. This generates a 2D electrochemical map of the region of space over the cell. Cross sections can be taken of theoretical and experimental (Figure 1A) depth scans to produce more conventional PACs. Depth scan images are comprised of hundreds of PACs at different locations over the cell surface. To analyze experimental PACs taken at positions displaced from the cell center requires full 3D simulations, as the symmetry axis required for the more common 2D axially symmetric method cannot be maintained. The experimental PACs extracted at these locations can be overlaid on the theoretical ones to obtain quantitative characterization of membrane permeability.

![Figure 1. (A) Experimental depth scan with PAC cross sections extracted along colored planes. Depth scan was acquired after 1 h incubation of the cell sample with 250 \( \mu \)M of CrCl3. Overlap of experimental and theoretical PACs corresponding to extraction planes seen in (A), at (B) the cell center, (C) 1/2 the cell radius from the center, and (D) the cell edge.](image-url)
may be attributed to the overwhelming of antioxidant defense concentrations of 5000 and 7500 μM, showing average P values of 31 ± 6 and 17 ± 8 μm/s, respectively. This decrease may be attributed to the overwhelming of antioxidant defense systems since Cr(III) toxicity can lead to increased ROS generation, followed by lipid peroxidation.6 Lipid peroxidation is known to affect changes in the bilayer thickness and membrane fluidity43 and is a likely mechanism by which membrane characteristics are altered.

After exposure to 10 000 μM Cr(III), relaxation in P occurs, returning to 83 ± 8 μm/s (Figure 2C). This relaxation resulted in the membrane permeability coefficient that is similar to the control samples (80 ± 6 μm/s). At this concentration, Cr(III) likely surpasses the trace essential requirements and becomes toxic. The resulting ROS production overwhelms the cellular defense systems, such as superoxide dismutase, catalase, and glutathione peroxidase, leading to oxidative damage and increased cell membrane damage.

Note that a complete data set (N > 4 cells) was obtained for each Cr(III) incubation concentration (example curves are shown in Figure 2A−C) and was used to determine its average ± standard error membrane permeability coefficients, which is summarized in Figure 2D. Three distinct regions of membrane response were observed when FcMeOH serves as the SECM mediator. Lower concentrations exhibited relative stability in membrane response with incubation concentrations below 50 μM (region a). Incubation of cells with 50–500 μM Cr(III) resulted in deviation from the previously observed membrane stability, and a decrease in permeability coefficient (region b). Higher concentrations, exceeding 500 μM Cr(III), (750 and 1000 μM) caused the permeability coefficient to increase, and return to a permeability coefficient value similar to the control (region c).

2.3. Cell Membrane Permeability to the Electrochemical Mediator FcCOO⁻. Under control conditions, SECM revealed that the membrane permeability coefficient to ferrocenecarboxylate (FcCOO⁻) was 0 μm/s (membrane impermeable). This was expected due to the charged nature of the mediator at pH 7.4. The membrane permeability
remained relatively stable around 0 μm/s after Cr(III) treatments less than 7500 μM (50–5000 μM, Figure 3A). Minor increases were observed after 100, 750, and 1000 μM treatments, showing average ± standard error membrane permeability coefficients of 29 ± 17, 25 ± 21, and 37 ± 18 μm/s, respectively. Due to the more hydrophilic and charged nature of the FcCOO⁻ mediator, it is unable to cross the hydrophobic interior of the phospholipid bilayer (cell membrane). As a result, FcCOO⁻ is largely impermeable to the cell membrane under normal (control) conditions. These results were in agreement with the SECM results previously described, as FcMeOH is partially permeable in nature, the decrease in membrane permeability trend is observable by FcMeOH but not observable by FcCOO⁻. FcCOO⁻, however, allows for more accurate imaging of cell topography since it is largely independent of membrane permeability under control conditions. The use of impermeable redox mediators when performing SECM on biological samples can indicate significant disruption in normal cell membrane performance. Therefore, the use of FcCOO⁻ as the SECM mediator can provide good indication of when Cr(III) no longer behaves as a trace essential metal, but rather as a toxin.

After T24 cells were treated with 7500–10 000 μM Cr(III), a trend of increasing permeability to FcCOO⁻ was observed (Figure 3B), which was similar to the trend determined with FcMeOH (Figure 2D). When incubated with 7500 μM Cr(III), the cell membrane permeability coefficient increased to 19 ± 16 μm/s. The highest concentration sample (10 000 μM Cr(III)) was observed to induce a membrane permeability coefficient of 260 ± 53 μm/s. This confirms a concentration-
dependent effect of Cr(III) on membrane permeability in T24 cells. We suspect, over longer exposure to Cr(III), that this may result in the selection of T24 cells with genetic mutations that offer greater resistance to Cr(III) poisoning. This permeability increase is likely attributed to the overwhelming of antioxidant defense systems as previously discussed. The effects of high Cr(III) concentration on the cell membrane were similar to that seen with the FcMeOH mediator. This confirms significant oxidative cell membrane damage, such that polar compounds (FcCOO\(^-\)) are capable of entering the cell. In particular, lipid peroxidation is known to drastically change the membrane characteristics by increasing the number of polar functional groups within the internal membrane region.\(^{65,67}\)

The membrane permeability trend for FcCOO\(^-\) was found to agree with those previously discussed for FcMeOH. Figure 3C shows the initial permeability coefficient to be 0 μm/s for FcCOO\(^-\). A trend of stability is observed (region a) at all concentrations occupied by regions a and b in the FcMeOH trend (Figure 2D). A concentration of 7500 μM Cr(III) appears to be the onset concentration at which T24 cells become permeable to FcCOO\(^-\). The permeability to FcCOO\(^-\) significantly increases following this concentration (region c), similar to that observed with FcMeOH.

A direct comparison of Figure 2D with Figure 3C also shows the apparent similarities in membrane responses following excessive CrCl\(_3\) exposure. Examination of the Cr(III) concentrations at which the membrane response is triggered in SECM can be used to judge relative toxicities.

2.4. Viability Study of T24 Cells Incubated with CrCl\(_3\).

Since changes in permeability were found to be dependent on concentration, the overall health of the Cr(III)-treated T24 cells was also considered. Here, cellular health was investigated by employing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, routinely used to measure cell viability of a bulk population of live cells (Figure 4). The MTT assay measures the reduction of the tetrazolium salt to a purple formazan product, which is quantitatively related to cell viability. FcCOOH dissociates at pH 7.4, forming ferrocenecarboxylic acid (FcCOOH, 97%), potassium chloride (KCl, 99%), and chromium chloride hexahydrate (CrCl\(_3\), 6H\(_2\)O, 99%) were purchased from Sigma-Aldrich (Mississauga, ON). Mediator solutions containing 0.9 mM FcMeOH with 0.1 M KCl were regularly prepared in deionized water (18.2 MΩ cm). The FcMeOH solution is diluted to half concentration with 1× phosphate-buffered saline (PBS) before used experimentally. A FcCOOH stock solution of 0.5 mM was prepared in PBS. At pH 7.4, FcCOOH dissociates into FcCOO\(^-\) in solution. Stock solutions of 0.5 M CrCl\(_3\) were prepared in autoclaved deionized water and syringe filtered through sterile 0.2 μm Supor Membranes (PALL Life Sciences, Mississauga, ON). The compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was acquired from R&D Systems Inc. (Minneapolis, MN) in the TACS MTT Cell Proliferation Assay Kit. Spectroscopic grade dimethyl sulfoxide (DMSO) was used to dissolve the formazan crystals (Caledon Laboratory Ltd., Georgetown, ON).

4. EXPERIMENTAL SECTION

4.1. Materials. Ferrocenemethanol (FcMeOH, 97%), ferrocenecarboxylic acid (FcCOOH, 97%), potassium chloride (KCl, 99%), and chromium chloride hexahydrate (CrCl\(_3\), 6H\(_2\)O, 99%) were purchased from Sigma-Aldrich (Mississauga, ON). Mediator solutions containing 0.9 mM FcMeOH with 0.1 M KCl were regularly prepared in deionized water (18.2 MΩ cm). The FcMeOH solution is diluted to half concentration with 1× phosphate-buffered saline (PBS) before used experimentally. A FcCOOH stock solution of 0.5 mM was prepared in PBS. At pH 7.4, FcCOOH dissociates into FcCOO\(^-\) in solution. Stock solutions of 0.5 M CrCl\(_3\) were prepared in autoclaved deionized water and syringe filtered through sterile 0.2 μm Supor Membranes (PALL Life Sciences, Mississauga, ON). The compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was acquired from R&D Systems Inc. (Minneapolis, MN) in the TACS MTT Cell Proliferation Assay Kit. Spectroscopic grade dimethyl sulfoxide (DMSO) was used to dissolve the formazan crystals (Caledon Laboratory Ltd., Georgetown, ON).

4.2. Cell Culture. Human urinary bladder cancer cells (T24 cells (HTB-4)) (ATCC, Manassas, VA) were grown and maintained in McCoy’s 5a medium (ATCC) containing 10% fetal bovine serum at 37 °C with 5% CO\(_2\). Experimental data were acquired using cells within a narrow passage window (45–48) to provide consistency among data sets.

Cell samples used for SECM experimentation were grown on uncoated 50 mm glass bottom Petri dishes (PS0G-0-30-F, MatTek Corporation, Ashland, MA). An hour prior to analysis, CrCl\(_3\) was injected directly into the growth media at the desired concentrations and incubated for 1 h. Immediately before examination, media were removed, and Cr(III)-treated T24 cells were washed thoroughly with sterile 1× PBS, before...
the addition of the mediator solution used for SECM analysis (FcMeOH or FcCOO−).

4.3. Cell Viability. The effects of CrCl3 on cellular viability were assessed using the MTT cell proliferation assay. Briefly, 2 × 10^5 cells per well were seeded onto Corning Scientific Costar 96-well polystyrene flat bottom plates. After 24 h, the growth medium was aspirated and replaced with new growth medium containing CrCl3. Sodium dodecyl sulfate (0.05, 0.10, 0.15, or 0.20 mg/mL) was used as a positive control for the assay.

After incubation for 1 h, the growth medium was carefully aspirated and replaced with 100 μL of fresh growth medium (absent of phenol red) and 10 μL of the MTT reagent. After 6 h, the MTT solution was carefully aspirated and the formazan crystals were dissolved with 50 μL of spectroscopic grade DMSO. The absorbance at 540 nm was read by an M1000 PRO plate reader (Tecan, Switzerland) following 1 s of shaking at 2 mm amp and 654 rpm. To remove the background media effects, empty (blank) wells were also treated according to the MTT protocol.

4.4. Instrumentation and Procedure. A detailed description of the SECM instrumentation and experimental procedures can be found elsewhere. Briefly, SECM experiments were carried out using a modified α-SNOM (WITec, Ulm, Germany). The α-SNOM set-up included a custom UME mount and a variable temperature Petri dish mount (Bioscience Tools, San Diego, CA). Optical imaging, and cell and electrode positioning were assisted by an inverted objective lens (50×, N.A. 0.55, W.D. 10.1 mm, Nikon, Japan) below the Petri dish. The UMEs were fabricated in-house following a previously developed methodology. Throughout this study, the UME consisted of a 10 μm Pt wire which was sheathed in insulating glass, with a ratio of glass to Pt diameter (RG) of approximately 3. Electrochemical instrumentation consisted of a CH Instruments Electrochemical Analyzer (CHI800B, CH Instruments, Austin, TX) with a CHI200 Picoamp Booster. An Ag/AgCl electrode was used as a combined counter and reference electrode. The CHI800B output channel was connected to the WITec α-SNOM data acquisition channel providing position specific electrochemical information.

The SECM is operated in feedback mode, where an electrochemical mediator (FcMeOH or FcCOO− in this study) is added to the live-cell sample. In the case of both of these, the mediators are oxidized at the UME tip by a 1e− reaction. Before each experiment, cyclic voltammetry (CV) was used to test for steady-state performance of the UME probe and electrochemical mediator. Identified through CV, the UME was biased at a probe potential of 0.3 V for FcMeOH and 0.4 V for FcCOO− for their respective steady-state oxidative currents (while in bulk solution, Figure S1). To limit the effects of forced convection, scan speeds did not exceed 21.4 μm/s.

Following incubation, the culture medium in the Petri dishes containing the CrCl3-treated cells was aspirated, and the cells were washed thoroughly with sterile 1× PBS. The electrochemical mediator solution was then added (FcMeOH or FcCOO−), and the dish was secured to the heated stage of the α-SNOM. The effects FcMeOH and FcCOO− have been investigated elsewhere and determined to be nontoxic over these experimental timescales. The samples were maintained at 37.0 ± 0.2°C to mimic physiological conditions for the cell line. Analysis of live-cell samples was conducted over a maximum duration of 60 min with individual scans taking 7:10 min. This allows multiple cells to be acquired from the same dish. No morphological changes are observed over the short scan time for the acquisition of the single cell depth scans. After location of a cell, the UME was then positioned near the cell of interest, followed by imaging of the cell using the depth scan imaging mode. This allowed for real time probe-to-cell distance information, reducing the probability of collisions. Manipulation of depth scan parameters, such as scan width, depth, image resolution, and integration time, was performed through the WITec software.

4.5. Simulation. Theoretical PAC data were generated using a COMSOL Multiphysics (v5.2) finite element method model as published elsewhere. A full 3D model was created to replicate the experimental geometry. A symmetry plane was present through the center of the UME and cell, allowing for the simplification of the model (Figure 5). The dimensions of the electrode (platinum radius of 5 μm and an RG of 3) were used to create the model of the UME. The average T24 cell size was determined optically (86 cells) to be 26.33 ± 0.62 and 18.23 ± 0.39 μm, in the long and short axis, respectively. T24 cell height was previously determined to be 8.7 ± 3.3 μm by SECM. This geometry was replicated in the model by a semiliposid with radii y = 13.5 μm, x = 9.0 μm, and z = 8.0 μm as a cell analog. The simulation model incorporated concentrations of mediator in the solution domain (Cm) and an independent cell interior domain (Cc). Initial concentrations of these domains were defined as C_B,T0 = 0.45 mM and C_C,T0 = 0 M for FcMeOH, or C_B,T0 = 0.50 mM and C_C,T0 = 0 M for FcCOO−. Mediator flux across the cell membrane boundary is dependent on the concentration

![Figure 5](https://i.imgur.com/3JZ8Z5F.png)

**Figure 5.** (A) Geometry of an SECM approach to an isolated cell with elements labeled (i.e., boundaries and domains). (B) Zoom in of the region between the UME and cell displayed in (A).
gradient as well as the permeability coefficient \( P \) (eqs 2 and 3).

\[
f_{\text{in}} = P(C_B - C_C) \quad (2)
\]

\[
f_{\text{out}} = P(C_C - C_B) \quad (3)
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The diffusion coefficients in both domains were set to \( 7.6 \times 10^{-10} \) and \( 5.7 \times 10^{-10} \) m²/s for FcMeOH and FcCOO⁻, respectively. The simulation was performed in two stages. The first stage allowed the two domains to reach equilibrium (over 10 min), which provides an initial concentration distribution for the UME approach. In the second stage, the electrode position is parameterized over its full experimental range of motion towards the cell.

In this study, the electrode position is moved in both the x and z axes, as a set of nested parametric sweeps. This allows the full simulation of depth scans over the cell. The theoretical depth scans can be treated as a 2D matrix of electrochemical current values with the UME tip position. One-dimensional PACs and horizontal sweeps can be extracted from the 2D matrix. This allows fitting of the experimental PACs or horizontal sweeps. The membrane permeability coefficients are parametrically simulated, ranging between 0 and 1000 \( \mu \)m/s. Nesting the UME position inside of the permeability parameterization generates a set of depth scans at these desired permeability coefficients (Figure 6). Please note that the permeability was assumed to be homogeneous across the entire cell membrane as we previously found with T24 cells challenged by Cd(II). This point can be further verified in Section 2.1. A membrane permeability coefficient of 0 \( \mu \)m/s produces the same depth scan profile as an ideally insulating surface (no flux) of the same geometry (Figure 6A). Low permeability coefficients restrict the transport of the electrochemical mediator, causing reduced current in close proximity to the cell surface (Figure 6B). With an increase in the permeability coefficient, less negative feedback is observed which leads to a higher electrochemical current profile (Figure 6C–G). A highly permeable cell membrane (Figure 6H) shows little electrochemical response from the cell as the mediator is able to move freely in and out of the cell towards the UME tip.

A complete report of the simulation methodology and COMSOL model parameters is provided in the Supporting Information.

### ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02113.

Cyclic voltammograms of FcMeOH and FcCOO⁻ electrochemical mediators and a COMSOL report that details model parameters, geometry, physics boundaries and corresponding equations, mesh, solver settings, and
post processing (electrode surface integration equation) (PDF)

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Notes
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