# Use of the thyrocyte sodium iodide symporter as the basis for a perchlorate cell-based assay

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Perchlorates are strong oxidants widely employed in military and civilian energetic materials and recently have been scrutinized as persistent environmental pollutants. The perchlorate anion, $ClO_4^-$, is a well-known and potent competitive inhibitor of iodide transport by the sodium iodide symporter (NIS) expressed in the basolateral membranes of thyroid follicular cells (thyrocytes). Iodide uptake by thyroid follicular cells is rapid and reproducible. The competitive radiotransporter assay in this study shows promise as a rapid and convenient method to assay for $ClO_4^-$ in water samples at the nM level. This work describes the initial efforts to define the assay conditions that enhance NIS selectivity for $ClO_4^-$. Experiments of 10 min co-incubation of $ClO_4^-$ and $^{125}I^-$ demonstrate a more significant effect on $^{125}I^-$ transport, with a quantifiable $ClO_4^-$ concentration range of 50 nM (5 ppb) to 2 μM (200 ppb), and $IC_{50}$ of 180 nM (18 ppb), nearly three-fold lower than previous reports. Since the $IC_{50}$ in our assay for other known competitor anions ($SCN^-$, $ClO_3^-$, $NO_3^-$) remains unchanged from previous research, the increased sensitivity for $ClO_4^-$ also produces a three-fold enhancement in selectivity. In addition to the possible applicability of the thyrocyte to the development of a cellular perchlorate biosensor, we propose that the high affinity of the NIS for $ClO_4^-$ also creates the potential for exploiting this membrane protein as a selective, sensitive, and broadly applicable biomechanical mechanism for controlled movement and concentration of perchlorate.

## Introduction

Ammonium and potassium perchlorate are strong oxidants widely employed in military and civilian energetic materials, *i.e.*, propellants, explosives and pyrotechnics.[1] Although its reduction is thermodynamically highly favorable, the perchlorate anion's reactivity is kinetically inhibited, primarily attributable to steric hindrance of access to the central chlorine atom within the tetrahedral $ClO_4^-$ structure.[2] This relative inactivity has historically made perchlorate a spectator anion of choice in aqueous solution chemistry and explains its classification as a persistent environmental contaminant.

The perchlorate anion has an identical charge and similar ionic radius to iodide ($I^-$), and it is a well-known and potent competitive inhibitor of $I^-$ transport by the sodium iodide symporter (NIS) expressed in the basolateral membranes of thyroid follicular cells.[3,4] Perchlorate is a more potent inhibitor of $I^-$ transport than other physiologically relevant anions in the environment, such as thiocyanate ($SCN^-$) and nitrate ($NO_3^-$), and its presence in drinking water supplies is thought to be harmful to certain vulnerable populations.[4,5] Perchlorate has no other adverse physiological effects and is normally excreted quantitatively.[5]

The discovery in 1997 of parts per billion (ppb) levels of $ClO_4^-$ in Western U. S. drinking water supplies has generated debate over the potential health effects of $ClO_4^-$ and the corresponding maximum safe exposure levels, and led to a spate of activity in the development of detection and measurement technologies.[6,7] In 1998, the U. S. Environmental Protection Agency (EPA) added the perchlorate anion to the Contaminant Candidate List (CCL) and in 1999, to the Unregulated Contaminants Monitoring Rule (UCMR).[8] In 2005, the National Academy of Sciences proposed a $ClO_4^-$ reference dose (RfD) of 0.7 μg $kg^{-1}$ $day^{-1}$, with this value being then adopted by the EPA.[9] This RfD for $ClO_4^-$ is the equivalent of a 24.5 ppb (250 nM) maximal concentration limit in drinking water for a 70 kg individual consuming 2 L of water per day as the only dietary source of perchlorate.[10]

Current analytical technologies, such as ion chromatography (IC), tandem electrospray ionization mass spectrometry (ESI-MS), and ion chromatography-electrospray ionization mass spectrometry (IC-ESI-MS) are all specified in EPA methods as able to quantify $ClO_4^-$ with low detection limits [approximately 2 nM (200 ppt)], but they require substantial sample pretreatment to ensure specificity of detection and are appropriate for laboratory, not field measurements.[8,10–12] As perchlorate has become a more visible environmental issue, other research has evolved for detection, with the goal of field portable, sensitive, selective, and inexpensive devices. Liquid membranes that use organic solvents containing ion-association complexes with cations, chelates, dyes and quaternary ammonium species have

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been studied in electrochemical schemes, but these have issues with selectivity and inadequate detection limits.[13–16] Optical schemes that employ a lipophilic pH indicator and an alkylammonium salt embedded in poly(vinylchloride) (PVC) that can transduce perchlorate concentrations to either an absorption or fluorescent signal change are also orders of magnitude too insensitive to reach the desired low concentrations.[17,18] Ion-selective electrodes (ISE) that incorporate ionophores in PVC, carbon paste or silica gel, have shown great promise as simple, inexpensive devices and have nearly reached the detection limits required by regulatory and action limits.[19–23] ISE still command great interest due to the ability to manipulate membrane chemistry, and hence selectivity and sensitivity, through the choice and added ratio of the macrocycle, plasticizer, and additives.[24–26] Our research program, instead, seeks to identify and develop technologies which could determine lower concentrations of $ClO_4^-$ in natural waters using biological mechanisms. A recent report uses perchlorate reductase from *Dechlorosoma* sp. perc1ace to build an enzymatic bioelectrode for $ClO_4^-$ in water.[27] Although the addition of an electron donor for regeneration is required, this system demonstrates excellent lower limits of detection and selectivity from the amperometric response of this enzyme. This paper reports the initial results using the inherent selectivity of a membrane channel protein and examining the possibility of exploiting the high affinity interaction of $ClO_4^-$ and the NIS as a highly specific molecular "filter" for a competitive radiotransporter assay. This work uses Fischer rat thyrocytes and radioactive $Na^{125}I$ to measure nM levels of $ClO_4^-$ due to inhibition of iodide uptake into the cell. We focused our initial efforts on determining if the response time, selectivity, and detectable limit of the NIS for $ClO_4^-$ warrants its further consideration as a basis for a highly selective and sensitive aqueous perchlorate biosensor *via* integration of either the entire functioning thyrocyte or this membrane protein as part of a sensing platform.

## Experimental

### Materials and reagents

Twenty-four well tissue culture plates were obtained from Sarstedt (Newton, NC, USA), fetal bovine calf serum from Hyclone (Logan, UT, USA), and carrier free sodium iodide from MP Biomedicals (Irvine, CA, USA). Trypsin-versene was from Bio-Whittaker. All other reagents were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA), and used as received.

### Cell culture technique

FRTL cells, CRL-1468 (ATCC, Manassas, VA, USA) were maintained in Kaighn's modified Ham's F12 medium (F12K) with 2 mM L-glutamine and 1.5 g $L^{-1}$ sodium bicarbonate. Medium was supplemented with 5% bovine calf serum and a six hormone mixture containing 10 ng $ml^{-1}$ somatostatin, 10 ng $ml^{-1}$ glycyl-L-histidyl-L-lysine acetate, 0.005 mg $ml^{-1}$ transferrin, 10 nM hydrocortisone, 10 mU $ml^{-1}$ thyroid stimulating hormone, and 0.01 mg $ml^{-1}$ insulin. Cells were maintained at 37 °C with 5% $CO_2$–95% air atmosphere in a humidified incubator, with a change of medium every 2–3 d. Cells were passaged using 0.05% trypsin and 0.02% EDTA every 6–7 d by seeding at 3–4 × 10 000 cells $cm^{-2}$. Cells were counted using a hemocytometer.

### Radioiodide uptake (RAIU) assays

Iodide uptake was measured essentially as described by Weiss *et al.*[28] with modifications. Briefly, cells were seeded at a density of $1 \times 10^5$ cells per well into 24 well plates and maintained in standard F12K hormone supplemented media. Cells were incubated overnight to allow adherence to the tissue culture plate but not long enough to allow the cells to divide. The medium was replaced with unsupplemented F12K medium. $^{125}I^-$ uptake was initiated by the manual addition of F12K medium containing 0.1 μCi of carrier free $Na^{125}I$ (approximately 75 pM $^{125}I^-$ in the final 500 μL well volume) and various concentrations of perchlorate or other test anions using a multichannel pipettor. Each 24 well plate consisted of six control wells, and six concentration increments performed in triplicate. All reactions were performed at room temperature over specified time intervals for the time course experiment as indicated or for 10 min for the competition assays. The media was aspirated and cells rapidly washed with ice-cold PBS to terminate $^{125}I^-$ uptake. Radioactivity was released from the cells by lysing with 0.1 mL of 0.1 M NaOH, with the entire termination procedure being completed within 45 s. Radioactivity released from the cell lysate was determined by γ-counting (Perkin-Elmer Packard Cobra II Autogamma, Waltham, MA, USA). All experimental assays were done in triplicate unless otherwise indicated and repeated at least twice. Relative $^{125}I^-$ uptake was recorded as percent of control (the percentage ratio of radioactivity in cells incubated with $^{125}I^-$ and the test anion relative to radioactivity in cells incubated with $^{125}I^-$ alone without inhibitor) to provide an internal control for plate-to-plate variability in cell number.

### Statistical analysis

All experiments were carried out in triplicate ($n = 3$ unless otherwise indicated). Statistical analyses and plotting were performed using Sigma Plot version 9.0 (Systat Software, San Jose, CA, USA). Curves were fit using the one-site competition model included with the software that is a form of the Hill model often used to model competitive inhibition.[29] The data is fit according to the following equation: $Y = \text{Min} + (\text{Max} - \text{Min})/(1 + 10^{(X - \log EC50)})$ where $Y$ is the radioactivity signal defined above, $X$ is the log(inhibitor concentration), Max and Min are the $Y$ values at the top and bottom plateau of the curve, and the effective concentration that causes a response change of 50% (EC50) is equivalent to our half-maximal inhibitory concentration ($IC_{50}$). The $IC_{50}$ can be visualized by dropping a vertical line to the abscissa where the curve crosses the 50% radioiodide uptake inhibition. Results were presented as means ± standard error of the mean (SEM) unless otherwise indicated. A Q test was used for identification and rejection of outliers. The paired Student's *t*-test was used to analyze possible differences between experimental and control groups. All $P$ values were two-sided, and $p < 0.05$ was considered to be significant.

## Results and discussion

The focus of this research was and continues to be the development of a rapid cell-based method for determining aqueous $ClO_4^-$. We seek to exploit the strong and selective preferential

uptake of $ClO_4^-$ anion by the sodium iodide symporter embedded within the cellular membrane of a rat thyrocyte. We consider the efficacy of the thyrocyte as a sensing element to be contingent upon a simplified RAIU assay protocol based on the function of the NIS as a quasi-selective molecular gate for $ClO_4^-$. The following results explore the NIS function with respect to desirable sensing features, such as speed, sensitivity, independence from perchlorate counterion and selectivity. Parameters that would affect cell viability and function, such as a wide range of temperature, pH and ionic strength, were not studied since these would merely have an adverse effect on uptake performance.

Initial experiments assessed the rapidity of the biomechanical response *via* thyrocyte based detection of perchlorate to justify use within a real time sensor. Fig. 1 shows a typical $^{125}I^-$ uptake curve in which radioiodide is quickly transported into thyroid cells; half maximal uptake is achieved in less than 3 min and maximal uptake is attained within 15 min. An uptake curve performed at 37 °C (data not shown) is very similar to the results obtained by Tonacchera *et al.*,[4] showing a higher count per min in each well but a longer time to reach a plateau (40 min). The fast and reproducible response characteristics at room temperature (23 °C) obviate the need for specialized temperature conditions for the assay. Furthermore, since mammalian cell lines are routinely shipped in cell culture flasks at room temperature (RT) with no great loss of cell viability; the cells are expected to survive only 10 min at RT but with a decrease in metabolism. Based on the steep response curve, incubation times shorter than 5 min, especially with a large sample set, would require rapid and precise automation for reproducibility and accuracy.[30] Based on these findings and the findings of Tonacchera *et al.*,[4] incubations at 37 °C for 45 to 60 min as used by other studies designed to reach iodide influx/efflux equilibrium appear to be unnecessary and an incubation time of 10 min at ambient temperature is sufficient for significant radioiodide uptake to occur.[3,29,31] Therefore, four fundamental experimental conditions were selected that differ from previous work for the development of a simple, cell-based biosensor assay for perchlorate: (1) co-incubation with $^{125}I^-$ only (no unlabeled NaI added) to achieve maximal sensitivity of the assay for competitor anions, (2) co-incubation time of 10 min for $^{125}I^-$ and the competitor anion, (3) incubation at room temperature, and (4) direct analysis of competitor anions in thyrocyte cell culture medium.[4,28]

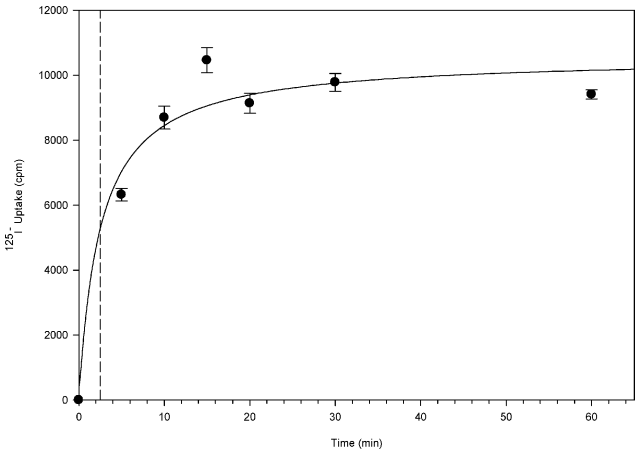


**Fig. 1** Time course of $^{125}I^-$ uptake in thyrocytes. $^{125}I^-$ uptake was measured at 5, 10, 15, 20, 30 and 60 min at ambient temperature. Half maximal uptake is achieved in less than 3 min (dashed line). Results represent means ± SEM of two separate quadruplicate experiments ($n = 8$).

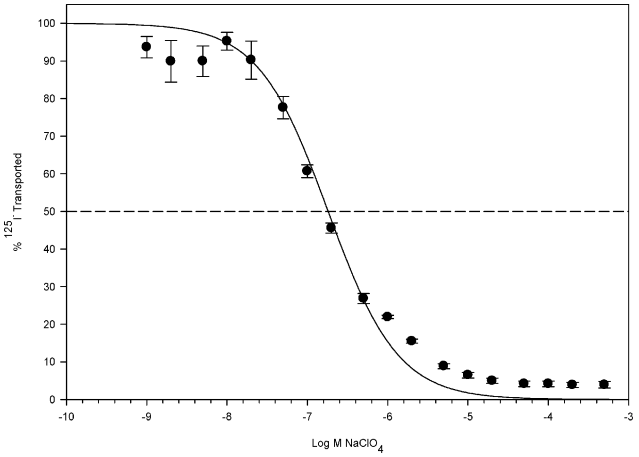


**Fig. 2** Concentration-dependent inhibition of $^{125}I^-$ transport by $ClO_4^-$ using a 10 min incubation time. Data are expressed as percent of the ratio of radioactivity in cells incubated with and without $ClO_4^-$. Each point represents an average of three separate triplicate experiments ± SEM ($n = 9$). Transport rates were fit by non-linear regression analysis for one site competition using the equation for a sigmoid plot with minimum and maximum values set to 0 and 100, respectively. The dashed horizontal line indicates 50% inhibition of $^{125}I^-$ uptake by $ClO_4^-$ ($IC_{50}$= 180 nM). Some error bars are obscured by data points.

As seen in Fig. 2, the sensitivity of the inhibition assay using the modified conditions was readily apparent by plotting the uptake of radioiodide *versus* increasing concentrations of perchlorate. Note that 100% of control iodide transport is not reached at very low concentrations of perchlorate. Both iodide efflux and perchlorate-induced iodide discharge are possible explanations for this effect. If physiological iodide efflux is constant, iodide uptake is slightly inhibited due to the perchlorate, and perchlorate-induced discharge of iodide occurs, transport that is close to, but not quite 100% of control will result. Based on these experiments, the half-maximal inhibitory concentration ($IC_{50}$) of perchlorate at which iodide uptake is inhibited by 50% is calculated to be 180 nM (18 ppb). This concentration is more than two-fold lower than that observed by Tonacchera *et al.*[4] who, using incubations of cold and radioactive NaI, found a 5 min incubation resulted in a representative $IC_{50}$ of 500 nM (50 ppb), and an $IC_{50}$ of 2.8 μM (280 ppb) for a 45 min incubation. This significant difference is due to the preferential uptake of $ClO_4^-$ over $I^-$. As $ClO_4^-$ is transported into the cells and its concentration in the medium decreases, there is greater potential for iodide transport and the inhibitory effect becomes muted at longer incubation times.[32] It must be pointed out that the goal of Tonacchera *et al.*[4] was not an optimized assay for perchlorate, but instead they used the NIS transfected into a different cell line, Chinese hamster ovary cells, to search for autoantibodies in diseased patient sera that inhibit iodide uptake. In our assay, the lowest concentration of $ClO_4^-$ resulting in

 

significant inhibition of $^{125}I$ uptake was 50 nM or 5 ppb ($p < 0.05$), five-fold lower than the RfD proposed by the NAS.[9] The data, therefore, demonstrates a quantifiable range over nearly a 100 fold concentration from 50 nM to 5 μM ($IC_{20}$ and $IC_{90}$ in Fig. 2). The inhibitory effect observed in Fig. 2 is not due to a toxic effect of perchlorate on the cell culture as perchlorate concentrations as high as 100 μM do not affect thyroid cell proliferation.[33]

Ammonium perchlorate and, to a lesser extent, potassium perchlorate are the most likely sources of perchlorate anion in natural waters. The effect of the counter cation on the assay sensitivity was investigated. The results of RAUI assays conducted using sodium, ammonium and potassium $ClO_4$ salts are shown in Fig. 3, which show the sigmoidal response curves nearly overlap regardless of the cation. The calculated $IC_{50}$ for sodium, ammonium, and potassium perchlorate are 180, 140 and 150 nM, respectively. These results demonstrate that in F12K medium containing 130 and 3.8 mM NaCl and KCl, respectively, the contribution of the monovalent cations of perchlorate salts play no significant role in the inhibition of iodide uptake by the perchlorate anion and the inhibition assay is therefore suitable for $ClO_4^-$ detection regardless of the monovalent cation.[34] Despite the reliance of the NIS on sodium, the excess sodium in the assay medium negates effects from minute quantities of additional sodium.

A key characteristic of the iodide transport system in thyroid cells is that the sodium iodide symporter protein has evolved with high specificity for iodide; a specificity that is surprisingly not exclusive, as seen in Fig. 4. It is well established that other monovalent anions may compete for transport by the NIS and thus inhibit iodide transport.[3,4] Using our modified assay, we establish the following order of inhibitory potency: $ClO_4^- > SCN^- \geq I^- > ClO_3^- > NO_3^-$, which is in agreement with previous reports.[3,29] The $IC_{50} \pm$ standard deviation for each anion as determined in our assay are as follows: $SCN^-$, 2.67 ± 0.46 μM; $I^-$, 11.9 ± 2.3 μM; $ClO_3^-$, 153 ± 27 μM and $NO_3^-$, 293 ± 98 μM.

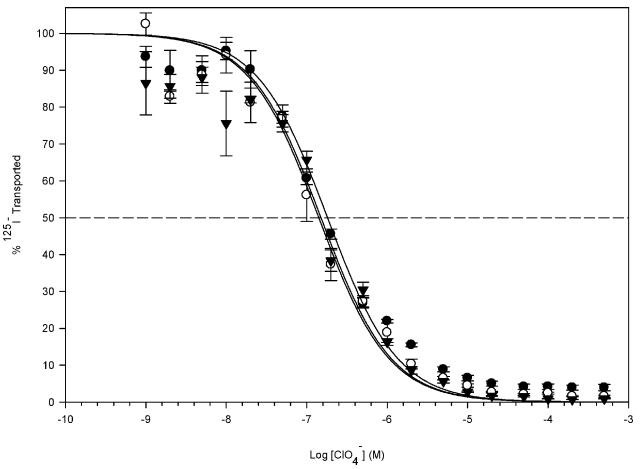


**Fig. 3** Comparison of common $ClO_4^-$ salts: ○ $NaClO_4$ ($n = 9$), ● $NH_4ClO_4$ ($n = 12$), ▼ $KClO_4$ ($n = 6$). Each point represents an average of at least two separate triplicate experiments ± SEM. The dashed horizontal line indicates 50% inhibition of $^{125}I^-$ uptake by $ClO_4^-$ ($NaClO_4$, $IC_{50}$= 180 nM; $NH_4ClO_4$, $IC_{50}$= 140 nM and $KClO_4$, $IC_{50}$= 150 nM). Some error bars are obscured by data points.

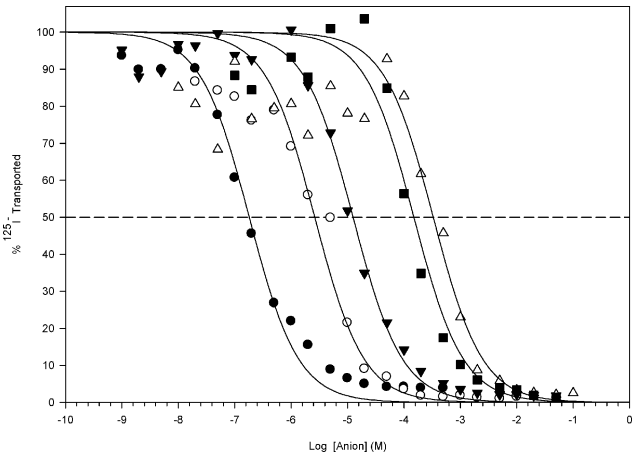


**Fig. 4** Analysis of anion selectivity by RAUI assay: ● $ClO_4^-$ ($n = 9$), ○ $SCN^-$ ($n = 6$), ▼ $I^-$ ($n = 9$), ■ $ClO_3^-$ ($n = 9$), △ $NO_3^-$ ($n = 6$). Data are expressed as percent of the ratio of radioactivity in cells incubated with and without competing anion present. Each point represents an average of at least two separate triplicate experiments. The dashed horizontal line indicates 50% inhibition of $^{125}I^-$ uptake. Error bars were omitted for clarity.

Due to the higher sensitivity of our optimized assay for perchlorate, the $IC_{50}$ of $ClO_4^-$ and $NO_3^-$ differ by a factor of 1600, significantly reducing the contributory effect of nitrate on inhibition of iodide uptake. In contrast, other studies have observed more moderate relative potencies for $ClO_4^-$ and $NO_3^-$ ranging between 240 and 550.[35] Environmental water samples are not expected to contain confounding levels of $SCN^-$, $I^-$, or $ClO_3^-$. $NO_3^-$, however, may be present at potentially interfering levels. If $NO_3^-$ is present at the drinking water maximum contaminant level of 45 mg $L^{-1}$ (725 μM) established by the EPA, the assay would determine a false positive of roughly 400 nM $ClO_4^-$and $^{125}I^-$ transport would be inhibited by 70%. While our assay has substantially increased the selectivity for $ClO_4^-$ over $NO_3^-$, this interference would require a separation technique prior to analysis.[10]

## Conclusions

This study was undertaken to investigate the use of the NIS protein within the membranes of thyrocytes as a selective transporter for perchlorate in water. A sensor that employs human thyroid cells can be envisioned to provide a measure of actual physiological response that eliminates the unknown link between contamination and risk. The advantage of cells is that they can be used to both measure perchlorate and serve as indicators of pathophysiology, while cell-free systems employing enzymatic bioelectrodes provide a measurement function only. Exploiting the behavior of the NIS, we have simplified and optimized the RAIU assay for rapid and accurate perchlorate detection. This preliminary data shows promising analytical figures of merit for NIS behavior in terms of response speed, sensitivity and selectivity. In addition, we have demonstrated an increased selectivity of the NIS for perchlorate over other competing anions serving to minimize potential interferences found in natural waters. Our simplified assay employing Fischer

rat thyrocytes convincingly demonstrates the potential viability of the NIS as the basis of a near real-time, highly sensitive and selective aqueous perchlorate biosensor. Although selective, the NIS is not specific for perchlorate which likely necessitates the front end use of a separation technique. Further studies will investigate the RAIU assay for determination of perchlorate in a military wastewater sample and the use of isolated NIS protein as a sensing or filtering structure for $ClO_4^-$.

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