A highly-selective chloride microelectrode based on a mercuracarborand anion carrier

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The chloride gradient plays an important role in regulating cell volume, membrane potential, pH, secretion, and the reversal potential of inhibitory glycine and GABAA receptors. Measurement of intracellular chloride activity, $a_{Cl}^i$, using liquid membrane ion-selective microelectrodes (ISM), however, has been limited by the physiochemical properties of Cl$^-$ ionophores which have caused poor stability, drift, sluggish response times, and interference from other biologically relevant anions. Most importantly, intracellular HCO$_3^-$ may be up to 4 times more abundant than Cl$^-$ (e.g. skeletal muscle) which places severe constraints on the required selectivity of a Cl$^-$–sensing ISM. Previously, a sensitive and highly-selective Cl$^-$ sensor was developed in a polymeric membrane electrode using a trinuclear Hg(II) complex containing carborane-based ligands, [9]-mercuracarborand-3, or MC3 for short. Here, we have adapted the use of the MC3 anion carrier in a liquid membrane ion-selective microelectrode and show the MC3-ISM has a linear Nernstian response over a wide range of $a_{Cl}$ (0.1 mM to 100 mM), is highly selective for Cl$^-$ over other biological anions or inhibitors of Cl$^-$ transport, and has a 10% to 90% settling time of 3 sec. Importantly, over the physiological range of $a_{Cl}$ (1 mM to 100 mM) the potentiometric response of the MC3-ISM is insensitive to HCO$_3^-$ or changes in pH. Finally, we demonstrate the biological application of an MC3-ISM by measuring intracellular $a_{Cl}^i$ and the response to an external Cl$^-$ free challenge, for an isolated skeletal muscle fiber.
usually difficult to de-convolute into accurate molar units, and in some conditions, sensors can alter the extent and kinetics of the ion concentration changes aimed to be measured. Fluorescence lifetime imaging circumvents some of these challenges, but requires specialized and expensive detection systems. In principle, an ISM is devoid of these limitations, and is expected to provide a direct determination of intracellular activity. In fact, ISMs are often used to measure the activities of ions in solutions that are used to calibrate photometric sensors. On the other hand, ISMs cannot track fast changes in ion activity (i.e. in the ms range, but see13,14) and in some cases selectivity is a limiting factor. The key component of an ISM is the ion-selective carrier or ionophore, which endows the electrode with the selectivity required to discriminate between ions of similar nature and the sensitivity (usually down to the sub-micromolar range). In contrast to the case for cations, there is a general lack of highly-selective naturally occurring or synthetic anion ionophores, and in particular for Cl−,15,16.

We tested ISMs made with several commercially available Cl− ionophores, and unlike our experience with cation ionophores (H+, Na+) we found their responses far from ideal. These Cl− ionophores included: tributyltin (TBT), chloride ionophores 1-IV (SelectophoreTM, Millipore-Sigma), and the antibiotic 3,4,4′-trichlorocarbanilide17. We found ISMs fabricated with these compounds had one or more of the following problems: lack of linearity, sub- or supra-Nernstian responses, drift, hysteresis, poor solubility in liquid membranes, sensitivity to blockers of chloride channels or transporters, and poor selectivity for Cl− over HCO3−. We also tested a chemically diverse group of recently published compounds described as Cl− carriers for biological membranes: compound-118, compound 4H19, cholapod-3, and decalin-13,20,21, and found them not appropriate to build ISMs, mostly demonstrating solubility problems.

Here we report that a Cl− selective anion carrier based on a trinuclear Hg(II) complex containing a carborane-based ligand, [9]mercuracarborand-3 (MC3), that was previously used in polymer membrane Cl− sensors22 is also ideal for liquid membrane Cl− selective ISMs. MC3 is a macrocyclic structure bearing a pre-organized Lewis acid cavity that selectively complexes with Cl− and functions as an anion carrier in the liquid membrane ISM. We show the MC3-ISM is highly selective for Cl− over other biologically relevant anions (HCO3−, lactate, PO43−) and is insensitive to Cl− channel blockers (9-anthracene carboxylic acid), inhibitors of cation–chloride co-transporters (bumetanide, furosemide), or changes in pH and is therefore well-suited for biophysical applications to quantitatively and accurately measure aCl−.

**Methods**

**MC3 synthesis.** To our knowledge, [9]mercuracarborand-3 (MC3, CAS Registry Number 146219-95-6) is not commercially available and so it was synthesized using air-free Schlenk techniques as described by Hawthorne and co-workers23. An oven dried Schlenk flask was charged with 144 mg (0.5 mmol) of ortho-carborane (Boron Specialties), filled with an N2 atmosphere, and 5 mL of anhydrous diethyl ether (Et2O) (Fisher, inhibitor free) were added via syringe. The reaction vessel was cooled to 0°C using an ice bath, 0.85 mL of 2.5 M n-BuLi in hexane (Sigma-Aldrich) was added dropwise to the rapidly stirring reaction mixture. After addition of the n-BuLi solution, the ice bath was removed and the reaction was stirred at room temperature for 2 hours. Next, 315 mg of Hg(OAc)2 (Mallinkrodt) was added to the reaction mixture under a flow of N2 and the reaction mixture was stirred at room temperature for 16 hours. Upon completion of the reaction, the reaction was quenched with 5 mL of H2O, the organic layer was collected, the aqueous portion was extracted with 5 mL Et2O. The organic portions were combined and washed with 5 mL H2O, then the organic layer was dried with MgSO4 and filtered through 2 cm of silica gel using Et2O as the eluent. The filtrate was evaporated to dryness using a rotary evaporator to yield a white solid. Due to difficulty with purifying the product by recrystallization from Et2O, a lipophilic cationic additive (tridodecylmethyl ammonium chloride, TDMAC, Sigma) in the water-immiscible organic solvent 2-nitrophenyl octyl ether (NPOE, Sigma). The reaction vessel was cooled to 0 °C using an ice bath, 315 mg of Hg(OAc)2 (Mallinkrodt) was added to the reaction mixture under a flow of N2 and the reaction mixture was stirred at room temperature for 2 hours. Next, 315 mg of Hg(OAc)2 (Mallinkrodt) was added to the reaction mixture under a flow of N2 and the reaction mixture was stirred at room temperature for 16 hours. Upon completion of the reaction, the reaction was quenched with 5 mL of H2O, the organic layer was collected, the aqueous portion was extracted with 5 mL Et2O. The organic portions were combined and washed with 5 mL H2O, then the organic layer was dried with MgSO4 and filtered through 2 cm of silica gel using Et2O as the eluent. The filtrate was evaporated to dryness using a rotary evaporator to yield a white solid. Due to difficulty with purifying the product by recrystallization from Et2O, an alternative purification method was developed as follows. The white solid was washed with hexanes, dissolved in 10 mL of 20% (v/v) hexanes in Et2O and left in a fume hood to evaporate the Et2O portion which resulted in precipitation of an off-white powder. The supernatant was decanted and the solids were loaded onto a 3 cm plug of silica gel wetted with 40% acetone (v/v) in hexanes and eluted with 40% acetone (v/v) in hexanes. The resulting filtrate was evaporated to dryness to yield 50 mg (14% yield) of an off-white solid which contains >95% MC3 by 11B NMR. 1H NMR (400 MHz, (CD3)2CO) δ 3.0–1.2 (m). 11B NMR (160 MHz, (CD3)2CO) δ 1.6 (d, 2B), −5.4 (d, 2B), −8.0 (d, 6B).

**Micropipette silanization.** Micropipettes were pulled from 1.5 mm borosilicate capillaries with micro-filaments (BF150-86-10, Sutter Instruments). A 4-step pulling protocol was optimized to obtain a short taper (~8 mm) leading to a sub-micron tip opening, using a horizontal puller (P97, Sutter Instruments). When filled with a saline mimicking the ion composition of myoplasm (see solutions), the microelectrodes had tip resistances of 10–12 MΩ, and are insensitive to Cl−-channel blockers (9-anthracene carboxylic acid), inhibitors of cation-chloride co-transporters (bumetanide, furosemide), or changes in pH and is therefore well-suited for biophysical applications to quantitatively and accurately measure aCl−.

**Cl− sensing liquid membrane and ISM half-cell saline.** The ISM liquid membrane consisted of a short column (200–300 μm) of a hydrophobic “chloride cocktail” lodged in the tip of the micropipette. The chloride cocktail was made by dissolving the MC3 and a lipophilic cationic additive (tridodecylmethyl ammonium chloride, TDMAC, Sigma) in the water-immiscible organic solvent 2-nitrophenyl octyl ether (NPOE, Sigma). We
used 10% MC3 and 2.5% TDMAC in NPOE (v/v). No other mixing ratios or solvents were studied. The ISM half-cell consisted of 100 mM NaCl, backfilled into the microelectrode and in contact with an Ag/AgCl reversible electrode.

**ISM fabrication.** We devised a simple and efficient method to fabricate ISMs. Starting with previously slanlized micropipettes, we routinely made six ISMs in less than 1 h. To fabricate an MC3-ISM both the chloride cocktail and the reference saline were sequentially backfilled into the micropipette, while viewing under a stereomicroscope. First, an excess amount of chloride cocktail (2–3 times the final volume of the lipid membrane) was delivered as close as possible to the micropipette tip using a 34G microneedle (Quickfil 34G-5, WPI, for which the polyimide coating was stripped from the end). The organic phase spontaneously moves towards the tip of the micropipette by capillary action and fills the shaft in 3–5 min. Initially using excess chloride cocktail speeds up this filling step. Once the tip is filled, the chloride cocktail volume is reduced as much as possible by suction with a new 34G needle. Finally, the saline solution is delivered (using another 34G needle), assuring that a clean interface is formed between the two liquids. Care must be taken to avoid air bubbles in the organic phase or interface, otherwise the ISM should be discarded.

Prior to use, each MC3-ISM was individually tested using a 3 point calibration including solutions of 1, 10 and 100 mM Cl (see solutions), with \( a_{Cl} \) of 0.64, 6.46, and 68.5 mM respectively. Tested MC3-ISMs were stored in closed jars (to avoid evaporation and dust) with tips submersed in a filtered (0.2 μm) solution containing 10 mM NaCl; and could be used after 1–2 weeks from fabrication with no detectable deterioration.

**Measurement of ISM potential.** A two-channel high input impedance (>10¹⁰ Ω) amplifier (FD223a, WPI) was used to measure the electrical potential of MC3-ISMs. One channel was connected to the Ag/AgCl wire of an MC3-ISM. The second channel was connected to a standard ("sharp") microelectrode having 10–15 MΩ tip resistance when filled with electrode solution (see solutions). This microelectrode served as a reference electrode, whose value \( (V_{\text{ref}}) \) reflects changes in junction potential that may occur upon solution exchanges. Three amplifier outputs, \( V_{\text{ref}}, V_{\text{ISM}}, \) and the analog difference \( V_{\text{ISM}} - V_{\text{ref}} \) were digitized with a 16-bit A/D converter and stored on a computer (LabVIEW, National Instruments). The figures show \( V_{Cl} \), defined as \( - \left( V_{\text{ISM}} - V_{\text{ref}} \right) \), because with this sign convention the potentiometric signal increases for an increase in \( a_{Cl} \).

**Solutions.** Solutions containing different [Cl\(^-\)] were prepared in two ways: (1) Calibration solutions devoid of any possible interfering anion were prepared by serially diluting a pure NaCl solution with distilled water (18 MΩ-cm, Millipore). (2) Calibration solutions suitable for use with mammalian cells were prepared by mixing. Solutions containing potential interfering anions were prepared by adding 10 mM of Na-lactate, NaHCO₃, Na₃HPO₄, NaSCN to a mixture of the 150 - Cl solution. Calibration solutions having a composition similar to that of Tyrode’s buffer but differing in the main anion content, and having the same pH and divalent cation content. These solutions differ in that one (150 - Cl solution) contained Cl\(^-\) as the main anion, and the other (0 - Cl solution) was devoid of Cl\(^-\), and contained SO₄\(^2-\) as the main anion. Both solutions have HEPES (a possible interfering anion) as the pH buffer.

150-Cl solution (mM): 140 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES; pH = 7.4 with NaOH.

0-Cl solution (mM): 115 Na₃SO₄, 2 K₂SO₄, 2 Ca(OH)₂, 1 Mg(OH)₂, 10 HEPES; pH = 7.4 with H₂SO₄.

Calibration solutions containing from 100 mM to 10 mM nominal Cl concentrations were made by serial dilutions of the 150 - Cl solution with the 0 - Cl solution.

Solutions containing potential interfering anions were prepared by adding 10 mM of Na-lactate, NaHCO₃, Na₃HPO₄, NaSCN to a mixture of the 150 - Cl and 0 - Cl solutions, and then performing a similar serial dilution as described above to obtain the desired [Cl\(^-\)]. Drug-containing solutions were made from highly concentrated stock solutions of 9-anthracene carboxylic acid (9-ACA, 500 mM in DMSO) or bumetanide (BMT, 50 mM in DMSO). DMSO final concentration was kept below 1:1000, and controls were run to exclude direct effects of solvent.

Solutions used for recording from isolated mouse muscle fibers were either standard (i.e. Cl-containing) Tyrode’s solution or Cl-free Tyrode’s in which the Cl\(^-\) was replaced with SO₄\(^2-\). Standard Tyrode’s (mM): 150 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES; pH = 7.4 with NaOH. Cl-free Tyrode’s (mM): 115 Na₃SO₄, 2 K₂SO₄, 2 Ca(OH)₂, 1 Mg(OH)₂, 10 HEPES; pH = 7.4 with H₂SO₄. A salt bridge was used to connect the bath of the recording chamber to the reference ground, so that shifts in the junction potential were minimized when bath exchanges were used.

The MC3-ISM was backfilled with a solution containing (in mM): 100 NaCl, 10 HEPES, pH = 7.4 with H₂SO₄. The reference (bath) and intracellular microelectrodes were filled with a solution (in mM): 60 K-aspartate, 5 di-Na ATP, 5 di-tris creatine phosphate, 40 EGTA, 20 MOPS, 5 MgCl₂, pH = 7.4 with KOH.

All chemicals were purchased and used as received from Millipore-Sigma, unless otherwise noted (vide supra).

**Cl\(^-\) activity calculation (a\(_{Cl}\)).** The value for the activity of an ion in solution, \( a_{ion} \), is often obtained from tables\(^a\), but these values were calculated from solutions of single salts (e.g. NaCl, KCl). Instead, we calculated \( a_{Cl} \) (in mM) for all simple and mixed solutions as: \( a_{Cl} = γ_{Cl^-} [Cl^-] \), where \( γ_{Cl^-} \) is the chloride activity coefficient (dimensionless). The activity coefficient was calculated from the extended Debye-Hückel\(^b\) formalism as:
where \( \delta_{\text{Cl}} \) is the effective \( \text{Cl}^- \) ion diameter (0.3 nm), \( A = -0.510 \), and \( B = 3.29 \). The ionic strength, \( I_s \), of a solution containing many ions, \( i_{\text{ion}} \), with valences \( z_i \) was calculated as:

\[ I_s = \frac{1}{2} \sum_i [i_{\text{ion}}] z_i^2 \]

"In cuvette" MC3-ISM characterization. A small circular plastic experimental chamber (8 mm in diameter, 1 mm depth, ~0.5 ml in volume) was used to study the response of MC3-ISMs to [\( \text{Cl}^- \)] changes and to determine selectivity. The chamber was continuously perfused with the test solution of choice and the entire chamber could be completely exchanged in ~1 sec. Perfusion flux (standard of 1–2 ml/min) was driven by gravity and the efflux was removed by aspiration, keeping the level of solution constant to minimize drifts in junction potentials. The chamber solution was connected to ground via a salt bridge (3% agar in 125 mM KCl, w/v containing a floating platinum wire) in contact with a 125 mM KCl solution and an Ag/AgCl electrode. This arrangement represented the second (electrical) half-cell across the MC3-ISM’s liquid membrane.

Muscle fiber ISM measurements. To test the MC3-ISM in a cellular context, we used enzymatically dissociated skeletal muscle fibers from the flexor digitorum brevis (FDB) of the mouse, as previously described. All mouse procedures were approved by the University of California Los Angeles Institutional Animal Care and Use Committee. All methods performed in this study were conducted in accordance with the guidelines and regulations of the IACUC. Fibers were plated in the same chamber as above, and impaled with a MC3-ISM and a reference micro-electrode. Before impalement, both MC3-ISM and reference micro-electrode were zeroed in the presence of \( 100^{-}\text{Cl}^- \) Tyrode.

Selectivity coefficient, \( K_{\text{Cl},X}^{\text{pot}} \). The relative selectivity of an MC3-ISM for \( \text{Cl}^- \) over a test interfering anion was determined by the fixed interference method. The concentration of the test interfering anion, \( [X^-] \), was maintained at a fixed value of 10 mM, and the MC3-ISM potential was measured as the \( [\text{Cl}^-] \) was varied from 10 nM to 100 mM. The MC3-ISM potential was plotted against \( \log(a_{\text{Cl}}) \) and the intersection of the two linear portions (Nernstian for high \( [\text{Cl}^-] \) and saturating for low \( [\text{Cl}^-] \)) determined the value of \( a_{\text{Cl}} \) that was used to calculate the selectivity coefficient for \( \text{Cl}^- \) over \( X^- \) as:

\[ K_{\text{Cl},X}^{\text{pot}} = a_{\text{Cl}}/a_X^{z_C/z_X} \]

Values are reported as the mean ± standard error of the mean.

Results
A cationic additive increases the \( \text{Cl}^- \) responsiveness an MC3-based ISM. We first tested the response of liquid membrane ISMs constructed using a cocktail containing only MC3 (10%) and NPOE. These electrodes were only weakly sensitive to [\( \text{Cl}^- \)] changes, as shown for an exemplary response in Fig. 1A. This response is sub-Nernstian, with a slope of 9.2 ± 0.67 mV/decade (n = 3) on a plot of \( V_{\text{Cl}} \) as a function of \( \log(a_{\text{Cl}}) \) (Fig. 1C, blue symbols). This result is in agreement with previous data, demonstrating that polymeric membranes containing MC3 are insensitive to \( \text{Cl}^- \) unless the membranes are doped with cationic compounds.
When the liquid membrane cocktail was supplemented with a lipophilic cationic additive, TDMAC (2.5%, w/w), the potentiometric response became highly sensitive to [Cl\(^-\)] changes between 1 and 100 mM (Fig. 1B). For brevity, we refer to microelectrodes with this complete liquid membrane cocktail as MC3-ISMs, but it should be recognized they are doped with TDMAC. The voltage response of MC3-ISMs was stable and showed no signs of hysteresis. Over this range of [Cl\(^-\)], the MC3-ISM response was nearly Nernstian, with a slope of 54.6 ± 0.18 mV/ decade (n = 24), as shown in Fig. 1C (black symbols). The reference electrode had very small voltage changes in response to these solution exchanges (2–3 mV, not shown), and these small deviations were subtracted from V\(_{\text{ISM}}\) to obtain V\(_{\text{Cl}}\) values shown in Fig. 1.

We did not explore other combinations of MC3 and TDMAC since the 10% and 2.5% (w/w), respectively, mixture tested gave optimal results. The need for a lipophilic cation stems from the fact that MC3 is a neutral carrier, and as such it is expected to produce a limited anion response unless a counterion for the mercuracarborand complex is added.22

**Linearity and Cl\(^-\) detection limit of the MC3-ISM.** The linear range for Cl\(^-\) responsiveness of the MC3-ISM was determined by changing the [Cl\(^-\)] over 7 orders of magnitude, in ten-fold steps from 100 mM to 10 nM. This calibration was performed with solutions prepared by serial dilution of 100 mM NaCl with water (Methods) to minimize the possibility of interfering anions at low concentrations of Cl\(^-\). A typical continuous recording of V\(_{\text{Cl}}\) during the decrease and then increase of [Cl\(^-\)] is shown in Fig. 2A. Jumps of V\(_{\text{Cl}}\) to new stable values are clearly evident with each solution exchange, and the dotted lines emphasize that no hysteresis was detected. The change in V\(_{\text{Cl}}\) became compressed (i.e. smaller jumps) for test solutions of less than 0.1 mM [Cl\(^-\)] as the linear range was exceeded.

A limited number of trials were also performed with solutions prepared by serially diluting 100 mM NaCl with a Na\(_2\)SO\(_4\)-based solution (Fig. 2B) that is commonly used for "chloride-free" conditions in studies on skeletal muscle fibers (Methods, 0 – Cl solution). This alternative method maintains a high ionic strength for all test values of [Cl\(^-\)] and provides a check for evidence of interfering anions in a mammalian physiologic buffer. A plot of steady-state V\(_{\text{Cl}}\) as a function of log\(_{10}(a_{\text{Cl}})\) shows that between 0.1 mM and 100 mM the response was linear (Fig. 2C). This range exceeds the expected values of intracellular a_{\text{Cl}} to be encountered in mammalian cells (3 to 20 mM, or even up to 100 mM in pathological contexts such as glioma).29 Moreover, the response for dilutions with pure water was near-linear down to an activity of 0.01 mM. The saturation of V\(_{\text{Cl}}\) at about −200 mV for the NaCl: Na\(_2\)SO\(_4\) mixture reveals an interfering anion effect of SO\(_4\)\(^2-\), although this was detectable only at low a_{Cl} levels (<0.1 mM) that are not encountered in cells.

The average slope of the linear response range was 52.8 ± 0.63 mV/decade (n = 14). While this slope is slightly smaller than the theoretical value of 58 mV, it is comparable to values reported for MC3 electrodes fabricated in solid membranes.22 Those electrodes also showed a sub-Nernstian response, with the slope being smaller for lower molar ratios of TDMAC to MC3.

**The settling time for an MC3-ISM is a few seconds.** Another desirable feature of ISMs for biological applications is a small time constant. To assess the time response of the MC3-ISM, we increased the flow rate of the solutions perfusing the experimental chamber (40 ml/min), as compared with those used in experiments in Figs 1 and 2. The continuous flow was briefly switched from a 100 mM Cl\(^-\) solution to a 10 mM Cl\(^-\) solution that also contained fluorescein (100 μM). Local spot illumination (100 μm diameter disc, λ = 488 nm) near the tip of the MC3-ISM was used to simultaneously measure the kinetics of the bath exchange while the MC3-ISM response was recorded. On average, the fluorescence transient decayed with a time constant of 1.1 ± 0.15 sec (n = 4) and after a brief lag, V\(_{\text{Cl}}\) decayed almost as quickly, 1.4 ± 0.26 sec (n = 4), as shown by the exemplary responses in Fig. 3. Thus even at this high flow rate, the response time of our MC3-ISM is limited by solution exchange, and we can conclude that the intrinsic time constant of the ISM is smaller than 1.4 sec.
The response time of a liquid-junction ISM is usually faster as the width of the organic phase decreases at the tip of the electrode\(^\text{13,14}\). The width for the liquid membrane of our MC3-ISMs was \(\approx 300\) μm, and we did not explore whether the time constant could be reduced by decreasing the width of the organic phase. In any event, a time response in the order of a few seconds is sufficient to study intracellular [Cl\(^-\)] changes expected to occur in several tens of seconds to minutes in biological settings.

The MC3-ISM is insensitive to changes in pH or \(\text{HCO}_3^-\). One of the biggest drawbacks of available Cl\(^-\) sensing ISMs is their sensitivity to \(\text{HCO}_3^-\).\(^\text{39}\) The problem posed by \(\text{HCO}_3^-\) is complex. This ubiquitous intracellular anion is more abundant than Cl\(^-\) in many cells (e.g. 3-fold higher in mammalian skeletal muscle), and the [\(\text{HCO}_3^-\)] varies greatly depending on pH and p\(\text{CO}_2\) changes. Consequently, for Cl\(^-\) sensing ISMs that are also sensitive to \(\text{HCO}_3^-\) there is no simple way to distinguish between real \(\alpha_{Cl}\) changes, and spurious potential shifts caused by changes in [\(\text{HCO}_3^-\)]. Early Cl\(^-\) sensing ISMs based on anion exchangers had notoriously poor selectivity for Cl\(^-\) over \(\text{HCO}_3^-\).\(^\text{15}\) Selectivity for Cl\(^-\) was greatly improved by using anion-selective carriers, e.g. Mn(III) porphyrins\(^\text{40}\), but the physiochemical properties of these first-generation carrier electrodes were unfavorable with instability, drift, and non-Nernstian behavior. The high selectivity for Cl\(^-\) over \(\text{HCO}_3^-\) and near-Nernstian behavior of MC3-based solid membrane electrodes\(^\text{29}\) showed tremendous promise for developing a Cl-selective liquid membrane ISM suitable for biological applications.

We measured the sensitivity of MC3-ISMs to interference from changes in pH or [\(\text{HCO}_3^-\)], as a test of suitability for these ISMs to be used for intracellular determination of \(\alpha_{Cl}\). First, we tested for MC3-ISM sensitivity to ∆pH from 6.5 to 8.5, which covers the entire physiologic range of expected cytoplasmic values. In these HEPES-buffered \(\text{HCO}_3^-\)-free solutions, a three-point Cl\(^-\) calibration response (1, 10, 100 mM) for an MC3-ISM was not affected by a pH shift from 7.4 to 6.5 or from 7.4 to 8.5 (Fig. 4A, left and right, respectively). Next, we measured the response of MC3-ISMs in constant 10 mM Cl\(^-\), a typical value for myoplasmic Cl\(^-\) in skeletal muscle fibers\(^\text{31,32}\), upon which was imposed step changes in \(\text{HCO}_3^-\) from 1 to 50 mM (Fig. 4B), a concentration range far beyond those expected to occur in vivo either in physiological or pathological conditions (i.e. \(\approx 13\) mM\(^\text{39}\)). The potentiometric response of MC3-ISMs was completely insensitive to the presence of \(\text{HCO}_3^-\) at concentrations as high as 50 mM. This is a remarkable feature of the MC3 Cl-selective carrier, and makes MC3-based ISMs the electrode of choice for biological purposes.

Chloride selectivity of the MC3-ISM. The selectivity of the MC3-ISM was assessed for Cl\(^-\) over various physiologically relevant anions including lactate, phosphate, thiocyanate and bicarbonate. The fixed interference method, as described by IUPAC was used\(^\text{37}\), wherein the concentration of the test interfering anion was held constant at 10 mM (as a Na\(^+\) salt), and V\(_{Cl}\) was measured in solutions with varying [Cl\(^-\)] between 100 mM and 1000 mM. We found that the MC3-ISM response is not significantly affected by the presence of lactate, phosphate, or bicarbonate for [Cl\(^-\)] from 100 mM to values as low as 100 μM (Fig. 5). On the other hand, the MC3-ISM was almost insensitive to Cl\(^-\) in the presence thiocyanate (Fig. 5, magenta diamonds). From the data in Fig. 5, we calculated the selectivity coefficient, \(K_{Cl,XY}^{\text{pot}}\), for each test anion (Table 1) which gives a selectivity series: thiocyanate > Cl\(^-\) > bicarbonate > phosphate > lactate. Importantly, the MC3-ISM was 125-fold more sensitive to Cl\(^-\) than to \(\text{HCO}_3^-\), which explains the lack of an effect for \(\text{HCO}_3^-\) in Fig. 4 and confirms the MC3-ISM is suitable for measuring intracellular \(\alpha_{Cl}\) without interference from fluctuating levels of \(\text{HCO}_3^-\).
Chloride channel blockers and inhibitors of Cl⁻ co-transporters have little effect on the MC3-ISM response. Chloride-selective electrodes are often sensitive to blockers of Cl⁻ channels or to inhibitors of Cl⁻ co-transporters. We assessed the MC3-ISM sensitivity for three commonly used Cl⁻ agents, the ClC-1 blocker 9-ACA and the NKCC1 inhibitors bumetanide (BMT) or furosemide (FUR). Our test for
interference was based on detecting a distortion of the Nernstian behavior of an MC3-ISM over an operating range of 1 mM to 100 mM [Cl\(^-\)], while the Cl\(^-\) agents were added at concentrations typically used for studies of muscle. We found that the MC3-ISM is almost insensitive to 9-ACA (Fig. 6A,D), with a very small effect (reduced V\(_{\text{Cl}}\)) in 200 μM 9-ACA when [Cl\(^-\)] was 5 mM or lower. Studies in skeletal muscle that are designed to eliminate the CIC-1 conductance typically use 100 μM 9-ACA\(^8\). With this concentration of blocker, the V\(_{\text{Cl}}\) response remained Nernstian down to a [Cl\(^-\)] of 2.5 mM (the lowest expected physiologically), demonstrating that MC3-ISMs can be used to measure intracellular Cl\(^-\) activity, \(a_{\text{Cl}}\), measured with an MC3-ISM. \(\text{Intracellular Cl}^-\text{ activity, } a_{\text{Cl}}\), measured with an MC3-ISM. \)

We verified the performance of the MC3-ISM as a sensor of intracellular Cl\(^-\), by recording \(a_{\text{Cl}}\) in a skeletal muscle fiber isolated from the flexor digitorum brevis of the mouse. The fiber was impaled with a standard reference electrode to measure the resting potential (\(V_m\), Fig. 7, upper panel) and also with a Cl-selective MC3-ISM (V\(_{\text{ISM}}\)) which reports the combined effect of \(V_m\) and V\(_{\text{Cl}}\). The offset for V\(_{\text{ISM}}\) = 0 mV was set in a calibration solution with 100 mM [Cl\(^-\)]. The difference between these two signals, V\(_{\text{Cl}}\) = \(V_m - V_{\text{ISM}}\) (Fig. 7, lower panel) is proportional to \(\log_{10}(a_{\text{Cl}})\).

In control Tyrode solution (140 mM Cl\(^-\)), the resting membrane potential was stable (\(V_m = -80\) mV), as was V\(_{\text{Cl}}\) = −65 mV, corresponding to \(a_{\text{Cl}} = 6\) mM. Upon switching the perfusion to SO\(_4\)-Tyrode (nominally zero Cl\(^-\)) the intracellular \(a_{\text{Cl}}\) exponentially fell to about 1.5 mM because of Cl\(^-\) efflux through the high resting conductance of CIC-1 channels. These changes are fully reversible, as shown by the recovery of \(a_{\text{Cl}}\) when the perfusion with Cl\(^-\) Tyrode was resumed at 500 sec in Fig. 7.

The transient response of \(V_m\) is also consistent with decrease of \(a_{\text{Cl}}\), followed by recovery\(^4\). At the onset of Cl\(^-\)-free Tyrode solution (Fig. 7, 80 sec), \(V_m\) depolarized because the equilibrium potential for Cl\(^-\) suddenly shifted from about −77 mV to a positive value. Intracellular Cl\(^-\) content then declined over the next 30 s, which shifted E\(_{\text{Cl}}\) to more negative potentials and caused \(V_m\) to relax back toward −80 mV. When extracellular Cl\(^-\) is suddenly
restored (Fig. 7, 500 sec), \(V_m\) had a hyperpolarizing undershoot because intracellular \(a_{Cl^-}\) was now lower than baseline. As \(a_{Cl^-}\) increased back to the normal basal level, \(V_m\) relaxes to the initial value of \(-80\) mV. Similar responses were observed in 8 muscle fibers.

**Discussion**

The development of a highly-selective and sensitive chloride sensor in polymeric membrane electrodes, based on the preorganized macrocyclic Lewis acid MC-3 as the anion carrier, was a major technological advance. Prior electrode designs using anion exchangers (e.g. TBT or TDMAC) lacked selectivity, which in these electrodes was dominated by anion lipophilicity (Hofmeister series). Improved Cl\(^-\) selectivity was achieved with metalloporphyrin carriers (e.g. Mn(III), chloride ionophore I Sigma-Aldrich), but interference from HCO\(_3\)\(^-\) was still problematic, \(\log K_{HCO_3/HCl}^{pot} = -1.4\), for intracellular determination of \(a_{Cl^-}\). Excellent selectivity for Cl\(^-\) over HCO\(_3\)\(^-\) was achieved with neutral carriers comprised of Lewis acids containing two or more covalently bonded Hg(II) (e.g. chloride ionophore II, Sigma-Aldrich), but these compounds were suboptimal for liquid membrane ion-selective microelectrodes because of sluggish response times \((t_{90\%} \sim 10\) s\), electrical instability with drift and non-Nernstian responses, or poor solubility in NPOE. In contrast, MC-3 is chemically stable, and MC-3 based polymeric membrane electrodes have more consistent Nernstian responses, high selectivity for Cl\(^-\), and faster settling times of a few seconds. The MC3 ionophore represents a unique platform for chloride sensing. These properties stem from the molecular architecture of this structure which includes a precise triatomic arrangement of Hg(II) sites separated by rigid ortho-carboranyl boron-rich cluster ligands. Such a trinuclear metal receptor arrangement allows MC3 to bind anions in a multivalent fashion. Furthermore, electron-withdrawing nature of the C-bound ortho-carborane clusters, similar in magnitude to the perfluoroaryl substituents\(^{40,41}\), enhances the Lewis acidity of the Hg(II) centers critical to the anion binding. Finally, the sterically encumbering nature of the boron cluster ligands enhances the kinetic stability of the corresponding organometallic complex and creates a well-defined pocket around the Hg-based centers modulating the anion binding selectivity.

We now show that the advantages of MC-3 based polymeric membrane electrodes can be realized in a liquid membrane microelectrode form factor. Following from the design of the MC-3 electrodes in PVC membranes, we used NPOE as the liquid organic phase in our ISMs and no other plasticizers were tested. Just as with the PVC membrane electrodes, the potentiometric response to Cl\(^-\) with MC-3 alone was sub-Nernstian (Fig. 1), and doping the liquid membrane with a lipophilic cationic additive (2.5% TDMAC) markedly increased the ISM sensitivity to near-Nernstian behavior (54.3 mV/decade). Increased amounts of cationic additive in PVC membrane electrodes extended the linear range for high \(a_{Cl^-}\) to about 100 mM, but with a cost of slightly reduced selectivity for Cl\(^-\) over other anions. Since our ISMs already had linear responses up to the highest concentration of the calibration solution (nominally 100 mM mixture, calculated \(a_{Cl^-} = 68.5\) mM), we did not test higher amounts of additive. Moreover, in a physiological context the expected intracellular \(a_{Cl^-}\) is in the range of 3 to 20 mM.

The potentiometric responses of MC-3 ISMs were remarkably consistent and stable over long recording periods. Except for the occasional failure in the fabrication of an ISM (e.g. air bubble at the liquid membrane/aqueous electrolyte interface), the variability was exceptionally small in the slope of the linear response range (0.1 mM to 100 mM). A sample of 24 MC-3 ISMs with an average slope of 54.6 mV/decade had a standard deviation of only 0.9 mV/decade and a range of 52.9 to 56.0 mV/decade. By comparison, a sample of 62 ISMs constructed with the “improved” Corning 477913 chloride ionophore was reported to have a mean slope of 32.8 mV/decade with a standard deviation of 5.1 mV/decade and the range of values extended from 35 to 64 mV/decade. The potentiometric response of MC-3 ISMs was stable with no significant drift over ten minutes or more, as shown.

**Figure 7.** Myoplasmic \(a_{Cl^-}\) measured with an MC3-ISM. Simultaneous intracellular recordings of membrane potential (\(V_m\), black trace) and the MC3-ISM response (\(V_{ISM}\), blue trace) were obtained by impaling a dissociated fiber from the flexor digitorum brevis muscle (upper panel). Digital subtraction (\(V_m - V_{ISM}\)) was performed to calculate the red trace in the lower panel, representing both linear changes of \(V_{Cl^-}\) (left ordinate) as a function of time and logarithmic changes in \(a_{Cl^-}\) as a function of time (right ordinate). At 80 s, the bath was exchanged to Cl-free Tyrode solution, and then back to normal Tyrode (150 mM Cl) at 500 s.
by examples in several Figs. (2–4, 6). Moreover, the intracellular response recorded from a skeletal muscle fiber was also stable, as shown by the return to the baseline value after recovery from a 10 min exposure to Cl-free conditions (Fig. 7).

The slow electrical response time of Cl-selective ISMs has been a limiting factor for use of some ionophores. The problem arises from the high resistivity of immiscible liquid membranes with neutral ion carriers in combination with the capacitance of the glass pipette. While reducing the ISM tip resistance with cationic additives, choice of the ionophore, and optimization of the liquid membrane thickness may be used to improve performance, the 10% to 90% response time was reported to be as long as 41 sec for Mn(III) porphyrin Cl-selective ISMs. For macroscopic MC-3 electrodes in PVC membranes, a more favorable rise time of about 10 sec was observed. In our MC-3 ISMs the 10% to 90% response time was about 3 sec (Fig. 3), which is sufficient to monitor changes of intracellular aCl in muscle fibers that typically occur over tens of seconds to minutes.

The most important advantage of MC-3 ISMs is the high selectivity for Cl over other anions, especially HCO3−. Historically, the Cl− ISMs used in several landmark papers showing intracellular aCl in muscle and heart is higher than expected from passive electrodiffusion were based on proprietary Cl− ionophores from Corning (477315 and later 477913) that had substantial interference from HCO3−. This limitation was compounded by the composition of the intracellular milieu, where aCl is typically 4 mM, and HCO3− is more abundant at 14 to 18 mM. With a Corning 477913 ISM, the selectivity for Cl over HCO3− was about 8:1, corresponding to a selectivity coefficient log10 (Kpot/Keff,Cl,HCO3−) of −0.9. With this degree of HCO3− interference, a true aCl of 4 mM in combination with an aHCO3− of 15 mM would cause a 9.3 mV error in the potentiometric value of the Cl− ISM, corresponding to an overestimate of aCl as 5.9 mM. In contrast, the high Cl-selectivity we observed for an MC3-ISM, log10 (Kpot/Keff,Cl,HCO3−) = −2, would result in only a 0.9 mV error with an estimated value of aCl = 4.14 mM. Insensitivity to HCO3− (Fig. 4) is also important because intracellular pH often changes or is intentionally manipulated during a study. The accompanying fluctuations of intracellular aHCO3− would render the potentiometric response uninterpretable for a poorly selective Cl− ISM. Sensitivity to pH is also a limitation for genetically encoded Cl sensors based on YFP variants, which has led to the development of ratiometric dual-sensor fluorescent proteins (ClopHensor) to monitor both [Cl] and pH simultaneously.

Another selectivity-based limitation of earlier Cl− ISMs was interference from drugs used to block Cl− channels (e.g. 9-ACA) or to inhibit Cl− exchangers and cotransporters (e.g. SITS, bumetanide, furosemide). These compounds are often used in studies of Cl transport, where drug-dependent changes in extracellular and intracellular Cl− activity are measured with ISMs. These inhibitors have high lipid solubility and are anions at physiologic pH, and so not surprisingly, substantial anion interference occurs for ISMs using poorly selective ionophores such as Corning 477413. For example, furosemide at 100 μM caused nonlinear potentiometric responses for aCl ≤ 30 mM, with a corresponding selectivity coefficient of KCl,Furo = 157. We tested three drugs that inhibit Cl transport: furosemide, bumetanide, and 9-AC. In furosemide, we detected mild deviations from linear Nernstian behavior for MC3-ISM electrodes when aCl ≤ 1.6 which is 20-fold lower than for Corning 477413 (Fig. 6C). In contrast, the potentiometric response of the MC3-ISM over the entire range of test solutions ([Cl] from 1 to 100 mM) was not affected by 20 μM bumetanide (Fig. 6B), which is ten times the IC50 to inhibit the Na-K-2Cl1 cotransporter. For the CIC-1 channel blocker, we detectable interference in 200 μM 9-ACA when [Cl] < 10 mM (Fig. 6A). Studies in skeletal muscle typically use 100 μM 9-ACA, and at this lower concentration we detected a change in the MC3-ISM response only for [Cl] < 2.5 mM. Overall, the MC3-ISM is highly selective for Cl with a linear Nernstian response over the biologically meaningful operating range of 1 to 100 mM, with no interference from endogenous anions (10 mM HCO3−, PO43−, or lactate) or bumetanide and very modest effects in high-dose 9-ACA well above the IC50.

Finally, we demonstrate that the MC3-ISM reliably measures aCl in a biological context, the intracellular activity in an isolated skeletal muscle fiber (Fig. 7). The response to an external Cl-free challenge is a stringent test of the MC3-ISM because the resulting decrease of intracellular aCl the MC3-ISM because the resulting decrease of intracellular aCl could reveal effects from interfering intracellular anions or other sources of distortion as an attenuation of the change in the potentiometric signal. The response in Fig. 7 clearly shows a large response for VCl = (VCl,MC3-ISM − VCl,ref), corresponding to a decrease of intracellular aCl to less than 2 mM. The record in Fig. 7 also shows the high quality of the signal-to-noise for measuring intracellular aCl as well as the stability of the response with no significant drift and a return to the same baseline when external Cl is restored 10 minutes later. Measurement of intracellular aCl is also of great interest in studies of the nervous system because the Nernst potential for Cl− determines the postsynaptic response to activation of glycine and GABA receptors. Moreover, intracellular Cl− varies over time, ranging from 3 to 20 mM or higher. Neuronal intracellular Cl− is high in the immature brain, in part due to activity of NKCC1 that promotes Cl− influx, and so receptor activation elicits post-synaptic depolarization (i.e. excitation). With development, NKCC1 decreases and is replaced by KCC that promotes Cl− efflux. The reduced intracellular Cl− shifts the Nernst potential for Cl− below the resting potential and receptor activation now produces hyperpolarization (inhibition). Derangements of the Cl− gradient in the neurons have been implicated in epilepsy and in anomalous regulation of cell volume that drives proliferation and migration of gliomas. The MC3-ISM offers an improved approach for measuring somatic aCl for the determination of basal levels and changes that may occur over seconds to minutes – for example in response to inhibition of specific transporters. Of course alternative methods, such as dye fluorescence lifetime or genetically encoded Cl-sensors, are required to measure local aCl changes within diffusion-restricted spaces in out in the dendrites or for fast transients less than 3 sec.
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**Author contributions**

The experimental design was conceived by M.D., S.C. and A.S. MC3 was synthesized by R.D. Measurement of ISM responses were performed by M.Q. and M.D. The paper was written by M.D., S.C. and A.S. All authors reviewed the final manuscript and approved the content.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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