Anal Bioanal Chem (2008) 390:1355–1359
DOI 10.1007/s00216-007-1804-z

TECHNICAL NOTE

Characterization of optimized Na^+ and Cl^- liquid membranes for use with extracellular, self-referencing microelectrodes

Mark A. Messerli · Ira Kurtz · Peter J. S. Smith

Received: 11 October 2007 / Revised: 6 December 2007 / Accepted: 12 December 2007 / Published online: 11 January 2008
© Springer-Verlag 2007

Abstract Self-referencing with ion-selective microelectrodes (ISMs) is a useful approach for monitoring near-real-time ion flux near single cells and across epithelia. While ISMs for H^+, Ca^{2+}, and K^+ have been optimized for use with self-referencing, ISMs for two other primary inorganic ions, Na^+ and Cl^-, have not. In this study, we have characterized ISMs based on three Na^+ ionophores (I, VI, and X) and one Cl^- ionophore to assess their suitability for use with self-referencing. ISMs constructed with Na^+ ionophore VI have short response times (≈100 ms) but possess nearly an order of magnitude less selectivity for Na^+ over K^+ than ISMs constructed with Na^+ ionophore X. The Na^+ ionophore X mixture was enhanced to give it a shorter response time while not compromising its selectivity. A Cl^--selective microelectrode was constructed and characterized with superior anionic selectivity compared with previously reported Cl^- ISMs used with self-referencing. This Cl^--selective microelectrode, however, has a relatively slow response time (≈3 s), thus requiring changes to the self-referencing protocol. Self-referencing with these ISMs will enable near-real-time ion flux measurements for Na^+ and Cl^-.

M. A. Messerli (✉) · P. J. S. Smith
BioCurrents Research Center, Program in Molecular Physiology,
Marine Biological Laboratory,
Woods Hole, MA 02543, USA
e-mail: mmesserli@mbl.edu

I. Kurtz
Division of Nephrology, David Geffen School Medicine at UCLA,
10833 Le Conte Ave.,
Los Angeles, CA 90095, USA

Keywords Ion-selective microelectrode · Self-referencing · Na^+ · Cl^-

Introduction

Self-referencing of ion-selective microelectrodes (ISMs) has been instrumental for detecting the very weak ion fluxes near single cells and tissues that arise as ions cross the plasma membrane through ion pumps, exchangers, and channels (recently reviewed in [1]). The use of ISMs in this manner is critically dependent on four parameters: (1) high selectivity for the primary ion, (2) insensitivity to pH, (3) short response time, and (4) insensitivity to pharmacological inhibitors of ion transport. ISMs for H^+, Ca^{2+}, and K^+ have proven reliable and have been used extensively to monitor trans-plasma membrane ion fluxes near plant and animal cells [1, 2]. Fluxes of two other commonly transported inorganic ions, Na^+ and Cl^-, have been studied less with self-referencing of ISMs due, in part, to poor selectivity or interference by components of the extracellular media. Previously we have presented critical evaluation of others' work: for example, use of self-referencing of a Cl^--selective liquid ion exchanger microelectrode, possessing poor selectivity, led to misidentification of the transported ion and misinterpretation of changes to the apparent Cl^- flux in response to 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid disodium (DIDS), a Cl^- transport blocker [3].

In this work we have screened and characterized three different Na^+-ionophore-based microelectrodes and one Cl^--ionophore-based microelectrode in order to determine their suitability for measuring extracellular ion fluxes near cells and tissues with the self-referencing technique. We identify the strengths of these liquid membranes over

previously used mixtures and list their potentials and limitations when used with self-referencing.

Materials and methods

Chemicals

All components of the Na^+- and Cl^--selective microelectrodes were purchased from Sigma-Aldrich (St. Louis, MO) including Na^+ ionophores I, VI, and X, Cl^- ionophore II, *ortho*-nitrophenyloctylether (*o*-NPOE), sodium tetraphenylborate (NaTPB), and tridodecylmethylammonium chloride (TDMACl). Na^+ transport inhibitors ouabain, bumetanide, and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) and Cl^- channel and transporter inhibitors DIDS, 5-nitro-2(3-phenylpropylamino)benzoic acid (NPPB), niflumic acid, and tamoxifen were also purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of inhibitors, dissolved in dimethyl sulfoxide (DMSO), were diluted to the point that the final amount of DMSO in the working solution was 0.1% or less. An equivalent amount of DMSO was placed in the ion standards when comparing the standards to an inhibitor that was dissolved in DMSO.

Microelectrode construction

ISMs were fabricated by simple modifications to the previous design [4]. Electrical connection between the liquid membrane and the high impedance headstage was made with 100 mM NaCl backfilling solution and a Ag/AgCl wire. Our standard pre-pulled, glass pipettes were 1.5-mm-outer-diameter, thin-walled (190 μm) borosilicate glass. Using glass with a thicker wall can reduce the time constant of the microelectrodes. When this was a critical feature, we constructed microelectrodes with 2-mm-outer-diameter, thick-walled (440 μm) glass pipettes. Both sizes of glass were pulled to an inner tip diameter of 2–3 μm using a P-97 Flaming/Brown Micropipette Puller (Sutter Instruments, Novato, CA).

Selectivity determination

Ionic selectivity coefficients were determined with a simplification of the separate solutions method [5], by comparing measured voltages of 100 mM solutions of the Cl^- salts of the tested cations, for the Na^+ microelectrodes and 100 mM of the Na^+ salts of the tested anions for the Cl^- microelectrode. NaOH was used to set the pH of 2-(*N*-morpholino) ethanesulfonic acid(MES) and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) solutions, while testing the Cl^--selective liquid membrane for its sensitivity to these organic H^+ buffers. The selectivity coefficients are presented in the Results using the log base 10 scale. Testing of the microelectrodes for pH sensitivity was performed in 1 mM HEPES, H^+ buffer, and 1 mM NaCl in order to increase the probability of measuring a change in voltage between pH 6 and 8, by reducing the background activity of the primary ion. KOH was used to set the pH of the two solutions.

Response time

Time responses of the microelectrodes were determined with a rapid exchange flow system as previously described [3]. Time responses are reported as the time it takes for the voltage to reach 95% of its steady state value ($t_{95\%}$).

Screening of ion transport inhibitors

Na^+-selective microelectrodes were introduced to three different Na^+ transport inhibitors, ouabain, bumetanide, and EIPA, using concentrations near their upper limit of application. Na^+-dependent voltages were measured by the microelectrodes in 1, 10, and 100 mM NaCl, in the absence and presence of each inhibitor. In a similar manner Cl^--dependent voltages were measured in the absence and presence of four Cl^- transport inhibitors, DIDS, NPPB, niflumic acid, and tamoxifen.

Results and discussion

Na^+ ionophores I, VI, and X

Three different Na^+ ionophores were chosen based on their optimal selectivity for Na^+ over K^+ in previous studies [6–8]. The Na^+-selective liquid membranes based on Na^+ ionophores I and VI were made at a Na^+ ionophore/*o*-NPOE/NaTPB ratio by weight of 10:89.5:0.5, whereas the optimized Na^+ ionophore X mixture was made at a Na^+ ionophore/*o*-NPOE/NaTPB ratio by weight of 10:89.75:0.25. Na^+ ionophore I possessed nearly an order of magnitude greater selectivity for Ca^{2+} than Na^+ (Table 1). This may make it suitable as an intracellular Na^+ detector [6] where resting Ca^{2+} is only around 100 nM, but not as an extracellular electrode where Ca^{2+} is between 1 and 5 mM; hence, no further studies were performed with ionophore I. The mixtures containing 10% Na^+ ionophore VI and X have mean responses of 56.4±0.3 mV and 57.5±1.0 mV, respectively, over a Na^+ range of 0.1–100 mM and possessed 1.7 and 2.6 orders of magnitude selectivity for Na^+ over K^+, respectively, but 3.5–4.0 orders of magnitude selectivity for Na^+ over other major inorganic interferents (Table 1). For comparison, Table 1 also contains published selectivity coefficients for Na^+ ionophore VI in *o*-NPOE alone [7] and for Na^+ ionophore X in *o*-NPOE with K^+ tetrakis(4-chloro-

Table 1 Selectivity coefficients of Na^+-selective microelectrodes

Cation	Na^+ ionophore							
	I	VI[a]	VI	X[b]	X[c]	X[d]	X[e]	X[f]
K^+	−2.1	−2.0	−1.7	−1.9	−2.3	−2.6	−2.6	−2.6
Ca^{2+}	+0.9	−4.0	−3.5	−2.5	−3.8	−3.5	−2.0	−4.1
Mg^{2+}	−1.8	−4.0	−3.9	<−6	−4.1	−3.7	−2.3	−3.9
NMG	−3.4	n.d.	−4.0	n.d.	−4.1	−3.7	−2.3	−4.0

NMG N-methyl-glucamine, *n.d.* not determined
[a] Values obtained from [7]
[b] Values obtained from [8]
[c] 1% ionophore X, 0.25% NaTPB
[d] 10% ionophore X, 0.25% NaTPB
[e] 10% ionphore X, 0.5% NaTPB
[f] Mixture in 'd' with 50% mol. ratio of ETH 500 to ionophore X

phenyl)borate [8]. Are selectivity coefficients of only 1.7 or 2.6 orders of magnitude sufficient to make accurate measurements of Na^+ efflux from a hypothetical Na^+/K^+ transporter with 1:1 stoichiometry under similar background concentrations (5 mM) of the two ions? By using our standard planar flux equation and a modification of the Nicolsky–Eisenman equation (listed in [2]) we calculate that the ISMs based on Na^+ ionophore VI and X would determine a Na^+ efflux that is only 3.3% and 0.4% smaller, respectively, than that determined with an ideal Na^+ ISM. The error will increase when the ratio of $[K^+]/[Na^+]$ increases. This indicates that the two Na^+-selective microelectrodes possess sufficient selectivity to monitor Na^+ fluxes with the extracellular self-referencing technique.

The high background $[Na^+]$ typically used in extracellular mammalian media, 80–140 mM, limits detection of the weak endogenous fluxes. However, we have identified that ISMs based on Na^+ ionophore VI and X have very poor sensitivity to a common organic substitute for Na^+, *N*-methyl-glucamine (NMG) (Table 1). Therefore, the background $[Na^+]$ can be reduced and replaced with NMG to increase the sensitivity of detection for Na^+. Care should be taken to ensure that reduction of Na^+ does not interfere with the transporter(s) being studied.

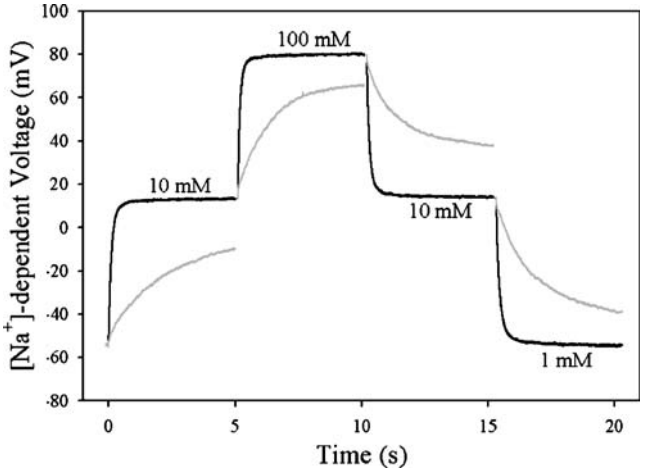

Fig. 1 Response of Na^+-selective microelectrodes, based on Na^+ ionophore X, to step changes in NaCl concentration. The microelectrodes were introduced to virtually instantaneous (≈7 ms) changes in NaCl. The recordings from the microelectrode with 1% ionophore have been aligned with the recordings made with the microelectrode with 10% ionophore so that close comparison could be performed. The response time of the microelectrode made with 10% ionophore X (*black*) is more than an order of magnitude shorter than the microelectrode made with 1% ionophore (*gray*)

The response time of the Na^+ ionophore VI mixture was the fastest of those tested (Table 2), with response times ($t_{95\%}$) between 100 and 130 ms for each of the order of magnitude changes in $[Na^+]$. Na^+ ionophore X has been used at less than 1% by weight in macroelectrode construction [8, 9]. We found that 10% Na^+ ionophore X increased response time by more than an order of magnitude (Fig. 1). Figure 1 shows comparison of response times of two identically constructed microelectrodes (2-mm thick-walled glass with a 25-μm column of ionophore mixture) with different amounts of Na^+ ionophore X, 1% (gray) and 10% (black). Our attempts to increase the response time of the mixture further by increasing the amount of NaTPB decreased the selectivity for Na^+ over Ca^{2+} and Mg^{2+} by more than an order of magnitude (Table 1). ISMs made with our standard thin-walled glass (190 μm) and longer columns of Na^+-selective solvent

Table 2 Response times of Na^+- and Cl^--selective microelectrodes

Ionophore	Mean column length (μm)	Response times (ms, $t_{95\%}$) for given concentration ranges			
		1–10 mM	10–100 mM	100–10 mM	10–1 mM
Na^+ VI[a]	37	120±14	110±13	117±14	127±17
Na^+ X[a]	24	1,488±223	843±130	1,215±203	1,877±212
Na^+ X[b]	17	584±201	495±171	552±143	679±172
Na^+ X[c]	25	335±100	293±77	438±102	461±116
Cl^- II[b]	25	2,795±582	3,119±681	3,213±686	3,397±697

[a] ISM constructed with 1.5-mm thin-walled glass (190 μm)
[b] ISM constructed with 2.0-mm thick-walled glass (440 μm)
[c] 50% mol. ratio ETH 500 to Na^+ X, with 1.5-mm thin-walled glass (190 μm)

produced response times two to three times longer than ISMs made with shorter columns and thicker walled glass, Table 2, indicating that the time constant of the microelectrode was still a limitation even for the optimized mixture. Reduced membrane resistance with ETH 500 also decreased the response time in thin-walled glass (Table 2) without impairing its selectivity (Table 1).

Neither of the Na^+ ISMs based on Na^+ ionophore VI or X showed a significant response (>0.5 mV) to $[H^+]$ over the common working pH range of 6–8 (data not shown).

Response to Na^+ transporter inhibitors

Both microelectrodes based on Na^+ ionophore VI and X were introduced to three common Na^+ transporter inhibitors to determine their effects on microelectrode response. Neither ouabain (100 μM), a Na^+/K^+ pump inhibitor, nor bumetanide (20 μM), an inhibitor of the $Na^+/K^+/2Cl^-$ exchanger, had an effect on the slope of response of the microelectrodes (Fig. 2). However, EIPA (100 μM), an inhibitor of the Na^+/H^+ exchanger, caused major interference on the Na^+ ionophore VI (Fig. 2a) and moderate interference on the Na^+ ionophore X in the 10–1 mM $[Na^+]$ range (Fig. 2b). This will complicate the study of other Na^+ transporters in cells that express high levels of the Na^+/H^+ exchanger as pharmacological knockout of the exchanger with EIPA will interfere with Na^+ detection. Keeping Na^+ at electrochemical equilibrium could minimize Na^+ flux through the exchanger in mammalian cells [10].

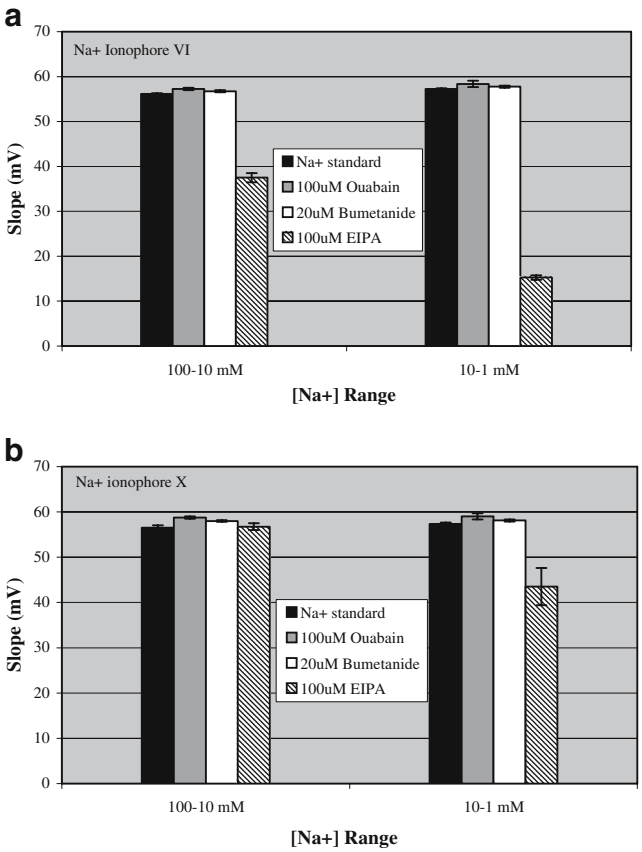

Fig. 2 Pharmacological blockers of Na^+ transport interfere with Na^+ detection. Na^+-selective electrodes based on Na^+ ionophore VI (**a**) and X (**b**) generate potential differences of 56–58 mV between baths of 100–10 and 10–1 mM NaCl. The Na^+ transport inhibitors, ouabain and bumetanide, did not alter these differences significantly. EIPA significantly decreased the response to changes in $[Na^+]$ for the ionophore VI mixture at both concentration ranges tested, but only decreased the response to $[Na^+]$ for the ionophore X mixture at the lower concentration range

Table 3 Selectivity coefficients for Cl^--selective microelectrodes

Anion	Cl^- II	TDMACl	24899[a]	24902[b]
HCO_3^-	−4.1	−1.0	−1.0	−0.9
$H_2PO_4^-$	−4.4	−1.2	−1.3	
Acetate	−5.3	−1.4	−1.3	−1.3
Gluconate	−1.2	0.0	−1.2	−3.0
NO_3^-	−2.6	+1.7	+2.0	
$H_2BO_3^-$	−3.9	−1.9	−1.3	
SO_4^{2-}	−3.1	−0.8		
MES (8.2)	−1.3	−0.5	−0.3	
MES (4.5)	−1.5	−0.9	−1.9	
HEPES (9.5)	−4.6			
HEPES (5.0)	−3.0			

[a] Values obtained from [3]
[b] Values obtained from [11]

Cl^- ionophore II

Cl^--selective microelectrodes were constructed with 2% wt. Cl^- ionophore II and 0.03% wt. TDMACl in *o*-NPOE.

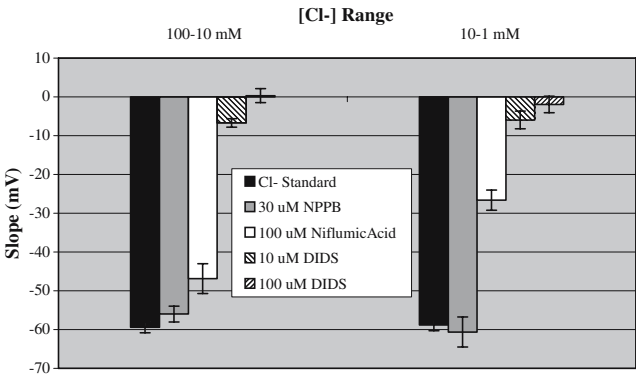

Fig. 3 Pharmacological blockers of Cl^- transport interfere with Cl^- detection. The Cl^--selective electrode based on Cl^- ionophore II generates potential differences of 58–60 mV between baths of 100–10 and 10–1 mM NaCl. Three of four Cl^- transport blockers interfered with Cl^- detection. NPPB had no effect on Cl^- detection. Niflumic acid showed minor interference with Cl^- detection at the high concentration range with significantly greater interference at the lower range. The low concentration of DIDS significantly impaired Cl^- detection, whereas the higher concentration, typically used, impaired detection even further

The Cl^--selective liquid membrane has a mean response of 59.3±2.2 mV over a Cl^- range of 0.1–100 mM and possesses better than four orders of magnitude selectivity for Cl^- over bicarbonate, phosphate, and acetate, but rather poor selectivity against gluconate, a common organic anion replacement for Cl^- (Table 3). The Cl^--selective liquid membrane possesses only about an order of magnitude selectivity for Cl^- over the organic H^+ buffer, MES (pK_a 6.1) but three to four orders of magnitude selectivity for Cl^- over the organic H^+ buffer, HEPES (pK_a 7.5). There is a difference of 1.6 in the selectivity coefficients between HEPES at pH 9.5 and 5.0; however, the difference occurs in the opposite manner of that expected if the microelectrode was sensing the anionic state of the buffer, indicating that some other parameter is influencing the voltage difference between the two pH levels.

For comparison, the selectivity coefficients are listed for the Cl^--selective anionic ion-exchanger, TDMACl, and the previously used Cl^--selective liquid membranes Fluka cat# 24902 [11] and 24899 [3] (Table 3). TDMACl has been reported to possess equal or better selectivity under specific electrode designs [12]. However under the conditions reported here, it possesses only about an order of magnitude selectivity for Cl^- over the other anions tested and even possesses two orders of magnitude selectivity for nitrate over Cl^-.

The response time ($t_{95\%}$) of the Cl^--selective microelectrode was relatively slow in our standard, thin-walled glass pipettes. Even with 25-μm lengths of ion-selective liquid membrane in thick-walled glass with 2- to 3-μm inner tip diameter, the microelectrode possesses response times from 2.5 to 3.5 s (Table 2).

Response to Cl^- transport inhibitors

NPPB (30 μM) was the only inhibitor that had no statistically significant effect on the slope of the responses to [Cl^-] (Fig. 3). While NPPB is a common anion transport inhibitor it is also relatively nonselective as it is used to block anion channels and transporters. Moderate interference to Cl^- detection occurred in the presence of niflumic acid (100 μM), whereas DIDS (10 μM) caused major interference near its lower range of application. Tamoxifen base and tamoxifen citrate (50 μM) immediately eliminated any sensitivity to Cl^-. These facts pose significant limitations to the use of pharmacological inhibition while using this ISM in the extracellular space.

Conclusions

Advances in electrochemical detection with ISMs are necessary to widen the scope of measuring near-real-time analyte flux. Here we have shown that two Na^+- and one Cl^--selective liquid membrane are suitable for use with self-referencing of microelectrodes, albeit with certain limitations. These microelectrodes can be used to measure near-real-time Na^+ and Cl^- flux for the purpose of characterizing plasma membrane transporters and understanding the physiological roles of Na^+ and Cl^- flux under normal and pathological conditions.

Acknowledgements This research was funded by NIH-NCRR grant P41 RR001395 to P.J.S. Smith and by NIH Grants DK077162, DK07789, and DK058563, and DK063125 to I. Kurtz.

References

1. Smith PJS, Sanger RH, Messerli MA (2007) Principles, development and applications of self-referencing electrochemical microelectrodes to the determination of fluxes at cell membranes. In: Michael AC, Borland LM (eds) Electrochemical methods for neuroscience. CRC, Boca Raton, pp 373–405
2. Messerli MA, Robinson KR, Smith PJS (2006) Electrochemical sensor applications to the study of molecular physiology and analyte flux in plants. In: Volkov AG (ed) Plant electrophysiology. Springer, Berlin, pp 73–107
3. Messerli MA, Smith PJS, Lewis RC, Robinson KR (2004) Chloride fluxes in lily pollen tubes: a critical reevaluation. Plant J 40:799–812
4. Smith PJS, Hammar K, Porterfield DM, Sanger RH, Trimarchi JR (1999) Self-referencing, non-invasive, ion selective electrode for single cell detection of trans-plasma membrane calcium flux. Micros Res Tech 46:398–417
5. Umezawa Y, Bühlmann P, Umezawa K, Tohda K, Amemiya S (2000) Potentiometic selectivity coefficients of ion-selective electrodes Part I. Inorganic cations. Pure Appl Chem 72:1851–1856
6. Steiner RA, Oehme M, Ammann D, Simon W (1979) Neutral carrier sodium ion-selective microelectrode for intracellular studies. Anal Chem 51:351–353
7. Tamura H, Kimura K, Shono T (1982) Coated wire sodium- and potassium-selective electrodes based on bis(crown ether) compounds. Anal Chem 54:1224–1227
8. Cadogan AM, Diamond D, Smyth MR, Deasy M, McKervey MA, Harris S J (1989) Sodium-selective polymeric membrane electrodes based on calix[4]arene ionophores. Analyst 114:1551–1554
9. Phillips F, Kaczor K, Gandhi N, Pendley BD, Danish RK, Neuman MR, Tóth B, Horváth V, Lindner E (2007) Measurement of sodium ion concentration in undiluted urine with cation-selective polymeric membrane electrodes after the removal of interfering compounds. Talanta 74:255–264
10. Noël J, Pouysségur J (1995) Hormonal regulation, pharmacology, and membrane sorting of vertebrate Na+/H+ exchanger isoforms, Hormonal regulation, pharmacology, and membrane sorting of vertebrate Na+/H+ exchanger isoforms. Am J Physiol Cell Physiol 268:C283–C296
11. Garber SS, Messerli MA, Hubert M, Lewis R, Hammar K, Indyk E, Smith PJS (2005) Monitoring Cl- movement in single cells exposed to hypotonic solution. J Memb Biol 203:101–110
12. Bratov A, Abramova N, Domínguez C (2004) Investigation of chloride sensitive ISFETs with different membrane compositions suitable for medical applications. Anal Chim Acta 514: 99–106