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Fluorinated tripodal receptors for potentiometric chloride detection in biological fluids

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ABSTRACT

Fluorinated tripodal compounds were recently reported to be efficient transmembrane transporters for a series of inorganic anions. In particular, this class of receptors has been shown to be suitable for the effective complexation of chloride, nitrate, bicarbonate and sulfate anions via hydrogen bonding. The potentiometric properties of urea and thiourea-based fluorinated tripodal receptors are explored here for the first time, in light of the need for reliable sensors for chloride monitoring in undiluted biological fluids. The ion selective electrode (ISE) membranes with tren-based tris-urea bis(CF_3) tripodal compound (ionophore I) were found to exhibit the best selectivity for chloride over major lipophilic anions such as salicylate ($\log K^{pot}_{Cl^-/Sal^-} = +1.0$) and thiocyanate ($\log K^{pot}_{Cl^-/SCN^-} = +0.1$). Ionophore I-based ISEs were successfully applied for chloride determination in undiluted human serum as well as artificial serum sample, the slope of the linear calibration at the relevant background of interfering ions being close to Nernstian (49.8 ± 1.7 mV). The results of potentiometric measurements were confirmed by argentometric titration. Moreover, the ionophore I-based ISE membrane was shown to exhibit a very good long-term stability of potentiometric performance over the period of 10 weeks. Nuclear magnetic resonance (NMR) titrations, potentiometric sandwich membrane experiments and density functional theory (DFT) computational studies were performed to determine the binding constants and suggest 1:1 complexation stoichiometry for the ionophore I with chloride as well as salicylate.

1. Introduction

Chloride is one of the most critical targets in biological fluids as its concentration, along with that of some other ions such as sodium, potassium, calcium, magnesium and lithium, is used for rapid patient care decisions (Dimeski et al., 2010). Accordingly, approaches to monitor these critical care species require development of sensors and devices for real-time monitoring with very high precision and accuracy.

Few methodologies are used in clinical laboratories for chloride determination, such as for example colorimetric, coulometric-amperometric and potentiometric procedures for serum analysis (Frost and Meyerhoff, 2015; Panteghini et al., 1986). Undeniably, potentiometric sensors offer one of the most convenient non-destructive way of determining ionic species due to their low cost, simple fabrication and miniaturization and low-energy consumption (Bakker and Telting-Diaz, 2002; Bobacka et al., 2008).

There exist two main types of anion-selective membranes for the potentiometric detection with ion-selective electrodes (ISEs). The first type, historically the most explored one, is the ISE membrane based on crystalline materials such as the AgCl-based solid-state electrode. However, the latter is not suitable for the analysis of biological samples since it suffers from protein adsorption to the AgCl surface (Bratov et al., 2004; Hulanicki and Michalska, 1995). The second type of membranes is based on polymeric matrices doped with ionophore and/or ion exchanger. The ISEs based on polymeric membranes have recently become an attractive tool for the direct monitoring of chloride in clinical analysis (Burtis and Bruns, 2014; Frost and Meyerhoff, 2015; Oesch et al., 1986; Yoon et al., 1998), however only few of the receptors reported so far possess adequate performance for practical application owing to challenges arising when analyzing biological fluids.

There are several issues in the development of the receptors adequate for the analysis of biological samples: i) leaching of active membrane components; ii) low biocompatibility of the membrane

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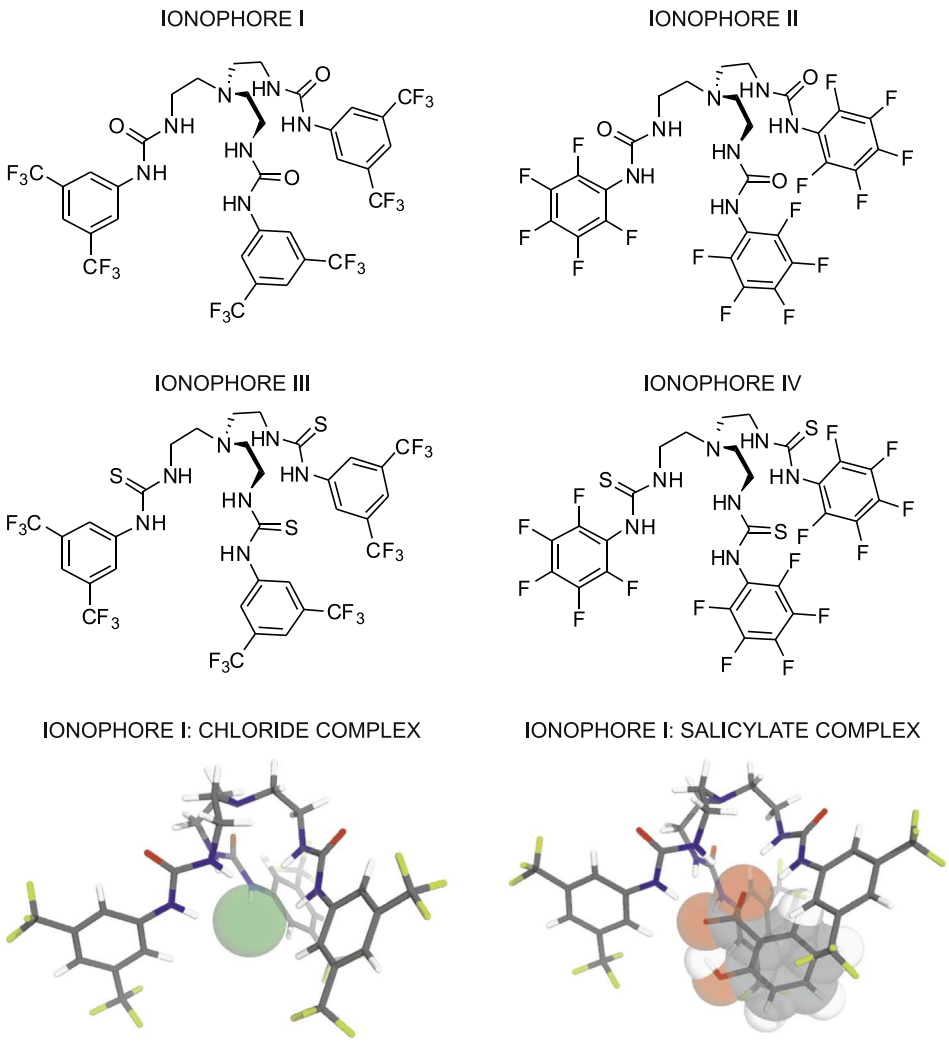

Fig. 1. (Top) Structures of the ionophores I-IV: (I) Tren tris-urea bis(CF_3) [$C_{33}H_{27}F_{18}N_7O_3$]; (II) Tren tris-urea pentafluoro [$C_{27}H_{18}F_{15}N_7O_3$]; (III) Tren tris-thiourea bis(CF_3) [$C_{33}H_{27}F_{18}N_7S_3$]; (IV) Tren tris-thiourea pentafluoro [$C_{27}H_{18}F_{15}N_7S_3$]. (Bottom) DFT (M06-2X/6−31+G(d)) optimized structures of the chloride and salicylate complexes of ionophore I without counter cation in vacuum; C (gray), H (white), N (blue), O (red), F (yellow-green), Cl (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

material with the sample and adsorption of proteins; iii) low selectivity of most receptors over interfering lipophilic anions such as salicylate, thiocyanate and bicarbonate commonly present in biological fluids (Bratov et al., 2004).

Certainly, salicylate is often the main interfering ion due to its lipophilicity, relatively high concentration and variable content in biological samples. For this reason, the determination of chloride in serum using ISEs often provides biased results owing to increased levels of salicylate in the samples from the patients who take aspirin (Yoon et al., 1998). Another challenge in potentiometric chloride detection concerns the upper detection limit of the ISEs since the high chloride concentration in clinical samples (*ca*., 100 mM in blood and serum) often causes strong complexation in the sensing phase, resulting in Donnan exclusion failure (Radu and Bakker, 2005).

While most chloride-selective ionophores reported in the past are organometallic compounds, some active membrane components reported recently are based on chloride complexation by hydrogen bonds (Sabek et al., 2015; Xiao et al., 1997; Zahran et al., 2010). Commonly used chloride-selective receptors with a metal center such as mercury, manganese or indium (Kondo et al., 1989; Park et al., 1991; Radu and Bakker, 2005; Rothmaier et al., 1996) often exhibit stability and/or toxicity issues (Sabek et al., 2015).

It is noted that most proposed receptors, both neutral and charged carriers, do not provide better selectivity and stability of chloride detection for clinical applications than traditional ion-exchanger based membrane (tridodecylmethylammonium chloride [TDMACl]) (Bratov et al., 2004). Consequently, the selectivity pattern of ISEs based on lipophilic quaternary ammonium salts (such as TDMACl) is fixed and follows the Hofmeister series since the selectivity of ion exchanger is mainly defined by the lipophilicity of the ion. The application of this type of chloride ISE is therefore limited to samples without significant concentrations of anions more lipophilic than chloride, and it would not be recommended for samples that contain salicylate or thiocyanate as in the case of blood or serum samples. Nevertheless, ISEs based on quaternary ammonium salts as active membrane component are still used commercially for chloride determination in biological samples despite the aforementioned limitations, owing to the lack of selective chloride receptors (Burtis and Bruns, 2014). To the best of our knowledge, only few studies published in the last two decades have reported on receptors with sufficiently improved potentiometric characteristics, close to those required for chloride detection in biological samples (Gupta et al., 2009; Sabek et al., 2015; Xiao et al., 1997; Zahran et al., 2010). However, even fewer studies demonstrated successful application of the investigated compounds for potentiometric chloride detection in undiluted non-spiked physiological samples (Sabek et al., 2015). Thus, further efforts are required to develop chloride selective ISEs with better selectivity, and adequate analytical performance and compatibility with biological fluids.

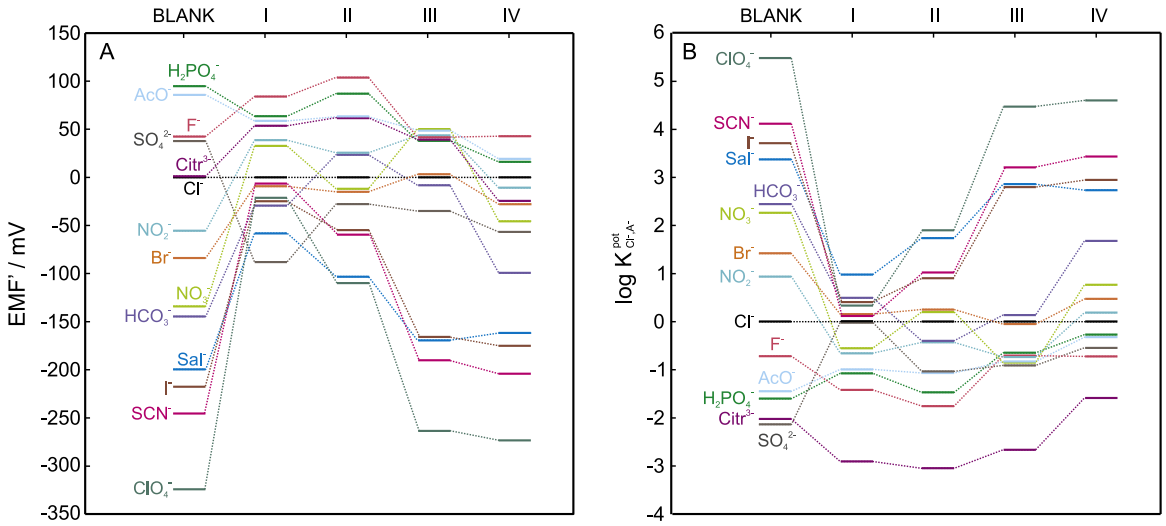

Fig. 2. Selectivity screening of ISEs based on the ionophores I-IV and a blank membrane (same composition but no ionophore). (A) Potential observed in 1 mM solution of corresponding sodium/potassium salt (EMF' is the potential corrected to EMF in 1 mM NaCl). (B) Potentiometric selectivity coefficients for chloride over different anions (A^-) at 1 mM concentration. Sal^-, AcO^- and $Citr^{3-}$ stand for salicylate, acetate and citrate anions respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The tren-based fluorinated tripodal compounds were recently shown to be efficient transmembrane transporters for chloride, nitrate, bicarbonate and sulfate anions in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) liposomal based assays (Busschaert et al., 2014, 2011). It was demonstrated that fluorinated transporters are more active anion transporters than their non-fluorinated analogues (Busschaert et al., 2014). To the best of our knowledge, the potentiometric properties of fluorinated tripodal compounds have not been reported in the past. This work therefore aims to examine a series of fluorinated tripodal compounds, both tris-ureas and tris-thioureas (Busschaert et al., 2011), as potential candidates for chloride analysis in biological samples.

2. Material and methods

2.1. Electrodes and membrane preparation

The membrane-based electrodes for potentiometric experiments were prepared according to well-established procedure (Bakker et al., 1997; Craggs et al., 1974). The amounts of synthesized receptors I-IV (see Fig. 1 for structures) taken for the preparation of ca. 200 mg of corresponding PVC-based membranes were as follows: 2.7 mg (15 mmol per kg of membrane) or 3.7 mg (20 mmol kg^{-1}) of ionophore I, 2.3 mg (15 mmol kg^{-1}) of ionophore II, 2.9 mg (15 mmol kg^{-1}) of ionophore III and 2.5 mg (15 mmol kg^{-1}) of ionophore IV. The above-indicated amount of the corresponding ionophore was mixed with 0.6 mg of ion exchanger TDMACl (5 mmol kg^{-1}), 66 mg of poly(vinyl chloride) (PVC) and 132 mg of dodecyl 2-nitrophenyl ether (DNPE) (PVC:DNPE [1:2]) and dissolved in 2 mL of tetrahydrofuran (THF). A blank membrane (in absence of ionophore) contained 5 mmol $kg^{-1}$of TDMACl, 66 mg of PVC and 132 mg of DNPE. The prepared cocktail was poured into a glass ring, (22 mm in diameter) placed on a glass slide and dried overnight at room temperature under a dust-free environment. After complete THF evaporation, a membrane of approximately 200 μm thickness was obtained. Small disks (diameter ca. 8 mm) were punched from the cast films. The obtained membranes were conditioned for two days in 1 mM KH_2PO_4 (pH~5) for selectivity screening and in 100 mM NaCl (pH~5.5) for all other experiments. After that, the membranes were mounted in Ostec electrode bodies that contain inner silver-silver chloride elements (Oesch Sensor Technology, Sargans, Switzerland). The inner compartment of the electrode was always filled with a solution containing 1 mM NaCl. The electrodes for human serum analysis were typically pre-conditioned for 1–2 days in 100 mM NaCl; long-term conditioning (for the stability experiment during 10 weeks) was performed in 1 mM NaCl in order to avoid super-Nernstian slopes.

2.2. Reference method for chloride detection

Argentometric titration with silver nitrate solution (0.1 M) and potassium chromate as indicator (so-called Mohr's method) was selected as the reference method for the determination of chloride in the human serum sample. Preliminary deproteinization was accomplished to facilitate the endpoint visualization using acetonitrile-NaOH treatment (Benzo et al., 2002). The restrictions regarding the acceptable protein concentration for accurate direct titration of biological fluids using chromate as indicator were emphasized in the past (Rose, 1936) and account for the interaction between the protein and the indicator. For more details, see 'Supporting information' section.

The detailed description of the reagents, material and equipment, ^{1}H NMR titration binding studies and the reference method for chloride detection is given in the Supporting Information.

3. Results and discussion

In this work, four different fluorinated tripodal receptors were examined as potential anion ionophores. Fig. 1 presents the chemical structures of these receptors (named as ionophores I, II, III and IV) and the density functional theory based structures of 1:1 complexes formed by ionophore I with anions such as chloride and salicylate.

In a first screening, the potential response of plasticized ionophore-based membranes (15 mmol kg^{-1} ionophore) together with the blank membrane (no ionophore) towards 1 mM concentration of common inorganic anions (chloride, salicylate (Sal^-), bicarbonate, thiocyanate, bromide, sulfate, acetate (AcO^-), dihydrogen phosphate, nitrate, nitrite, fluoride, citrate ($Citr^{3-}$), perchlorate, iodide) was evaluated. Fig. 2A shows the normalized electromotive force values in 1 mM solutions of different anions EMF'_{A^-} calculated by subtracting the readout potential value in 1 mM chloride solution EMF_{Cl^-} from the EMF measured in the solution of the corresponding anion (EMF_{A^-}) as follows: $EMF'_{A^-} = EMF_{A^-} - EMF_{Cl^-}$

From the EMF' values presented in Fig. 2A, the selectivity coefficients for chloride over other anions were estimated by applying the separate solutions method (see Supporting Information for details)

and schematically illustrated in Fig. 2B (Umezawa et al., 2000). Positive values in Fig. 2A indicate a less interfering effect than chloride (i.e., for the blank membrane, EMF_{F^-} is + 43 mV) whereas a positive value in selectivity coefficient plots means the opposite (i.e., for the blank membrane, the selectivity coefficient for chloride over perchlorate is + 5.5 indicating that perchlorate is ~300.000 times more interfering over chloride at the same concentration).

While the blank membrane behavior follows the typical Hofmeister selectivity pattern ($ClO_4^- > I^- > NO_3^- > Br^- > Cl^- > F^- > H_2PO_4^- > SO_4^{2-}$), the ionophores I-IV exhibit, in general, a suppressed selectivity for chloride over sulfate ($\log K^{pot}_{Cl^-/SO_4^{2-}}$ from −1 to 0 vs. $\log K^{pot}_{Cl^-/SO_4^{2-}} = -2.1$ for the blank membrane) and to a lesser extent over phosphate and acetate ($\log K^{pot}_{Cl^-/H_2PO_4^-}$ and $\log K^{pot}_{Cl^-/AcO^-}$ both from −1.1 to −0.3 vs. $\log K^{pot}_{Cl^-/H_2PO_4^-} = -1.6$ and $\log K^{pot}_{Cl^-/AcO^-} = -1.5$ for the blank membrane). In contrast, the selectivity for chloride over the majority of other inorganic anions increases remarkably, including highly lipophilic anions such as perchlorate, thiocyanate, salicylate, bicarbonate and nitrate. The significantly improved selectivity for chloride over most lipophilic anions in comparison to the blank membrane is especially pronounced for ionophore I. For example, the values of $\log K^{pot}_{Cl^-/ClO_4^-}$, $\log K^{pot}_{Cl^-/SCN^-}$, $\log K^{pot}_{Cl^-/Sal^-}$, $\log K^{pot}_{Cl^-/HCO_3^-}$ decrease from +5.5 to +0.4, from +4.1 to +0.1, from +3.4 to +1.0 and from +2.4 to +0.5, respectively.

Accordingly, it has been demonstrated (see Fig. 2) that ionophore I may be plausible for the detection of chloride in the presence of lipophilic anions, which is in fact highly desirable for the analysis of biological fluids since some of the anions mentioned, such as salicylate, thiocyanate and bicarbonate, are often present at significant concentrations and may therefore interfere with chloride determination. The concentration ranges for major anions in human serum are listed in Table 1. For instance, the concentration of salicylate is relatively high and variable, from a few μM to the mM level (Blacklock et al., 2001; Borthwick et al., 2006; Duh and Cook, 2005; Wong et al., 2016), which makes it extremely difficult to calibrate the sensor by adjusting the background concentration of standard solutions as it would require additional knowledge of salicylate concentration in every particular sample prior to chloride analysis. Hence, to enable chloride determination it is essential to provide a receptor with a suppressed response towards salicylate.

In this context, a more careful study of ionophore I selectivity for chloride over salicylate was performed by recording a complete set of calibrations for chloride and salicylate anions. As observed in Fig. 3, ionophore I exhibits the best selectivity for chloride over salicylate compared to ionophores II, III and IV. The selectivity coefficients calculated from the full calibration curves are similar to those obtained in the preliminary screening (see Fig. 2B). The $\log K^{pot}_{Cl^-/Sal^-}$ equals to + 3.7 for the blank membrane and + 1.0, + 1.9, + 3.0 and + 3.1 for ionophores I, II, III and IV respectively, suggesting that ionophore I may so far be the most promising candidate for chloride detection. Note that a value of + 1.0 indicates that the same ISE, in two different solutions containing the same concentration level of either Cl^- or Sal^-, gives a larger response for Sal^- compared to Cl^- (by approximately −60 mV). As chloride in serum samples is at least 100-fold more concentrated than salicylate (see Table 1), chloride detection using ISEs based on ionophore I should be possible.

Table 1
Reference range values of major anions present in human serum for healthy people.

Anion (A^-)	Concentration, mM
Chloride	98–107 (Duh and Cook, 2005)
Bicarbonate	22–29 (Duh and Cook, 2005)
Salicylate[a]	0.5 (Borthwick et al., 2006); 0.2–0.7 (Wong et al., 2016); 1.09–2.17 (Duh and Cook, 2005)
Phosphate	0.87–1.45; 1.45–1.78[b] (Duh and Cook, 2005)
Sulfate	0.2–0.5 (Blinn et al., 2005)
Thiocyanate	0.017–0.069; 0.052–0.206[c] (Duh and Cook, 2005)

[a] Therapeutic level.
[b] For children.
[c] For smokers.

The results of molecular modelling using density functional theory (DFT) calculations (M06-2X/6−31+G(d)) (Zhao et al., 2008) for the chloride and salicylate complexes with ionophore I demonstrated the optimized 1:1 host:guest geometry. The structures of the corresponding complexes obtained from the DFT calculation are presented in Fig. 1. The 1:1 complexation stoichiometry is consistent with previously reported crystal structures of receptors I, III and IV with various anions (chloride, sulfate, nitrate and carbonate) (Busschaert et al., 2011). The NMR titration data with TBA-chloride (in DMSO-d_6/0.5% H_2O mixture) fit best to the additive 1:2 binding model (see Supporting Information, Figs. S1-S8), thenceforth deriving the stepwise association constants (K_1 and K_2) (Howe et al., 2014). The derived association constants of binding to chloride for receptors I-IV ($\log K_1$ & $\log K_2$) are 4.1 & 0.8, 3.4 & 0.9, 4.3 & 0.9, and 3.5 & 1.0 respectively, the weak K_2 for all receptors indicating that the formation of 1:2 host:guest complex at the working concentration of the sensor is negligible. Generally, the thiourea analogues bind chloride stronger than the urea equivalents (receptors I c.f. III and II c.f. IV) due to the more acidic NH hydrogen bond donors of thiourea (Gomez et al., 2005). Interestingly, a remarkable difference was found for bis(CF_3) and pentafluoro functionalities which resulted in significantly larger association constants for the bis(CF_3) substituent for both urea and thiourea analogues.

The derived association constants ($\log K_a$) from fitting the NMR titration data with TBA-salicylate to the 1:1 binding model for salicylate complexation for receptors I-IV were found to be 1.8, 1.4, 1.9 and 1.7 respectively. While following the same trend as observed for chloride complexation (receptor III > I > IV > II), these values are lower than those calculated above for chloride. In addition, NMR titration studies with TBA-perchlorate indicate no binding (see Supporting Information, Figs. S9-S12). The logarithmic binding constants estimated from selectivity coefficients (Ceresa and Pretsch, 1999) for the receptors I, II, III and IV were found to be respectively 5.8, 4.3, 1.7 and 1.6 for chloride complex and 3.4, 2.7, 1.2 and 1.0 for salicylate complex, assuming no substantial binding of the ionophores with the perchlorate ion confirmed by the 'sandwich membrane experiment' (Mi and Bakker, 1999) (see Supporting Information, Table S1, Eq. S7). Therefore, similarly to NMR-based binding constants, the complex formation constants obtained by potentiometry suggest stronger binding of the receptors with salicylate. However, significant discrepancy between the absolute values is observed and the trend is different in comparison with NMR-studies (potentiometry-based trend: receptor I > II > III > IV). The latter accounts for the difference in hydration energy of the anions (Morf, 1981) along with the difference in organic solvent environments employed in two independent studies (from DMSO-d_6 to plasticized PVC media).

Having determined that ionophore I is the most selective one for chloride over salicylate, the composition of ionophore I-based anion selective membrane was optimized and a membrane containing 20 mmol kg^{-1} of ionophore I and 5 mmol kg^{-1} of ion exchanger (to give a higher slope at high concentrations of chloride with salicylate background) was chosen for further experiments.

To evaluate the suitability of the ionophore I for chloride detection in biological fluids, calibrations of the relevant ISE were performed in solution with salicylate in background. The choice of the salicylate background concentration was determined by the salicylate levels in human serum which relies heavily on the diet and drug consumption since salicylic acid is a common component of anti-inflammatory drugs. A typical range of few μM or lower has been reported for serum samples of the patients not taking aspirin (Blacklock et al., 2001) while

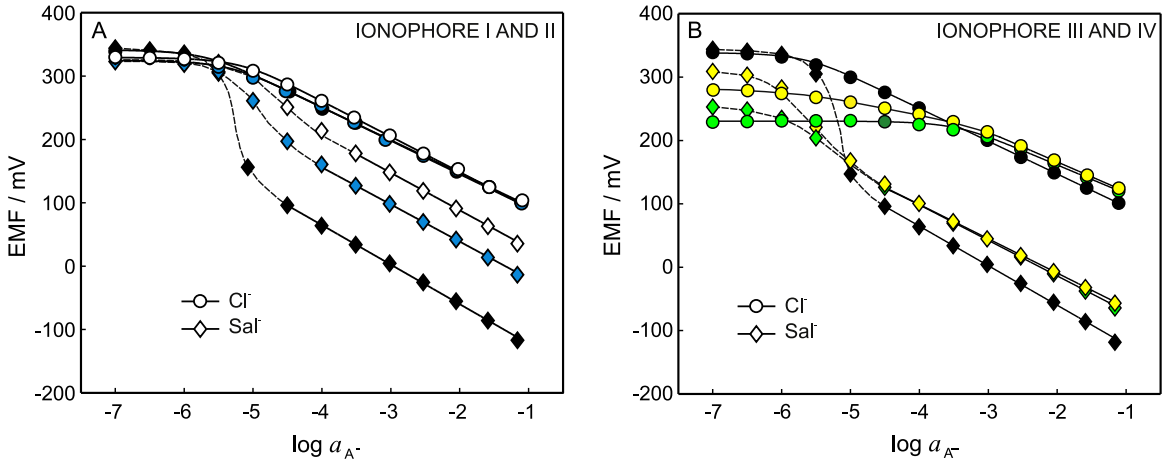

Fig. 3. Calibration curves for chloride (circles) and salicylate (diamonds) for ISEs based on different receptors. (A) Ionophore I (white), ionophore II (blue), blank membrane (black), (B) ionophore III (yellow), ionophore IV (green), blank membrane (black). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

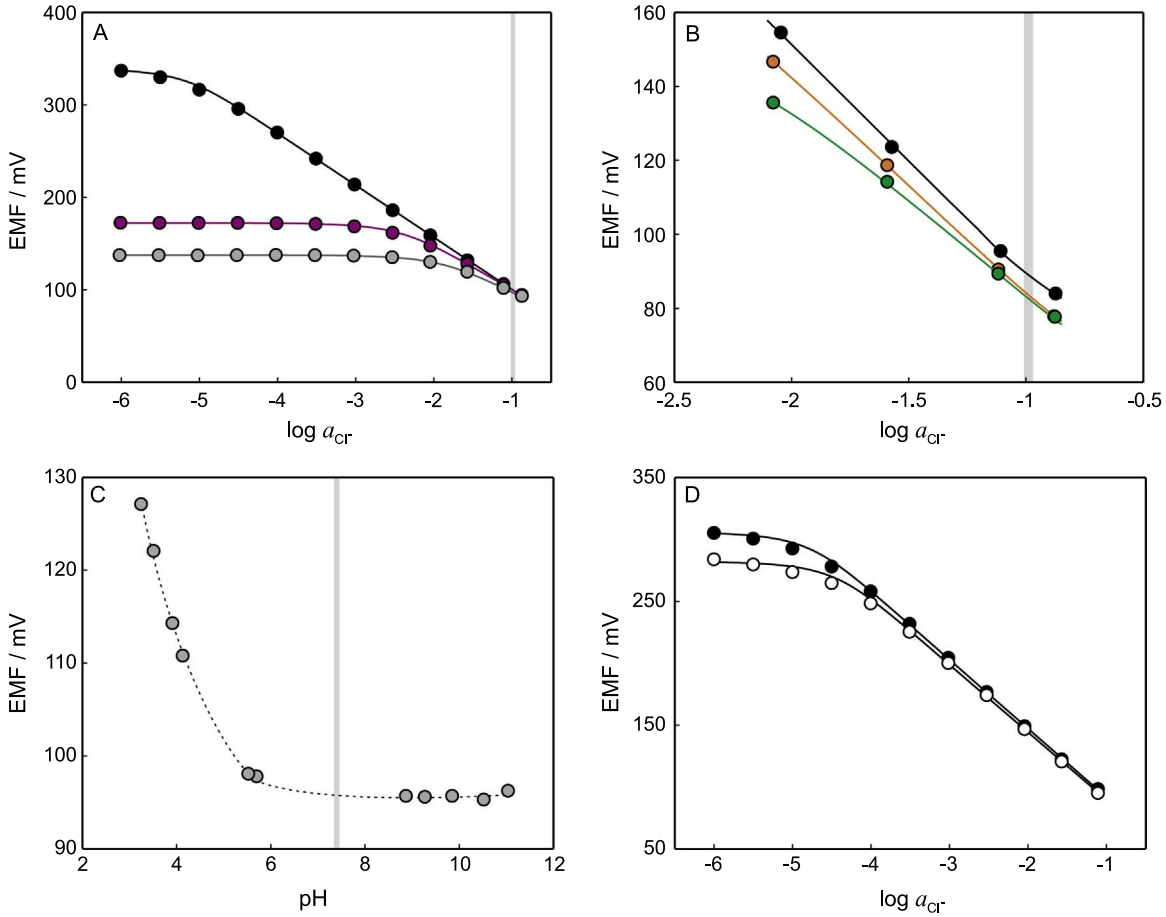

Fig. 4. Influence of the background and pH on the performance of the ISE based on ionophore I. (A) Calibration curves for chloride with different salicylate backgrounds: no salicylate (black), 0.5 mM NaSal (purple) and 2 mM NaSal (gray). (B) Calibration curves for chloride with different backgrounds: no background (black), 30 mM $NaHCO_3$ acidified to pH 7.6 (orange), 30 mM $NaHCO_3$ and 0.5 mM NaSal acidified to pH 7.5 (green). (C) Potential changes upon addition of sodium hydroxide to the solution of NaCl 100 mM acidified with HCl. Dashed line is shown to guide the eye. (D) Calibration curves in water at pH 5.5 (black) and 10 (white). The gray window in the figures indicates the reference chloride range in human serum for healthy people. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the values reported for the patients taking drugs containing salicylic acid vary significantly for different studies (see Table 1). Normal therapeutic salicylate level for analgesic purpose is found to be about 0.2–0.7 mM (Wong et al., 2016). However, in severe cases of salicylate poisoning salicylate serum levels may reach the concentration of several mM; the salicylate levels higher than 2 mM lead to salicylate poisoning and require appropriate treatment (Blacklock et al., 2001; Cotty et al., 1965; Goto et al., 1998; Wong et al., 2016; Wrathall et al., 2001) Considering the aforesaid, the calibration curves for chloride analyte (Fig. 4A) were recorded at two salicylate levels: 0.5 mM (normal therapeutic level of patients taking aspirin) (Wong et al., 2016) and 2 mM (highest limit of asymptomatic patients or patients with mild salicylate poisoning) (Dargan et al., 2002).

As expected, the calibration curve without background electrolyte

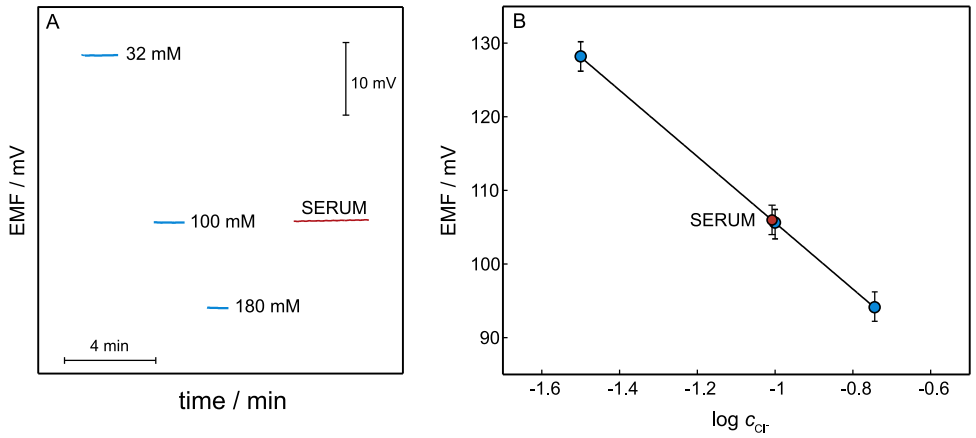

Fig. 5. Chloride detection in human serum sample. (A) Potential readings for three calibration solutions with 0.5 mM salicylate background and the serum sample. Concentration of chloride in the calibration solutions is indicated in the Figure. (B) Calibration curve corresponding to the results presented in A.

results in a Nernstian slope of 57.3 ± 1.8 mV (n = 12) with a lower detection limit (LDL) of ~10 μM. Deterioration of the electrode performance is observed both at 0.5 mM and 2 mM salicylate background resulting in slopes of 46.7 ± 1.8 mV and 35.8 ± 4.3 mV (n = 12 and 6 respectively) accompanied with an increment of the lower detection limits up to 3 and 10 mM respectively, yet satisfactory for the measurement of chloride in the required range (approx. 100 mM).

Due to the fact that pH of blood and serum normally ranges from 7.35 to 7.45 (Duh and Cook, 2005), it was important to characterize the influence of pH on the performance of the electrode. Fig. 4C illustrates the potential changes (EMF) of the ionophore I-based ISE upon increasing the pH by successive additions of 2 M NaOH to NaCl solution preliminary acidified with HCl to pH~3.3 (total chloride concentration 100 mM). A well-defined plateau (96.3 ± 1.3 mV) is observed above pH~5.5 until pH~11.5 (higher pH values were not accomplished within the experiment) meaning that the performance of the ISE is nearly pH-independent in the given pH range at 100 mM chloride concentration. The EMF increase below pH~5.5 might possibly account for protonation of the tertiary amine of the tren motif resulting in the decrease of ionophore I partitioning within the PVC-based membrane. Interestingly, this observation correlates with the transmembrane anion transport activity of tren-based receptors, which were completely inactive in POPC liposomes at acidic pH (Wu et al., 2016). To confirm the applicability of the membrane at higher pH values, the calibration in water at pH 5.5 was compared to the calibration curve obtained at increased pH (Fig. 4D). A pH of 10 was chosen, as it was difficult to achieve a constant pH of 7.4 without buffering the sample. It must be noted that the experiments described here were performed in non-buffered aqueous solutions (unless specified, in the case of bicarbonate as background electrolyte). This is due to the fact that the presence of organic buffers (calibrations in Tris, MES and HEPES were performed, results not shown) significantly deteriorates the electrode performance (lower limit of detection and the slope of the calibration curve). The latter has been already reported in the past for different types of chloride sensors (Bratov et al., 2004; Xiao et al., 1997) based on ionophores with hydrogen bond donor groups and accounts for the complexation between the highly lipophilic buffer anions and the receptor (Xiao et al., 1997). By comparing both calibration curves, it can be seen that the increase of pH from 5.5 to 10 deteriorates the lower detection limit of the electrode only to a very modest extent ($\log a_{LDL} = -4.8$ and -4.5 respectively), and more importantly, it does not influence the electrode performance in the concentration range required for the analysis of undiluted biological fluids.

As it follows from the selectivity coefficients presented in Fig. 2 and the reference range values given in Table 1, apart from the salicylate interference, the bicarbonate interference should also be considered for the analysis of serum samples. Therefore, a calibration at 30 mM bicarbonate background was performed, along with the calibration in the solution containing both 0.5 mM salicylate and 30 mM bicarbonate (Fig. 4B). The initial chloride concentration for the calibration with bicarbonate background was much higher than in Fig. 4A (ca. 10 mM chloride) due to the necessity to acidify the bicarbonate solution to pH ca. 7.5 which was accomplished by spiking with hydrochloric acid. The latter was performed to achieve the required pH without introducing extraneous anions (such as sulfate etc.) that might deteriorate the electrode response. Importantly, as can be observed in Fig. 4B, the presence of bicarbonate in the solution does affect the electrode performance resulting in the slope 51.9 ± 2.4 mV (n = 6) and a shift of the intercept (E°) of ca. 7 mV. The addition of 0.5 mM salicylate to the bicarbonate at pH 7.5 affects the performance only to a small extent resulting in the slope of 49.8 ± 1.7 mV (n = 12). Thus, a very small difference in the electrode performance is observed with bicarbonate background only and the bicarbonate with the additional amount of 0.5 mM salicylate suggesting the adequacy of analyzing the samples both with no salicylate and therapeutic level of salicylate using the same external calibration.

Hereafter, the chloride detection in human serum sample was accomplished using external calibration in the solution containing 0.5 mM salicylate and 30 mM sodium bicarbonate (Fig. 5). For simplicity, the calibration curve in Fig. 5B is shown in concentration scale (instead of activity) where the slope corresponds to 46.7 ± 1.6 mV in log c-scale (n = 12). The method was first validated by analyzing the artificial serum sample (see Supporting Information for the composition) containing 112.6 mM chloride level. An acceptable recovery of 98.8 ± 4.3% was accomplished resulting in a chloride concentration of 110.8 ± 4.7 mM (n = 7). The potentiometric chloride detection in the human serum sample was performed multiple times at different days, every time with a set of 2–4 electrodes, pre-conditioned in the 100 mM NaCl solution for at least 1 day. The chloride concentration was found to be 98.6 ± 3.8 mM (n = 10).

To confirm the reliability of the potentiometric results, argentometric titration was chosen as a reference method for chloride detection in the sample (Rose, 1936). The chloride concentration in the artificial serum sample (112.6 mM chloride) was found to be 111.1 ± 4.0 mM (n = 8) whereas the chloride concentration in the human serum sample after deproteinization was found to be 109.3 ± 5.9 mM (n = 6). Even though the deproteinization leads to better endpoint visualization, it was observed that the titration endpoint in the real sample even after deproteinization was much less pronounced compared to the artificial sample, thus the slightly elevated (in comparison to potentiometric measurements) value obtained titrimetrically accounts most probably for the difficulties of analyzing the colored serum sample with a complex matrix. The results obtained using both

potentiometric and volumetric titration methods were compared by applying the F-test at NC = 95% and the *t*-test at NC = 99%. The calculated F and t values (F = 2.41 and t = 3.98) did not exceed the theoretical values (F = 6.68; t = 4.03), indicating that there are no significant differences in accuracy or precision between the two methods at the considered confidence level.

Finally, the long-term stability of ionophore I-based ion-selective membrane electrode was evaluated over a period of 10 weeks (see Supporting Information, Fig. S13). No change in electrode performance within 3 weeks of different experiments (including multiple calibrations and exposure to real serum samples) and a moderate deterioration of the electrode slope over the period of 10 weeks were observed (long-term conditioning was performed in 1 mM NaCl to avoid super-Nerstian slopes). It should be mentioned that such excellent long-term stability is rarely achievable with anion-selective receptors.

4. Conclusions

The investigation of potentiometric properties of ion selective electrodes (ISEs) based on fluorinated tren-based tripodal compounds, previously reported as efficient transmembrane transporters for chloride, nitrate, bicarbonate and sulfate anions facilitated by hydrogen bonding complexation, showed this new class of receptors to be promising for potentiometric anion sensing. ISEs based on tren-based tris-urea bis(CF_3) tripodal compound (ionophore I) were found to exhibit significantly improved selectivity for chloride over major lipophilic anions (such as salicylate, thiocyanate, bicarbonate and nitrate) compared to the blank ISE (without ionophore) as well as to the majority of chloride-selective receptors reported so far. The results of DFT computational studies showed the binding geometry of ionophore I complex with chloride as well as with the main interfering anion salicylate to exhibit 1:1 stoichiometry. Sufficient selectivity over salicylate and bicarbonate along with high upper limit of detection enable direct analysis of chloride in undiluted human serum. The chloride detection in human serum as well as artificial serum sample was accomplished using a poly(vinyl chloride)-based ISE, the results of potentiometric measurements were confirmed using argentometric titration. Remarkably, ionophore I was shown to exhibit a good long-term stability of potentiometric performance over the period of 10 weeks of application which is a rare success for solvent polymeric ionophore-based anion-selective membrane electrodes.

Associated content

Detailed description of the reagents, materials and equipment, ^{1}H NMR titration binding studies, DFT computational studies and the reference method for chloride detection. Figs. S1-S8. Fitted binding isotherms of ^{1}H NMR titration binding studies. Figs. S9-S12. Selected partial ^{1}H NMR titration spectra of ionophores I- IV with TBA-perchlorate. Fig. S13. Stability of performance of the ISE based on Ionophore I. Calculation of selectivity coefficients and ion activities. Estimation of formal complex formation constants from potentiometric data.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2017.07.001.

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