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Tweezer-Type Neutral Carrier-Based Calcium-Selective Membrane Electrode with Drastically Reduced Anionic Interference

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A new calcium-selective ionophore *N*,*N*-dioctyl-3α,12α-bis(*N*-heptyl-*N*-methylcarbamoyl-methoxyacetamidoacetoxy)-5*β*-cholan-24-amide (denoted as BACA), was synthesized, and its potentiometric performance has been evaluated in comparison with that of the best known calcium-selective neutral carriers, ETH 129 and ETH 1001. The ^{1}H NMR spectra of BACA titrated with Ca(SCN)$_2$ suggest that BACA forms a 1:1 complex, tweezing a calcium ion between the two parallel diamide groups substituted on a rigid bile acid frame. The calcium-selective membrane based on BACA was less selective to calcium (log $K^{POT}_{Ca^{2+},j}$ = −4.2, −4.2, −4.6, and −4.8, respectively, for *j* = Mg^{2+}, Li^+, Na^+, and K^+) than those based on ETH 129 (log $K^{POT}_{Ca^{2+},j}$ = −4.4, −4.3, −5.4, and −5.4, respectively, in the same order) and ETH 1001 (log $K^{POT}_{Ca^{2+},j}$= −4.4, −4.4, −5.4, and −5.4), implying that BACA forms a weaker calcium complex than the other two ETH compounds. In our experimental conditions, potentiometrically determined complex formation constants of calcium-selective neutral carriers (log $\beta_{Ca^{2+}L}$) were 15.2, 14.0, and 8.6 for ETH 129, ETH 1001, and BACA, respectively. A slightly reduced calcium selectivity of BACA, however, affects the anionic interference-free calcium-selective membrane; the BACA-based membrane exhibits a Nernstian response up to 10^{-1} M Ca^{2+} in the presence of lipophilic anions (e.g., SCN^-, ClO_4^-, salicylate, and I^-) and anionic surfactant, whereas the ETH 129- and ETH 1001-based ones suffer from serious anionic interference showing a curvature or leveled off response over about 10^{-4} M. It was demonstrated that such a trade off does not affect the analytical performance of BACA-based electrode in most applications, including clinical analysis.

The calcium ion, one of the most important electrolytes in physiological systems, is known to form 1:2 and 1:3 complexes with the noncyclic diamides, for example, (−)-(*R*,*R*)-*N*,*N*′-(bis(11-ethoxycarbonyl)undecyl)-*N*,*N*′-4,5-tetramethyl-3,6-dioxaoctanediamide (ETH 1001) and *N*,*N*,*N*′,*N*′-tetracyclo-3-oxapentanediamide (ETH 129), respectively.[1−4] Both ETH compounds have been most widely used in the construction of calcium-selective electrodes, as well as in the model study of calcium transport phenomena in biological systems.[5−10]

The calcium-selective electrodes based on these ionophores, however, exhibit a large interfering response to lipophilic anions (e.g., ClO_4^-, SCN^-, I^-, Br^-, and salicylate). For example, they result in an anionic response or considerably decreased sensitivity in the presence of such anions.[11] Clinical analyzers based on a neutral carrier-based calcium-selective electrode are known to report reduced ionized calcium levels in the presence of a toxic level of thiocyanate.[12] The theory of ISE predicts that such an anionic interference response could be diminished by formulating the ISE membrane with a new ionophore that has a smaller complex formation constant than the existing ones for the primary ion.[13] However, the compromise should be made without costing the excellent calcium selectivity of ETH 1001 and ETH 129. In this report, we introduce an intelligently designed calcium-selective neutral carrier that satisfies such requirements.

7-Deoxycholic acid derivatives are useful frame molecules having two hydroxyl linkers at the C3 and C12 carbons, which are aligned in the same direction, approximately parallel, and ∼6.1 Å apart on the conformationally rigid steroidal ring structure.[14−19]

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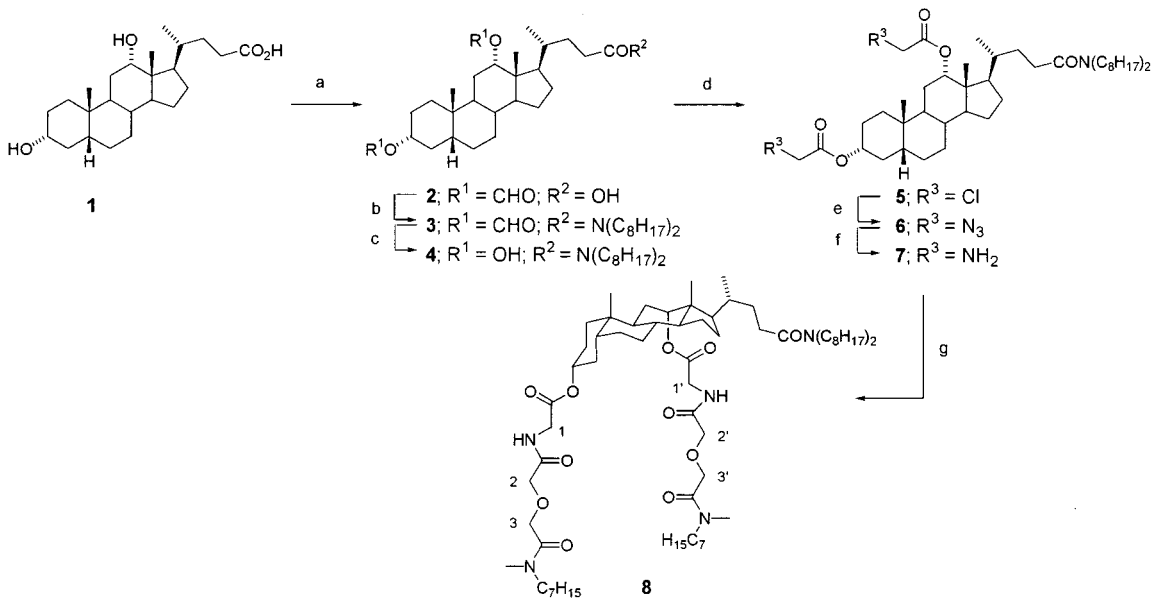
Scheme 1[a]



[a] (a) HCOOH, $HClO_4$ (cat.), Ac_2O, 55 °C, 1.5 h, 99%; (b) Et_3N, $ClCO_2Me$, CH_2Cl_2, 0 °C, 1 h, then $HN(C_8H_{17})_2$, 0 °C, 2 h, 89%; (c) 3% K_2CO_3 in MeOH/H_2O (4:1) solution, 60 °C, 24 h, 98%; (d)CaH_2, *n*-Bu_4NBr, $ClCH_2COCl$, toluene, 90 °C, 5 h, 80%; (e) NaN_3, DMF, 70 °C, 20 h, 88%; (f) PPh_3, H_2O, THF, room temp, 18 h, 100%; and (g) $C_7H_{15}N(CH_3)COCH_2OCH_2COOH$, EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride), HOBT (1-hydroxybenzotriazole hydrate), CH_2Cl_2, DMF, room temp, 24 h, 51%.

Linking ion-recognizing moieties, such as glycolic diamides for calcium ion, to those hydroxyl groups, it is possible to design ion-selective tweezers.[20] A preliminary molecular modeling study showed that compounds with two glycolic diamides linked to a 7-deoxycholic acid (e.g., compound **8** in Scheme 1) may capture the calcium ion within the binding cavity formed by six oxygen atoms (Ca^{2+}−oxygen distance, 233−276 pm), resulting in a 1:1 complex without undergoing large conformational changes.[21] On the other hand, the calcium coordination sphere formed by three ETH 129 molecules contains nine oxygen atoms; that formed by two ETH 1001 molecules, eight oxygen atoms. Hence, it is expected that the tweezer-type ionophore would form a less-tight calcium complex and exhibit reduced anionic interference, as compared to ETH 129 or 1001. On the basis of this theory, we synthesized a new calcium-selective ionophore based on a rigid bile acid frame, *N*,*N*-dioctyl-3α,12α-bis(*N*-heptyl-*N*-methylcarbamoylmethoxyacetamido-acetoxy)-5*β*-cholan-24-amide (**8**; hereafter, denoted as BACA), and attempted to bridge the ion recognition concept with the theory of ISE by comparing the potentiometric properties of three different calcium-selective electrodes based on ETH 129, ETH 1001, and BACA, respectively, in the presence of various anions, surfactants, and protein.

EXPERIMENTAL SECTION

Reagents. The sources of reagents used were as follows: poly(vinyl chloride) (PVC), bis(2-ethylhexyl) adipate (DOA), 2-nitrophenyl octyl ether (NPOE), potassium tetrakis(4-chlorophenyl)borate (KTpClPB), poly(vinyl alcohol) (PVA; MW 22000), [(−)-(*R*,*R*)-*N*,*N*′-bis-[11-(ethoxycarbonyl)undecyl]-*N*,*N*′-4,5-tetramethyl-3,6-dioxaoctane-diamide (ETH 1001), and [*N*,*N*,*N*′,*N*′-tetracyclohexyl-3-oxapentanediamide] (ETH 129), 9-(diethylamino)-5-octadecanoylimino-5H-benzo[a]phenoxazine (ETH 5294) were from Fluka (Buchs, Switzerland); polyoxyethylenesorbitan monooleate (Tween 80), polyoxyethylene 23 lauryl ether (Brij 35), sodium dodecyl sulfate (SDS), tris[hydroxymethyl]aminomethane (Tris), and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO); and *t*-octylphenoxypolyethoxy ethanol (Triton X-100) was from FischerBiotech Co. (Fair Lawn, NJ). All other chemicals used were analytical reagent grade. Standard solutions and buffers were prepared with deionized water (18 MΩ·cm).

Preparation of *N*,*N*-dioctyl-3α,12α-bis(*N*-heptyl-*N*-methylcarbamoylmethoxyacetamido-acetoxy)-5*β*-cholan-24-amide. A new calcium-selective neutral carrier (**8**) *N*,*N*-dioctyl-3α,12α-bis(*N*-heptyl-*N*-methylcarbamoylmethoxyacetamidoacetoxy)-5*β*-cholan-24-amide (BACA) was synthesized as described in Scheme 1. Because two long alkyl chains are required for sufficient hydrophobicity of the compound in ion-selective membranes,[22] the acid moiety of **1** was first converted into a long-chain-containing dialkylamide (**4**) via formylation of hydroxyl groups (**2**), introduction of the amide moiety (**3**), and deformylation to regenerate hydroxyl groups (**4**). With this key intermediate in hand, chloroacetyl groups were introduced on two hydroxyl groups and were transformed into **7**. Coupling with $C_7H_{15}N(CH_3)COCH_2OCH_2CO_2H$ furnished the requisite **8** with 31% of reasonable overall yield. Further synthetic and structural details, including spectroscopic data on each intermediary and the final compound, BACA, are given in the Supporting Information.

Preparation and Evaluation of Calcium-Selective Membranes. To optimize the potentiometric performance of the

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calcium-selective electrodes, various PVC membranes based on BACA were prepared from the 100-mg mixtures of the following compositions [listed in the order of the weight % of PVC, plasticizer (a−c, NPOE; and d−f, DOA), BACA, and the lipophilic additive, KTpClPB, in mol % with respect to the ionophore]: (a) 32.9/66.0/1.0/25; (b) 32.9/65.9/1.0/50; (c) 32.9/65.6/1.0/100; (d) 32.9/66/1.0/30; (e) 32.9/65.9/1.0/50; and (f) 32.9/65.7/1.0/100. The compositions of ETH 129- and ETH 1001-based membranes, 32.8/65.7/1.0/69 and 32.8/65.6/1.0/53, respectively, were optimized with NPOE plasticizer as described in the literature.[3] To determine the potentiometric complex formation constants, four different membranes were formulated with 10 mmol/kg of lipophilic pH indicator ETH 5294, 10 mmol/kg of calcium-selective neutral carrier, and 3 mmol/kg of KTpClPB in a 1:2 PVC/NPOE matrix (total weight, 100 mg). The cocktail solutions dissolved in 0.5 mL THF were then poured into a glass ring (i.d., 22 mm) placed on a slide glass, and dried for a day at room temperature. Small disks were punched from the cast films and mounted in Philips electrode bodies (IS-561; Glasblaserei Möller, Zürich, Switzerland). For all electrodes, 0.1 M $CaCl_2$ was used as the internal reference electrolyte.

Potentiometric evaluation of the electrodes was carried out as described previously using a static arrangement: potential differences between the ISEs and the Orion sleeve-type double-junction Ag/AgCl reference electrode (model 90-02) were measured using an IBM-compatible computer equipped with a custom-built high-impedance input 16-channel analog-to-digital converter. The response curves and calibration plots were obtained by adding standard solutions to 200 mL of magnetically stirred background electrolyte (0.05 M Tris−H_2SO_4, pH 7.4) every 100 s to vary the concentrations of each ionic species stepwise from 10^{-6} to 10^{-1} M, and the measurements of potentials were taken every second at room temperature. All measurements and plots are reported on a concentration scale. Selectivity coefficients, $K^{POT}_{Ca^{2+},j}$ ($j = Li^+$, Na^+, K^+, NH_4^+, and Mg^{2+}), were determined by the separate solution-matched potential method.[23−25] Detection limits were estimated at the cross of two linear lines, the one extrapolated from a high concentration range (10^{-5}−10^{-1} M Ca^{2+}) and the other parallel to the *x* axis drawn through the mean potential value at the lowest concentration of Ca^{2+}.[24] The effects of various interfering substances on the potentiometric responses of the calcium-selective electrodes were measured in the presence of salts containing a lipophilic anion (0.01 M $NaClO_4$, NaBr, NaI, NaSCN, and sodium salicylate), surfactants (0.05 w/v % of nonionic surfactants, Tween 80, Triton X-100, and Brij 35, and 0.1 mM anionic surfactant SDS), and protein (0−60 μg/mL of BSA) in the background electrolyte. Potentiometric formation constants were estimated using the method proposed by Bakker et al.[28] Potentiometric pH response curves were obtained by titrating 0.05 M $CaCl_2$ solution buffered to pH 3.0 in 1.0 mM citric acid and 1.0 mM boric acid, with a concentrated LiOH solution, while simultaneously monitoring the sample pH with a pH glass electrode.

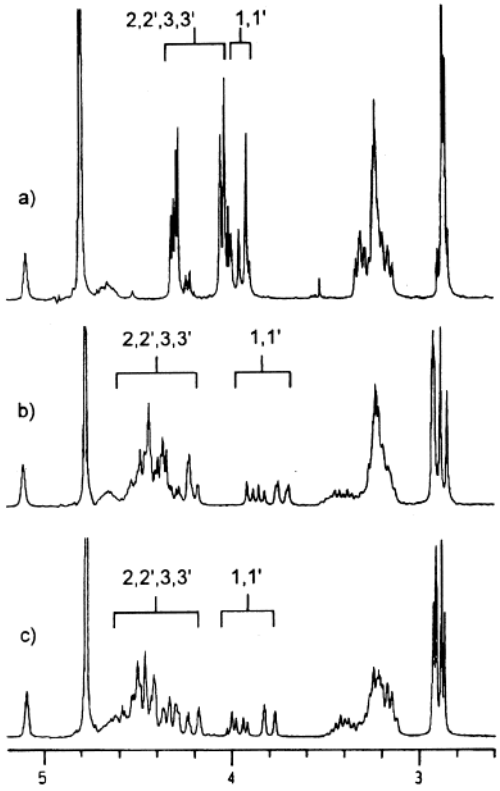

Figure 1. 300 MHz ^{1}H NMR Spectra of the Ca^{2+} ionophore BACA measured in methanol-d_4: (a) free ionophore, (b) ionophore after addition of 1 equiv of $Ca(SCN)_2$, and (c) ionophore after addition of 2 equiv of $Ca(SCN)_2$.

RESULTS AND DISCUSSION

The recognition properties of the new ionophore BACA toward Ca^{2+} ion were studied through ^{1}H NMR titration with $Ca(SCN)_2$ in methanol-d_4 solvent. In the ^{1}H NMR spectrum of free ionophore, diastereotopic methylene protons of 2, 2′, 3, and 3′ appeared as multiplets at ∼4.2 and ∼4.8 ppm (Figure 1a). Following the addition of 1 equiv of $Ca(SCN)_2$ to the BACA, significant downfield shifts of the 2, 2′, 3, and 3′ protons and upfield shifts of the 1 and 1′ protons were observed, with the changes of coupling patterns of diastereotopic methylene protons of 2, 2′, 3, and 3′ (Figure 1b); these changes in NMR peaks evidence the complexation between Ca^{2+} ion and six oxygen atoms of glycolic diamides in BACA. An increment of the amount of $Ca(SCN)_2$ up to 2 equiv merely affected the chemical shifts of 2, 2′, 3, and 3′ protons (Figure 1c). Clearly, the result strongly implies that BACA forms a 1:1 complex with the Ca^{2+} ion.

To determine the composition of BACA-based membranes, as described in the Experimental Section, we prepared six different types of solvent polymeric membranes with two different plasticizers, NPOE and DOA, and varying amounts of a lipophilic additive, KTpClPB, from 25 to 100 mol % relative to the concentration of the neutral carrier. As listed in Table 1, it was observed that the calcium selectivity of the DOA-plasticized membranes over sodium and potassium was about 100 times poorer than the NPOE-plasticized ones. When the content of lipophilic additive, KTpClPB, was increased over 50 mol % relative to the ionophore in the

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Table 1. Calcium Response Slopes, Detection Limits, and Selectivity Coefficients of the Electrodes Based on BACA[a]

membrane[b]	slope[c]	detection limit[d]	selectivity coefficient[e] Mg^{2+}	Na^+	K^+
a	30.0	−6.5	−4.3	−4.5	−4.5
b	31.7	−6.6	−4.2	−4.6	−4.8
c	30.1	−7.4	−3.9	−4.4	−4.1
d	30.5	−6.1	−4.0	−2.7	−2.8
e	31.0	−6.1	−4.0	−2.9	−3.0
f	30.7	−5.9	−3.8	−2.6	−2.3

[a] Range: 10^{-5}−10^{-1} M. [b] For compositions, see Experimental Section. [c] mV/decade. [d] Log [Ca^{2+}], M. [e] Log $K^{POT}_{Ca^{2+},j}$.

Table 2. Calcium Response Slopes, Detection Limits, and Selectivity Coefficients of the Electrodes Based on Various Calcium Ionophores[a]

ionophore	slope[b]	detection limit[c]	selectivity coefficient[d] Mg^{2+}	Li^+	Na^+	K^+	NH_4^+
ETH 1001	31.5	−6.7	−4.4	−4.4	<−5.4	<−5.4	−5.0
ETH 129	31.0	−6.6	−4.4	−4.3	<−5.4	<−5.4	−5.1
BACA	31.7	−6.6	−4.2	−4.2	−4.6	−4.8	−4.2

[a] Range: 10^{-5}−10^{-1} M. [b] mV/decade. [c] Log [Ca^{2+}], M. [d] Log $K^{POT}_{Ca^{2+},j}$.

membrane, the electrodes exhibited deteriorated calcium selectivities over Mg^{2+}, Na^+, and K^+. Hence, further potentiometric evaluations were made with the membrane comprising PVC (32.9 wt %), NPOE (65.9 wt %), 50 mol % KTpClPB, and BACA (1.0 wt %).

We then compared the potentiometric performances of three different calcium-selective membranes based on BACA, ETH 1001, and ETH 129. As indicated in Table 2, the potentiometric performance of the BACA-based electrode is nearly the same as that of the ETH 1001- or ETH 129-based electrodes, providing a Nernstian response in the 10^{-5}−10^{-1} M range, and a low detection limit (2.5×10^{-7} M). However, the calcium selectivities of the BACA-based electrode over Na^+, K^+, and NH_4^+ are 5−7 times inferior to those of the ETH-compound-based electrodes. As suggested at the beginning of this paper, it is presumed that the BACA neutral carrier forms a less-tight complex with calcium than does ETH 1001 or ETH129, resulting in a slightly reduced calcium selectivity. Potentiometric complex formation constants of calcium-selective neutral carriers have been determined using the method proposed by Bakker et al.;[28] they were 15.2, 14.0, and 8.6 on a logarithmic scale, respectively, if we assume 3:1, 2:1, and 1:1 ligand−calcium complex formation with ETH 129, ETH 1001, and BACA. However, the calcium-binding ability and selectivity of BACA are still applicable for clinical analysis with some precautions about the variations in sodium concentration between calibrations and measurements. For example, when the level of sodium was varied from 90 to 140 mM in the presence of 1.30 mM Ca^{2+} (background electrolyte: 0.05 M Tris-H_2SO_4, pH 7.4), the BACA-based electrode exhibited a positive potential shift of ~1 mV (equivalent to a 6% error in calcium determination),

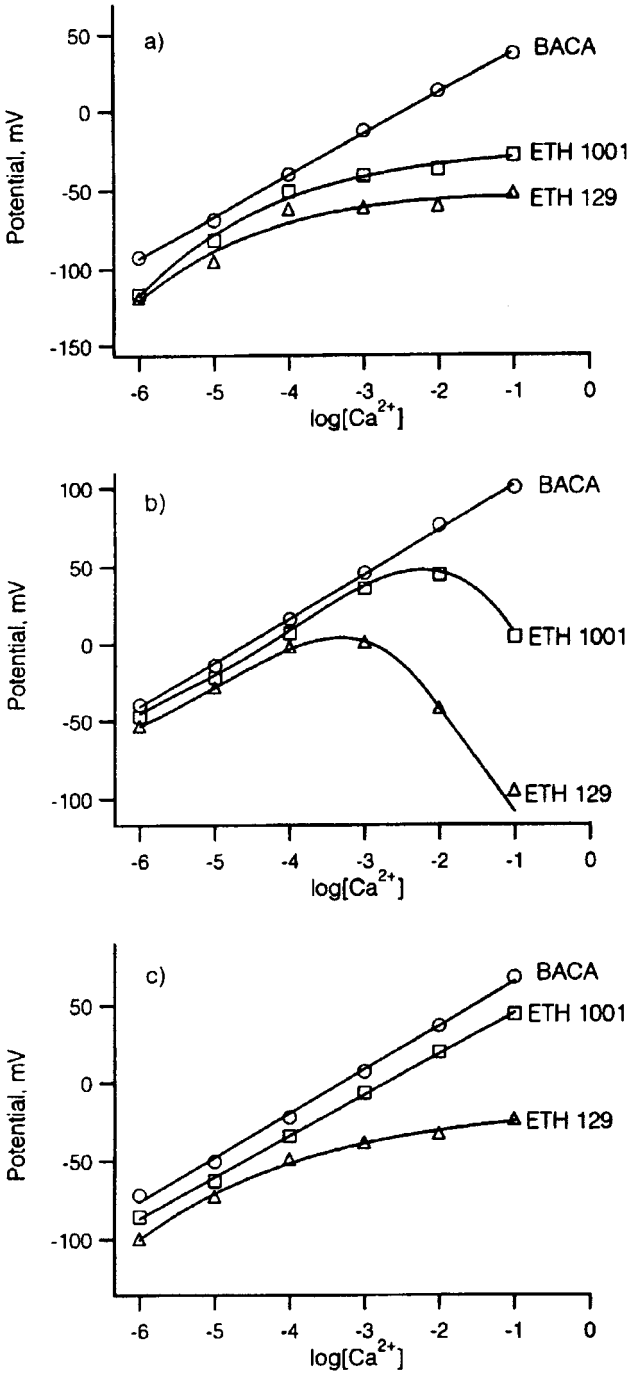

Figure 2. Potentiometric responses of the PVC membranes containing BACA, ETH 1001, and ETH 129 to $CaCl_2$ in the presence of 0.01 M (a) $NaClO_4$, (b) $Ca(SCN)_2$, and (c) NaI. Background electrolyte, 0.05 M Tris−H_2SO_4, pH 7.4.

whereas the ETH 1001-based electrode was 0.6 mV under the same conditions.

The moderate calcium-binding ability of BACA, however, may be advantageous for reducing the anionic interference problem, which is a frequently observed phenomenon in the use of ETH 1001 or ETH 129-based electrodes in the presence of lipophilic anions, for example, SCN^-, ClO_4^-, salicylate, and I^-. As shown in Figure 2a, the BACA-based electrode exhibited little anionic interference when it was titrated with $CaCl_2$ in the presence of 0.01 M ClO_4^-, but the calcium response of ETH 129- and ETH

Table 3. Effect of Surfactants on the Potentiometric Responses of Calcium-Selective Electrodes[a]

	ETH 1001				ETH 129				BACA			
			selectivity[d]				selectivity[d]				selectivity[d]	
surfactant	slope[b]	detection limit[c]	Na^+	K^+	slope[b]	detection limit[c]	Na^+	K^+	slope[b]	detection limit[c]	Na^+	K^+
Tween 80[e]	33.0	−6.0	−3.8	−3.3	31.9	−5.5	−3.8	−3.4	32.0	−6.0	−2.7	−1.9
Triton-X-100[e]	32.5	−5.4	−2.2	−1.1	30.9	−5.3	−2.9	−1.7	28.7	−5.3	−0.1	+1.4
Brij 35[e]	32.4	−5.9	−3.7	−2.7	29.8	−5.3	−3.8	−3.1	31.5	−5.9	−1.9	+0.2
SDS[f]	12.2				6.2				28.7	−5.7	−0.9	+0.8

[a] Range: 10^{-5}—10^{-1} M. [b] mV/decade. [c] Log [Ca^{2+}], M. [d] Log $K^{POT}_{Ca^{2+},j}$. [e] 0.05%. [f] 0.1 mM.

1001-based electrodes with optimized compositions[3] leveled off in the $10^{-4} - 10^{-3}$ M range. The effect of anionic interference is further demonstrated in Figure 2b, using $Ca(SCN)_2$ salt: the response of ETH ionophore-based electrodes became anionic in the 10^{-3}—10^{-2} M range, whereas that of the BACA-based electrode is linear up to 10^{-1} M. When the same test was performed in the presence of 0.01 M iodide, as shown in Figure 2c, the ETH 129-based electrode suffered from a large anionic interference, the ETH 1001-based one slightly over 10^{-2} M Ca^{2+}, and the BACA-based one negligibly up to 10^{-1} M. Salicylate also induced anionic effects similar to those observed in the presence of iodide. These results confirmed that the BACA-based electrode does not suffer from an anionic interference problem, even in the presence of a high concentration of lipophilic anion.

ISEs are often used in flow-injection analyzer systems equipped for automated sampling, measuring, and cleaning. A small amount of surfactant is added to the cleaning solution or calibration solutions to clean the membrane surfaces of ISEs. However, ISEs may exhibit potential drifts or reduced responses in the presence of some surfactants. Hence, we examined the effect of surfactants on the potentiometric responses of calcium-selective electrodes. As summarized in Table 3, although the neutral surfactants added to the sample solution (0.05%) increased the background potential ($E°$) and resulted in increased detection limits as high as a decade, they did not change the response slopes of calcium-selective electrodes based on ETH 1001, ETH 129, and BACA. On the other hand, the anionic surfactant SDS (10^{-4} M) added to the background electrolyte made the ETH 129- and ETH1001-based electrode hardly responsive to calcium, while causing little effect on the response slope of the BACA-based electrode.[26] The most noticeable adverse effect of surfactants on all Ca^{2+}-ISEs is the deterioration of their calcium selectivity;[27] such an effect was more pronounced for the BACA-based electrode, resulting in a reversed Ca^{2+}/K^+ selectivity in the presence of Triton X-100, Brij 35, and SDS. The moderated calcium-binding ability of BACA, which is advantageous for reducing anionic interference, becomes a disadvantage for maintaining high calcium selectivity in the presence of surfactants. In general, our experimental results indicate that the use of surfactant-containing calibrants or samples is not recommended for all calcium-selective electrodes, including the BACA-based electrode.

The addition of bovine serum albumin (BSA, 60 μg/mL) to the background electrolyte shifted the potential up by +12 to +17 mV for all electrodes, which increased the detection limits of the electrodes by about one-half of a decade. However, the responses of three calcium-selective electrodes to calcium over 10^{-5} M were virtually the same as those observed in the absence of BSA.

In summary, the new calcium-selective neutral carrier based on a rigid bile acid frame, BACA, provides potentiometric performance comparable to that of the well-known ETH 1001 or ETH 129. The ^1H NMR spectra of BACA titrated with $Ca(SCN)_2$ suggest that BACA forms a 1:1 complex, tweezing the calcium ion with six oxygen atoms between the two diamide groups. It was observed that the ISE membrane based on BACA was 5−7 times less selective to calcium over other alkali and alkali earth metal cations than those based on ETH 129 and ETH 1001, implying that BACA forms a weaker calcium complex than the other two ETH compounds. In accordance with this observation, the potentiometric complex formation constants were determined to be 15.2, 14.0, and 8.6 for ETH 129, ETH 1001, and BACA, respectively, in logarithmic scale. However, the calcium selectivities of BACA (log $K^{POT}_{Ca^{2+},j} = -4.2, -4.2, -4.6$, and -4.8, respectively, for $j = Mg^{2+}$, Li^+, Na^+, and K^+) are still sufficient to use for most analytical applications, including clinical analysis. A slightly reduced calcium selectivity of BACA affects the anionic interference-free calcium-selective membrane; the BACA-based membrane exhibits a Nernstian response up to 10^{-1} M Ca^{2+} in the presence of lipophilic anions (e.g., SCN^-, ClO_4^-, salicylate, and I^-) and anionic surfactant, whereas the ETH 129- and ETH 1001-based ones suffer from serious anionic interference, showing a curvature or leveled off response over about 10^{-4} M.

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SUPPORTING INFORMATION AVAILABLE

Synthetic and structural details, including spectroscopic data on each intermediary and the final compound, BACA is available in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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