New Lipophilic Rhodamines and Their Application to Optical Potassium Sensing

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New lipophilic fluorescent rhodamines were synthesized directly from 3,6-dichlorofluoresceins and the respective long-chain amines with excellent solubility in lipids and lipophilic membranes. Spectrophotometric and luminescent properties of the dyes are reported and discussed with respect to their application in new optical ion sensors. One rhodamine (2a) was applied in a poly(vinyl chloride)-based sensor membrane for continuous and sensitive optical determination of potassium ion, using valinomycin as the neutral ion carrier.

INTRODUCTION

Rhodamines are widely used as fluorescent probes and labels, in dye lasers, for dyeing polymers, and for several other purposes [1–4]. Along with fluoresceins, they are frequently applied as fluorescent labels in immunological techniques [3,4]. Due to their high fluorescence quantum yield and photostability [5–7], rhodamines are also of interest for use in optical sensors ("optrodes") [8]. Other applications include use for labeling of proteins and as polarity-sensitive probes, reference dyes, and potential-sensitive probes. We are particularly interested in optical probes with special properties such as pH-dependent emission or analyte-dependent fluorescence lifetimes. In addition, optrodes for chemical sensing can be envisaged that are based on the use of probes sensitive to potential or polarity.

The major goal of this work was the design of long wave-absorbing and stable lipophilic fluorescent probes for use in optical sensors [8]. Rhodamines and fluoresceins have been used successfully in optrodes using proper ion carriers [9–11]. Two approaches for carrier-based sensing are known: In the first, local changes in potential (as a result of local rearrangement of molecules) are measured, while in the other an ion-exchange mechanism or an ion coextraction mechanism is operative [11–14].

One of the major problems in the design of optical sensors for chemical species results from the insufficient stability of indicators or labels. Both leaching and photobleaching can severely compromise the use of fluorescent probes in optical sensors as well as in biomedical studies. Although sensing via measurement of fluorescence lifetime can strongly reduce this limitation, highly stable dyes are still desirable. Since most rhodamines are rather photostable, we attempted to chemically modify them in order to make them soluble in lipophilic pvc[3]-based optical sensors membranes. In addition to their stability, some rhodamines have long-wave absorptions and emissions, which is advantageous in view of the available solid-state material LEDs and lasers that emit in the long-wave region of the spectrum only [4,8]. We report here (i) on the synthesis and application of new

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[3] Abbreviations used: FAB, fast atomic bombardment; ^{1}H-NMR, proton nuclear magnetic resonance; PTCB, potassium tetrakis-(4-chlorophenyl) borate; pvc, poly(vinyl chloride); THF, tetrahydrofuran; TOP, tris-(2-ethylhexyl)-phosphate.

fluorescent rhodamines with (a) excellent solubility in plasticized pvc and similar lipophilic materials and (b) long-wave absorption and emission wavelengths and (ii) on their application to a potassium-sensitive optrode.

EXPERIMENTAL

Chemicals and Reagents

Poly(vinyl chloride) (pvc; high molecular grade), the ion carrier potassium tetrakis(4-chlorophenyl) borate (PTCB), tris-(2-ethylhexyl)-phosphate (TOP), and tetrahydrofurane (THF) were obtained from Fluka AG (Buchs, Switzerland). The high-purity potassium chloride and earth alkali halides were obtained from Merck (Darmstadt, Germany). Doubly distilled water was used throughout.

Syntheses of Fluorescent Probes

Fluorescein-3,6-dichloride (1a). This was prepared according to Ref. 15. In essence, fluorescein was heated with PCl_5 at 100°C for 2 h. After the addition of water, the mixture was heated to 70°C, centrifuged, sucked off, and washed with hot water. The procedure was repeated, and the residue treated with 3% aqueous sodium hydroxide. The insoluble residue was extracted with hot ethanol and, finally, recrystallized from toluene. Yield, 27%; m.p., 251–253°C.

4,5-Dibromo-fluorescein-3,6-dichloride (1b). This was prepared by analogy to 1a to give white crystals (45%); m.p., 262–264°C. Elemental analysis (calc./found) for $C_{20}H_8Br_2Cl_2O_3$ (527.0): C, 45.58/45.58; H, 1.52/1.55; halogens, 43.77/43.86.

N,N'-Dioctadecyl-rhodamine Perchlorate (2a). Three and seven-tenths grams (10 mmol) of compound 1a, 5.4 g (20 mmol) of octadecylamine, and 3.0 g of anhydrous zinc chloride were heated to 185°C for 4 h. The resulting melt was washed six times with 30 ml of hot distilled water. The residue was dissolved in 25 ml ethanol, then acidified with 2 ml 70% perchloric acid, and the perchlorate salt precipitated by the addition of 15 ml ice-cold water. The crude product was purified by recrystallization from 50 ml 85% ethanol containing 1 ml perchloric acid. Five and four-tenths grams (29%) of a red product was obtained. A small part was further purified by flash chromatography on silica using a mixture of 80% light petroleum (b.p., 60–80°C) and 20% ethyl acetate as the eluent; m.p., 168–171°C. Elemental analysis (calc./found) for $C_{56}H_{87}N_2O_7Cl$ (935.8): C, 71.88/71.96; H, 9.37/9.27; N, 2.99/3.07, Cl, 3.79/3.82. (The mass spectrum is shown in Fig. 3.)

Carboxy-Substituted Rhodamines (2b–2e), General Method. A mixture of 10 mmol of fluorescein-3,6-dichloride (1a) or 4,5-dibromo-fluorescein-3,6-dichloride (1b) and 20 mmol of the corresponding ω-amino-substituted fatty acid was heated with 3.0 g of anhydrous zinc chloride to 185–200°C for 5–7 h. The obtained melt was treated with hot distilled water as described above. The residue was dissolved in 0.1 *M* sodium hydroxide. After filtration, rhodamines 2b–2f were precipitated by the addition of ice water and acidification with perchloric acid. Further purification was accomplished by recrystallization from 80% aqueous ethanol containing 1% (v/v) perchloric acid.

N,N'-Di-(11-carboxyundecyl)-rhodamine Perchlorate (2b). This was obtained in a 39% yield (3.2 g) as red crystals of m.p. 106–108°C. Elemental analysis (calc./found) for $C_{44}H_{59}N_2ClO_{11}$ (827.4): C, 63.87/62.65; H, 7.19/7.34; N, 3.39/3.49; Cl, 4.28/5.31. FAB mass spectrum: $m/z = 827$ (mol peak).

N,N'-Di-(11-carboxyundecyl-4,5-dibromo-rhodamine Perchlorate (2c). This was obtained as fine red crystals of m.p. 165–169°C in a 42% yield (4.1 g). Elemental analysis (calc./found) for $C_{44}H_{57}N_2Br_2ClO_{11}$ (985.2): C, 53.64/52.77; H, 5.83/5.99; N, 2.84/2.56; halogen, 19.82/20.73. FAB mass spectrum: $m/z = 985$ (mol peak).

N,N'-Di-(7-carboxyheptyl)-rhodamine Perchlorate (2d). This was obtained in a 43% yield (3.1 g) as red crystals of m.p. 121–124°C. Elemental analysis (calc./found) for $C_{35}H_{43}N_2ClO_{11}$ (715.2): C, 60.46/58.99; H, 6.06/6.61; N, 3.92/4.08; Cl, 5.96/6.05.

N,N'-Di-(5-carboxypentyl)-rhodamine Perchlorate (2e). This was obtained in a 27% yield (1.8 g) as red crystals of m.p. 132–134°C. Elemental analysis (calc./found) for $C_{32}H_{35}N_2ClO_{11}$ (659.1): C, 58.32/58.00; H, 5.35/5.11; N, 4.25/4.57; Cl, 5.38/7.38.

Instruments

Fluorescence excitation and emission spectra and the response curves of membranes were measured using an Aminco SPF 500 spectrofluorometer equipped with a 250-W tungsten halogen lamp as a light source or a Shimadzu RF 5001 instrument. Long-term tests of membranes were performed with a fiber-optic fluorometer coupled to a computer-controlled data acquisition unit. The arrangement consisted of an autosampler (Besta, Heidelberg, FRG), a peristaltic pump (Gilson, France), a self-constructed flow-through cell with the sensor membrane mounted inside a fiber optic photometer (Oriel 3090, Chelsea Instruments, London, UK), a data acquisition unit (Keithley 575, Taunton, USA), and a personal computer. FAB mass spectra were obtained with

a VG ZAB-HSQ instrument (from Vacuum Generators) at room temperature and 70 eV using a glycerol/thioglycerol matrix.

Preparation of the Potassium-Sensitive Membrane

A mixture of 2.4 mg pvc, 0.4 mg PTCB, 1.8 mg valinomycin, 0.8 mg N,N'-dioctadecyl-rhodamine perchlorate (2a), and 6.0 mg TOP was dissolved in 1.5 ml freshly distilled tetrahydrofurane. One-tenth milliliter of this solution was pipetted onto a 12 × 50-mm dust-free 175-μm Mylar plate (Du Pont de Nemours & Co., Brussels), which was stored in a THF-saturated atmosphere in an exsiccator. After about 5 h the Mylar plate with an approximately 1- to 3-μm-thick sensing membrane on it was removed and placed in ambient air for 15 min in order to dry. Before measurements, the membrane was placed in 0.1 M potassium chloride solution for activation.

Experimental Procedure for Testing Sensor Membranes

The sensor membrane was mounted in a flow-through cell contained in the fluorometer. Standard solutions prepared from twice-distilled water were pumped over the sensing membranes. The excitation beam was directed onto the sensing membrane in contact with the aqueous sample passing by, and fluorescence was collected at an angle of about 55°. Fluorescence was excited at 514 nm using an interference filter (in addition to the excitation monochromator) for isolating the excitation wavelength. Fluorescence intensity was measured at 550 nm after passing a 530-nm cutoff filter (Schott). The temperature was kept constant to 25 ± 1°C. The experimental setup is shown in Fig. 1.
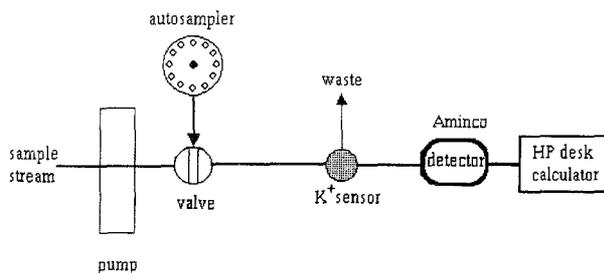

Fig. 1. Experimental setup.

RESULTS

Synthesis of Rhodamines

Common methods for synthesis of rhodamines [5–7, 15–17] start from phthalic acid anhydride, which is reacted with two equivalents of a 3-aminophenol at temperatures of about 200°C. However, properly substituted rhodamines are difficult to obtain via this route because of the poor accessibility of the respective 3-aminophenols. In addition, numerous by-products are formed in this process.

A rather simple and straightforward route to rhodamines is based on the reaction of fluorescein-3,6-dichlorides with primary or secondary amines. This approach has the potential of providing an easy access to side-chain functionalized rhodamines. In particular, we have found that lipophilic rhodamines are obtained in good yields but with insufficient purity so that extensive purification is necessary.

The dichlorides 1a and 1b can be easily prepared from fluorescein or 4,5-dibromofluorescein, respectively, and PCl_5, as described by Bayer [15]. The chloro atoms in 1a and 1b are highly reactive and easily substituted when reacted with two equivalents of the primary amine (Fig. 2). Secondary amines also react, but the products are difficult to purify. Zinc chloride and aluminium chloride are useful catalysts, with working temperatures range from 170 to 230°C.

Rhodamines 2a–2e are obtained as red crystals, insoluble in water but soluble in ethanol, wherein they form orange solutions displaying intense yellow fluorescence. Purification of the ω-carboxy derivatives 2b–2e is also possible by dissolving them in alkaline solution and precipitating the product by acidification. While 2a was obtained in pure form, the results of elemental analyses of 2b and 2c are dissatisfying, but there are no doubts as to the structures in view of the characteristic spectral properties of the probes and other spectral evidence. We assume that small amounts of zinc complexes of rhodamines are formed and cannot be removed during the usual purification process.

Spectral Properties and Structural Assignment

The absorption maxima of 2a–2e range from 523 to 528 nm in ethanol, with molar absorbances of typically 55,000 $M^{-1} \cdot cm^{-1}$. The excitation maxima are the same as the absorption maxima. Fluorescence emissions maximize at 555 to 567 nm. The quantum yield of 2a in ethanol has been determined to be 0.70 relative to the
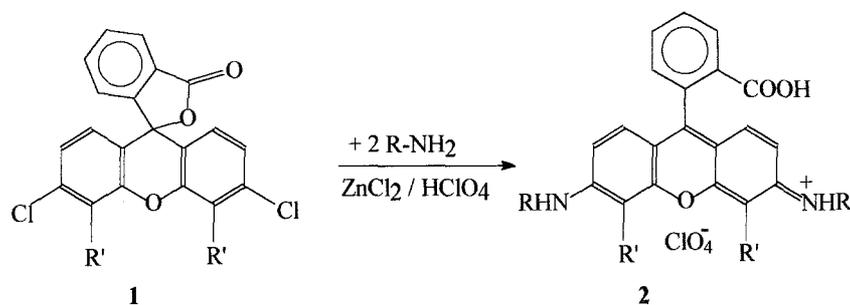
number	R	R'
1a	-	H
1b	-	Br
2a	∼∼∼∼∼∼∼CH₃	H
2b	∼∼∼∼∼∼COOH	H
2c	∼∼∼∼∼COOH	Br
2d	∼∼∼∼COOH	H
2e	∼∼∼∼COOH	H

Fig. 2. Synthetic pathway to rhodamines 2a–2e.

monoprotonated form of rhodamine B (quantum yield, 0.51 at 21°C) [18].

The signals in the ¹H-NMR spectra appear in the expected area and support the structures given in Fig. 2. Particularly significant are the mass spectra which were obtained by the FAB technique. Figure 3 shows a re-
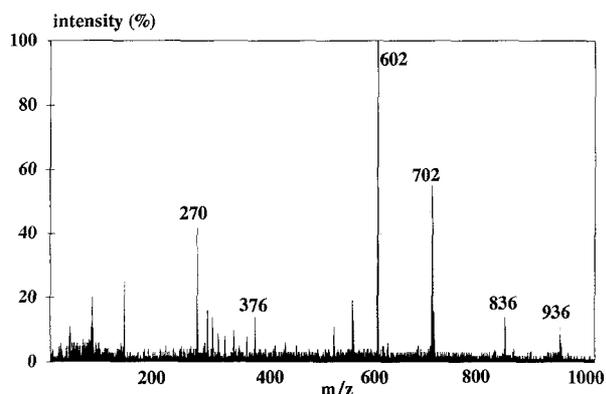

Fig. 3. Mass spectrum of 2a using the FAB technique. The molecular peak is at m/z = 936; intensities are expressed in terms of normalized ion abundance.
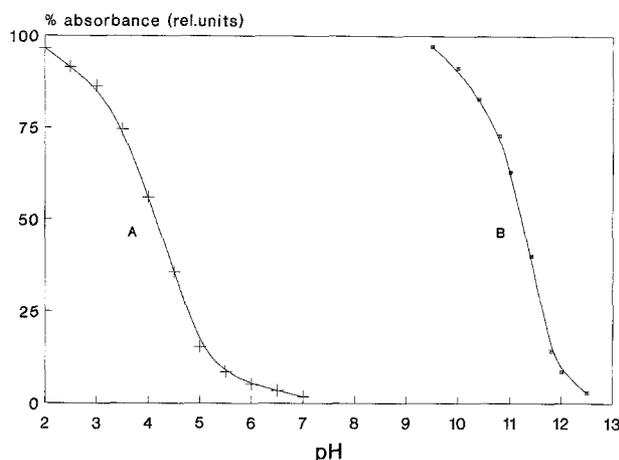
presentative spectrum, including a distinct mol peak (M⁺) at m/z = 936, the loss of perchloric acid (836), and a base peak at 602 mass units.

pH Dependence of the Optical Spectra

Absorbance and fluorescence spectra of 2a were measured both in aqueous/ethanolic solution and in plasticized pvc membranes at different pH's. Figure 4 shows the effect of the solvent (matrix) on the pH-dependent absorption of 2a. Similar effects are found in fluorescence intensity. Most striking is the difference in the pK_a values, being 4.3 in aqueous ethanol (alcohol/buffer mixtures) but 11.3 in the plasticized pvc. Such dramatic changes are, however, not uncommon [19] and can be attributed to effects caused by solvent and additives such as plasticizers. In protic solvents, for instance, interactions of solvent hydroxy groups with the hydroxy and basic nitrogen functions of the dye are favored [18], while in a lipophilic solvent or matrix the solvent tends to solvate the lipophilic part of the dye, e.g., its long alkyl groups. In addition, it has been found that the addition of PTCB has a dramatic effect on the pK_a of a

Fig. 4. Normalized absorbance vs pH curves of **2a** in different media. (A) In a pvc membrane plasticized with TOP; (B) in ethanol/buffer mixtures (9:1).

cationic dye. This, however, will be reported elsewhere [20].

Application to Optical Sensor Membranes

Given its properties, rhodamine **2a** is considered to be a suitable proton carrier for use in optical sensor membranes based on the ion-exchange mechanism. Consequently, potassium-sensitive sensor membranes have been prepared by dissolving **2a**, along with valinomycin and other additives (see Experimental), in a pvc/plasticizer mixture. Valinomycin selectively carries potassium into the pvc membrane. With respect to the sensing mechanism, it is assumed [11–14] that, in order to maintain electroneutrality of the membrane, a proton is released from **2a** upon which it undergoes a change in fluorescence intensity [12] as shown in Fig. 4.

The maxima of the excitation and emission spectra of **2a** in a plasticized pvc membrane are at 540/562 nm. However, when used in the sensor, the rhodamine was excited at 514 nm and fluorescence intensity measured at 560 nm in order to minimize interference by stray light. Using such a sensing membrane, potassium can be continuously monitored over the 0.001 to 100 mM concentration range, with an analytically useful range of 0.1–10 mM potassium concentration. Figure 5 shows a typical signal track obtained when exposing the sensor membrane to various levels of potassium ion and the resulting calibration graph at pH 7.38. The sensor membrane fully reversibly responds to potassium ion over the 10 μM to 100 mM range, and response times are generally shorter than 1 min. Because of the stability and

lipophilicity of the dye, bleaching and leaching are lower than reported for rhodamine B C_{18} ester [9,10]: The decrease in fluorescence after 1 h was less than 2% under intense illumination by a xenon flash lamp, compared to 3–4% in the case of the C_{18} ester.

The broad dynamic range of the sensor (compared to Fig. 4) is rather surprising. We assume that not only an ion-exchange mechanism, as postulated in Ref. 12 (and supported by the spectral changes), is operative. Rather, there must be additional processes and effects involved which are difficult to prove at present. Nevertheless, the extended dynamic range is welcome in practical applications of such sensor materials.

DISCUSSION

The synthetic path to rhodamines presented here is rather straightforward and particularly well suited for functionalized rhodamines with long-chain alkyl substituents, although yields are moderate. Conceivably, this pathway can be applied to numerous other substituted rhodamines as well. While some of the dyes are not obtained with analytical purity after simple crystallization (but only after flash chromatography), the impurities seem to be of a nature that does not interfere in many applications including optical sensing.

The alkyl side chains do not significantly perturb the optical properties of **2a** when compared with rhodamine 6G. Both the fluorescence quantum yield (0.70) and the molar absorptivity (55,000) of **2a** are high, which is important when it is used as an optical probe Rhodamine. **2a** is shown to be a useful probe in optical potassium sensing and gives a membrane material which shows an excellent analytical performance and a wide dynamic range. Conceivably, by replacing the carrier (in this case, valinomycin) with carriers for other ions, sensors will be obtained for a whole set of other ionic species, without the need for changing the optical system of the instrument, because the dye can be the same. This will simplify considerably the design of analytical instrumentation.
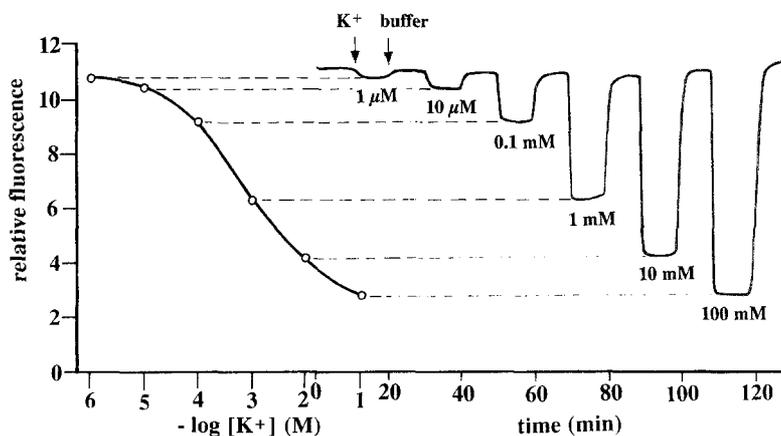
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Fig. 5. Calibration graph and response curve of the potassium-sensitive pvc membrane containing **2a** as proton carrier and TOP as plasticizer, at pH 7.38.

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