Cation-Selective Color Sensors Composed of Ionophore−Phospholipid−Polydiacetylene Mixed Vesicles

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Received July 12, 1999

Abstract: Supramolecular assemblies of vesicles composed of ionophores and phospholipids embedded in a matrix of polymerized diacetylene [PDA] lipids are shown to undergo visible color changes in the presence of ions in solution. The blue-to-red color transitions of the vesicles are directly related to binding of the cations to the ionophores, and their association with the lipids. The system detects cations in submillimolar concentrations and demonstrates a significant ionic selectivity, in particular between the physiologically important ions Na^+ and K^+.

Introduction

Development of selective ion-sensors is highly desirable because of the physiological importance of ions such as potassium, sodium, calcium, and others. Various ion-sensors have been described, which are based upon electrochemical determination of ions in solution,[1] fluorescence detection,[2] and other methods.[3] Currently available ion assays, however, are somewhat limited due to low ionic selectivity, for example, between K^+ and Na^+.[4] Recent studies report the synthesis of highly selective Na^+ fluorescent dyes.[5]

We describe here a simple supramolecular self-assembly that exhibits visible and rapid color changes in the presence of specific cations in solution. The color sensors consist of mixed vesicles, composed of conjugated polydiacetylene [PDA] lipids and natural phospholipids, to which ionophores are added. The vesicles exhibit blue-red color changes in the presence of ions, induced through interaction between the ionophore-ion complexes and the phospholipid/PDA vesicles. The ionic selectivity is determined by the affinities of the ionophore molecules added to the vesicles, thus allowing for general applicability of the system as an ion sensor.

Materials and Methods

Vesicle Preparation. The vesicles were prepared by sonication of an aqueous mixture of synthetic dimyristoylphosphocholine [DMPC] [Avanti Polar Lipids, Alabaster, AL] and 10,12-tricosadiynoic acid [GFS Chemicals, Powell, OH] [4:6 mol ratio] at a temperature of around 70 °C. Following sonication, the solution is cooled at 4 °C overnight and irradiated at 254 nm for 10−20 s to induce polymerization of the polydiacetylene [PDA] backbone. The ionophore molecules have been added after the polymerization step.

Colorimetric Measurements. Vesicle concentrations used in the experiments were generally 1 mM. Ionophores [dissolved in 10 μL of trifluoroethanol, (TFE)] were added to the solution after polymerization to a total concentration of 0.7 mM. Prior to addition of the cations, a Tris solution at pH 8.5 has been added, to a final concentration of 2 mM. UV−vis spectroscopy measurements were carried out at 27 °C on a Hewlett-Packard 8452A diode-array spectrophotometer, using a 1 cm optical path cell.

A quantitative value for the extent of blue-red color transition is given by the colorimetric response [CR], which is defined as[6]

$$CR = [PB_0 - PB_I]/PB_0$$

where $PB = A_{blue}/[A_{blue} + A_{red}]$. A is the absorbance at either the "blue" component in the UV−vis spectrum (≈640 nm) or the "red" component (≈520 nm). (Note: "blue" and "red" refer to the visual appearance of the material, not its actual absorbance.) PB_0 is the red/blue ratio of the control sample [without ions], while PB_I is the value obtained for the vesicle/ionophore/ion solutions.

NMR. C-13 NMR experiments were carried out in a 11.7 T magnetic field on a Bruker DMX500 NMR spectrometer fitted with a broad-band probe. DMPC/PDA vesicles were prepared using DMPC isotope labeled with C-13 at both carbonyl positions. The vesicle concentration was 1 mM, and the solution also included 2 mM Tris at pH 8.5. Five thousand scans were accumulated for each spectrum using a Bloch-decay pulse-sequence with a recycle delay of 1 s. A 10 Hz Lorentzian window function was applied to all spectra. TMS was used as the external reference.

Fluorescence Experiments. The fluorescence probe *N*-[[4-(6-phenyl-1,3,5-hexatrienyl)phenyl]propyl]trimethylammonium-*p*-toluenesulfonate (TMAP-DPH) [Molecular Probes Inc., Eugene, OR] was added to the polymerized vesicles to a final concentration of 0.1 μM [phsopholipid:probe ratio of approximately 400:1]. Addition of the fluorescence probe did not affect either the initial blue color of the vesicles or the color transitions induced by the ionophore/ion pairs. The vesicle solutions containing the fluorescent probe were allowed to equilibrate at 25 °C for around 1 h before the experiments.

Steady-state fluorescence anisotropy and lifetime measurements were carried out at a wavelength of 430 nm and λ_{ex} of 356 nm, using a K-2 ISS spectrofluorimeter. All experiments were conducted in ratio mode in which the anisotropy was measured against a control. Contribution of light scattering to fluorescence intensity was confirmed to be less than 5%. The temperature was maintained at 25 °C. The reported

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10.1021/ja9924288 CCC: $19.00
Published on Web 01/22/2000

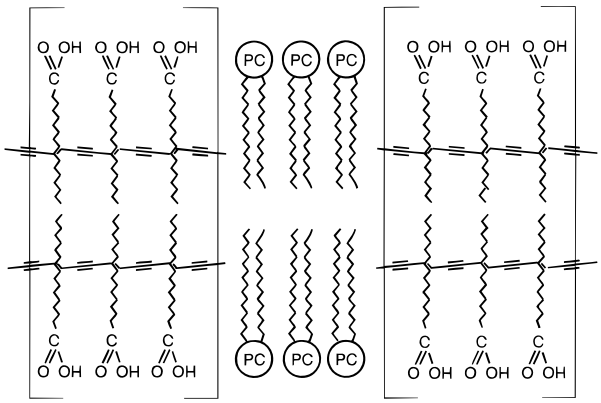

Figure 1. Schematic representation of a portion of the DMPC/PDA vesicles used in the experiments. The figure shows part of the vesicle interface, which consists of a polymerized network of 10,12-tricosadiynoic acid and inserted phospholipids.

anisotropy values are an average of four independent measurements. The fluorescence anisotropy is defined as

$$A = (I_v - I_h)/(I_v + 2I_h)$$

Where I_v and I_h are the vertically polarized and horizontally polarized fluorescence intensities, respectively.

In the lifetime measurements, fluorescent phase shift and modulation were evaluated at a modulation frequency ranging from 1 to 150 MHz, relative to TiO_2 scatterer. The excitation source was a xenon lamp (300 W). A cutoff T_2 filter was used in all experiments. Each phase and modulation value was an average of five measurements, with uncertainties of 0.5° and 0.01, respectively. Least-squares analysis was carried out to obtain the decay values.

Results and Discussion

Figure 1 presents a schematic picture of a portion of the vesicle assembly used in this study, which consists of PDA and DMPC. The vesicles contain approximately 40% mol percent DMPC and appear intense-blue to the eye due to the alternating triple-bond/double-bond conjugated PDA backbone.[7] Addition of ionophores, such as valinomycin[8] or monensin,[9] does not affect the blue color of the vesicles. PDA-based assemblies have been known to exhibit colorimetric transitions due to a variety of environmental perturbations, such as temperature,[10] pH,[11] surface pressure,[12] and molecular recognition.[6] We have shown recently that chromatic transitions can be induced following biological processes involving natural lipids embedded within the PDA matrix in mixed phospholipid/PDA vesicles.[13,14]

Figure 2 shows a photograph depicting the color changes induced by monovalent cations in the ionophore/DMPC/PDA vesicle solutions. Figure 2 clearly demonstrates that the extent of the blue-red color transition of the vesicles depends on the type of cation added to the solution. For example, when valinomycin is the ionophore employed in the experiment, Figure 2A, the vesicle solution exhibits the most pronounced red color in the presence of rubidium ions, followed closely by potassium. Furthermore, a more moderate colorimetric transition is induced in the valinomycin/DMPC/PDA vesicle solution after addition of Cs^+ ions, while Li^+ and Na^+ ions do not seem to initiate a blue-red color change at all. Similarly, in an aqueous DMPC/PDA solution containing the ionophore monensin, Figure 2B, the greatest color change is observed in the presence of Na^+ ions. The color changes occur within seconds after addition of the ions to the vesicle solutions, and they reach stability within tens of seconds. The counterions do not seem to affect the extent of color changes.

The ionic selectivity demonstrated in Figure 2 is consistent with the established binding affinities of the ionophore molecules used in the experiments. Ionophores are generally hydrophobic cyclic molecules, which selectively bind cations and transport them across membranes.[8,9,15] Valinomycin, for example, forms complexes with cations in the following decreasing affinity: $Rb^+ > K^+ > Cs^+ \gg Na^+ > Li^+$.[8] Indeed, the color changes observed in Figure 2A correlate the relative strengths of the ion-bound complexes of valinomycin. Monensin, on the other hand, displays the highest affinity toward Na^+ ions.[9] The colorimetric results shown in Figure 2B are consistent with this property, and confirm that the most significant blue-red color change within the monensin/DMPC/PDA vesicles is induced by addition of sodium cations to the solution.

The general applicability of the concept described here is further demonstrated in Figure 3, depicting quantitatively the blue-red color changes of ionophore/DMPC/PDA vesicles in the presence of various cations. The graphs presented in Figure 3 describe the colorimetric responses [CR],[6] which gauge the extent of blue-red transitions of the vesicles. Figure 3 indicates, for example, that colorimetric transitions can be detected even in the presence of low, submillimolar concentrations of ions [a CR of less than 20% can be already detected by the naked eye].

The ionic selectivity is clearly determined by the type of ionophore incorporated into the vesicles. Figure 3C, for example, shows that the colorimetric responses of DMPC/PDA vesicles, to which the divalent-pyrrole ionophore denoted A23187[16] has been added, decrease in the order $Co^{2+} > Ba^{2+} > Sr^{2+} > K^+$. This ionic selectivity is again consistent with the published ionic affinities of A23187.[16] The data shown in Figure 3 indicate that the ionic selectivity of the vesicles can be adjusted by choosing the appropriate ionophore. The results also indicate that the technique is not limited to detection of monovalent alkali metals, and point to its potential application for screening ionophore-type molecules for their ionic binding.

Elucidating the physical and chemical basis of the chromatic transitions of PDA systems is still an active area of research.[17] Previous studies have indicated that the blue-to-red chromatic transitions in polydiacetylene-based vesicles are due to interfacial perturbations, which induce strains and distortions within the pendant side-chains of the PDA.[7,17] The structural perturbations are believed to give rise to a gauche−trans conformational transition of the PDA backbone, resulting in shortening of the conjugation network and absorption in a shorter wavelength [and accordingly the red appearance compared to the initial blue color].[18] Furthermore, it has been reported that the irreversible nature of the blue-red color transformations is due to a transition from a meta-stable blue phase into a more thermodynamically stable red phase.[19] A similar mechanism has been previously

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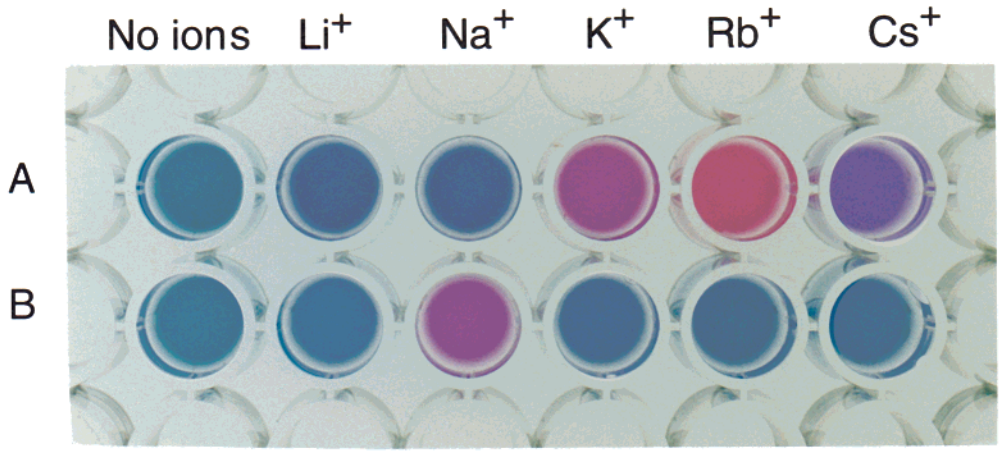

Figure 2. Photograph of a portion of a 96-well plate containing: (A) valinomycin/DMPC/PDA solutions after addition of ions and (B) monensin/DMPC/PDA solutions after addition of ions. Total volume in wells was 0.25 mL. Control vesicle solutions [left-hand wells] contained ionophore/DMPC/PDA vesicles without addition of ions. Cation concentrations in all wells were 10 mM.

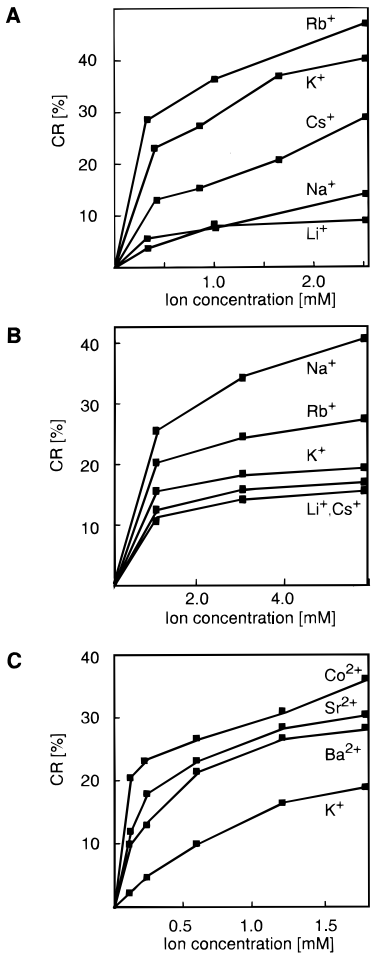

Figure 3. Graphs depicting the colorimetric response [CR, defined in the Methods section] of ionophore/DMPC/PDA vesicles titrated with cations: (A) valinomycin/DMPC/PDA; (B) monensin/DMPC/PDA; and (C) A23187/DMPC/PDA.

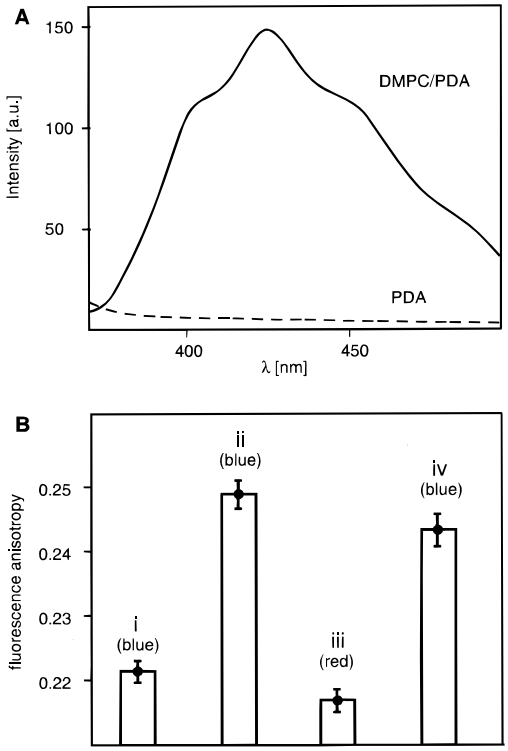

Figure 4. Fluorescence data acquired using TMAP-DPH inserted into DMPC/PDA vesicles. (A) Fluorescence absorption spectrum of TMAP-DPH mixed with DMPC/PDA [4:6 mole ratio] and pure PDA [no phospholipid] vesicles, respectively, and (B) steady-state fluorescence anisotropy measurements of valinomycin/DMPC/PDA vesicles to which KCl and NaCl have been added, respectively: (i) control DMPC/PDA vesicles; (ii) DMPC/PDA vesicles + valinomycin [0.3 mM]; (iii) DMPC/PDA vesicles + valinomycin + KCl [ion concentration 3 mM]; (iv) DMPC/PDA vesicles + valinomycin + NaCl [ion concentration 3 mM]. The color of the vesicle solution is indicated in parentheses.

ascribed to the color changes observed in mixed DMPC/PDA vesicles, in which bio-molecular events occurring exclusively within the phospholipid domains indirectly affect the PDA backbone. It has been demonstrated, for example, that the disruption of phospholipid moieties following chemical cleavage by phospholipases,[13] or through association of membrane peptides,[14] induces structural modifications within the PDA backbone, resulting in observations of blue-red color transitions.

Fluorescence data shown in Figure 4, acquired using the fluorescent dye *N*-[[4-(6-phenyl-1,3,5-hexatrienyl)phenyl]propyl]trimethylammonium-*p*-toluenesulfonate (TMAP-DPH), shed light on the molecular and dynamical changes within the ionophore/phospholipid/PDA system. DPH is widely used for probing the hydrophobic interior of lipid membranes.[20] TMAP-DPH is anchored by the charged TMAP moiety, which is located

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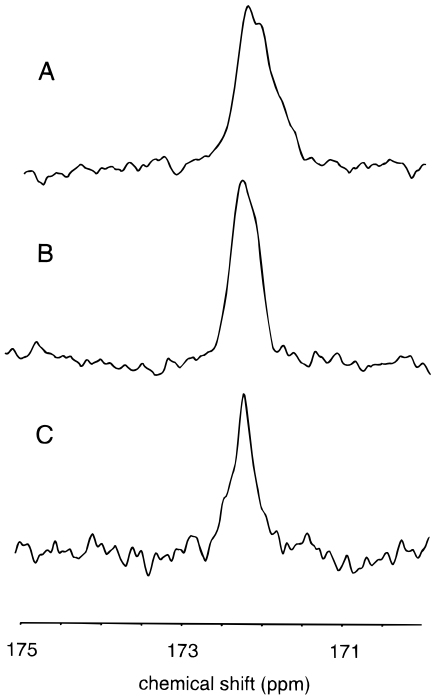

Figure 5. C-13 NMR spectra of mixed vesicles prepared using DMPC isotope-labeled with C-13 at both carbonyl positions. Vesicle concentration was 1 mM, and the solutions included 2 mM Tris at pH of 8.5: (A) DMPC/PDA vesicles; (B) DMPC/PDA vesicles to which valinomycin has been added [0.7 mM]; and (C) valinomycin/DMPC/PDA vesicles after addition of KCl [10 mM].

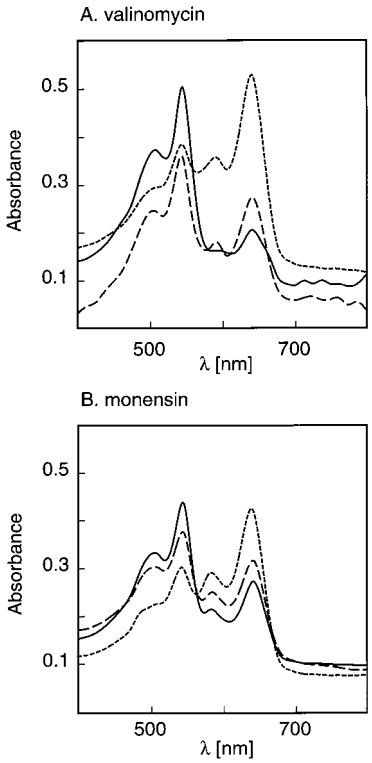

Figure 6. UV−vis absorption spectra of ionophore/DMPC/PDA vesicle solutions containing K^+ and Na^+ ions. *Valinomycin*: solid line, 10 mM KCl; short dash, 10 mM NaCl; long dash, 9.97 mM NaCl + 0.03 mM KCl [around 350:1 molar ratio]. *Monensin*: solid line, 5 mM NaCl; short dash, 5 mM KCl; long dash, 4.87 mM KCl + 0.13 mM NaCl [around 40:1 molar ratio].

at the lipid−water interface.[21] Figure 4A clearly demonstrates that the typical absorption spectrum of DPH is detected only in the presence of phospholipid-containing PDA vesicles, and not in pure PDA vesicles. This result confirms that the DPH probe is embedded within the DMPC domains.[22]

Fluorescence anisotropy measurements shown schematically in Figure 4B indicate that distinct changes occur in the "microviscosity" [or order-parameter] of the phospholipid domains after addition of the ionophores and ions, respectively, to the DMPC/PDA vesicles. Figure 4B reveals that initial addition of valinomycin to the vesicles gives rise to an increase in the fluorescence anisotropy from approximately 0.22 to 0.25, indicating higher lipid microviscosity.[23] The increased microviscosity is most likely ascribed to the incorporation of the hydrophobic valinomycin molecules, in a relatively large quantity, within the phospholipid domains, thereby reducing the rotational reorientation of the DPH probes.[23] Reduced fluidity of biological membranes has been previously observed upon similar association with membrane proteins.[24]

Figure 4B, however, points to a higher fluidity [the fluorescence anisotropy of the DPH is *reduced* to around 0.22] in conjunction with the blue-red color transition induced by addition of K^+ ions. The changes in fluorescence anisotropy depicted in Figure 4B, of around 15%, are significant; changes of similar magnitudes have been observed in a variety of cellular and vesicle systems in which membrane fluidity has been modified.[25] Figure 4B also indicates that, in contrast to K^+, addition of Na^+ ions to the valinomycin/DMPC/PDA vesicles does not induce a similar decrease in viscosity. This observation is consistent with the absence of a significant blue-red colorimetric transition following addition of Na^+ to valinomycin/DMPC/PDA vesicles [Figures 2 and 3].

Fluorescence-lifetime measurements do not register a significant variation in the average lifetime, of around 6.8 ns, of the excited state of DPH between the control vesicles and vesicles to which valinomycin, KCl, or NaCl have been respectively added. The relative lack of change in the lifetime implies that the observed changes in fluorescence anisotropy reflect real modifications of microviscosity within the lipid environment, and are not due to changes in rotational relaxation times.[24,25]

The correlation between the decrease in fluorescence anisotropy of DPH within the phospholipid moieties, shown schematically in Figure 4, and the blue-red color transition of the vesicles could help to elucidate the mechanisms responsible for the colorimetric transitions observed in the DMPC/PDA system studied here. Light-scattering and NMR diffusion measurements [data not shown] could not detect changes in the overall size distribution and mobility of the DMPC/PDA vesicles following addition of ionophores and ions. Therefore, the lower microviscosity of the phospholipid domains in the presence of valinomycin and potassium is attributed to "contraction" of the surrounding PDA matrix, due to the shorter conjugated backbone in the "red" phase. Compression of PDA matrices, induced by external factors, has been detected in other studies.[26,27] The

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structural transformation of the PDA matrix most likely occurs because of the rejection between the lipid chains and the potassium/valinomycin complexes.[28] In contrast to the K^+/valinomycin pair, Na^+ ions do not exhibit strong binding to valinomycin in the ionic concentrations examined here, and accordingly, the addition of sodium ions to the valinomycin/DMPC/PDA vesicles does not induce a significant change in microviscosity [Figure 4B].

C-13 NMR data shown in Figure 5 support this interpretation. Figure 5 depicts C-13 NMR spectra acquired for solutions of mixed DMPC/PDA vesicles, in which the DMPC molecules have been isotope labeled with C-13 at the two carbonyl positions. The C-13 spectrum shown in Figure 5A corresponds to the two overlapping carbonyl signals from the DMPC molecules embedded within the PDA matrix. Addition of valinomycin to the vesicles does not significantly affect the position or line-width of the C-13 signal, as shown in Figure 5B. However, further addition of KCl to the vesicle solution induces a noticeable narrowing of the C-13 resonance, Figure 5(C). The significant reduction of the line width of the C-13 signal indicates a higher mobility of the carbonyls, which occurs in parallel with the blue-red color transition. This result is consistent with the fluorescence data shown in Figure 4, indicating a decrease in viscosity within the phospholipid domains in DMPC/PDA vesicles, in the presence of the valinomycin/K^+ complex.

Figure 6 depicts the extent of K^+/Na^+ selectivity of the ionophore/DMPC/PDA vesicle assemblies, and again shows that the blue-red color transitions are highly dependent upon the cation−ionophore pair employed. Figure 6A, for example, features the UV−vis absorption spectra of valinomycin/DMPC/PDA solutions containing mixtures of sodium and potassium ions at different molar ratios. The UV−vis spectra indicate that, even at a Na^+ to K^+ molar ratio approaching 350, the vesicles still successfully discriminate between potassium and sodium ions. The sensitivity toward the K^+ ions is apparent in the UV−vis spectra from the more intense "red band" at around 520 nm, compared to the "blue-band" at around 640 nm. The K^+/Na^+ selectivity observed in the mixed vesicles is significantly higher than values reported for various commercially available fluorescent ion-sensors.[4] Similarly, Figure 6B demonstrates that DMPC/PDA vesicles containing monensin exhibit selectivity of approximately 40 between Na^+ and K^+. The smaller Na^+/K^+ discrimination of the monensin/DMPC/PDA vesicles, in comparison with valinomycin/DMPC/PDA, is ascribed to the lower Na^+/K^+ selectivity of monensin compared to valinomycin.[8,9]

The colorimetric sensor system described here is robust and versatile. The vesicle solutions can be stored at 4 °C and retain their color sensitivity for long periods of time [months]. This assay could be applied for rapid determination of physiological ionic species, and might be extended for microscopic colorimetric determination of intra- and extra-cellular ion concentrations, as well as for evaluation of the performance and selectivity of putative ion-binding compounds and metal-binding peptides.

Acknowledgment. R.J. is grateful to the US-Israel Binational Science Foundation for financial support. We are grateful to Dr. C. Hariharan and Prof. A. Parola for assistance with the fluorescence experiments and analysis.

JA9924288

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