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Flow Cytometric Ion Detection with Plasticized Poly(Vinyl Chloride) Microspheres Containing Selective Ionophores

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The use of flow cytometry as a rapid, high-throughput diagnostic tool for the analysis of ions is described. Monodisperse, uniform microspheres, which obey bulk optode theory and are governed by bulk extraction processes rather than surface phenomena, were prepared under mild, nonreactive conditions using a sonic stream particle casting apparatus. As an initial example demonstrating the utility of this approach, microspheres that contained a H^+-selective fluorescent chromoionophore (ETH 5294), a cation-exchanger (NaTFPB), and either a highly sodium-selective (sodium ionophore X) or a potassium-selective ionophore (BME-44) were prepared. Separate solution analysis of sodium- and potassium-selective microspheres resulted in the generation of functional response curves using peak channel fluorescence intensities. The selectivity observed for both types of particles is sufficient for the clinical determination of Na^+ and K^+. Furthermore, sodium- and potassium-selective microspheres were analyzed in parallel using sodium sample solutions, resulting in the successful determination of sodium ion concentrations and providing important information about the selectivity of the potassium-selective sensors over sodium. This work demonstrates the potential applicability of flow cytometry as a means for developing multiplexed, rapid, high-throughput analyses for clinically relevant ions.

The analysis of complex biological fluids, such as whole blood, serum, and urine, is of paramount importance in clinical chemistry. Electrolytes, such as Na^+ and K^+, are routinely assessed using carrier-based ion-selective electrodes (ISEs).[1,2] With more than one billion ISE measurements being performed annually worldwide in clinical laboratories,[1] this class of chemical sensors plays a crucial role in laboratory diagnostics. Trends in analytical chemistry continue to move toward the development of miniaturized systems, and there is a great interest in streamlining all available assays into one common method. It appears that optical readout methodologies are most attractive to achieve this goal.

Several approaches have been developed for ion analyses that use fluorescence transduction.[3−17] This is primarily due to the high signal-to-noise ratio afforded by this detection method, making it an attractive choice for creating sensors of reduced size. Most ion-selective optical sensors use the same carriers previously developed for use in ISEs, and they obey bulk extraction principles consistent with traditional optode theory.[2] For a thorough review of bulk optode theory, refer to the work of Seiler and Simon.[18] Typically, an optode membrane is composed of a plasticized poly(vinyl chloride) (PVC) matrix, an ionophore that selectively binds the primary ion, ionic sites that facilitate mass transfer of ions from the aqueous sample to a hydrophobic sensing phase, and a H^+-selective fluorescent chromoionophore (fluoroionophore), which is responsible for signal transduction. Deprotonation of the chromoionophore occurs when protons are exchanged by target ions entering the membrane phase; changes in chromoionophore protonation result in measurable changes in its optical behavior.

Many sensing formats have been developed that exploit the manifold advantages of ionophore-based sensing. One approach employs optical fibers that have an ion-sensing film attached at

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their distal end.[10,14] The fluorescence emitted by the fluoroionophore travels through the optical fiber by means of total internal reflection. These types of sensors are able to interrogate intracellular and extracellular matrixes; however, they are limited by the lengthy preparation procedures required and the inability to precisely control the thickness of the sensing membrane. Other approaches for ion determinations have utilized particle-based technologies. Arguably, the first optical nanoprobes were reported by Lubbers et al. and were used for measuring the pH and pO_2 of physiological structures.[19] More recently, the group of Kopelman has reported both acrylamide and PVC-type nanometer-sized sensing spheres that have proven to be quite useful in interrogating intracellular environments.[3−5,20] Meanwhile, micrometer-sized particles have been prepared in our group by various methods, including heterogeneous polymerization,[13]solvent casting,[17]and very recently, with the use of a sonic stream particle casting apparatus.[16] Spatial and spectral characterization of the particles was performed by fluorescence microscopy/spectroscopy. This approach, albeit an excellent technique for single particle analysis, is not yet suitable for high-throughput screening applications.

In an attempt to increase the throughput of ion determinations, Kim et al. have described a 96-well plate-format absorbance-based optode that required microvolume samples and that could be read using existing clinical laboratory instrumentation.[21] An even more promising technique, however, that offers rapid, high-throughput analyses with multiplexing capabilities is flow cytometry. Microspheres have been used for flow cytometric applications for more than 25 years[22] and they are commonly used for multiplexed analyses. It has been demonstrated with microsphere-based technologies that as many as 64 different analytes can simultaneously be screened using this technique.[23−25] With a large surface area available for attaching numerous molecular recognition chemistries[6] and a core that can be impregnated with encoding dyes,[26] microspheres have played an important role in the development of suspension array technologies.[27] Numerous biologically relevant analytes have been detected using microsphere-based flow cytometry.[7,8,11,12,25,28−30] Electrolytes, however, are a class of analytes that have never been assessed with this technique.

In this work, monodisperse microspheres that are selective for either Na^+ or K^+ were prepared using a sonic stream particle-generating apparatus. For the first time, it is demonstrated that flow cytometry can be used for the high-throughput analysis of these plasticized poly(vinyl chloride) sensing microspheres and that multiplexed measurements are capable using this analytical method.

EXPERIMENTAL SECTION

Reagents. Poly(vinyl chloride) (PVC), bis(2-ethylhexyl) sebacate (DOS), *tert*-butylcalix[4]arene tetraethyl ester (sodium ionophore X), 2-dodecyl-2-methyl-1,3-propanediyl bis[*N*-[5′nitro-(benzo-15-crown-5)-4′-yl]carbamate] (potassium ionophore III, BME-44), 9-(diethylamino)-5-octadecanoylimino-5H-benzo[*a*]phenoxazine (chromoionophore I, ETH 5294), and sodium tetrakis-[3,5-bis(trifluoromethyl)phenyl]borate (NaTFPB) were Selectophore quality from Fluka (Milwaukee, WI). The ring-locked cyanine (RLC) reference dye 2-[2-[2-chloro-3-[(1,3-dihydro-3,3-dimethyl-1-decyl-2H-benzoindol-2-ylidene)ethylidine]1-cyclohexen-1-yl]ethenyl]3,3-dimethyl-1-decylbenzoindolium iodide was obtained from Beckman Coulter, Inc. (Brea, CA). Dichloromethane (DCM) (EM Sciences), xylenes (EM Sciences), cyclohexanone (JT Baker), and dimethyl sulfoxide (DMSO) (Aldrich) were ACS grade and were obtained from the indicated suppliers. Chloride salts of sodium and potassium were reagent grade and were obtained from Mallinckrodt and Sigma, respectively. Tris(hydroxymethyl)aminomethane (Tris) was reagent grade from Sigma. A reverse-osmosis water filtration system (US Filter, Philadelphia, PA) was used to distill and deionize the water (18 MΩ cm) from which sample solutions were prepared.

Particle Preparation. Potassium particles contained PVC (33 wt %), DOS (66 wt %) ETH 5294 (0.2 mmol/kg), NaTFPB (0.3 mmol/kg), and BME−44 (21.1 mmol/kg). Sodium particles contained PVC (33 wt %), DOS (66 wt %), ETH 5294 (0.2 mmol/kg) and either Na^+-ionophore X (10.0 mmol/kg) and NaTFPB (0.3 mmol/kg) (type 1) or Na X (89.3 mmol/kg) and NaTFPB (27.1 mmol/kg) (type 2). For segregation of particle subsets in the parallel analyses, type 1 sodium particles also contained RLC (3.9×10^{-3} mmol/kg). Particles were prepared using a particle casting apparatus that has been described elsewhere.[16] To summarize, a polymer cocktail containing PVC, DOS, ionophore, ETH 5294, and NaTFPB was dissolved in 5 mL of cyclohexanone and diluted with 100 mL of DCM. The addition of 1 mL of xylenes to the solution aided in the aesthetic appearance of the cast particles. The polymer solution and an aqueous sheath liquid (deionized water) are hydrostatically directed from storage bottles into a mixing chamber. The polymer cocktail enters the proximal end of the mixing chamber assembly, where it is directed toward a ceramic orifice located at the distal end of the assembly. By adjusting the operating frequency of a piezoelectric transducer that is mounted on the proximal end of the assembly, the polymer stream is broken off into uniform droplets. The aqueous sheath liquid surrounds the droplets and by hydrodynamic focusing maintains the single file order of the droplets. As the droplets travel through the sheath liquid, solvent slowly partitions out of the droplets and into the catch liquid, ultimately causing the precipitation of uniform, monodisperse microspheres of homogeneous composition. During particle formation, a stock solution of a nonionic surfactant (3% (v/v) Tween 20) is added to reduce clumping. The microspheres are collected in a ∼20 L receptacle and left to cure. This step of the process is needed to remove solvent that remains in the microspheres, and it allows the

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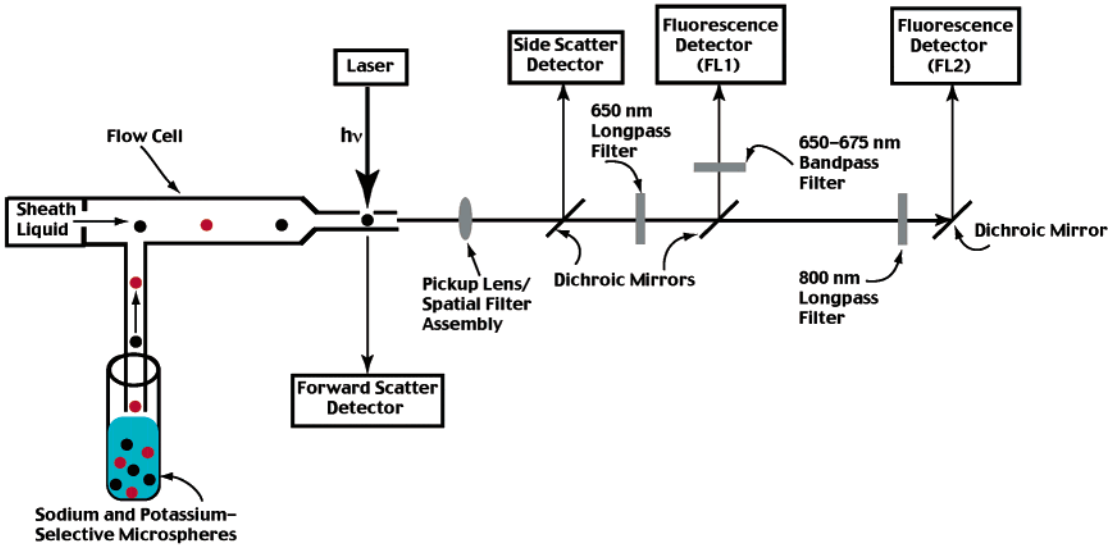

Figure 1. Optical configuration of the flow cytometer used in this work.

particles to sediment. Following the curing process, the excess catch liquid is decanted and the microspheres are concentrated.

The following settings and specifications were used in this work: tip diameter, 38.1 μm; frequency, 21.5 kHz; polymer flow rate, 0.5 mL/min; water flow rate, 52 mL/min; surfactant flow rate, 1 drop/10 s; curing duration, 4 d. In contrast to a recent report,[16] the surfactant was added using a model 352 syringe pump (Sage Instruments, Boston, MA).

Instrumentation and Measurement. The absorption behavior of ETH 5294 in both its protonated and deprotonated forms was determined using a DU 70 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). For fluorescence characterization, a 1×10^{-5} M solution of ETH 5294 in DMSO was mixed with a 1% (v/v) aqueous solution of either HCl or NaOH and excited at 635 nm using a Fluorolog 3 fluorometer (Instruments SA, Edison, NJ) to determine the emission behavior of the protonated and deprotonated forms at this wavelength. Optical characterization of RLC in methanol found $\lambda_{ex} = 820$ nm and $\lambda_{em} = 860$ nm.

A Beckman Coulter EPICS XL flow cytometer modified for both 635- and 785-nm excitation was used to interrogate the microspheres. A 650-nm long-pass emission filter and a 660 (±15)-nm band-pass filter were used to collect fluorescence emitted between 650 and 675 nm, and an 800-nm long-pass emission filter was used to collect fluorescence emitted above 800 nm. A schematic representation illustrating the optical setup of the cytometer that was used appears in Figure 1. Sensing microspheres were equilibrated for 30 s in sodium or potassium sample solutions containing 4 mM Tris buffer, pH 7.4.

RESULTS AND DISCUSSION

Uniform, monodisperse microspheres were prepared from plasticized PVC using a high-throughput particle casting technique that has previously been described elsewhere.[16] Plasticized PVC was selected as the polymer matrix, because it provides a lipophilic environment conducive for retention of active sensing components, and it has repeatedly been proven to be a suitable material for ionophore-based sensing.[1,18] The particles were incorporated with a highly selective ionophore, a neutral H^+-selective fluoroionophore, and a cation exchanger during the casting process. An ion-exchange process, consistent with classical optode theory, is the sensing mechanism employed to impart sensor function. In this process, target ions, here either sodium or potassium, are extracted into the bulk of the microsphere where they are complexed by selective ionophores. Influx of positive charge into a microsphere results in deprotonation of the fluoroionophore and a concerted expulsion of a proton from within the microsphere. The equilibrium describing this process has been reported repeatedly[1,18] and appears in eq 1.

$$I^+ (aq) + L (org) + CH^+ (org) + R^- (org) \rightleftharpoons IL^+ (org) + C (org) + R^- (org) + H^+ (aq) \quad (1)$$

In eq 1, I^+ is the sample ion, L and IL^+ are the uncomplexed and complexed forms of the ionophore, respectively, C and CH^+ are the deprotonated and protonated forms of the fluoroionophore, respectively, and R^- is the cation-exchanger. Parenthetical notations (aq) and (org) denote the aqueous and organic phases, respectively. If the sample pH remains constant (i.e., via buffering) the concerted ion exchange allows for the quantitative determination of the target analyte by measuring changes in the optical activity of the fluoroionophore (i.e., fluorescence intensity). Ionophore-mediated sensing strategies are far superior to sensing approaches that use surface-attached indicators[6] because of the high selectivity that they impart.

The absorbance and fluorescence emission characteristics of the fluoroionophore used in this work, ETH 5294,[31] appear in Figure 2. In DMSO, the protonated form of ETH 5294 has absorption maxima at 280, 324, and 635 nm, whereas the deprotonated form has maxima at 272, 514, and 615 nm and a shoulder at 305 nm. It is apparent from Figure 2a that the deprotonated form of the fluoroionophore does not strongly absorb at 635 nm, which may explain the absence of a fluorescence signal

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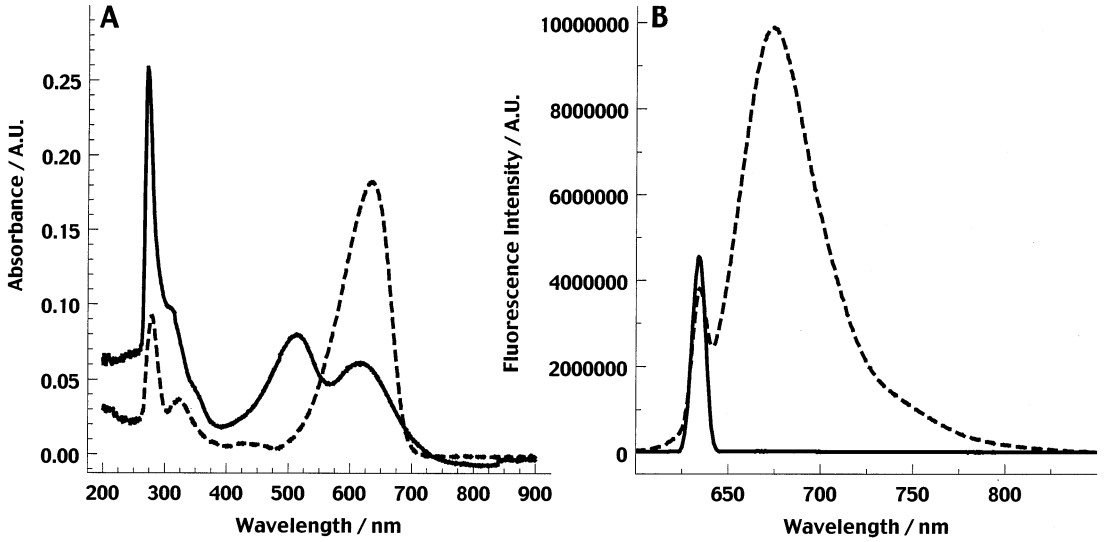

Figure 2. (A) Absorbance behavior of the protonated (dashed line) and deprotonated (solid line) forms of ETH 5294 in DMSO. (B) Fluorescence emission characteristics of ETH 5294 in both the protonated (dashed line) and deprotonated (solid line) forms (λ_{ex}= 635 nm).

for this form of the indicator in Figure 2b. The protonated form of the fluoroionophore, however, exhibits an emission maximum at 674 nm. In Figure 2b, the emission peak observed at 635 nm for both forms of ETH 5294 is due to elastic laser scattering and is only an artifact. When excited at 635 nm, the emission behavior of ETH 5294 is substantially different from that observed when it is excited at 560 nm.[17] A loss of ratiometric capabilities results, which is consistent with data reported for another self-referencing fluoroionophore.[32] Unlike most fluorescence-based assays, which quantitate a directly proportional relationship between analyte concentration and fluorescence intensity, the approach reported here uses an inverse relationship: an observed decrease in fluorescence correlates to an increase in ion concentration. This can be explained by eq 1, which shows that the deprotonated form of the fluoroionophore predominates at higher target ion concentrations.

Implementation of microsphere-based technologies for ion analyses makes it possible to use existing instrumentation commonly found in the clinical laboratory to determine this class of analytes. A suitable technique that is commonly used for microsphere-based assays is flow cytometry. When dealing with biological samples, a primary concern is the biasing of results due to sample autofluorescence. This obstacle may be circumvented by judiciously selecting a fluoroionophore that has appropriate spectral characteristics and by using long-wavelength excitation. Under these circumstances, the emission behavior of the sensing dye is usually beyond the wavelength range over which autofluorescence typically occurs.[3] Furthermore, encoding dyes, such as RLC, and optical filters may also be used to eliminate undesired fluorescence wavelengths. Flow cytometry also offers a means for high-throughput screening, which is a capability not found with microscopic techniques. This is primarily due to differences in the detectors commonly employed with each technique. In fluorescence microscopy, charge-coupled devices that offer excellent spatial and temporal resolution, but suffer from longer data acquisition times on the order of the millisecond time scale are typically used. Flow cytometry, on the other hand, uses photomultiplier tubes, which allows for measurements to be collected on the microsecond time scale.[30] This inherent advantage in conjunction with multiplexed measurement capabilities makes flow cytometry an attractive analytical method for clinical applications.

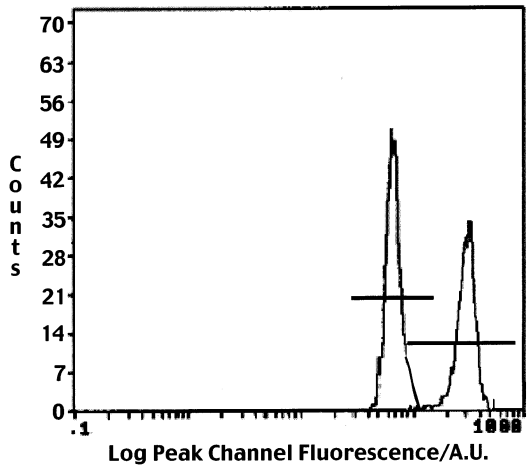

Figure 3. Single-parameter histogram depicting peak channel fluorescence intensity variations in type 1 sodium-selective microspheres equilibrated in 10^{-1} M (left) and 10^{-4} M (right) sodium samples containing 4 mM Tris, pH 7.4. Horizontal bars represent the full width at half-maximum, from which CVs were determined.

A cytometric histogram that illustrates the observed fluorescence change that results from changes in sample ion concentration is shown in Figure 3. This single-parameter histogram shows the number of counts as a function of the log of the peak channel fluorescence. The horizontal distance between the Gaussian curves, which represents sample sodium concentrations of 10^{-1} (left) and 10^{-4} M (right), is indicative of a substantial change in the fluorescence behavior of the fluoroionophore. An increase in target ion concentration results in an increase in the proportion of fluoroionophore in the deprotonated state, which does not emit (see Figure 2). Response curves depicting the fluorescence changes over a wide range of sample concentrations appear as shown in Figure 4. Type 2 sodium particles containing Na^+

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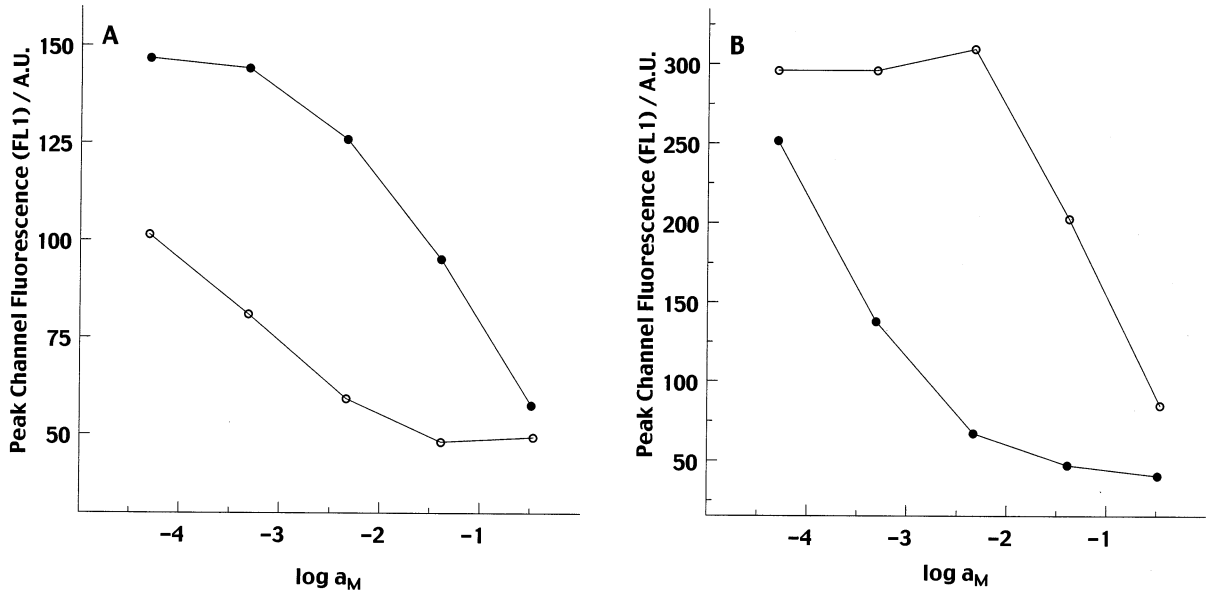

Figure 4. (A) Response and selectivity of type 2 sodium-selective microspheres in sodium (open circles) and potassium (solid circles) sample solutions containing 4 mM Tris, pH 7.4. (B) Response and selectivity of potassium-selective microspheres in potassium (solid circles) and sodium (open circles) sample solutions containing 4 mM Tris, pH 7.4.

ionophore X were used to generate the curves in Figure 4a. Separate sample solutions were used for each respective ion. Notice the higher concentrations of potassium required to deprotonate the fluoroionophore, indicating the selective discrimination of potassium by the sodium ionophore. A parallel shift in the location of the potassium response curve of ~2.5 orders of magnitude is consistent with that previously reported using other detection platforms.[16]

Cytometric measurement of the fluorescence intensities from individual particles produces a coefficient of variation (CV) in the estimate of the mean fluorescence equal to the CV of the particle population. The CV in the estimate of the mean can be improved by averaging the measurements from multiple particles. Repeat subsampling of a read of 10 000 sensors gave precision improvement consistent with Poisson statistics. For mean peak fluorescence channel values of 109.3, the CV in the estimate of the mean improved from 12.5 to 1.2 to 0.4% when the sample size was increased from 1 to 100 to 1000, respectively. Conversely, potassium-selective particles containing the ionophore BME-44 demonstrate function and selective discrimination of sodium ions in Figure 4b. The selectivity observed, which is indicated by the 2.5 orders of magnitude parallel shift of the sodium response curve is consistent with reports of another particle-based detection method.[17] The data points shown in Figure 4 are the mean values of 2000 sensors. The precision of microsphere-based flow cytometry is ultimately contingent upon the monodispersity of the particles. The particle fabrication method used here has been reported to produce uniform particles with a diameter variation of 1.5%.[33] Furthermore, CVs representing the particle-to-particle reproducibility of fluorescence flow cytometric measurements have been reported to be as low as 3.6%.[33] Although this signal variation may appear to be a potential limitation of this technique, ratiometric capabilities of the readout will greatly improve

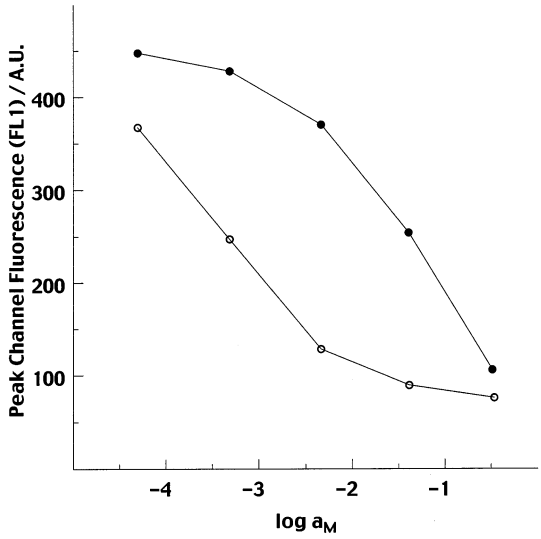

Figure 5. Parallel analysis of type 1 sodium (open circles) and potassium-selective (solid circles) particles in the presence of sodium sample solutions containing 4 mM Tris, pH 7.4.

reproducibility.[16] Moreover, the ease of measurement of large numbers of particles makes possible excellent precision, even with poor particle uniformity.

When considering the utility of flow cytometry as a diagnostic tool for the clinical determination of ions, one must consider whether this approach will work for analyzing complex biological fluids. A precursor to that step, however, is the parallel analysis of more than one type of sensor. Both sodium and potassium-selective particles were suspended in samples containing various sodium ion concentrations. Cytometric analysis of the mixed particle suspension resulted in a single region of particle density on a forward-scatter versus side-scatter plot, which implies homogeneity of particle size (data not shown). The region of particle density was gated and analyzed using FL2, which separated the particle subsets into two distinct regions on the basis

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of their long-wavelength fluorescence behavior. Because the type 1 sodium particles were also doped with the RLC long-wavelength active dye, particle subsets could easily be segregated. Each particle subset was then gated and analyzed using FL1, which is the fluorescence channel corresponding to the fluorescence signal generated by ETH 5294. Single-parameter histogram analysis of FL1 resulted in the response curves shown in Figure 5. A functional response curve was obtained for sodium, whereas the curve for potassium is indicative of the selectivity behavior of BME-44 over sodium. The selectivity behavior of sodium ionophore X agrees with that found in Figure 4a, in which the microspheres were measured in a serial manner (i.e., each particle subset was measured separately). Preliminary lifetime studies of these microsphere-based sensors have shown that type 2 sodium particles have remained fully functional for periods as long as 6 weeks. This first step toward the clinical determination of ions lays the foundation for the potential applicability of this technology for the multiplexed analysis of complex biological fluids.

CONCLUSIONS

Ionophore-mediated microsphere-based sensors selective for sodium and potassium were prepared using a high-throughput particle casting apparatus that utilizes a sonic droplet formation process. Flow cytometry was demonstrated to be a useful analytical detection platform for the analysis of sample ion concentrations. Microsensors were analyzed serially and in parallel via long-wavelength flow cytometry and were shown to possess acceptable sensitivity, selectivity, and precision for the potential clinical determination of these ions.

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