Published in final edited form as:

Biomaterials. 2011 November ; 32(33): 8574–8583. doi:10.1016/j.biomaterials.2011.07.081.

Triazacryptand-Based Fluorescent Sensors for Extracellular and Intracellular K⁺ Sensing

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Abstract

A 4-amino-naphthalimide derived fluorophore with a triazacryptand moiety ligand was synthesized as a potassium ion (K⁺) sensor (KS1). This sensor is a monomer possessing a polymerizable vinyl group. By taking advantage of the polymerizable characteristics of the vinyl group, KS1 was polymerized with 2-hydroxyethyl methacrylate (HEMA) and acrylamide (AM) to form K⁺-sensing films for extracellular sensing. The sensitivity of the films to potassium ions can be further tuned through the adjustment of the HEMA and AM weight ratios as well as introduction of positive or negative charge-containing segments. KS1 and its poly(2-hydroxyethyl methacrylate)-co-poly(acrylamide) (PHEMA-co-PAM) thin films show high selectivity for K⁺ over competing sodium ions (Na⁺) at physiological concentrations. Extracellular sensing was demonstrated using a KS1-conjugated PHEMA-co-PAM thin film to measure the K⁺ efflux of *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*) stimulated by lysozyme. Meanwhile, KS1 itself permeates human glioblastoma U87MG and human esophagus premalignant CP-A cell lines. KS1 was used to monitor K⁺ efflux stimulated by adenosine-5'-triphosphate (ATP), amphotericin, and a mixture of nigericin, bumetanide and ouabain, demonstrating application of this material as an intracellular potassium ion sensor.

Keywords

Potassium ion sensor; Amino-naphthalimide; Intracellular sensing; Extracellular sensing; Fluorescent probe

1. Introduction

Potassium makes up about 0.4 percent of the mass of the human body and is the most abundant intracellular cation. Intracellular potassium ion (K⁺) concentration is about 150 mM for mammal cells, while extracellular concentration is around 5 mM [1,2]. K⁺ plays diverse roles in biological processes including muscle contraction [3], heartbeat [4], nerve transmission [5] and kidney function [6]. Abnormal K⁺ flux is an early sign onset for a number of diseases including alcoholism [7], eating disorders such as anorexia and bulimia [8], heart disease [4], diabetes [9], AIDS [10] and cancer [11]. The molecular mechanisms

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of K⁺ physiology and pathology are insufficiently understood, however, owing in part to the lack of tools for measuring changes in extracellular and intracellular K⁺ concentrations with high spatial and temporal fidelity. Therefore, measurement of the extracellular and intracellular K⁺ concentrations is very important. Common analytical techniques, such as the K⁺ electrode [12], produce reliable data with high sensitivity, but can't be applied for non-invasive study in biological systems, nor are they suitable for small volumes (few to tens of pico-liters) with small cell numbers, and especially at the single cell level. A fluorescence method stands out as it is non-invasive, disposable, and easily miniaturized. One of the earliest and best known intracellular fluorescent K⁺ probes is a potassium-binding benzofuran isophthalate (PBFI), which uses a diaz-18-crown-6 as an ionophore and a substituted benzofuran as the fluorophore. Yet it is limited by its poor selectivity against sodium ions (Na⁺) [13]. To improve the selectivity, He *et al* developed a ligand based on the triazacryptand (TAC) platform, which features excellent selective responses for K⁺ over competing Na⁺ at physiological concentrations [14]. The sensor was based on an amino-naphthalimide fluorophore, chemically grafted on amino-cellulose, and was used to measure the K⁺ concentration in blood and serum [14]. Verkman's group developed other fluorescent K⁺ sensors based on the triazacryptand ligand and bodipy, crimson, or xanthylum fluorophores [15–17]. However, these small molecule-based sensors are significantly taken up by many cell types and thus their utility for extracellular K⁺ sensing is limited [15,16]. To alleviate this problem, Verkman *et al* grafted their sensors onto amino-dextran for extracellular sensing [16]. Very recently, Verkman's group synthesized a series of small molecules as K⁺ sensors using a novel synthetic approach through a ligand design with reduced synthesis effort [18]. Yet, these sensors were not reported for either intracellular or extracellular investigations.

We have been developing fluorescent sensors including pH, O₂, Zn²⁺, DNA, and temperature sensors [19–28] for not only materials but also applications for intracellular and extracellular sensing, especially at the single cell level [27,28]. Our long-term goals are cellular metabolism investigation, disease/cancer detection and diagnosis using a multi-sensor platform. Herein we report the synthesis and investigation of a compound with the triazacryptand moiety as the K⁺ ligand and a polymerizable vinyl bond-containing amino-naphthalimide fluorophore as a K⁺-sensing fluorescent probe (KS1, Figure 1). The KS1 was copolymerized with a series of monomers, including 2-hydroxyethyl methacrylate (HEMA), acrylamide (AM), 2-(methacryloyloxy)ethyltrimethyl ammonium chloride (METAC), and 2-(methacryloyloxy)ethylsulfonic acid sodium salt (MESA) to form a series of extracellular potassium-sensing films or membranes with tunable potassium ion sensitivity. The extracellular K⁺ sensing ability of the KS1-immobilized sensing membranes was demonstrated through the measurement of K⁺ efflux of *E. coli* and *B. subtilis* stimulated by lysozyme. Meanwhile, we demonstrated that the KS1 is cell permeable to human glioblastoma U87MG and human esophagus premalignant CP-A cell lines, and can be used to monitor the K⁺ efflux through a stimuli.

2. Experimental Section

2.1. Materials

An amino-substituted triazacryptand ionophore (TAC-NH₂) and *N*-2-propenyl-4-bromonaphthalimide (compound **1**) were synthesized according to the known procedures [14, 29]. HEMA, AM, METAC, trimethylsilylpropyl acrylate (TMSPA), azobisisobutyronitrile (AIBN), *N,N*-diisopropylethylamine (DIPEA), *N*-methyl-2-pyrrolidone (NMP), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), amphotericin, nigericin, bumetanide, and ouabain were purchased from Sigma-Aldrich (St. Louis, MO). MESA was ordered from TCI America (Portland, OR). Ethoxylated trimethylolpropane triacrylate

(SR454) was a product of Sartomer (Exton, PA). Adenosine-5'-triphosphate (ATP), Hoechst 33342, LysoTracker Red[®], and lysozyme were ordered from Invitrogen (Carlsbad, CA).

2.2. Instruments

A Varian liquid-state NMR operated at 400 MHz for ¹H NMR and at 100 MHz for ¹³C NMR was used for NMR spectra measurements. High resolution mass spectrometry (HRMS) was performed by the ASU Mass Spectrometry Laboratory. An oxygen plasma cleaner (Harrick Plasma, Ithaca, NY) was used for quartz glass surface activation. A Shimadzu UV-3600 UV-Vis-NIR spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD) was used for absorbance measurements. A Shimadzu RF-5301 spectrofluorophotometer was used for fluorescence measurements. For easy measurement of the films in liquid solutions, quartz glass was cut with a dicing saw into squares of 1.31 cm × 1.31 cm, which can fit diagonally into a quartz fluorescence cuvette to enable the sensing film to be positioned at an angle of 45° to the excitation light.

2.3. Synthesis of KS1

100 mg (135.7 μmol) of TAC-NH₂, 51.5 mg (149.27 μmol) of compound **1**, and 21.1 mg of DIPEA were suspended in 1 mL NMP and microwaved to 150 °C for 4 h. The mixture was poured into 10 mL water. The resulting precipitate was filtered off, and then washed with 10 mL water. The crude product was purified using a silica gel column to yield 36 mg yellow gum. Yield: 27.3%. ¹H NMR (400 MHz, DMSO-d₆) δ (ppm): 2.18 (s, 6H, CH₃), 2.93 (t, 2H, CH₂), 3.00–4.05 (m, 41H, OCH₃, OCH₂ and NCH₂), 4.62 (d, 2H, NCH₂), 5.00–5.10 (m, 2H, CH₂=), 5.8–6.0 (m, 1H, CH=), 6.65–8.70 (m, 14H, phenyl). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 163.93, 154.59, 153.17, 152.24, 150.90, 142.04, 138.89, 137.17, 135.08, 134.65, 132.91, 131.19, 130.10, 127.75, 127.49, 124.53, 124.43, 122.32, 122.02, 121.87, 121.47, 120.98, 120.68, 116.74, 114.97, 113.90, 113.37, 71.10, 70.86, 70.45, 69.97, 68.61, 68.26, 67.93, 66.97, 66.60, 61.82, 58.60, 55.96, 53.80, 53.32, 53.00, 52.56, 44.70, 42.05, 41.38, 34.40, 31.90, 31.10, 30.14, 29.68, 29.34, 22.67, 21.50, 21.03. HRMS (APCI+): m/e calculated for C₅₆H₆₉N₅O₁₀ (M+H): 972.1746; found. 972.3068.

2.4. Typical procedure for extracellular sensing film preparation

Thin films were prepared according to our published protocols [21,23]. A schematic drawing of the film preparation is given in supplementary data (S-Figure 1). F4 film is described as a typical example. The components of other films are given in Table 1. 1 mg of the monomeric KS1, 800 mg of HEMA, 150 mg of AM, 50 mg of SR454, and 10 mg of AIBN were dissolved in 1 mL DMF as a stock solution. 15 μL of the stock solution was added onto the surface of the TMSPA-modified quartz glass and covered with a clean but untreated cover slip to make a sandwich structure. Using TMSPA to modify the quartz glass was to enable the sensors and matrices to be chemically grafted onto a quartz substrate [23]. The thickness was controlled using 25 μm Kapton tape (DuPont, Wilmington, DE). The sandwich set-up was placed into a vacuum oven, which was then evacuated and refilled with nitrogen three times. Polymerization was carried out under nitrogen at 80 °C for 1.5 h in the oven. The quartz glasses with polymer membranes were removed from the oven, then the Kapton tape and non-surface modified glass was removed from the polymerized membrane surface. The polymerized membranes on the quartz glasses were washed three times with methanol to remove any remaining non-polymerized monomers and residual DMF. The films were dried and stored away from light at room temperature.

2.5. Culture of *E. coli* and *B. subtilis* for potassium release study

E. coli JM 109 and *B. subtilis* 168 were cultured overnight in LB medium (Difco®, Becton and Dickinson Company, Sparks, MD) by shaking at 180 rpm at 37 °C. The concentration of

E. coli and *B. subtilis* in culture was estimated by measuring the optical density at 600 nm (OD_{600}). OD_{600} value of 1 indicated a concentration of 5.0×10^8 cfu/mL. After the microorganism density was determined, bacterial cells were collected by centrifuge at $2000 \times g$ for 20 minutes at room temperature. Then appropriate densities of bacteria were made using fresh buffer (150 mM NaCl, pH 7.4) to achieve the desired initial concentrations for experiments.

The F4 film was placed diagonally into quartz cuvettes with 2 mL of bacteria suspension at different densities. Potassium release from the bacteria was stimulated by adding 100 μ L of 20 mg/mL lysozyme. Emission measurements were taken every 5 min at the sensor's peak emission (525 nm) using an excitation wavelength of 450 nm. The potassium ion concentration was calculated from the measured fluorescence ratio (F/F_0).

2.6. Cell culture and imaging

U87MG cells (American Type Culture Collection, ATCC, Manassas, VA) were cultured in Eagle's Minimal Essential Medium (EMEM) supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, 2 mM L-glutamine (Sigma-Aldrich), and incubated at 37 °C in a 5% CO₂ atmosphere. CP-A cells (kindly provided by Dr. Brian J. Reid at Fred Hutchison Cancer Research Center, Seattle, WA) were cultured in Keratinocyte-serum free medium (Invitrogen, Carlsbad, CA) supplemented with Bovine Pituitary Extract (BPE) and human recombinant Epidermal Growth Factor (rEGF, Invitrogen) at 37 °C in a 5% CO₂ atmosphere. Cells were seeded onto 96 well plates at 10,000 cells per well, and incubated for 1 day. Sensors dissolved in DMSO were added to the medium to prepare concentrations in a range of 0.5 – 5 μ M. 10 min of internalization was found to be sufficient for achieving satisfied images. To confirm the sensor's subcellular distribution in cells, Hoechst 33342 dissolved in fresh medium and subsequently incubated with cells for 30 minutes to stain nuclei. Concentrations of LysoTracker Red[®] and Hoechst 33342 were 100 nM and 10 μ M, respectively. Using a Nikon Eclipse TE2000E inverted microscope with a C1si confocal laser scanner (Melville, NY), Hoechst 33342 was excited at 402 nm and its blue emission was collected using a 450/35 nm filter set. LysoTracker Red[®] was excited at 561 nm and its red emission was collected using a 605/75 nm filter set. Sensor (KS1) was excited at 440 nm and the green emission was collected using a 515/30 nm filter set.

2.7. Monitoring the intracellular K⁺ efflux using KS1

U87MG and CP-A cells were seeded onto 96 well plates at 10,000 cells per well and maintained in 100 μ L medium overnight. The following day, the cells were loaded with KS1 (2 μ M) for 20 min, then the excess KS1 was removed by washing with medium. The test substances were added and the experiment was terminated after 2.5 h by washing the cells with physiological NaCl solution. A microplate reader (Molecular Device, Downingtown, PA) was used to read the fluorescence intensities from the intracellular KS1. 444 nm was used to excite the sensor and the emission was measured at 538 nm. The measured fluorescence intensities were corrected with background fluorescence. Each experiment was repeated in triplicate.

3. Results and Discussion

3.1. Design of sensors

KS1 was constructed using an amino-naphthalimide as the fluorophore and a triazacryptand moiety as the ligand. KS1 was synthesized through a reaction of TAC-NH₂ and compound 1. Different from the K⁺ sensor reported by He *et al* [14], the KS1 possesses a polymerizable vinyl bond (Figure 1). The polymerizable sensors, together with their structural tunability, make them to be intriguing precursors for broad applications. Herein,

the KS1 was polymerized with a series of HEMA, AM, METAC, and MESA to form a series of polymer sensing films with different weight ratios among the monomers and charge densities. Different from the post-functionalization of the amino-cellulose and amino-dextran with carboxylic acid group(s)-containing potassium ion probes for extracellular sensing [14,16,17], the polymerization characteristics of KS1 opens an avenue for extracellular sensor preparation.

3.2. Extracellular sensor film preparation

The sensing thin films composing the KS1 moiety and its polymer matrices derived from PHEMA-co-PAM were chemically grafted on transparent optical substrate (herein, quartz glass) (Figure 1). To achieve a stable film on the substrate, the quartz glass was modified with an acrylate-containing silane, TMSPA [23,30]. Detailed schematic drawing of the film preparation procedure is outlined in supporting material (S-Figure 1). The silane groups were anchored on the quartz surface by the reaction of active hydroxyl groups of the quartz surface with the silane groups. The free acrylate segments enable the chemical grafting of the KS1, HEMA, AM, and other monomers on the quartz surfaces. To further produce the polymer thin film with good mechanical stability, SR454 was used as a crosslinker to form robust polymeric matrices. Under thermal polymerization condition using AIBN as an initiator, the sensing films were prepared. Thickness of the sensing films was controlled to be 25 μm . The sensing moieties were chemically immobilized into the polymer matrices, and the polymer matrices were then chemically grafted on the substrate, alleviating the leaching of the sensing moieties from the matrices on the substrates to increase the long-term stability of the sensing films. The sensing films were stable in water for at least 3 months without any leaching of the sensing moieties or change in sensing performance.

Eight films (Table 1) were prepared for investigation of the influence of various weight ratios of PHEMA, PAM, and charge densities on the sensing performances. F1 is a PAM sensing film without PHEMA components. F2 is a PHEMA sensing membrane without PAM segments. F3 and F4 are two sensing films with different weight ratios of PHEMA to PAM. F5 to F8 are PHEMA-co-PAM derived films with tunable charge densities by the copolymerization of the neutral HEMA and AM with the charge-containing METAC or MESA.

3.3. Sensing performance of KS1 in buffer

Figure 2A and 2B gave the UV-Vis and emission spectral changes upon the interaction of KS1 with potassium chloride (KCl) in 10 mM HEPES buffer (pH 7.4). The absorption spectra before and after interaction with K^+ did not change, while emission intensities increased with increasing K^+ concentrations. This fluorescence change was attributed to photo-induced electron transfer (PET, Figure 1). The lone electron pairs of the amino groups in the triazacryptand ligand quench the emission of the fluorophore of the amino-naphthalimide through PET. After interaction with K^+ , the lone electron pairs of the ligand were occupied due to the formation of the ligand- K^+ complex. This change restores the emission of the amino-naphthalimide fluorophore. Fluorescence intensity change at 525 nm was plotted against K^+ concentration, which was illustrated in Figure 2C. Binding constant K could be determined by the fluorescence spectral change using the Benesi-Hildebrand equation (equation 1) [31–33].

$$F_0/(F_0 - F) = F_0/(F_0 - F_{\text{complex}}) + F_0/(F_0 - F_{\text{complex}}) \times 1/K \times 1/[M] \quad (1)$$

where K is the binding constant, F_0 is the integrated fluorescence intensity of free sensor, F is the observed integrated fluorescence intensity, F_{complex} is the emission of the ligand-

metal complex, and $[M]$ is the concentration of the added metal ion. When $F_0/(F_0 - F)$ is plotted against the $1/[M]$, the binding constant is given by the ratio intercept/slope.

Curve fitting of the fluorescence intensity of KS1 against the reciprocal of the K^+ concentration ($1/[K^+]$), the Benesi-Hildebrand plot [31–33], gives a linear fit (Figure 2D). This is a characteristic of a 1:1 complexation behavior from which the binding constant (K) is estimated to be 0.133 mM^{-1} , corresponding to the disassociation constant (K_d) of 7.53 mM.

KS1 shows a high selectivity against a few biologically relevant metal ions (Figure 2E) including Na^+ . Na^+ is usually an interference ion to K^+ for the typical K^+ probes including PBFI, a 1,10-diaza-18-crown-6 chelating group coupled with a coumarin fluorophore (CD18), and 6,7-(4-methyl)coumaro-[2,2,2] cryptand (MCC) [13,29,34].

3.4. Sensing performances of KS1 in the membranes of F1 to F8

Typical K^+ sensing using film F4, made of PHEMA-*co*-PAM with 80:15 by weight, was given in Figure 3. Figure 3A shows the emission change upon titration with KCl. Its corresponding fluorescence intensities at 525 nm were given in Figure 3B. The sensing response to Na^+ was also given in Figure 3B, showing the high selectivity of K^+ to Na^+ . This trend is in accordance with that of pure KS1 in buffer. However, the sensitivity of this sensing film F4 is better than that of pure KS1, which may possibly be due to the fact that this sensing film can be swollen by water, resulting in a better interaction of the sensing moiety in the PHEMA-*co*-PAM hydrogel with K^+ . Curve fitting of the fluorescence intensity of F4 against the reciprocal of K^+ concentration ($1/[K^+]$) using the Benesi-Hildebrand equation 1 [31–33] gives a linear fit (Figure 3C). This suggests the characteristic of a 1:1 complexation behavior from which the association constant (K) is estimated to be 0.138 mM^{-1} , corresponding to the disassociation constant (K_d) of 7.25 mM.

For K^+ sensors, besides the interference by Na^+ , pH value is another factor to be concerned. Figure 3D plots the pH dependent fluorescence changes at different pH values. Results showed that this sensing membrane before the complexation with K^+ had no response to pH from 6 to 8. After the complexation with the K^+ , the sensing membrane did not respond to pH from 5 to 8. These results are in accordance with other K^+ sensors derived from the triazacryptand ligand [14,16,17]. These results showed our K^+ sensors are minimally influenced by biological pH values.

Figure 4 gives the comparison of the responses of the eight thin films to K^+ . The sensing membrane made of PAM matrix (F1) did not response to K^+ , indicating its poor K^+ permeability. Sensing membrane F2 made of PHEMA has better response to K^+ than that of F1. Interestingly, the two films of F3 and F4 show much better sensitivity than F2, indicating the chemical composition of the PHEMA-*co*-PAM improves K^+ permeability. It is known that PHEMA can have good swelling properties and ion permeability. However, its swelling and ion permeability could be further improved by the copolymerization with other water soluble polymers, such as PAM. The composite materials can increase the swelling degree (W_∞) and therefore increase the range of pore sizes and size distribution in the hydrogel film [35].

Charges of polymers also affect the sensing activities of sensing films. Increasing the density of negative charge on the film by MESA obviously improved the sensitivity of K^+ response. In contrast, increasing the density of positive charge by METAC decreased the response sensitivity to K^+ . At present we do not understand the detailed mechanism completely, but we believe it should be related to ion permeability, charges, and water swelling properties of the various composite materials.

The above studies demonstrate that by taking the advantages of the polymerizable vinyl group, different sensing membranes could be prepared through the tuning of the structures of monomeric precursors for matrices and their composited ratios. Through the tuning of the chemical composites, the sensing performances were also manipulated.

3.5. Demonstration of the applicability of KS1 for intracellular sensing

Two cell lines (U87MG and CP-A) were used for intracellular sensing. It is well known that cells usually contain about 150 mM of K^+ , which is within the sensing range of KS1. Figure 5 shows the confocal fluorescence images of KS1. KS1 can internalize with cells within a short time, as few as 10 minutes. A KS1 concentration of 0.5 μ M in cell culture medium was able to stain cells, but the signal was weak. To achieve images with satisfactory signal-to-noise ratios, a sensor concentration of 2 μ M was used for intracellular study. Bright green fluorescence was not uniform throughout the cells, implying the nonuniform K^+ distribution of the intracellular K^+ in the cytoplasm area. It should be noted here that there was no any autofluorescence was observed under the same optical set-up, indicating the fluorescence observed under the confocal fluorescence microscopy was from KS1 and its potassium ion complex. The subcellular distribution of the sensor in the cytoplasm region was further confirmed by a minimum colocalization of the green emission of KS1 with blue emission from the nuclear staining probe (Hoechst 33342). For intracellular sensing, a significant concern is whether the sensor is affected by the intracellular pH value, especially the low pH value (around 4.5 to 5.5) of the lysosome [36]. To assess this, we co-stained cells with KS1 and LysoTracker Red[®], which is a specific staining reagent for lysosome with red emission. Figure 5D and 5H showed the overlays of the green channels from KS1 and red channels from LysoTracker Red[®]. The green emissions were distributed in the whole cytoplasm area. The yellow colors in the overlapped images indicated the exact subcellular locations of KS1 and LysoTracker Red[®]. Therefore, it can be seen that most of the green emissions were not in the lysosomal area, showing that KS1 is a good optical sensor for intracellular potassium ion imaging. It is interesting to note that in the green channel, some of the locations that coincide with lysosomal staining are stained green more highly, especially in the CP-A cells. These may be due to the possibilities of that the fluorescence of KS1 is enhanced in lysosomes or KS1 is more concentrated in lysosomes.

Cytotoxicity of the sensor KS1 in its monomeric and polymeric forms to cells was studied using trypan blue staining and/or 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays according to our published protocols [20, 22, 23]. In its monomeric form at a KS1 concentration of 2 μ M, after 30 minutes internalization with cells, more than 97% cells were viable; after 24 hours internalization of KS1 with cells, higher than 90% of cells were viable. In its polymeric form after 24 hours interaction with cells, higher than 93% of cells were variable. These results showed the biocompatibility of the sensor in both monomeric and polymeric forms under our experimental conditions.

To monitor the stimuli-responsive change of the intracellular K^+ level, cells internalized with 2 μ M KS1 for 20 minutes were treated with ATP, nigericin (Nig), amphotericin B (Ampho B), and a mixture of nigericin, bumetanide, and ouabain (Figure 6). The decrease of the fluorescence intensities indicates the efflux of the intracellular potassium ions from the cells. ATP is stimulating molecule of ATP-gated K^+ channels. Amphotericin B (AmphoB) is an efflux stimulator. Nigericin is an ionophore. The relative percentages of the fluorescence intensities before and after the addition of the stimuli materials are given in Figure 6. It was found that nigericin ionophore was not an efficient K^+ efflux stimulator. However, the combination of the ionophore with influx inhibitors bumetanide (B) (inhibitor of Na^+ , K^+ , $2Cl^-$ -cotransport) and ouabain (O) (inhibitor of Na^+ , K^+ , ATPase pump) has been shown to induce an obvious K^+ efflux in the two cell lines. These results are in accordance with the trends of other studies [37]. Therefore, KS1 was demonstrated to be a candidate for the

monitoring of intracellular potassium ion balance. It should be pointed out here that, because of the difficulty in calibrating intracellular K^+ concentration, the application of single emission sensor KS1 is still qualitative.

3.6. Application of the K^+ sensing films for studying antimicrobial reagents

There is growing interest in the role of antimicrobial reagents in innate immunity [38]. Because the internal ionic environment of bacterial and eukaryotic cells is generally potassium-rich, leaking of K^+ has been used to monitor membrane lytic events in erythrocytes [39], yeast cells [40], liposomes [41], and bacteria [42,43]. Herein, film F4 was used as a typical example for extracellular K^+ sensing. We applied the F4 film for both gram positive (i.e. *B. subtilis*) and negative (i.e. *E. coli*) bacteria and used lysozyme as a model drug to stimulate K^+ efflux from the organisms. Lysozyme is a ubiquitous enzyme in antibacterial defense strategies in nature and was shown to permeabilize the cytoplasmic membrane [44]. The bacteria were cultured at 37 °C overnight, centrifuged, and washed three times with potassium-free buffer with 150 mM NaCl at pH of 7.4. The washed organisms were resuspended in 2 mL of the potassium-free buffer in quartz cuvettes at a concentration range of 1.5×10^{10} to 1.0×10^{11} cfu/mL. F4 was immersed into the above prepared mixtures, and then lysozyme was added into the cuvettes to stimulate the K^+ efflux, which was monitored through the time dependent fluorescence intensity changes of F4 film. With the K^+ releasing from the bacteria into the NaCl solution, the fluorescence intensity of F4 increases. Figure 7 plots the time and bacterial concentration-dependent K^+ concentration changes measured by using F4 film. For *E. coli*, K^+ efflux increased slowly over time; while for *B. subtilis*, K^+ efflux was very fast, within the first 10 minutes, and then very slow within the rest of time course. The difference between *E. coli* and *B. subtilis* is due to the antibacterial properties and action mode of lysozyme, which is more facile and faster to *B. subtilis* (gram-positive bacteria) than to *E. coli* (gram-negative bacteria). The bactericidal properties of lysozyme are primarily ascribed to its *N*-acetylmuramoyl hydrolase enzymatic activity, resulting in peptidoglycan hydrolysis and cell lysis. Gram-negative bacteria are relatively insensitive to lysozyme by the virtue of their outer membrane that acts as a physical barrier preventing access of the enzyme [44].

Thus, using the sensing membranes, it may be possible to establish a simple cell-based fluorescence assay for antimicrobial drugs/peptides screening and understanding the environmental stimuli-to-potassium efflux/influx kinetics.

3.7. Comparison with other reported K^+ sensors

The sensor KS1 and its sensing films F2 to F8 exhibit higher selectivity of K^+ to Na^+ , better than that of PBFI. This good selectivity for K^+ is in accordance with those sensors derived from the triazacryptand and its analogues [14,16,18]. The intracellular K^+ sensor KS1 shows a larger K_d than that of PBFI (6.6 mM) [45]. Although the K_d values were not reported for the other triazacryptand-containing small molecules-based K^+ sensors [15,18], according to their figures, the K_d values might be smaller than KS1. These may be due to (1) structural difference and (2) sensing mechanism difference. KS1 follows a PET sensing mechanism. A TAC coupled fluorescing xanthylum chromophore (TAC-Red) and *N*, *N'*-(2,2'-(2,2'-(1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7,16-diyl)bis(1*H*-imidazole-2,1-diyl))-bis(ethane-2,1-diyl))-5-(*N*-(6-(dimethylamino)-9-(3*H*-xanthen-3-ylidene)-*N*-methyl-methanaminium) pyrimidin-2-amine chloride (**Sensor 1**) reported by Verkman *et al* [15–17] are based on an intramolecular charge transfer (ICT) mechanism. Ideal intracellular K^+ sensors should have a relative large K_d , since the intracellular K^+ concentration is around 150 mM. Ideal extracellular K^+ sensor should have small K_d since the extracellular concentration is around 5 mM. The extracellular sensing films F2 to F8 show tunable K_d values in a range of from 4.96 mM to 26.3 mM. The K_d values of the extracellular sensors

using the triazacryptand ligand grafted in cellulose and dextran matrices [14, 16] were not reported. Basing on the reported figures by He *et al* and verkman *et al* [14, 16], we believe the K_d values are in the similar range, suggesting the close sensitivity to K^+ .

3.8. Future development

The herein reported potassium ion sensors show high selectivity of K^+ versus Na^+ , and minimum pH influence at a biological pH range of 5 to 8. The sensors derived from KS1 exhibit a single emission color. When applied in complicated biological environments, medium additives, cellular autofluorescence, and other biological components may affect the calibration and measurement accuracy for the sensors with single emission colors. Consequently, single-emission detection is sometimes problematic for precise fluorometric analysis under biological conditions [46]. Ratiometric measurement approach was devised to circumvent these unfavorable effects by simultaneous measurement of two fluorescence signals followed by calculation of their intensity ratios. Verkman's group developed a ratiometric K^+ sensor by introducing a rhodamine derivative into a TAC-Lime conjugated dextran for extracellular sensing [17]. Preliminarily, we have demonstrated the preparation of extracellular ratiometric K^+ sensor by a copolymerization of KS1 with a K^+ insensitive fluorophore (Ref-Probe-1, structure was given in supplementary data, S-Figure 2) with red emission in the PHEMA-co-PAM matrix. The typical fluorescence change is given in S-Figure 2. The emission intensity ratios at 525 nm from the K^+ sensor to 705 nm from the Ref-Probe-1 can be used to calculate the K^+ concentrations. We are using the ratiometric sensors for single cell potassium influx and efflux investigation under the stimulation of ATP. On the other hand, using the polymeric characteristics of KS1, dual potassium and oxygen sensors can also be prepared through a copolymerization of KS1 with a monomeric oxygen sensor in the PHEMA-co-PAM matrix (S-Figure 3). Therefore, we believe the polymerizable characteristics of the K^+ sensor will have broad applications by integrating with other polymerizable monomers for various biopolymers and biosensors. In addition, since we can tune the extracellular sensing films' charge densities, further investigation will evaluate the influence of cell attachments/detachments and fouling/non-fouling properties on cellular morphologies, cell fate specification, and cell migration [47,48]. With the integration of the sensing capacity and charged/non-charged sensing films, more biological information relevant with cellular metabolism and disease/cancer is expected to be obtained.

4. Conclusion

A polymerizable and highly selective potassium ion sensor KS1 was synthesized and investigated. KS1 itself shows a comparable dissociation binding constant (K_d) with the popularly investigated PBFI potassium ion sensor. KS1 is permeable to cells, showing its applicability as an intracellular potassium ion sensor, which was further confirmed by stimulation with ATP, amphotericin B, and a mixture of nigericin, bumetanide and ouabain to U87MG and CP-A cells. By using the polymerizable characteristics, KS1 was copolymerized with a series of PHEMA and PAM copolymers as extracellular potassium sensors. Sensitivity could be tuned through adjusting of the co-monomer's compositions and weight ratios. It is expected that using the polymerization approach, polymeric and nanomaterials-based potassium ion sensors can be prepared, which will broaden the application of potassium ion sensors. A typical sensing film F4 was used for extracellular sensing. Lysozyme was used as a stimulus to *E. coli* and *B. subtilis* to release their intracellular potassium ions. Time and bacteria species dependent K^+ efflux was observed. Under the stimuli of lysozyme, the K^+ efflux of the gram-negative *E. coli* was much slower than that of the gram-positive *B. subtilis*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support was provided by the Microscale Life Sciences Center, a NIH Center of Excellence in Genomic Sciences at Arizona State University: Grant 5P50 HG002360, Dr. Deirdre Meldrum, PI, Director. Dr. Brian J. Reid and Dr. Tom Paulson at Fred Hutchison Cancer Research Center (Seattle, WA) were acknowledged for kindly providing us the CP-A cell line.

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Figure 1. Synthesis of KS1 and its sensing membranes as well as the illustration of the photo-induced electron transfer (PET) mechanism.

Figure 2.

A) UV-Vis spectra of KS1 at 0 mM and 200 mM potassium chloride (KCl) conditions. B) Fluorescence spectral change of KS1 in 10 mM HEPES buffer containing different KCl concentrations using an excitation wavelength at 450 nm. C) Change of fluorescence intensity at 525 nm at different potassium ion concentrations. F is the fluorescence intensity at various conditions. F_0 is the emission intensity before the titration (with 0 mM K⁺). D) Benesi-Hildebrand plot of KS1. E) Fluorescence intensity change of KS1 in the presence of various biological cations at their physiological concentrations: Na⁺ (15 mM), Ca²⁺ (0.5 mM), Mg²⁺ (2.5 mM), Fe³⁺ (18 mM), Zn²⁺ (45 mM), Mn²⁺ (0.9 mM), and Cu²⁺ (16 mM).

Figure 3.

A) Emission intensity change of the KS1-conjugated PHEMA-co-PAM (F4) with KCl in buffer. B) Change of fluorescence intensity at 525 nm at different KCl and NaCl concentrations. F is the fluorescence intensity at various conditions. F_0 is the emission intensity before titration (with 0 mM K^+). C) Benesi-Hildebrand plot of F4. D) Influence of pH values on the fluorescence intensities at 525 nm of F4 film before and after the complexation with variable potassium ions.

Figure 4.

A) Fluorescence intensity changes of F1, F2, F3, and F4 titrated using KCl in the concentration range of 0 to 10 mM. B) Fluorescence intensity changes of F4, F5, F6, F7, and F8 titrated using KCl in the concentration range of 0 to 10 mM.

Figure 5.

Confocal fluorescence images of CP-A (A–D) and U87MG (E–H) cells. A and E depict the blue channel, showing nuclei stained with Hoechst 33342. B and F depict the green channel, showing the loading of KS1 into the cells. C and G depict the red channel, showing lysosomes loaded with LysoTracker Red[®]. D is the overlay of A, B and C. H is the overlay of E, F and G.

Figure 6.

Fluorescence change of cells loaded with KS1 stimulated with ATP, amphotericin (amphoB), nigericin (Nig), and the mixture of nigericin, bumetanide, and ouabain (N+B+O). A represents the U87MG cell line. B represents the CP-A cell line. Concentrations of the stimuli materials were: ATP (100 μ M), nigericin (5 μ M), amphotericin B (3.25 μ M), bumetanide (10 μ M), and ouabain (10 μ M). 444 nm was used as the excitation wavelength and emission was measured at 538 nm using a microplate reader.

Figure 7.
Effect of lysozyme on K^+ -release from *E. coli* (A) and *B. subtilis* (B) visualized by F4 film.

Table 1

Compositions of the films F1 to F8, their sensitivity, and K_d values.

Films	Polymer compositions and their weight ratios ^{a)}	Sensitivity (F/F_0) ^{b)}	K_d (mM)
F1	PAM	1.02 (± 0.01)	N/A
F2	PHEMA	1.77 (± 0.03)	18.5
F3	PHEMA:PAM (50:40)	2.03 (± 0.03)	14.9
F4	PHEMA:PAM (80:15)	2.34 (± 0.02)	7.25
F5	PHEMA:PAM:PMETAC (80:15:20)	1.59 (± 0.02)	26.3
F6	PHEMA:PAM:PMETAC (80:15:5)	1.90 (± 0.02)	15.8
F7	PHEMA:PAM:PMESA (80:15:5)	2.69 (± 0.03)	6.04
F8	PHEMA:PAM:PMESA (80:15:20)	3.03 (± 0.03)	4.96

^{a)} Each film contains a 5%-weight percent SR454 crosslinker.

^{b)} The sensitivity of the sensing films to K^+ was at the 0 and 10 mM KCl conditions for comparison because this range is usually enough for extracellular K^+ sensing. F_0 is the fluorescence intensity before the titration using KCl. F is the fluorescence intensity at 10 mM KCl.