Supporting Information

Potassium ion fluorescent sensing in chemically stimulated mammalian cells using a GINKO2-encapsulated sensor fibremat

Azumi Sako,[a] Yusuke Nasu,[b,c,d] Akiko Obata[a] and Toshihisa Mizuno [a,e] *

[a]*Department of Life Science and Applied Chemistry, Graduate School of Engineering, Nagoya Institute of Technology, Gokiso-cho, Showa-ku, Nagoya, Aichi 466-8555, Japan.*
[b]*Institute of Biological Chemistry, Academia Sinica, Nankang, Taipei 115, Taiwan.*
[c]*Institute of Biochemical Sciences, National Taiwan University, Da'an, Taipei 106, Taiwan*
[d]*Department of Chemistry, School of Science, The University of Tokyo, Bunkyo-ku, Tokyo, 113-0033, Japan.*
[e]*Department of Nanopharmaceutical Sciences, Graduate School of Engineering, Nagoya Institute of Technology, Gokiso-cho Showa-ku, Nagoya, Aichi 466-8555, Japan*

Corresponding Author: Toshihisa Mizuno (toshitcm@nitech.ac.jp)

EXPERIMENTAL

Materials

Unless stated otherwise, all the chemicals and reagents were commercially obtained and used without further purification. Methacryloyl Chloride, (±)-1-Amino-2-propanol, 2,2,2-trifluoroethanol (TFE), Methacrylonitrile, 2,2′-azobis(isobutyronitrile) (AIBN), disodium phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), and fluorescein were purchased from Wako Pure Chemical Industries, Ltd. (Japan). Diacetone alcohol and adipic acid dihydrazide (ADH) were purchased from Tokyo Chemical Industry Co., Ltd. (Japan). Polycaprolactone (M_n 80,000)(Cat. No. 440744**)** were purchased from Sigma–Aldrich (USA). Synthesis of the 2-hydroxypropyl methacrylamide (HPMA) was conducted according to the previous report. Green fluorescent protein was produced by *E. coli* BL21(DE3) strain using the pET-3b vector, harbouring the superfolder GFP gene. GINKO2 was produced by *E. coli* DH10B strain (Thermo Fisher Scientific) using pBAD-GINKO2.

Synthesis of diacetone methacrylamide (DAMA)

Methacrylonitrile (7.5 mL, 89.4 mmol) was added dropwise to diacetone alcohol (11.2 mL, 89.4 mmol) in *con*-H_2SO_4 (10 mL) with keeping the temperature below 10˚C using ice bath. After further 1 h stirring at 4˚C, the resulting solution was extracted with dichloromethane. The obtained organic layer was dried over Na_2SO_4 and solvent was removed *in vacuo*. The oily residue was subjected to silica gel chromatography (eluent; CH_2Cl_2 to CH_2Cl_2/MeOH = 10/1) and gave the target product as colourless oil. Yield 8.1 g (49%). ^{1}H-NMR (400 MHz, $CDCl_3$) 1.41 (s, 6H, C<u>H</u>$_3$-), 1.89 (s, 3H, C<u>H</u>$_3$-C(=CH$_2$)-C(=O)-), 2.11 (s, 3H, C<u>H</u>$_3$-C(=O)-), 2.91 (s, 2H, -C<u>H</u>$_2$-C(=O)), 5.72, 5.79 (s, 1H each, CH$_3$-C(=C<u>H</u>$_2$)-), 6.30 (brs, 1H, -C(=O)N<u>H</u>-).

Synthesis of the copolymer of HPMA and DAMA, poly(HPMA /DAMA)

DAMA (1.29 g, 7.0 mmol), HPMA (4.00 g, 27.9 mmol), and AIBN (8.70 mg, 53.0 μmol) were dissolved in DMSO (5 mL) and by freeze-pump threw the dissolved oxygen was removed. Then this mixture was heated at 90 ˚C for 5 h under N_2 atmosphere to progress polymerization. To precipitate the target copolymer from the resulting reaction mixture, it was added to acetonitrile. By successive reprecipitation using acetonitrile as a poor solvent the purification of the target copolymer was performed. Yield 4.1 g (78 %). ^{1}H-NMR (400 MHz, $CDCl_3$) 0.5 – 2.1 (m, 33H, -[CH_2-C(C$\underline{H}_3$)-C(=O)]$_n$-, -NH-C(C$\underline{H}_3$)$_2$-CH_2- in DAMA unit, and C$\underline{H}_3$-C(OH)- in HPMA unit), 2.10 (s, 3H,C$\underline{H}_3$-C(=O)-CH_2- in DAMA), 2.52-3.1 (brs, 10H, -NH-C$\underline{H}_2$-CH(OH)- in HPMA and -C(CH_3)$_2$-C$\underline{H}_2$-C(=O)- in DAMA), 3.70 (s, 4H, CH_3-C$\underline{H}$(OH)-CH_2-), 4.70 (s, 4H, CH_3-CH(O$\underline{H}$)-CH_2-), 6.52 (brs, 1H, -C(=O)N$\underline{H}$- in the DAMA unit), 7.18 (brs, 4H, -C(=O)N$\underline{H}$- in the HPMA unit). From the proton ratio at 7.18 ppm (for amide NH in HPMA) and 6.52 ppm (for amide NH in DAMA), we could estimate the molar ratio of HPMA to DAMA in the obtained copolymer to be 8:2. The M_n and polydispersity index (PDI) were determined by gel permeation chromatography by referring the poly(ethylene glycol) (PEG) standards and were 131 kDa and 2.64, respectively.

Construction of the core-shell fibermats, consisting of the core nanofibers of poly(HPMA/DAMA)/ADH and the shell layer of PCL

ADH [0.5 molar equivalent with respect to the DAMA unit in poly(HPMA/DAMA)] was added to a poly(HPMA/DAMA) solution [0.25 g dissolved in 1 mL of phosphate buffer (100 mM; pH 7.4)] and transferred to a Luer-Lok syringe (1 mL). To encapsulate GINKO2, GINKO2 (639.4 nmol) was added to this solution. To encapsulate fluorescent low MW molecule such as fluorescein, 7.52 μmol of fluorescein was added to this

solution. Concomitantly, 8 wt% PCL solution dissolved in TFE was prepared and transferred to another Luer-Lok syringe (5 mL). Both syringes were then placed in different syringe pumps fitted with a linear actuator (KDS-100, KD Scientific, USA) and connected to a co-axial spinneret (MECC Co., Ltd., Japan) equipped with 27 G needle (Terumo Corp.) for passing the poly(HPMA/DAMA)/ADH) core solution through a polytetrafluoroethylene (PTFE) tube. The poly(HPMA/DAMA)/ADH and nylon6 solutions were then electrospun (SD-02, MECC Co., Ltd., Japan) at linear extrusion velocities of 0.3, 1.2 mL h^{-1}, respectively, at a high voltage (20 kV). The obtained fibermat core and shell components were poly(HPMA/DAMA)/ADH and PCL, respectively. The distance between the spinneret tip and electrically grounded collector (aluminium plate; 150 × 200 mm) was set to 180 mm for constructing the poly(HPMA/DAMA)/ADH–PCL fibermats.

Scanning electron microscopy (SEM)

The samples were coated with amorphous osmium by plasma chemical vapor deposition using an OPC60A vacuum evaporator (Filgen, Inc., Japan). The sample morphologies were examined using field-emission SEM (JSM-7800F, JEOL, Japan) operating at 3-kV acceleration. The fibermat nanofibre mean diameters and corresponding standard deviations were evaluated using the SEM images of 30 nanofibres and Image J software.

Transmission electron microscopy (TEM)

The TEM samples were prepared by directly collecting the electrospun core–shell nanofibres from the co-axial spinneret on a TEM grid comprising a formvar carbon film on a 100-mesh copper wire grid (50) [Okenshoji Co., Ltd., Japan]. To obtain adequate image contrast between the core and shell, sodium phosphotungstate was mixed at a final concentration of 0.0001% (w/v) with the core precursor solution comprising

poly(HPMA/DAMA) and ADH. The TEM images were acquired using a JEM-1400Plus instrument (JEOL, Japan) operating at 100-kV acceleration.

Leakage experiment of the preloaded fluorescein or GINKO2 from the fluorescein or GINKO2-encapsulated core-shell fibermat to an immersing buffer or incubation medium

The loaded fibremats (1 mg) were cut in square pieces (5 × 5 mm; 1 mg) and placed in a 24-well plate and immersed in 1.0 mL of phosphate buffer (pH 7; 50 mM) (for fluorescein) or incubation medium (for GINKO2) for defined periods at 25 ˚C and gently orbitally shaken at 100 rpm. The fibremats were then removed from the buffer, and the F_{520} values of the remaining solutions were measured using a plate reader (Varioskan LUX, ThermoFischer Scientific Inc., U.S.A.). By comparison to the standard curve, the fluorophore release degree was determined during the assay period.

Fluorescence response of GINKO2 in fibermat or in solution

The GINKO2-encapsulated fibermat was cut into a round shape (6.4 mm in diameter) using a steel punch and placed at the bottom of a 96-well black plate. The fibermat was initially pre-immersed in 200 μL of 10 mM HEPES buffer (pH 7.4) and then alternately immersed in 200 μL of HEPES buffer (10 mM, pH 7.4), containing various concentrations of KCl (0 – 300 mM) for 10 min. The fluorescence emission at 520 nm (F_{520}) from the fibermat (excited at 485 nm) was measured using a fluorescence microplate reader (Varioskan LUX, ThermoFischer Scientific Inc., U.S.A.). For the time-course measurements of F_{520} for the GINKO2-encapsulated fibermat after immersion in a 150 mM KCl solution, fluorescence observations were performed every minute for the first 10 min following immersion in 200 μL of HEPES buffer (10 mM, pH 7.4), containing 150 mM KCl. Subsequently, the solution was replaced with HEPES buffer (10 mM, pH

7.4) without KCl, and the decrease in F_{520} was recorded every minute for the next 100 min. This process was repeated five times.

In a separate experiment involving trypsin treatment, the GINKO2-encapsulated fibermat in a 96-well plate was initially immersed in 100 μL of 10 mM HEPES buffer (pH 7.4) containing trypsin (0.5 mg/mL) for 2 hours at 37˚C. Then, successive additions of 100 μL of 10 mM HEPES buffer (pH 7.4), containing KCl (resulting in final concentrations of 150 mM), were made, and the time-course observation of the initial increase in F_{520} was conducted. The subsequent processes were performed similarly to the samples without trypsin treatment. To observe the changes in fluorescence emission of GINKO2 in solution by KCl, 1 μM of GINKO2 in 100 μL of 10 mM HEPES buffer (pH 7.4) was mixed with 100 μL of 10 mM HEPES buffer (pH 7.4), containing different concentration of KCl (0 – 300 mM). After 10 min of incubation, fluorescence emissions were measured using a fluorescence microplate reader (Varioskan LUX, ThermoFischer Scientific Inc., U.S.A.). For the time-course alterations of F_{520} of GINKO2 in solution with 150 mM KCl, fluorescence observations were performed every minute following successive additions of 100 μL of HEPES buffer (10 mM, pH 7.4), containing 300 mM KCl (resulting in a final concentration of 150 mM) to GINKO2 in 100 μL of HEPES buffer (10 mM, pH 7.4). Similarly, in the experiment with trypsin treatment, GINKO2 (1 μM) in 100 μL of 10 mM HEPES buffer (pH 7.4) was treated with trypsin (final concentration of 0.5 mg/mL) for 2 hours at 37˚C. Then, successive additions of 100 μL of 10 mM HEPES buffer, containing 300 mM KCl (resulting in a final concentration of 150 mM), were made, and the time-course observation of F_{520} was performed.

Fluorescence response of the GINKO2-encapsulated fibermat after chemical stimulation of HeLa and A549 cells

The GINKO2-encapsulated fibermat was cut into circular pieces (6.4 mm in diameter)

using a steel punch and placed at the bottom of a black 96-well plate. The circle fibermats set in the 96-well plate were sterilized by gas sterilization. The fibermats were washed in the imaging buffer (25 mM HEPES, pH 7.4; 1.8 mM $CaCl_2$; 0.8 mM $MgSO_4$; 25 mM D-glucose) thrice and subsequently, HeLa or A549 cells (1×10^5 cells/well) were seeded onto each well. After preculture, 20 μL of a valinomycin solution in DMSO was added to give a final concentration of 10 μM. Fluorescence intensity (λ_{ex} = 485 nm, λ_{em} = 520 nm) was recorded every minute for 30 min using a fluorescence microplate reader (Varioskan LUX, ThermoFischer Scientific Inc., U.S.A.). As a reference experiment, 20 μL of DMSO alone was added, and fluorescence was measured under the same conditions.

ICP analysis of the secreted K^+ ions from HeLa and A549 cells after valinomycin stimulation

HeLa or A549 cells (1×10^5 cells/well) were seeded to 96-well plate. After preculture, 20 μL of a valinomycin solution in DMSO was added to give a final concentration of 10 μM. Cell culture supernatants after 30 min valinomycin stimulation were collected and centrifuged to remove cellular debris. The potassium ion concentration in the supernatants was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES, ICPS-7510, Shimadzu Corp.). Samples were diluted to 1/20 with ultrapure water prior to measurement. Calibration curves (as shown in Fig. S4) were prepared using potassium standard solutions (0, 0.25, 0.5, 1.0, 2.5, 5, 10 ppm), and all measurements were performed in triplicate.

Evaluation of the Durability of GINKO2 Fluorescence Response to Thermal Treatment

The GINKO2-encapsulated fibermat was cut into circular pieces (6.4 mm in diameter) using a steel punch and suspended in 2 mL of 10 mM HEPES buffer (pH 7.4). The

samples were heated at 50, 60, 70, 80, 90 and 100 °C for 30 min using a dry aluminum block thermostatic bath (MG-2200, Tokyo Rikakikai Co., Ltd., Japan) and subsequently cooled on ice for 30 min. The fibermats were then placed at the bottom of a black 96-well plate and immersed in 200 μL of HEPES buffer (10 mM, pH 7.4) containing 300 mM KCl for 10 min. Fluorescence emission at 520 nm (F_{520}) was recorded upon excitation at 485 nm using a fluorescence microplate reader (Varioskan LUX, Thermo Fisher Scientific Inc., USA) and compared with the fluorescence intensity measured before thermal treatment. As a reference, a GINKO2 solution (1 μM in 2 mL of 10 mM HEPES buffer, pH 7.4) was subjected to the same heating (100 °C, 30 min) and cooling (ice bath, 30 min) procedure. After treatment, 100 μL of the solution was transferred to a black 96-well plate and mixed with an equal volume (100 μL) of HEPES buffer (10 mM, pH 7.4) containing 600 mM KCl, resulting in a final KCl concentration of 300 mM. Fluorescence emission at 520 nm (λ_{ex} = 485 nm) was then measured under identical conditions and compared with the fluorescence intensity prior to thermal treatment.

Evaluation of the Durability of GINKO2 Fluorescence Response to organic solvent exposure

The GINKO2-encapsulated fibermat was cut into circular pieces (6.4 mm in diameter) using a steel punch and suspended in 2 mL of a 1:1 (v/v) mixture of 10 mM HEPES buffer (pH 7.4) and either methanol or diethyl ether. The suspension was subjected to orbital shaking for 30 min. The fibermats were then transferred to the bottom of a black 96-well plate and immersed in 200 μL of HEPES buffer (10 mM, pH 7.4) containing 300 mM KCl for 10 min. Fluorescence emission at 520 nm (F_{520}) was recorded upon excitation at 485 nm using a fluorescence microplate reader (Varioskan LUX, Thermo Fisher Scientific Inc., USA) and compared with the fluorescence intensity measured before solvent exposure. As a reference, a GINKO2 solution (1 μM) was prepared in 2 mL of the same

1:1 (v/v) mixture of 10 mM HEPES buffer (pH 7.4) and either methanol or diethyl ether, and subjected to orbital shaking for 30 min. Subsequently, 100 μL of the solution was transferred to a black 96-well plate and mixed with 100 μL of 600 mM KCl, resulting in a final KCl concentration of 300 mM. After incubation for 10 min, fluorescence emission at 520 nm (λ_{ex} = 485 nm) was measured under identical conditions and compared with the fluorescence intensity prior to solvent exposure.

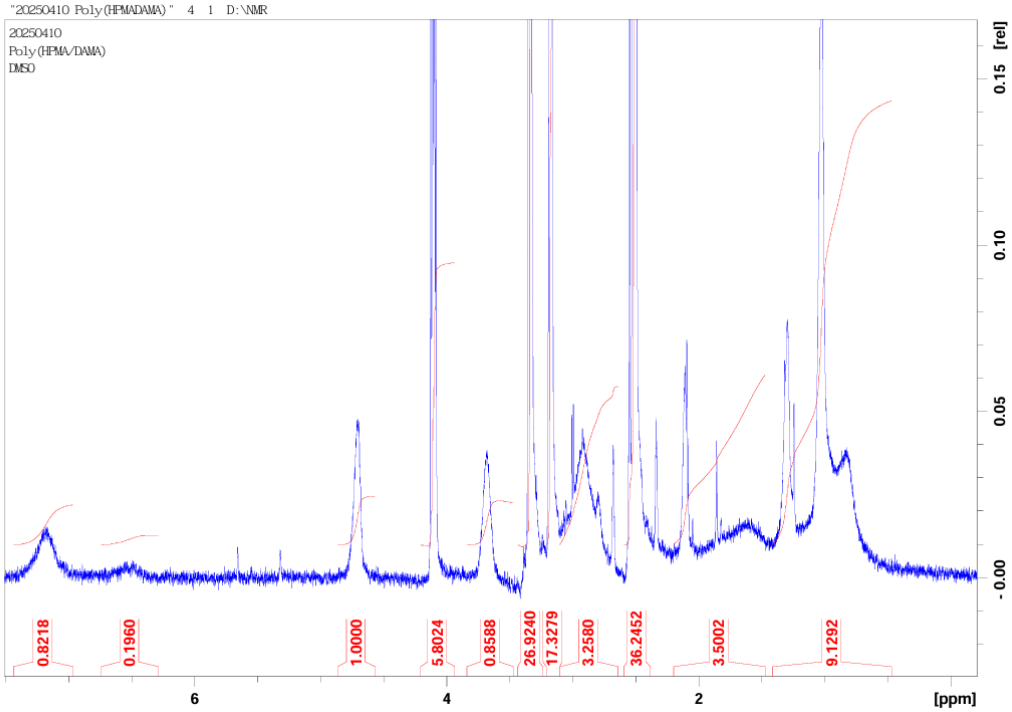

Fig. S1 1H-NMR spectrum of poly(HPMA/DAMA)

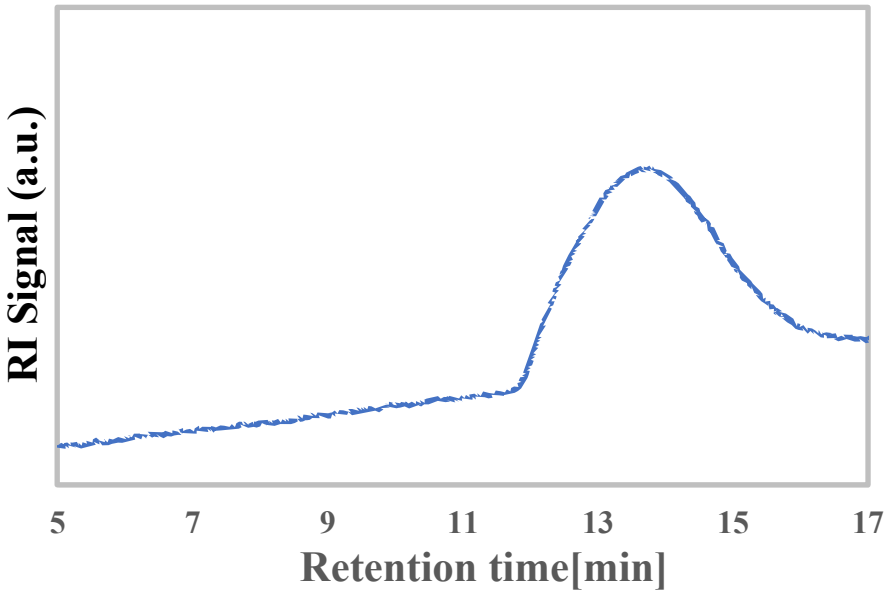

Fig. S2 GPC chromatogram of poly(HPMA/DAMA)

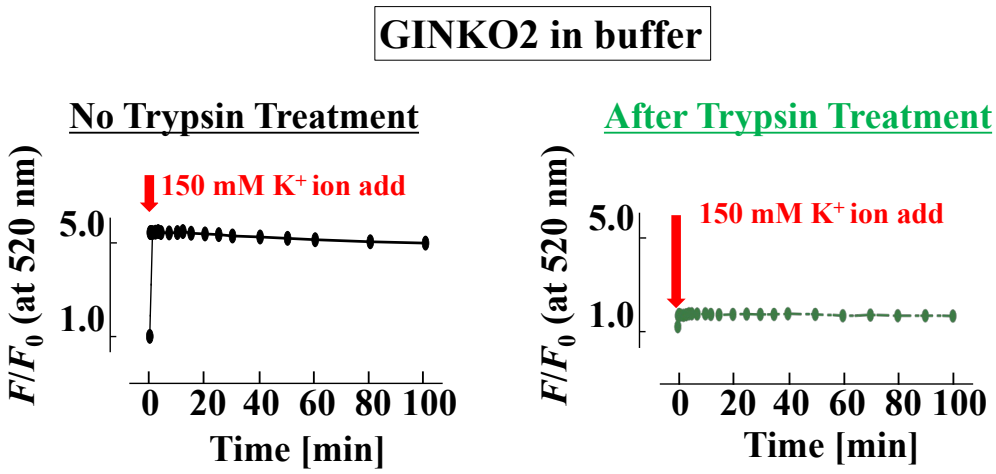

Fig. S3 Fluorescent responses of the GINKO2 with or without trypsin treatment in solution to KCl addition (150 mM). (e) Comparison of fluorescent responses of GINKO2, encapsulating in fibermat, to NH_4Cl (5mM), $ZnCl_2$ (5 μM), $CaCl_2$ (10 mM), NaCl (150 mM), $MgCl_2$ (10 mM), lactate (10 mM), urea (20 mM), and KCl (150 mM).

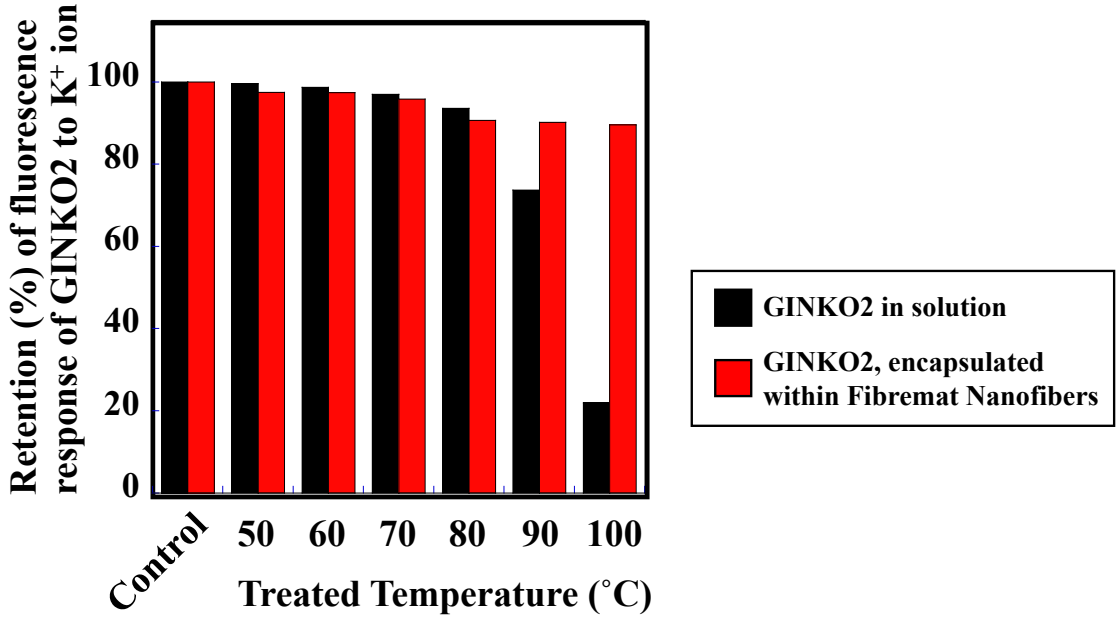

Fig. S4 Comparison of the retention (%) of the fluorescence response to K^+ ions for GINKO2 encapsulated within fibremat nanofibers (red bars) and GINKO2 in solution (black bars) after heat treatment at 50, 60, 70, 80, 90, and 100 °C for 30 min. The control corresponds to the fluorescence response measured before heat treatment at 25 °C.

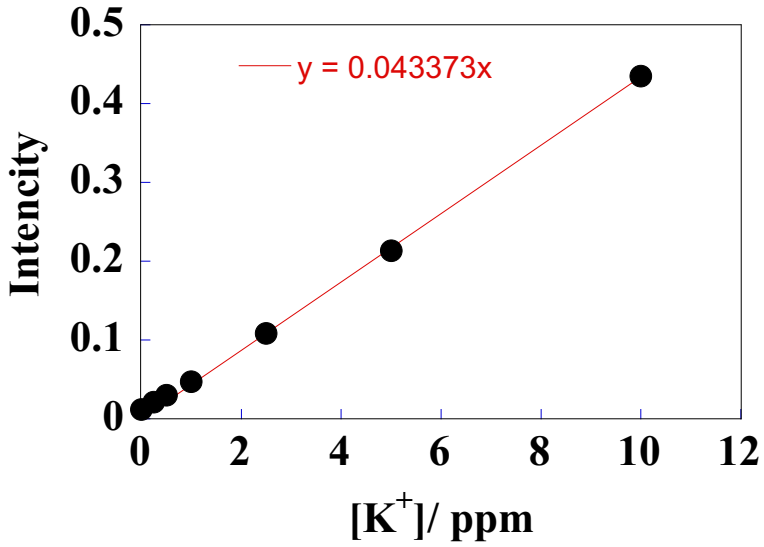

Fig. S5 Calibration curves for the potassium standard solutions (0, 0.25, 0.5, 1.0, 2.5, 5, 10 ppm).