In this study, a novel resorufin thionocarbonate-based $\text{Hg}^{2+}$-selective signaling probe (RT) for microfluidic paper-based analytical device ($\mu$PAD) applications is reported. The designed probe, RT, was readily synthesized by the one-step reaction of resorufin with phenyl thionochloroformate. The RT probe displayed a prominent color change from yellow to pink and a marked turn-on fluorescence signaling behavior exclusively toward the $\text{Hg}^{2+}$ ion. The signaling of RT was due to $\text{Hg}^{2+}$-induced hydrolysis of the phenyl thionocarbonate moiety to form the parent resorufin dye, which restored its spectroscopic properties. In addition, RT exhibited the $\text{Hg}^{2+}$-selective signaling behavior without interference by coexisting environmentally relevant metal ions. The detection limit for $\text{Hg}^{2+}$ in simulated wastewater samples was estimated to be $5.8 \times 10^{-8} \text{M}$. In particular, an RT-equipped $\mu$PAD prepared using a wax printing technique enabled simple and convenient determination of $\text{Hg}^{2+}$ ions in simulated wastewater samples, with a detection limit of $5.9 \times 10^{-6} \text{M}$.

Mercury, which is released into the environment as vapor or inorganic and organic mercurial species, has been reported to cause serious damage to human health\(^1\). It has harmful impacts on the respiratory system, causing chest pain, cough, dyspnea, and hemoptysis\(^2\), and damages the nervous system, leading to e.g., severe behavioral and personality changes, increased excitability, loss of memory, and insomnia\(^3\). Hence, many environmental and health-related departments, such as the United States Environmental Protection Agency (US EPA) and the World Health Organization (WHO), have designated mercury one of the most dangerous metal species\(^4\). Therefore, the development of a fast and convenient method for mercury determination has become an important issue in analytical and environmental sciences.

Several quantitative $\text{Hg}^{2+}$ determination methods based on analytical instrumental techniques, such as atomic absorption spectroscopy (AAS)\(^5\), X-ray fluorescence spectroscopy (XRF)\(^6\), differential pulse anodic stripping voltammetry (DPASV)\(^7\), and capillary electrophoresis (CE)\(^8\) have been reported. Nevertheless, due to their high costs and technical difficulties in operation, the development of selective and sensitive signaling methods employing simple colorimetric or fluorescence changes is highly desirable. In this context, many UV–vis and/or fluorescence signaling methods using reaction-based probes have been developed\(^9-14\).

In particular, a large number of elaborately designed probes containing sulfur-based moieties have been designed to exploit the highly thiophilic nature of mercury in the determination of $\text{Hg}^{2+}$ ions. Following the pioneering research of Czarnik's anthracene thioamide-based $\text{Hg}^{2+}$ signaling system\(^15\), many $\text{Hg}^{2+}$-selective signaling probes using $\text{Hg}^{2+}$-induced desulfurization of sulfur-based compounds, such as thiocarbonyl\(^16,17\), thioamide\(^18\), thioimide\(^19,20\), and thiourea derivatives\(^21-23\), have been reported. Furthermore, a number of probes exist which use the $\text{Hg}^{2+}$-induced desulfurization-based cyclization of thioureas\(^24-29\), and cleavage of thioacetal\(^30\), thioethers\(^31\), thiophosphinates\(^32\), and thionocarbonates\(^33\). Sulfur-based functional groups in many of these probes effectively suppress the fluorescence of signaling subunits, and desulfurization results in the restoration of the absorption and fluorescence properties of the parent dyes\(^33\).

Following the introduction of microfluidic paper-based analytical devices ($\mu$PADs)\(^34\), many paper-based kits for the determination of biologically and environmentally important materials have been reported. Paper-based devices manifest many features such as desirable mechanical properties (flexibility, stiffness, lightness, and
type fluorescence signaling behavior for the Hg²⁺-ated prominent, selective colorimetric and fluorescence signaling behavior toward Hg²⁺ of resorufin by thionocarbonate, the probe exhibited a yellow color and weak fluorescence emission in a 1:1 measurement, and high-resolution mass spectrometry. As expected, due to the protection of the phenolic moiety presence of Hg²⁺ for Ag⁺. Application of resin printing fabrication techniques such as drawing, stamping, cutting, and photolithography, a number of μPADs have been developed for the determination of important species including nitrates, Hg²⁺, Cu²⁺, and Fe³⁺. However, some of these methods suffer from high instrument costs and technical difficulties, and mass production is difficult in some cases. To overcome the drawbacks of μPAD fabrication methods, the wax printing fabrication technique has been developed, and successfully used for the preparation of μPADs for the determination of biologically and environmentally important species including Cu²⁺, Ni²⁺, Hg²⁺, Ca²⁺/Mg²⁺, and organophosphate pesticides.

Herein, we report a new resorufin thionocarbonate-based Hg²⁺-selective signaling probe and its application to μPADs. The RT probe demonstrated a prominent color change from yellow to pink as well as remarkable turn-on type fluorescence signaling behavior for the Hg²⁺ ion. We confirmed that the Hg²⁺ signaling of RT was due to Hg²⁺-induced hydrolysis of the thionocarbonate moiety to form the parent resorufin dye, using 1H NMR and mass spectrometry. Furthermore, use of an RT-equipped µPAD prepared using a wax printing technique enabled the simple and convenient determination of Hg²⁺ ions in simulated wastewater samples, with a detection limit of 5.9 × 10⁻⁶ M.

Results and Discussion
Preparation of RT. The designed probe, RT, was synthesized by simple reaction of resorufin, which acts as a reporting chromophore as well as fluorophore, with phenyl thionochloroformate (triethylamine, dichloromethane, yield = 77%) (Fig. 1). The chemical structure of RT was characterized by 1H NMR and 13C NMR measurements, and high-resolution mass spectrometry. As expected, due to the protection of the phenolic moiety of resorufin by thionocarbonate, the probe exhibited a yellow color and weak fluorescence emission in a 1:1 mixture of acetone and citrate buffer solution (pH 6.2, 20 mM) and acetone.

Hg²⁺-selective UV–vis and fluorescence signaling of RT. First, we investigated the UV–vis signaling behavior of RT in the presence of representative metal ions. In an acetone/citrate buffer solution (1:1, pH 6.2), RT exhibited a broad absorption band centered around 448 nm (Fig. 2). The treatment of RT with common metal ions did not result in any significant changes in the absorption profile, except in the case of Hg²⁺ ions. In the presence of Hg²⁺, RT showed a strong absorption band at 578 nm and a prominent color change from yellow to pink. Based on this observation, the Hg²⁺ signaling of RT was assessed using the absorbance changes at 578 nm (Fig. S1, Supplementary Information). Absorbance enhancements (A/A₀) at 578 nm clearly showed the prominent Hg²⁺-selective signaling behavior (A/A₀ at 578 nm = 320 for RT in the presence of Hg²⁺), while the response to other metal ions was negligible (A/A₀ varied between 0.1 for K⁺ and 1.4 for Ag⁺).

Next, the fluorescence signaling behavior of RT in the presence of environmentally relevant metal ions was measured (Fig. 3). The RT showed a weak fluorescence emission centered around 587 nm due to the protection of the resorufin moiety by the phenyl thionocarbonate functionality. However, the treatment of RT with Hg²⁺ ions resulted in a prominent fluorescence enhancement at 591 nm (I/I₀ at 591 nm = 101 for Hg²⁺). Other tested metal ions caused minor changes in fluorescence emission (i.e., I/I₀ at 591 nm varied between 0.92 for Na⁺ and 2.29 for Ag⁺) (Fig. S2, Supplementary Information). These observations imply that the designed probe, RT, exhibited prominent, selective colorimetric and fluorescence signaling behavior toward Hg²⁺ ions. However, as this...
research is aimed at the development of paper-based Hg\textsuperscript{2+} determination tools, we focused on the colorimetric signaling behavior, which is more convenient for application in paper-based devices for use in the field than fluorescence signaling.

**Determination of Hg\textsuperscript{2+} by the RT probe.** To detect Hg\textsuperscript{2+} ions in environmental and industrial samples, the selective signaling response to Hg\textsuperscript{2+} ions in the presence of relevant metal ions is a requisite. The Hg\textsuperscript{2+} signaling of RT was not affected by the presence of environmentally relevant metal ions (Fig. S3, Supplementary Information). The ratio of absorbance changes (A_{Metal\textsuperscript{2+}}/A_{Hg(II)}) at 578 nm only varied in a narrow range between 0.84 (Fe\textsuperscript{3+}) and 1.0 (Li\textsuperscript{+}). The pH-dependency of Hg\textsuperscript{2+} signaling by RT showed that signaling became more pronounced as the solution pH increased, up to pH 6.2, and was subsequently not influenced significantly by pH variation between pH 6.2 and 9.4 (Fig. S4, Supplementary Information). Therefore, signaling experiments were performed in citrate buffer solutions at pH 6.2, where the most prominent Hg\textsuperscript{2+} signaling contrast was observed. The Hg\textsuperscript{2+} signaling of RT was fast and completed within 5 min. From the signaling time course, we estimated that the rate constant of Hg\textsuperscript{2+} signaling under pseudo-first order conditions was 0.874 min\textsuperscript{-1} (Figs S5 and S6, Supplementary Information).

The Hg\textsuperscript{2+} signaling of RT was due to the Hg\textsuperscript{2+}-induced cleavage reaction of thionocarbonate moiety of RT to yield the parent resorufin dye and non-fluorescent phenol as a side product (Fig. 4). The postulated Hg\textsuperscript{2+} signaling process of RT was confirmed by \textsuperscript{1}H NMR and mass measurements. As shown in Fig. 5, we found that the \textsuperscript{1}H NMR spectrum of the signaling product (RT + Hg\textsuperscript{2+}) was nearly identical to that of the postulated signaling product resorufin. Furthermore, the mass spectrum of the signaling product of RT with the Hg\textsuperscript{2+} ion revealed a diagnostic peak at m/z = 213, which is consistent with the proposed signaling product, resorufin (calcd. for [C\textsubscript{37}H\textsubscript{29}N\textsubscript{6}O\textsubscript{3}]\textsuperscript{+}, m/z = 213.0) (Fig. S7, Supplementary Information). We also characterized the signaling side product, phenol, by \textsuperscript{1}H NMR and \textsuperscript{13}C NMR measurements (Figs S8 and S9, Supplementary Information).

Next, to estimate the detection limit of RT for the Hg\textsuperscript{2+} ion, the Hg\textsuperscript{2+} concentration-dependent UV–vis absorbance change was measured. As shown in Fig. 6, the absorbance at 578 nm increased linearly with the increase of Hg\textsuperscript{2+}, up to 1.0 \times 10\textsuperscript{-5} M. From this measurement, the detection limit of RT for Hg\textsuperscript{2+} ion was calculated to be 6.3 \times 10\textsuperscript{-8} M, according to the IUPAC guideline (3s\textsuperscript{0}/m\textsuperscript{53}). In addition, because the determination of mercury pollution is one of the most critical challenges in environmental and industrial fields, we attempted to determine Hg\textsuperscript{2+} in simulated wastewater samples\textsuperscript{24}. The titration of RT with Hg\textsuperscript{2+} showed a satisfactory calibration plot up to 1.0 \times 10\textsuperscript{-8} M. From these concentration-dependent experiments, the detection limit for Hg\textsuperscript{2+} in simulated wastewater was estimated to be 5.8 \times 10\textsuperscript{-8} M (Fig. S10, Supplementary Information).

**Development of the RT-based \muPAD.** Recently, due to their convenience and cost-effectiveness, many probes constructed on \muPADs have been developed for the determination of a variety of biological and environmental species\textsuperscript{36–37}. Herein, we utilized the newly developed Hg\textsuperscript{2+} probe as a \muPAD-based colorimetric signaling tool to increase its convenience in practical applications by eliminating the need for complicated instruments operated by well-trained professionals\textsuperscript{41}. The designed \muPAD tool was easily prepared by wax printing, based on a diagnostic peak at m/z = 213, which is consistent with the proposed signaling product, resorufin (calcd. for [C\textsubscript{37}H\textsubscript{29}N\textsubscript{6}O\textsubscript{3}]\textsuperscript{+}, m/z = 213.0) (Fig. S7, Supplementary Information). We also characterized the signaling side product, phenol, by \textsuperscript{1}H NMR and \textsuperscript{13}C NMR measurements (Figs S8 and S9, Supplementary Information).

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on the design shown in Fig. 7. First, using the μPAD kit, we investigated the effect of pH on the Hg^{2+} signaling behavior in various buffer solutions. As shown in Fig. S11 (Supplementary Information), the paper kit exhibited the most prominent color change in response to Hg^{2+} ions in tris-HCl buffer solution at pH 8.0. Next, the colorimetric signaling behavior of the μPAD kit in the presence of representative metal ions was studied. An RT-equipped μPAD kit is light-yellow in color, and no significant color change was observed in the presence of common metal ions except in the case of Hg^{2+} ions (Fig. 8(a)). Upon treatment with Hg^{2+} ions, the μPAD kit underwent a pronounced color change from yellow to pink. Subsequently, we investigated the Hg^{2+} signaling behavior of the paper kit by RGB color analysis. As shown in Fig. 8(b), the ΔL_{RG} value, assessed by the difference between the red and green channel levels (L_{red} − L_{green}), clearly revealed the Hg^{2+}-selective signaling behavior. For instance, the control ΔL_{RG} value of the μPAD kit was 11.83 for distilled water, whereas the value increased to 85.47 on exposure to Hg^{2+} ions. In the presence of thiophilic Ag^{+}, noticeable interference was observed due to the reactivity of the thionocarbonate moiety of probe RT with Ag^{+} ions. However, interference from Ag^{+} was dramatically suppressed by use of a tris-HCl buffer as an Ag^{+} masking agent, due to the formation of an insoluble AgCl precipitate (K_{sp} = 1.8 × 10^{-10}) by interaction of Ag^{+} with chloride ions in the tris-HCl buffer solution (ΔL_{RG} = 21.35 for Ag^{+}). However, considerable interference was still observed, as the residual AgCl in the measurement solutions released a small amount of Ag^{+} which induced a considerable colorimetric response in the μPAD. We believed that Ag^{+} ions released from AgCl subsequently react with RT to form much more insoluble Ag_{2}S (K_{sp} of Ag_{2}S = 8.0 × 10^{-51}) which induced a colorimetric response in the RT-equipped μPAD. Thus, we removed the residual AgCl precipitate using a syringe filter and found that the interference from Ag^{+} decreased significantly (ΔL_{RG} = 13.12 for Ag^{+}, filtered) (Fig. S12, Supplementary Information). Furthermore, we confirmed that the suppression of Ag^{+} interference on the RT-equipped μPAD by syringe filtration was maintained for at least 30 min during the signaling experiment (Fig. S13, Supplementary Information). We also confirmed that the Hg^{2+} signaling by the RT-equipped μPAD kit was not affected by the filtration process (Fig. S14, Supplementary Information). Other metal ions showed insignificant changes (ΔL_{RG} values ranged from 10.99 for Li^{+} to 17.55 for...
Finally, the Hg\(^{2+}\) signaling by the \(\mu\)PAD was evaluated quantitatively, by plotting \(\Delta L_{RG}\) values as a function of [Hg\(^{2+}\)] (Figs S15 and S16, Supplementary Information). The \(\Delta L_{RG}\) value steadily increased with an increase in Hg\(^{2+}\) concentration, exhibiting a linear relationship up to \(2.0 \times 10^{-4}\) M Hg\(^{2+}\) ions. In fact, it might be possible to obtain a distorted Hg\(^{2+}\) titration plot in the presence of a low concentration of Hg\(^{2+}\) as the Hg\(^{2+}\) interacts first with the bottom part of the spotted probe. However, a satisfactory calibration curve with a good linear relationship \((R^2 = 0.9945)\) for Hg\(^{2+}\) in the concentration range between 0 and 50 \(\mu\)M was obtained (Fig. S16, Supplementary Information). That might be because the time required for the expression of a stable Hg\(^{2+}\)-induced response from the probe was approximately 5 min (Fig. S5, Supplementary Information), while that for the full elution of the analyte up to the top of the \(\mu\)PAD reservoir was approximately 1 min. Thus Hg\(^{2+}\) ions are eluted quite evenly over the entire spotted probe area before the chromogenic signaling occurs. From the Hg\(^{2+}\)-concentration-dependent signaling profile, the detection limit for Hg\(^{2+}\) ions by the paper kit was estimated as \(4.5 \times 10^{-6}\) M, according to IUPAC guidelines (Fig. S16, Supplementary Information). Based on these results, to demonstrate practical application, the determination of Hg\(^{2+}\) in a simulated wastewater sample was conducted using an RT-equipped \(\mu\)PAD kit, and the detection limit for Hg\(^{2+}\) in simulated wastewater was calculated to be \(5.9 \times 10^{-6}\) M (Fig. S17, Supplementary Information). In fact, the detection limit of RT-equipped \(\mu\)PAD for the Hg\(^{2+}\) ion is not superior to those of previously reported \(\mu\)PAD systems\(^{40,47}\). However, the RT-equipped \(\mu\)PAD is more easily prepared and can determine the mercury ions by a simple elution procedure compared with previously reported \(\mu\)PAD systems, which required extra treatment of the analytes.

**Conclusion**

We have developed the new resorufin thionocarbonate-based Hg\(^{2+}\) signaling probe RT which operates via the selective hydrolysis of thionocarbonate with Hg\(^{2+}\) ions. RT showed a significant color change from yellow to pink as well as prominent fluorogenic signaling behavior exclusively toward Hg\(^{2+}\) ions. Selective Hg\(^{2+}\)-signaling by RT was unaffected by the presence of common metal ions as a background, and the detection limit of Hg\(^{2+}\) in a simulated wastewater sample was calculated to be \(5.8 \times 10^{-8}\) M. From the \(^1\)H NMR, \(^{13}\)C NMR, and mass spectral measurements, we confirmed that the Hg\(^{2+}\) signaling of RT was due to Hg\(^{2+}\)-induced hydrolysis of the phenyl thionocarbonate moiety of RT into its parent resorufin dye and phenol. To demonstrate the practical application of the probe, the determination of Hg\(^{2+}\) ion using an RT-equipped \(\mu\)PAD was performed. With the RT-equipped \(\mu\)PAD, the convenient determination of Hg\(^{2+}\) ions in simulated wastewater was realized with a detection limit of \(5.9 \times 10^{-6}\) M, using a readily available office scanner as an image capturing tool.
Methods

General. Resorufin, phenyl thionochloroformate, and triethylamine were purchased from Aldrich Chemical Co. All solvents were of spectroscopic grade and obtained from Aldrich Chemical Co. 1H NMR and 13C NMR spectra were acquired on a Varian VNS NMR spectrometer (1H NMR: 600 MHz, 13C NMR 150 MHz). UV–vis and fluorescence spectra were measured using a Scinco S-3100 spectrophotometer and a Scinco FS-2 fluorescence spectrophotometer, respectively. High-resolution mass spectrometry (HRMS) results were obtained on a JEOL JMS-700 mass spectrometer using fast atom bombardment (FAB) ionization. Column chromatography was performed using silica gel (Merck, 240 mesh).

Preparation of Hg$^{2+}$ signaling probe, RT. Resorufin (0.21 g, 1.0 mmol) was dissolved in dichloromethane (30 mL), and triethylamine (0.27 mL, 2.0 mmol) was added to the solution. After 30 min of stirring, phenyl thionochloroformate (0.21 mL, 1.5 mmol) was added dropwise, and the reaction mixture was stirred at room temperature for 12 h. The reaction mixture was washed with distilled water and brine several times. The solution was evaporated under reduced pressure and the remaining residue was purified by column chromatography (silica gel, eluent: dichloromethane) to yield RT (0.27 g, 77%) as a scarlet powder. 1H NMR (600 MHz, CDCl3): δ 7.87 (d, $J$ = 8.4 Hz, 1 H), 7.48 (dd, $J$ = 8.6, 7.3 Hz, 2 H), 7.44 (d, $J$ = 9.8 Hz, 1 H), 7.35 (t, $J$ = 7.4 Hz, 1 H), 7.27–7.20 (m, 4 H), 6.87 (dd, $J$ = 9.8, 2.0 Hz, 1 H), 6.34 (d, $J$ = 2.0 Hz, 1 H); 13C NMR (150 MHz, CDCl3): δ 193.5, 186.2, 155.4, 153.4, 149.1, 148.9, 144.4, 135.4, 134.8, 131.9, 131.4, 129.8, 127.1, 121.6, 119.7, 110.5, 107.4; HRMS (FAB$^+$, m/z): calcd for C19H12NO4S $[M+H]^+$: 350.0482, found 350.0483.

Preparation of stock solutions. A stock solution of RT (5.0 × 10$^{-4}$ M) was prepared in spectroscopic grade acetonitrile. Stock solutions of the metal ions (10 mM) were prepared by dissolving the metal perchlorate salts in distilled water. Caution: metal perchlorates are highly explosive, thus should be handled carefully and used in small quantities. The simulated wastewater was prepared by following the reported literature procedure$^{54}$. Composition of the simulated wastewater: [NaClO$_4$] = 2.39 mM, [NaHCO$_3$] = 1.23 mM, [NaCl] = 987 μM, [NaNO$_3$] = 484 μM, [Mg(ClO$_4$)$_2$] = 288 μM, [KClO$_4$] = 281 μM, [Ca(ClO$_4$)$_2$] = 250 μM, [Na$_2$SO$_4$] = 208 μM, [Na$_2$HPO$_4$] = 105 μM, [NaF] = 161 μM.

Evidence of the Hg$^{2+}$ signaling process of RT. A solution of RT (0.035 g, 0.01 mmol) in 10 mL of methanol was slowly added to mercury perchlorate (0.12 g, 0.03 mmol) in methanol. The signaling progress
monitored by TLC. Due to the low solubility of resorufin in methanol, a simple filtration was sufficient for the separation of the primary signaling product resorufin and side product phenol in the filtrate. 1H NMR and mass spectral measurements of the solid product (resorufin) were performed after column chromatography. The 1H NMR and 13C NMR of phenol were obtained after the evaporation of the filtrate.

**Determination of Hg²⁺ using the μPAD.** The μPAD was designed using Microsoft Office Power Point. The μPAD was printed on Whatman® cellulose chromatography paper using a commercial wax printer (Fuji Xerox, ColorQube 8570). After printing, the paper was baked in a 150 °C oven for 90 s and cut into separate μPADs. The RT was dissolved in dichloromethane (8.0 mM) and loaded onto the middle of the circle in the prepared μPAD using a capillary tube (Aldrich, Z114952) (applied volume = 1.6 μL). Varying concentrations of Hg²⁺ in simulated wastewater sample were added to the RT-equipped μPAD kit, which resulted in a color change from yellow to pink. The analyte solution was prepared by adding 1% (v/v) of tris buffer solution (pH 8.0, 1.0 M) to the wastewater stock solution. Synthetic wastewater samples with varying concentrations of Hg²⁺ were prepared, and each sample was added to a separate vial. The bottom of an RT-equipped μPAD kit was placed in each vial and the analyte solution was eluted to the top of the μPAD. The μPAD was removed and dried under ambient conditions for 10 min. The results were recorded using a flatbed scanner (Epson, Perfection V550 Photo Color Scanner), and the RT spot on the μPAD, encircling 4.0 mm from the center, was analyzed using the Photoshop program (Adobe, Photoshop CS6). We chose to analyze a subsection of the spot due to the change in the size of the spot to approximately 4.2–4.3 mm in diameter, caused by broadening of the wax barrier during the baking step, and to the absence of a clear boundary line between the RT spot and the wax barrier of the μPAD.

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Author Contributions
S.K.C. and M.G.C. designed the experiment and wrote the manuscript. M.G.C., S.Y.P. and K.Y.P. carried out all the experiments. All the authors analyzed the results and reviewed the manuscript.

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