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Highly sensitive detection of E2 activity in ubiquitination using an artificial RING finger

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The ubiquitin-conjugating (E2) enzymes of protein ubiquitination are associated with various diseases such as leukemia, lung cancer, and breast cancer. Rapid and accurate detection of E2 enzymatic activities remains poor. Here, we described the detection of E2 activity on a signal accumulation ISFET biosensor (AMIS sensor) using an artificial RING finger (ARF). The use of ARF enables the simplified detection of E2 activity without a substrate. The high-sensitivity quantitative detection of E2 activities was demonstrated via real-time monitoring over a response range of femtomolar to micromolar concentrations. Furthermore, the monitoring of E2 activities was successfully achieved using human acute promyelocytic leukemia cells following treatment with the anticancer drug bortezomib, which allowed the assessment of the pathological conditions. This strategy is extremely simple and convenient, and the present detection could be widely applied to specific E2s for various types of cancers. Copyright © 2017 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: E2 activity; cancer; artificial RING finger; biosensor; diagnosis; ubiquitination

Introduction

Protein ubiquitination is a post-translational and enzymatic cascade reaction consisting of ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes [1,2]. Most RING fingers function as E3 enzymes and transfer activated ubiquitin (Ub) from E2 enzymes to the substrate Lys (Figure 1 A) [3–5]. This ubiquitination induces diverse effects such as protein degradation, protein interaction, and subcellular localization on the substrate [1,2]. In addition, the enzymatic activities of E2 and E3 enzymes are associated with various diseases such as leukemia, lung cancer, and breast cancer [6–9]. In leukemia, the fusion proteins AML1-ETO [acute myeloid leukemia (*RUNX1*)-eight twenty-one (*MTG8*)] and PML-RARα (promyelocytic leukemia-retinoic acid receptor α) contribute to leukemogenesis [10]. Increasing production of the fusion proteins in cells promotes their degradation by the ubiquitination system. The fusion proteins are degraded by the E2 enzyme and the E3 enzyme (SIAH1) possessing the RING finger [11,12]. Thus, assessing E2 activity during the ubiquitination reaction leads to the monitoring of tumor development for diagnostic and prognostic purposes. There is growing interest in the highly sensitive detection of E2 activities for capturing the pathological conditions of cancers.

Previously, we reported the 'α-helical region substitution' method for creating the artificial RING finger (ARF) that enables the detection of Ub-transferring activity from E2-Ub conjugates (Figure 1B) [13–15]. The ARF reaction provides the simplified detection of E2 activities in the ubiquitination. ARF is ubiquitinated upon itself without using a substrate. Here, ARF was engineered on the basis of the SIAH1 RING finger that mediates PML-RARα degradation using the method of α-helical region substitution (Figure 2) [10,13].

It was recently reported that a signal accumulation ISFET biosensor (AMIS sensor) allows the detection of proton changes in an ultra-low volume of 20 μl [16]. This technique is based on polarization in a semiconductor, in which the proton change caused by the reaction affects the flowing current of gate channels. The system does not require labeling of fluorescent dye molecule as compared with bioluminescence resonance energy transfer method.

In this study, in the reaction with ARF, we present, for the first time, the detection of E2 activities as the altering of the electron polarization in the sensing layer of the AMIS sensor. The present method demonstrates the high-sensitivity quantitative analysis of E2 activities, which leads to the rapid and accurate monitoring of the pathological conditions of acute promyelocytic leukemia (APL)-derived NB4 cells. This study opens the new diagnostic technique of E2 activities in the ubiquitination.

Materials and Methods

Peptide Synthesis

ARF was designed using the method of α-helical region substitution, and it was synthesized by the standard F moc solid-phase

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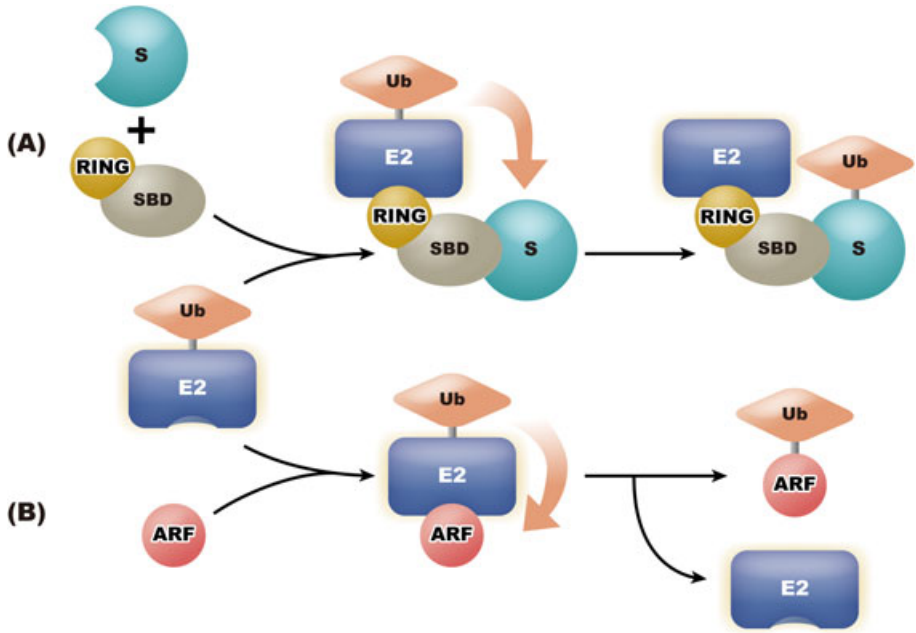

Figure 1. Schematic representation of the ubiquitination reaction. (A) Ubiquitin (Ub) is transferred from E2 to a substrate (S) that binds to a substrate-binding domain (SBD). RING does not bind to Ub, and it indirectly catalyzes the ubiquitination of the substrate. (B) An artificial RING finger (ARF) created by α-helical region substitution directly promotes the Ub transfer, and it is ubiquitinated upon itself. [Colour figure can be viewed at wileyonlinelibrary.com]

method (Figure 2). SynProPep products were purchased from Shimadzu Corp. (Kyoto, Japan) for peptide assembly including amide resin. After cleavage with trifluoroacetic acid, the peptide was purified using reverse-phase HPLC with Shim-pack C18 column equipment (Shimadzu Corp.). The purity of the peptides was >98%, and the molecular mass was analyzed by MALDI-TOF MS on a Shimadzu AXIMA-TOF2. The peptide was dissolved in 1 ml of 8 M guanidine–HCl, and subsequently, it was dialyzed against degassed Solution A (20 mM Tris–HCl [pH 6.9], 50 mM NaCl, 1 mM dithiothreitol, and 50-μM $ZnCl_2$) overnight at 4 °C using a Slide-A-Lyzer dialysis cassette (Thermo Scientific, Rockford, IL, USA). For the ARF peptide, the zinc : ARF stoichiometry (2 : 1) was confirmed as described previously [13].

Detection of AMIS Signals in Substrate-independent Ubiquitination

The ubiquitination solution consisted of 1.5 mM Tris–HCl (pH 6.9), 375-μM Mg-ATP, 75-μM dithiothreitol (DTT), 1.5 U/ml inorganic pyrophosphatase (Sigma, St. Louis, MO, USA), and 4.0-μM $ZnCl_2$, containing 3.0-μM Ub, 9.0-nM human recombinant E1, and human recombinant E2 (UbcH6 or UbcH8). The solutions (16 μl) were subjected to both Chamber A and B of a two-chambered device of the AMIS sensor on an AMIS-101 Micro Bioactive Analyzer (Bio-X Inc., Kyoto, Japan) [16]. The solutions were allowed to stand for 10 min for the formation of Ub thioester-linked E2 conjugates. Subsequently, 4-μl volumes of Solution A and ARF (50 μM) were added into Chamber A and B, respectively. The mixtures (20 μl) in the chambered devices were gently mixed. The final concentrations of Tris–HCl, Mg-ATP, DTT, inorganic pyrophosphatase, Ub, E1, and ARF were 1.2 mM, 300 μM, 60 μM, 1.2 U/ml, 2.4 μM, 7.2 nM, and 10 μM, respectively. The AMIS data for the Ub transfer onto ARF were obtained by subtracting the AMIS signals of Chamber A from those of Chamber B. The AMIS signals at 37 °C were recorded for 600 s at 5-s intervals.

WSTF PHD finger:
ARCKVCRKKGEDDKLILCDECNKAFHLFCLRPALYEVPDGEWQCPACQPAT

The method of α-Helical Region Substitution

Artificial RING finger (ARF):
ARCKVCRKKGEDDKLILCDECNKAFHLFCLPKLTC CPACQPAT

Figure 2. Strategy for the creation of the artificial RING finger. ARF was designed as a chimeric finger between the WSTF PHD finger and the SIAH1 RING finger. ARF was engineered using the method of α-helical region substitution (underlined). RPALYEVPDGEWQ of the WSTF PHD finger was removed. Instead, PKLTC (α-helical region) of SIAH1 RING finger was inserted.

Cell culture

The NB4 human APL cell line and the proteasome inhibitor bortezomib were kindly provided by Dr. Mariko Takenokuchi (Himeji Dokkyo University, Japan). Bortezomib was dissolved in DMSO to prepare a 1.0 mM stock solution and stored at −20 °C. NB4 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. NB4 cells were used for experiments after 3 h of growth in FBS-free RPMI-1640 medium. NB4 cells (1×10^5 cells/ml) were treated with 0, 10, and 100-nM bortezomib for 48 h at 37 °C. Viable cell counts were estimated by trypan blue exclusion. After centrifugation at 300×*g* for 5 min, the supernatant of NB4 cell culture was collected for the ubiquitination experiments (Supernatant A).

Detection of E2 Activity in NB4 Cells

For western blot analysis, 5 μl of 200 mM Tris–HCl (pH 7.5) and 1 μl of 50 mM DTT were added into a 24-μl volume of Supernatant A. The ubiquitination experiments were performed by

Journal of PeptideScience

adding 20 μl of ARF (25 μM). The mixtures were thoroughly mixed and then incubated at 37 °C for 60 min. The reactions were stopped by the addition of sodium dodecyl sulfate (SDS) sample buffer. The obtained solutions were subjected to Tris–tricine SDS-PAGE (15%) and then transferred to a polyvinylidene difluoride membrane. To detect ARF biotinylated at the N-terminus, the membrane was reacted with streptavidin–horseradish peroxidase solution (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA) and then developed by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK) using western blotting detection reagent. The emitted signals were captured using a Luminescent Image Analyzer (LAS-3000; Fujifilm, Tokyo, Japan).

Next, for the AMIS experiments, Supernatant A was diluted fivefold with water, and then the diluted samples (16 μl) were subjected to a two-chambered device of the AMIS sensor [16]. Detection of the AMIS signals in the ubiquitination reactions at 37 °C was conducted as described above.

Results and Discussion

Detection of E2 Activity Using the AMIS Sensor

We assessed the real-time detection of E2 activities using ARF on a Micro Bioactive Analyzer (AMIS-101) equipped with an AMIS sensor (Figure 3a). In the ubiquitination, ARF is ubiquitinated by specifically cooperating with the E2 enzymes UbcH5, UbcH6, and UbcH8 but not UbcH1, UbcH2, UbcH3, UbcH7, UbcH10, or UbcH13/Mms2 [13]. Addition of ARF in the AMIS sensor prompted the Ub transfer from the E2 conjugates, which led to the accumulation of Ub–ARF conjugates [13]. The aminolytic cleavage of the E2-Ub thiol ester yields the free thiol group (−SH) of the active-site cysteine of E2s [17]. Accordingly, the ARF reaction induces the free protons by the Ub-transferring reaction. The proton changes in the ARF reaction were observed as the altering of the electron polarization in the sensing layer on the AMIS-101, and thus, E2 activity was detected as the AMIS signal. The increase of free protons derived from the ubiquitination reaction allows the activity of E2 UbcH8 to be monitored in real time (Figure 3b). After the peak of the injection shock, the AMIS signal could be detected based on the ARF reaction from approximately 150 s. Subsequently, the AMIS signal reached a plateau at approximately 300 s, and then it was extremely stable until 600 s. The intensity (volts) of the AMIS signal increased proportionately with the logarithm of concentration of UbcH8, and removal of the indispensable Ub for the ubiquitination system resulted in the abolishment of the AMIS signal. The AMIS signal displayed concentration-dependent behavior and a linear relationship between its intensity (volts) and the logarithm of concentration of UbcH8 over the range of 140.0 fM–1.4 μM with a detection limit of 50.4 fg (Figure 3c). Moreover, concerning UbcH6, the detection limit of E2 activity was 30.0 fg, and the linear range was 70.0 fM–1.4 μM (Figure 3c). The slopes of the linear plots for the standard curve were 0.044 and 0.018 for UbcH8 and UbcH6, respectively. These results were in agreement with those evaluated by the western blot analyses presented previously [13]. Taken together, these findings demonstrated that the E2 activities for Ub transfer from the E2 enzymes to ARF are intimately interrelated with the polarization behavior of the electron as the AMIS signal. Thus, the E2 activities in the ubiquitination reaction were detected quantitatively via real-time monitoring in a matter of minutes.

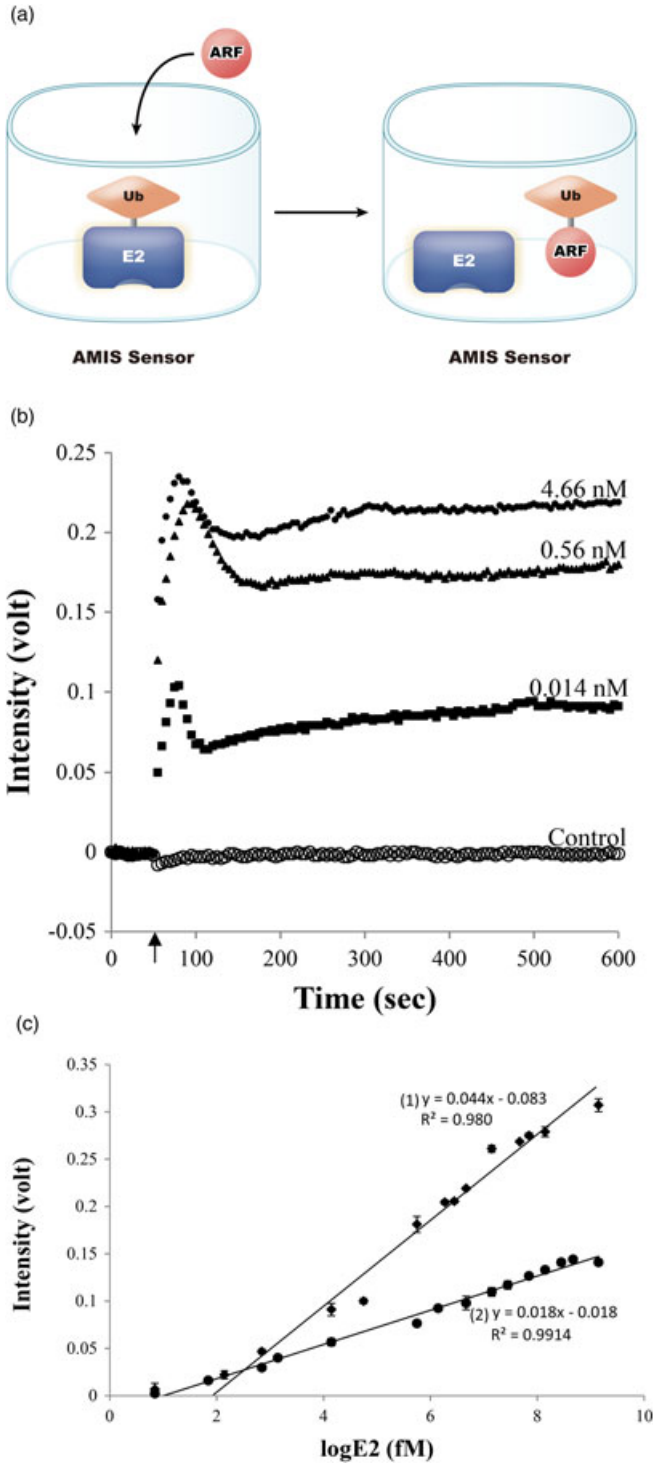

Figure 3. Real-time detection of E2 activity using the AMIS sensor. (a) The artificial RING finger (ARF) was subjected to the chambered device of the AMIS sensor on a Micro Bioactive Analyzer (AMIS-101). The ubiquitin (Ub) transfers from the E2 conjugate onto the ARF. (b) The representative AMIS signals of 0.014 (■), 0.56 (▲), and 4.66 (●) nM UbcH8 were collected at 5-s intervals (37 °C), and the AMIS measurements in the absence of Ub were used as the control (○). The arrow shows the injection time of the ARF. (c) The calibration curves of (1) UbcH8 and (2) UbcH6 for the intensity of the AMIS signal (y) and the concentration (x). The intensities of the AMIS signals were collected at the plateau stage (600 s). The data are presented as the mean ± SD of the results of five independent experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

Ubiquitination of ARF in the Culture Supernatant

Bortezomib is a specific inhibitor of the Ub proteasomal system that induces cell death in various cancers such as leukemia, multiple myeloma, and breast cancers [18–20]. Treatment with bortezomib reduced NB4 cell viability (Figure S1, see Supporting Information) [[12]], whereby the Ub thioester-linked E2 conjugates escaped from cells into the culture supernatant [12]. In this experiment, we tested the ARF reaction in the culture supernatant of NB4 cells by Western blotting (Figure 4a). ARF specifically cooperated with the diapedesis of the Ub thioester-linked E2. The application of ARF allowed the detection of E2 activities in crude extracts such as the culture supernatant of cells. However, E2 activities following treatment with 10 and 100-nM bortezomib could not be distinguished from each other. These E2 activities will be examined subsequently using the AMIS detection.

Monitoring of E2 Activity in the Culture Supernatant Using the AMIS Sensor

Next, using the AMIS sensor, we tested the real-time detection of E2 activities in the culture supernatant of NB4 cells following treatment with bortezomib. ARF was added to the culture supernatant, and then the AMIS signals for the activities of bortezomib-evoked E2 emigration were detected on the AMIS-101 (Figure 4b). The reaction time of ARF was longer than that of the reaction system for the recombinant E2 indicated in Figure 3b. This is due to the condition of the crude system extracted from NB4 cells. After the shock peak of the injection, the AMIS signals of the ARF reaction were observed from approximately 150 s and then plateaued at approximately 500 s. The average intensities (V) of the AMIS signals at 600 s following treatment with 0, 10, and 100 nM bortezomib were 0.021, 0.065, and 0.089 V, respectively. These findings from the AMIS signals are summarized in Figure 4c.

We previously confirmed that ubiquitinated E2 is associated with the pathogenesis of leukemia in NB4 cells [12]. Bortezomib treatment elevates the diapedesis of the Ub–UbcH8 conjugate from cells, but not the UbcH6 conjugate [12]. Accordingly, the intensities of the AMIS signals were defined as (α), (α + β), and (α + γ) according to treatment with 0, 10, and 100-nM bortezomib, respectively, as shown in Figure 4c. The intensity α = 0.021 V indicates the total level of E2 activity in NB4 cells in the absence of bortezomib, whereas the intensities β = 0.044 V

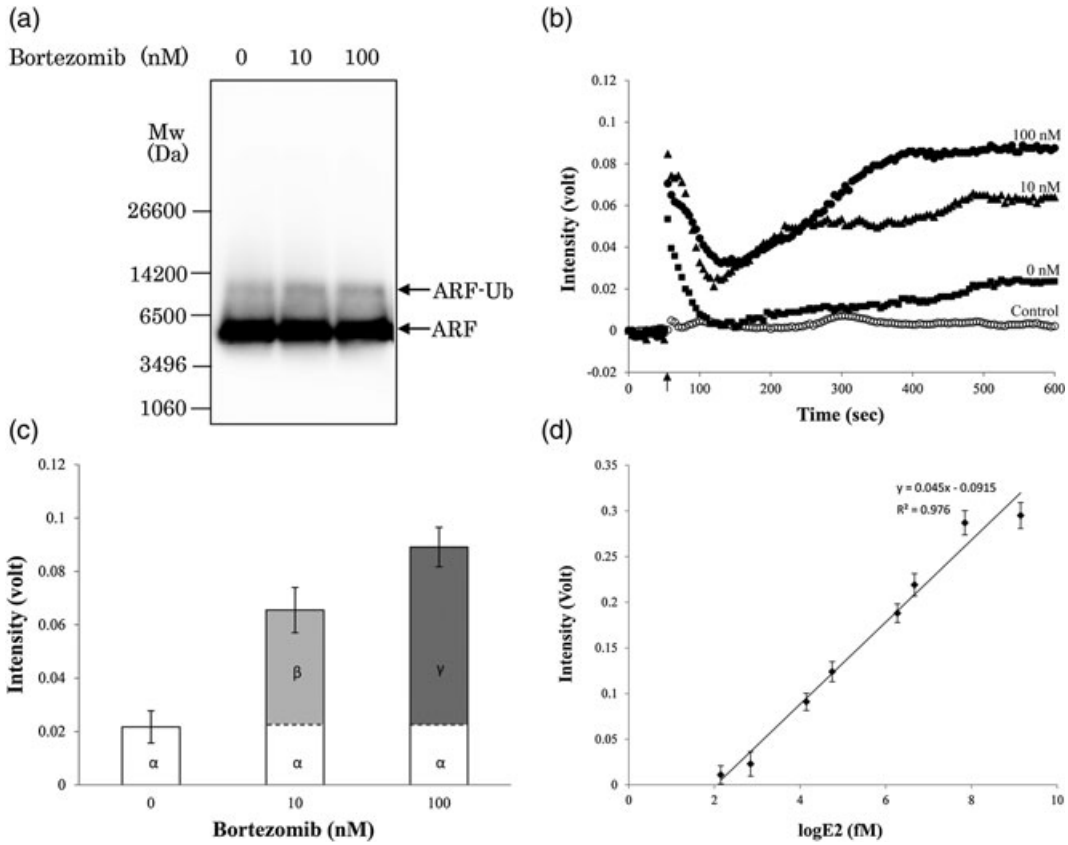

Figure 4. Monitoring of E2 activity in NB4 cells after treatment with bortezomib. (a) ARF was added to the supernatant of NB4 cells treated with 0, 10, or 100 nM bortezomib for 48 h at 37°C. ARF was ubiquitinated upon itself. The biotinylated ARF on the polyvinylidene difluoride membrane was reacted with the streptavidin–horseradish peroxidase solution and developed with enhanced chemiluminescence reagent. The emitted signals were detected as E2 activities on a LAS-3000 luminescent image analyzer. (b) The representative AMIS signals in the supernatants of NB4 cells treated with bortezomib. After the addition of 4 μl of ARF to the sample diluted fivefold with water, the E2 activities of NB4 cells treated with 0 (■), 10(▲), or 100 (●) nM bortezomib were detected at 5-s intervals (37 °C) on an AMIS-101. The arrow shows the injection time, and the AMIS detection for the culture medium was used as a control. (c) The AMIS measurements of the E2 activities were specified as (α), (α + β), and (α + γ) according to the concentration of bortezomib. The data are presented as the mean ± SD of the results of three independent experiments. (d) The calibration curve of UbcH8 for the intensity of the AMIS signal (y) and the concentration (x) in FBS-free RPMI-1640 medium containing 300-μM Mg-ATP, 60-μM DTT, 1.2 U/ml inorganic pyrophosphatase, 7.2-nM E1, and 2.4-μM Ub. The intensities of the AMIS signals at 600 s were calculated as the mean ± SD of the results of three experiments.

Journal of PeptideScience

and $\gamma = 0.068$ V indicate the increment of UbcH8 activity evoked by bortezomib. The standard curve of UbcH8 in FBS-free RPMI-1640 medium was built up (Figure 4d), and it was similar to that shown in Figure 3c. Thus, the curve was hardly affected by the medium components. The increasing levels of UbcH8 activity were calculated using the standard curve (Figure 4d) in the culture supernatant of NB4 cells (1×10^5 cells/ml), resulting in 5.1 pM (8.9 pg/100 μl) and 17.3 pM (30.4 pg/100 μl) for treatment with 10 and 100 nM bortezomib, respectively. These findings lend credence to the detection of E2 activities in the culture supernatant of cells. Moreover, concerning the reaction kinetics, the real-time detection presented in Figure 4b illustrates that the E2 reaction velocity of the Ub transfer increased in a concentration-dependent manner with drug treatment. Taken together, these data indicate that E2 activities augmented by bortezomib in a concentration-dependent manner were monitored in real time and quantified with high sensitivity and specificity.

The aim of this study was to elucidate that E2 activities are captured as the altering of the electron polarization in a semiconductor. NB4 cells treated with bortezomib were used as a model for demonstrating the E2 detection. Western blot analysis was limited in the study of E2 enzymes due to the lack of a high quantitative capability, as shown previously. The reaction of ARF changed the current flowing through gate channels in the sensor of the AMIS-101. The current, which was accumulated and amplified in the detector, was detected as the AMIS signal, allowing for the highly sensitive detection of E2 activities in ubiquitination. The present method enabled the highly sensitive quantitative detection of E2 activities over a response range from femtomolar to micromolar concentrations. Compared to western blotting, this detection was rapidly achieved in real time within several minutes.

Leukemia is characterized by the existence of leukemia cells at a level of more than 1×10^9 cells/body, which is equivalent to approximately 2×10^5 cells/ml of blood [21]. The activities of the E2 enzymes that emigrated from NB4 cells (cultured NB4 cells, 1×10^5 cells/ml) into the culture supernatant were monitored successfully using the ARF reaction. The present approach could be applicable to the detection of E2 activity for leukemia patients, and therefore, it is helpful for designing suitable treatment programs for patients. Moreover, many diseases are associated with E2 enzymes, such as UbcH10 with lung tumors and UbcH13 with breast cancer [22,23]. The previously engineered ARF specifically cooperates with UbcH13 [15]. Thus, the present detection method could be widely applied to specific E2s for various types of cancers.

In conclusion, we discovered that E2 activities are intimately interrelated with the polarization behavior of the electron in a semiconductor. By taking advantage of the simplified reaction with ARF, we have succeeded in the high-sensitivity detection of E2 activities via real-time monitoring. Furthermore, the rapid and accurate monitoring of E2 activity was successfully achieved using human acute promyelocytic leukemia cells following treatment with the anticancer drug bortezomib. This strategy is extremely simple and convenient for the assessment of the pathological conditions.

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Author Contributions

K.M. designed this study. K.M., M.S., M.Y-S., and K.S. performed all the experiments. K.M. wrote the main manuscript text and prepared all the figures. All authors reviewed the manuscript.

Competing Interest Statement

The authors declare no competing financial interests.

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Supporting information

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Figure S1. NB4 cells were cultured with or without 10 or 100 nM bortezomid for 48 h. The viable cell counts at 48 h were determined using trypan blue dye exclusion. Bortezomib reduced NB4 cell viability in a dose-dependent manner. The data are presented as the mean ± SD of the results of three independent experiments in triplicate.