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RESEARCH ARTICLE

Development of a Portable Biosensor System for Pesticide Detection on a Metal Chip Surface Integrated with Wireless Communication

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Abstract A novel low-cost ubiquitous sensor system was built on a microminiaturized electrochemical chip positioned on a gold electrode using a low-frequency wireless communication chipset to detect pesticides *in situ*. Different residual organophosphorus pesticides can be detected based on an enzyme reaction with electron transfer in the portable sensor system using a sensor array chip. This disposable, low-cost, and microminiaturized biosensor chip can be used to detect harmful residual agrichemicals within 3-4 min on a solid surface.

Keywords: agrichemicals, electrochemistry, biosensor, convergence, portable

Introduction

A miniaturized portable biochemical sensor system that allows on-site detection of hazardous agents, including toxic chemicals and agents, is desirable (1). Such a system would allow detection of residual harmful agrichemicals and toxic chemicals in agricultural products (2,3).

A sensor system consists of a sensor chip composed of sensor arrays for detection of residual chemicals, and a sensor platform for signal management and wireless communication in connection with the sensor chip. Government, industry, and the public should all be interested in development of real-time portable chemical sensor technology that can be used to protect the public from environmental pollutants and other hazardous agents, including toxic agrichemicals that remain on agricultural products (4,5).

Residual toxic agrichemicals are typically present on produce at low concentrations so development of a sensitive sensing technology is essential. To improve the performance of a whole sensor system, a robust yet inexpensive sensor platform, chemical functionalization of the sensor surface for selective sensing, a sensitive detection module using an immobilized enzyme, and a suitable I/O signal system with electric current or conductivity should be developed in an integrated manner. Once developed, this platform technology can be expanded for detection of other chemicals of interest using different detection modules.

A ubiquitous sensor platform is needed so that wireless signals can be easily analyzed and transmitted for different applications. For this reason, metal-micropatterned sensor chips are of particular interest. A sensor module can be designed in such a way that receptor molecules immobilized on a gold substrate specifically capture a particular agrichemical of interest (6). Then, the gold-based nanobiosensor chip triggers electric signal changes when a chemical binds to a receptor molecule.

Development of an inexpensive disposable agrichemical

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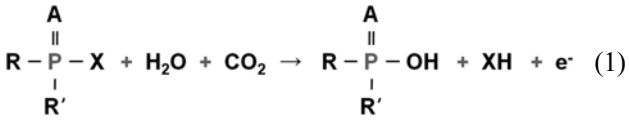
biosensor system integrated with wireless communication based on immobilization of a gold binding polypeptide (GBP) and an organophosphorus hydrolase (OPH) fusion protein on a gold electrode surface is herein reported. Immobilization of proteins on a solid surface is based on specific interactions between GBP and the gold substrate chip in which a GBP-OPH fusion protein is immobilized onto the gold surface, creating a sterical environment based on lifting of the OPH to the top of the protein for hydrolysis of organophosphorus (OP) chemicals. The fusion protein can be assembled with a correct orientation based on protein binding affinities. This strategy can be used to successfully identify several important pesticides with high specificities and sensitivities. Finally, the electrochemical detection signal can be inverted to the numerical values on a display panel of a portal device, which allows the readout of residual pesticides with simplicity and low-coat, and holds great promise for point-of-care detections.

Materials and Methods

Materials All restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA). Phosphate-buffered saline (PBS, pH 7.4) from BD Biosciences (San Jose, CA, USA) was used as a flow solution and as a dilution buffer for surface plasmon resonance (SPR, BIAcore® 3000; Biacore Life Sciences, Uppsala, Sweden) analysis. Deionized water was obtained using an ultrapure water system (Milli-Q; Millipore, Billerica, MA, USA). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of bioreceptor and sensor chips OPH was used as a model protein for immobilization on a gold electrode and hydrolysis of an OP substrate. Six histidine (6His) residues were used to facilitate purification of fusion proteins based on metal affinity chromatography. The DNA fragment encoding incomplete 6His-GBP was first obtained by PCR amplification using the primers P1 (5'-GAAACAGCATATGCACCATCACCATCACCACCAC GGCAAAACCCAGGCGACCAG-3') and P2 (5'-GGAA TTCAGACTGAATGGTACCGCTCGTCGCTTGGGTTT TACCGTGCATAGATT-3'), and plasmid pTGE as a template (7). For cloning of a mature OPH (mOPH) gene, PCR amplification was carried out using the primers P3 (5'-GAAATTCCATATGCATCATCACCACCACCACGG ATCGATCGGCACAGGCGA-3') and P4 (5'-AAAAC TCGAGTGACGCCCGCAAGGTCGGTGA-3'), and the genomic DNA of *Flavobacterium* sp. (ATCC 27551) as a template. The PCR product of 6His-GBP was digested using *Nde*I and *Eco*RI, and the PCR product of the mOPH gene was digested using *Eco*RI and *Xho*I, then ligated into the same sites of pET-22b(+) (Novagen, Darmstadt, Germany) to construct pET-6HGBP-mOPH. For production of the 6His-GBP-OPH fusion protein, *Escherichia coli* BL21(DE3) (F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3), Novagen, Darmstadt, Germany) harboring pET-6HGBP-mOPH was cultivated in 100 mL of Luria-Bertani medium supplemented with ampicillin (100 μg/mL) in a shaking incubator (Jeiotech, Daejeon, Korea) with 200 rpm at 37°C. After addition of isopropyl-β-D-thiogalactopyranoside for protein expression, *E. coli* cells were further cultivated at shaking condition with 200 rpm at 37°C for 6 h, then disrupted by using ultrasonicator (Braun Ultrasonics, Danbury, CT, USA) for 1 min at a 20% output. After centrifugation (16,000×*g*, 10 min, 4°C), the insoluble pellet fraction was saved for protein purification. The recombinant 6His-GBP-OPH fusion protein was purified using Ni-NTA His bind resins (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's standard protocol. In order to immobilize the enzyme on the gold surface, Au electrodes were immersed in a PBS solution containing 100 μg/mL of the GBP-OPH fusion protein for 12 h, then rinsed with PBS to remove loosely bound enzyme molecules.

Chemical reaction The enzymatic reaction of pesticides with OPH on the electrochemical sensor module was (8):



(1)

where R represents an alkoxy group and R' represents either an alkoxy or a phenyl group, A represents either O (oxygen) or S (sulfur), and X represents a phenoxy, thiol, cynanide, or a fluorine group.

System design Design and production technology for wireless networking has emerged based on high-level communication protocols, such as Zigbee, using the IEEE 802.15.4-2003 standard for small, low-powered devices (9). A handheld device for agrichemical detection is operated with software containing a mono-scheduler, user timer, network layer, media access control (MAC) layer, wireless and sensing modules, and a hardware abstraction layer.

The agrichemical sensor system in this study contained a fast, cycle-accurate modeling environment for design and validation of advanced system-on-chip (SoC) designs with a verification process (Fig. 1). Additionally, validation and deployment of system-level models were integrated into all leading system-level platforms, including SoC designer simplifying the virtual platform creation. The easy-to-use graphical user interface (GUI) of SoC designer allows users to rapidly assemble models to create the virtual program. Moreover, debugging of interfaces can be provided

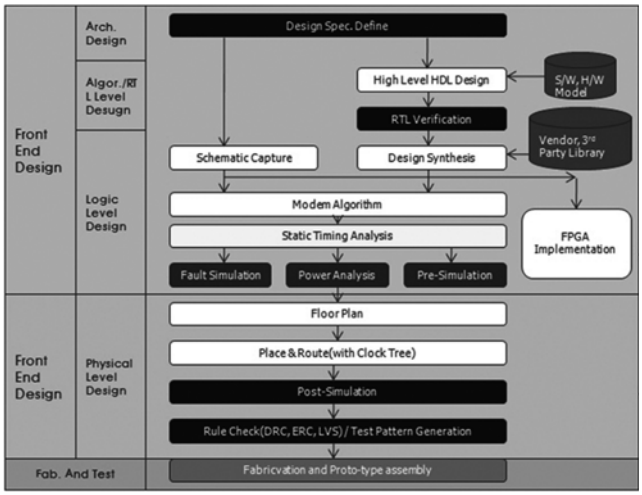


Fig. 1. SoC design and verification process. An algorithm with static timing analysis and post-simulation generation was designed for processing of a sensing signal.

using SoC designer, and analysis tools of system architecture can be obtained with the accuracy to model system characteristics by SoC designer (10).

Working algorithm and architecture The architectural structure of the software for the wireless agrichemical sensor device is shown in Fig. 2. The MAC layer and the sensing module ensure fast and stable operation with a mono-cross layer scheduler and a user timer (11). The growing complexity of service-centric systems has increased the need for pertinent and reliable software and trusted system solutions. Systematic approaches to measurement of sensor data in software architectures are needed in order to obtain sufficient and credible proactive evidence of the biosensor level or performance of a system. The systematic definition of biosensor metrics and security assurance metrics is a relatively new field that still lacks widely accepted definitions of metrics and applicable measuring techniques for design-time and run-time biosensor monitoring. Network layer data operates with the function of forwarding data along an accurate route. A hardware abstraction layer (HAL) serves as an application programming interface (API) for higher level components. An operating system with a defined HAL is easily portable across different hardware platforms, which is especially important for embedded systems (Fig. 2A).

MAC layer MAC layer architecture (MLA) provides a component-based architecture for MAC protocols in wireless sensor networks. MLA extends the unified power management architecture to provide the hardware-independent interfaces that are required by timing-sensitive MAC protocols, and defines platform-independent reusable components that implement the MAC layer logic on top. The MLA architecture was developed for a large number of platform-independent MAC implementations with little or no further

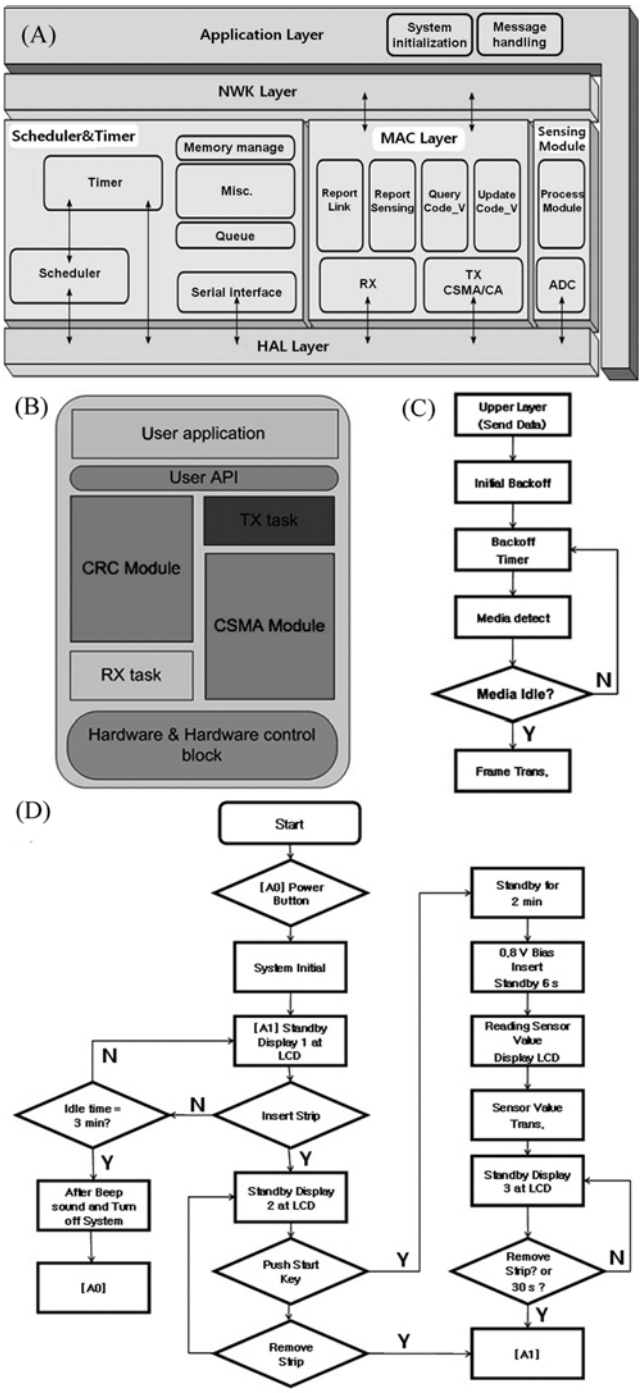


Fig. 2. Software architecture for MAC and sensing. (A) Software block diagram communication with the MAC layer and the HAL; (B) Component parts for the MAC layer with a user API and a hardware control block; (C) a MAC layer functional chart; (D) Flow chart of component parts for the sensing module from the start of sensing to signal display

effort required to adapt these implementations to new hardware platforms. Here, a wireless network multiple access method was used with carrier sense multiple access and collision avoidance (CSMA/CA) (Fig. 2B, C). In this method of data transmission, it is first necessary to listen to

a channel for a predetermined amount of time to determine whether or not another node is transmitting on the channel within the wireless range. If the channel is determined to be idle, then the node is permitted to begin the transmission process. If the channel is determined to be in use, the node defers transmission for a random period of time (Fig. 2D). Once the transmission process begins, it is still possible that the actual transmission of application data will not occur (12,13).

Measurement of surface plasmon resonance spectroscopy Binding of the GBP-OPH fusion protein on a gold surface was characterized based on SPR using a BIAcore3000 (Biacore Life Science). A gold sensor chip was attached to a separate chip carrier for easy assembly after surface coating, and was inserted into the BIAcore SPR system. All experiments were conducted in a PBS solution at a flow rate of 5 mL/min at room temperature, and all sensorgrams were fitted globally using BIA evaluation software (Biacore Life Science). Fifty mL of GBP-OPH fusion protein (0.1 mg/mL) was loaded onto the chip using a liquid-handling microneedle. After protein binding, the surface of the SPR gold chip was washed with PBS flow solution at a flow rate of 5 mL/min at room temperature for 20 min and equilibrated with the PBS solution.

Measurement of electrochemical reactions CV scans were recorded using a CHI750E electrochemical workstation (CH Instruments, Austin, TX, USA) with a conventional 3-electrode electrochemical cell using an Au working electrode, a KCl-Ag/AgCl reference electrode, and a platinum wire counter electrode. Electrochemical measurements were performed over a range of −1.0 to +1.0 V to investigate typical cyclic voltammograms of electrodes and the baseline of the background current. All potentials were collected vs. a KCl-Ag/AgCl reference electrode. Chronoamperometric responses were performed in a N_2-saturated PBS solution at a constant potential. All electrochemical measurements were performed at room temperature.

Results and Discussion

Immobilization of the GBP-OPH fusion protein on the gold chip The fusion protein comprising GBP and mOPH as a bifunctional crosslinker was produced using *T7* promoter expression in *E. coli* BL21(DE3). The expressed GBP-OPH crosslinker was simply immobilized on the gold surface with correct orientation (14). In order to determine the best concentration of the fusion protein as a bioreceptor for detection of organochemicals, the GBP-OPH fusion protein was functionally immobilized on the gold surface and directly monitored in real-time based on Biacore3000 SPR spectroscopy analysis (Fig. 3). When the dynamic and

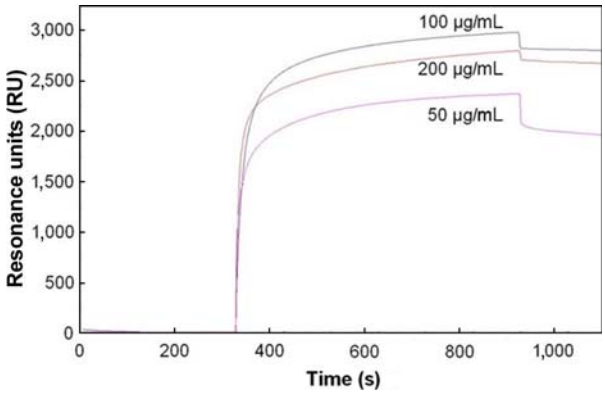


Fig. 3. SPR sensorgrams of the GBP-OPH fusion protein using concentrations of 50, 100, and 200 μg/mL. Increases in the SPR signal were 1,935 RU, 2,850 RU, and 2,640 RU on the gold chip surface, respectively.

specific binding of GBP-OPH was immobilized onto the gold surface, the best increase in the SPR signal up to 2,850 resonance units (RU) was observed upon introduction of the GBP-OPH solution of 100 μg/mL onto the chip surface, and 94% of the GBP-OPH remained on the surface after washing with PBS flow on the microchannel in SPR machine. Thus, most of the fusion protein was strongly immobilized onto the chip surface. The 2,850 RU value indicated that 2.85 ng of the GBP-OPH fusion protein was immobilized on a gold surface area of 1 mm^2. One RU was determined as 0.0001° of resonance angle shift and was equivalent to a mass change of 1 pg/mm^2 on the sensor surface (15-17). From this result, the concentration of the fusion protein was set at 100 μg/mL for effective immobilization on the gold surface. Furthermore, the GBP-fusion protein was readily immobilized on the gold chip surface without any chemical modification and remained on the chip surface even after washing with PBS, indicating that the GBP-fusion protein was strongly bound to the gold chip surface through the GBP portion, which has a high affinity for gold substrates. The standard Gibbs free energy value of the GBP monolayer on gold surfaces is lower than the self-assembled monolayer (SAM) value of thiol compounds, which demonstrates that GBP binds more strongly to the gold surface than thiol-based molecules (17). Polar groups exposed *via* the M, K, T, Q, and S residues in the GBP sequence and the GBP physical structure probably played an important role in cumulative binding to the gold surface, although the exact mechanism of binding remains unknown.

Rapid and sensitive electrochemical detection of pesticides The capacity for detection of OP pesticides (with a phenoxy, thiol, cynanide, or fluorine group) through an enzymatic reaction using OPH is shown in Fig. 4. Electrons generated through the reaction with pesticides produced a signal that was analyzed in order to measure the quantities of OP

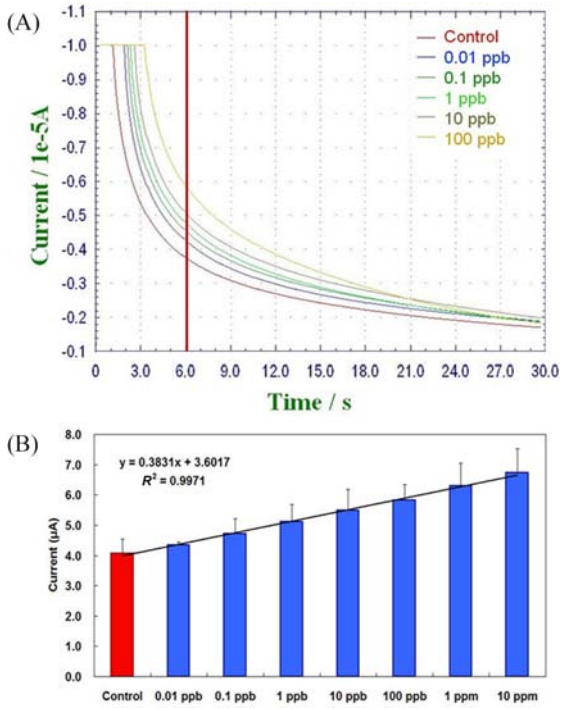


Fig. 4. Electrochemical detection with different concentrations of OP pesticides and paraoxon. (A) Time profiles for electric currents with paraoxon concentrations. An electric voltage was supplied to the chip electrode for 6 s (red line) after the OPH reaction for 3 min. (B) Linear regression of current responses for different concentrations of paraoxon vs. increasing electron current signals. A standard curve has a correlation coefficient (R^2 value) of 0.9971 as shown in inset.

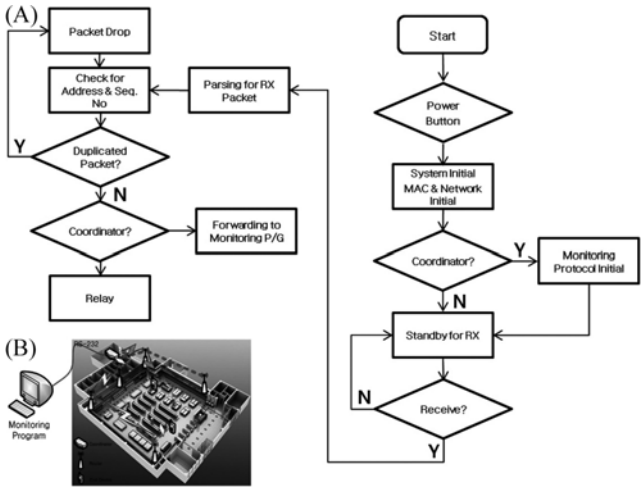


Fig. 5. Network process for data monitoring. (A) Flow chart of MAC and receiving layers from the start of sensing to display steps; (B) Network topology with routers between the coordinator and the end device

agricultural chemicals. Based on equation 1, OPH enzymes induced hydrolysis reactions that released electrons, which were used as visible numerical signals based on analog to digital signal conversion in the electrochemical biosensor system.

After an enzymatic reaction for 3 min, an electric voltage was subsequently supplied to the chip electrode for 6 s at room temperature (Fig. 4A). Each amperometry measurement was performed at +0.8 V for detection of paraoxon pesticides at different concentrations from a linear calibration of concentration vs. current (Fig. 4B). Detection values for OP pesticides were derived from a highly sensitive fast response system. Acceptable limits for agricultural pesticide residues in regulations of local governments and ministry of food and drug safety in Republic of Korea are 0.05 ppm for potatoes, 0.1 ppm for corn and rice, and 0.3 ppm for most other agricultural products, including apples, pears, and cucumbers (18). Therefore, the detection system described herein within a sensitive linear range of pesticides can be used for actual samples.

Network structures and physical layers The inner network layer structure and the defined network topology were integrated in the wireless agrichemical sensor system (Fig. 5), which contained a coordinator, a router, and a wireless end device (19). Each node is shown transmitted data. The key difference among the nodes was that an end device cannot route traffic, but routers can route traffic, and the coordinator, in addition to routing traffic, was responsible for forming the network. Every network must have a one and only one coordinator. The end device that is directly connected with the coordinator receives and transfers data and connects sequentially via the router relays to a database in the monitoring program. The physical layer is the first and lowest layer in the 7-layer open systems interconnection (OSI) model of computer networking (20). The physical layer consists of the basic hardware transmission technologies of a network and is a fundamental layer upon which the logical data structures of higher-level functions in a network are based. Due to a plethora of available hardware technologies with widely varying characteristics, this is perhaps the most complex layer in the open systems interconnection architecture.

The physical layer defines the means of transmitting raw bits rather than logical data packets over a physical link that connects network nodes. The bit stream may be grouped into code words or symbols and is converted into a physical signal that is transmitted over a hardware transmission medium. The physical layer provides an electrical, mechanical, and procedural interface to the transmission medium. The shapes and properties of electrical connectors and the frequencies and modulation of low-level parameters have been used in a portable device (21).

For normal hardware operation in this study, each component was integrated on a printed circuit board (PCB)

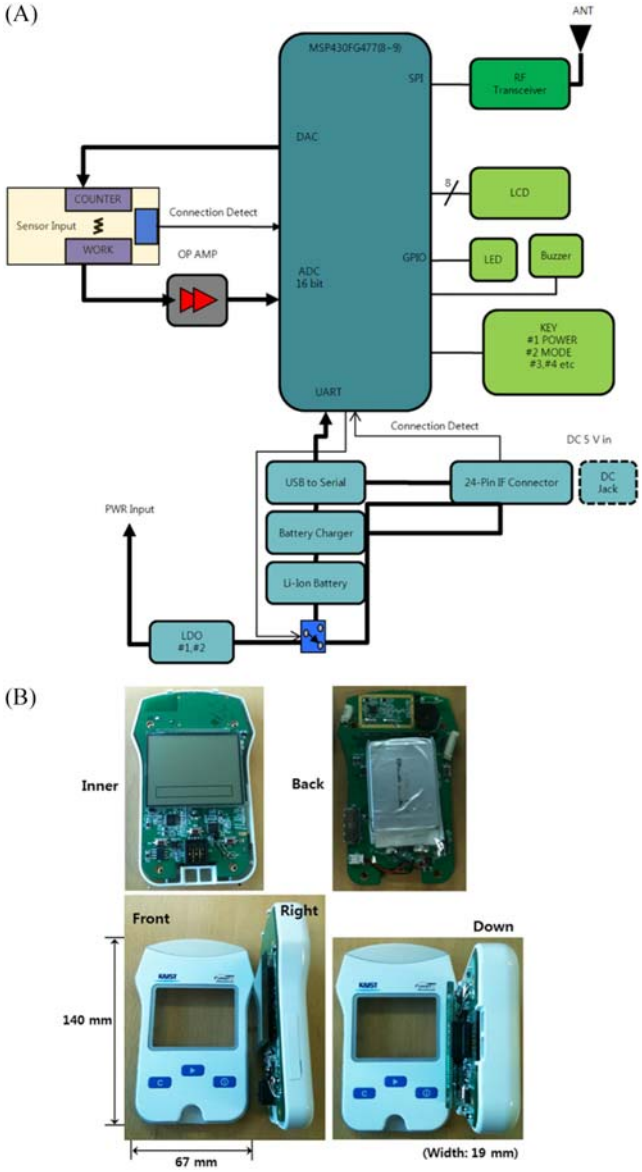


Fig. 6. Hardware structure of a personal device. (A) Hardware block diagram; (B) Real images of the agrichemical-sensing electrochemical device composed of a PCB panel, CPU, LCD guide, wireless communication module, and sensor module. The blue square and right-side end represent a socket for insertion of a gold strip.

prototype (Fig. 6) with a rechargeable and connectable 24-pin port. The prototype incorporated the 7 independent units of an analog-to-digital converter (ADC) and a signal converter, an input section, a microcontroller unit (MCU), a signal amplifier, a radio-frequency (RF) receiver, display, and charging circuit in a package (Fig. 6A). The MCU was used for the multicontroller function with I/O, rapid communication, ADC, and other circuits as a central processing unit (CPU), mainly integrated for sensor data processing, display, and ZigBee wireless communication. All circuit noise was minimized in order to avoid signal-coupling opportunities through inclusion of ground and power planes, and system shielding techniques. Sensor input signals from biosensor strips were converted into digital signals, then processed for display and transfer. This procedure was sequentially performed by the internal order flow of the installed program and the firmware in the device hardware.

Housing and user interface of the portable system The goal of a user interface design is to make user interaction as simple and efficient as possible, which is often called a user-centered design. Good user interface design facilitates completion of a task at hand without drawing unnecessary attention to the interface itself. Graphic design can be used to support usability. The design process must balance technical functionality and visual elements to create a system that is operational and also usable and adaptable to changing user needs.

Interface design is involved in a wide range of programs. All signals involve many biosensing interactions, yet signals also require some unique knowledge for detection of agrichemicals. A mock-up design was defined for ease of operation (Fig. 6B). As a result, specialized data, whether for software design or industrial design, could be displayed. Software and hardware designs must be improved for non-professional users, such as agricultural producers, distributors, and general consumers. Nonetheless, the device size must be smaller, and the featured color should be favorable to facilitate commercialization.

Biosensing signaling for detection of pesticides When a gold strip was inserted into the socket on the device, auto-calibration was performed using a reference sensor chip. Following the calibration step of the internal order flow (Fig. 5) of the installed program and the firmware, the OPH reaction and the voltage supply were started for reading of the sensor signal. Wireless sensing networks (WSNs) for signal processing and communications were combined with biosensing data for transfer to the end device via the router RF mode. The end device in a central server system can monitor local sensing data. Furthermore, a central node using a monitoring program and a serial interface with an active mode were used for the coordinator module. A flow chart of the sensing process within approximately 3 min from insertion of a sensor module to completion of detection is shown in Fig. 2D. The biosensing result was displayed on the LCD monitor, complete with information regarding the toxicity and concentration (integrated using a specific equation for concentration as shown in Fig. 4B) of agrichemicals. In addition, a GUI was embedded with visible test procedures to enhance convenience for an individual user. Special processes were designed for wireless communication based on the agrichemical biosensor system.

The electrochemical sensor module for detection of

agricultural chemicals was integrated using an enzymatic reaction and a metal-fabricated sensor chip to sense almost any phosphorus-containing compound, especially when dealing with OP neurotoxins. OP pesticides degrade rapidly by hydrolysis upon exposure to sunlight, air, and soil, although small amounts can be detected in food and drinking water (22). Organo-pesticides have great acute toxicity, posing risks to people who are exposed to large amounts. They are one of the most common causes of poisoning worldwide and are frequently intentionally used in suicides in agricultural sectors. Pesticide toxicity is not limited to an acute phase, however; and chronic effects have long been noted (23). Neurotransmitters, such as acetylcholine (which is affected by OP pesticides), are profoundly important in brain development, and many OP chemicals have neurotoxic effects in developing organisms, even at low levels of exposure. OP pesticides, the main components of agrochemicals, are used as direct substrates for OPH (22,24). Therefore, OPH can be used for detection of OP agrichemicals.

The 6 histidine tag allows simple purification of the fusion protein using immobilized metal affinity chromatography. Thus, the system described herein allows efficient immobilization of pesticide-detecting bioreceptors on a gold substrate. As a result, a gold sensor chip with microfluidics was investigated, which led to the observation that the detection limits of target chemicals were approximately 1 ppb in specific tests using a model pesticide. Furthermore, the multiplex chip for OP chemical detection can be prepared at a price less than $0.5 per gold sensor chip. The strategy and the platform system reported herein can be used for study of simple portable systems for detection of harmful materials.

In conclusion, an electrochemical detection system for agrichemicals was developed based on lifting the OPH enzyme on top of a fusion protein. The process involved on-site detection and a central monitoring program, and a wireless communication capability. A data set containing the ID of the sensor, the detection time, and the site, kind, concentration, and toxicity of chemicals was transferred to a central database GUI system through data routing machinery. This total system can be used on a farm, in a large-size supermarket, or in a distribution center. This BT-IT fusion technology contributes to enhancement of human health.

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Disclosure The authors declare no conflict of interest.

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