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Proton-sensing transistor systems for detecting ion leakage from plasma membranes under chemical stimuli

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ABSTRACT

The membrane integrity of live cells is routinely evaluated for cytotoxicity induced by chemical or physical stimuli. Recent progress in bioengineering means that high-quality toxicity validation is required. Here, we report a pH-sensitive transistor system developed for the continuous monitoring of ion leakage from cell membranes upon challenge by toxic compounds. Temporal changes in pH were generated with high reproducibility via periodic flushing of HepG2 cells on a gate insulator of a proton-sensitive field-effect transistor with isotonic buffer solutions with/without NH_4Cl. The pH transients at the point of NH_4Cl addition/withdrawal originated from the free permeation of NH_3 across the semi-permeable plasma membranes, and the proton sponge effect produced by the ammonia equilibrium. Irreversible attenuation of the pH transient was observed when the cells were subjected to a membrane-toxic reagent. Experiments and simulations proved that the decrease in the pH transient was proportional to the area of the ion-permeable pores on the damaged plasma membranes. The pH signal was correlated with the degree of hemolysis produced by the model reagents. The pH assay was sensitive to the formation of molecularly sized pores that were otherwise not measurable via detection of the leakage of hemoglobin, because the hydrodynamic radius of hemoglobin was greater than 3.1 nm in the hemolysis assay. The pH transient was not disturbed by inherent ion-transporter activity. The ISFET assay was applied to a wide variety of cell types. The system presented here is fast, sensitive, practical and scalable, and will be useful for validating cytotoxins and nanomaterials.

Statement of Significance

The plasma membrane toxicity and hemolysis are widely and routinely evaluated in biomaterials science and biomedical engineering. Despite the recent development of a variety of methods/materials for efficient gene/drug delivery systems to the cytosol, the methodologies for safety validation remain unchanged in many years while leaving some major issues such as sensitivity, accuracy, and fast response. The paper describes a new way of measuring the plasma membrane leakage in real time upon challenge by toxic reagents using a solid-state transistor that is sensitive to proton as the smallest indicator. Our system was reliable and was correlated to the results from hemolysis assay with advanced features in sensitivity, fast response, and wide applicability to chemical species. The downsizing and integration features of semiconductor fabrication technologies may realize cytotoxicity assays at the single-cell level in multi-parallel.

1. Introduction

There is growing interest in the delivery of functional molecules and nanomaterials to the cytosol for cell therapy and bioimaging [1]. Translocation of these molecules across the

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plasma membrane with high efficiency and without cytotoxicity is challenging. Studies on viruses have identified a series of short oligopeptides for efficient cytoplasmic invasion [2]. In addition, functional nanomaterials have been developed for direct penetration without disrupting the plasma membrane integrity [3–5]. Experimental and computational observations have shown that the size, shape, charge balance, amphipathicity, structure, alignment, and conformation of the nanomaterials are essential for translocation [6–8]. However, the mechanism of translocation has not been fully determined, and the occurrence of membrane damage during internalization has not been evaluated. This is because of the lack of a suitable sensing method with high spatiotemporal resolution for detecting the formation of extremely small pores that are accessible to low-molecular-weight electrolytes [9].

The plasma membranes of eukaryotic cells are flexible and stable lipid bilayers composed mainly of phospholipids and fatty acids, and are 6–10 nm thick. These extremely thin molecular films separate the intracellular space from the external world, and control the distributions of the ions, nutrients, and biomolecules that are essential for cell survival. Intact plasma membranes pass gas and hydrophobic molecules, but block ions and large molecules. The apparent permeability of plasma membranes to electrolytes is several orders of magnitude lower than their permeability to gas [10]. Ions are passively delivered via transporter, and are actively transported via ion pumps for maintaining the cell's homeostasis [11]. Large molecules are encapsulated in vesicles for translocation via energy-dependent membrane fusion processes such as endocytosis and exocytosis [12]. Plasma membranes can be disrupted by physicochemical stimuli, or interaction with exogenous toxicants. Membrane injury results in uncontrolled diffusion of mobile ions and macromolecules across the lipid bilayers in both directions, leading to acute cytotoxicity. Existing cytotoxicity assays determine biomarkers or indicators released from the cytosol optically, but this approach has some issues. First, a biomarker of interest cannot detect pores smaller than itself, which makes it difficult to evaluate the true safety of newly developed functional nanomaterials [3]. Second, indicators are quantified at a set endpoint, and this type of measurement lacks information about the progressive damage of plasma membranes. Third, a unique combination of indicator and sample may cause false-positive signals by inducing the permeation of indicator across plasma membranes with altered hydrophobic/hydrophilic balances [13]. Fourth, cationic samples may react with anionic indicators to form insoluble complexes, and spoil assays [14].

This study aimed to address these issues by developing a pH-sensing ion-sensitive field-effect transistor (ISFET) system that monitored the loss of ion-barrier properties of plasma membranes. ISFETs have been used as non-destructive sensors for measuring cellular activities in real time [15–27]. As previously reported, the ISFET system measured the stepped responses of free $[H^+]$ in the cell–gate insulator interspace during periodic exchanges of ammonium chloride (NH_4Cl), which produced pH changes without suffering from the time-dependent pH drift that occurs in stagnant conditions [28]. In this study, we elucidated the molecular mechanisms of the pH transients during the solution exchanges surrounding cells adhered on ISFET by numerical simulation. Based on the observation that the pH transients originated in the ion-barrier property of healthy plasma membranes, the ISFET system was applied for a membrane-toxicity assay using model chemical reagents. The ISFET assay was compared with a conventional hemolysis assay, which measures the amount of hemoglobin protein leaked from sheep erythrocytes at a set endpoint.

2. Experimental sections

2.1. Materials

Poly-L-lysine solution (Mw 150–300 kDa) and 1,3-bis(tris(hydroxymethyl)methylamino)propane (BTP) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Human Caucasian hepatocyte carcinoma (HepG2) and A498 Homo sapiens kidney carcinoma cell lines were purchased from DS Pharma Biomedical Japan (Osaka, Japan). The Madin-Darby canine kidney (MDCK), NIH/3T3 mouse embryo fibroblast cell lines, human embryonic kidney 293 (HEK293), and HeLa cells were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Fetal bovine serum was purchased from MP Biomedicals Japan (Tokyo, Japan). All the other reagents were from commercial sources and were used as received without further purification unless otherwise stated.

2.2. Cell culture

Cells were cultured on a 75 cm^2 tissue culture polystyrene dish in Dulbecco's modified eagle medium (high glucose) supplemented with 10% FBS and penicillin/streptomycin (100 $\mu g\ mL^{-1}$, Wako Pure Chemical Industries, Osaka, Japan) at 37 °C in a 5% CO_2 atmosphere. Subconfluent cultures (70–80%) were prepared using 0.25% trypsin/EDTA at the density of 1.3×10^4 cells cm^{-2}.

2.3. ISFET system for measuring extracellular pH

An open gate *n*-channel depletion type ISFET with a 40 nm-thick Ta_2O_5/140 nm-thick Si_3N_4/125 nm-thick SiO_2 layer as gate insulator was obtained from Isfetcom Co. Ltd. (Saitama, Japan). For sensor readout, the FET was operated as a source-drain follower, as described earlier [29,30]. The ISFET was operated at a drain-source voltage of 0.5 V with a drain-source current of 0.5 mA and no DC bias potential against the Ag/AgCl pellet (Warner Instruments, Hamden, CT) as a reference electrode. A LabJack U6-Pro (LabJack Corp., Lakewood, CO) was connected for recording the voltage between drain and reference. A cylindrical glass tube (inner diameter 5 mm) was fixed around the rectangular gate area (10 μm × 340 μm) of the ISFET chip by thermosetting epoxy resin to make a small well in which the gate electrode was directly in contact with the cells in the solution. To secure the cell attachment, the gate was immersed in 0.01% *w*/*v* poly-L-lysine solution at room temperature for 10 min, followed by rinsing with water and drying at 60 °C for 2 h. Then, HepG2 cells (1×10^5 cells) were seeded on the sensor and cultured at 37 °C in 5% CO_2 overnight before the measurements. The BTP buffer (1 mmol L^{-1} [BTP], 140 mmol L^{-1} [NaCl], 4 mmol L^{-1} [KCl], 1 mmol L^{-1} [$MgCl_2$], and 20 mmol L^{-1} [sucrose]; pH 7.4) was used for conditioning and washing the cells during the measurements. A BTP buffer containing 10 mmol L^{-1} [NH_4Cl] or [CH_3COONa] instead of 20 mmol L^{-1} [sucrose] (pH 7.2) was used for quickly modulating the intra/extra-cellular pH of the cells adhered on the gate insulator by loading with membrane-permeable NH_3 or CH_3COOH via passive diffusion. The two buffers with the same osmolality (313 mOsm L^{-1}) were injected alternately into the cells–ISFET well, both at a flow rate of 120 $\mu L\ min^{-1}$, using a program-controlled fluidic system with syringe pumps and solenoid valves at 37 °C. The vertical distance between the gate insulator and the outlet of the microfluidic tube was set at approximately 80 μm to instantly exchange the solution surrounding the cells. We used the sodium salts of polystyrene sulfonate (PSS) and the acid type of polyacrylic acid (PAA), and the pH of these solutions was adjusted to 7.4 prior to uses in the ISFET assay. The pKa of PAA is 4.5 [31], which means that the degree of dissociation of PAA is close to 1 at pH 7.4.