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Development of a Whole-Cell-Based Biosensor for Detecting Histamine as a Model Toxin

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A novel whole-cell potentiometric biosensor for screening of toxins has been developed. The constructed biosensor consists of a confluent monolayer of human umbilical vein endothelial cells (HUVECs) attached to an ion-selective cellulose triacetate (CTA) membrane modified with a covalently attached RGD (arginine-glycine-aspartic acid) peptide sequence. When the HUVECs form a confluent monolayer, ion transport is almost completely inhibited, thereby reducing the response of the ion-selective electrode (ISE). When the monolayer is exposed to agents that increase its permeability (e.g., toxins), ions can diffuse through the membrane, and a potential response from the ISE is achieved. Histamine, a model toxin that increases the permeability of HUVEC monolayers, was used in this study. When the cell-based membranes are exposed to varying concentrations of histamine, the overall response increases with increasing histamine concentration. Thus, the measured potential is an indirect measurement of the histamine concentration. Further experiments were performed for a similar molecule, L-histidine, to test for selectivity. The cell permeability was unaffected by L-histidine, and the sensor response remained unchanged. This type of sensor should find multiple applications in medical, food, and environmental fields and in homeland security.

Conventional cell-based biosensors consist of microorganisms incorporated into amperometric or potentiometric devices with many applications including detection of pesticides and monitoring water quality by measuring biological oxygen demand.[1] These simple cellular systems have been studied extensively, and more recent research has focused on more complex systems such as using animal cells or tissues as the biological recognition elements.[1,2] Some general types of cell-based biosensors include biochemical sensors that are used to detect biological products delivered to the medium from cell metabolism, sensors that measure cell−cell contact or cell−substrate contact, and sensors that measure the electrical response of neural networks or cells that produce an electrical signal (i.e., heart cells).[2] Many of the cell-based biosensors constructed to study cell−cell contact utilize endothelial cells as the recognition element to study such topics as drug delivery across the blood−brain barrier and wound healing.[2] In this study, the barrier properties of endothelial cells were used to facilitate toxicology testing.

In vivo, human umbilical vein endothelial cells (HUVECs) line blood vessels by attaching to proteins on the interior wall. Specifically, the arginine-glycine-aspartic acid (RGD) segment of fibronectin is one of the most widely recognized protein sequences to which HUVECs bind.[3−9] Previous studies have shown that endothelial cells readily attach to and proliferate on various substrates with immobilized RGD peptides.[3−12] In particular, the attached HUVECs grow to form a monolayer that mimics the interior of the blood vessels.[3−12] When the monolayer forms, tight junctions develop between adjacent HUVECs that prevent the passage of molecules across the monolayer.[13−19]

A current need exists for a simple, reliable, and quick screening method for toxins. This paper describes the development of a whole-cell-based biosensor for detecting toxins that could find multiple applications in the medical, food, and environmental industries and in homeland security. In this study, a HUVEC-based ion-selective electrode (ISE) was used to measure the presence of histamine, a model toxin. Histamine resides in both the blood of patients with problems such as severe allergic reactions and in foodstuffs such as seafood.[20] Research has shown that histamine alters the permeability of HUVEC monolayers by causing the

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formation of gaps along the intercellular contacts in recently confluent cultures. This result was found both in vivo and in cultured endothelial cells.[21,22] Although there are conflicting theories regarding the precise mechanism by which this process occurs, small gaps are created between cells that resemble pores, and cell−cell adhesion is altered.[23−26]

The biosensor consists of a confluent monolayer of HUVECs attached to a modified cellulose triacetate (CTA) membrane of an ISE, selective for potassium ions. In the presence of a fixed concentration of potassium in the sample, the confluent HUVEC monolayer blocks the interface and no appreciable ISE response is obtained. When the monolayer is exposed to agents that affect the permeability of the endothelial cells (e.g., toxins), K^+ ions are allowed to reach the ISE membrane, resulting in a change in the potential of the ISE. Therefore, when the cell-based biosensor is exposed to histamine, an ISE response is obtained. Potassium ions transport through paracellular spaces to the CTA membrane surface with ease since the pore-to-molecule diameter ratio is large.[25] Hence, the response of the ISE serves as an indirect measurement of the presence of the toxin. These results are compared to those of the biosensor after exposure to L-histidine, a molecule similar in structure to histamine, to determine the selectivity of the sensor for histamine versus L-histidine.

EXPERIMENTAL SECTION

Reagents. Cellulose triacetate pellets were purchased from Eastman Kodak (Rochester, NY). Valinomycin was obtained from Calbiochem (San Diego, CA). The plasticizer *o*-nitrophenyl octyl ether (NPOE) and the lipophilic salt potassium tetrakis(chlorophenyl)borate (KTClPB) were from Fluka (Ronkonkoma, NY). Carbonyldiimidazole (CDI), tris(hydroxymethyl)aminomethane (Tris), L-histidine, and histamine were purchased from Sigma (St. Louis, MO). Methylene chloride, chloroform, 1,1,2,2-tetrachloroethane, and all chloride salts were from Aldrich (Milwaukee, WI). All aqueous solutions were prepared with deionized water obtained with a Milli-Q Water Purification System from Millipore (Bedford, MA).

The synthetic RGD peptide sequence used in this work was Gly-Arg-Gly-Asp-Ser (GRGDS) and was purchased from Bachem (King of Prussia, PA). The endothelial cells used in these experiments were HUVECs obtained from Cambrex BioScience (East Rutherford, NJ) and were cultured in an EGM-2 media system from Cambrex BioScience supplemented with fetal bovine serum, hydrocortisone, insulin-like growth factor, basic fibroblast growth factor, vascular endothelial growth factor, human epidermal growth factor, ascorbic acid, human fibroblast growth factor, gentamicin sulfate, amphotericin-B, and heparin.

Preparation of the Asymmetric Cellulose Triacetate Membrane. Hydrolyzed, asymmetric CTA[27−30] with a valinomycin ionophore has been shown to result in functional membrane ISEs. The hydroxyl groups on the outer surface of the hydrophilic base layer provide sites for further surface activation and attachment of biomolecules.[29−31] The procedure for preparing the CTA membranes for endothelial cell attachment is illustrated in Figure 1.

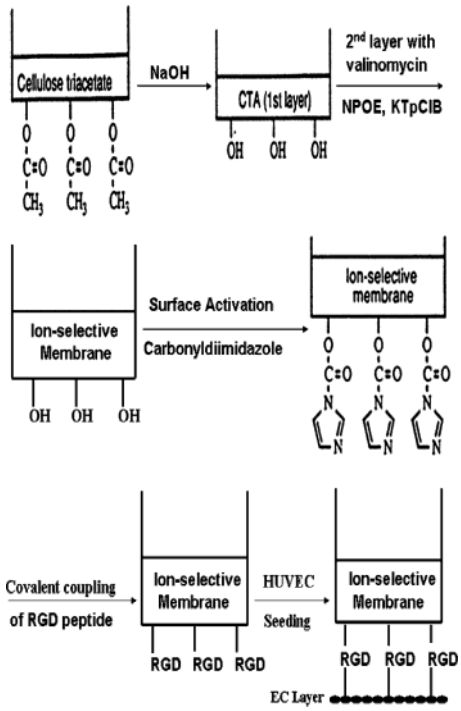

Figure 1. Preparation of the modified ion-selective cellulose triacetate membrane for attachment of endothelial cells (EC).

Casting the Base Layer of the CTA Membrane. The base layer of the membrane was prepared by dissolving 74 mg of CTA pellets in 1.1 mL of methylene chloride, 0.4 mL of chloroform, and 0.40 mL of 1,1,2,2-tetrachloroethane. The mixed solution was then cast in a 31-mm-i.d. glass ring placed on a Teflon plate. After the 2 days allowed for solvent evaporation, the membrane was removed from the glass ring and floated on 1.0 M sodium hydroxide for 4.5 h. Raised edges of the membrane allowed only the bottom side to be hydrolyzed. The membrane was then removed from the sodium hydroxide solution and immediately rinsed with deionized water.

Casting the Second Layer of CTA Membrane. The second layer was cast using an ionophore cocktail to make the membrane selective to potassium. The particular cocktail used in this study was composed of 1 mg of valinomycin, 100 μL of NPOE, and 0.42 mg of KTClPB, along with 35 mg of CTA. This mixture was dissolved in a solvent mixture composed of 0.80 mL of methylene chloride and 0.80 mL of chloroform. The solvent was allowed to evaporate for 2 days as the two layers fused into one single asymmetric membrane.

Surface Immobilization with RGD Peptide. After the basic membrane was obtained, the bottom side (with the free OH

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groups) was immersed in cold deionized water. Next, 324 mg of CDI was added in five increments over a 15-min period, giving a final concentration of 0.10 M CDI to activate the surface for peptide coupling. Following this activation, the membranes were immediately incubated overnight in a 0.1 M sodium carbonate solution (pH 9.5) containing 400 μg/mL RGD peptide to promote covalent peptide attachment from the N-terminal amine. The membranes were then removed from the coupling solution and rinsed sequentially with 0.10 M $NaHCO_3$ (pH 8.5), deionized water, acetate buffer (pH 4.0), and deionized water. Protein immobilization was confirmed using a micro-BCA protein assay kit obtained from Pierce Biotechnology (Rockford, IL).

Cell Seeding onto Membranes. Following peptide attachment, the electrode pieces were autoclaved, and the membranes were placed under germicidal UV for 24 h. HUVECs were then seeded onto the bottom surface of the membrane with a cell seeding density of 1×10^5 cells/mL. The cells were allowed to spread and form a confluent monolayer over the membrane surface for 24 h at 37 °C in a humidified incubator with 5% CO_2. HUVECs were used experimentally up to passage 5.

Evaluation of Electrode Response. Following the membrane preparation, 6-mm-i.d. disks were cut from them and mounted in Philips IS-561 electrode bodies (Glasblaserei Möller, Zurich), with the RGD-modified surface facing the sample solution. It should be noted that when cells were seeded onto membrane pieces, the pieces were cut and mounted prior to cell seeding so as to not disrupt the formed monolayer. Following cell attachment and spreading, the control membranes were tested to confirm the inhibited ion response due to the presence of the confluent monolayer. The internal filling solution consisted of 0.01M KCl, and the internal reference electrode was Ag/AgCl. The external reference electrode consisted of a double-junction Ag/AgCl electrode (Orion Model 90-02-00) with an Orion (90-02-02) internal filling solution and with 0.1 M Tris buffer (pH 7.5) in the outer compartment. Potentiometric responses were measured with a four-channel high impedance amplifier interface (World Precision Instruments) connected to a Model 100 Instrunet A/D converter. The data were analyzed using Instrunet software on a Macintosh Power PC. Prior to initial use, the electrodes were conditioned in sterile 0.01 M KCl solution. A series of incrementally sized aliquots of sterile KCl solutions were then sequentially added to the stirred initial sterile buffer solution. Calibration plots were constructed by plotting the measured potential (mV) versus the logarithm of the concentration of potassium ions present in the bulk solution.

The response was first evaluated after protein immobilization and as a function of cell confluency. After confirmation of the inhibited response following 24 h of cell growth, the cell-based membranes were exposed to various concentrations of histamine for 20 min, and the membrane electrode response was immediately recorded. The histamine concentrations tested ranged from 1×10^{-5} to 0.1 M (pH 7.5 in Tris buffer). The electrode response was measured for the following conditions at a final concentration of 0.1 M KCl: (1) the membrane without cells and without histamine, (2) the membrane without cells and with histamine (0.1 M), (3) the membrane with cells and without histamine, and (4) the membrane with cells and with varying concentrations of histamine. To investigate optimal exposure times, experiments were also performed for the membrane with cells after 5, 15, 40, and 60 min of exposure to 0.1 M histamine. Experiments were repeated with L-histidine to compare these results to those with histamine.

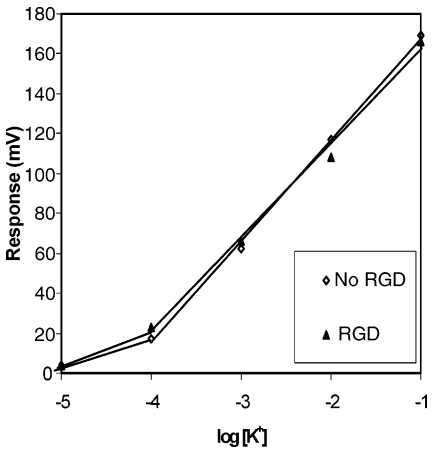

Figure 2. Typical ISE calibration plot for ISE membranes with and without immobilized RGD peptide.

Data Analysis. Data analysis was conducted by first evaluating the potential response obtained at a final concentration of 0.1 M KCl for each membrane with cells and/or histamine. The final data are reported as a ratio of the potential response for ISEs with HUVECs and/or histamine to the potential response of the ISEs without HUVECs or histamine for a final concentration of 0.1 M KCl to account for slight variations between fabricated membranes. The purpose of reporting the ratios of the potentials measured at 0.1 M KCl is to illustrate the total increase in potential after exposure to particular concentrations of histamine and show how much of the original signal is regained. Hence, these ratios are provided as comparisons but are not presented as analytical quantities. Data are reported as mean ± SEM. Multiple pairwise comparisons were made using one-way ANOVA and the Student-Newman-Keuls test for post-hoc comparisons of the means with $P < 0.05$. Calculations were performed using SigmaStat V2.0 software.

RESULTS AND DISCUSSION

Initial experiments were conducted for the membranes without cells or histamine to ensure a good sensitivity for the CTA membrane electrode itself. A typical sensitivity value for the membranes without cells or histamine was approximately 55 mV/decade, which is consistent with literature values for a near-Nernstian response for similar CTA membranes.[29,30] Upon peptide immobilization, the response was evaluated and compared to the control without peptide. Figure 2 reveals that the peptide itself does not create mass transfer resistance since the response profile matches that of the control. Thus, the results from the biosensor are unaffected by the presence of the peptide on the membrane. The microBCA protein assay confirmed an immobilization density of 0.15 μg/mm^2.

Control experiments were also performed for the response of the ISE with cells but without histamine. To obtain the response as a function of HUVEC growth, calibration plots were constructed for various cell seeding times. The results along with the corresponding phase contrast micrographs are illustrated in Figure 3. After the cells are seeded, the response decreases as

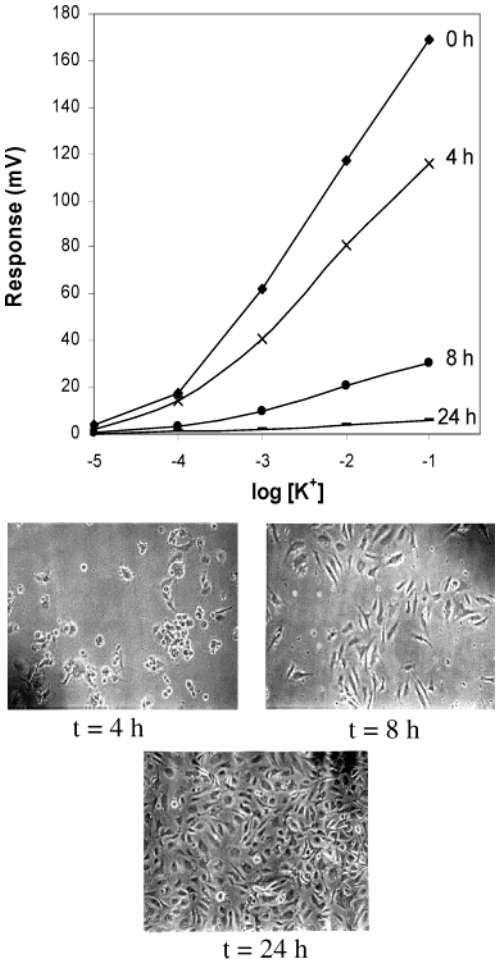

Figure 3. ISE calibration plots for ISE membranes as a function of the cell seeding times. The corresponding phase contrast micrographs are shown to illustrate cell growth. As the time of cell seeding increases from 0 to 24 h, the response decreases as the cell monolayer becomes more confluent.

the cells begin to spread, and the baseline potential tends to more positive values. After 24 h of growth, the response of the sensor is almost completely inhibited. The inhibited ion transport is caused by the barrier function of the HUVEC monolayer. In vivo, the monolayer is primarily responsible for the endothelial barrier function since it restricts the passage of molecules through blood vessel walls.[13−19] Thus, in vitro, when the monolayer forms, strong adherens junctions between HUVECs, which normally serve to restrict transport, also inhibit the transport of ions (in this case K^+) to the electrode surface. A major component of adherens junctions is vascular endothelial (VE)-cadherin, which is a type II cadherin and consists of two main regions: the cytodomain and the ectodomain. The cytodomain is directly connected to the plasma membrane of cells and adheres to α-catenin, plakoglobin, p120 subfamily of the armadillo proteins, and other essential components, which aid in stabilizing adherens junctions by firmly attaching to the actin cytoskeleton. The ectodomain contains proteins involved in cell−cell adhesion that prevent the passage of molecules through the junctions.[15,32−35] A simplified model of adherens junctions is shown in Figure 4.

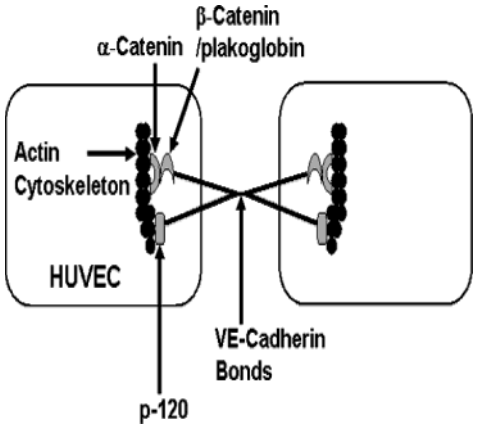

Figure 4. A simplified diagram depicting the role of VE-Cadherin in HUVEC adherens junctions. VE-Cadherin extends from the inside of the cells (cytodomain) to the outside (ectodomain) where it binds to VE-Cadherin from an adjacent cell. Within the cytodomain, VE-Cadherin forms multiprotein complexes with cytoplasmic proteins, which are strongly supported by the actin cytoskeleton.

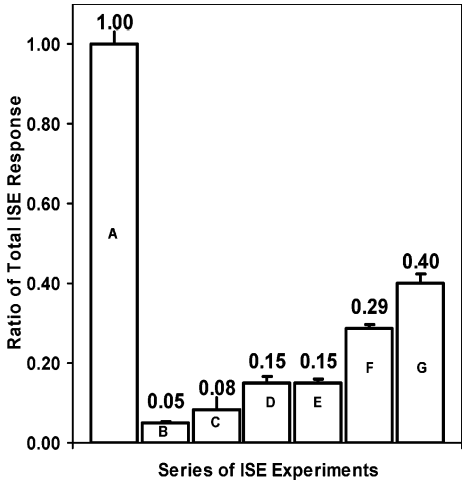

Figure 5. Plot of the ratio of the ISE response obtained at 0.1 M KCl for the following conditions to the ISE response for the membrane without HUVECs or histamine at 0.1 M KCl: (A) with histamine only at 0.1 M, (B) with cells only, (C) with cells and 1×10^{-5} M histamine, (D) with cells and 1×10^{-4} M histamine, (E) with cells and 1×10^{-3} M histamine, (F) with cells and 1×10^{-2} M histamine, and (G) with cells and 0.1 M histamine. The error bars represent the standard error of the mean for data obtained from five experiments.

After confirming the inhibited ion response due to the HUVECs, experiments were performed with histamine and with cells in the presence of a fixed concentration of K^+. Since the largest histamine concentration investigated in these experiments is 0.1 M, control experiments for this concentration without cells were conducted to ensure that histamine alone does not affect the ISE response. The results in Figure 5 (Bar A) illustrate that histamine alone does not affect the overall response of the sensor, and once again, Figure 5 (Bar B) confirms a 95% decrease in the response due to 24 h of growth of endothelial cells. Furthermore, an increase in the overall response is observed with increasing histamine concentration for the HUVEC-coated electrodes. As the

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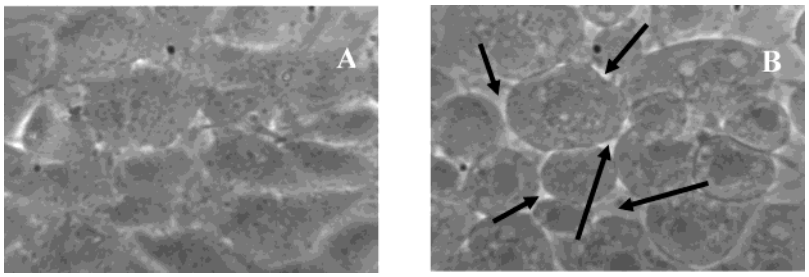

Figure 6. Phase contrast micrographs (512× magnification) of (A) HUVECs before histamine treatment and (B) HUVECs after histamine treatment (1×10^{-3} M) for 20 min. Arrows indicate the formation of paracellular spaces from the effects of histamine.

concentration of histamine is increased from 1×10^{-5} to 0.1 M, the inhibition in the response is decreased from 92% to 60%. Phase contrast micrographs, shown in Figure 6, indicate that the increase in the sensor response can be attributed to the formation of gaps between adjacent cells upon exposure to histamine (10^{-3} M, 20 min). Whether the cells actually contract due to forces of tension within the cellular cytoskeleton or whether they retract, "or round up", due to physically altered cell−cell adherens junctions from tyrosine phosphorylation of adhesion proteins,[23−26] small spaces or gaps are created between the cells that allow the passage of potassium ions to the sensor surface so that a potential response is obtained. This effect is magnified with increasing histamine concentrations.

According to the literature, the optimal exposure time for a recently confluent HUVEC culture to 1×10^{-4} M histamine is 25 min instead of 20 min.[23] Even though gaps begin to form along the intercellular contacts within 1−5 min of exposure to 1×10^{-4} M histamine, the permeability increase reportedly reaches a maximum at 25 min.[23] On the basis of this information, optimal exposure times were investigated to determine if the maximum permeability increase depends on the concentration of histamine used or if the optimal exposure time is 25 min for all concentrations. Experiments were performed for the membrane with cells after 5, 15, 20, 40, and 60 min of exposure to 0.1 M histamine. These results are shown in Figure 7. The data reveal the time dependence of exposure to histamine since the potential response at a final concentration of 0.1 M KCl after 15 min of contact with 0.1 M histamine is approximately 5 times the response after 5 min. This result is attributed to the time required for gap formation between cells. Larger gaps permit the transport of more ions to the sensor surface, thereby resulting in a larger potential response. Furthermore, increases in the exposure time beyond 15 min do not affect the response of the biosensor for 0.1 M histamine. This result indicates that the optimal exposure time for this concentration appears to exist between 5 and 15 min. At 15 min, the cells have already responded to the presence of histamine and formed the corresponding gaps for 0.1 M. Thus, an increased response beyond this threshold value for 0.1 M histamine cannot be obtained even if the exposure time is increased due to the presence of the HUVECs on the membrane surface. The cells create a blocked interface[36,37] at the membrane surface. The HUVECs cause mass transfer resistance because they physically block ion exchange at the membrane surface where gaps are not present, thereby making the rate of ion exchange slower[37] at the membrane interface. As long as the cells reside on the membrane surface, only a fraction of the response from the control membranes can be recovered.

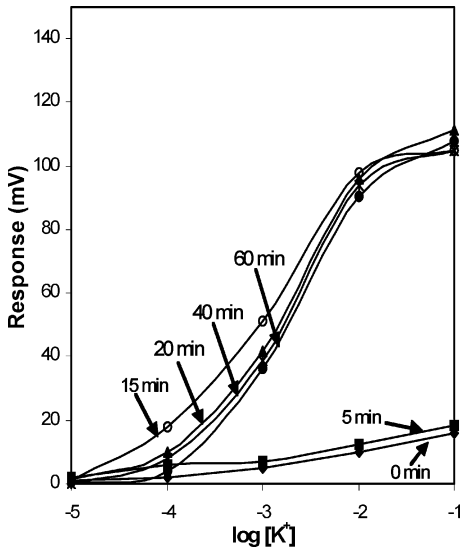

Figure 7. ISE calibration plots after exposure times of HUVEC-covered electrodes to 0.1 M histamine for 5, 15, 20, 40, and 60 min.

Statistical analysis reveals that there is no significant difference between the potential response for controls with cells only and the response obtained at a concentration of 1×10^{-5} M histamine (Figure 5), but a significant difference does exist at a concentration of 1×10^{-4} M histamine. This indicates that the detection limit of this sensor is between 1×10^{-5} and 1×10^{-4} M histamine. Given that the detection limit lies in this range, this sensor can be used to detect large quantities of histamine in seafood since the histamine concentration (4.5×10^{-4} M) stipulated by the U.S. Food and Drug Administration would result in a response from the sensor.[20] The detection limit may be reduced even further if the exposure times for the lower concentrations are optimized, which may further extend its applicability to the physiological detection of histamine.

One concern in using these sensors in biological applications is the inhibition of the ion response due to adsorption of other proteins onto the membrane surface. Preliminary studies have shown that when these biosensors are exposed to proteins in the cell culture media, inhibition of the response was not observed. Future studies will focus on the biocompatibility of these sensors to further confirm that they can be used in biological applications without external interference.

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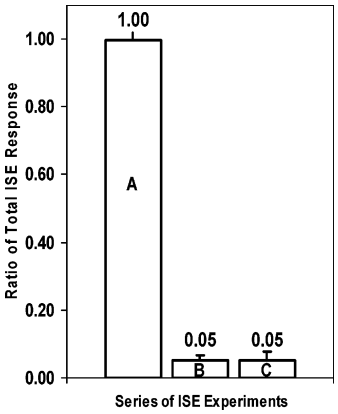

Figure 8. Plot of the ratio of the ISE response obtained at 0.1 M KCl for the following conditions to the ISE response for the membrane without HUVECs or L-histidine at 0.1 M KCl: (A) with 0.1 M L-histidine only, (B) with cells only, and (C) with cells and 0.1 M L-histidine. The error bars represent the standard error of the mean for data obtained from five experiments.

It should be noted that another technique has been used in the past to study the effect of toxins on cells. The electric cell−substrate impedance sensor (ECIS) quantitatively detects changes in cell activities by measuring fluctuations in impedance.[38,39] Although this method is complimentary, the complexity of the signals from ECIS can make the interpretation of the cellular response to chemical agents difficult and the equipment required is more complicated and expensive. The ISE measurements used in this study only require a simple electrode and can be easily adapted to small-scale use through planar electrodes. This especially has advantages in using this type of sensor in in vivo applications. Future work will focus on adapting these sensors to small-scale planar systems.

L-Histidine is a molecule very similar in structure to histamine. To the best of our knowledge, L-histidine has not been reported as an agent that increases the permeability of HUVEC monolayers. Thus, experiments conducted with L-histidine can verify the selectivity of the biosensor to histamine. The results of the experiments performed with 0.1 M L-histidine are illustrated in Figure 8. On the basis of the above results, the response of the cell-based ISE after exposure to L-histidine is similar to the response obtained with the control membranes containing cells only. Furthermore, control experiments were conducted to determine the effect of L-histidine on the HUVEC-based ISEs without cells. These results indicate that L-histidine does not directly affect the membrane performance itself. Therefore, it can be concluded that the sensor can discriminate histamine from L-histidine, and further supports that the response to histamine is due to a modification of monolayer permeability and not a nonspecific effect from a chemically similar molecule.

In summary, a novel whole-cell biosensor has been developed that has potential for simple, reliable, and quick screening of toxins. This biosensor has the general ability to test for a specific class of compounds, which increase the permeability of HUVEC monolayers. It has been demonstrated that when a confluent HUVEC monolayer is formed on the CTA membrane of a K^+-selective electrode, the sensor response is inhibited due to the decrease in ion transport across the cell monolayer. When exposing the cells to the model toxin, histamine, the adherens junctions between the cells are disrupted, and a response from the ISE is achieved. Hence, this response provides an indirect measurement of the presence of the toxin. In addition, it has been shown that the magnitude of the response is dependent on the concentration of histamine and, hence, a correlation could be developed that would be used to determine concentrations of the toxin in samples with unknown histamine concentrations. While the detection limit of the current sensor is between 1×10^{-5} and 1×10^{-4} M, it is possible that this could be improved by optimizing the exposure time at the lower concentrations. Current studies are focusing on this optimization along with sensor stability and storage issues. Finally, since other toxins are known to also increase the permeability of HUVEC monolayers, studies on the applicability of this sensor for detecting other environmental and physiological toxins are in progress.

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