A novel and simple cell-based detection system with a collagen-encapsulated B-lymphocyte cell line as a biosensor for rapid detection of pathogens and toxins

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Cell-based biosensors (CBBs) are becoming important tools for biosecurity applications and rapid diagnostics in food microbiology for their unique capability of detecting physiologically hazardous materials. A multi-well plate-based biosensor containing B-cell hybridoma, Ped-2E9, encapsulated in type I collagen matrix, was developed for rapid detection of viable cells of pathogenic *Listeria*, the toxin listeriolysin O, and the enterotoxin from *Bacillus* species. This sensor measures the alkaline phosphatase release from infected Ped-2E9 cells colorimetrically. Pathogenic *L. monocytogenes* cells and toxin preparations from *L. monocytogenes* or *B. cereus* showed cytotoxicity ranging from 24 to 98% at 3–6 h postinfection. In contrast, nonpathogenic *L. innocua* (F4247) and *B. subtilis* induced minimal cytotoxicity, ranging only 0.4–7.6%. Laser scanning cytometry and cryo-nano scanning electron microscopy confirmed the live or dead status of the infected Ped-2E9 cells in gel matrix. This paper presents the first example of a cell-based sensing system using collagen-encapsulated mammalian cells for rapid detection of pathogenic bacteria or toxin, and demonstrates a potential for onsite use as a portable detection system.

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Detection of biological entities using cell-based assays (CBAs) has already found its niche in biomedical research as well as in clinical, environmental, and bioprocess industries.^{1–5} The rapid detection methodologies applicable to these fields using CBAs have had enormous impact on the emergence of cell-based biosensors (CBBs).^{5–8} In a CBB, the normal physiological response of a living cell or cellular component resulted by one or more external factors (stimuli) is recorded.^{9,10} This ‘response’ can be qualitatively or quantitatively analyzed to deduce the nature and character of the external ‘stimuli’.^{5,6,8–11} In some of these CBBs the sensing elements could be the vegetative cells of bacteria,^{5,12} or eukaryotic¹³ or mammalian cells.^{8,14,15} Different types of platforms or materials have been used to ‘pattern’ or lay different types of higher eukaryotic cells to design cell-based sensors.^{5,10,16–20} Interfacial engineering to couple biological cell (or cellular components) to signal transducers or detection platforms is critical for successful cell-based biosensing.^{21–24} The types of materials used for immobilization and entrapment of living cells vary

widely, encompassing biocompatible polymers (such as polyethylene glycol, alginate, synthetic peptides or silica gel)^{25–28} to biologically derived substances such as collagen or fibronectin, to name a few.^{29,30}

The viability of the living cell after immobilization determines the actual working life of the sensor. For that reason, materials that best mimic the actual physiological conditions would be the most effective material to use as a matrix for cell harboring. Cellular entrapment in biomaterials such as collagen has distinct advantages, including the unparalleled biocompatibility of collagen, which makes it one of the most versatile biomaterials used for tissue culture known so far.^{31,32} In addition, collagen gel can be prepared with different pore sizes that trap different size cells by varying the concentration.^{33,34} Collagen gel has high mechanical strength and has excellent gelling properties.³³ Three-dimensional (3D) encapsulation of different cell types in collagen gel has become a standard method in tissue engineering applications.^{31,32} Application of mammalian cells

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in collagen-entrapped form for use as biosensors is still in its infancy,^{34,35} but offers significant promise.^{34–36} Previous studies from our laboratory have shown that a mouse B-cell hybridoma (Ped-2E9)-based cytotoxicity assay can be used to detect the presence of pathogenic *Listeria* species and the enterotoxins from *Bacillus* species.^{16,37,38} This cytotoxicity assay can also differentiate the presence of viable pathogenic *Listeria* species from nonpathogenic ones.^{16,39–42}

Adherent and nonadherent mammalian cell lines of various tissue origins were used previously to distinguish differential cytotoxic response of virulent *Listeria* species from avirulent ones.^{16,43–45} The cytotoxic properties were determined by monitoring the changes in cell morphology,⁴² formation of plaques in a monolayer,^{43,45} or dye uptake due to loss of membrane permeability.⁴⁴ The drawbacks of many of these assays are prolonged assay time (16–24 h) and lack of quantitative data; thus, they are undesirable for incorporation into biosensor platform for detection of *Listeria*. In contrast, the Ped-2E9 cell-based cytotoxicity assay for biosensing has several advantages: cells are highly sensitive to cytotoxin, thus the cell-based assay is very rapid (1–6 h),^{39,46,47} and provides both quantitative as well as qualitative information about the presence of pathogens or toxins. This assay measures the activity of enzymes such as alkaline phosphatase (ALP) or lactate dehydrogenase released by the hybridoma cells infected with pathogen or toxins^{16,37–39,46,47} making the Ped-2E9-based assay a robust assay system that can easily be integrated into an automated sensing platform. The rapid detection of viable pathogenic *Listeria* species and enterotoxin from *Bacillus* from food, clinical and environmental samples are important from commercial and biosecurity perspectives.

In this paper, we report a new design fabrication mode of a portable CBB platform using collagen-encapsulated Ped-2E9 cells for rapid detection of pathogenic *Listeria* and *Bacillus* species and the toxins from these organisms. By applying a novel method using laser scanning cytometry we determined that the bacteria- or toxin-mediated death of gel-entrapped mammalian cells can be directly correlated to an analytical cytotoxicity assay.

MATERIALS AND METHODS

Reagents and Biochemicals

Type I collagen (rat tail, collagen-I) was purchased from BD Bioscience (San Jose, CA, USA). Dulbecco's modified Eagle's mediums with phenol red (DMEM) or without phenol red (DMEM-phenol red free, DMEM-PRF) were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Norcross, GA). Fluorescence dyes, propidium iodide (PI), Hoechst 33342, acridine orange (AO) and a ready-to-go ALP liquid substrate were purchased from Sigma-Aldrich (St Louis, MO, USA). All media and reagents for bacteriological growth studies were purchased from Difco Laboratories (Sparks, MD, USA).

Preparation of Collagen Gel

Collagen type I gels were prepared by mixing appropriate volumes of collagen solution with $10 \times$ phosphate-buffered saline ($10 \times$ PBS, Hyclone, UT, USA) and 1 N NaOH in precooled eppendorf tubes. The final concentrations of the collagen in the gel matrix were varied from 0.7 to 2.5 mg/ml to obtain an appropriate collagen sieve diameter. An average diameter of 5–7 μm is considered as optimal for the present application (average bacterial diameter, 2 μm and Ped-2E9 cell diameter, 10 μm). After addition of the reagents the gel solutions were kept at 37°C for 15–30 min to complete the gelation.

Determination of Gel Matrix Pore Size by Cryo-Scanning Electron Microscopy

To determine the pore size of the gel matrix sieves (with different collagen concentrations from 0.7 to 2 mg/ml), we performed a cryo-scanning electron microscopy (SEM) analysis with minimal dehydration of the gel matrix. The gel preparations were mounted on a slit holder and plunged into liquid nitrogen slush. A vacuum was pulled and the samples were transferred to the Gatan Alto 2500 (Pleasanton, CA, USA) prechamber (cooled to approximately –60°C). After the samples were cut with a microtome with a cold knife, the samples were sublimated at –85°C for 30 min followed by sputter coating for 100 s with platinum. The samples were then transferred to the microscope cryostage (approximate temperature: –130°C) for imaging. The SEM images were taken with an FEI NOVA nanoSEM (FEI, Hillsboro, OR, USA) using ET and TLD detectors operating at 5 kV accelerating voltage, with approximately 6 mm working distance and the magnifications were varied between $\times 2500$ and $\times 50\,000$.

Mammalian Cell Culture

Lymphocyte origin murine Ped-2E9 cells³⁹ were routinely cultured in DMEM supplemented with 10% FBS (DMEM + FBS) at 37°C in 7% CO₂. Liquid-nitrogen-stored Ped-2E9 cells (passage numbers between 5 and 10) were suspended at a ratio of 1:10 in DMEM + FBS and grown for 72 h. Ped-2E9 cells were passaged again in DMEM with or without phenol red supplemented with 2.5% FBS¹⁶ in T-25 and T-75 flasks (Nunc, Denmark) until cells reached to the logarithmic phase of growth (usually 72 h) after which they were used for experiments.

Encapsulation of Ped-2E9 Hybridoma Cells in Gel Matrix

The general approach of encapsulating the Ped-2E9 cells in collagen gel for biosensing is described in Figure 1. Log-phased grown Ped-2E9 cells were collected, counted, washed, and about 2.5×10^7 viable cells/ml were resuspended in complete serum-free medium (SFM). The cell suspension was mixed with neutralized type I collagen (final pH was adjusted approximately to 7 by adding 1 N NaOH) at a final concentration of 1.5 mg collagen/ml. A volume of 30–100 μl /

Figure 1 Strategy for cell-based biosensing using collagen-encapsulated Ped-2E9 hybridoma cells. Schematic design of the gel-entrapped Ped-2E9 cell in multi-wells and the assay procedure.

well of cell suspensions in collagen were dispensed in 96- or 48-well plates to obtain a gel thickness of 30–50 μm , respectively, and the plates were maintained at 37°C for 15–30 min to form a firm gel. The gel thickness of 30–50 μm was used throughout this study because the laser scanning cytometer (LSC), which was used to verify the viability of collagen-trapped Ped-2E9 cells, has a vertical scanning (penetration) depth range of 30–50 μm . DMEM-PRF with 2.5% FBS was added to each well and the plates were maintained at 37°C in a humidified incubator with 7% CO_2 .

Viability of the collagen-suspended cells was enumerated and recorded at every 24 h for a period of 5 days by Trypan blue (TB) dye (0.4%) exclusion test or with incorporation of fluorescence dyes, Hoechst and PI. For enumeration of viability using TB, the cells were dislodged by treating the collagen matrix by 100 $\mu\text{g}/\text{ml}$ *Clostridium histolyticum* collagenase (Sigma) for 30 min at 37°C. The dislodged cells were then incubated with 0.4% TB solution for 1–3 min. The hybridoma cells were counted using an inverted light microscope (Olympus, Tokyo, Japan). The viable cells excluded the dye whereas the nonviable ones took up the dyes and appeared blue.

Laser Scanning Image Cytometry

Image cytometry was used⁴⁸ to evaluate the absolute count and the percentage of viability of the gel-encapsulated Ped-2E9 cells *in situ*. The live or dead cell counts were obtained directly from gel matrix without physical disruption of the collagen gel. Briefly, a 50 μl of mixed dye (containing 50 $\mu\text{g}/\text{ml}$ PI and 20 $\mu\text{g}/\text{ml}$ Hoechst) was added directly to the wells of a 96-well plate (Costar 3603, Corning, NY, USA) containing collagen-encapsulated Ped-2E9 cells and incubated for 1 h at 37°C. Subsequently, the wells were washed twice with SFM, and after the second wash, 50 μl of SFM was added to each well containing gel-encapsulated cells and the laser scanning image cytometry (LSC) analysis of live/dead cell counts were performed with an iCys research imaging cytometer (CompuCyte, Cambridge, MA, USA). This instrument is based on an inverted fluorescent microscope and

enables the quantitative analysis of adherent or immobilized cells.^{48,49}

For the current analysis, the nuclei of both, viable and dead cells were stained using Hoechst 33342 and were excited using a violet laser (405 nm). The nuclei of nonviable (dead) cells were exclusively stained using PI and excited with an Argon laser (488 nm). Both PI and Hoechst were used as parameter for identifying gel-encapsulated cells in 96-well assay plates. Within each well an area of 2500 μm^2 was analyzed, yielding a sufficient cell count for a statistical comparison.

Bacterial Cultures and Toxin

Frozen stock of *Listeria monocytogenes* strains Scott A (serotype 4b), V7 (serotype 1/2a) and *L. innocua* F4247; *Bacillus cereus* strains (A926, MS1-9, F4810) and *B. subtilis* were grown in brain heart infusion (BHI) broth at 37°C and subcultured twice. Fresh cultures (1 ml each) were centrifuged (13 000 g for 10 min) at room temperature. The cell pellets were washed twice in filter sterilized (filter pore size 0.45 μm) cell phosphate-buffered saline (C-PBS, 0.14 M NaCl, 5 mM KCl, 3 mM Na_2HPO_4 , 4 mM NaH_2PO_4 , 10 mM glucose (pH 7.2)), or in presterilized serum-free DMEM-PRF and resuspended in 1 ml of C-PBS or in DMEM-PRF, respectively.

Crude toxins from *Bacillus* species, prepared from bacterial cell-free culture supernatants as described previously,³⁷ were used for cytotoxicity assays immediately or stored at 4°C for not more than 48 h.

Listeria cultures were grown in 50 ml of BHI broth treated with 0.5% charcoal for 24 h at 37°C under shaking conditions (140 r.p.m.). Cells were pelleted by centrifugation and the cell-free supernatant was filter-sterilized (0.45 μm) and was concentrated to 1 ml using a 50-kDa cutoff membrane in Amicon protein concentrator (Millipore, Bedford, MA, USA). The presence of listeriolysin O (LLO) protein was confirmed by SDS-PAGE analysis and by examining the zone of hemolysis on sheep blood agar plate (BD-BBL, Franklin Lakes, NJ, USA). The protein concentrations of the *Bacillus* crude toxin preparation and LLO were determined by using a Bio-Rad protein assay kit following the manufacturer's protocol (data not shown).

Measurement of Cytotoxicity

Collagen-I-encapsulated Ped-2E9 cells were grown for different incubation times (24–96 h) in 48 or 96-well plates. Spent medium from each well was aspirated without disturbing the gel and resuspended in serum-free DMEM-PRF. The cytotoxicity effects of bacterial cells or the toxin preparations on collagen-encapsulated Ped-2E9 cells were measured by an ALP release assay as described previously.⁴⁰ Briefly, 50–100 μl of bacteria cell suspension (1×10^9 CFU/ml) or crude toxin preparations (~ 20 $\mu\text{g}/\text{ml}$, 50–100 μl) were added to each well of the 96- or 48-well microtiter plate, respectively, and held at 37°C in a humidified incubator with

7% CO₂ for a period of 3–6 h. The supernatant from the 48-well plate was collected, centrifuged (1800 g for 5 min) and placed in a mini-cuvette containing a ready-to-go ALP liquid substrate (Sigma). The color development was recorded at 3–5 min intervals at absorbance of 405/595 nm using a handheld USB2000 mini-spectrophotometer (Ocean Optics, Dunedin, FL, USA). For direct measurement of cytotoxicity on plates, a 100- μ l of ALP liquid substrate (Sigma) was added to each well of 96-well plate and were read after 3–5 min of incubation at room temperature at absorbance of 405/595 nm using a Bio-Rad plate reader (Hercules, CA, USA). The plates were further incubated at 37°C for about 5–15 min to observe for the development of a visible yellow color (positive cytotoxicity). The percent values of cytotoxicity were obtained by a formula described earlier.^{16,40}

To confirm the functional significance of the choice of optimal concentration of collagen (1.5 mg/ml with an approximate gel sieve or pore diameter of 6–7 μ m, as per our SEM data) for encapsulation of the Ped-2E9 cell and for the cytotoxicity assay, we tested two different concentrations of collagen, one lower (1 mg/ml with an approximate diameter of 9–10 μ m) and one higher (2 mg/ml with an approximate pore diameter of 1–2 μ m). Also, to assess how the different multiplicity of infections (MOIs) ratio of *L. monocytogenes* may affect the Ped-2E9 cell cytotoxicity results, two lower MOIs (50:1 and 10:1) were tested along with the 100:1 ratio, at 3 and 6 h postinfection time points. Furthermore, a comparison of two-dimensional (2D) conventional Ped-2E9 cell assay^{16,37–40} was done with the three-dimensionally encapsulated Ped-2E9 cells in collagen matrix to assess whether 3D encapsulated system produces similar assay results. We also performed cryo-SEM analysis with infected hybridoma cells following the same procedures mentioned above.

LSC analysis was carried out in 96-well plates following the same procedure as described above for viability measurement. A parallel TB live–dead count (described earlier in the present article) was taken in duplicate 96-well plates for comparisons with the live–dead counts obtained from LSC analysis.

Determination of Cell Death by Apoptosis or Necrosis

Ped-2E9 cell death by apoptosis or necrosis were determined by using a fluorescence microscopy described previously^{16,50,51} with some modifications.^{52,53} Briefly, a cell staining solution containing 20 μ g/ml of AO and 100 μ g/ml of PI (Sigma) was prepared in C-PBS. The collagen-entrapped Ped-2E9 cells (exposed to bacterial cells or toxins, as described in previous sections) were dislodged by collagenase treatment as above. Aliquots of 100 μ l of cell suspension (1 to 2 $\times$ 10⁶/ml) was mixed with 100 μ l of staining solution and analyzed immediately with a fluorescence microscope (Leica, model DMLB, Wetzlar, Germany, with SPOT software, version 4.6.4.2, Diagnostic Instruments, Sterling Heights, MI, USA) using green (for AO) and red filters (for PI). The

detection of live (L), apoptotic (A) and necrotic (N) cells were carried out in the following manner, green (live), yellow or orange (apoptotic), red (necrotic), both by visual scoring on a hemacytometer and by using image analysis softwares, SPOT, version 4.6.4.2 (images acquisition) and ImageJ v1.38 (NIH, USA) with ‘color counter’ (v2001) and ‘color histogram’ plug-ins (v2007) to analyze and enumerate the images.

Statistical Analysis

Data are expressed as mean $\pm$ standard error of mean (s.e.m.). Statistical analyses of data were performed using GraphPad Prism (version 3.02, GraphPad Software, San Diego, CA, USA). Comparisons of cytotoxicity values between single control and pathogen- and toxin-exposed sample means were made using two-tailed Student’s *t*-test. The limit for statistical significance was set at either *P* < 0.05 or *P* < 0.01 as mentioned.

RESULTS

Viability of Ped-2E9 Cells in Collagen Microenvironment

The collagen matrix microenvironment was fabricated such that the pore diameter of the gel matrix sieve is maintained between 5 and 7 μ m. The cryo-SEM photographs confirmed that a collagen gel concentration of 1.5 mg/ml optimally achieves the desired pore size.

After encapsulation of the hybridoma cells in the collagen matrix, we determined the viability of the gel-encapsulated Ped-2E9 cells by TB staining and LSC methods. Approximately 82–85% Ped-2E9 cells remained viable after 48 h of encapsulation in collagen I matrix (Figure 2). A progressive decrease in viability was observed for the period of 48–96 h. Overall, the viability of the gel-encapsulated cells over a period of first 96 h was found to be comparable to cells grown in DMEM-PRF with 2.5% FBS without gel matrices (data not shown).

Figure 2 Viability assay of gel-encapsulated Ped-2E9 cells. Viability assessment of the gel-encapsulated Ped-2E9 cells over a period of 5 days (120 h). The percent viable cells count were obtained from Trypan blue dye exclusion method (TB) and by laser scanning cytometry (LSC) as described in the Materials and methods section. Values are presented as mean $\pm$ standard error of mean (s.e.m.) of three experiments carried out in duplicate.

Pathogen- or Toxin-Induced Cytotoxicity of Gel-Encapsulated Ped-2E9 Cells

The cytotoxic response of collagen gel-encapsulated hybridoma cells was assessed by assaying ALP released from the damaged hybridoma cells (as described in Materials and Methods). Along with the quantitative enzyme assay, an SEM visualization of Ped-2E9 cell damage and LSC quantification of cytotoxicity based on fluorescence imaging data was also performed.

Measurement of cytotoxicity by ALP release assay

Collagen-encapsulated Ped-2E9 cells in 48-well plates exposed to either bacterial cells or the toxin preparations were assayed for ALP-based cytotoxicity at two different time points postinfection (Figure 3a). Toxin preparations from pathogenic *L. monocytogenes* Scott A and *B. cereus* A926 showed approximately 92 and 98% cytotoxicity, respectively in 3 h; whereas toxin preparations from nonpathogenic *L. innocua* F4247 and *B. subtilis* showed only 2 and 8% cytotoxicity, respectively (Figure 3a). When whole bacterial cells were used (at an approximate MOI of 100 bacteria to 1 Ped-2E9 cell, MOI=100:1), the cytotoxicity caused by *L. monocytogenes* strains Scott A and V7 was found to be approximately 38 and 34% (3 h postinfection) and 72 and 68% (6 h postinfection), respectively. In contrast, the nonpathogenic *L. innocua* F4247 induced only 0.06 or 0.4% cytotoxicity during the same time period (Figure 3a). A visual examination of the 96-well plate also revealed that the intensity in the change of color with pathogenic vs nonpathogenic bacteria or toxins was obvious (data not shown). This observation was confirmed by the direct reading of the plate in a plate reader (Figure 3a). These data are in agreement with our previous study.⁴⁰ Our data also indicate that *B. cereus* F4810 shows lower hybridoma cytotoxicity (24%) as compared to *B. cereus* strains A926 and MS1-9. This difference may be due to strain variation and in agreement with the previous report.³⁷

In the present study, we also tested lower MOIs, 50:1 and 10:1. With *L. monocytogenes* strains Scott A and V7 at MOI of 50:1 and 10:1, at 3 h postinfection, the cytotoxicity values obtained from treatments of these pathogenic strains were significantly higher ($P<0.05$ or $P<0.01$) when compared with negative control, nonpathogenic *L. innocua* F4247 at all postinfection time points (3 and 6 h; Figure 3b) but significantly lower ($P<0.05$ or $P<0.01$) than MOI of 100:1.

Measurement of cytotoxicity by LSC studies

Cellular viability data (as depicted by, PI and Hoechst staining) of the gel-entrapped Ped-2E9 cells (1, 3 and 6 h postinfection) obtained from LSC analysis also supported the cytotoxicity data (Figure 3a–c) obtained by ALP assay. When the percent PI-positive Ped-2E9 cell counts, designated as 'LSC (PI+)', were compared with percent TB positive, 'TB(+)' they showed a very close numerical values. For

example, at 3 and 6 h postinfection, *L. monocytogenes* V7 caused approximately 23 and 81% of Ped-2E9 cells to become PI positive in LSC, whereas it caused approximately 19 and 75% of Ped-2E9 cells to become TB-positive (Figure 3c). Similarly, the crude toxin preparations from *B. cereus* MS1-9 showed very close numerical values of LSC (PI+) and TB (+) (Figure 3c). The percent values of the cytotoxicity (ALP released, calculated as per formula described in the Figure 3a legend) were in agreement with the PI-positive or TB-positive cell numbers (Figure 3c), indicating that higher the cellular death (ie, higher the PI+ and TB+ values), higher is the calculated values of cytotoxicity. Representative laser scanning images (as depicted in Figure 4a–f) show an increase in PI-positive (dead) hybridoma cells (stained red) over PI-negative but Hoechst-positive live cells (blue). While Figure 4 (g–i) are representative scattergrams for numerical analysis of the live and dead counts of cells in the x - y positions as depicted by Hoechst and PI channels.

3D vs 2D system of Ped-2E9 cell cytotoxicity assay: effect of collagen entrapment and gel matrix pore size (gel concentrations)

When compared to the conventional 2D Ped-2E9 assay system (without collagen), the 3D collagen matrix system showed similar characteristics (Figure 5a). For example, when *L. monocytogenes* Scott A cells were used at MOI 100:1, the percent cytotoxicity values in 2D and 3D assay formats were 46.2 and 37.9 (at 3 h postinfection), respectively. At 6 h postinfection the values were 85.8 (2D) and 72.2 (3D) (Figure 5a). In the case of *B. cereus* toxins, we observed the same trend; for example, at 1 h postinfection time point, toxin of *B. cereus* MS1-9 strain showed percent cytotoxicity values of 45.8 (2D) and 33.7 (3D), at 3 h postinfection, the same strains showed 97.7 (2D) and 88 (3D) percent cytotoxicity (Figure 5a). We found no statistically significant differences ($P>0.05$) when we compared the percent cytotoxicity values by 2D vs 3D assay formats.

In the present study, we measured the pore size of the collagen matrices with different concentrations of collagen by cryo-nano SEM studies, and it was found that a collagen gel containing 1.5 mg/ml collagen would have an average gel sieve or pore diameter of about 5–7 μ m (mean = 6 μ m) as described above. Further, we examined the functional suitability of the selected collagen concentration by performing a comparative study with two different collagen concentrations, 2 and 1 mg/ml at three different postinfection time points (Figure 5b). Our data revealed that when whole bacterial cells were used, the cytotoxicity values obtained from a collagen gel matrix with 1.5 mg/ml collagen at 3 and 6 h postinfection time points were significantly ($P<0.05$) higher than those of the other two gel concentrations (Figure 5b). When LLO and toxins from *B. cereus* MS1-9 were tested, we found no significant difference ($P>0.05$) in cytotoxicity values with 1.5 or 2 mg/ml collagen gels at 1, 3 and 6 h

Figure 3 Assessment of Ped-2E9 cell cytotoxicity induced by pathogenic *Listeria* or *Bacillus* toxin. (a) Quantitative values of the cytotoxicity were calculated by alkaline phosphatase (ALP) released from damaged Ped-2E9 cells resulted from exposure to bacteria cells or toxin preparations (tox). The x axis legends are: LmSA, *L. monocytogenes* Scott A; LmV7, *L. monocytogenes* V7; Lin, *L. innocua* F4247; LmSA (tox), *L. monocytogenes* Scott A toxin; Lin (tox), *L. innocua* F4247 toxin preparation; Bc1, *B. cereus* A926; Bc2, *B. cereus* MS1-9; Bc3, *B. cereus* F4810 and Bs, *B. subtilis*. The percent cytotoxicity was reported based on ALP release which was calculated using formula:

$$\left[\frac{A_{\text{experimental value}} - A_{\text{control (media)}}}{A_{\text{control (Triton-X)}} - A_{\text{control (media)}}} \right] \times 100.$$

Values are average of two separate experiments carried out in duplicate; values are presented as mean $\pm$ standard error of mean (s.e.m.). (b) Comparisons of Ped-2E9 cell cytotoxicity induced by *Listeria* whole cells infected at different multiplicity of infections (MOIs). Cytotoxicity assessment of three different MOIs, 10:1, 50:1 and 100:1 at two different postinfection time points (3 and 6 h). The percent cytotoxicity values (ALP assay) were calculated by the above mentioned formula. Columns, mean; bars, s.e.m. * $P < 0.05$; ** $P < 0.01$. (c) Quantitative comparisons of pathogen or toxin induced Ped-2E9 cell cytotoxicity by three methods, laser scanning cytometry (LSC), Trypan blue dye exclusion (TB) and ALP release cytotoxicity assay. On y axis, percentage values of gel-entrapped PI-positive Ped-2E9 cells obtained from LSC analysis, reported as LSC (PI+); percent values of Trypan blue-positive Ped-2E9 cell, designated as TB (+); and percent cytotoxicity values (ALP assay) as calculated by the above mentioned formula and designated as Cyt (%). The x axis legends are: uninfected, Ped-2E9 cells which are not infected with pathogens or toxin; Lm V7, *L. monocytogenes* V7; and Bc toxin, crude toxin preparation from *B. cereus* MS1-9. The measurements were carried out and reported at 1, 3 and 6 h postinfection. Values are presented as mean $\pm$ standard error of mean (s.e.m.) of three experiments carried out in duplicate.

Figure 4 Representative laser scanning cytometry images of Ped-2E9 cells entrapped in gel. The images (a–f) are shown as merged and virtually colored images of blue (Hoechst 33342, 405 excitation line) and red (PI, 488 excitation line) as obtained by the photomultiplier of the iCys LSC. The blue cells were counted as ‘live’ whereas the red cells were counted as ‘dead’. (a) Control, uninfected Ped-2E9 cells; (b) and (c) infected with *L. monocytogenes* V7 cells (approximate multiplicity of infection of 100 bacterial cells to 1 Ped-2E9 cell) 3 and 6 h postinfection, respectively; (d) control (without toxin); (e and f) infected with crude toxin from *B. cereus* MS1-9 (approximate total protein concentration of the crude toxin preparation was 20 µg/ml, and used in 1:2 dilution) 1 and 3 h postinfection, respectively. The scattergrams (g–i) represent the x–y positions of cells identified as live (as indicated by the presence of a Hoechst signal, upper row) and cells identified as dead (as indicated by propidium iodide signal, lower row); (g) control cells (high viability) (h) infected with *L. monocytogenes* V7 cells, 3 h postinfection (medium viability); (i) infected with crude toxin from *B. cereus* MS1-9, 3 h postinfection (low viability).

postinfection time point, but, the cytotoxicity values with lower gel concentrations (1 mg/ml) were significantly ($P < 0.05$) low at the same time points (Figure 5b), and this was attributed to the fact that some Ped-2E9 cells may have been lost during washing and medium replenishment step (data not shown, the cell retention within the collagen matrix was enumerated by cell count methods as described earlier).

Visualization of Ped-2E9 cell membrane damage by pathogens or toxins and cytotoxicity by SEM study

The time dependence of the pathogen and toxin induced damage of the gel-encapsulated Ped-2E9 cells were evident by SEM images (Figure 6). A progressive damage of the cell membrane can be observed with the increase of the infection time. Figure 6b depicts the attachment of *L. monocytogenes* V7 cells on gel-encapsulated Ped-2E9 cells, while a complete disruption of cell membrane and

morphology can be observed at 3 h postinfection with *B. cereus* MS1-9 toxin (Figure 6f).

Assessment of apoptosis vs necrosis of Ped-2E9 cells by pathogens or toxins

The results of the apoptosis and necrosis analysis revealed that exposure to viable *L. monocytogenes* Scott A cells caused apoptosis in approximately half (50%) of the Ped-2E9 cell population (Figure 7b), while about 84% Ped-2E9 cells underwent necrotic death when exposed to *B. cereus* MS1-9 toxin (Figure 7d). LLO from *L. monocytogenes* Scott A induced approximately 63% necrotic and 26% apoptotic cell death (Figure 7c). In control cells (Figure 7a), a majority of Ped-2E9 cells were viable (90%) and 7% cells were necrotic and only 3% were apoptotic. The propensity of Ped-2E9 cells to undergo apoptotic cell death when exposed to *Listeria* cells as revealed by the current study is in agreement with previous observations.^{16,54}

Figure 5 Three-dimensional (3D) vs two-dimensional (2D) system of Ped-2E9 cell cytotoxicity assay—effect of collagen entrapment and gel matrix pore size (gel concentrations). **(a)** Comparison of percent cytotoxicity values in 3D culture system with 2D controls in three different time points, 1, 3 and 6 h. The percent cytotoxicity values (alkaline phosphatase (ALP) assay) were calculated by the above mentioned formula. The legends are: LmSA, *L. monocytogenes* Scott A cells; LmV7, *L. monocytogenes* V7 cells; Bc1, *B. cereus* A926 toxin and Bc2, *B. cereus* MS1-9 toxin. The suffixes, 2D, two dimensional formats without the gel; 3D, three dimensional formats with collagen gel. Bars, s.e.m. **(b)** Three different collagen concentrations were tested as encapsulating matrices for Ped-2E9 cells. The x axis legends are as follows: LmSA, *L. monocytogenes* Scott A; LmSA (tox), *L. monocytogenes* Scott A toxin; Bc1 (tox), toxin from *B. cereus* MS1-9. The values in parenthesis is the approximate diameter of the collagen sieve (pore size in μm) as estimated by scanning electron microscopy (described in Materials and Methods section). The percent cytotoxicity values (ALP assay) were calculated by the above-mentioned formula. Columns, mean; bars, s.e.m. * $P < 0.05$.

DISCUSSION

The advent of biosensor-based technology has created an immense opportunity to probe various biochemical and cellular interactions more vividly. Cell-based sensors are often preferred to other kinds of recognition system as living cells best mimic a physiological situation.^{9,55} Due to the inherent advantages of CBBs in screening of ‘active’ (eg, live

pathogenic bacteria) vs ‘nonactive’ (eg, heat killed pathogenic bacteria) biological entities, the application of this types of biosensors are increasing rapidly, specially in clinical and environmental diagnostics, food safety, biosecurity and in advanced pathological applications.^{4,5,9,10,16}

In our study, we have shown that a B-cell hybridoma, which normally grows in suspension, can be immobilized in collagen matrices. Further, we have also shown qualitatively as well as quantitatively that these gel-encapsulated cells can be effectively used as a sensor device to detect the presence of pathogenic microorganisms or their toxins. This study also incorporates a novel method, used for first time to determine the viability of gel-encapsulated cells *in situ* using a LSC-based technique. This application of hybridoma cells instigates further studies to improve the detection limit by modulating collagen concentrations or altering the encapsulation procedures.

In the present study, while encapsulating the mammalian cells in collagen matrix, we found that there is a progressive decrease in cell viability for the period of 48–96 h (Figure 2). This decrease could probably be attributed to nutrient limitation, accumulation of toxic metabolites or diffusion limitation in the gel matrix.^{56,57} Second, when the cytotoxicity data collected, after 3 h of exposure to toxin or whole bacterial cells, showed lower cytotoxicity value when compared with 6 h postinfection values (Figure 3a). It was also observed that the cytotoxicity values were slightly lower but not statistically significant in 3D collagen-entrapped assay format as compared to 2D control without collagen (Figure 5a). This may be due to the slower diffusion of toxin molecules or released enzymes and substrates in the gel as compared to the liquid system.⁵⁷ Moreover, the gel matrix sieve size is a limiting factor for bacterial entry (Figure 5b). The requirement of a longer postinfection incubation time with intact bacterial cells, and the lower concentrations of detectable ALP may be attributed to a collagen concentration, which may hinder bacterial passage in the gel matrices and to reach to hybridoma cells. Thus, we indirectly quantified the effect of 3D gel matrix on the cytotoxicity by comparing cytotoxicity data with 2D system in absence of collagen matrix (Figure 5a). The observations made in the comparative 2D and 3D systems revealed a slight decrease in cytotoxicity values, although statistically insignificant, for 3D system, is thought to be due to possible diffusion limitation (Figure 5a). This diffusion limitation can be attributed to the entry of bacteria and toxin into the gel matrices, the interaction of bacteria and toxin with the Ped-2E9 cells and thereby causing cell damage, and the release of cellular ALP and its diffusion out of gel matrix.

Overall, our data suggest that B-lymphocyte origin Ped-2E9 hybridoma cell line can effectively be encapsulated in the collagen matrix. The advantage of using collagen type I is that it is readily available and is used widely by many investigators and has been standardized as a gel scaffold.^{31–34} Both collagen I and collagen II are fibrillar collagens and share high degree

Figure 6 The cryo-SEM images of pathogen- or toxin-induced damage of Ped-2E9 cells in collagen gel matrix. (a) Uninfected Ped-2E9 cell encapsulated in collagen gel; (b and c) depict the bacteria and mammalian cell interactions; (d-f) show the damage of Ped-2E9 cells upon exposure to toxins from *B. cereus* MS1-9 and listeriolysin O (LLO) of *L. monocytogenes* Scott A in different time intervals.

Figure 7 Quantitative analysis of apoptotic and necrotic Ped-2E9 cells induced by pathogenic *Listeria* or *Bacillus* toxin as depicted by intake of fluorescence dyes acridine orange (AO, green) and propidium iodide (PI, red). Cells were dislodged from collagen matrices by treating with collagenase and were mixed immediately with dye solutions (Materials and methods section). Fluorescence micrograph images (merged images of green and red panel) are from different treatments such as (a) uninfected Ped-2E9 cells; (b) *L. monocytogenes* Scott A whole cells; (c) listeriolysin O (LLO) of *L. monocytogenes* Scott A; (d) toxins from *B. cereus* MS1-9. Green fluorescence (AO) indicates viable or live cell population (marked as L), red fluorescence (PI) indicates dead cells (necrotic, marked as N), and yellow or orange fluorescence (merger of AO + PI) indicates either proapoptotic or late apoptotic cells (marked as A). An average cell count of 400 ($n = 400$) per sample per treatment was counted manually (hemacytometer) and by ImageJ software. Values reported are mean of three independent experiments. Bar = 20 µm.

of similarities in amino-acid sequence and physiological properties^{31–34} further aided in our decision to use collagen type I. We also used Ultra Pure™ agarose (Invitrogen) as an entrapment agent, but the results were unsatisfactory. Agarose (0.7–1.5%)-encapsulated Ped-2E9 cell viability was very poor; greater than 75% of the Ped-2E9 cells were dead after 24 h of encapsulation, and 98–100% cell death was observed after 48 h (data not shown). Thus, agarose was not considered for further evaluation.

Pathogenic microorganisms or toxins have already been shown to cause membrane disruption and apoptotic cell death in lymphocytes⁵⁸ and in B cells.⁵⁴ Previously, we have shown *Listeria*-mediated apoptotic cell death of Ped-2E9 cell, B-cell origin,⁵⁴ which is verified further in this study (Figure 7). As revealed by the SEM images (Figure 6), the cell membrane damage of gel-encapsulated Ped-2E9 cells caused by bacterial cells or toxins show close similarity with our earlier studies,^{47,54} indicating that, the cellular and

physiological characteristics of both the Ped-2E9 cells and the pathogens remained unaltered in a collagen gel micro-environment. This finding is particularly important as the viability of mammalian cells after immobilization in biocompatible matrices is the key factor for cell-based assays.^{15,27,32,35}

In the present study, we have shown that Ped-2E9 cells (which grow in suspension) can be immobilized three-dimensionally into a biocompatible collagen gel matrix without losing their biological and physiological properties of sensitivity to pathogenic *Listeria* and *Bacillus* cells or toxins. This 3D immobilization also reduces the normal 2D assay steps such as centrifugation, critically important for the integration of these cell types in a biosensor platform. Further, we speculate that, this immobilization technique will pave the way for convenient designs of future CBBs and easy to use cell-based assay systems. Finally, we conclude that gel-immobilized mammalian cells have a tremendous potential to be used as cell-based sensing system to interrogate with pathogenic organisms, toxins and toxic chemicals for food safety, biosecurity, clinical and pathological applications.

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