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Chemical toxicity detection using quantum dot encoded *E. coli* cells

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ABSTRACT

Bioconjugated quantum dots (QDs) with *E. coli* cells (bioconjugates) were employed as fluorescent switches that turn-off instantly against any cellular-stress caused by a toxic chemical. Paraquat (PQ), H_2O_2 and triton X-100 were used as models for assessing their toxicities on bioconjugates. These chemicals interacted on the cell-surfaces where QDs are harbored. The extent of toxicity imposed by chemicals on bioconjugates was successfully probed by (i) real-time fluorescence signals, (ii) visible changes upon UV-light illumination and (iii) scanning electron microscopic (SEM) analysis. Hierarchical cluster analysis using kinetic data of fluorescence and viable cell numbers showed a close relationship between structurally different compounds having similar toxic effects, such as PQ and H_2O_2, both induced toxicities through generating reactive oxygen species (ROS). In contrast, triton X-100 disrupted the cell–wall integrity and thus showed distinct response due to the loss of cell-bound QDs. Increasing cellular toxicity with chemicals thus followed the order PQ < H_2O_2 < TX100 confirming the inherent nature of model chemicals to induce cellular toxicity. Our results demonstrated a facile optical strategy that enables rapid and real-time cytotoxicity screening of potentially hazardous chemicals, such as new drugs that lead to ROS generation.

1. Introduction

QDs have been successfully used as labeling probes for fluorescent resonance energy transfer (FRET) [1], *in vitro* and *in vivo* imaging [2,3], immunoassay [4,5] and DNA hybridization [6] that hold promising biological and biomedical applications. Carboxylated QDs are of great demand as these can be conjugated to the amino groups of biomolecules, such as proteins, enzymes or antibodies. This linking chemistry is simple, rapid and it is widely used in certain biosystems [7]. Conjugation efficiency can be improved by chemically modifying either surface charge states of proteins [8] or surface modification of QDs [9]. Repulsion between QDs and proteins can be prevented by providing a high ionic strength or higher concentration of protein in conjugation processes, and in this case, the luminescence intensity of QDs partly be quenched in solution with high ionic strength [10]. Labeling of QDs on living cells without affecting their cellular integrity can provide valuable information on their behavior, interaction with their surroundings and external stimuli or perturbations. QD-cell bioconjugates can serve as mobile fluorescent switches whose responses can be easily traced by their fluorescence emission properties.

Several *in vitro* cytotoxicity tests have been reported for more direct measurements of cell rupture or biomarkers of cell leakage. These include Neutral red assays (NRU) [11], Red blood cell lysis assay [12], Fluorescein leakage assay [13], Neutral red release [14] and use of Chinese Hamster lung (CHL) and ovary (CHO) cell lines [15,16]. These methods require cell-breakage and pretreatment of the samples. Intact bacterial cells can be used as whole-cell sensors because of their sensitivity and susceptibility toward external stress (stimuli) or toxic chemicals. Such responses can be utilized as signature algorithms to assess or predict toxicity of chemicals that could replace the animal models [17]. Toxicity response generated by bacterial cells is often determined in terms of various stress responses that induce change in cellular structure and morphology. The stress responses in bacteria are classified into different types based on the nature of toxicity exerted by a chemical compound [18,19]. Systemic approaches such as by use of animal models often fail to provide such information on cellular stresses that often are reflected as undetectable side-effects. In order to address these side-effects, there is a strong need to develop a simple, rapid and robust method to assess the cytotoxicity of chemicals or drugs that potentially exhibit side-effects.

This study provides a proof-of-concept using developed bioconjugates for rapidly assessing toxicities of potentially hazardous

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and membrane permeable chemicals. In our previously published work, we reported on testing the toxicity of CNTs using bioconjugates that mechanically disrupted the cells, but the chemical or biochemical toxicities in cells remained unclear [20]. Therefore, in this paper, similar bioconjugates were utilized to detect chemical toxicity that not necessarily required collapsing the cell-structure for detection. The paper also reports on the detection of variety of cellular-stresses using bioconjugates upon exposure to carefully chosen model chemicals, such as PQ, H_2O_2 and triton-X100. Our method also enables a rapid qualitative analysis of cellular toxicity with UV-light illumination of bioconjugates in presence of toxic chemicals.

2. Experimental

2.1. Cells, chemicals and reagents

Wild-type *E. coli* DH5α strain was used as model living bacterial cells. Luria-Bertani broth (LB-broth) and Luria-Bertani agar (LB-agar) were obtained from Difco (MI, USA). *N*-hydroxysuccinimide (NHS), N-ethyl-N′-(3-(dimethylamino) propyl) carbodiimide (EDC), PQ and H_2O_2 were purchased from Sigma–Aldrich. Triton-X 100 was procured from Merck, Germany. Qdot® 585 ITK™ carboxyl quantum dots (Invitrogen) were used as labeling probes having emission maxima at ~585 nm. All other reagents used in this study were of analytical grade.

2.2. Apparatus

Bioconjugation process was carried out under sterile conditions in microbiological safety cabinet. The fluorescence spectral studies were carried out by using a NanoDrop 3300 Fluorospectrometer (Thermo Scientific). LEO Supra 35VP Scanning Electron Microscope (SEM) was used to acquire images of bioconjugates. All optical measurements were performed at room temperature under ambient conditions.

2.3. E. coli culture preparation

E. coli DH5α cells were freshly grown overnight in LB-broth at 37 °C with constant shaking at 125 rpm in an incubating orbital shaker and the cells were harvested by centrifugation at 3000 rpm for 5 min at 4 °C. The cells thus obtained were washed thrice with sterile PBS, pH-7.4 followed by centrifugation for 5 min at 3000 rpm at 4 °C. The cell pellet was resuspended in same buffer and colony forming units (CFU) were determined.

2.4. Bioconjugation of E. coli with QDs

Bioconjugation of QDs with *E. coli* cells was performed using EDC/NHS coupling chemistry as described previously [20]. Briefly, first ratio of QDs attachment to cells was optimized using following procedure. A mixture of 50 mM EDC and 5 mM NHS was first incubated with different concentration of Qdot® 585 ITK™ ranging 0–32 nM for 5 min at 25 °C (Table S1). This mixture was quickly added to the cell-suspension and the entire reaction mixture was incubated at 25 °C for 15 min. The reaction mixture was centrifuged at 1500 rpm for 3 min and the supernatant was removed. The cell-pellet thus obtained was washed thrice with PBS, pH-7.4 followed by centrifugation and finally stored at 4 °C until further use. The optimized conditions for covalent coupling of QDs with cells was determined to be 16 nM of QDs for 1.2×10^9 colony forming units (CFU)/mL of *E. coli* cells. Thus obtained QD-conjugated cells were labeled as bioconjugates. Control reaction was carried out without adding EDC/NHS in cell-suspension in PBS.

2.5. Cell viability

Cell viability tests with bioconjugates were carried out after serial dilutions of cell-suspension in PBS followed by plating on LB-agar plates. Cell viability and shelf life of bioconjugates was measured by counting the CFUs.

2.6. Fluorescence measurements.

Relative fluorescence unit (RFU) signal from bioconjugates was measured using NanoDrop Fluorospectrometer scanning in range of 500–750 nm. The shelf-life of bioconjugates was also determined by fluorescence measurement after storing at 4 °C over a period of 2–4 months for their ability to emit fluorescence.

2.7. Chemical treatment

All the reagents used in present study were freshly prepared before use. Model chemicals, such as H_2O_2, PQ and Triton-X 100 were prepared in different concentrations (0.5, 1 and 5 mM) in PBS pH-7.4. These model chemicals were incubated with bioconjugates at different time intervals (1–3 h). After the incubation, fluorescence signal in the reaction mixture and cell-viability (CFU) were measured along with respective controls. Scheme 1 illustrates bioconjugation of QDs with *E. coli* and their stress responses to model chemicals.

2.8. Hierarchical clustering analysis

Hierarchical clustering using similarity matrix analysis was constituted with respect to kinetics of fluorescence emission (RFU) and number of surviving bioconjugates (CFU) after the chemical treatment. To analyze the severity of toxicity of model chemicals (PQ, H_2O_2 and Triton-X 100) in bioconjugates, the most effective conditions (0.5, 1 and 5 mM for 3 h) were employed. Thus obtained data was further applied to clustering analysis based on similarity matrix using Cluster 3.0 program and visualized with Java TreeView 1.14r3. The hierarchical relationship was generated using RFU and CFU data from most effective conditions and heatmap was obtained. The color green to red in the heatmap represent the scale bar for low to high toxicity, respectively.

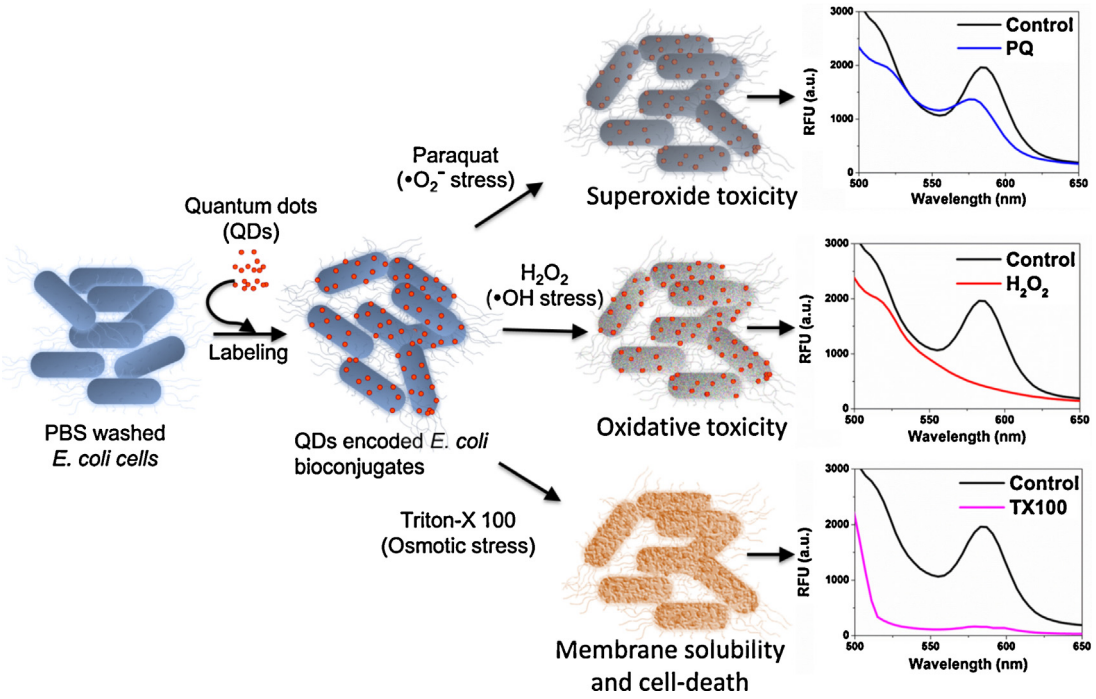
2.9. Scanning electron microscopic examination

To examine the morphological changes by SEM, the bioconjugates before and after incubated with model chemicals (5 mM for 1 h) were mounted on pre-cleaned SiO_2 substrates and dehydrated at room temperature.

3. Results and discussion

3.1. QDs to E. coli optimization for bioconjugation

QDs chosen in this study were made of CdSe core encapsulated in a crystalline shell of ZnS with an amphophilic polymer coating that prevented from any damage or toxicity in cells, which is consistent to earlier reports [21]. Bioconjugation of QDs with *E. coli* cells was performed using EDC/NHS coupling chemistry as described in experimental section. It was estimated that 16 nM QDs was required for $\sim 1.2 \times 10^9$ *E. coli* cells/mL for stable bioconjugation that yielded reproducible fluorescence signals (final RFU at 585 nm = 2100; Table S1). Therefore, this optimized ratio of QD:cells were labeled as bioconjugates and used for further toxicity studies with model chemicals. The bioconjugates were also examined after UV-illumination that showed bright fluorescent emission originating from the cells (Fig. 1 insets a and b). This



Scheme 1. Schematic illustration of bioconjugation of QDs with *E. coli* and their stress responses to toxic chemicals, such as paraquat and H_2O_2 (oxidative stress) and triton X-100 (osmotic stress).

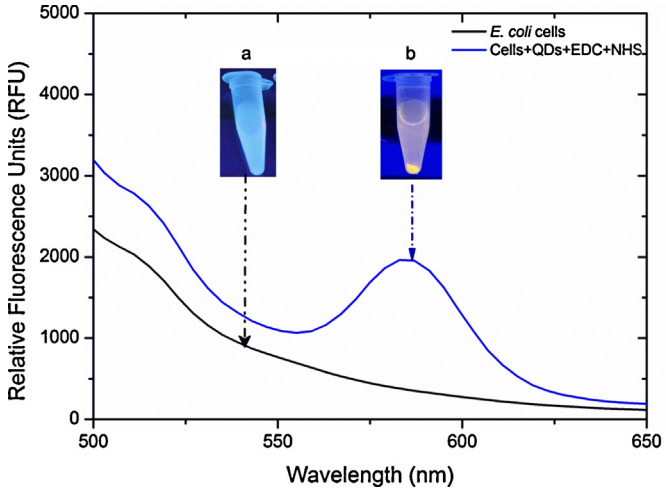


Fig. 1. Fluorescence emission spectra of normal *E. coli* cells (black), cells incubated with QDs + EDC + NHS (blue). The inset figure shows respective images of tubes exposed to the UV light corresponding to control cells (a) and cells + QDs + EDC + NHS (b) after centrifugation at 1500 rpm for 3 min and separated cells from free QDs and cells conjugated with QDs that were pulled down forming bioconjugates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

enabled direct visualization of developed bioconjugates as these brightly fluoresced with UV illumination that may turn-off their light when any cellular stress or cell damage occurs. The developed bioconjugates may therefore interact and respond to any small perturbations on the cell-structure that alter the cell integrity. These bioconjugates can serve as living fluorescent switches against any cellular perturbations at their membrane interfaces.

3.2. Real-time fluorescence measurement and cell viability test of bioconjugates upon exposure of model toxic chemicals

Model chemicals such as PQ, H_2O_2 and Triton-X 100 were exposed to bioconjugates to demonstrate the use of bioconjugates as whole-cell living baits. Each of these chemicals has a unique ability to induce cellular toxicity. For example, PQ (1,1-dimethyl-4,4-bipyridinium), a widely used nonselective pesticide induces oxidative stress by generating superoxide anion radical ($\bullet O_2^-$) [22]. H_2O_2 induces bactericidal effect by generating hydroxyl radical (•OH) [23]. Triton-X 100, a well-known surfactant, has the ability to irreversibly permeabilize the cell-membrane [24]. Such responses are often tested using classical assays, such as toxicity of silver nanoparticles using mitcochondrial function (MTT assay), reactive oxygen species (ROS) and DNA fragmentations assay [16]. Here, real-time fluorescence emission spectra of bioconjugates were measured upon exposure of model chemicals with different concentrations (0–5 mM) at 1 h (Fig. 2A–C). These concentrations of model chemicals were chosen to cover the minimum to maximum lethal effect on cells [22–24]. The fluorescence emission spectra of bioconjugates showed drop in response with increasing concentration of model chemicals. This result indicated that bioconjugates experienced cellular stress upon exposure of model chemicals, whose severity increases with increased concentrations.

The fluorescence emission of chemical exposed bioconjugates was also found to diminish with time (1–3 h) indicating that interaction of chemicals on cells occurred that not only depended on concentration, but also on the exposure time (Figs. 2D–F and S2–S7). It was observed that all three model chemicals at lethal concentration (5 mM) showed maximum diminished fluorescence response of bioconjugates as expected within 1–3 h exposure time (Fig. 2F). Cell viability tests were performed using bioconjugates before and after chemical exposure by plating on LB-agar plates and counting the number of CFUs. The ability of bioconjugates to respond to each of the model chemicals tested was reflected on their viability which was consistent to fluorescence responses (Figs. S8 and 2A–C).

3.3. Hierarchical cluster analysis

Hierarchical cluster analysis was performed with respect to RFU and CFU data of bioconjugates after the chemical treatment at

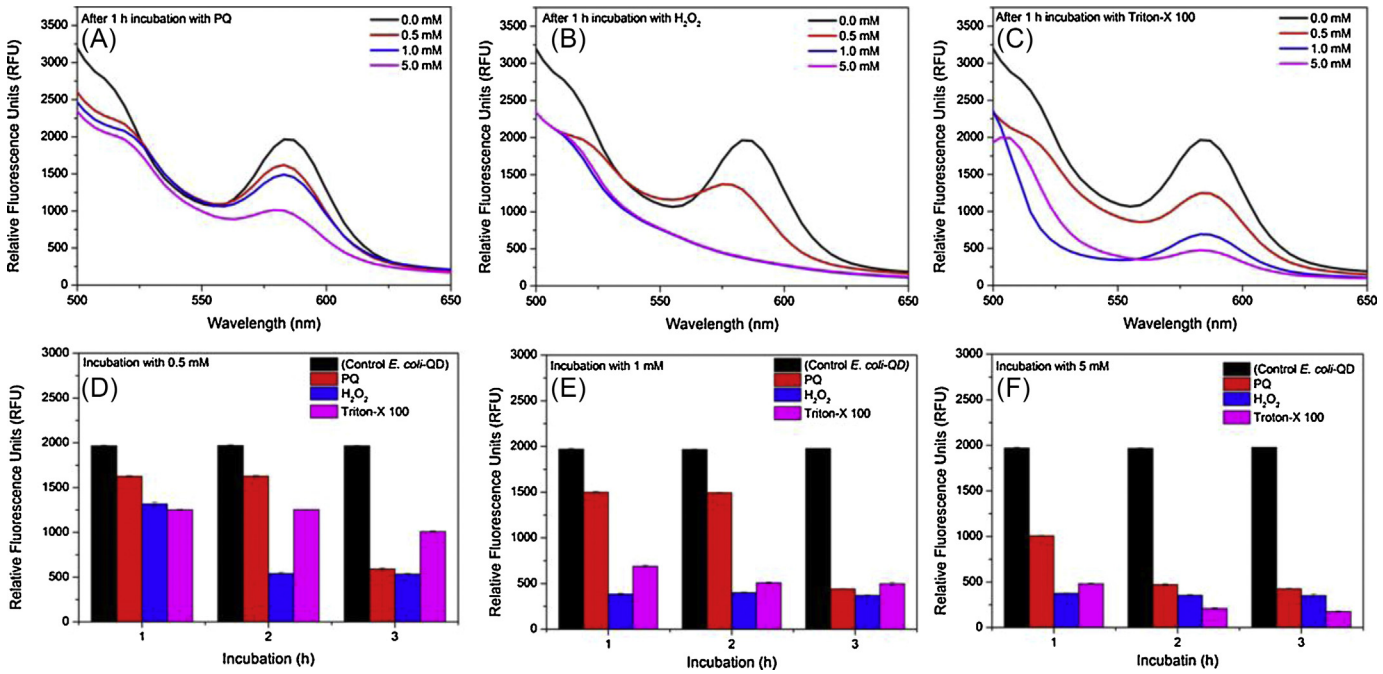


Fig. 2. Fluorescence emission spectra of bioconjugates before and after incubation with different chemicals: (A) PQ, (B) H_2O_2 and (C) triton-X 100. The relative fluorescence responses ($n = 3$, at peak maxima at 585 nm) of bioconjugates against PQ, H_2O_2 and triton X-100 as a function of incubation time at different concentrations of: (D) 0.5 mM, (E) 1 mM and (F) 5 mM, respectively.

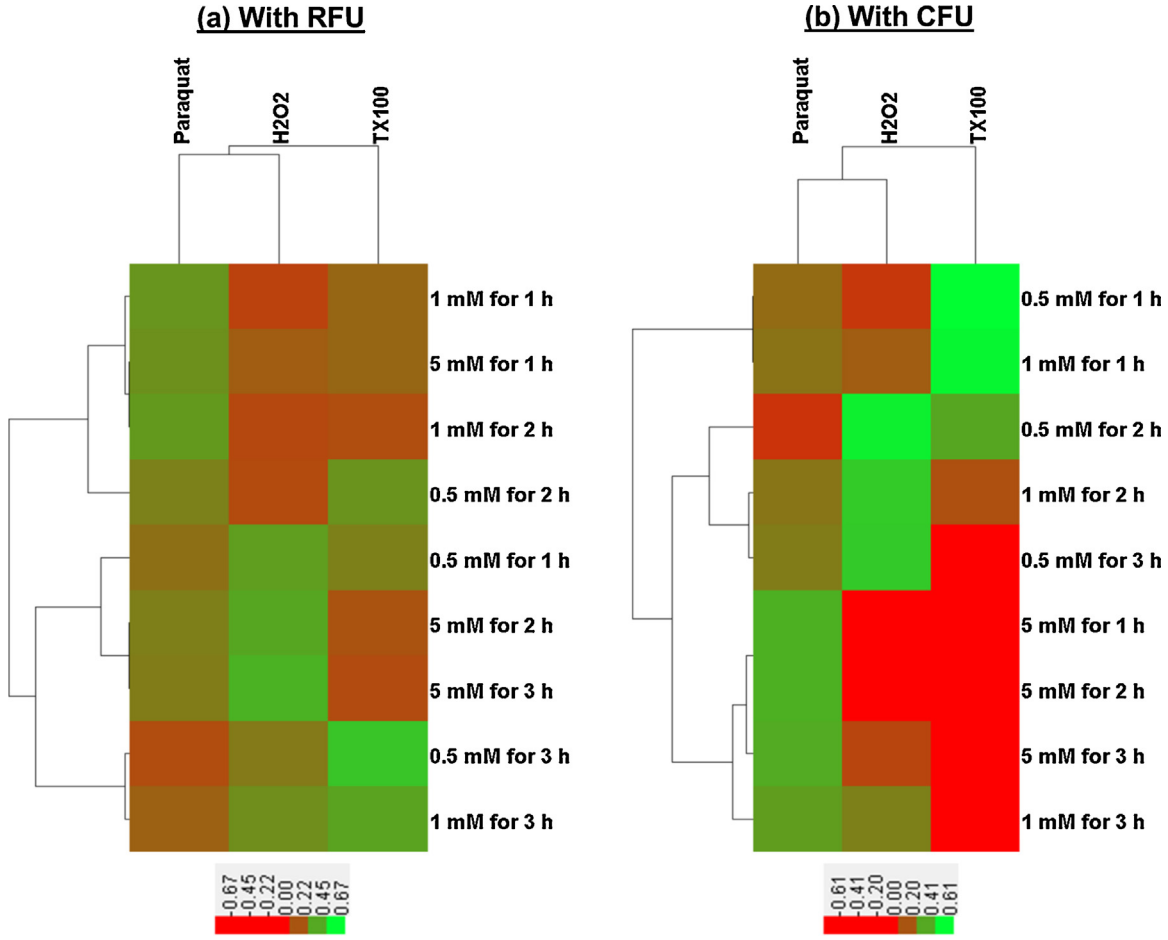


Fig. 3. Hierarchical clustering using similarity matrix was constituted and severity of toxicity with PQ, H_2O_2 and triton-X 100 incubated for 3 h at different concentration is shown with respect to (a) RFU and (b) CFU. The results were color coded for visual inspection of the impact on RFU and number of cells survived (CFU). A color generated from similarity matrix for both RFU and CFU data was normalized to indicate green for unaffected conditions and red for severe toxicity or cellular death. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

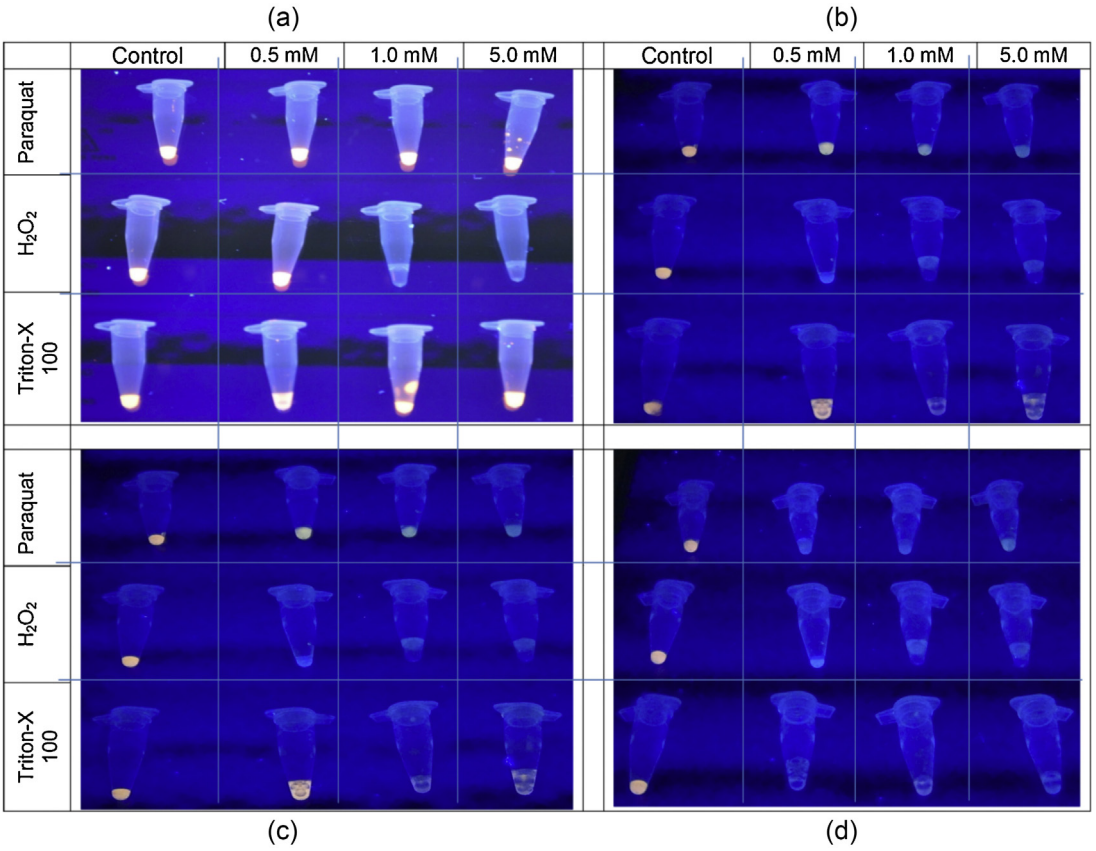


Fig. 4. UV-illuminated images of bioconjugates incubated with different concentrations of chemicals at: (a) 5 min, (b) 1 h, (c) 2 h and (d) 3 h.

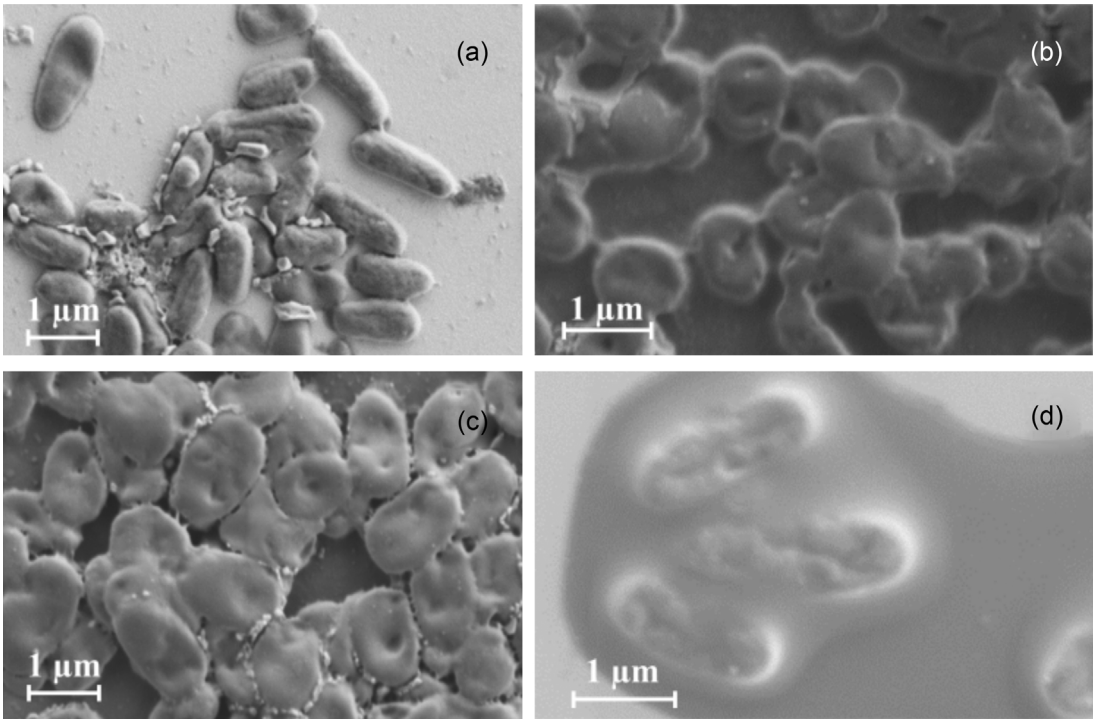


Fig. 5. Scanning electron micrographs of (a) only bioconjugates (QD encoded *E. coli* cells) and bioconjugates treated with (b) PQ, (c) H_2O_2, (d) Triton X-100 for 1 h at 5 mM concentration.

different time and concentrations derived from results shown in Fig. 2D–F and S8. This analysis enabled partitioning the data into groups based on the type of chemical and mode of toxicity (Fig. 3a and b). The dendrogram showed relationships between the nature of model chemicals tested on bioconjugates, where responses with PQ and H_2O_2 were closely similar in their effects. In contrast, results with triton X-100 had a distinct effect that remain separated in clustering analysis. It is clear from the Fig. 3a and b that increasing cellular toxicity with test chemicals in bioconjugates followed the order of PQ < H_2O_2 < TX100. The cluster similarity of bioconjugates against PQ and H_2O_2 with respect to decline in RFU can be explained with the type of radical they introduce in the medium, such as superoxide and hydroxyl radicals respectively that induces potent cellular stress (Fig. 3a). Triton-X 100, however had a distinct mechanism of inducing cellular damage through membrane permeabilization [24] and it was clearly distinguishable from the responses of PQ and H_2O_2 in heatmap (Fig. 3). These results demonstrated that it is possible to distinguish the nature of a chemical and type of cellular stress in bioconjugates through their RFU/CFU clustering analysis.

3.4. Qualitative test for cytotoxicity

Qualitative (Yes/No) cytotoxicity tests of model chemicals on bioconjugates were carried out by visualizing changes in samples illuminated with UV light. UV-illuminated images of bioconjugates incubated with different concentrations (0–5 mM) of model chemicals for 1–3 h are shown in Fig. 4a–d. The fluorescence responses of bioconjugates with UV illumination clearly indicated dependency on concentration and incubation time of model chemicals. The visible fluorescence signal from bioconjugates diminished at higher concentration (5 mM) of model chemicals as well as with increasing incubation time. This approach enabled rapid assessment of chemical cytotoxicity in living cells. These results were corroborated with real-time fluorescence measurements and cell viability test of bioconjugates upon exposure of model toxic chemicals as described in Section 3.2.

3.5. Morphological studies with bioconjugates before and after exposure of model chemicals

SEM images of bioconjugates were acquired before and after exposure of higher concentrations (5 mM) of model chemicals incubated for 1 h. This condition was chosen because of significant effect occurred in bioconjugates as per the results obtained with real-time fluorescence measurement and cell viability test. SEM images showed significant morphological changes in bioconjugates after exposure of model chemicals that probably may lead to the loss of cell-bound QDs (Fig. 5a–d). It is clear that significant cell injuries were occurred with PQ and H_2O_2 as they formed lesions and flaccid structures compared to normal cells (Fig. 5a–c). The bioconjugates treated with Triton-X 100 appeared to be solibilized in micelles, and thus formed bubble pocketed entrapment of bioconjugates with profusely injured membrane (Fig. 5d). QDs in bioconjugates may have been covalently attached on a protein embedded in lipid bilayer membrane of cells which is more susceptible to surfactant such as Triton-X 100 and therefore, extensive loss of fluorescence was evident from cytotoxicity results.

4. Conclusions

In summary, we demonstrated the use of bioconjugates for detecting toxicities imposed by membrane permeable chemicals, such as PQ, H_2O_2 and Triton X-100. The bioconjugates contained QDs on their surfaces that are the primary contact points for chemicals before they permeabilize into the cells. Therefore, the ability of fluorescence emission from QDs on cell-surfaces depended on two possible mechanisms: (i) reactions associated with interaction of chemicals with cell-bound QDs, such as quenching of fluorescence or (ii) chemical induced partial disruption of cell-membrane and associated loss of cell-bound QDs. At extreme conditions, such as prolonged exposure or high levels of chemicals, bioconjugates tend to lose cell-bound QDs probably due to partial dispersion (dilution) of QDs in the extracellular medium, which can be correlated to the morphological changes or loss of cell viability. This result suggested that quenching of QDs on bioconjugates may not be taking place, or cellular enzymes released in response to test chemicals may have participated in quenching of QDs on bioconjugates that diminished the fluorescence. Changes due to cytotoxicity in bioconjugates can be visually observed by illuminating with the UV-light and thus, enabling rapid and robust toxicity screening for large number of samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2014.02.027.

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