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Determining the fate of fluorescent quantum dots on surface of engineered budding *S. cerevisiae* cell molecular landscape

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ABSTRACT

In this study, we surface engineered living *S. cerevisiae* cells by decorating quantum dots (QDs) and traced the fate of QDs on molecular landscape of single mother cell through several generation times (progeny cells). The fate of QDs on cell-surface was tracked through the cellular division events using confocal microscopy and fluorescence emission profiles. The extent of cell-surface QDs distribution among the offspring was determined as the mother cell divides into daughter cells. Fluorescence emission from QDs on progeny cells was persistent through the second-generation time (~240 min) until all of the progeny cells lost their cell-bound QDs during the third generation time (~360 min). The surface engineered yeast cells were unaffected by the QDs present on their molecular landscapes and retained their normal cellular growth, architecture and metabolic activities as confirmed by their viability, scanning electron microscopy (SEM) examinations and cytotoxicity tests, respectively. Our results demonstrated that QDs on mother cell landscape tend to distribute among its progeny cells that accompanied with concomitant reduction in QDs' fluorescence, which can be quantified. We suggest that surface engineered cells with QDs will enable investigating the cellular behavior and monitoring cell growth patterns as nanobiosensors for screening of drugs/chemicals at single cell level with fewer side effects.

1. Introduction

QDs are used as smart emitters because of their superior optical properties compared to traditional organic fluorophores (Medintz et al., 2005; Sun et al., 2007; Weng and Ren, 2006). QDs exhibit better photochemical stability, high quantum yield, size-tunable photoluminescence from visible to near-IR and resistance to chemical and photochemical degradation (Jamieson et al., 2007; Yong et al., 2008). In recent years, surface modified QDs containing biomolecules such as DNA, protein, and small peptide like molecules have been widely used in number of applications, such as cellular imaging, drug delivery and as different forms of nanosensors (Liu et al., 2008; Michalet et al., 2005; Somers et al., 2007). The cell surface modification is carried out with sophisticated and complicated procedures, such as the addition of non-biogenic functional groups by metabolic or genetic engineering (Boonyarattanakalin et al., 2006; Chen et al., 2005; Laughlin et al., 2008; Liu et al., 2007). Such methods have evolved into biocompatible and bioorthogonal strategies, which causes significant perturbations to cell membranes. Non-covalent attachment of macromolecules by chemical approaches to cell surface engineering have emerged as powerful tools (Wilson et al., 2009). The layer-by-layer (LbL) technique has been utilized to introduce various functionalities, including fluorescent and magnetic properties, catalytic moieties and supporting templates to the living cells (Fakhrullin et al., 2010; Hillberg and Tabrizian 2006; Zamaleeva et al., 2010). It is a prerequisite for any application that the functionalization is ensured along with the mechanical robustness of the artificial materials (Yang et al., 2011). Labeling the QDs on yeast cell membrane has been documented, which utilized saccharide/lectins specific recognition (Coulon et al., 2010) and concanavalin-A protein has been used to mediate chemical coupling at the terminal carbohydrate residues (Zem et al., 2006). These methods have postulated the binding strategies, but failed to explain the effect of growth after the conjugation at cellular levels.

Over the decades, yeast has been widely used in research fields mainly because of its cellular structure and functional organization which share many similarities with human cells. Yeast has become a powerful discovery platform for modeling the cellular toxicities caused by elements that induce human disease related proteins, such as neurodegenerative disease proteins (Khurana and Lindquist, 2010). Yeast has been used as a model for human cells to screen thousands of chemical compounds that are capable of rescuing cells from disease in humans (Matlack et al., 2014). However, the mechanism of toxicity of nanoparticles (NPs) toward

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yeast is unclear (Garcia-Saucedo et al., 2011). There have been very few studies reported on the potential impact of nanomaterials/NPs on yeast cells (Garcia-Saucedo et al., 2011; Hadduck et al., 2010; Kasemets et al., 2009; Schwegmann et al., 2010). Internalization of NPs into yeast cells is although not well understood, but it is supposed that NPs are unable to enter the yeast cell under normal conditions because of its rigid cell-wall (Nomura et al., 2013). However, dissolved ions and oxidative stress induced by NPs may cause disruption of the cell wall (Kasemets et al., 2009). The non-/less-toxic nature of NPs in yeast could enable engineering for resurfacing the molecular landscape of cells with NPs. The unique physico-chemical properties of NPs, such as in QDs can therefore be coupled on yeast cell surface that can be used as smart nano-biosensors for high throughput screening applications. Quantum dots (QDs) are widely studied as luminescence probes in biological and medical research in recent years. The unique properties of QDs have attracted tremendous interest in exploiting them in a variety of biological applications (Alivisatos et al., 2005; Medintz et al., 2005; Michalet et al., 2005; Weng et al., 2015).

In present study, we have surface engineered the living yeast *S. cerevisiae* cells by decorating QDs and demonstrated the fate of QDs on molecular landscape of cells. These QDs on cell surfaces were tracked through several generation times upon division when allowed to proliferate under normal laboratory conditions. Fluorescence and absorbance spectral analysis of engineered cells provided useful information on the growth pattern of a mother cell to its progeny. The cell morphology and toxicity evaluation studies demonstrated the non-toxicity of QDs on yeast cells that served as a powerful tool for high-throughput screening of compounds that affect cellular growth and division. These findings addresses first report towards the fate of QDs conjugated on molecular landscape of cells after cell division, which helps to design series of smart bioconjugates with less toxic effects for high-throughput screening.

2. Experimental

2.1. Cells, chemicals and reagents

Budding yeast *S. cerevisiae* (BY-4741) cells were used as a model eukaryotic unicellular organism. Yeast extract, peptone, dextrose broth/agar (YPD) media was purchased from Difco (MI, USA). *N*-hydroxysuccinimide (NHS), N-ethyl-N′-(3-(dimethylamino) propyl) carbodiimide (EDC), cysteamine, tris(2-carboxyethyl)phosphine (TCEP) were purchased from Sigma-Aldrich. Qdot® 625 ITK™ carboxyl quantum dots (Invitrogen Co.) were used as labelling probes having emission maxima at 625 nm. Lactate dehydrogenase (LDH) cytotoxicity assay- kit was purchased from Pierce Biotech., Inc. USA. All other reagents used in this study were of analytical grade and filtered through 0.22 μm sterile filters.

2.2. S. cerevisiae culture preparation

S. cerevisiae cells were freshly grown overnight in YPD-broth at 30 °C and 100 rpm in an orbital shaker and incubated for 18 h. The cells at early stationary phases were harvested and centrifuged at 5000 rpm for 3 min at 15 °C. Cells thus obtained were washed thrice with sterile phosphate buffered saline (PBS, pH 7.4) followed by centrifugation for 5 min at 5000 rpm at 4 °C. The cell pellets were resuspended in same buffer and colony forming units (CFU) were determined. Aliquots were made that carried 2×10^9 CFU mL^{-1} for test and control experiments and divided into several sub-aliquots for replicates that carried same number of cells.

2.3. S. cerevisiae cell surface engineering with QDs

Cell surface engineering was carried out through bioconjugation of Qdot® 625 ITK™ (QDs) on freshly grown yeast cells as reported previously (Chouhan et al., 2014). Briefly, covalent coupling of carboxylated QDs with cysteamine was carried out and the reaction mixture contained optimized concentration of 8 nM QDs, 8 mM cysteamine, 50 mM EDC and 5 mM NHS in a final volume of 1 mL. This reaction mixture was allowed to stand at RT for 30 min for covalent coupling between $-NH_2$ of cysteamine and –COOH of carboxyl-QDs (as shown in Scheme S1). Thus formed cysteamine activated QDs suspension was centrifuged at 14,000 rpm for 5 min and stored until use for bioconjugation. In a separate set of reaction, *S. cerevisiae* cells ($\sim 2 \times 10^9$ CFU mL^{-1}) were suspended in solution containing 100 μL of 5 mM TCEP and incubated for 20 min at RT to reduce the disulfide bridges of cell-surface membrane proteins. The TCEP treated cells were centrifuged for 5 min at 5000 rpm at 4 °C and washed thrice with PBS (pH 7.4). TCEP reduced the cell-surface disulfide-containing protein motifs and generated free –SH groups that facilitated immobilization of cysteamine activated QDs on cells. Immobilization of TCEP treated cells was carried out by mixing them with SH-activated QDs and the resulting bioconjugates were centrifuged and the pellets were washed thrice with PBS, pH 7.4 and stored for further studies.

2.4. Fluorescence emission measurement

Real-time fluorescence emissions from QDs on engineered *S. cerevisiae* cells (bioconjugates) were measured by scanning emission wavelengths ranging from 500–750 nm, after exciting with a blue LED. The characteristic fluorescent emission peak at 625 nm corresponded to the presence of QDs on cell-surfaces. The fluorescence spectral studies were carried out by using a NanoDrop 3300 Fluorospectrometer (Thermo Scientific NanoDrop Products).

2.5. Confocal and scanning electron microscopic analysis

Fluorescence microscopy images of bioconjugates were acquired with a Carl-Zeiss LSM 710 confocal microscope equipped with a Plan-Apochromat 63x/1.40 oil objective. QDs on cell-surfaces were excited with a 405 nm laser and images were collected using a 553–718 nm filter. The morphological features of yeast cells occurred were examined by a LEO Supra 35VP Scanning Electron Microscope (SEM). For this, samples were fixed by dropping washed cell-suspensions on silicon wafer chips and air dried. The fixed samples were sputter coated with gold (10 s, 50 mA) for SEM examinations. SEM was operated at an accelerating voltage of 5 keV depending on the sample type.

2.6. Cell viability of surface engineered cells

Viability tests of cells were performed by spread-plating the cells on YPD agar or alternatively by calculating the number of cells corresponding to the measured absorbance at 600 nm. First, the initial concentration of the bioconjugates was adjusted to 2×10^9 cells mL^{-1} in YPD broth and incubated at 30 °C in an orbital shaker. Samples were withdrawn from cultures at different generation times, considering each generation time corresponds to 120 min at which a cell divides into two daughter cells (Marcand et al., 2000; Schade et al., 2004). Calculated numbers of bioconjugated and normal cells were withdrawn from respective cultures at different generation times (0, 120, 240 and 360 min) and arrested their growth by placing them on ice-bath at 4 °C. Diluted suspensions (100 μL each) were spread on YPD agar plates and incubated for two days at 30 °C and the number of living cells were determined by counting the number of colony-forming units

(CFUs). Viability (%) was evaluated by comparing the number of CFUs in normal cells (control) and surface engineered yeast cells (test).

2.7. LDH cytotoxicity assay for surface engineered cells

LDH cytotoxicity assay was performed to assess the cytotoxicity or cell membrane integrity of surface engineered cells with QDs as described in Supporting Information (SI) section. The absorbance in blank and maximal cell death samples were subtracted from all the test samples and calculated the % cell death using the following equation 1.

$$\%\ \text{cytotoxicity} = \frac{(\text{experimental OD}_{490} - \text{untreated OD}_{490})}{\text{maximum death OD}_{490}} \times 100 \quad (1)$$

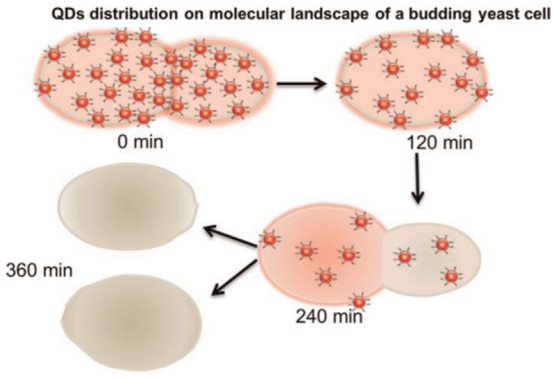
3. Results and discussions

3.1. Engineering S. cerevisiae landscape by decorating with QDs and tracing

Wild-type yeast *S. cerevisiae* cells were surface engineered with QDs using a unique chemical coupling strategy exploiting the cell-surface or outer transmembrane protein disulfide bridges. Cell-surface protein disulfide bridges were coupled with –SH modified QDs through TECP reduction as described in experimental methods. This process enabled decoration of QDs on cellular landscapes without altering the normal cellular functions, such as metabolic activities, viability or cell morphology. The doubling time of a typical *S. cerevisiae* cell is reported to be approximately 120 min under optimum conditions in YPD broth at 30 °C (Diaspro et al., 2002; Marcand et al., 2000; Schade et al., 2004). We hypothesized that if a normal cell divides, part of the mother cell composition likely to be distributed to the progeny/daughter cells after the division (Scheme 1). This hypothesis was tested by allowing the surface engineered (QD decorated cells) to grow and divide in YPD medium for defined time intervals corresponding to their average generation/doubling times (0, 120, 240 and 360 min). The cell growth and associated fluorescence emission profiles originating from the QDs present on the surface engineered cells were compared. Fig. 1(a)–(c) shows growth/division of QD engineered cells and fate of QDs at different generation times as determined by the changes measured at OD_{660} and fluorescence emission at 625 nm, respectively.

Stable decoration of QDs on cellular landscapes and their fate was tracked through the cellular division with respect to time. A typical *S. cerevisiae* cell doubles in approximately 120 min under optimum conditions in YPD broth at 30 °C (Diaspro et al., 2002; Marcand et al., 2000; Schade et al., 2004). Therefore, the surface engineered (QD decorated) cells were allowed to grow and divide in YPD medium for defined time intervals corresponding to every 120 min doubling times (0, 120, 240 and 360 min). Therefore, fate of QD effective emission could be tracked as cells multiply/divide in to subsequent offspring through measuring associated fluorescence emission and growth profiles. Fig. 1(a)–(c) shows change in fluorescence emission of QDs on engineered cells at 625 nm and associated absorbance at OD_{660}, respectively.

It is clear from Fig. 1(a) and (b) that QD-engineered mother cell (bioconjugate) tends to distribute its cell-surface QDs among its progeny (daughter) cells. This can be evidently seen with concomitant reduction in fluorescence as the cells start to divide and thus increase in number of cells through 0–360 min. This type of genetically unaltered phenotypic changes observed in daughter cells indicated the inheritance of partial cellular components in daughter cells from a mother cell after the completion of division



Scheme 1. Distribution of QDs on molecular landscapes of mother cell (0 min) and its subsequent offspring (progeny cells) through three generation times (doubling times, 120, 240 and 360 min) respectively.

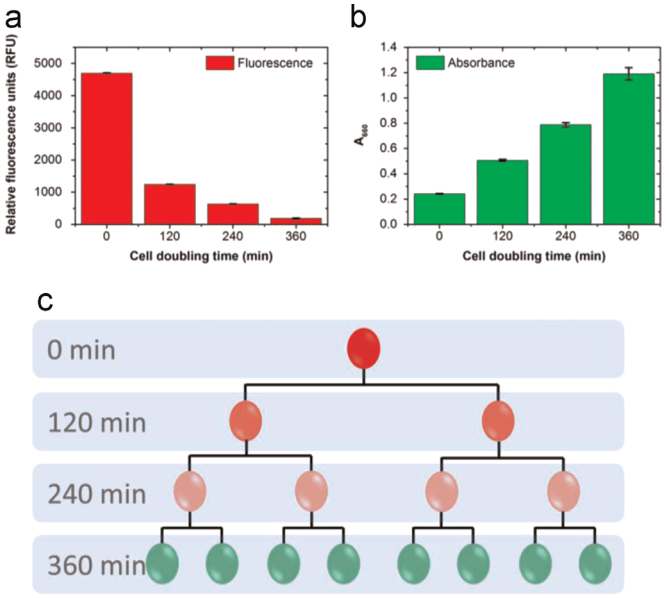


Fig. 1. Growth and fluorescence emissions from the surface engineered *S. cerevisiae* with QDs (bioconjugates): (a) fluorescence emissions (RFU) measured at 625 nm in surface engineered cells with QDs after different doubling times, (b) growth of cells measured at OD_{660} of bioconjugates in YPD medium at 30 °C at different time intervals corresponding to approximate doubling times (0, 120, 240 and 360 min) and (c) schematic illustration of fate of QDs on surface engineered yeast cells with respect to doubling times.

process (Fig. 1(a)–(c)). The fact that fluorescence emission was persistent till 240 min in growth media suggests that the QDs on engineered mother cell was distributed to a maximum of four daughter cells (at around two generation times). These daughter cells upon further division are likely to lose all of the QDs or at least they were undetectable upon the cells entering the third generation time at 340 min. Cumulative fluorescence and absorbance patterns with surface engineered cells indicated the heterogeneity due to the presence of QDs in population with normal cellular growth compared to control cells. It also implies that engineered cells were able to divide and proliferate with phenotypic distribution of cell-surface QDs among immediate and subsequent daughter cells (Fig. 1a-b).

3.2. Confocal microscopic examination of surface engineered cells

Yeast cells ($\sim 2 \times 10^9$ cells mL^{-1}) were initially coupled with 8 nM of –SH modified QDs yielded relative fluorescence units, RFU = 6048 at 0 min. Loss of fluorescence from QDs decorated cells

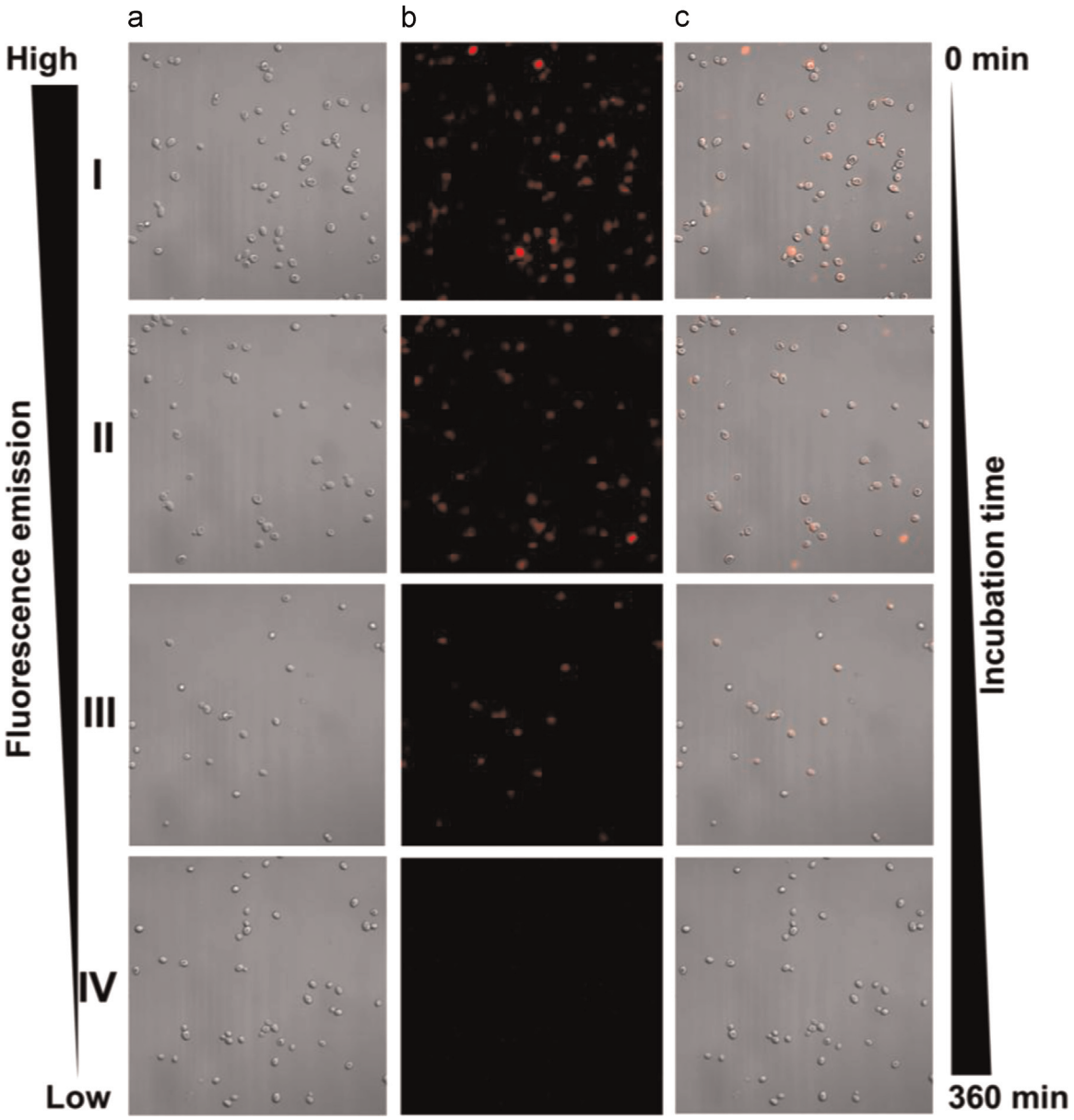


Fig. 2. Confocal images of QDs-*S.cerevisiae* bioconjugates at different time intervals. Stages I–IV, refer the time corresponding to 0, 120, 240 and 360 min, respectively. Columns represent different image types acquired: (a) bright-field images, (b) fluorescence images and (c) overlayed images of (a) and (b).

occurred at 120, 240 and 360 min (RFU = 1241, 632 and 90, respectively) that was accompanied by the increase in cell number (OD_{660} = 0.5; 0.8 and 1.2, respectively). The engineered cells were further subjected to confocal studies in order to evaluate the distribution patterns and topography associated with surface bound QDs. Fig. 2(a)–(c) shows real-time confocal images of QD decorated cells acquired at different generation times (0–360 min). Confocal images of mass of cells at different growth cycles showed the difference between fluorescent and non-fluorescent daughter cells (rows I–IV in Fig. 2(a)–(c)) with constant numbers of cells taken from respective generations (2×10^9 cells mL^{-1}). It was observed that the fluorescence intensity from the cells diminished with time from 0 to 360 min, which was consistent to the trend observed with the measured fluorescence from the cell-suspension (Fig. 1a).

The fluorescence images when closely observed with a single engineered cell revealed that QDs were well distributed along the outer cellular landscape (Fig. 3(a) and (b)). As the surface engineered cell population allowed to proliferate in YPD medium, a few budding mother cells also carried QDs on emerging bud surface (undetached daughter cell) that exhibited low illumination (Fig. 3(c)–(h)). At each stage of the developmental/growth cycle, the QDs' fluorescence associated with the cells concomitantly diminished. At 0 min, the mother cell had a maximum of ~60.23 QDs as calculated based on concentration of QDs/number of cells used for bioconjugation, which is evident from the confocal images (Fig. 3(a) and (b)). As the growth stage entered in to the next phase of cycle (120 min, doubling time of yeast), a new cell-bud emerged to from a daughter cell (Fig. 3(c) and (d)). A fully-grown bud or just detached cell can be visualized at 240 min of cell cycle due to the presence of cell-surface QDs (Fig. 3 (e) and (f)). At around 360 min, the cells were completely devoid of any QDs on their surfaces and therefore no detectable fluorescence was observed in confocal images (Fig. 3(g) and (h)).

3.3. Scanning electron microscopic examination of engineered cells

SEM images of surface engineered cells with QDs

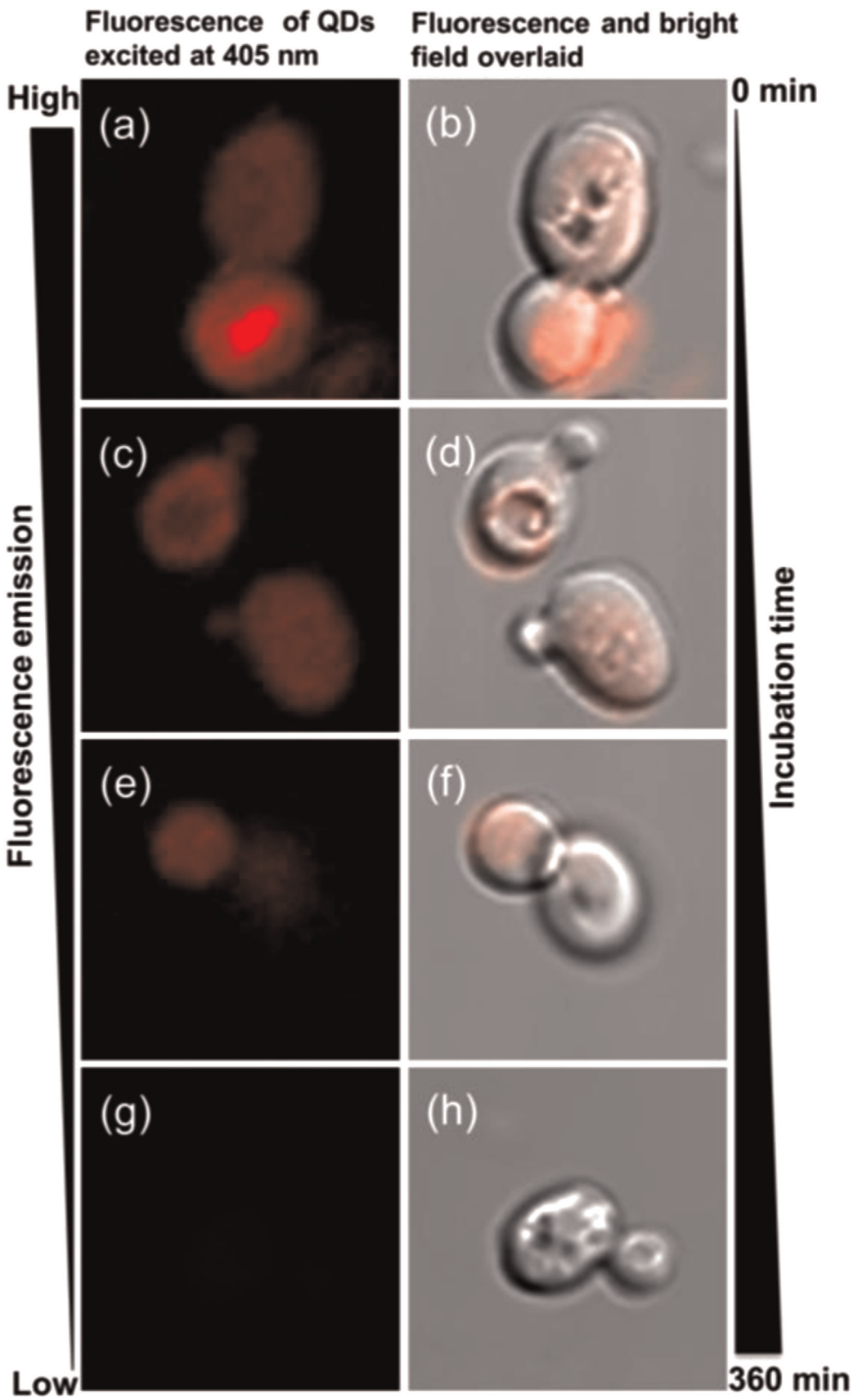


Fig. 3. Confocal microscopic images of individual yeast cell surface engineered with QDs at their molecular landscapes. Images were acquired after exciting with 405 nm laser and the emitted fluorescence at 625 nm at three sequential doubling times (generation times). Fluorescence emissions from surface engineered mother cell with QDs at different generation/doubling times: (a) and (b) 0 min. (c) and (d) 120 min (e) and (f) 240 min and (g) and (h) 360 min. Left panel shows the fluorescence images of surface engineered cells and the right panel shows the overlaid florescence and bright field images.

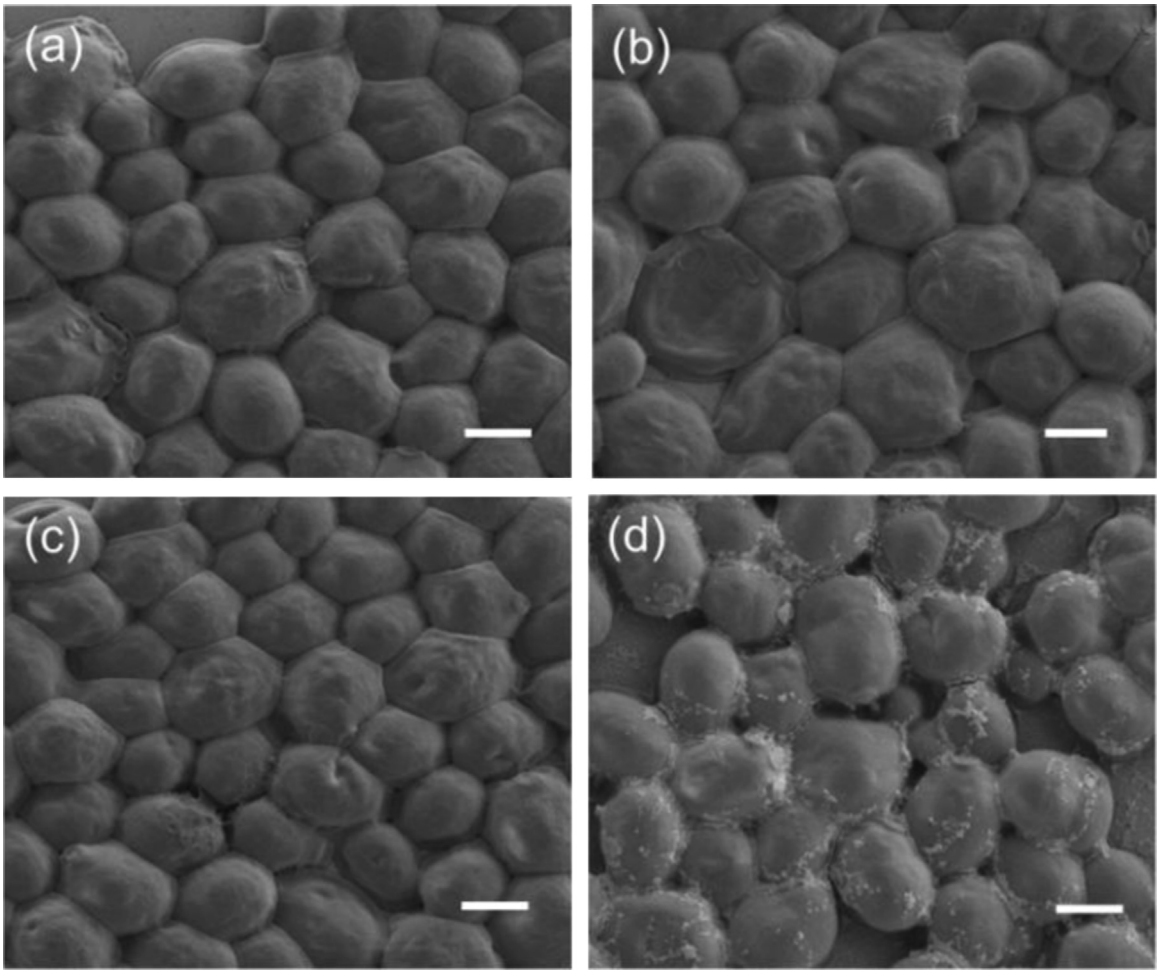


Fig. 4. SEM images of surface engineered *S. cerevisiae* cells at different doubling times, such as: (a) 0 min, (b) 120 min, (c) 240 min and (d) 360 min. The scale bar highlighted in the images represents 2 μm lengths.

(bioconjugates) were acquired in their native forms and after their proliferation in the culture media to determine any morphological changes that may have occurred after each growth cycle. It is evident from Fig. 4(a)–(d) that the surface engineered cells were morphologically healthy during different growth cycles with occasional small enlargement in size. The SEM examination of cells indicated that the increase in absorbance through 0–360 min was mainly associated with the increase in cell number, but not originated from the dead cell or cell-debris (Fig. 1b). This result also implies that the cells were unaffected by the chemical conjugation using the TCEP chemistry and no obvious difference between the surface engineered and the control cells (Fig. 4(a)–(d)).

3.4. *In vitro LDH cytotoxicity assay using surface engineered cells*

Measuring release of LDH is a commonly used method to assess the integrity of cell membrane and cell viability. LDH is a stable cytosolic enzyme that functions to catalyze the interconversion of lactate and pyruvate concomitantly with the oxidation/reduction of nicotinamide adenine dinucleotide (NADH and NAD^+). LDH plays an important role in carbohydrate metabolism, and at least one of its five isoforms is expressed in most tissues and cells. Upon loss of membrane integrity following cell death, LDH is released into the media. The release of LDH due to leakage in the media is a first sign of toxicity exerted by cells. Interestingly, we observed that the surface engineered cells with QDs did not induce but restrained the LDH leakage and the cells remained stable through the three generation times (0–360 min) (Fig. 5a). The LDH levels of bioconjugates remained same as that of the control cells (10–12%) indicating that the QDs had negligible influence on the LDH leakage. This was partly due to the protective polymer layer coated on QDs used in this study (see experimental methods).

Cell viability tests were also carried out by counting the number of surviving *S. cerevisiae* cells after surface engineering cells with QDs (Fig. 5b). At the initial incubation time (0 min), cells were healthy and retain 100% viability both in control and test cell-suspensions (Fig. S1). We observed that as the cell cycle progressed, there was no obvious reduction or loss of viable cell numbers (Fig. 5b; S1). At 120 min both control and test cell-suspension showed 86 and 83% viability, respectively and this can be explained by the fact that cells need an initial adaptation to grow in a new micro-environment, such as that with altered cellular landscape. Further, cell division and proliferation continued through 240 min incubation, during this stage, there was no reduction in the number of cells. This result suggested that no toxicity occurred on surface engineered cells or its internal organelles in presence of cell-surface bound QDs and retained normal metabolic function and division.

QDs have unique physicochemical properties and are applied in various areas. However, their biological properties in organisms will finally determine their destiny in future. Carbon based nanomaterials found to exhibit toxicity in yeast (Chang et al., 2011), while the results presented in this study did not reveal toxicity with QDs in yeast cells suggesting that the QDs used in this study were biocompatible in nature. We systematically studied to evaluate the toxicity/biocompatibility of QDs to yeast cells, which is

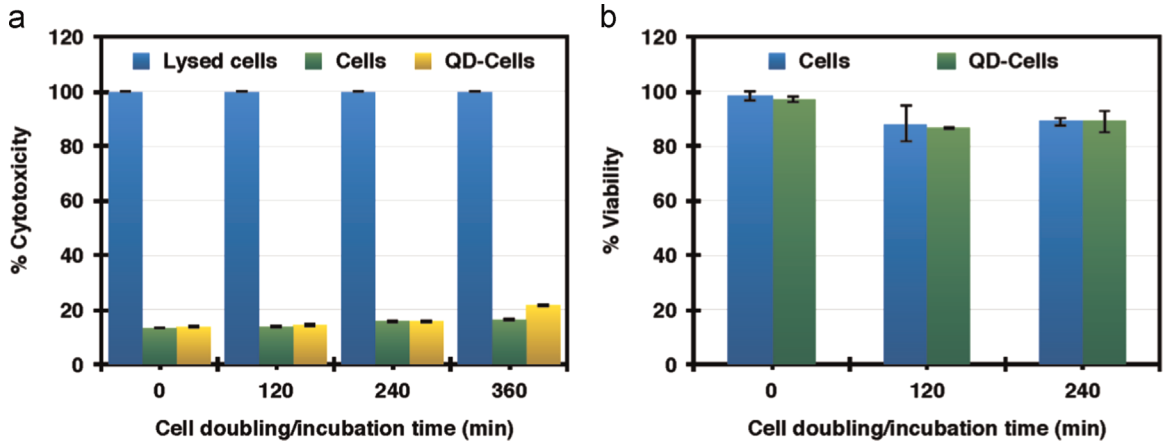


Fig. 5. (a) LDH cytotoxicity assay using lysed *S. cerevisiae* cells (dead cells, positive control), normal *S. cerevisiae* cells and surface engineered *S. cerevisiae* cells with QDs and (b) Viability tests using 2×10^9 cells mL^{-1} of normal *S. cerevisiae* cells and equal number of surface engineered *S. cerevisiae* cells QDs at three subsequent doubling times shown in *x*-axis.

widely used as model organism for the toxicity screening. We observed no evidence of internalizing QDs within the cells. This feature of yeast cells contributed to the high biocompatibility of luminescent QDs that have profound applications in biomedicine and high-throughput screening. Contrastingly, the uptake of bare carbon nanomaterials by cells is a widely observed phenomenon (Lewinski et al., 2008) and non-functionalized fullerene or CNTs can easily diffuse into different cells that causes cytotoxicity (Li et al., 2008; Wang et al., 2011). The interaction on the cellular surface may be reflected by evaluating the membrane integrity. Interestingly, the LDH leakage levels from QD engineered cells were same as the control cells, which suggest that the outer membrane surface was intact in surface engineered cells. We found that QDs tethered on the membrane was not toxic to the cells, which was evident from SEM and cytotoxicity analysis (Figs. 4(a)–(d) and 5(a) and (b)). However, a few studies have shown the potential impact of other NPs on yeast cells, but these studies exhibit low or no toxicity towards yeast compared with bacteria (Garcia-Saucedo et al., 2011; Kasemets et al., 2009; Lee et al., 2009; Schwegmann et al., 2010). We hypothesized that NPs cause toxic effect towards cells only when they are in intimate contact with the membrane. QDs conjugated on the cell surface with appropriate space, limits direct contact with the membrane and eventually less toxic to cells. Our results suggested that the surface engineered cells maintain their internal metabolic activity and hamper the QDs' fluorescence as the cell division occurs. The findings of this study allow in situ targeting of cells while preserving their native protein expression and cell division mechanisms for implications in high-throughput screening.

4. Conclusion

In this study, we reported on development of surface engineered yeast cells with QDs on their landscapes. The surface engineered cells enabled visually tracking fate of QDs through the progression of cell-division, which accompanied by the QD's fluorescence emission to probe precise *in vitro* information of cellular activity. The presented method of evaluating single cell activity is a cost effective way compared with current labelling techniques. One of the most striking features of the presented technique is the ability to distinguish the changes occurred between the mother cell and emerging progeny cells, at respective growth phases. Therefore, QD engineered cells can be employed to study the cellular behaviour at a single-cell level. Influence of QDs on cell-surfaces had no effect and thus providing mechanical support on cell membranes. LDH cytotoxicity assays and cell viability tests evidently showed the non-toxic nature of QDs to cells. Our findings provided a new insight and perspective to evaluate the biological effects and safety of QDs or similar nanomaterials. Therefore, with a simple and effective QD's assembly on cell-surfaces, these smart living nano-indicators (nanobiosensors) can provide a sensitive tool for high-throughput screening and detection of toxic chemicals, nanomaterials, drugs or environmental agents.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.02.017.

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