Bioconjugate Chemistry

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Yeast Populations Evolve to Resist CdSe Quantum Dot Toxicity

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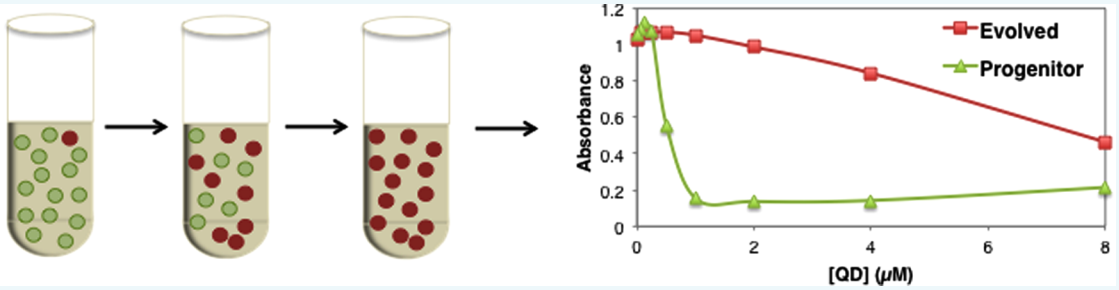
Supporting Information



ABSTRACT: Engineered nanomaterials are used globally in biomedical, electronic, and optical devices, and are often discarded into the environment. Cell culture experiments have shown that many inorganic nanoparticles are toxic to eukaryotic cells. Here, we show that populations of eukaryotic cells can evolve to survive chronic exposure to toxic CdSe semiconductor quantum dots (QDs). We grew yeast *Saccharomyces cerevisiae* for 24 days in liquid medium containing QDs prepared daily at half the minimum inhibitory concentration (MIC_{50}) of the progenitor yeast cells. After 24 days, the cells grew normally under constant exposure to QDs. We concluded that these cells evolved to resist QD toxicity. Surprisingly, when we removed QDs from the growth medium, some of the evolved cells grew poorly, i.e., they grew better in the presence of QDs. Finally, genetic analysis confirmed that the ubiquitin ligase gene *bul*1 was mutated in the evolved cells, which suggests that this gene may be implicated in increased CdSe QD tolerance. This study shows that chronic exposure to QDs can exert selective pressure causing irreversible genetic changes leading to adaptation.

The broad usage of nanoparticles (NPs) will lead to exposure and accumulation of nanomaterials in the environment, posing potential risks for both wildlife and humans.[1] Studies of animal exposure to NPs have shown that nanomaterial toxicity may be related to the physicochemical properties of NPs and the route of exposure.[2−4] Toxicity can arise from the NP core. For example, quantum dots (QDs) often contain metalloid cores comprising CdSe and CdTe. Dissolution of the core into its constituent metals, such as Cd and Se, results in ionic species that are toxic to higher organisms.[5−7] CdSe quantum dots are now an integral component in many electronic devices, such as light-emitting diodes, photovoltaic cells, and flat television screens; thus, the increasing prevalence of these nanomaterials in society is cause for concern over the risk of environmental exposure. Here, we asked whether the genome of eukaryotic cells can adapt and mutate to confer resistance to the toxicity associated with nanoparticles.

In order to determine whether cells become resistant to NP toxicity, we first selected a model nanoparticle with a well-known cytotoxicity profile. We used CdSe QDs, as these QDs are highly toxic to mammalian cells.[2−4,8−10] We hypothesized that incubation of CdSe QDs with eukaryotic cells would exert selective pressure, leading to adaptation. In cell culture experiments, almost all studies have shown that Cd-based NPs can be toxic based on three mechanisms: (1) free Cd^{2+} present in particle solution, (2) leaching of Cd^{2+} from the NP's core, and (3) generation of reactive oxygen species.[2] Derfus et al. were the first to show that CdSe QDs break down in oxidative environments and kill hepatocytes by metal poisoning.[10] Another mechanism for toxicity is NP interaction with proteins on and/or in a cell, causing altered protein conformation, disruption of plasma membrane integrity, and production of harmful reactive oxygen species in a size, surface-chemistry, and composition-dependent manner.[3,11−13] In vivo, some degradation of NPs can occur within a whole animal, and NPs can persist in an organism and accumulate in organs of the reticuloendothelial system.[14−16] In short-term studies, QDs have been shown to be nontoxic to animals; however, many of these QDs are too large to be excreted, and the long-term toxicity of quantum dots remains unknown.[14,17−19] Therefore,

Received: February 1, 2017
Published: February 2, 2017

DOI: 10.1021/acs.bioconjchem.7b00056
Bioconjugate Chem. XXXX, XXX, XXX−XXX

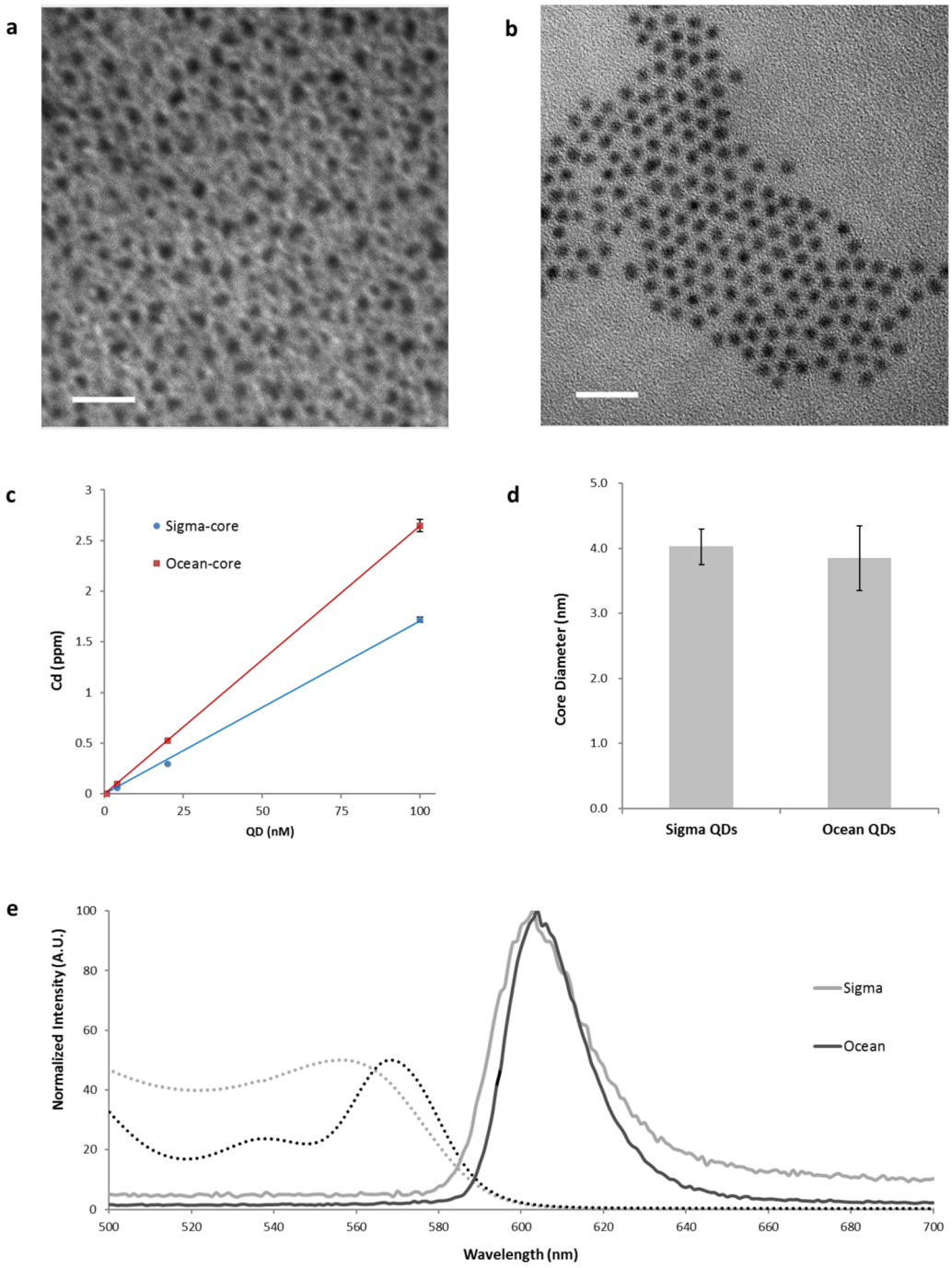


Figure 1. Characterization of QDs. Representative electron micrographs for CdSe QDs from Sigma-Aldrich (a) and Ocean NanoTech (b). Scale bar, 20 nm. (c) Cadmium content of the two different QD types with error bars (n = 3). Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) demonstrates that the QDs from Ocean NanoTech have ∼1.5 times higher cadmium content than QDs from Sigma-Aldrich. (d) Core diameters of the two QD types as determined by electron microscopy. (e) Optical Characterization of QDs. Fluorescence (solid line) and absorbance (broken line) spectra. Both QDs showed similar fluorescence properties with peak emission at 605 nm, but different absorbance profiles with peak absorbances at 555 and 570 nm for QDs from Sigma-Aldrich and Ocean NanoTech, respectively.

there is a need for studies that characterize an organism's responses to long-term exposures to QDs.

We purchased CdSe QDs from two vendors, Sigma-Aldrich and Ocean NanoTech, rather than synthesizing them in-house: we rationalized that most QDs used in current device development come from commercial sources, and we sought to compare the effects of NPs—marketed as identical by different vendors—on cellular evolution. Our in-house characterization showed that these quantum dots had diameters of 4.0 nm (SD = 0.27) and 3.8 nm (SD = 0.49) from Ocean NanoTech and Sigma-Aldrich, respectively (Figure 1). We surface-modified these QDs using methods that were previously optimized by our group.[20] We first modified the QD surfaces with mercaptohexadecanoic acid to render them soluble in relatively polar solvents, such as DMSO and THF: this then allowed the QD solvents to be miscible with water, and then coating the QDs with bovine serum albumin (BSA) enabled them to be soluble in aqueous solvents. We needed to use the

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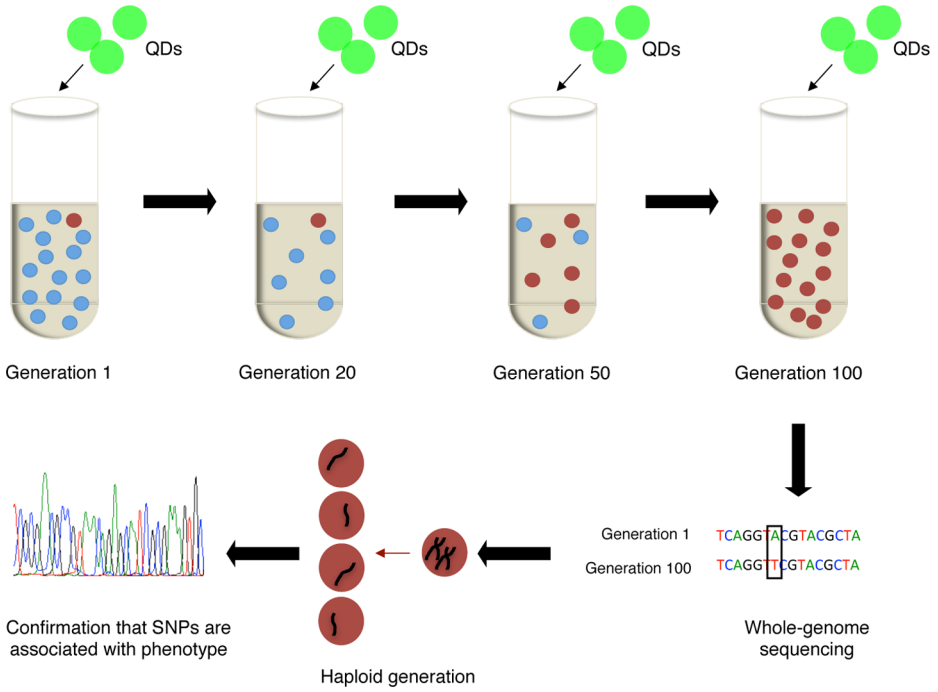

Figure 2. Schematic of overall evolution and quantitative genetics experiments. The QDs (lime circles) are incubated with the yeast (blue circles) in liquid medium. QD concentration used was determined by MIC_{50}. At Generation 1, the progenitor strain is present (blue circles). As evolution progresses in the presence of QDs, mutated yeast begin to dominate the population (red circles; a red circle is depicted in Generation 1, because we cannot be certain that the mutation conferring growth advantage was not present in the initial pool). Quantitative genetics follows: mutations in the evolved strain's genome are identified by genomic sequencing. Sporulation and haploid generation allow examination of offspring to determine linkage of a mutated gene (genotype) to the growth of yeast under constant exposure to QDs (phenotype). Resequencing confirms that the identified SNP is associated with the gene responsible for the change in phenotype.

same batch of QDs—with the same properties—throughout the entire evolution study; thus, we BSA-coated the QDs, so that they can be stored in solution with retention of monodispersity. The use of a BSA coating reduced "aggregation" properties as a factor in the experiments, which can influence cell uptake and transport.[21]

The results that we present here may not necessarily be adapted to other particle surface chemistries and compositions, as particle transport into cells, stability, and breakdown will vary. However, this study presents an overflow and strategy to examine evolution of yeast in the presence of nanoparticles: this strategy can be applied to mammalian cells. Evolution studies must be done for different particle designs in order to generalize conclusions.

Next, we identified a cellular system that would enable us to monitor cellular response to QDs over 100−300 generations. We measured the minimum inhibitory concentration (MIC) values, the lowest concentration at which no growth of cells was observed, of both vendors' CdSe QDs on both *Escherichia coli* (bacteria) and *Saccharomyces cerevisiae* (yeast) using established methods.[22] Initial MIC determination was necessary because (1) MIC assays were used to establish that our model cellular system was susceptible to the toxic effects of the QDs, and (2) MIC values were used to determine a suitable QD incubation concentration that would provide a stressful growth environment for the cells, yet would still allow survival of some of the population. Because both bacteria and yeast rapidly replicate, we hypothesized that they could be used as model organisms to directly explore the evolutionary impact of QDs over several hundred generations. Both the Sigma-Aldrich and Ocean NanoTech QDs had virtually no effect on bacteria up to a concentration of 1.2 μM, which indicated high tolerance toward these QDs. We could not establish MIC values in bacteria, and therefore, we chose not to use bacteria for these studies, as the amount of QDs needed to conduct numerous generations of evolutionary growth would prove extremely costly.

With yeast, however, we were able to determine MIC values for the Sigma-Aldrich and Ocean NanoTech QDs: 1300 nM and 950 nM, respectively (data not shown). The difference in MIC values suggests that the Ocean NanoTech QDs had a more deleterious effect on yeast growth—by approximately 1.4 times—than those from Sigma-Aldrich, despite these QDs being marketed as the same. Using inductively coupled plasma-atomic emission spectroscopy (ICP-AES),[23] we measured total Cd content for both Sigma-Aldrich and Ocean NanoTech QDs, and found that the Ocean NanoTech QDs contained ~1.5 times more total Cd than the Sigma-Aldrich QDs (Figure 1c). Moreover, we performed transmission electron microscopy (TEM) and observed that QDs are transported into cells; however, TEM did not conclusively show that QDs entered the cell nuclei.

Next, we assessed whether the model organism can adapt to quantum dot exposure. We incubated yeast cells with a concentration of quantum dots around the MIC_{50}, i.e., the concentration of QDs at which cell population growth is reduced by half. We surmised that this concentration would favor population-level adaptation and select for those cells harboring mutations that confer advantage toward growth and survival, without killing the entire population.[24] Using methods developed by Anderson and co-workers,[25] we exposed two populations of *S. cerevisiae* to 500 nM Sigma-Aldrich QDs, and another two populations of *S. cerevisiae* to 500 nM Ocean

DOI: 10.1021/acs.bioconjchem.7b00056
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NanoTech QDs (Figure 2). This QD concentration is close to the MIC_{50} values: 650 nM for Sigma-Aldrich, 475 nM for Ocean NanoTech.

We express QD concentration in molar units: our 500 nM QD concentration is equivalent to 10 ppb (parts per billion, # nanoparticles/# H_2O molecules), which is a unit often used to express concentrations of environmental contaminants. Yeast populations were initiated from a single haploid progenitor and evolved for 160 generations in liquid environments (6.6 generations/day); the 500 nM QD concentration was held constant over the duration of the evolution experiment.

After 160 generations of growth (24 days), MIC testing was repeated to identify any changes in the evolved cells' tolerance to QDs (Figure 3). The MIC_{50} value for populations evolved in

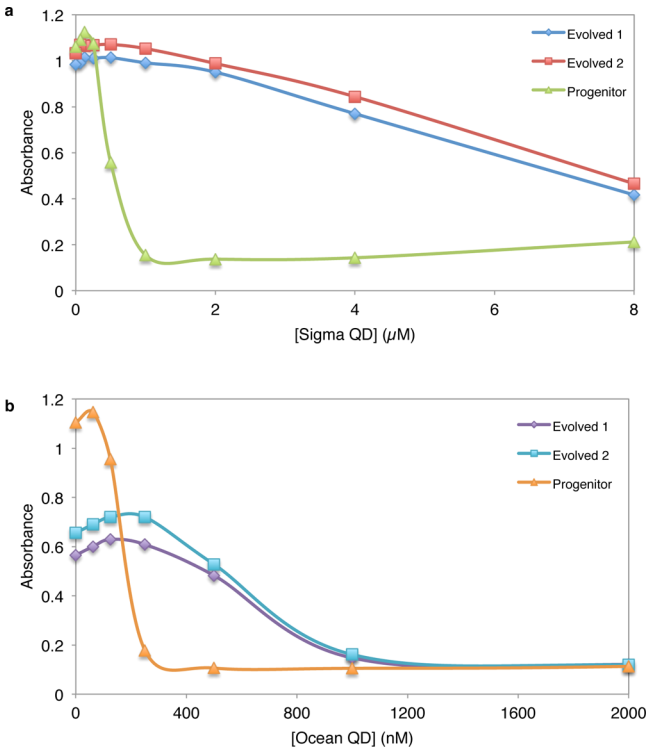

Figure 3. QD MIC determination for evolved and progenitor populations. MICs were measured for all Sigma-evolved (a) and Ocean-evolved (b) populations and the progenitor population, Sce13. Concentrations of cells were measured by absorbance at 600 nm. MICs were determined as averages from three biological replicates from each population.

QDs from Sigma-Aldrich was 7000 nM, a greater than 10 times increase over the MIC_{50} value 500 nM determined for the progenitor cells in this set of measurements (Figure 3a). The MIC_{50} value for populations evolved in Ocean NanoTech QDs was also markedly higher after evolution; the MIC_{50} of the evolved cells was determined to be 700 nM, while the MIC_{50} measured for the progenitor was ~200 nM, based on post-evolution measurement (Figure 3b). These results demonstrate that the yeast adapted to the toxicity of the CdSe QDs during prolonged exposure, as higher QD concentrations were required to inhibit cell growth post-evolution.

Notably, evolved yeast cells were only slightly more tolerant to free Cd^{2+}, with MIC values that ranged 80−160 μM for cells evolved in Sigma-Aldrich QDs, and 40−80 μM for cells evolved in Ocean NanoTech QDs; in comparison, the yeast progenitor's MIC to Cd^{2+} is 20 μM (Figure 4). This indicates that there may be a mechanistic difference in how the cells

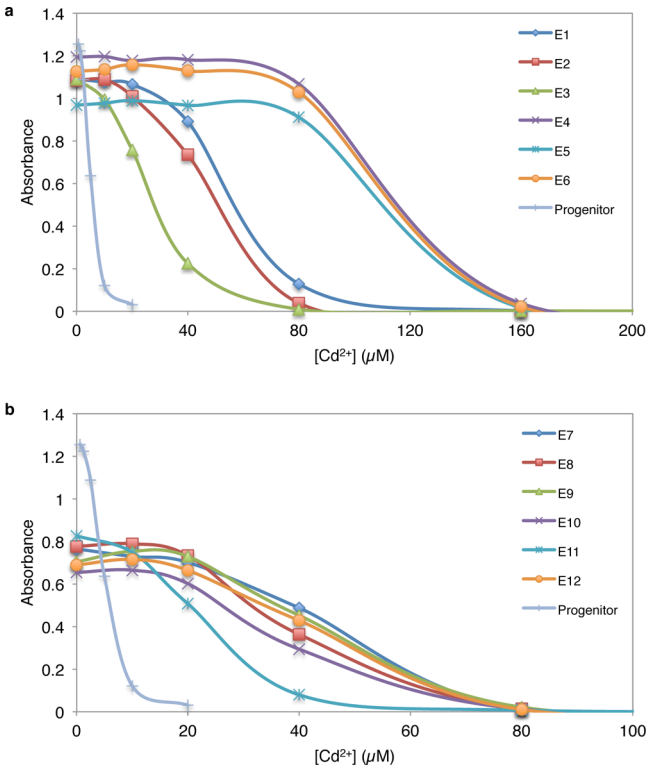

Figure 4. Cd^{2+} MIC determination for progenitor and evolved populations. MICs were measured for six Sigma-evolved (a, E1−E6) and six Ocean-evolved (b, E7−E12) strains and the progenitor population, Sce13. Absorbance measurements were made at 600 nm. MICs were determined as averages from two biological replicates from each Sigma- and Ocean-evolved population and three biological replicates from the progenitor population. The progenitor was only tested up to 20 μM Cd^{2+}. Note that Sigma-evolved populations (population 1, E1−E3; population 2, E4−E6) showed more variation with respect to Cd^{2+} tolerance: population 2 strains tolerate more Cd^{2+} than population 1 strains after evolution. In contrast, the two Ocean-evolved populations evolved more similarly and had lower Cd^{2+} tolerance overall. This may indicate that the post-evolution phenotype observed for Sigma populations was more influenced by Cd, while that of Ocean populations was more influenced by the QDs themselves (see text for details).

handle exposure to Cd-based QDs vs free metal ions. Because we found the Ocean NanoTech QDs to contain more total Cd (discussed above) and any free Cd^{2+} leaching from the QDs was not removed during the evolution experiment, one might expect that the cells exposed to Ocean NanoTech QDs would evolve higher tolerance to Cd^{2+} if free Cd ions exerted any selective pressure during the evolution. Surprisingly, this was not the case. We emphasize that because MIC values can have a 2 times range of error, the change we describe earlier of the yeast progenitor's MIC to Cd^{2+} of 20 μM increasing to 20−40 μM after prolonged exposure to the Ocean NanoTech QDs is not remarkable (Figure 4; Supporting Information Figure 3). We note that these results strongly suggest that the resistant phenotype observed for Ocean NanoTech populations post-evolution was caused by the QDs themselves and was not simply due to Cd exposure. We also measured the progenitor's MIC to Se; this value was 8 mM indicating a high tolerance

DOI: 10.1021/acs.bioconjchem.7b00056
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toward selenium, and thus, we did not further examine the effect of selenium. Based on our results, we can conclude that the adaptation of yeast to nanoparticles differs from adaption to their individual components.

Other studies show varied results when comparing Cd-based QDs to free Cd^{2+} ions. Tang et al. previously found that soluble Cd^{2+} led to more DNA damage than damage from CdSe/ZnS QD exposure, suggesting mechanistic differences between the toxic effects of soluble Cd^{2+} vs Cd-based QDs (we note that our results are based on use of uncapped CdSe QDs, whereas the QDs used by Tang et al. are capped with ZnS).[26] The authors showed in another study that Cd^{2+} concentrations in zebrafish liver cells after exposure were proportional to the concentration added, independent of whether the Cd was supplied as salts or CdTe QDs.[27] In general, CdTe QDs led to significantly more cellular accumulation of Cd^{2+} than $CdSO_4$. For example, Su et al. used CdTe QDs and reported a QD-based toxicity that was greater than the toxicity associated with a comparable amount of free cadmium ion.[11,28−30]

Thus, there appears to be a mechanistic difference in how cells handle exposure to Cd-based QDs vs the free metal ion: in the Tang et al. work,[26] the Cd^{2+} ions caused more DNA damage than the CdSe/ZnS QDs, but the Su et al. study found that CdTe QDs were more toxic than Cd^{2+} ions.[26,28] This suggests that the yeast adaptation to QD exposure may not necessarily also confer increased tolerance to free Cd^{2+} ions, similar to our observations discussed already.

Using light microscopy, we found no gross morphological changes in the evolved strains, and no noticeable differences in budding pattern or frequency of vacuole formation when compared to the progenitor (data not shown). Our results contrast with those from zebrafish liver cells, which began to exhibit alterations in cellular morphology when exposed to only 50 nM CdTe QDs.[26] In comparison, HeLa cells containing CdSe/ZnS QDs were found to maintain biological function and remain viable for several generations; moreover, the cells remained in good condition and continued to endocytose QDs for up to 20 days after incubation, with no observed morphological changes.[31]

Although we observed no morphological changes, in the case of yeast cells evolved with Ocean NanoTech QDs, their overall fitness was markedly less than that of the progenitor, evident by the lower absorbance measurements for Ocean-evolved populations (vs the progenitor) at a QD concentration of 0 nM in Figure 3b. This trend is also apparent in Figures 4b and 5b. That is, evolved yeast cells incubated for 24 h in liquid yeast-extract peptone dextrose (YPD) medium containing no CdSe QDs showed much poorer growth in comparison with the progenitor yeast cells; the strain evolved in Ocean NanoTech QDs required approximately twice as much time to produce a saturated culture than did the progenitor yeast strain. This is a clear example of an evolutionary trade-off: an increase in fitness in one environment is coupled with a decrease in fitness in another.[32]

We next performed whole-genome sequencing (WGS) on all evolved cell populations. WGS revealed five single nucleotide polymorphisms (SNPs) in known genes in the experiments conducted with Sigma-Aldrich QDs. We did not uncover any indels (insertions and deletions), in particular, small indels. More complex changes, e.g., large indels and chromosomal rearrangements, cannot be definitively called from WGS data.[33] Cells evolved with Ocean NanoTech QDs only exhibited one SNP in a known gene (see Supporting Information Table 1).

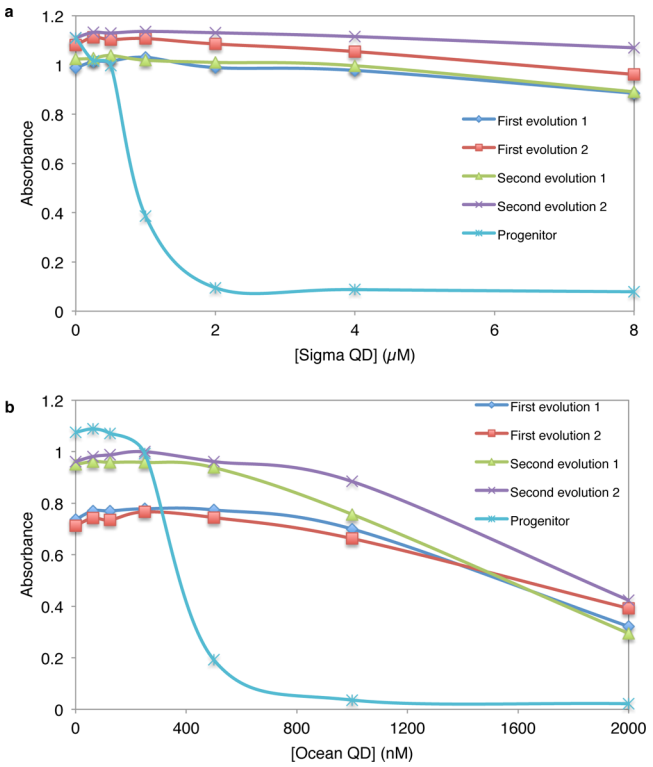

Figure 5. QD MIC determination for progenitor and evolved populations after the second evolution without the selective pressure of the QDs. MICs were measured for Sigma-evolved (a) and Ocean-evolved (b) populations and the progenitor population, Sce13. For each QD, MICs were measured from populations obtained after the first evolution with QDs ("First evolution") and populations obtained after the second evolution without QDs ("Second evolution"). Absorbance measurements were made at 600 nm. MICs were determined as averages from two biological replicates from each population. Note that after the second evolution without QDs, the stunted growth observed in Ocean replicates appears to have recovered (indicated by similar culture optical densities for progenitor and second evolution samples at a QD concentration of 0 nM); however, these cells retained their ability to tolerate the QDs: MIC values for Ocean samples with and without selective pressure from QDs are comparable.

Of the known genes, the majority were involved in gene expression (transcription and translation) and cell growth (cell wall synthesis). It is not uncommon for parallelism to be observed in evolved populations, where similar regulatory genes acquiring mutations are favored in multiple populations. Even a single mutation in a critical regulatory gene can result in large-scale changes in the cell.[24] In contrast, phenotypic changes can be the result of mutations in multiple genes working in concert. As anticipated, the majority of SNPs were identified in multiple readouts from the same population, as serial passage experiments favor homogeneous populations due to strong selective pressures that drive sweeps, in which favorable alleles rapidly rise in frequency to nearly 100%. In contrast, most mutant alleles never rise in frequency, or they are lost during the daily dilution associated with our batch-culture protocol.[24,25]

We confirmed the SNPs identified by WGS through PCR amplification of the locus of each SNP from DNA freshly extracted from each evolved strain followed by Sanger sequencing. All six SNPs in known genes were confirmed (see Supporting Information Table 1); that is, the mutation detected by WGS was present at the specified position of each

DOI: 10.1021/acs.bioconjchem.7b00056
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SNP (another seven SNPs in unknown genes were also confirmed). Next, we investigated whether the post-evolutionary change in QD tolerance could be attributed to a single mutation. We monitored the segregation of the mutations at the position of each SNP, so that they could be assessed for QD tolerance separately. We mated the evolved *S. cerevisiae* strains (mating type MAT**a**) with a compatible strain, SCE3 (MATα), and induced sporulation. We isolated tetrads: two spores are expected to carry the mutant nucleotide, and two spores the wild-type nucleotide (Supporting Information Table 1). We confirmed the expected two-to-two segregation by Sanger sequencing and found one mutation present in two strains from one Ocean NanoTech QD-evolved population that segregated. All other SNPs did not segregate as anticipated.

The segregated mutation, N239K, occurred in *bul1* (GenBank: BAA08787.1), which encodes the ubiquitin-binding component of the Rsp5p E3-ubiquitin ligase complex in *S. cerevisiae*.[34] Bul1 is a transferase with ubiquitin−ubiquitin ligase activity (reviewed in ref 35). Bul1 therefore plays an important role in polyubiquitination in yeast, as it mediates the transfer of ubiquitin (Ub) from one protein to an existing Ub chain. Interestingly, the uncovered mutation converts Asn239 to Lys: the Lys side chain serves as the link to ubiquitin when a protein is post-translationally modified. Using its PY-motif, Bul1 has been shown to interact with the WW domain of the Ub ligase Rsp5.[34,36] The N239K mutation in Bul1 is not in this critical PY-motif; it is 79 amino acids C-terminal to this motif.

Deletion of *bul1* has been previously associated with increased Cd^{2+} sensitivity. In one study, a *bul1* deletant was found to exhibit moderate sensitivity to Cd^{2+}, as well as to other heavy metals and H_2O_2, suggesting a role for Bul1 in handling of Cd^{2+} and oxidative stress in yeast.[37] Another study showed that a double deletant of *bul1* and its homologue, *bul2*, was impaired in polyubiquitination of the general amino acid permease, Gap1, resulting in aberrant intracellular transport of Gap1.[38] Additionally, Bul1 and Ub-dependent signaling have been implicated in the control of nutrient-responsive transcription under nitrogen starvation.[39] Thus, the mutation we uncovered in *bul1* could potentially affect any number of ubiquitination-dependent processes. Similar to the involvement of *bul1* in Cd^{2+} tolerance and response to other stressors, the mutation we uncovered may lead to downstream cellular events important for increased QD resistance.

To examine the importance of the N239K mutation in Bul1, we conducted MIC tests on the spores harboring the mutation to determine whether the mutant genotype was responsible for the resistant phenotype; after chromosomal segregation, spores harboring the N239K mutation in Bul1 did not tolerate greater QD concentrations than spores without the mutation (Table 1; Supporting Information Figure 2). Thus, we did not detect a simple link between the N→K mutation and a resistant phenotype.

Because we did not establish a definitive relationship between one specific genetic mutation and the resistant phenotype, we questioned whether the increased tolerance to the QDs could have been caused by epigenetic changes that occurred during QD exposure. Epigenetic changes are functionally relevant changes, such as DNA methylation or chromatin remodeling, that are not caused by alteration of the nucleotide sequence (reviewed in refs 40, 41). Such an event is not detectable by DNA sequencing, as it is not a genetic change, but can still contribute to increased QD tolerance exhibited by the evolved cells due to potential changes in gene expression profile.

Table 1. Ocean NanoTech QD MICs[a]

strain	sample	Ocean QD MIC (nM)	mutation present?
O1−1	b1	200	N
	b2	100	Y
	b3[b]	200	N
	b4	100	Y
	Sce13	100	N
	O1−1 evolved[b]	800	Y
O1−3	s1[b]	100−200	N
	s2	100	N
	s3	200	Y
	s4	100	N
	m1[b,c]	200	Y
	m2[c]	200	N
	Sce13	100	N
	O1−3 evolved[b]	800	Y

[a]O1-1 haploids from one tetrad (b1−b4), O1-3 haploids from two tetrads (s1−s4; m1, m2), evolved O1-1 and O1-3 strains, and Sce13 progenitor. [b]Exhibited inhibited growth in the absence of QDs (i.e., in YPD broth alone). We note that the differences between 100 nM and 200 nM values are not significant. [c]The "m" samples resulted after dissection of a tetrad that did not harbor four spores capable of growth on YPD agar; i.e., after plating, only two spores formed colonies, while the other two did not. Of the two colonies that did grow, one grew to a normal size (m2), while the other exhibited inhibited growth on a YPD agar plate (m1). Because m1 was found to contain the mutation and m2 was not, these samples were subjected to MIC testing.

Therefore, we subjected the evolved strains to a second evolution during which the evolved strains were grown for an additional 80 generations (12 days) without the selective pressure of the QDs. If the tolerance detected was in fact due to epigenetic factors, we anticipated that this second evolution might result in reversion of the resistant phenotype—i.e., the yeast would regain susceptibility to QD toxicity. Such a result would indicate that the phenotypic change observed after the first evolution was independent of the genetic changes we uncovered and could therefore be attributed to an epigenetic effect.

We repeated the MIC tests after the second evolution and observed that the resistant phenotype persisted for strains evolved in both Sigma-Aldrich and Ocean NanoTech QDs (Figure 5). Interestingly, Ocean NanoTech QD-evolved populations appear to have recovered during this second evolution from the stunted growth exhibited after the original evolution (Figure 5b). Despite this, these yeast retained their ability to tolerate the QDs. These findings strongly suggest that the changes initially observed in the evolved strains are both permanent and genetic. Although this is not a conclusive experiment that completely eliminates the possibility of epigenetic effects, this result leads us away from surmising that epigenetic changes contributed to the strains' increased resistance to toxic QDs.

Our results clearly demonstrate that yeast cells are capable of genetic adaptation to the toxicity associated with nanoparticles. Notably, the role that *bul1* has already been shown to play in cellular handling of free Cd ions (discussed above) is captured in our results that demonstrate that a genetic mutation in *bul1* may affect the cell's ability to cope with the CdSe quantum dots. Although Bul1 may have a role in the adaptive response, it does not account for the full increase in resistance observed; the *bul1* mutation may be interacting with another undetected determinant(s). The likelihood of epigenetic effects being the

DOI: 10.1021/acs.bioconjchem.7b00056
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cause of the evolved yeast's ability to withstand prolonged exposure to QDs appears to be low.

We note that no effort was made to assess the resistance of genotypes combining more than one SNP. Instead, we attempted to assess mutations at each SNP individually. It is possible that the resistant phenotype of a haploid is a manifestation of the combined effect of multiple mutations. It is plausible that exposure to a complex structure with known cytotoxic effects, such as a Cd-based QD, could lead to global genomic/proteomic changes, rather than a single, critical mutation that alone accounts for the change in QD tolerance. This is consistent with the findings of Serero et al., who propose that *S. cerevisiae* respond to Cd^{2+} toxicity by global modification of their proteome and sulfur metabolism to produce massive amounts of the antioxidant glutathione.[37] An alternate possibility is the presence of further mutations that remain undetected in the genome-wide screen for variation. These mutations, possibly along with *bul1*, may result in a polygenic explanation for the observed tolerance to QDs.

Interestingly, ubiquitin is expressed in virtually all eukaryotic tissues, but prokaryotes do not carry any system similar to ubiquitination. We discussed above that bacteria (*E. coli*) showed high tolerance to CdSe QD exposure, and this may be due to prokaryotes being simpler organisms than eukaryotes, which possess more complicated genetics and structure that can be affected by foreign substances.[41] The differences in complexity between prokaryotes vs eukaryotes offers an explanation for why we did not observe any effect on bacteria from QD exposure, and may indicate the necessity of exploring nanomaterials' effects on various members of our ecosystem with a special focus on the more complex members. Yeast, however, is the simplest eukaryote and provides a reasonable starting point.

Our study illustrates that chronic QD exposure can direct evolution toward increased QD tolerance: only 24 days of exposure to 10 ppb CdSe QDs resulted in permanent genetic adaptation in *S. cerevisiae*. SNPs that might otherwise be lost from the population during evolution are retained to increase fitness in a toxic environment, sometimes by sacrificing fitness in the original environment.

Moreover, our results suggest that two similarly classified QDs, available commercially, may have drastically different effects on the biology of evolution and tolerance, lending credibility for the systematic assessment of the environmental impact of each QD. Nanotechnology is a burgeoning field in the research and manufacturing communities; nanomaterials, including QDs, are part of our everyday lives. It is critical that we understand how these materials can permanently change our environment and its inhabitants.

Given that this study opens the door to many questions about nanomaterials, evolution, and environmental responses, we anticipate that our future experiments will explore parallel evolution, where more replicates will be evolved to detect common mutations conferring the largest benefits and the uncovering of other genetic determinants that can indicate exposure to nanomaterials. Fitness benefits offered by a specific mutation can be assessed through the use of allelic replacement,[42] which is a type of gene conversion where one allele replaces another allele of the same gene. Allelic replacement will also reveal whether the mutations work individually, have an additive effect, or work synergistically with other mutations to increase fitness.

METHODS

Quantum Dot Preparation and Characterization. CdSe core-only quantum dots that are soluble in organic solvents and with emission maxima at 580−590 nm were purchased from two venders: Ocean NanoTech (cat# QCO-580−0050, Little Rock, AK, USA) and Sigma-Aldrich (cat# 662607, St. Louis, MO, USA). Quantum dots were solubilized by diluting stock solutions in chloroform and adding 16-mercaptohexadecanoic acid (Sigma-Aldrich, cat# 674974) dropwise until the solution became turbid. The QD solution was stirred in the dark at room temperature for 3 h. Quantum dots were purified from unbound ligand by three acetone washes, and the QDs were resuspended in 50 mM borate buffer with additional 16-mercaptohexanoic acid (pH ∼ 8). An equivalent volume of chloroform was added, and the solution was stirred for an additional 2 h in the dark at room temperature. The aqueous (colored) fraction was retrieved, and a further acetone wash step was performed. The QDs were again resuspended in 50 mM borate buffer, filtered using a 0.22 μm pore size syringe filter (Millipore, cat# SLGP033RS) and stabilized with filtered bovine serum albumin (1% w/v final concentration; Sigma-Aldrich, cat# A9418). The QD solution was aliquoted and lyophilized for long-term storage. Quantum dots were reconstituted in sterile 1× PBS prior to use and characterized using standard techniques. Optical characteristics were measured using UV−vis spectrometry (Shimadzu UV-1601PC) and fluorescence spectroscopy (Jobin-Yvon Fluoromax-3). Core size was determined using transmission electron microscopy (FEI Tecnai 20, Advanced Bioimaging Center, Mount Sinai Hospital, Toronto, Canada).

Evolution. Four experimental populations were propagated in 1.5 mL liquid medium (yeast extract peptone dextrose, YPD) each with aeration (shaking at 300 rpm) and with daily serial transfers (1/100 dilution) for 160 generations (24 days). Two populations were evolved in media containing QDs purchased from Sigma-Aldrich, and two populations were evolved in media containing QDs purchased from Ocean NanoTech.

Minimum Inhibitory Concentration. Minimum inhibitory concentration was determined using methods described elsewhere.[22] Briefly, 2 times dilutions of QDs were added to yeast growth media and a yeast starter culture. The cells were allowed to propagate for 48 h. The absorbance at 600 nm was assessed to determine cell density and determine the concentration at which growth was inhibited (MIC). Three colonies were chosen from each population after 160 generations to assess MIC prior to sequencing. Based on an absorbance and concentration plot, MIC was determined to be the concentration at which no cell growth occurred, and MIC_{50} was the concentration at which 50% of the growth was inhibited. Growth or no growth was assessed both visually and using the PowerWave XS2 plate reader (BioTek).

DNA Extraction, Sequencing, and Analysis. DNA extraction was carried out on 12 evolved samples (three per population) and the progenitor using the Gentra Puregene cell kit (Qiagen) following the manufacturer's instructions. DNA libraries were built using the Illumina TruSeq DNA library preparation protocol. The library was sequenced on an Illumina HiSeq 2500 using paired-end recipe (2 × 100 bases) with TruSeq v3 chemistry (TCAG Sequencing Facility, Toronto). Sequence analysis was performed using Geneious software (v 6.1.5, Biomatters Limited). SNPs were identified in comparison to a reference sequence of *S. cerevisiae* (R64−1−1); SNPs

DOI: 10.1021/acs.bioconjchem.7b00056
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occurring in more than one sample per population were chosen for further analysis.

Mating, Sporulation, and Haploid Assessment. Evolved strains were mated with a compatible strain, Sce3, in YPD. Mating was confirmed by visual inspection for flocculation and the presence of zygotes (via light microscopy) after approximately 6 h. Diploid yeast were sporulated in 1% potassium acetate. The resulting tetrads were treated with yeast lytic enzyme (BioShop) plated on YPD agar, and 3−5 tetrads per strain were dissected using a micromanipulator (Carl Zeiss Axiolab) and grown on YPD agar for 3−5 days. DNA was extracted from each of the four haploid colonies from the tetrads; regions containing the SNP of interest were amplified by PCR. PCR products were sequenced to assess segregation of mutations. Tetrads harboring mutations that segregated were cultured and subjected to MIC testing.

Transmission Electron Microscopy (TEM). Purified yeast cells, with or without QDs, were spun down, fixed with 2% glutaraldehyde fixative solution, and stored at 4 °C until sectioning. Processing and sectioning of cells was performed at the Advanced Bioimaging Centre, Mount Sinai Hospital, Toronto, Canada. Sections of 100 nm thickness were cut on an RMC MT-6000 ultramicrotome and placed on copper grids for TEM imaging without further staining. Cell images were obtained using a Hitachi H7000 TEM at the Centre for Nanostructure Imaging, Department of Chemistry, University of Toronto, Canada.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.7b00056.

Schematic of haploid assessment; MIC determination; Table of SNPs observed (PDF)

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Author Contributions

S.S. and A.S. designed and performed experiments, analyzed data, and wrote the paper. P.T., K.M.T., and F.S. performed experiments and analyzed data. J.B.A., W.C.W.C., and J.A.S. conceived the project idea, designed experiments, and wrote the paper.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors would like to thank Cindy Short for her assistance with the DNA extraction protocol, Caroline Sirjusingh for her assistance with the microscopy, Dr. Ichiro Inamoto for his helpful advice and discussion of this work, and Sherif Saleh for experimental assistance with MIC assays. J.A.S. and W.C.WC. are grateful for funding from NSERC Discovery Grant and the Collaborative Health Research Program (CHRP).

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DOI: 10.1021/acs.bioconjchem.7b00056
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