Biosynthesis

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Construction and Potential Applications of a Functionalized Cell with an Intracellular Mineral Scaffold**

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Highly efficient natural biosystems, such as yeast and M13 bacteriophage, have potential for the fabrication of minerals.[1–2] In particular, it is innovative to synthesize various minerals inside a biosystem because such a biosynthesis can avoid problems such as environmental pollution, energy loss, chemical safety, and noncompatibility with biological systems. Since cadmium sulfide quantum semiconductor crystallites were synthesized inside yeast in 1989,[3] a few similar studies have investigated the biosynthesis of other minerals.[4] Our objective was to prepare a functionalized cell inside which a mineral scaffold could be built. Most importantly, such a functionalized cell could be used as a biosupport or for drug delivery in order to meet the purposes of various applications in the fields of medicine, and environmental and material sciences.

Herein we report the preparation of functionalized cells that could produce a biogenic $CaCO_3$ nanoparticle scaffold inside *S. cerevisiae* through the endogenous reaction of Ca^{2+} ions and CO_2 under normal growth conditions. A study using the functionalized cells as an anticancer drug carrier for doxorubicin hydrochloride (DOX) demonstrated the advantages for localized drug release by the pH-sensitive structure, and enhanced cytotoxicity by increasing the cellular uptake. Further investigations also revealed the desirable properties of the functionalized cells for removing heavy metals from aqueous solutions.

The functionalized cells could be easily obtained. In a typical synthesis, yeast cells and maltose were placed in a beaker and thoroughly mixed. A saturated aqueous solution of $Ca(OH)_2$ was then added into the beaker and the resulting mixture was kept under moderate stirring for 10 min. Subsequently, the products were collected and washed. The possible generation mechanism of the functionalized cell is shown in Figure 1. Firstly, *S. cerevisiae* was reactivated in maltose solution, and carbon dioxide was produced by respiration. Secondly, addition of a saturated solution of $Ca(OH)_2$ led to Ca^{2+} and OH^- ions entering the yeast cell. In the basic environment, the carbon dioxide inside the yeast cell could form CO_3^{2-} ions. Meanwhile, Ca^{2+} ions could interact with the biomolecules derived from the cell, such as proteins and polysaccharides. Finally, the $CaCO_3$ crystals were formed and could be stabilized by the biomolecules.

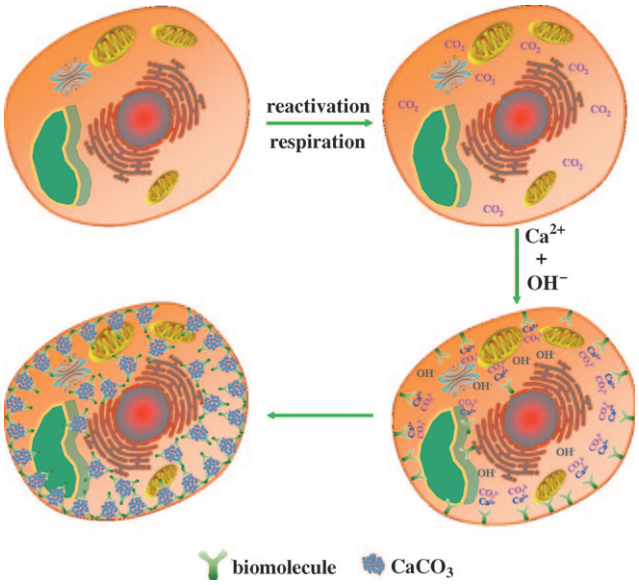

Figure 1. Formation mechanism of the functionalized cell with endogenous production of a $CaCO_3$ nanoparticle scaffold.

Figure 2a shows the typical SEM image of the functionalized cell. Compared with the control *S. cerevisiae* (Figure S1 in the Supporting Information), it was found that the surface of *S. cerevisiae* remained unchanged before and after the formation of $CaCO_3$. Figure 2b shows the in situ high-resolution TEM (HRTEM) image of several individual intracellular biogenic $CaCO_3$ nanoparticles. It was found that these $CaCO_3$ nanoparticles had an average size of 4–6 nm, with lattice spacings of $d = 3.823$, 3.064, and 2.481 Å, which correspond to the (012), (104), and (110) facets, respectively, of the calcite phase of $CaCO_3$. X-ray energy dispersive spectroscopy (EDS) analyses of individual functionalized cells and control *S. cerevisiae* cells showed that

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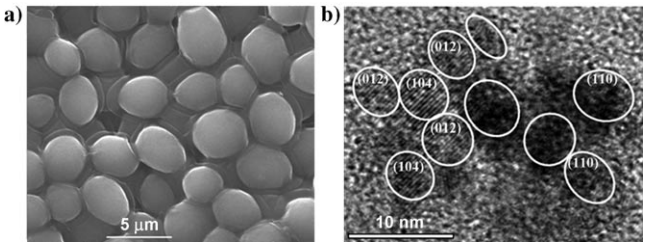

Figure 2. a) Scanning electron micrograph of the functionalized cells. b) In situ HRTEM image of several individual biogenic $CaCO_3$ nanoparticles.

Ca^{2+} ions were present in the functionalized cells, but not in the control *S. cerevisiae* cells (Figure S2). These data proved clearly that $CaCO_3$ nanoparticles could be endogenously produced by the biochemical reactions in yeast cells.

The XRD pattern of the functionalized yeast cells demonstrated that the phase of the biogenic $CaCO_3$ was calcite (Figure S3). Furthermore, the composition of the mineral phase was also confirmed by FTIR spectroscopy (Supporting Information, Figure S4).[5] Thermogravimetric analysis (TG) curves of the functionalized cells and control *S. cerevisiae* showed that the functionalized cells contained about 15% $CaCO_3$ by weight (Figure S5).

The presence of $CaCO_3$ inside the functionalized cells was further confirmed by using confocal laser scanning microscopy after the cells were stained using tetracycline hydrochloride. The results showed the stained $CaCO_3$ scaffold exhibited a fluorescent blue color under UV light (360–370 nm), thus indicating the $CaCO_3$ scaffold was indeed synthesized inside the yeast cell (Figure S6a). In contrast, the fluorescent color could not be visualized in the control yeast cells (Figure S6b), thus suggesting the absence of $CaCO_3$ nanoparticles. The presence of $CaCO_3$ inside the functionalized cells was also further determined by the *z*-axis focal plane confocal images, thus demonstrating that the fluorescence of biogenic the $CaCO_3$ scaffold was located inside the cell membrane (Figure 3).

Yeast *S. cerevisiae* cells were still viable in the presence of the biogenic $CaCO_3$ scaffold. A trypan blue exclusion assay was used to detect the metabolic status of the functionalized cells. Figure 4a shows the light micrograph of the functionalized cells stained with trypan blue. The functionalized cells were not stained blue, thus showing they were still metabolically viable. Additionally, flow cytometry analysis showed that the functionalized cells were 93.41% viable (Figure S7).

The growth curves of the control *S. cerevisiae* and the functionalized cell were characterized by measuring their turbidity (optical density at 600 nm, OD_{600}). As shown in Figure 4b, curves of the percentage of living cells in YPD broth (2% tryptone and 2% glucose) were plotted against the growth time. It was observed that the functionalized cell could not proliferate until after 60 h incubation (see curve 1). In contrast, the proliferation of the control *S. cerevisiae* cells was faster under the same conditions (see curve 2). In order to further confirm the observation, the cell density was also monitored by light microscopy. From the microscopy images, the proliferation of the functionalized cell was not seen after culturing for 24 h in YPD broth at 37°C (Figure 4b, right-hand image). In contrast, it was found that the cell density of the control *S. cerevisiae* increased rapidly under the same conditions (Figure 4b, left-hand image), thus suggesting that the proliferation of the functionalized cell could be delayed. This delay in proliferation of the functional cell was probably attributable to the biogenic $CaCO_3$ scaffold, which could reduce mass transport and biological communication between the cells and the surrounding environment.[6]

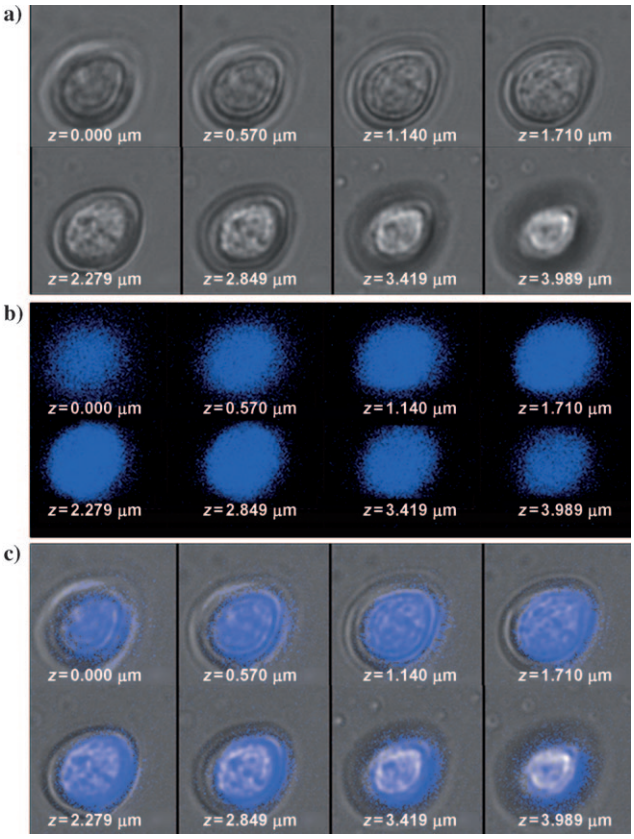

Figure 3. Confocal laser scanning images of the functionalized cells. a) Different *z* axis focal planes under visible light. b) Different *z* axis focal planes excited by UV light. The biogenic $CaCO_3$ scaffolds are stained fluorescent blue. c) Combination of the above fluorescence results.

Drug delivery has been greatly improved in recent years.[7] A cell-based delivery system exhibits a number of advantages, including increased bioavailability, improved pharmacokinetics, and reduced toxicities.[8] In this regard, the $CaCO_3$ produced in yeast cells possesses the potential for application in the development of efficient drug carriers because of its ideal biocompatibility and biodegradability.

We took advantage of the autofluorescence of doxorubicin (DOX) to monitor its loading into both the functionalized cells and the control *S. cerevisiae* cells. The results showed there was strong autofluorescence in the functionalized cell, but not in the control *S. cerevisiae* cell after 17 h of co-incubation with DOX, thus indicating that the biogenic $CaCO_3$ could induce DOX to enter into the functionalized cell (Figure S8a, b). It was therefore reasonably assumed that the interactions of the Ca^{2+} ions and the OH and CO groups in DOX led to an efficient DOX loading into the functionalized cells.

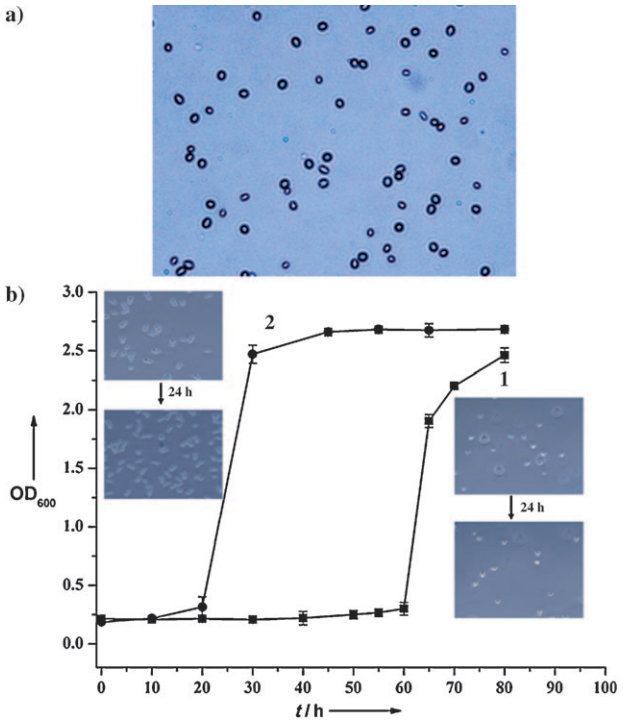

Figure 4. Effects of the $CaCO_3$ nanoparticles scaffold on the viability and proliferation of *S. cerevisiae* cells. a) Light micrograph of the functionalized cells stained by trypan blue. b) Growth curve of the functionalized cells (curve 1) and the control *S. cerevisiae* cells (curve 2); the light micrographs of the functionalized cells (right-hand images) and the control *S. cerevisiae* cells (left-hand images) before and after incubation for 24 h in YPD broth.

Time- and pH-dependent release of DOX were also investigated. The amount of DOX released from the functionalized cells gradually increased with decreasing pH value and time (Supporting Information, Figure S8c). However, only a small quantity of DOX was released under physiological conditions (pH 7.0–7.5). The results demonstrated that the release of DOX from the functionalized cell into tumor tissues (pH 6) or lysosomes inside cancer cells (pH 4.5) was pH-sensitive.[9] In order to further evaluate the functionalized cells as pH-sensitive drug carriers, the functionalized cells in an acidic environment (pH 4.0) were collected at the end of the DOX release test and characterized by XRD. All the peaks indexed to the $CaCO_3$ (calcite) disappeared, thus revealing that the cells are pH-sensitive carriers, and thus suggesting DOX could be favorably released at the target site by adjusting the pH value.

In order to determine the cytotoxic effect of the functionalized cells loaded with DOX on PC-12 cells, the cell density was monitored by light microscopy. Figure 5 shows the cell densities of PC-12 treated with the functionalized cells without and with DOX, respectively. As shown in Figure 5a, the functionalized cell without DOX showed almost no cytotoxicity to PC-12 cells, thus indicating good biocompatibility. In contrast, when compared to the effect of DOX alone, the death rate of PC-12 cells was greatly increased when the functionalized cells were loaded with DOX (see Figure 5b). It could be speculated that the functionalized

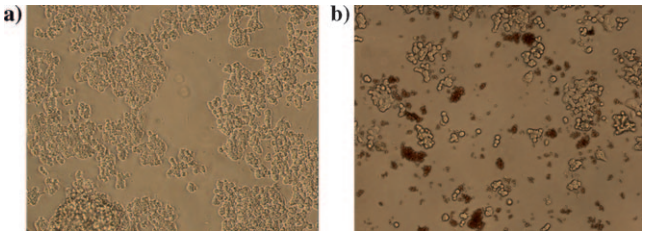

Figure 5. Light microscopy images of PC-12 cells treated with a) the functionalized cell and b) the functionalized cell loaded with DOX for 56 h, respectively.

cells, which act as pH-sensitive drug carriers, could promote the release of DOX and enhance its cytotoxicity by increasing cellular uptake.

The reduction of the amounts of heavy metals in polluted and waste water to an acceptable level is a huge challenge.[10] Compared with conventional methods for removing heavy-metal ions from aqueous solutions, biosorption is ideal for the treatment of large volumes of wastewater that contain low concentrations of metal complexes.[11] We further investigated the properties of the functionalized cells in removing heavy metals by using Pb^{2+} ions as an example. The experiment was conducted using solutions containing 15.2 mg L^{-1} Pb^{2+} ions at pH 5.37 with the functionalized cells, and the control test was performed using control yeast cells. As seen in Figure 6, we observed that the sum of Pb^{2+} ion uptake capacities of the functionalized cells was greater than that of the control yeast cells. It is possible that precipitation transformation between $CaCO_3$ and $PbCO_3$ occurred only in the functionalized cells but not in the control yeast cells, in addition to biosorption of Pb^{2+} ions. The results demonstrated the functionalized cells had a greater advantage over the control yeast cells in removing heavy-metal ions. Most importantly, this method is efficient in removing trace impurities from aqueous solutions.

In summary, we have successfully prepared functionalized cells that can endogenously produce a $CaCO_3$ scaffold under normal physiological conditions. The results demonstrated

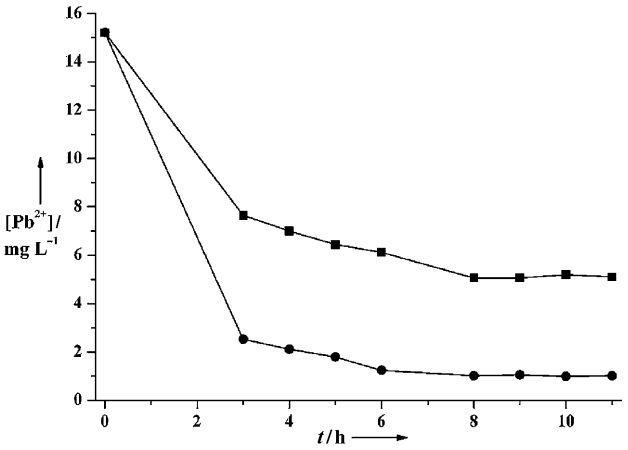

Figure 6. Biosorption of Pb^{2+} ions (pH 5.37) by the functionalized cells (●) and the control *S. cerevisiae* cells (■) at initial metal concentrations of 15.2 mg L^{-1}.

that the functionalized cells were able to not only localize drug release because of their pH-sensitive nature but also enhance the drug cytotoxicity because of their ability to increase the DOX uptake of cancer cells. In addition, the functionalized cells were effective in removing heavy metals from aqueous solutions and thus have an important application in environmental protection. Future studies will address the preparation of functionalized cells with possible endogenous synthesis of other mineral scaffolds (e.g., other carbonates and hydroxyapatite) in order to develop a large number of drug carriers for various biological and pharmaceutical applications.

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