Bioinspired Mineralization

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Bioinspired, Cytocompatible Mineralization of Silica–Titania Composites: Thermoprotective Nanoshell Formation for Individual *Chlorella* Cells**

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The cytocompatible nano-encapsulation of individual living-cells with ultrathin (< 100 nm) and robust shells promises the potential of chemically manipulating cellular activities (e.g., cell division) at the single-cell level and protecting cells from external stressors, by mimicking both structural and functional characteristics of bacterial endospores found in nature.[1–3] In the manipulation of cellular activities, the mechanical rigidity of the artificial shell encasing a single cell is found to be a requirement for control of cell-division behaviors,[4] and a few methods have been developed for formation of robust nanoshells. The examples include layer-by-layer assembly,[5] polydopamine coating,[6] and bioinspired mineralization.[7,8] In some cases, the artificial shells endow the encapsulated cells with improved tolerance against lytic enzymes,[6] nutrient deprivation,[8] osmotic pressure,[9] shear force,[10] or heat.[11]

The inorganic (or inorganic–organic hybrid) materials generally surpass the organic ones in robustness and rigidity, and their physicochemical properties can be tuned further by composite formation. For example, the composite of SiO_2 and TiO_2 has been studied intensively owing to the synergistic combination of amorphous SiO_2 and crystalline TiO_2.[12] The SiO_2–TiO_2 nanocomposites showed a higher thermal stability than pure SiO_2 or TiO_2,[12b] and the thermo-oxidative stability of polystyrene was enhanced by the addition of titanium-containing polyhedral oligomeric silsesquioxane.[13] In this regard, it also can be thought that the cellular thermo-resistance could be achieved by encapsulating a cell with an SiO_2–TiO_2 composite shell. However, conventional chemical processes for inorganic materials generally cannot be applied to living cells because of the harsh reaction conditions that are lethal to cells.

On the other hand, the mild conditions developed for bioinspired mineralization[14] have been adopted for single-cell encapsulation with silica[7,8] or titania (TiO_2).[15,16] In pursuing our aim of bioinspired TiO_2 formation, we have previously designed a peptide, $(RKK)_4D_8$ (R: arginine; K: lysine; D: aspartic acid), for cell-surface deposition of abiological TiO_2 derived from titanium(IV) bis(ammonium lactato)dihydroxide (TiBALDH) on *Chlorella* cells;[15] the cationic R and K residues induce the deposition of TiO_2 onto the *Chlorella* cells after being adsorbed electrostatically onto the negatively charged cell-surface, and the D moiety diminishes the cytotoxicity of the positively charged peptide. Herein, we report the bioinspired formation of SiO_2–TiO_2 composites with $(RKK)_4D_8$ from both TiBALDH (TiO_2 precursor) and silicic acid (SiO_2 precursor). The mild mineralization conditions enable the cytocompatible nano-encapsulation of individual *Chlorella* cells, and the SiO_2–TiO_2 shell greatly improves the cellular thermo-tolerance.

The formation of the SiO_2–TiO_2 composites was first investigated with the $(RKK)_4D_8$ peptide in solution (Figure 1 a). The reaction conditions were set to be physiologically mild conditions (pH 7.4 and room temperature). To a tris(hydroxymethyl)aminomethane (TRIS) buffered solution of TiBALDH and silicic acid (1:1, molar ratio) was added the TRIS-buffered solution of $(RKK)_4D_8$. Precipitates were observed right after the addition, and collected after 30 min. The X-ray photoelectron spectroscopy (XPS) spectrum confirmed that the nanoparticulates (5–50 nm in diameter from the scanning electron microscopy (SEM) analysis) contained both SiO_2 and TiO_2. The Raman and X-ray diffraction (XRD) spectra indicated that the majority of TiO_2 was amorphous (See the Supporting Information for the peak assignments, Figure S1a–c). In addition, the high-resolution transmission electron microscopy (HR-TEM) image showed that the

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approximately 3 nm anatase TiO_2 was embedded in the nanoparticulates (Figure 1b; Also see the Supporting Information, Figure S2).[17] When applied to a solid substrate, the mineralization approach formed a SiO_2–TiO_2 film, the thickness of which could be tuned by a layer-by-layer process. For this method a gold substrate coated with carboxylic acid-terminated self-assembled monolayers (SAMs) was alternately immersed in the $(RKK)_4D_8$ and the precursor solutions. Each deposition time was minimized to 2 min for direct application to cell encapsulation. The resulting films were characterized by XPS and time-of-flight secondary ion mass spectrometry (TOF-SIMS) depth profiling, confirming the coexistence of SiO_2 and TiO_2 in the films (See the Supporting Information, Figure S3a and b). After 2-by-2 deposition, the thickness was about 30 nm and increased linearly with the number of depositions (Figure 1c). Taken all together, the conditions for SiO_2–TiO_2 formation were suitable for cell encapsulation, because the reaction was fast under mild conditions, and the film thickness could be controlled.

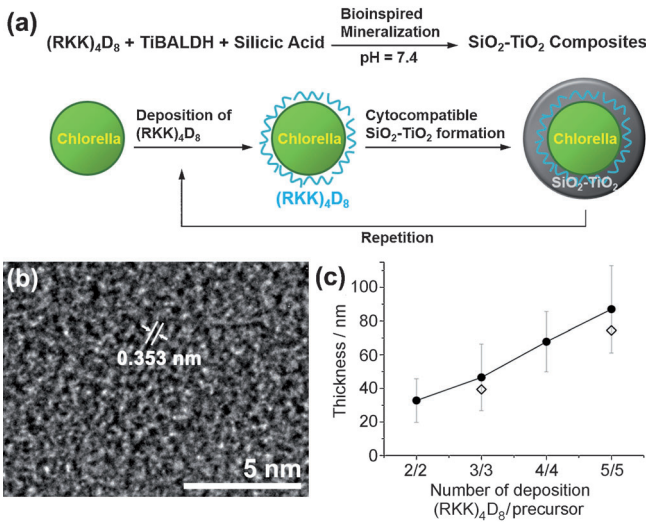

Figure 1. a) Scheme for formation of SiO_2–TiO_2 composites and for nano-encapsulation of individual *Chlorella* cells within the SiO_2–TiO_2 composite shell. b) HR-TEM image of SiO_2–TiO_2 particulates formed in solution. c) A graph for the average thickness of the SiO_2–TiO_2 film on gold versus the number of depositions. For comparison, the thickness of the SiO_2–TiO_2 shell on *Chlorella* is added to the graph (◇).

Figure 1a depicts the experimental procedure for nano-encapsulating individual *Chlorella* cells in a SiO_2–TiO_2 shell. Briefly, $(RKK)_4D_8$ was deposited onto the *Chlorella* surface for 2 min in the TRIS buffer by electrostatic interactions, and the resulting *Chlorella* suspension was treated with TiBALDH and silicic acid (1:1, molar ratio) in the TRIS buffer for 2 min. This cycle was repeated three times, leading to the formation of *Chlorella*@SiO_2–TiO_2. The resulting *Chlorella*@SiO_2–TiO_2 was characterized by SEM, energy-dispersive X-ray (EDX) spectroscopy, and TEM. The SEM micrographs showed that the *Chlorella* cells were encapsulated individually with a shell of SiO_2–TiO_2 nanoparticulates (Figure 2a and d). The polycondensation of silicic acid and TiBALDH was confirmed by the elemental and line-scan analysis of EDX spectroscopy (Figure 2b and e). The TEM micrographs of microtome-sliced *Chlorella*@SiO_2–TiO_2 indicated that the shell was about 40 nm thick (Figure 2c and f; Also see Figure 1c). The EDX mapping with TEM also indicated that the encasing layer was composed of SiO_2 and TiO_2, and the outmost particulate structures were mainly TiO_2 (Figure 2g).

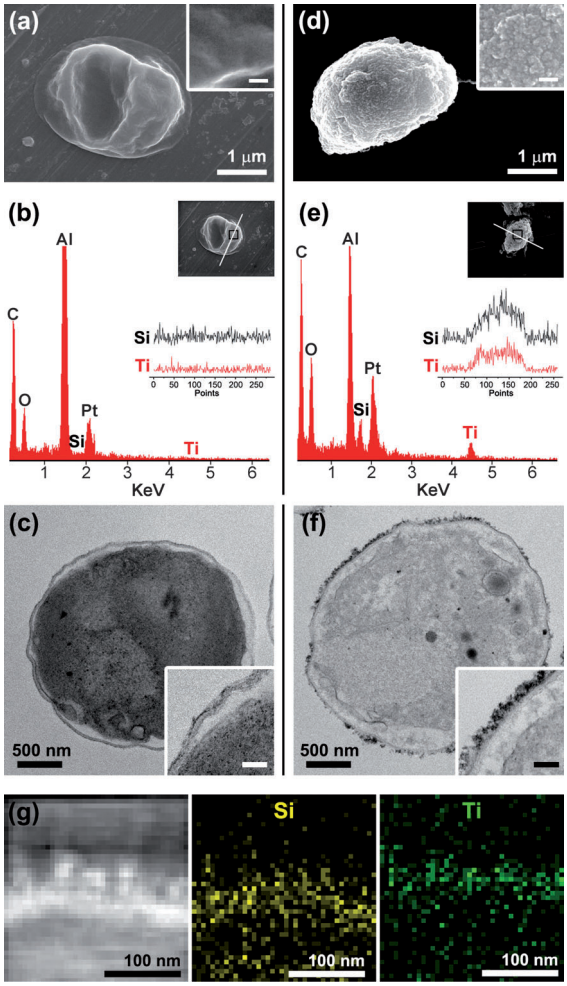

Figure 2. a–c) Native *Chlorella* and d–f) *Chlorella*@SiO_2-TiO_2: SEM micrographs, EDX elemental analysis (Inset: Si and Ti line profile), and TEM micrographs (Scale bar in the inset: 100 nm). g) EDX mapping of microtomed *Chlorella*@SiO_2-TiO_2 by TEM.

The encapsulation process was found to be highly cytocompatible. The effects of SiO_2–TiO_2 shell-formation on cell viability were investigated by measuring esterase activities in a cell with fluorescein diacetate (FDA) and monitoring cell growth. FDA is hydrolyzed to green-fluorescent fluorescein,[18] and *Chlorella* emits bright red autofluorescence;[15] therefore, in the merged image, greenish- and reddish-yellow *Chlorella* cells were considered alive, and the red ones were

considered dead (see Figure 4). The FDA test showed that the cell viability was about 87% after 3-by-3 deposition (See the Supporting Information, Figure S4). This value was noteworthy, because the *Chlorella* encapsulation with only TiO_2 under the same conditions (*Chlorella*@TiO_2) had only 55% viability.[15] We think that the amorphous and porous nature of SiO_2 in the SiO_2–TiO_2 shell leads to a more facile diffusion of gases and other small molecules for survival, compared with the TiO_2 shell.[16] Another possibility of the increased viability was the reduced quantity of the toxic chemicals, such as ammonium lactate, that were liberated during the TiO_2 formation (See the Supporting Information, Figure S5).[17a,b] In addition to the unperturbed enzymatic activity, the resulting *Chlorella*@SiO_2–TiO_2 was metabolically active and capable of growing after encapsulation. The cell growth at 23 °C under a halogen lamp was monitored by measuring the absorbance at 600 nm (OD_{600}), and the graphs of $\log_{10}(OD_{600}\times100)$ versus time were plotted for the exponential growth phase.[19] The rate constant, k, was calculated to be 0.38 day^{-1} for *Chlorella*@SiO_2–TiO_2 or 0.42 day^{-1} for native *Chlorella*. The k value also implied that the encapsulation processes did not harm the metabolic activities for cell growth. Of interest, the formation of the SiO_2–TiO_2 shell profoundly retarded the cell growth progression, which is one of the characteristics of artificial spores:[1,2] compared with native *Chlorella*, the division for *Chlorella*@SiO_2–TiO_2 was retarded by 32 h (● in Figure 3a), and a 44 h retardation was observed for cells encapsulated by a 5-by-5 deposition (▲ in Figure 3a). The cell-division processes have been studied previously, which showed that the encasing layer was ruptured presumably due to the division force.[4b,20] The suppression of cell division in this study was believed to result from the uniformity and mechanical stiffness of the shell, which resisted the division force and/or slowed down the cellular activities.[4]

Not only the ultrathin SiO_2–TiO_2 shell had the potential to control the cell division, but also the shell was found to effectively dissipate heat energy. Thermo-protection of green algae is practically important, because they have intensively been used in biosensors and bioenergy,[21] and electrical heating would cause cell death. The thermo-protective properties of the SiO_2–TiO_2 shell were investigated by applying a thermal stress to the *Chlorella* cells. The normal growing temperature for *Chlorella* was 23 °C,[22] and the native and *Chlorella*@SiO_2–TiO_2 cells were heated at 45 °C. After 2 h, a majority of native *Chlorella* were dead (ca. 81%), but more than a half of the encapsulated *Chlorella* (56.0%) survived the elevated temperature (Figure 3b). The survival ratio for *Chlorella*@SiO_2–TiO_2 was calculated to be 0.64 (=56.0%/87.2%) and that for native *Chlorella* was to be 0.20 (=19.3%/96.3%). In other words, the SiO_2–TiO_2 shell led to an approximately three-fold enhancement of thermo-protection. For comparison, the survival ratio for *Chlorella*@TiO_2 was 0.56 (=31.1%/55.4%). These results are indicative of a synergistic effect of the composites of SiO_2 and TiO_2. The SiO_2–TiO_2 shell almost completely protected *Chlorella* from heat especially for 1 h at 45 °C (viability only decreases from 87.2% to 85.4%; Figure 4).

In summary, a bioinspired approach was successfully applied to the formation of SiO_2–TiO_2 composites by using an $(RKK)_4D_8$ peptide. The cytocompatible process made it possible to encapsulate individual *Chlorella* cells within a SiO_2–TiO_2 shell. The formed *Chlorella*@SiO_2–TiO_2 was enzymatically and metabolically active, and showed an enhanced tolerance to thermal stress. Considering that many eggs are protected by their outmost inorganic shells and biological metabolism is controlled tightly by layered organic–inorganic shells, we believe that the formation of artificial inorganic shells would be a promising approach for the protection of cells from external stressors. In addition, the combination of biological and abiological inorganic materials also would increase the number of tools available for the manipulation of the artificial shells.

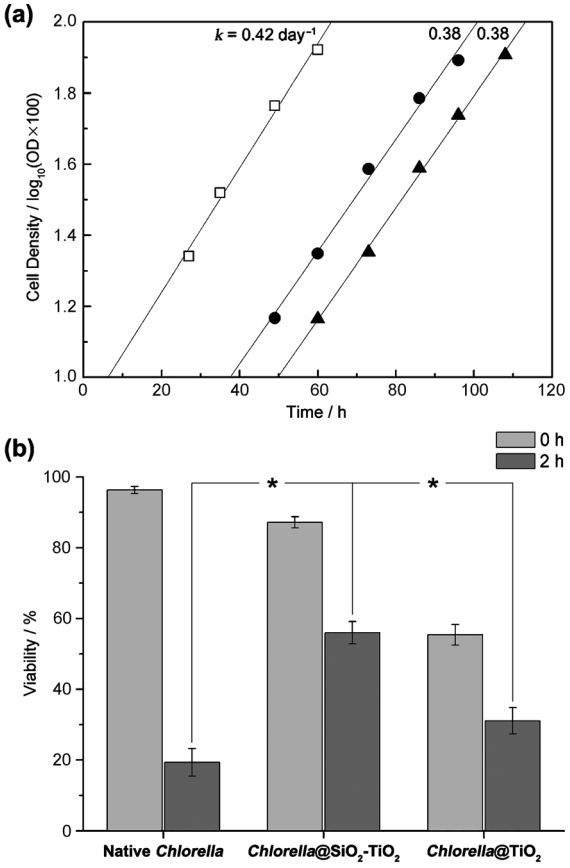

Figure 3. a) Growth curves of native *Chlorella* (□), *Chlorella*@SiO_2–TiO_2(3-by-3 deposition) (●), and *Chlorella*@SiO_2–TiO_2(5-by-5 deposition) (▲). b) Viability (± standard error) of native *Chlorella*, *Chlorella*@-SiO_2–TiO_2, and *Chlorella*@TiO_2 before (0 h) and after 2 h thermal treatment at 45 °C. The viabilities after 2 h were analyzed statistically by one-way ANOVA, followed by the Bonferroni's multiple comparison test. There are significant differences (*$p < 0.001$).

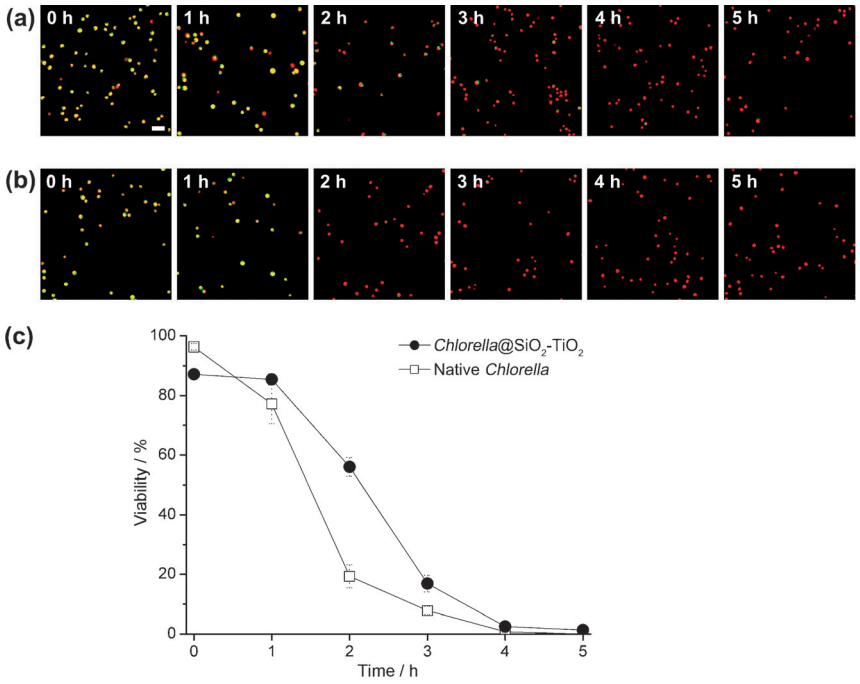

Figure 4. Viability of a) *Chlorella*@SiO_2–TiO_2 and b) native *Chlorella* after thermal treatment at 45 °C. *Chlorella* cells in greenish- and reddish-yellow *Chlorella* cells are considered alive, and the red ones are considered dead. The scale bar is 10 μm. c) Viability curves of *Chlorella*@-SiO_2–TiO_2 (●) and native *Chlorella* (□) after thermal treatment at 45 °C.

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