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Cell-in-Shell Hybrids: Chemical Nanoencapsulation of Individual Cells

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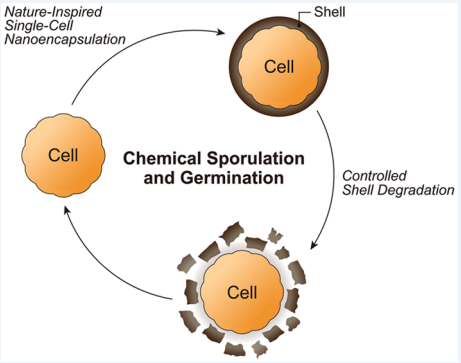
CONSPECTUS: Nature has developed a fascinating strategy of cryptobiosis ("secret life") for counteracting the stressful, and often lethal, environmental conditions that fluctuate sporadically over time. For example, certain bacteria sporulate to transform from a metabolically active, vegetative state to an ametabolic endospore state. The bacterial endospores, encased within tough biomolecular shells, withstand the extremes of harmful stressors, such as radiation, desiccation, and malnutrition, for extended periods of time and return to a vegetative state by breaking their protective shells apart when their environment becomes hospitable for living. Certain ciliates and even higher organisms, for example, tardigrades, and others are also found to adopt a cryptobiotic strategy for survival. A common feature of cryptobiosis is the structural presence of tough sheaths on cellular structures. However, most cells and cellular assemblies are not "spore-forming" and are vulnerable to the outside threats. In particular, mammalian cells, enclosed with labile lipid bilayers, are highly susceptible to *in vitro* conditions in the laboratory and daily life settings, making manipulation and preservation difficult outside of specialized conditions. The instability of living cells has been a main bottleneck to the advanced development of cell-based applications, such as cell therapy and cell-based sensors. A judicious question arises: can cellular tolerance against harmful stresses be enhanced by simply forming cell-in-shell hybrid structures? Experimental results suggest that the answer is yes. A micrometer-sized "Iron Man" can be generated by chemically forming an ultrathin (<100 nm) but durable shell on a "non-spore-forming" cell.



Since the report on silica nanoencapsulation of yeast cells, in which cytoprotective yeast-in-silica hybrids were formed, several synthetic strategies have been developed to encapsulate individual cells in a cytocompatible fashion, mimicking the cryptobiotic cell-in-shell structures found in nature, for example, bacterial endospores. Bioinspired silicification and phenolics-based coatings are, so far, the main approaches to the formation of cytoprotective cell-in-shell hybrids, because they ensure cell viability during encapsulations and also generate durable nanoshells on cell surfaces. The resulting cell-in-shell hybrids extrinsically possess enhanced resistance to external aggressors, and more intriguingly, the encapsulation alters their metabolic activity, exemplified by retarded or suppressed cell cycle progression. In addition, recent developments in the field have further advanced the synthetic tools available to the stage of chemical sporulation and germination of mammalian cells, where cytoprotective shells are formed on labile mammalian cells and broken apart on demand. For example, individual HeLa cells are coated with a metal−organic complex of ferric ion and tannic acid, and cellular adherence and proliferation are controlled by the programmed shell formation and degradation. Based on these demonstrations, the (degradable) cell-in-shell hybrids are anticipated to find their applications in various biomedical and bionanotechnological areas, such as cytotherapeutics, high-throughput screening, sensors, and biocatalysis, as well as providing a versatile research platform for single-cell biology.

1. INTRODUCTION

Tardigrades, commonly known as water bears, are special. These tiny, eight-legged invertebrates, about 1.5 mm long at most, cope effectively with the extremes of environmental conditions by forming a tough, dry husk called a tun.[1] Studies show that the tardigrade in the tun state can survive at 150 °C, in liquid helium (−272 °C), under intense radiation (α, γ, and UV), and at high pressure (600 MPa) in laboratory settings and even under the indescribably harsh conditions in outer space.[2] The shell-bearing, cytoprotective state of cryptobiosis ("secret life"), although not common, is also observed in bdelloid rotifers, eggs of brine shrimp and killifish, and seeds of some plants, to name a few.[3]

Bacterial sporulation is probably the most intensively studied and best-known biological process of cryptobiosis. For example, *Bacillus subtilis* forms a cytoprotective, tough, and metabolically dormant endospore in response to environmental stresses, such as nutrient deprivation, in the sporulation process.[4] As with the tun structure of tardigrades, the spore is encased in and protected by a tough shell, which is hierarchically composed of a peptidoglycan cortex and a proteinaceous coat (and in some cases an additional exosporium layer). The germination process involves rupture of the shell, leading back to a metabolically active, vegetative state. The cell-in-shell structure is also found in phylum ciliophoran, *Maryna umbrellata*, which construct an outermost silica-particulate layer at the resting cyst state by adapting themselves to external stresses.[5]

The intriguing features of cryptobiosis found in nature have inspired researchers, including us, to mimic the natural cell-in-

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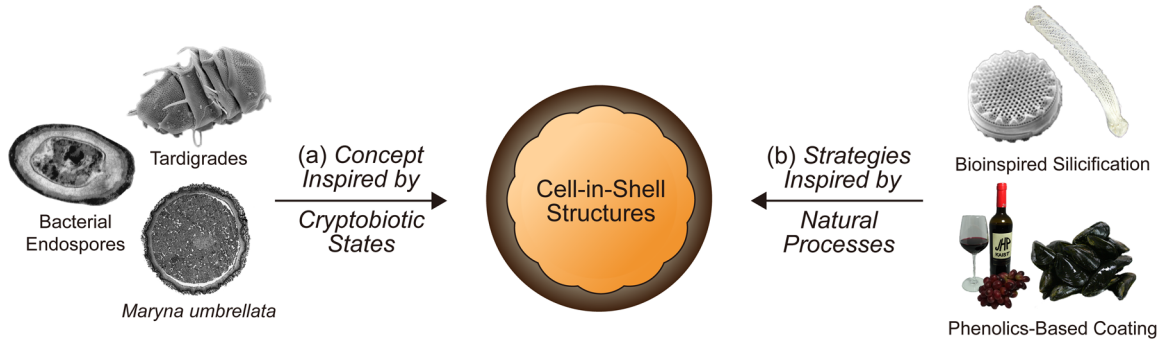

Figure 1. Nature-inspired formation of cell-in-shell hybrids.

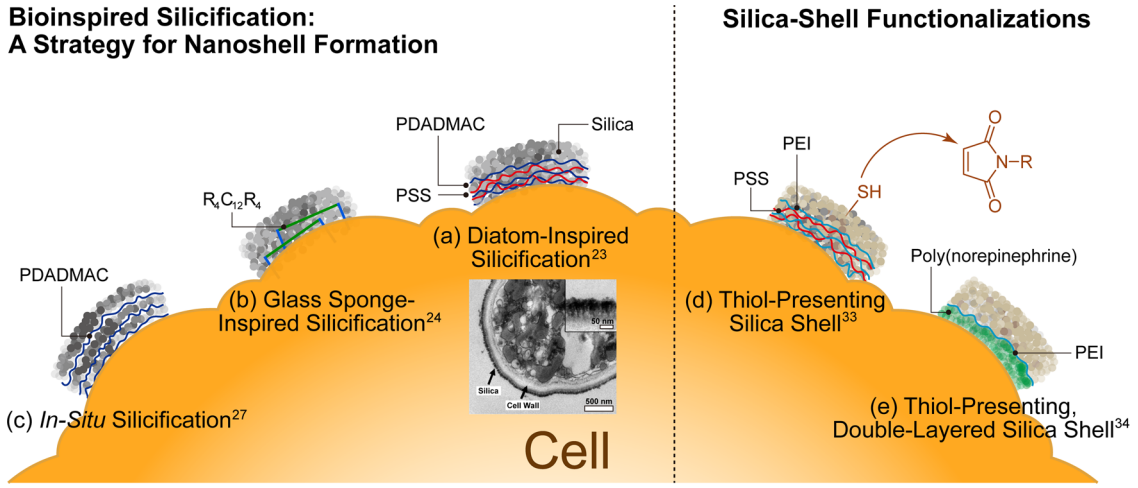

Figure 2. Bioinspired silicification for cytocompatible formation of cell-in-shell hybrids. TEM image adapted with permission from ref 23. Copyright 2009 Wiley-VCH.

shell structures by chemically forming a thin (<100 nm) but tough shell on individual living cells (Figure 1a). These chemically generated cell-in-shell hybrids, coined artificial spores, are reported to acquire an enhanced tolerance, compared with native, nonencapsulated cells, against various harmful stressors.[6−9] In addition to cytoprotection, recent developments in the field also make it possible to break up the artificially formed, tough shells in a controlled fashion while maintaining cell viability.[10,11] The *chemically driven* sporulation and germination of non-spore-forming cells are believed to significantly contribute to advances in applications where cell survival under daily life conditions is a critical issue, as well as to fundamental studies in single-cell biology. Exogenous properties, which are not innate to cells, such as magnetism, are also introduced to the shell chemically for certain applications.

The bedrock properties of artificial shells have been proposed for the realization of artificial spores:[6] (1) durability—the shell should be sufficiently robust to withstand physical impacts caused by, for instance, osmotic pressure and dehydration; (2) permselectivity—the shell should possess selective permeability in such a fashion that nutrients and gases pass through the shell to maintain cell viability, but foreign aggressors, such as macrophages and lytic enzymes, are blocked physicochemically; (3) functionalizability—the shell should be functionalizable to manipulate the cell−cell and cell−material interactions at the single-cell level; (4) degradability—last but not least, controlled shell degradability is crucial and unquestionably required for application of the artificial spores to both fundamental and applied areas, where cells themselves act as functionally operating entities, for example, cell therapy and cell-based sensors. The cells are protected from harmful stresses (under laboratory settings or daily life conditions), and the shells are broken apart on demand when the cells inside are needed for intended purposes. This active taking-off process is explicitly superior to the natural germination that occurs passively, typically induced by the presence of nutrients, because the timing of shell degradation can be tightly controlled in the artificial spores. In this sense, the artificial spore can be considered as a micrometric Iron Man: what is important is not the shell but the cell inside the shell.

Since our concept introduction,[6] much effort has been made to develop synthetic strategies to meet the suggested shell properties, especially degradability,[10−12] as well as to widen the process applicability to various cell types (microbial and, more importantly, mammalian cells[11,13]). In this Account, we discuss the current status of strategic developments in the realization of artificial spores and also deliver future perspectives on the cell-in-shell hybrid structures.

2. STRATEGIES

2.1. Bioinspired Silicification

Cytocompatibility or noncytotoxicity should be the first priority in the selection of encapsulation materials and processes; it is absolutely of no use if any elegantly designed chemistry kills biological cells during the formation of cell-in-shell hybrids. As in the inception of artificial spores, nature, again, gives us inspiration (Figure 1b).

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We were inspired by mechanically durable silica structures found in nature, especially the silica wall of diatoms and the silica exoskeleton of glass sponges, to construct inorganic siliceous shells. In addition to mechanical toughness, biosilica was also proposed to have thermal resistance, exemplified by ancient thermophiles[14] and hot-spring bacteria,[15] which also would be beneficial in the cytoprotective construction of artificial spores.

Studies on biosilicification suggested that proteins or peptides play key roles in the formation of biogenic silica.[16−19] For example, the biosilicification in diatoms is thought to involve *in vivo* polycondensation of silicic acid derivatives by cationic polypeptides, named silaffins, containing long-chain polyamines.[16,17] In bioinspired silicification, silica thin films were formed *in vitro* on a solid substrate under physiologically relevant conditions by using tertiary or quaternary amine-containing polymers, which had been grafted onto the substrate by surface-initiated polymerization, as a catalytic template,[20−22] suggesting a promising bioinspired approach to chemically dealing with living entities in a cytocompatible fashion. Based on these findings,[20−22] the synthetic protocols of bioinspired silicification were directly applied to the silica nanoencapsulation of individual cells (Figure 2).

2.1.1. Shell Formation. In the diatom-inspired formation of silica shells, the first step was the introduction of polyamines, catalytic templates, onto cell surfaces. An approach of surface-initiated polymerization[20] was found to be cytotoxic, killing cells, but the approach of layer-by-layer (LbL) assembly proved sufficiently cytocompatible for silica nanoencapsulation. With microbial *Saccharomyces cerevisiae* (baker's yeast) as a model cell, we first coated individual cells with LbL multilayers of poly(diallyldimethylammonium chloride) (PDADMAC) and poly(styrenesulfonate) (PSS), where PDADMAC acted as a biomimetic catalyst for bioinspired silicification.[23] A mechanically durable siliceous shell, 50 nm in thickness, was generated uniformly on the cell wall with the use of PDADMAC/PSS(11/10) (Figure 2a). The endurance of the resulting cell-in-shell hybrid against nonphysiological conditions (without any nutrients in pure water at 4 °C for 30 days) increased 3-fold compared with native yeast; the viability of the encapsulated yeast decreased by 27% after 30-day incubation, while that of native yeast dropped sharply by 76% under the same conditions. The biosilicification in glass sponges was also mimicked (Figure 2b).[24] An arginine/cysteine (R/C)-rich peptide, $R_4C_{12}R_4$, was rationally designed as a biomimetic catalyst: the positively charged R parts acted as an anchoring moiety toward negatively charged cell surfaces, and the C parts catalyzed the hydrolysis and polycondensation of tetraethyl orthosilicate in the imidazole buffer.[25,26]

The LbL process was modified slightly to achieve control over the thickness of silica shells (Figure 2c).[27] After the first deposition of positively charged, catalytic PDADMAC to the negatively charged yeast-surface, a negatively charged silica layer was formed *in situ* by incubating the PDADMAC-coated yeast cells in the aqueous solution of silicic acid derivatives. This deposition step was repeated up to 7 cycles to generate silica shells with different thicknesses. It was found that cell growth, determined with microbial growth curves and colony-forming units, was suppressed in a shell thickness-dependent manner, suggesting a chemical toolbox for tightly tuning cellular activities.

The cell types for silica encapsulation were expanded to chemically weaker but functionally more applicable mammalian cells, including Jurkat, fibroblast, and HeLa cells, which are encased with labile lipid bilayers. In addition to enhanced tolerance against external stresses, cell cycle progression was also suppressed by the silica coat, as observed with HeLa cells, which remained inside the coat without any adherence onto culture plates for up to 12 h.[13] The formation of silica shells has also been applied to a cyanobacterium (*Synechocystis sp.*) to reduce its light-induced photoinhibition.[28] The silica-encapsulated *Synechocystis* retained more than 50% of its maximum photosynthetic activity after 2-h irradiation of intense light (500 $\mu E/(m^2 \cdot s)$), while the activity of native cyanobacteria fell to zero. Although not a cell *per se*, viruses, such as human enterovirus type 71 (EV71) and human poliovirus Sabin type II, were also encapsulated with silica for the development of heat-resistant viral vaccines.[29]

The method of bioinspired silicification was also utilized to form abiological titanium dioxide (titania, TiO_2) shells on living cells based on previous reports showing that the similar synthetic protocols could be used for titania formation.[30] With $(RKK)_4D_8$ (R arginine, K lysine, D aspartic acid) as a biomimetic catalyst and titanium bis(ammonium lactato)-dihydroxide (TIBALDH) as a titania precursor, individual *Chlorella* cells were encapsulated with titania (Figure 3a).[31] Additionally, heat-tolerant *Chlorella* cell-in-shell hybrids were generated by performing the co-condensation of TIBALDH and silicic acid (Figure 3b).[32]

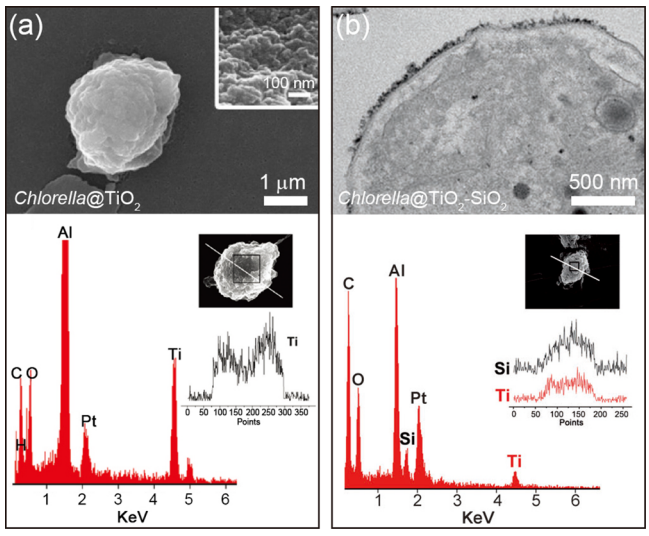

Figure 3. Nanoencapsulation of *Chlorella* with (a) titania and (b) titania−silica. Panel a adapted with permission from ref 31. Copyright 2012 American Chemical Society. Panel b adapted with permission from ref 32. Copyright 2013 Wiley-VCH.

2.1.2. Shell Functionalization. We found that silane chemistry (i.e., formation of silane-based self-assembled monolayers) was deadly toxic to cells and therefore modified the silicification protocols for shell functionalization:[33] mercaptopropyl trimethoxysilane was used as a coprecursor with tetramethyl orthosilicate during the silicification step, leading to the formation of thiol-presenting silica shells on yeast (Figure 2d). The cytocompatible thiol-maleimide coupling reaction was then utilized for introduction of various functional groups, and as a demonstration, the avidin-functionalized yeast cells were spatioselectively immobilized onto biotin-patterned solid substrates for sensor development.

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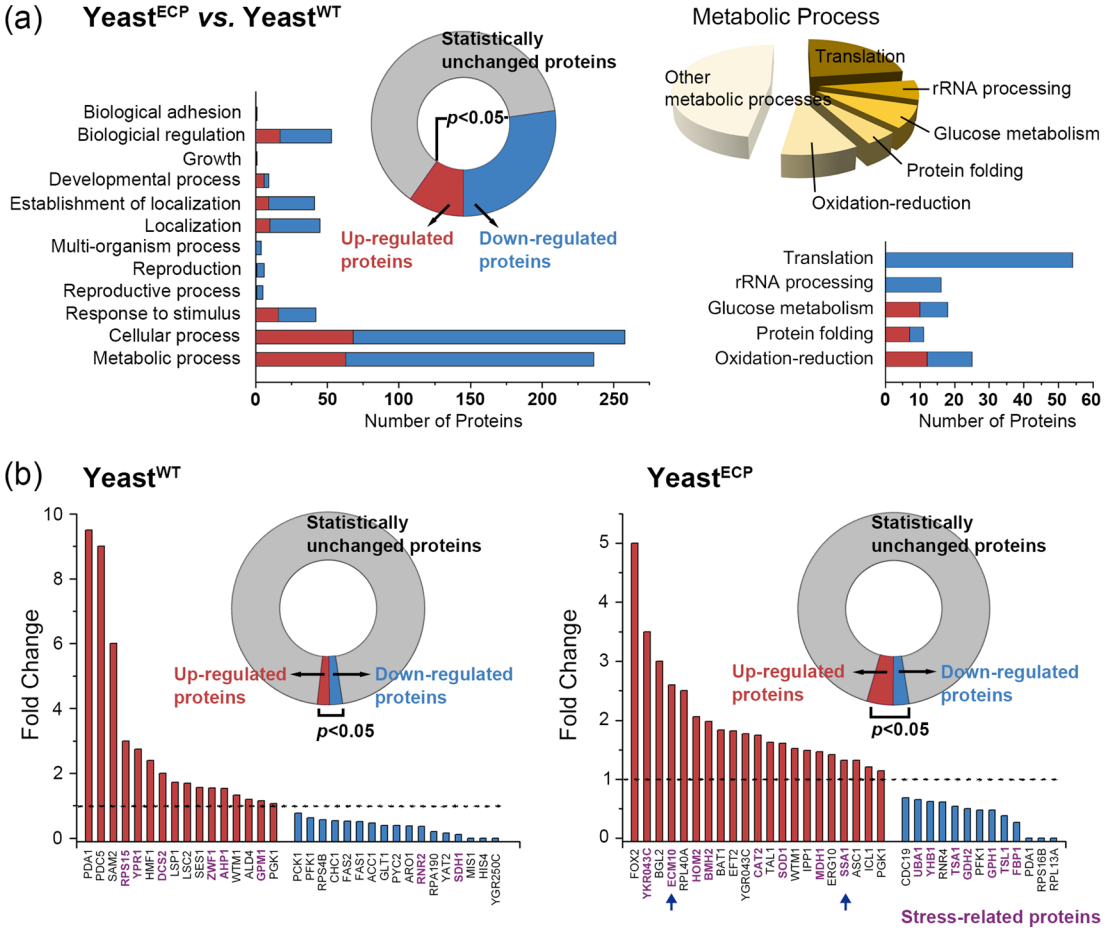

Figure 4. (a) Mass spectrometry-based, proteomic analysis of encapsulated yeast (Yeast^{ECP}) with native yeast (Yeast^{WT}) as a reference. Right pie chart shows subcategorization of the metabolic process. The expression change was evaluated on the fold change (F_c) value, defined as the spectral count of Yeast^{ECP} divided by that of Yeast^{WT}. $F_c > 1$ = up-regulated; $F_c < 1$ = down-regulated. (b) Proteome-wide analysis of Yeast^{WT} and Yeast^{ECP} after UV−C irradiation. Used with permission of Dr. Choi, copyright 2015.

The thiol-presenting silica shell was also constructed on a poly(norepinephrine) nanofilm encasing yeast cells, resulting in the formation of an organic/inorganic hybrid shell (Figure 2e).[34] The double-layered silica hybrid shell supported greatly enhanced resistance against lytic enzymes, desiccation, and UV−C irradiation. For example, most of the uncoated yeast cells (~90%) were dead after 2-h drying at 30 °C, but the encapsulation increased cell viability to 86%, indicating more than a 12-fold enhancement of survival ratio against desiccation. In this particular work, mass spectrometry-based, proteome-wide analysis was additionally performed to gain insight into the enhanced tolerance and the suppressed cell growth in the cell-in-shell hybrids. The analysis showed that, compared with native yeast, a majority of proteins involved in translation were down-regulated, while certain proteins of glucose metabolism and protein folding were partially up-regulated, for the encapsulated yeast (Figure 4a). This metabolic adaptation seemed to contribute to the enhanced resistance: for example, upon UV−C irradiation of yeast, we observed the up-regulation of proteins ECM10 and SSA1, which belong to the molecular-chaperone HSP70 family, only for the UV−C-tolerant encapsulated yeast (Figure 4b). These results suggest metabolism-level connectivity between the natural sporulation and the chemical cell-in-shell construction, and recruit further investigations from different directions and disciplines.

2.2. Phenolics-Based Coating

2.2.1. Tannic Acid (TA). Inspired by nature, phenolics-based coatings have been developed as a substrate-independent strategy for multifunctional surface engineering.[35−39] The coating characteristics of Fe^{III}−TA complexes (especially, fast reaction time and film degradability) have been taken advantage of in single-cell coating to demonstrate chemical mimicry of bacterial sporulation and germination.[10,11] Compared with the approach of bioinspired silicification, which did not allow for the cytocompatible breakup of the inorganic shells, the on-demand degradability of the Fe^{III}−TA complexes gave an advanced manipulability of living cells at the single-cell level.

Individual yeast cells were encased within the Fe^{III}−TA shell (Figure 5a), and the assays showed that cell viability was more than 98% after four depositions (yeast@[Fe^{III}−TA]$_4$; shell thickness ≈ 40 nm). The Fe^{III}−TA shell exhibited common shell properties for artificial spores: ultrathin thickness (<100 nm), mechanical toughness, permeability, resistance against harmful stresses (UV−C irradiation, lyticase, and silver nanoparticles in this study), and functionalizability (demonstrated by yeast micropatterning and magnetic capture). More importantly, tailor-made control over cell-division characteristics was realized by shell formation and degradation.[10] The logarithmic value of colony-forming unit per mL, log(CFU/mL), of native yeast was calculated to be 7.12, but the value dropped to 4.47 for yeast@[Fe^{III}−TA]$_4$, indicating that the

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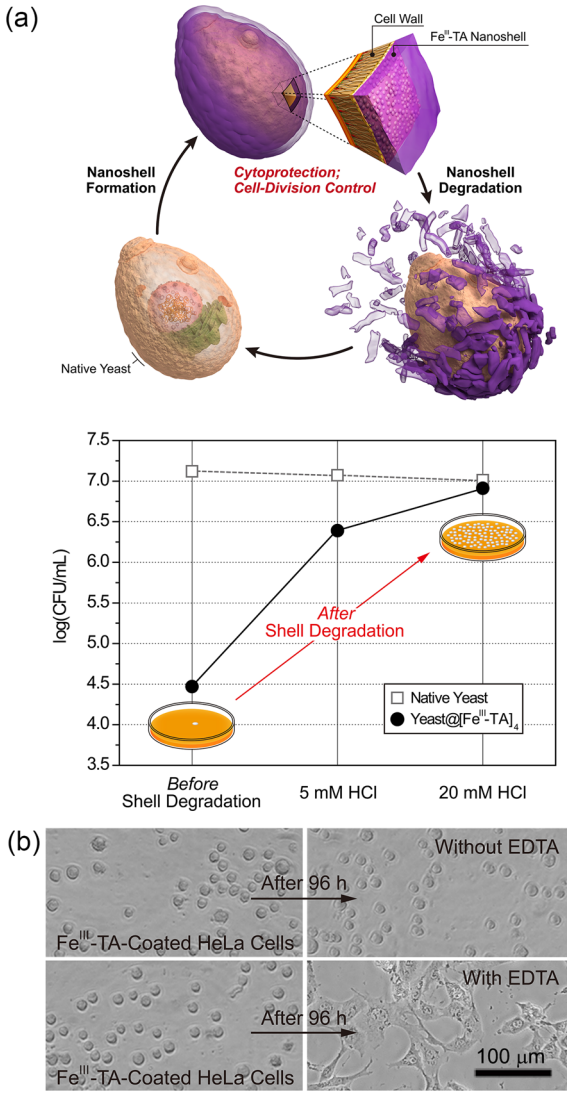

Figure 5. Phenolics-based coating and control over cellular activity of (a) yeast and (b) mammalian cells. Panel a adapted with permission from ref 10. Copyright 2014 Wiley-VCH. Panel b adapted with permission from ref 11. Copyright 2015 The Royal Society of Chemistry.

Fe^{III}–TA shell rendered the encapsulated yeast cells inactive or significantly less active in cell division by at least 450-fold (Figure 5a). However, mild acid treatment degraded the Fe^{III}–TA shell, and the log(CFU/mL) value was recovered to 6.91, which was similar to that of native yeast (7.01) under the same conditions. Chemical sporulation and germination were also demonstrated with mammalian cells, such as HeLa cells, via Fe^{III}–TA coatings (Figure 5b).[11] Coated HeLa cells, which are typically anchorage-dependent, floated without any adherence onto the culture surface in culture medium for 96 h, implying that the Fe^{III}–TA coat decreased the cell cycle progression (cell growth and proliferation). However, treatment with ethylenediaminetetraacetic acid (EDTA), cleaving the Fe^{III}–TA linkages, led to the adherence and proliferation of the HeLa cells.

2.2.2. Dopamine. Dopamine (and its derivative, norepinephrine) has proved highly versatile in the functional coating of various substrates including living cells under mild conditions.[38,39] This mussel-inspired coating method was taken to coat individual yeast cells by simply immersing them in a dopamine solution at pH 8.5 (Figure 6a).[40] The protective

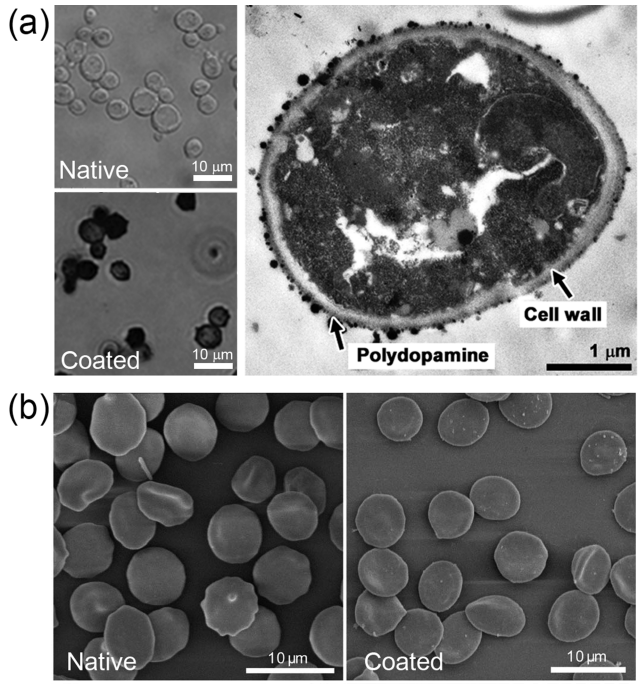

Figure 6. Polydopamine coating of (a) yeast and (b) red blood cells. Panel a adapted with permission from ref 40. Copyright 2011 American Chemical Society. Panel b adapted with permission from ref 41. Copyright 2014 The Royal Society of Chemistry.

capability of the polydopamine shell was also utilized to develop universal blood that does not require blood-type matching for blood transfusion (Figure 6b).[41] The polydopamine-coated red blood cells (RBCs) were antigenically shielded and showed unperturbed physicochemical properties compared with native RBCs. In a related study, the polydopamine coating has been applied to the protection and metabolic control of multicellular microbial entities[42,43] (multicellular cytoprotection was also demonstrated with bioinspired silicification[44]).

2.3. Electrostatic Deposition

Simple electrostatic depositions of positively charged materials onto cell surfaces (and subsequent LbL multilayer formations) have been used in the area of cell-surface engineering.[45−47] Polyamine-stabilized magnetic nanoparticles were used for magnetic functionalizations of yeast,[48] *Chlorella*,[49] and HeLa cells.[50] Additionally, the magnetically functionalized cells have been used for the construction of 3D tissue-mimicking cell clusters (Figure 7).[51−53] Other functional nanomaterials, such as graphene, were also deposited onto cell surfaces to endow the coated cells with new properties.[54−56]

Although successful in cell-surface functionalizations, the LbL multilayers are typically formed electrostatically, and the mechanical durability generally has not been ensured for the realization of artificial spores.[45,57] However, mechanical durability could be increased by a cross-linking LbL approach, suggesting the LbL strategy as a simple but versatile method for forming artificial shells.[58,59] Catechol-grafted poly(ethylenimine) and hyaluronic acid were crossed-linked after the multilayer formation, which controlled the division characteristics of yeast cells (Figure 8a).[58] Additionally, *in situ*

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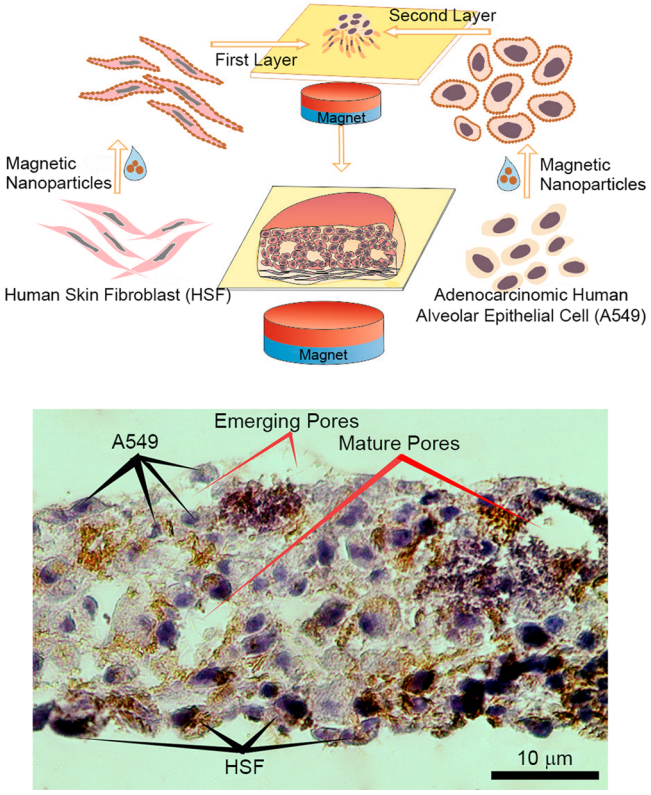

Figure 7. Magnetic functionalization of cells and construction of 3D cellular assemblies. Adapted with permission from ref 52. Copyright 2013 The Royal Society of Chemistry.

cross-linking between multilayers has been developed for tuning the mechanical durability of the cell-encasing films (Figure 8b).[59]

3. CONCLUSIONS AND FUTURE PERSPECTIVES

Recent years have witnessed unforeseen research activities for the generation of cytoprotective cell-in-shell hybrid structures, namely, artificial spores. A microbial or mammalian cell (or a virus) is dressed up in an ultrathin (<100 nm), durable coat, mainly based on a bioinspired, chemical process, bioinspired silicification or phenolics-based coating. It is also demonstrated that the cytoprotective shell can be degraded on demand in a cytocompatible fashion, reminiscent of the sporulation and germination processes in nature. These cell-in-shell hybrids would provide a versatile research platform for single cell-based analysis, for example, on cell−cell communications, by confining individual cells in a 3D microspace and can also be applied to various biotechnological and biomedical areas by dispatching micrometric Iron Men in which a cell inside the shell is protected securely and ready for action after shell degradation. This research area has passed in part, if not entirely, the stage of concept development, but some fundamental and technical challenges still remain and elicit multidisciplinary efforts from various disciplines for its further advancements.

3.1. Challenges in Fundamental Studies

In addition to the anticipated basic properties of the shells, mechanical durability maintaining cell morphology against physical impacts and chemical permeability ensuring the diffusion of small molecules (e.g., nutrients and wastes) and gases, the encapsulation process, rather unexpectedly, alters metabolic activities, exemplified by retarded cell cycle

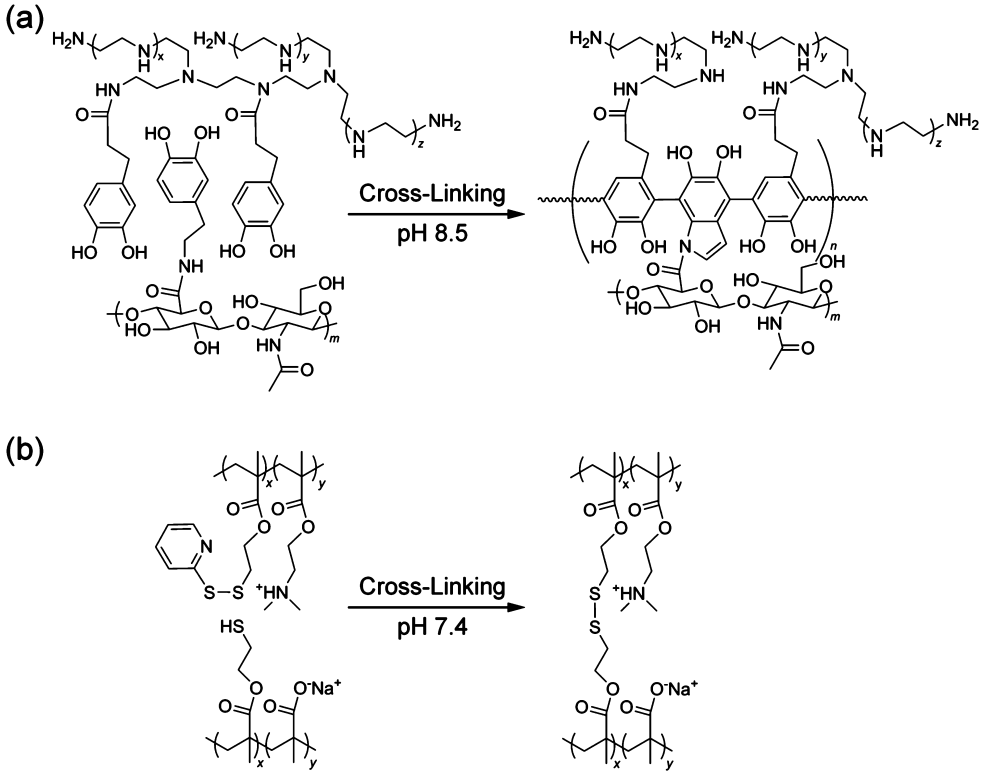

Figure 8. Cross-linking strategies in LbL coating of cells.

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progression. For example, yeast cells are arrested in the lag phase by the shell formation,[10,23] and the retardation degree in cell division is determined by the toughness and thickness of the encapsulating shells (or diffusivity of the shells).[27,58,60] Although an inkling of this phenomenon might be given by nature's cryptobiotic process, further biochemical and biophysical investigations should follow to resolve the fundamental issues, for example, the effects of encapsulation on cellular activities and the biological origins of the enhanced cytoprotection.

3.2. Challenges in Applications

Previous studies focused primarily on feasibility demonstrations of the encapsulation process for sustaining the cell viability and rudimentarily mimicked the sporulation and germination processes. Although potential applications of cell-in-shell hybrids were reported in the areas of universal blood[41] and biocatalysis,[61] none have been close to commercial production. In this respect, advancements in shell fabrication will be beneficial for the seamless application of artificial spores. (1) Multifunctional shells: There have been demonstrations that cells could be functionalized magnetically or the artificial shells are postfunctionalized with molecules of interest, but the introduced functionalities are singularly functional not multiplexed. Considering the multifaceted roles of natural cryptobiotic coats, we envision multifunctional shells that better adapt to application settings, in which the conditions change dramatically and sporadically. Different organic and inorganic functional materials can be incorporated into a shell or multifunctional building blocks can be used for shell formation. Examples of functional shells also include "active" shells that control cellular activities at a single-cell level. For example, an external input is applied to the active shell, and time-controlled modulation of cellular metabolism could be achieved for certain applications.[62,63] (2) Multilayered shells: The functional shells can be formed by integrating a multitude of functional shells into one as aforementioned. The integration also might lead to synergistic outcomes to the swathed cells, as in case of hierarchical shells of bacterial endospores. (3) Dynamic shells: The shells demonstrated so far are typically rigid and static. Shell rigidity (i.e., mechanical durability) is one of the required shell properties in artificial spores, but the shell could be dynamically rigid, exemplified, in part, by recent attempts to form "self-repairing" shells.[64] In addition to self-assembly approaches,[64,65] innate biochemical activities of cells, such as dephosphorylation,[66] H_2O_2 production,[67] and mineral precipitation,[68−72] could be utilized, after genetic modifications if needed, for *self-adapted*, dynamic formation of cytoprotective shells. The self-adaptive shell formation and degradation would suggest a next direction for autonomous artificial spores.

In addition to the development of shell materials and processes, it is also time to put a practical focus on the direct application of encapsulation strategies to cells that are functionally beneficial to industrial sectors but are, at present, biologically vulnerable under real implementation settings, such as T cells, stem cells, neurospheres, and erythrocytes in cell therapy, keratinocytes in cosmetics and wound healing, and even probiotics in dairy food. Shell materials and properties [e.g., characteristics and time duration (permanent or temporal) of cytoprotection] should be varied, depending upon the pursued applications, and will be optimized to meet the required specifications.

In conclusion, recent efforts in cell-in-shell hybrids clearly show that biological cells are not just research targets to explore at a distance but can become living objects to manipulate, as chemists typically do with molecules. The cryptobiosis-inspired formation of artificial spores, initiated by scientific curiosity, will greatly contribute to technological developments in cell therapy, cell-based sensors, biocatalysis, high throughput screening, or even cosmetics, as well as to our fundamental understanding in single-cell biology. Cryptobiosis also could be regarded as just one end point in the continuous spectrum of hibernation, which would give us limitless inspiration for formation of cell-in-shell hybrids with different degrees and chemical manipulation of living cells at a single-cell level.

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Notes

The authors declare no competing financial interest.

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