"Face-Lifting" and "Make-Up" for Microorganisms: Layer-by-Layer Polyelectrolyte Nanocoating

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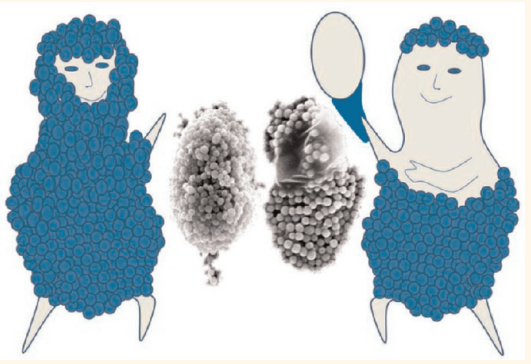
ABSTRACT



Layer-by-layer encapsulation of living biological cells and other microorganisms *via* sequential adsorption of oppositely charged functional nanoscale components is a promising instrument for engineering cells with enhanced properties and artificial microorganisms. Such nanoarchitectural shells assembled in mild aqueous conditions provide cells with additional abilities, widening their functionality and applications in artificial spore formation, whole-cell biosensors, and fabrication of three-dimensional multicellular clusters.

Fabrication of biomimetic structures based on living cells in order to improve cell functionality and ability to survive harsh conditions is a great challenge. Biological cells have micrometer dimensions, and in order to modify them using engineering methods, one has to operate at the nanoscale. Genetic methods, although effective, do not allow for equipping the cells with independently produced nanodevices. Microbial cells are living entities, and they have to be processed with nonharmful engineering methods in order to preserve their ability to reproduce. Nanolithographic, beam epitaxial, and other physical methods are not applicable; thus, one has to rely on mild aqueous chemical self-assembly methods. In this Perspective, we propose methods for surface modification to make living cells "healthier": to enhance their nutrition paths, to protect them against phagocytosis/digestion, to provide them with the ability to surpass a harmful media (*e.g.*, acidic stomach on the way to the higher pH digestion tract), to protect them against ultraviolet (UV) radiation with a layer of polyphenols (*i.e.*, bacteria sunscreen), and to supply cells with additional instrumentation for their functionality (*i.e.*, magnetic function and mechanical robustness). We will "dress" cells with a functional "cloth", preserving these newly acquired properties for one to two generations.

Current nanoassembly methods allow individual cell encapsulation by coating 2−10 layers of polyelectrolytes with predetermined compositions (nanoarchitectural approach). A variety of cells, including bacteria, yeast, normal and cancerous human cell lines,[1−4] stem cells,[5] and even microscopic multicellular species (*e.g.*, worms),[6] have been used for surface functionalization with polyelectrolytes and nanoparticles. Encapsulation of cells in calcium phosphate and silica has been reported, and an application of magnetically responsive cells in biomimetic devices required an even more "bio-friendly" approach.[7−9] This nanocoating can be patched, providing cells with orientation properties.[10]

In this Perspective, we concentrate on cell encapsulation with layer-by-layer (LbL) self-assembly *via* sequential adsorption of oppositely charged components: polyelectrolytes, nanoparticles, and proteins. This LbL method for encapsulation is based on the consecutive deposition of polycations and polyanions, bound together through electrostatic interactions and applied first for planar film production and later for encapsulation of colloids, including biocells.[11,12] The typical polycation/polyanion bilayer thickness in a swollen state is 4−5 nm, and

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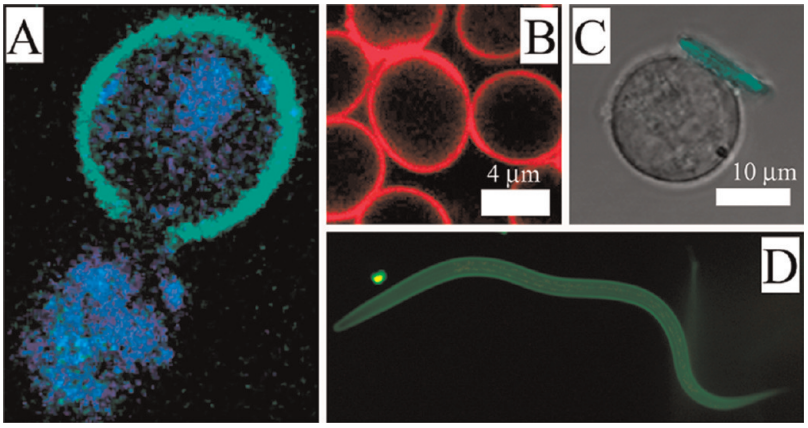

Figure 1. Layer-by-layer (LbL) coating of living microbial cells, human cells, and microworms with 40 nm thick polyelectrolyte shells. (A) $(PAH/PSS)_6$ encapsulated yeast cell (green) shown along with a coating-free daughter cell. Reprinted from ref 2. Copyright 2002 American Chemical Society. (B) Hydrogen-bonded LbL assembly on yeast cells of $(PEI(TA/PVPON)_4)$ shells. Reproduced with permission from ref 18. Copyright 2011 The Royal Society of Chemistry. (C) Lymphocyte cells partially coated with LbL patches. Reprinted from ref 10. Copyright 2008 American Chemical Society. (D) $(FITC\text{-}PAH/PSS)_5$ polyelectrolyte-coated living nematode Caenorhabtidis elegans. Reprinted from ref 6. Copyright 2011 American Chemical Society.

shell thicknesses are 30−100 nm.[13] This simple encapsulation technique enables researchers to process many biological cells in parallel. The ability to design shells of any composition, containing nanosized layers of polymers, proteins, and nanoparticles in a predetermined order enables control of the capsules' properties, such as sensitivity to temperature and pH, permeability, and structural stability. These surface-functionalized cells thus have their intrinsic functions enhanced or altered.

Cells as Disposable Templates for LbL Microcapsules. Initial reports focused on using biological cells like other colloid microparticles (nonviable cells).[12] These templates for LbL assembly were advantageous because the selected cells have similar sizes and shapes, can be easily harvested in large numbers, are present in a great variety of sizes and geometries, and have unique natural functionalities that are as-yet impossible to realize in man-made machines. *Escherichia coli* bacteria and erythrocytes (red blood cells) were employed as sacrificial microcores to fabricate polymeric capsules with alternating layers of cationic poly(allylamine)hydrochloride (PAH) and anionic poly(styrene) sulfonate (PSS).[14] Then, the cell interior was decomposed with NaOCl to fabricate hollow replicas of the cells, such as erythrocytes (disk-shaped), bacteria (rod-shaped), or echinocytes (multiray star shaped).[15] The applications of such cell-templated microcapsules are limited by their reservoir properties.

Electrostatic LbL Assembly on Living Cells. Polyelectrolyte shells deposited on living microbial or human cells provide these cells with properties not readily found in wild-type cells, widening their biotechnological applications. Polymer coating is a chemical engineering process that does not require genetic manipulation. Layer-by-layer surface functionalization of living yeast cells *via* their sequential incubation in aqueous saline solutions of polycations and polyanions produced four to six bilayers of polyelectrolyte coating on the cells.[2] Because the cell surfaces are negatively charged at physiological pH, the shell assembly begins with the deposition of a polycation, then a polyanion is deposited, and so on, until the planned shell architecture is realized. Having a negative polyanion layer outermost on the modified cells provides better colloidal stability (typical ζ-potential of *ca.* −30 mV). Usually, cells are washed after every LbL deposition cycle to remove nonreacted polyelectrolytes; however, an improved technique now allows for nonwashing shell assembly by the sequential addition of a determined amount of polyelectrolytes that are needed to recharge the cell surface.[16] The sequential multilayer shell assembly is monitored by alternating the electrical ζ-potential of the coated shells, and each new polyelectrolyte layer inverts the cells' surface potential (switching between +30 and −30 mV).[17] The process was demonstrated with fluorescent or confocal microscopy, where fluorescently tagged polyelectrolytes were incorporated into the coating (see Figure 1). The LbL shells were found to be quite soft, thus allowing for budding, and the daughter cells were able to pierce the multilayers and carry some of the shell.

There are four main research directions in bioencapsulation: (1) LbL functionalization of isolated microorganisms (*e.g.*, fungi, algae, and bacteria); (2) functionalization of isolated mammalian cells, including human cells; (3) encapsulation of cell aggregates, tissue sections, and microorganisms (*e.g.*, microworms); and (4) encapsulation of viruses. To date, a few publications have described virus decoration with gold or silica nanoparticles,[19] but we have focused on the complete shelling of viruses to modify their surface properties and eliminate specific immune responses for intercellular drug delivery.

Cell Encapsulation. Cationic PAH, poly(ethylene imine) (PEI), poly-L-lysine (PLL), and poly(dimethyldiallyl ammonium chloride) (PDDA) and anionic PSS, poly(glutamic acid) (PGA), and poly(acrylic acid) (PAA) were used for encapsulation, which was accomplished with natural polyelectrolytes (chitosan, hyaluronic acid, glutamic acid, and heparin) and proteins (gelatin, albumin, and lysozymes). Our experience has shown that higher molecular weight polyelectrolytes bind better and allow minimal penetration of polyelectrolytes into the cell interior. Hydrogen-bonded LbL assembly of nonionic polymers was also demonstrated.[18] Decisions related to the design of the shell composition and shell thickness are based on the applications envisaged for the functionalized cells (surface chemistry, selective permeability, and triggered release).

The surface LbL engineering of living cells has employed both eukaryotic and prokaryotic unicellular species. Widely available and affordable microbial cells are valued in biotechnology and biomedicine, and attention has been focused on LbL functionalization of yeast and bacteria cells. These microorganisms were protected from the environment with semipermeable polysaccharide shells.[20] Along with yeast, dormant bacterial spores and stationary-phase bacteria cells were LbL coated.[17,21] Layer-by-layer encapsulation of microbes is a versatile approach for adjusting their properties, studying physiological processes, and preparing for long-term cell storage.

Layer-by-layer assembly on living animal and human cells is more difficult due to the nature of their cellular membranes. Mammalian cells have fragile cell membranes, unlike microbes, which have thick cell walls. Nevertheless, the preserved viability of LbL-coated human cells was demonstrated.[3] Biogenic and biocompatible natural polyelectrolytes, such as polypeptides and polysaccharides, work better at preserving cell nativity. Surface engineering of human cells with LbL films allows us to control the performance of cell membranes for whole-cell biosensors, to assemble tissues from functionalized cells, and, ultimately, will lead to advances in cell-based therapies.[10,22] The surface modification of mouse mesenchymal stem cells was demonstrated using poly-L-lysine and hyaluronic acid as the polyion pairs.[5] Mouse fibroblast cells were coated with gelatin/fibronectin, and then the coated cells were employed in fabrication of tissue-mimicking cellular clusters, where up to five layers of fibroblasts were assembled on supporting substrates.[22] Human erythrocytes were coated with alginate/chitosan grafted with phosphorylcholine and encompassed by two bilayers of alginate and poly-L-lysine grafted with poly(ethylene glycol).[3] Breast cancer cells were also LbL coated with polyelectrolytes.[23]

Functionalization of live lymphocytes using a photolithographic patterning technique supplemented with LbL assembly yielded the selective functionalization of cells coated with LbL patches (see Figure 1C), whereas the neighboring regions remained intact.[10] Such patch coating may find applications for cell orientation or attachment of single nanodevices.

Small Cell Clusters and Organisms. Layer-by-layer assembly was also applied for larger tissue clusters, such as pancreatic islets, for applications as transplants with increased resistance to the immune system for the therapy of type-I diabetes.[24] Even microscopic multicellular organisms, such as *Caenorhabtidis elegans* nematodes, can be effectively coated with polyelectrolyte shells.[6] This technique can be further extended to millions of other small organisms, including worms, insect larvae, and eggs, among others, thus outlining a facile way to manipulate tiny living organisms. An interesting feature of LbL assembly is that it can be deposited on heterogenic areas, such as microorganism surfaces or wounds. Polycation/polyanion assembly does not need a uniform surface; through cooperative electrostatic interaction, polyelectrolytes can be anchored to chemically non-uniform objects, attaching to favorable areas and bridging over others.

Viruses. We encapsulated the 78 nm diameter bacteriophage T7 with a PAH/PSS/PAH/5 nm silica shell and, using transmission electron microscopy (TEM), demonstrated an even coating of bacteriophage capsid and its short tail with polyelectrolyte/silica shell. The encapsulation was performed with the nonwashing LbL assembly technique, and the goal was to demonstrate the possibility of virus encapsulation, which follows from efforts to encapsulate spherical viruses for drug delivery (*e.g.*, 200 nm diameter modified herpes).

Non-electrostatic LbL Assembly on Living Cells. The possible toxic effects of polycations have stimulated the search for non-electrostatic LbL assembly of polymers on cells. A recent report demonstrates the hydrogen-bonded assembly of uncharged polymer pairs. Yeast cells were coated with poly(*N*-vinyl pyrrolidone)/tannic acid multilayers, still using a cationic poly(ethyleneimine) first layer to facilitate the film adhesion.[18] Formation of the non-electrostatic LbL shells around the yeast was confirmed by confocal and electron microscopy, and the thickness of the wall was compared to conventional LbL polyelectrolyte shells.

LbL Functionalization of Cells with Nanoparticles. Polyelectrolytes facilitate adhesion of nanoparticles to biocells, thus providing the stability of the sandwich-like polyelectrolyte/nanoparticle coating and suppressing nanoparticle internalization through the cell walls into the cytoplasm. Layer-by-layer assembly with metal nanoparticles (Au, Ag) was demonstrated for fungi and bacteria.[1,25] The LbL nanoparticle coating is similar to deposition of linear polycations/polyanions, but some of the polyelectrolyte layers are instead replaced by a layer of properly charged nanoparticles. It is often necessary to finish shells with linear polyelectrolyte outer layers to

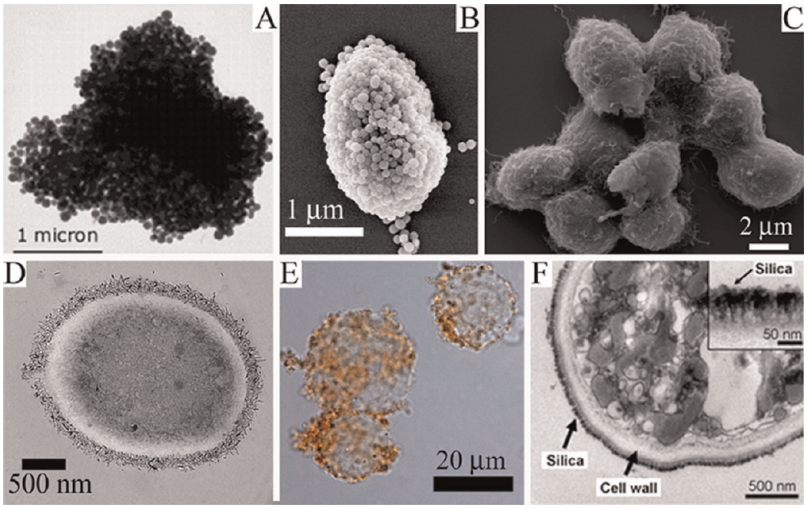

Figure 2. Layer-by-layer-mediated deposition of nanoparticles on living cells. (A) Transmission electron micrograph of a blood platelet coated with a shell of poly(dimethyldiallyl ammonium chloride) (PDDA)/ poly(styrene)sulfonate (PSS)/PDDA + (silica/PDDA)$_2$. Reprinted from ref 26. Copyright 2002 American Chemical Society. (B) Scanning electron micrograph image of Bacillus subtilis spores coated with poly-L-lysine/poly(glutamic acid) layers: (PLL/PGA)$_4$ + PLL + 72 nm silica particles. (C) Scanning electron micrograph image of yeast cells encapsulated with poly(allylamine)hydrochloride (PAH)/PSS doped with carbon nanotubes. Reprinted from ref 27. Copyright 2002 American Chemical Society. (D) Transmission electron micrograph of a single yeast cell coated with PAH/PSS doped with magnetic nanorods. Reprinted with permission from ref 28. Copyright 2010 RSC Publishing. (E) Optical microscopy image of human HeLa cells coated with PAH-stabilized magnetic nanoparticles. Reprinted from ref 4. Copyright 2011 American Chemical Society. (F) Transmission electron micrograph of microtome-sliced yeast cell coated with 50 nm SiO_2 facilitated by PAH/PSS deposition prior to silica layer formation. Reprinted with permission from ref 8. Copyright 2009 Wiley.

fix the multilayer architecture and to prevent nanoparticle loss. Electron microscopy images have demonstrated the incorporation of nanoparticles into the shells on human blood platelets and bacterial spores.[17,26] Carbon nanotubes and magnetic nanorods have also been deposited on cells (see Figure 2).[27,28] Nanotubes loaded with drugs could potentially provide long-lasting cell treatment and sustained drug release. We are currently working on biocompatible natural clay nanotubes of 50 nm diameters for functionalization of biocells.

Functional particles, such as magnetic nanospheres and nanorods were LbL attached to yeast cells.[28] Furthermore, human blood platelets were functionalized with polyelectrolytes doped with 80 nm silica particles, 45 nm fluorescently labeled latex, and with immunoglobulin-G for targeting in blood vessels.[26]

The functionalization procedure should be performed within minutes, not hours, as in conventional LbL assembly. To achieve this, a single-step deposition of polyelectrolyte-stabilized nanoparticles was used, where the polycation-modified nanoparticles readily adhered to the negatively charged cell membrane. This is similar to LbL technique optimization for industrial high-performance liquid chromatography products where a multilayer silica shell ("halo") is deposited on microcores through fast multilayer deposition *via* "exponential" LbL mode, using concentrated partially discharged high-molecular-weight polycations. Twenty nanometer poly(allylamine)-stabilized magnetic nanoparticles were used to modify human cells and living nematodes magnetically.[4,6,28] Nanoparticles were arranged as a uniform monolayer coating the intact cell walls. The procedure is fast (30 min) in comparison with usual LbL deposition (4–5 h). This direct technique is not limited to poly(allylamine); other cationic polymers can also be used.

Calcium phosphate shells on yeast cells required the preliminary assembly of an LbL precursor followed by the subsequent co-precipitation of calcium and phosphate. The LbL multilayers facilitated the electrostatic accumulation of Ca^{2+} ions on the yeast cells' surface followed with octacalcium phosphate, resulting in a 1 μm amorphous calcium phosphate layer.[7] Polyelectrolyte-stimulated silication was also used to form a uniform 50 nm silica layer on individual yeast cells.[8]

Viability and Toxicity Issues: Are They Alive Down There? Layer-by-layer films assembled onto living microbial cells produce a 20–60 nm thick flexible, hydrogel-like coating that can be regarded as an artificial structure mimicking a cell wall. The toxic effects of the polyelectrolyte layer may be caused by blockade of nutrients/ions transportation, destruction of cellular membranes, direct penetration of polyelectrolytes into cellular membranes, or retardation of the reproduction cycle (*i.e.*, cells are "arrested" in the shells and cannot grow). Microbial cells survived LbL deposition of polyelectrolytes because their walls protected them from osmotic pressure effects. A few reports have suggested that synthetic polyelectrolyte coatings cause some level of cell death and suppress green fluorescent protein

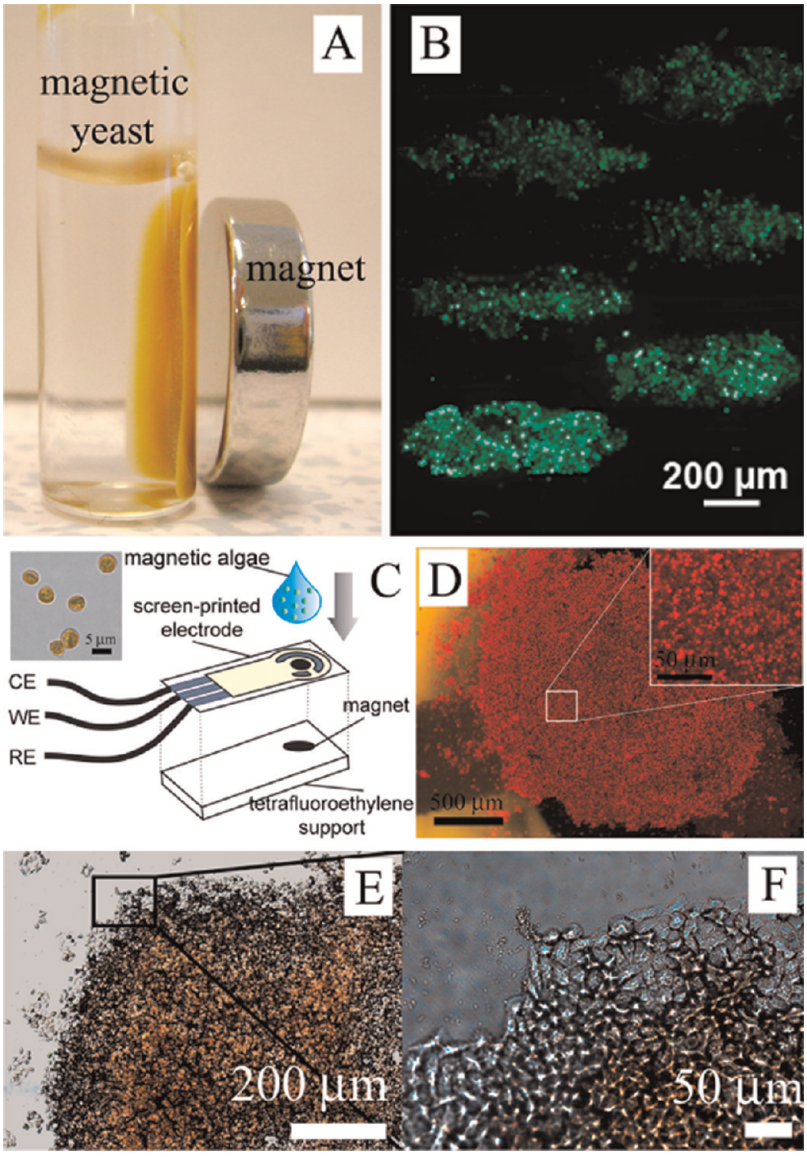

Figure 3. Functional devices based on magnetically functionalized living cells. (A) Magnetic behavior of poly(allylamine)-hydrochloride (PAH)/poly(styrene)sulfonate (PSS)/magnetite-functionalized yeast cells. (B) Magnetic retention of green fluorescent protein (GFP) reporter yeast in a microfluidic toxicity screening device. Reprinted with permission from ref 9. Copyright 2011 Springer. (C) Scheme and (D) fluorescent micrograph of utilization of magnetically modified microalgae cells in electrochemical whole-cell biosensors. Reprinted with permission from ref 32. Copyright 2010 The Royal Society of Chemistry. (E,F) Optical micrographs demonstrating micro-organization and growth of magnetically functionalized human cells (the inset shows the photograph of multicellular clusters formed above the cylindrical magnets). Reprinted from ref 4. Copyright 2011 American Chemical Society.

(GFP) synthesis, while other results indicate very low toxicity for LbL-coated microorganisms.[18,24,25] Viability of encapsulated cells improved when natural polysaccharides were used for the shelling.

The situation is more complex with human cells, suggesting that the fragile cellular membrane can be affected by the deposition of extracellular polymer multilayers. A study of cytotoxic effects of polycations on HeLa human cancer cells indicated that polyphosphoric acid, (poly)ethylene imine, and poly-L-lysine severely affected the viability of LbL-coated cells.[23] The increased number of layers also affected the polyelectrolyte toxicity.[24] Poly(allylamine)hydrochloride (PAH) did not affect the viability and reproduction in *C. elegans* nematodes, which are protected by a thick skin-like cuticle;[6] however, PAH did cause cell death in encapsulated pancreatic islets.[29] The apparent toxicity of polycations can be explained by their electrostatic interaction with the cellular membrane, which causes pore formation and the subsequent cell death. Intrinsic cell walls or cuticles protect microbes' and nematodes' cellular membrane from direct contact with the polycations.

Layer-by-layer assembly with natural biocompatible polyelectrolytes, polysaccharides, poly(amino acids), DNA, and polyphenols aims

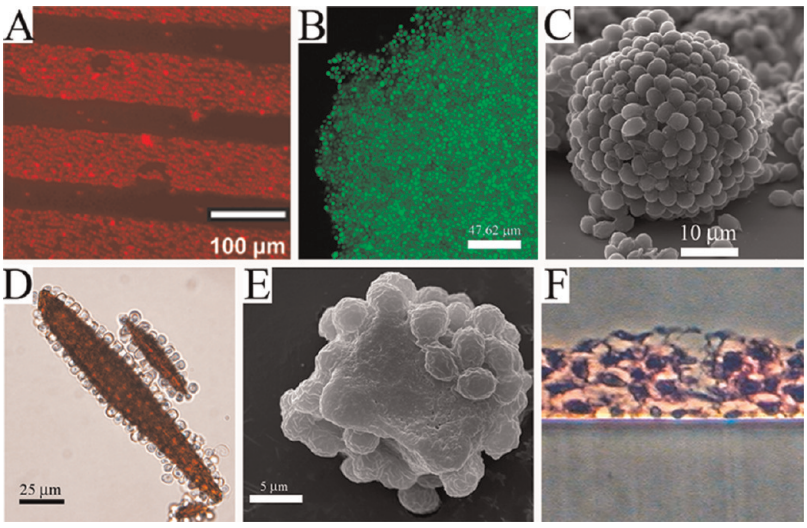

Figure 4. Layer-by-layer-facilitated assembly of cells into two- and three-dimensional hybrid structures. (A) LbL-coated yeast cells self-assemble on oppositely charged patterned surfaces. Reprinted from ref 20. Copyright 2005 American Chemical Society. (B) Confocal image of an artificial, free-standing yeast biofilm supported by the LbL polyelectrolyte multilayer. Reprinted with permission from ref 30. Copyright 2011 Elsevier. (C) Scanning electron micrograph of a spherical yeastosome demonstrating the assembly of LbL-coated yeast on air microbubbles. Reprinted with permission from ref 33. Copyright 2010 RSC Publishing. (D) Needle-like and (E) cubic-like cellosomes built from LbL-coated yeast. Reprinted with permission from ref 32. Copyright 2010 RSC Publishing. (F) Hematoxylin and eosin-stained image of four-layered LbL-coated cells (scale bar 30 μm). Reprinted with permission from ref 22. Copyright 2007 Wiley.

to improve encapsulated shell viability (especially evident in encapsulation of stem cells). Mouse mesenchymal stem cells that were LbL coated with polylysine/hyaluronic acid were not harmed and were able to produce colonies.[5]

Applications. Polyelectrolyte/nanoparticle-functionalized cells may find a number of practical applications, including as biosorbents, in spore formation, and in tissue engineering. The advantage of using polymer-modified cells is the almost unlimited quantity of different combinations (wall architectures) possible. Functional nanoparticles and nanodevices can be embedded into wall multilayers, which, in turn, may further attenuate the functionality of the composite shell. The modified cells not only act as microtemplates but also exhibit intrinsic functionality, including proliferation. Here, we focus on recently reported applications of LbL-coated cells for enhanced storage, in biosensors and in tissue engineering.

Artificial Spore Formation. Building nano-organized shells around living cells provides additional protection against harsh environments (extreme pH, oxidizing media, and UV radiation) while not disturbing cell functionality. An LbL-assembled shell may not only serve as a diffusion barrier for passive protection but may also include active catalytic elements (*e.g.*, shells enriched with catalase or magnetite nanoparticles decomposed hydrogen peroxide, decreasing its concentration within the capsule by a factor of 1000). By including elements that are sensitive to light, heat, or magnetic fields within an LbL shell, one could open the capsules with external signal-releasing biological cells, thus mimicking bacterial spore functionality.[13,17]

Biosensors. Conventional cell immobilization approaches based on cross-linking or matrix incorporation frequently reduce the viability of cells, cause side effects (*i.e.*, false responses), and are irreversible. In contrast, LbL-mediated magnetic functionalization of biocells promises the reversible immobilization of cells on biosensor surfaces. Yeast cells coated with carbon nanotubes/polyelectrolytes were immobilized on glassy carbon electrochemical electrodes in a toxicity sensor prototype (see Figure 3). Magnetic behavior rendered by LbL-coated yeast bioreporter cells can be used in fabrication of whole-cell biosensors. Magnetically modified microbial cells were immobilized *via* magnet-attracting microfluidic optically transparent chambers or on electrochemical screen-printed electrodes (see Figure 3) and then used for genotoxicity and triazine herbicide screening.[9,32]

Tissue Engineering—Building Artificial Microorganisms. Layer-by-layer-coated yeast cells were assembled on geometrically defined two-dimensional patterns that were fabricated using microcontact printing of polymers on glass substrates (see Figure 4A). Free-standing artificial biofilms were constructed from yeast contained between polyelectrolyte layers that reconstituted the shapes of the original templates and were additionally functionalized with silver nanoparticles, latex spheres, and magnetite.[30]

Layer-by-layer coating of certain compositions attracts or rejects cell adsorption, and these results lead to the formation of multicellular systems connected through polyelectrolyte multilayers.[31] Advanced tissue engineering, where the isolated LbL-coated human cells were assembled in five-layer tissue-like

material, was an impressive application in which fabrication of 25 μm films of mouse fibroblasts was reported.[22] Layer-by-layer interlayers glued biological cells together, enabling multilayer tissue formation.

Fibronectin and gelatin were used to mimic an extracellular matrix on glass surfaces, which stimulated the attachment of fibronectin-coated fibroblasts. Potentially, heterogeneous populations of live cells can be directed into xenogeneic multicellular clusters, having the complex structure characteristic of organs and tissues. Magnetically coated "ironclad" human cells were able to colonize and to grow on substrates, repeating the round shape of permanent magnets placed below the cell culture wells, suggesting that a similar method may be utilized in cell delivery and tissue formation (see Figure 4).

Fabrication of three-dimensional multicellular clusters mimicking the structure of primitive multicellular organisms is yet another avenue for LbL-coated cells. Functionalization of simple microbial model cells with polyelectrolytes helps to re-enact the likely environmental conditions facilitating the transition from unicellularity to multicellularity. With LbL-coated yeast cells, spherical, needle-shaped, and cubic-shaped multicellular living assemblies (cellosomes) were produced, representing a man-made artificial model of a multicellular organism.[32] Polyelectrolyte-coated cells self-assembled on air bubbles or calcium carbonate microcrystals, yielding a membrane built up from cells. These cellosomes were viable for several weeks and resembled natural colonial microorganisms, that is, *Volvox* species. One may expect that similar approaches might be employed to fabricate more complex cellular structures consisting of different types of cells, including human cells.

CONCLUSIONS

In this Perspective, we have described recent progress in LbL nanocoating to modify biological cells in order to provide them with new structural and functional features. Cell shells with defined multicomponent compositions may contain polyelectrolytes, proteins, DNA, and nanoparticles in locations defined with nanometer precision, thus making assemblies with predetermined functionality. This vision was inspired in part by bacterial spore formation, and additional composite shells enabled long-time survival of cells in harsh conditions and allowed cells to flourish in favorable environments. Our LbL shells also can provide cell protection, additional selectivity in cellular membrane permeability, and new magnetic, electrical, and mechanical properties for microorganisms. In the near future, we foresee the production of nanosize robotic devices that may be embedded or attached to biocells through architectural shell formation to provide cells with new instrumentation for better and more productive living.

Conflict of Interest: The authors declare no competing financial interest.

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