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Complete List of Authors:	Zeng, Jinfeng; Osaka University, Graduate School of Engineering, Department of Applied Chemistry Matsusaki, Michiya; Osaka University, Graduate School of Engineering, Department of Applied Chemistry

Review

Layer-by-Layer Assembly Nanofilms to Control Cell Functions

Jinfeng Zeng[a], Michiya Matsusaki[a,b]*

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Controlling cell functions, including morphology, adhesion, migration, proliferation and differentiation, in the cellular microenvironments of biomaterials is a major challenge in the biomedical fields such as tissue engineering, implantable biomaterials and biosensors. In the body, extracellular matrices (ECM) and growth factors constantly regulate cell functions. For functional biomaterials, the layer-by-layer (LbL) assembly technique offers a versatile method for controllable bio-coating at micro-/nano-meter scale to mimic ECM microenvironments. In this review, an overview of recent research related to the fabrication of cell-function controllable nanofilms via LbL assembly in the form of sheet-like nanofilms and customized nanocoatings around cells or implants is presented. Firstly, the components, driving forces, and especially the new approaches for high-throughput assembly of a nanofilm library are introduced. Moreover, we focus our attention on the control of cell functions via LbL nanofilms in the form of nanofilms and nanocoatings respectively. The effects of tunable growth factor release from multilayers on cell functions are also discussed.

1. Introduction

In the biomedical field, the ability of biomaterials to control cell functions is a key factor for tissue engineering. It is well known that the bulk properties of biomaterials play an important role in the control of constructs architecture and cell functions.[1] However, the surface properties are the keys for controlling cell behaviors of functional constructs,[2,3] including adhesion, morphology, proliferation, migration and differentiation. In the body, various types of cells are surrounded with a micrometer- or nanometer-sized fibrous meshwork of extracellular matrix (ECM) which not only acts as a physical support for cells but also induces cell-cell contacts and cell-matrix interactions to regulate their functions.[4,5] In the last few decades, researchers have focused on the fabrication of native ECM-like scaffolds,[1,2,6–8] to mimic the sophisticated cellular environment as a way of improving the interactions between cells and bio-constructs. Furthermore, the design or formation of biocompatible surfaces with micro-/nano-meter scale have attracted much attention.

In the first half of the 20th century, self-assembled monolayer (SAM)[9,10] and Langmuir-Blodgett (LB)[11,12] deposition showed remarkable capabilities in the design and deposition of hierarchical nanofilms. However, neither of these techniques were widely used in practical applications due to their intrinsic drawbacks including their high time-consumption, complicated operation, limited availability of materials and instability over a long period.[13] In order to overcome these limitations, Decher[14,15] *et al.* reported a layer-by-layer (LbL) assembly technique as a promising surface coating approach, where the fabrication of multilayered nanofilms was achieved by immersing the substrates such as glass slides into oppositely charged polymer solutions. Due to the simplicity and versatility of LbL technique and the ability to control the components and nanometer-sized thickness, basic research to understand the assembly mechanisms[16–20] and applied studies to observe the interactions between cells and multilayers have been conducted, especially in the biomedical field.[13,21–27] In the last decade, Shukla and Almeida[22]. wrote a review highlighting the significant impact of LbL assembly multilayers on biomedical applications. They suggested that LbL assembly coating would enhance the multi-functionality of biomaterials, making them more suitable in cellular and tissue engineering. Additionally, Picart[28] and co-workers reviewed a variety of biomimetic matrices along with controllable nanostructures and controllable physical, chemical, and biochemical properties to support similar cellular microenvironments for cells. Another review reported by Picart[23] further discussed various ways to spatially and temporally control the biochemical and mechanical properties of multilayers and introduced the applications of LbL multilayers in the biomedical field. Latterly, Li[21] *et al.* also reviewed the applications of LbL assembly multilayers in the form of nanofilms, scaffold nanocoatings, and 3D scaffolds in the field of tissue engineering.

Although remarkable progress in the development of LbL assembly scaffold has been made in the field of tissue engineering, the impact of the mechanical, physical and biochemical properties of LbL multilayers on cell functions are yet to be clearly understood. Studies of cell-function controllable LbL multilayers will guide the better design and fabrication of biomimetic scaffolds and promote their development in the biomedical field.

In this review, we intend to provide a systematic overview of recent efforts toward the progress of LbL assembly nanofilms in tissue engineering and discuss various approaches to control cell functions via the LbL assembly strategy (Table 1). This review begins with an overview of the fabrication of bio-nanofilms via the LbL assembly method, including the components, driving forces and assembly techniques. In particular, we will highlight the new

[a] *Department of Applied Chemistry, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan.*
[b] *JST-PRESTO, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan.*
*E-mail: m-matsus@chem.eng.osaka-u.ac.jp

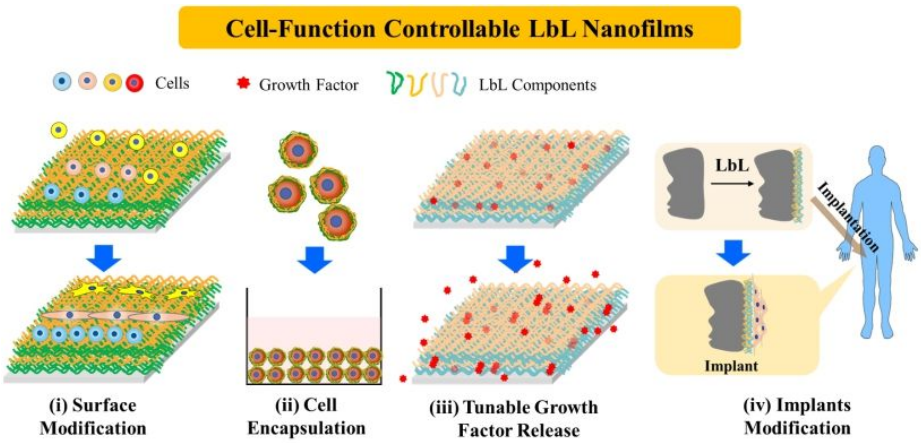

Fig. 1 Schematic illustration of different forms of LbL nanofilms with cell-functions controllability.

approaches for high-throughput assembly and screening of a nanofilm library. In subsequent sections we will discuss the regulation of cell-functions through LbL assembly nanofilms from the following aspects: (i) 2D nanofilms onto the surface of substrates for 2D cell culture; (ii) cell encapsulation by LbL nanofilms; (iii) tunable growth factor release from nanofilms; and (iv) combination of the LbL assembly technique, cells and scaffolds for tissue or organ repair (Fig. 1). An essential understanding of the impact of LbL nanofilm properties on cell functions will contribute to various research areas, from fundamental material science to applications such as tissue engineering, biosensors and cancer therapy.

2. Preparation of Cell-Function Controllable LbL Nanofilm

LbL assembly is a versatile method of fabricating highly customized polymer multilayers or modified surfaces with micro- or nanoscale, because of its efficient, simple, adjustable and operationally repeatable approach.[27,29–31] Typically, LbL assembly depends simply on the alternate adsorption of oppositely charged molecules without expensive instruments, complicated operations or harsh environmental requirements. After each adsorption process, necessary washing and drying steps are usually performed to remove the loosely bound molecules to avoid contaminating the solution for the subsequent steps.[14,15,30] Due to the easy and flexible operation of LbL assembly, accurate tailored hierarchical architectures at micro- or nanoscale can be obtained by controlling the assembly conditions, composition, and deposition layer number regardless of the type or size of the substrates.[19–21] Studies related to LbL assembly have demonstrated the utility of an extensive range

Table 1 Summary of the cell functions controlled by LbL nanofilms in this paper.

Properties	Purposes	Methods	References
Stiffness	For cell adhesion, proliferation and differentiation	Cross-linking	35, 36, 42, 46
		Thickness	55
Morphology	For cell morphology and differentiation	Component	57
		Substrate	43, 59
Biological Property	For adsorption of bio-molecule	Component	32-34, 61, 62, 86, 87
	For cell adhesion	Coating on cell monolayer	21, 53, 54, 88-93
		Coating on single cell, spheroid and cell sheet	23, 37, 54, 70, 71, 88, 94-98
Release Property	For cell proliferation and differentiation	Thickness	63, 99-102
		External stimulus	65, 103

of complementary molecules that can be driven via electrostatic or non-electrostatic interactions,[13,16] which are described later in this review. The broad availability of building blocks for LbL fabrication heralds the potential development of a wide array of multilayered devices with distinct shape, size and functionality.

2.1 Classes of Materials for Nanofilm

The easy subsequent deposition of materials allows the use of many types of components, including synthetic polymers,[32–36] proteins,[37–41]polysaccharides,[42–47] dyes[48] and nano-structures[49–51]. However, the nature and intrinsic properties of constituents play a crucial role in the performance of the final constructs. To mimic the complicated properties and structures of ECM in the body, materials with properties such as biocompatibility, nontoxicity, similarity to human tissues, and possession of cell recognition sites could be promising candidates for the application of supporting cell survival in tissue engineering, thereby controlling cell adhesion, morphology, migration, proliferation and differentiation.

2.1.1 Protein. *In vivo*, cells are surrounded and supported by ECM which is composed of protein and proteoglycan, for example fibronectin (FN), collagen, and laminin.[27] These proteins secreted by cells and assemblies closely associated with cell surfaces, have been used to construct LbL assembly nanofilms for biomedical applications.[37,52] Basically, the ability of native ECM to attach cells and control cell functions can be mostly preserved by employing ECM proteins as building blocks of assembly multilayers.[27] FN is a well-known ECM protein containing arginine-glycine-aspartic acid (RGD) sequences that can interact with other proteins such as collagen, gelatin (G) and integrin receptors on the cell membrane, playing an important role in cell adhesion, migration, proliferation and differentiation.[37] Our group initially constructed cellular multilayers by sequentially depositing FN/G nanofilms and mouse fibroblasts layers[53] or directly accumulating FN/G coated cells.[54] We discovered that cellular multilayers coated with FN/G nanofilms showed high functionality and stability against physical stress, but with less inflammation. They also exhibited high biological properties and response to drugs, probably because of the similar surrounding to native-ECM.[54] Apart from fibronectin and gelatin, collagen and protamine sulfate[55] can also improve the association of materials and cells, and maintain or enhance cell functions in vitro. They have thus attracted considerable attention with respect to the construction of LbL nanofilms in tissue engineering.

2.1.2 Naturally-Derived Polymer. *Polypeptides*. Polypeptides are short chains of amino acid monomers linked by peptide bonds, including poly(l-lysine) (PLL), poly (d-lysine) (PDL) and Poly (l-glutamic acid) (PGA).[27] Due to their ability to form secondary structures (α helix or β sheet), biocompatibility, tuned biodegradability, specific biomolecule recognition, designability and abundant functional groups, polypeptides have been widely used for LbL assembly, forming biologically active surfaces.[27] Of these, PLL is usually considered as a model protein because arginine and lysine are the major positively charged amino acids. The research groups of Ji[42,45], Picart[46] and Hammond[47] all used PLL as building blocks to construct LbL nanofilms that exhibited excellent bioactivity and cell adhesion. *Polysaccharides*. Polysaccharides consisting of repeated monosaccharides linked by O-glycosidic bonds usually come from animals, plants, and microorganisms. Polysaccharides including chitosan (CHI)[34,56], alginate (Alg)[32,43,57], hyaluronic acid (HA)[35,42,46], dextran sulfate (Dex)[27], heparin (Hep)[58] and chondroitin sulfate (CS)[59] with excellent biocompatibility, biodegradability and easy modification, have been demonstrated to be potential candidates for LbL assembly to mimic native ECM. For example, CHI with abundant primary amines is a kind of positively charged polyelectrolyte and has been widely used as a polycation during LbL assembly. It is also well known that HA can interact with CD44 which is involved in the interactions of cells and surroundings, benefiting cell adhesion and migration.[27]

Additionally, in order to meet the intended purpose or improve some properties of materials, such as the mechanical properties and biological activities, polysaccharides can be modified with various functional molecules to create functional constructs.[34–36] Dopamine derived from mussels is well known to adhere to a wide range of substrates. Dopamine modified polysaccharides prepared by carbodiimide chemistry have been widely used to form thin and surface-adherent dopamine films.[60] Moreover, multilayers modified with dopamine can not only promote cell adhesion and proliferation, but also improve the mechanical properties of nanocoatings.[61,62] For example, multilayer films developed with chitosan and dopamine-modified hyaluronic acid (HA-DN) using the LbL method have been reported to exhibit enhanced cell adhesion, proliferation and viability.[60] These examples suggest the great application potential of polysaccharides serving as LbL assembly building blocks for controlling cell functions in the biomedical field.

2.2 Molecular Interactions Driving the Nanofilm

LbL assembly is a simple, flexible, efficient and versatile strategy for producing highly customized polymeric nanofilms, involving the simple immersion of the substrate into complementary polymer solutions.[16] Initially, LbL was simply based on the sequential adsorption of oppositely charged polymers via electrostatic interaction. Researchers subsequently extended the LbL assembly adsorption of polymers not only based on polyelectrolytes, but also uncharged materials including biomolecules[37,54] and nanoparticles[63]. Solely electrostatic interaction has already been extended to hydrogen bonding[64,65], coordinate bonding[66], covalent bonding[67], host-guest interaction and biologically specific recognition.[25,54] In this section, we will comprehensively review the application of electrostatic interaction and biologically specific recognition in the fabrication of nanofilms controlling cell functions via LbL assembly and give a brief introduction to the potential application of supramolecular LbL assembly in tissue engineering.

2.2.1 Electrostatic Interaction. Since the pioneering research on LbL assembly by Decher[14,15] and co-workers, electrostatic interaction has been demonstrated to be one of the most important and most explored assembly driving force within the LbL technique. Based on electrostatic interaction, by means of repeatedly immersing a substrate into charged polymer solutions, LbL assembly provides a versatile strategy for the fabrication of well-controlled nanostructures. It is well known that the physicochemical parameters of polyelectrolyte are strongly influenced by pH, ionic strength, temperature and solvent. Furthermore, polyelectrolyte concentration, molecular weight and charge density also play important roles in the architecture as well as the mechanical and physicochemical properties of LbL assembly

multilayers. Cell functions, especially cell adhesion are greatly influenced by the surface charge, wettability, roughness and stiffness of contact surfaces.[3] Thus, it is very easy to form tailored constructs with customized shape, thickness and topological structure by controlling polyelectrolyte assembly conditions to control cell adhesion, morphology, or migration. In 2014, Borges and Mano reviewed several factors and intrinsic properties of polyelectrolyte for controlling the growth, structure and properties of LbL assembly multilayers.[16] Recently, Elizarova and Luckham[68] comprehensively described the current understanding of the important effects of polyelectrolyte choice and LbL assembly process including the deposition of the first polyelectrolyte layer onto the selected substrates and the adsorption of the second layer to the oppositely charged polymer layer on the properties and structures of the resultant multilayers. In the present review, we will also discuss the influences of surface properties of polyelectrolyte-based LbL nanofilms on cell adhesion, morphology, proliferation and differentiation. Electrostatic interaction is the first and the most widely studied molecular driving force for the LbL assembly technique. Multilayers with well-controlled thickness, physicochemical and mechanical properties can be easily obtained, making it the most powerful and extensive assembly approach via Electrostatic interaction. However, the LbL assembly approach based on Electrostatic interaction is only applicable to charged materials. Films with strong negatively charged strength will weaken the attachment of cells, but strong positively charged strength will damage cell structure, factors ignored by the majority of reports. Additionally, due to its susceptibility to assembly conditions (e.g. ion strength, pH), the structures and properties of obtained multilayers are easily affected by environmental stimuli. The films would even undergo disassembly.

2.2.2 Biologically Specific Recognition. Biologically specific recognition is the interaction of biomaterials, including avidin-biotin, protein-polysaccharide, antibody-antigen and protein-ligand interactions. It requires high steric matching, supporting high specificity to the target molecules, and involves multiple molecular interactions, such as electrostatic, hydrogen bonding and hydrophobic interactions.[54] In our group, we have reported LbL multilayers constructed based on FN using biologically specific recognition for the first time.[69] FN can interact with heparin, dextran sulfate and gelatin at some specific binding domains even though they all have a negative charge under physiological conditions.[13] We have been focusing on the study of encapsulating cells by LbL nanofilms using biological interaction, with particular emphasis on attempts to apply FN to control cell functions.[13,54] Moreover, 3D tissue constructs have been successfully fabricated by enhanced cell-cell interactions based on FN LbL nanofilms.[70,71] Borges and Mano[16] also reviewed some of the progress based on biologically specific recognition including avidin-biotin, antibody-antigen, lectin-carbohydrate interactions, as well as DNA hybridization. They concluded that biologically specific recognition further widened the range of materials for functional LbL assembly nanofilms with enhanced stability, specificity and orientation for application to biosensing, drug/gene delivery, and tissue engineering. In general, although biologically specific interaction is a complicated recognition process along with various interactions, it provides a new molecular driving force, making nonionic polymers, even polymers with the same charges, applicable for LbL assembly. Moreover, these biomaterials interact with each other specifically *in vivo*, guiding or influencing the activities of cells and even organisms to some degree. LbL assembly based on biologically specific recognition provides a readily available strategy for the fabrication of functional nanofilms specifically controlling cell functions in tissue engineering.

2.2.3 Supramolecular LbL Assembly. Apart from electrostatic interaction and biologically specific recognition, other non-covalent bonds such as hydrogen bonding, coordinate bonding and host-guest interaction are also widely used in LbL assembly. Supramolecular LbL assembly film refers to polymers assembled on the basis of the abovementioned intermolecular non-covalent interactions. Among them, hydrogen-bonding is one of the most studied driving forces for incorporating unchanged polymer into supramolecular multilayer nanofilms.[16] Recently, Sukhishvili[72] *et al.* reviewed the fabrication of supramolecular films and capsules by LbL assembly of native polymer and weak polyelectrolyte based on hydrogen bonding. Free-standing hydrogen-bonded films exhibited excellent pH and temperature responsiveness making hydrogen-bonded films and capsules attractive candidates for biosensors and controlled drug release. Similarly, in 2018, Akiba[73] *et al.* overviewed the synthesis of LbL assemblies containing calix[n]arene (CA[n]) and cucurbit[n]uril (CB[n]) and their application in separation and purification, sensors for ions and molecules, and controlled release. CA[n] and CB[n] are well known for their specific self-assembly with guest molecules forming supermolecules, a process known as host-guest interaction. Due to its highly selective, strong but dynamic interaction, host-guest interaction has also been used to construct stimuli-responsive multifunctional bio-interfaces by LbL assembly of two determined molecules, such as cyclodextrins, cucurbiturils, calixarenes and their complementary guest molecules.[16] The fabrication of multilayers based on host-guest interaction was demonstrated to be an alternative approach in the field of reversible, healing and programmable soft surfaces.[16] Hydrogen bonding plays an important role in the specific biochemical properties and unique construction of proteins and nucleic acids. Moreover, ECM and cells are inherent in coordinating and adapting to each other during embryonic development, wound healing and so on. Stimuli-responsive biointerfaces based on host-guest interaction or hydrogen bonding have shown great potential to engineer the cell niche where cell functions, such as adhesion and morphology, are largely affected by dynamically changeable substrate rigidity and roughness. Thus, supramolecular LbL assembly based on hydrogen bonding and host-guest interactions have great potential in active molecules delivery and reversible adsorption of proteins and cells in tissue engineering. However, stimuli-responsive films based on specific supramolecular recognition are limited to specific structures, even only synthetic material. Further studies should pay attention to exploring other molecular structures of multilayers with dynamic properties which are suitable for cells culture.

2.3 New Methods for Control of Nanofilm Deposition

During the last two decades, various new developments have demonstrated the widespread application of LbL assembly. Nevertheless, different substrates with different standards and

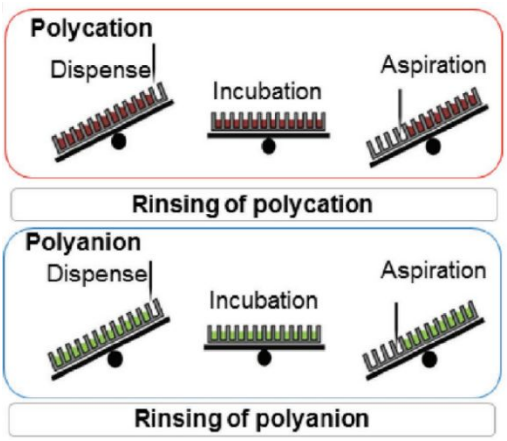

Fig. 2 Schematic representation of the layer-by-layer deposition at high throughput in multiple well cell culture microplates. The automated process for the polycation and polyanion deposition consists of dispensing and aspiration steps: when tilting is used, the microplate is tilted during all dispensing and aspiration steps. Reproduced with permission from ref. 76. Copyright 2018 WILEY-VCH.

various requirements of assembly processes have promoted the development of a wide range of LbL assembly techniques. In order to meet these requirements, various deposition methods have been developed, including (i) immersion, (ii) spin, (iii) spray, (iv) electromagnetic driven, and (v) fluidic assembly.[17] In 2015, Richardson[17] *et al.* summarized the current technologies for LbL assembly and emphasized that innovation was still necessary in the coating of microscopic substrates. For example, the need to increase assembly speed and reduce materials waste during the assembly process, especially for valuable materials like expensive biomolecules and tailored polymers were highlighted. To address these challenges and enable more thorough investigations of LbL film assemblies, some simple approaches for the high-throughput construction of LbL nanofilms in biomedical applications have been developed in the past five years.

2.3.1 Liquid Handling Robot. The liquid handling robot is a new liquid handler that enhances the reproducibility and efficiency of laboratory operations by assisting researchers with fully unattended automated pipetting. High-throughput assays can be easily achieved by a liquid-handling robot, especially for biomaterials screening.[74] For the rapid investigation of interactions between a variety of LbL nanofilms and cells to optimize biomaterials, high-throughput methods utilizing cell culture materials and commercially available liquid handling equipment were first reported by Jaklenec and co-workers.[75] They verified the direct effect of key parameters, including deposition time and assembly pH on the thickness of films. The results are consistent with the traditional LbL assembly approach. Their works demonstrated the viability of this automated high-throughput method to fabricate

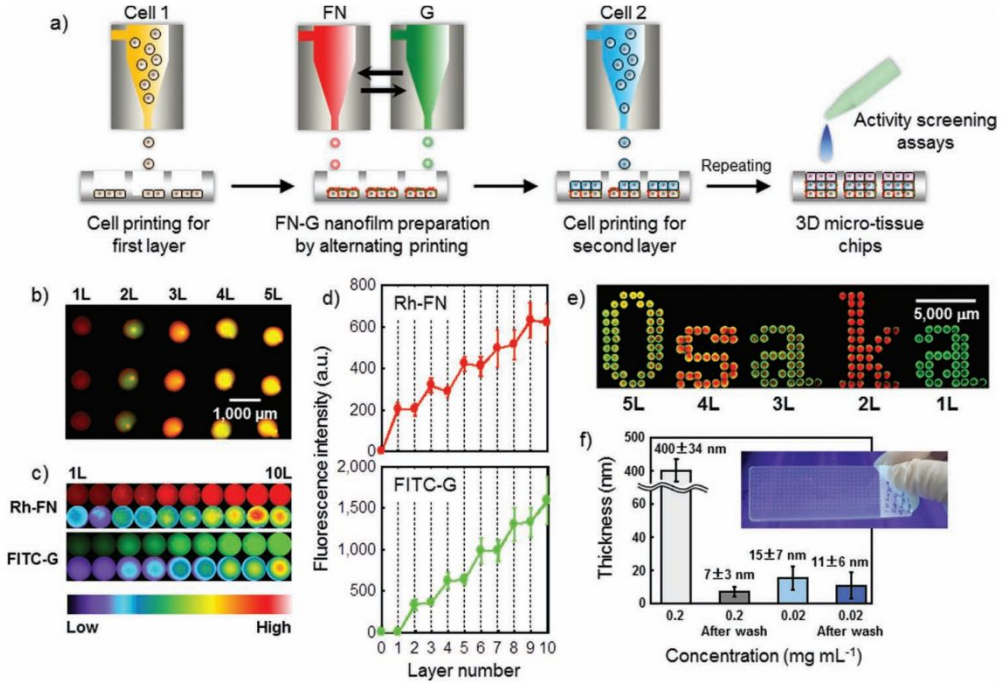

Fig. 3 a) Schematic illustration of the development of 3D micro-tissue arrays by the LbL printing of single cells and proteins. b) Fluorescence merged image of monolayered (1L) to five layered (5L)-spots prepared by the LbL printing of 0.2 mg/mL FN and G in 50 mM Tris-HCl buffer (pH=7.4). The FN and G were labelled with rhodamine (Rh-FN) and fluorescein isothiocyanate (FITC-G), respectively. c) Fluorescence and luminance images of Rh-FN and FITC-G in 1L to ten layered (10L)-FN-G nanofilm spots. The luminance distribution bar is shown at the bottom. d) Dependence of the layer number of the FN-G nanofilm spots on the fluorescence intensity (n=4, over 10 spots per image). The stepwise increase in fluorescence intensity was measured alternately. e) Fluorescence merged image of "Osaka" letters consisting of the 1L to 5L-FN-G nanofilm spots. f) Thickness of the 10L-FN-G nanofilm spots using 0.2 and 0.02 mg/mL solutions before and after 1 hour of incubation in ultrapure water (n=3). The inset shows a photograph of 300 spots from 10L-FN-G nanofilms prepared on a slide glass under UV light. The solution concentration was 0.02 mg/mL. The diameter of the FN-G nanofilm spots in b), c), d), and f) was 750 μm and in e) 500 μm. The center-to-center spacing of the spots in b), c), d), and f) was 1,000 μm and ine) was 750 μm. Reproduced with permission from ref. 80. Copyright 2013 WILEY-VCH.

various nanofilms for rapid characterization and optimization of desirable material properties. In order to further improve automation, Picart[76] and co-workers used a robotic arm equipped with multiple channels to deposit homogeneous thin films into a 96-well plate, forming multilayers with a few tens of nanometers to a few micrometers. In order to ensure the homogeneity of film, a tilting (T) of the microplate carrier was introduced during assembly (Fig. 2). Cell adhesion and stem cell differentiation on peptide-grafted polyelectrolyte nanofilms obtained by this high-throughput method were further assessed. They showed that this new method could be adaptable to other types of microplates and any type of robotic arm, paving the way for future applications in regenerative medicine and high-throughput drug screening. The liquid handling robot is an automatic process for the operation of LbL assembly, which has greatly enhanced the assembly speed, making the high throughput assembly possible. However, this method has complex equipments requirements and simplifying these requirements s will be the goal for its wider application in the future.

2.3.2 Inkjet Printing. Inkjet printing is a type of computer printing that recreates a digital image by propelling droplets of ink onto paper, plastic, or other substrates. Because of its precise deposition, inkjet printing has been widely used to fabricate customized constructs based on polymers, nanoparticles, and even expensive biomaterials, like growth factors, DNA and cells.[76,77] Therefore, there is great potential to integrate inkjet printing with LbL assembly for high-speed nanofilm fabrication, a process known as high-throughput assembly. Hong[78] and co-workers first demonstrated the viability of inkjet-based LbL assembly by the successful fabrication of nanofilms with controlled film shape, composition and functionality on various substrates. The same group subsequently used stabilized basic fibroblast growth factor (bFGF) as an ink and successfully incorporated it into multilayers of bFGF/heparin on the same substrate. Released bFGF was confirmed to enhance the proliferation of human dermal fibroblast (HDF) and maintain the undifferentiated state of induced pluripotent stem (iPS) cells.[79] Our group reported simplified 3D-liver structures, produced by alternately inkjet printing single cells and FN-G solutions, forming hundreds of multilayered micro-tissues in one micro-array, used for high-throughput drug evaluations (Fig.3).[80]

Taken together, LbL assembly based on inkjet printing provides a practicable approach for high-throughput construction of nanofilms. It also plays an important role in the construction of specific patterned nanofilms for cells co-culture, even for organized 3D tissues construction in tissue engineering. Nevertheless, due to the high print speed and the tiny droplets, rinsing steps were ignored because of the rapid drying of deposited samples. Thus, the stability of multilayers via inkjet printing will remain a great challenge due to the residue of unassembled polymers. In further studies, methods to enhance the stability of multilayers should be given more attention.

2.3.3 Capillary Flow. Hammond's group[81] developed a new microfluidic approach for high-throughput preparation and screening of nanometer-sized multilayers in parallel, and named this approach "capillary flow layer-by-layer" (CF-LbL), as shown in Fig. 4. This platform with micro-channels can be designed based in both 96- and 384-well plate dimensions for high-throughput screening of available material properties. The nanofilm assemblies were formed by polymer solutions automatically flowing through these microfluidic channels and then depositing. Various LbL nanofilms with a variety of compositions, morphologies, and architectures could be formed by easily controlling the solution flowing state. They have already demonstrated that the highly controllable LbL multilayers formed by this simple technology possessed the same properties as those obtained from the traditional LbL assembly method. Nevertheless, this CF-LbL LbL approach significantly reduced material waste, requiring only 0.1% material per film compared to conventional methods. Based on this technology, Dong[82] *et al*. introduced benchtop CF-LbL systems using

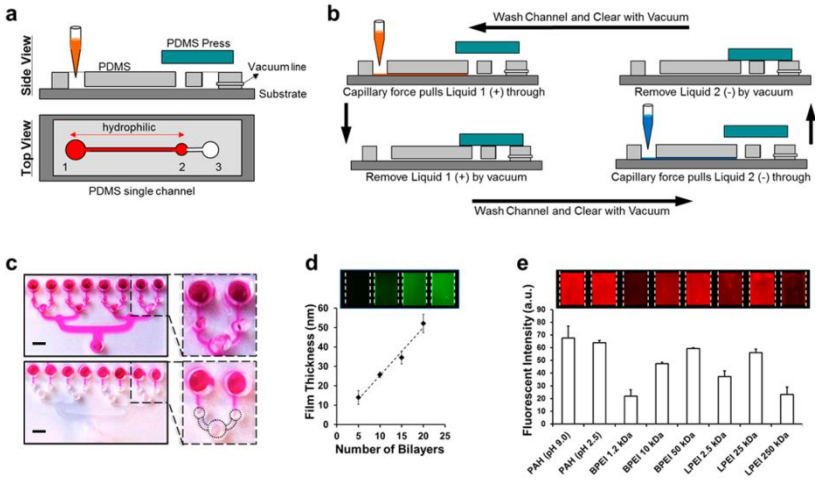

Fig. 4 Design of capillary flow layer-by-layer (CF-LbL) device. (a) Top and side view of a single channel within a CF-LbL device; the red region is O_2 plasma treated. (b) The process of alternating adsorption of material inside the microfluidic channels, (+) polycation and (-) polyanion species. (c) Multiple independent channels within a single CF-LbL device. The top image is fully O_2 plasma treated; the bottom image selectively treated; scale=3 mm. (d) Measurement of film thickness for a sample PAA/PAHFITC LbL film. (e) Screening LbL film architectures for material incorporation. Fluorescently labeled PAA is incorporated into bilayer LbL films with the polycations PAH, branched polyethylenimine (BPEI), and linear polyethylenimine (LPEI). Data shown as mean ±SD. Channels used were 800 μm wide and 2.0 mm long. Reproduced with permission from ref. 81. Copyright 2014 American Chemical Society.

standard multichannel pipettes instead of an automated liquid handling system, obviating the need for significant capital investment and specialized computing skills. Results showed that channel geometry and flow conditions had a noticeable effect on the thickness and roughness of the resultant films. Bioactive polymer multilayers were deposited inside the micro-channels using this simple platform, demonstrating its potential for high-throughput LbL assembly.

Capillary flow has been demonstrated to be a powerful, high throughput tool for the fabrication and optimization of various multilayers. The designing of the fluid channel, including size, geometry, and precisely manipulating picoliter volumes of fluid will be the directions of future developments. For the transition from laboratory-scale bio-fabrication to commercialization, reproducible production, inexpensive and simple operation are areas that will need to be studied.

Despite these challenges, in the past reports, high-throughput LbL assembly methods allow for new ways of rapidly observing and investigating cell functions based on arrays of LbL nanofilms and could be used to screen interactions between molecularly distinct LbL films and cells.[75] High-throughput LbL assembly also significantly reduces the amount of materials and shortens the assembly time[81], both of which are beneficial for expensive and customized materials or biomaterials with a short half-life. The high-throughput LbL assembly approach has played a significant role in a broad range of fields including tissue engineering and drug screening systems.

3. Surface Properties of LbL Nanofilm Controlling Cell-Function

In vivo, ECM is a complex system, communicating with cells in a complicated way, including mechanical and biochemical signals.[83] Recreating native ECM *in vitro* will play an important role in improving cell functions for 2D cell culture, benefiting the development of tissue engineering. The versatility of the LbL assembly strategy has attracted much interest in the production of hierarchical nanofilms with controllable film architecture and surface properties, such as stiffness, thickness, topography, surface hydrophilicity and charge.[83,84] In this section, we will discuss the control of cell functions by the surface properties of substrate based on the LbL assembly technique.

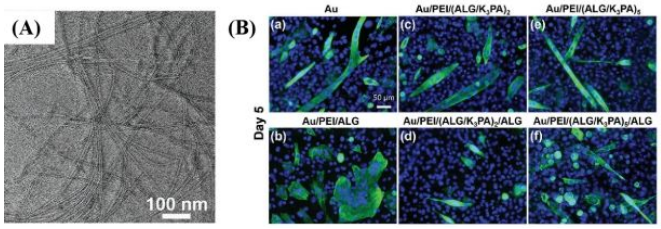

Fig. 5 (A) Representative TEM image of the ALG/K3PA nanofibrous structure. (B) Representative immunofluorescence images of C2C12 cells with myotubes stained with troponin T (green) and nuclei with DAPI (blue) at 5 d of culture on uncoated and coated Au surfaces, using differentiation medium (DM): a) bare Au, b) Au/PEI/ALG, c) Au/PEI/$(ALG/K3PA)_2$, d) Au/PEI/$(ALG/K3PA)_2$/ALG, e) Au/PEI/$(ALG/K3PA)_5$, and f) Au/PEI/$(ALG/K3PA)_5$/ALG. The scale bar is 50 μm in all image. Reproduced with permission from ref. 57. Copyright 2017 WILEY-VCH.

3.1 Stiffness of Nanofilm

Most types of cells are sensitive to the mechanical properties of substrates. It has been demonstrated that substrates rigidity and roughness have a great impact on controlling cell functions, such as adhesion, spreading, proliferation and morphology.[27] In recent years, several efforts to control the mechanical properties of materials by adjusting LbL assembly parameters have been reported.[35,46,55] Cross-linking of multilayers and nanofilm layer number have been applied successfully to adjust the stiffness of the substrate surface. Ji's group[42] constructed LbL films with varying stiffness by controlling cross-linking degree to observe the process of endothelial-to-mesenchymal transition. The results demonstrated that endothelial cells (EC) exhibited stronger adhesion and lost their markers on the stiffer films even without growth factors, but kept their phenotype with a softer substrate.[55] Meanwhile, Picart[46] *et al.* reported that LbL assembly offered a convenient approach to keep human muscle stem cells in a quiescent state on biomimetic multilayers with optimized stiffness even though these cells didn't express optimal adhesion to substrates. However, it is worth noting that the cellular microenvironment is dynamic and undergoes constant remodeling. Biomaterials with mechanical adaptability to cells are desirable.[35] Ji and co-workers fabricated LbL nanofilms with dynamically adjustable stiffness by controlling the degree of cross-linking. The adhesion of endothelial cells decreased subsequently with the decrease of stiffness, but the functions of the formed EC monolayer and endothelial status were obviously improved.[35,36] Therefore, LbL assembly as a simple approach for adjusting the stiffness of substrates has great potential in the development of cell-based functional biomaterials.

3.2 Surface Morphology of Nanofilm

LbL assembly has great versatility to precisely control the polymer deposition, adapting to fabricate films with a patterned geometry at nanoscale which plays an important role in cell functions and morphology.[23] At first, Mano[57] *et al*. fabricated a supramolecular multilayered nanofilm based on Alg and self-assembled a 1D nanofiber of low-molecular-weight peptide amphiphiles. The nanofibrous structure was observed to improve C2C12 cell adhesion, proliferation, and even enhance C2C12 cell differentiation into tube-like myotubes (Fig.5). Similarly, a pioneering strategy of nanofilm fabrication with well-defined grooves at sub-micrometer resolution was performed by Mano's group to control cell functions using the LbL assembly method.[59] Mouse fibroblasts and C2C12 cells both showed better viability and proliferation when they were cultured on patterned surfaces than on flat membranes. Interestingly, cell morphology was also induced distinctly along the grooves or other finely-defined wells[43], which is important in the regeneration of some special tissues, such as orientated capillaries and muscle structures.[85]

3.3 Biochemical Properties of Nanofilm Surface

In the last few decades, countless efforts have demonstrated that cell functions are influenced strongly by the biochemical properties of the substrates.[3] In the following section, we will discuss the

influence of key parameters of the substrate surface on cell behaviors. Cell membranes are negatively charged due to the presence of phosphates which are easily attracted by the positively charged surface. It has also been demonstrated that appropriate surface wettability improved cell adhesion.[3] Due to the powerful ability to modulate surface electronegativity and wettability by depositing polyelectrolytes alternately, the LbL assembly approach has been widely used to fabricate a suitable surface for cell culture and tissue model construction.[86,87] Additionally, one of the particularly attractive features of LbL nanofilms is the incorporation of biochemical signals to mimic the communication of cells and surroundings, such as proteins.[27] One such example is dopamine secreted by mussels, exhibiting outstanding adhesion to a variety or substrates due to the presence of catechol groups. Nanofilms modified with dopamine can not only promote cell adhesion and proliferation, but also improve the mechanical properties of the nanocoating, enhancing the repair of bone and the wound healing process.[61,62] Hammond's group[32] and Yu's group[33] also applied the LbL assembly approach to create multilayers modified with antibodies on the surface of microchips to specifically catch circulating tumor cells from the bloodstream and then release, collect cancer cells, providing viable cancer cells for downstream analyses. Together, these works suggest that the LbL technique is a relatively simple yet powerful technology for constructing stable nanofilms with controllable biochemicals to adjust cell behaviors and functions.

Overall, cell functions, such as adhesion, proliferation and differentiation depend strongly on the mechanical and physicochemical properties of substrates. Based on the LbL methodology, it is possible to construct films with matched biomaterials or well-defined patterned motifs engraved on the surface with a sub-micrometer resolution. However, there are few reports on the incorporation of several favorable biological factors into one LbL system similar to natural ECM. If suitable mechanical and physicochemical properties could all be applied to one substrate surface, it would be easier to adjust cell functions to be similar to those *in vivo*.

4. LbL Nanofilm Encapsulating Cells

Encapsulation of cells through the artificial modulation of extracellular microenvironments to mimic native ECM offers an effective strategy to control cell functions. Encapsulating cells has been demonstrated to effectively modulate the communication to the cellular environment[54] and provide protection for cells against physical damage[88] or attenuate deleterious host responses.[21,54] LbL assembly is an appropriate method for the preparation of nanometer sized films on various substrates under mild environments and is adapted to not only polyelectrolytes, but also viruses, proteins, and even cells.[54] LbL assembly may be an ideal technique to encapsulate cells or cell aggregates for mimicking native-ECM *in vitro* to maintain or improve cell functions. For example, Oliveira *et al*. summarized the research of cellular encapsulation by LbL multilayers around individual cells, cell aggregates and even the cell-laden materials, respectively via the direct coating technique and indirect method. They highlighted the possibility of LbL assembly to overcome the limitations of the use of bulk hydrogels, such as low permeability and instability. They also introduced some crucial features of LbL systems for cell encapsulation in different fields of application.[89]

At the beginning, our group developed a simple but unique bottom-up strategy for 3D tissue construction by coating fibroblast cell layers with fibronectin (FN)/gelatin (G) LbL nanofilms that are served as nano-ECM (Fig. 6a).[53] We demonstrated that the FN/G nanofilm could offer a stable adhesive surface for the adhesion of a second layer of cells. This bottom-up strategy offered an efficient

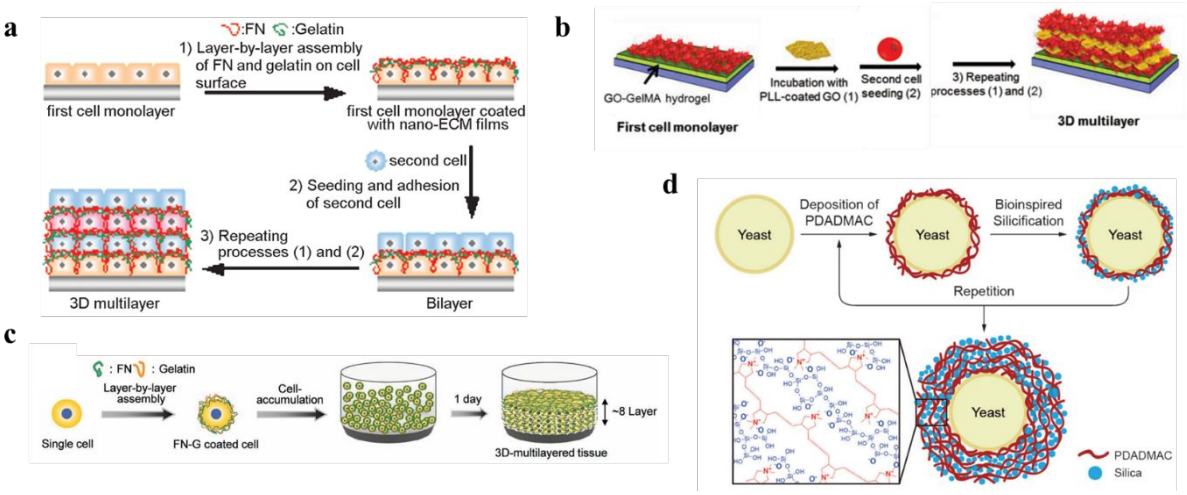

Fig. 6 (a) Fabrication process of 3D cellular multilayers composed of cells and nano-ECM films. The nano-ECM films were composed of FN and gelatin, and the outermost surfaces of all films were FN, which allowed cell adhesion. Reproduced with permission from ref. 53. Copyright 2007 Wiley-VCH. (b) Schematic illustration of the multilayer tissue constructs fabricated via LbL cell seeding and deposition of PLL-coated GO particles. Reproduced with permission from ref. 90. Copyright 2014 WILEY-VCH. (c) Schematic illustration of the rapid construction of 3D multilayered tissues by the cell-accumulation technique using FN-G nanofilms on a single cell surface. Reproduced with permission from ref. 93. Copyright 2011 WILEY-VCH. (d) Scheme for thickness-controllable encapsulation of individual S. cerevisiae cells within an ultrathin silica shell via LbL-based in situ silicification. Reproduced with permission from ref. 94. Copyright 2015 Wiley-VCH.

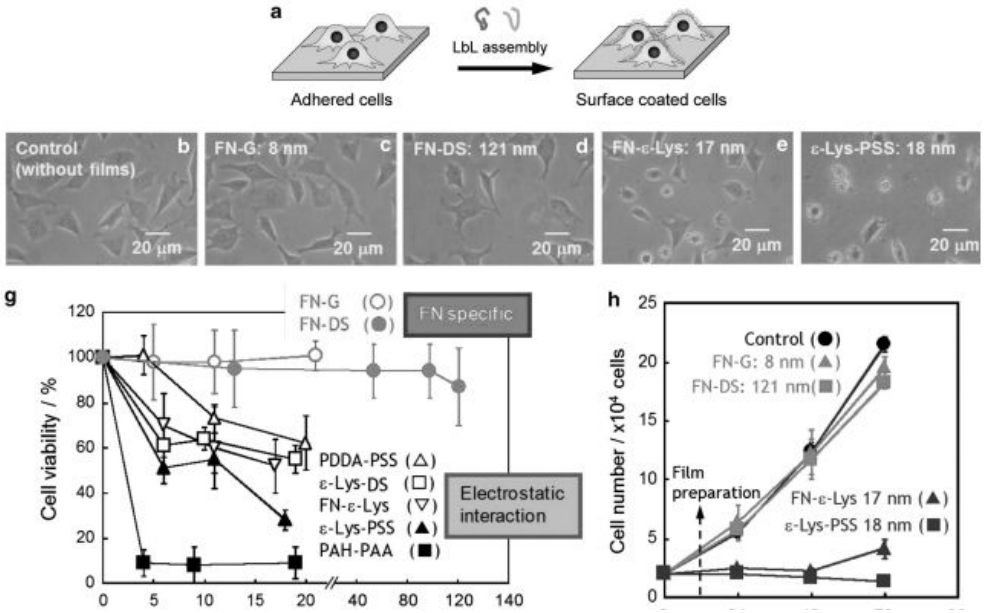

Fig. 7 (a) Schematic illustration of layer-by-layer (LbL) assembly on adhered cell surfaces (b-f) Phase contrast (Ph) images of the L929 fibroblasts (b) without or with (c) 8-nm-thick fibronectin (FN)-gelatin (G), (d) 121-nm-thick FN-dextran sulfate (DS), (e) 17-nm-thick FN-e-Lys and (f) 18-nm-thick e-Lyspoly (sodium styrenesulfonate) (PSS) nanofilms on the cell surfaces after 24 h of incubation. DS and e-Lys represent sodium DS and e-poly (lysine hydrochloride), respectively. (g) The relationship between cell viability and thickness of various nanofilms prepared on the cell surfaces after 24h of incubation (n¼ 3). PAH and PAA represent poly (allylamine hydrochloride) and poly (acrylic acid), respectively. (h) Cell proliferation versus various nanofilms prepared on the L929 cell surfaces during 72 h of incubation. Reproduced with permission from ref. 54. Copyright 2014 WILEY-VCH.

approach for the fabrication of artificial 3D-tissue models.[90] Shin[91] *et al*. also successfully constructed multilayered cardiac tissue by alternately depositing cardiomyocytes and PLL-coated graphene oxide (PLL-GO), as shown in Fig. 6b. The architecture of the multilayered cell construct was easily controlled by cell number and the location or PLL-GO layer's number, which showed enhanced biological activity, adjustable mechanical properties and ease of handling. Similarly, Perry[92] *et al.* reviewed the fabrication of engineered basement membrane using LbL assembly. Basement membranes are thin layers of specialized ECM which provide support to cells, act as barriers between different types of cells and regulate cell phenotypes and functions. A co-cultured system based on LbL nanofilms which are located between different types of cells will be the easiest method of recreating relevant basement membranes by inducing the secretion of ECM components.[93] Additionally, compared with ECM hydrogel scaffolds, the LbL co-culture was better able to maintain the cellular phenotype, mainly because of the secretion of basement membrane (BM) components and signal molecules.

Our group have been active in the development of 3D artificial tissues or angiogenesis models *in vitro* based on multilayered cell constructs. Besides the alternation of cells and LbL nanofilm as shown in the above, we also tried to encapsulate single cells with LbL FN/G nanofilms and then used them to construct 3D tissues, namely the cell-accumulation technique (Fig. 6c).[94] Encapsulating around a single cell via ECM proteins not only offered native ECM-like support for cells, but also isolated cells from each other avoiding contact inhibition to maintain cell proliferation.[23] In 2014, we reviewed the efforts in our group to construct nanometer-sized artificial ECM films by encapsulating ECM proteins on a single cell surface for tissue engineering, especially vascularized tissues.[54] We discovered that cells coated with LbL nanofilm similar to natural ECM showed high functionality, less inflammation, and high cell viability even after 18 cycles of centrifugation.[88] Additionally, compared with cell encapsulation using polyelectrolyte (PE) multilayers, FN-based multilayers around cell surfaces provided a more comfortable microenvironment for cells. From our research, we knew that cells encapsulated by FN-based multilayers exhibited extended morphologies similar to the normal cells (Fig.7b-f). The FN-based multilayers also exhibited thickness-independent cytotoxicity, with extremely high cell viability, while the viability of PE-encapsulated cells decreased quickly with the increase of nanofilm thickness (Fig.7g-h). Furthermore, cells encapsulated with PEM nanofilm exhibited strong inflammation, most likely because of the cationic cytotoxicity, with the result that cell death or growth stagnation was profoundly affected by the thickness, cationic species and cationic charge. Thus, LbL nanofilms provides an appropriate environment for the survival of cells, while appropriate selection of components toavoid cytotoxicity is the key to successful cell encapsulation. FN-based nanofilms constructed through the biological recognition driving force provide a more cytocompatible microenvironment than polyanion and polycation.[54] These LbL coating techniques present a new way to fabricate 3D multicellular architectures with high biological properties in the fields of regenerative medicine, pharmaceuticals and pathology.

Thus, in recent years, we have maintained our focus on the study of various functional 3D tissues using the cell-accumulation technique, such as 3D human colon epithelial tissue,[70] full-thickness human skin equivalents with blood and lymph-like capillary networks,[37] and 3D-pancreatic cancer tissues.[71] Similarly, inspired

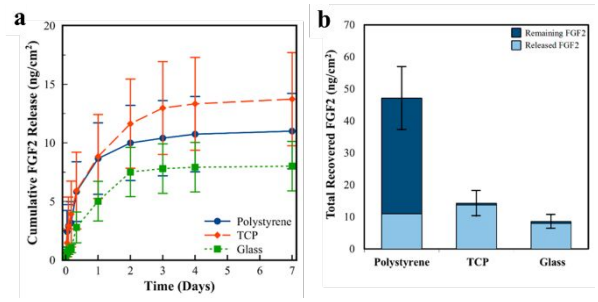

Fig. 8 (a) FGF2 release from PEM-coated polystyrene, TCP, and glass over 7 days. (b) Comparison of released and remaining FGF2 recovered after the release study and acid/base wash. The data are arranged to show the total FGF2 recovered from the system. In all cases, FGF2 was adsorbed to the substrate from a 100 µg/mL solution, and then the PEM was assembled on the FGF2-coated surface. Error bars represent the standard deviation (n=5).

by the structure of bacterial endospores that can survive under hostile conditions, Choi's group also fabricated "artificial spores" by encapsulating cells individually within biocompatible, thin and tough shells.[95,96] These 3D "cell-in-shell" structures enable the modulation and control of cellular metabolism, such as control of cell division, resistance to external stresses, and surface-functionality, providing a useful platform for cell communication and cell therapy. As with 2D cell culture, functions and metabolism are affected and controlled by the properties of artificial shells, for example thickness, stiffness and the biochemical properties.[8,96] Choi and co-workers encapsulated yeast cells with controllable thickness of silica shells by combining the LbL technique with a process of bioinspired silicification (Fig. 6d) and investigated the relevance of cell metabolic activity and shell thickness.[95] Their results showed that cell growth was not affected by the LbL silicification, but showed a shell-thickness dependency.

Additionally, the process of cellular therapy was largely limited due to the poor functional cell survival after cell transplantation.[97] Inspired by the effective protection of abovementioned nanocoatings encapsulated onto single cells against physical damage, transplanted cells coated with LbL nanofilm could also be protected against hostile surrounding environments *in vivo*.[97] Moreover, LbL nanocoatings can not only protect cells, but also maintain the volume of transplanted cells and enhance their mechanical properties[98]. Zhi *et al*. deposited biocompatible nanofilms around islet cells using the LbL assembly technique to maintain cell viability and normal insulin secretory function.[97] Moreover, nanocoatings could also be designed as carriers to deliver bioactive molecules or anti-inflammatory drugs avoiding instant blood-mediated inflammatory reaction post-transplantation. These results suggest that considering cells as building blocks in the fabrication of multicellular constructions is a very appealing strategy. Nanometer-sized artificial ECM films offer a similar extracellular microenvironment with native ECM to maintain and improve cell functions for constructing 3D tissue models *in vitro*. On the other hand, nanocoatings around cells also enhance the mechanical properties of transplanted cells, increasing the possibility of cell therapy in tissue engineering.

5. Tunable Growth Factor Release from LbL Nanofilm

Growth factors are well known for their specific ability to control cell proliferation, and differentiation.[38] However, the high concentration or rapid consumption of growth factors will result in inconsistent and even slowed cell proliferation rates.[99] To a certain degree, cell functions are greatly dependent on the sustained release and long-term storage of growth factors. Among the well-known nanofilms fabrication methods, building blocks of LbL assembly nanofilms enables the incorporation of many bioactive molecules, including growth factors under mild, non-denaturing, aqueous conditions.[23] Moreover, properties of LbL nanofilms can be adjusted easily for tunable growth factor release.[99]

5.1 Impacts of Assembly Condition on Growth Factor Release

Properties of nanofilms are dependent on many processing and environmental variables. Some reports have shown that the assembly pH is as important as the external pH in affecting the properties of LbL nanofilms.[100] Furthermore, different assembly pH, temperature and solution concentration result in varying stability of nanofilms and greatly influence the release of growth factors.[63,99,100] Another study showed that the chosen cell culture substrate also exhibited different influences. Plasma-treated polystyrene had the highest cumulative fibroblast growth factor (FGF2) release and glass had the lowest. The authors concluded that the first layer greatly affected the adsorption of subsequent layers resulting in different nanofilm structures that influenced the release of growth factors (Fig. 8).[99] Additionally, growth factor release not only relies on the diffusion, but can also be induced by the dynamic swelling of nanofilms. Thus, the location of growth factor,

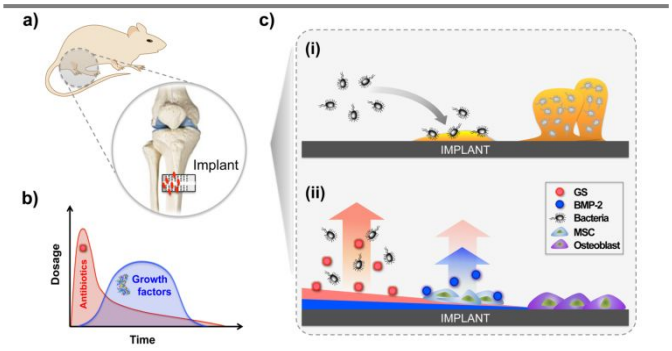

Fig. 9 (a) Illustration of rat tibia model with induced osteomyelitis. (b) Desired release profile of an antibiotic and a growth factor and illustration of the top-down degradation of a LbL coating on an orthopedic implant. (c) Possible scenarios following in vivo application (i) In an uncoated implant, the residual bacteria in the defect and avascular tissue act as foreign bodies and can cause reinfection and form biofilm (represented by the yellow area). (ii) In our dual therapy LbL coating, however, local delivery of an antibiotic (red circles) controls infection until the implant is vascularized and immune-competent. The subsequent release of a growth factor (blue circles) induces the osteogenic differentiation potential of endogenous precursor bone marrow stem cells, resulting in optimal bone healing and bone-implant integrity. Reproduced with permission from ref. 101. Copyright 2016 American Chemical Society.

determined by the thickness of nanofilms plays a significant role in their release. Hammond and co-workers constructed a degradable multilayered coating onto transplants which could sequentially release antibiotic and BMP-2 in a time-staggered manner (Fig.9).[101] Antibiotics loaded in the top layers of the coating could be modulated to release quickly for rapid elimination of infection, followed by sustained release of underlying BMP-2 for several weeks. The rodent model demonstrated quantifiable differences in intraosseous bacterial survival and bone remodeling for multiple weeks, and BMP-2 induced bone regenerated interface with host tissue 15 times more effectively than in an uncoated one. Hong *et al.* also investigated and compared the loading and release of bFGF from nanofilms composed of different repeating polycation/polyanion/bFGF structures.[102] Positively charged bFGF can serve as a ‘‘building block’’ for multilayers assembly, marked as tetralayer, whereas polycation directly adsorbed onto a polyanion layer with bFGF as loading agent is marked as trilayer. Results indicated that the amount of cumulative bFGF released from the trilayer film was 10 times greater than that from the tetralayer films.

5.2 Impacts of Nanofilm Architecture on Growth Factor Release

The architecture of LbL nanofilms, such as porosity, modulus, and crystallinity affect molecular pathways of the initially incorporated active molecules, presenting a different growth factors release kinetic.[65] Hong’s group discovered that only about 1/6 of basic fibroblast growth factor (bFGF) was released from heat-treated starch nanofilms within the first 24 h compared with untreated multilayers. Molecular rearrangement of starch was induced at high temperature, resulting in a narrowing of the molecular pathways of bFGF, which exhibited a linear erosion release profile over a long period. Kumorek[103] *et al.* indicated that crosslinking chemistry also affected the absorption and release of FGF2. With the increase of cross-linking, it becomes more difficult for films to swell, resulting in slow diffusion of growth factors.

Together, the controllable release and concentration of growth factors have a significant impact on desired cell functions. LbL assembly provides an efficient strategy for the prolonged storage of growth factors to maintain their activity and sustain their release by adjusting the multilayers’ parameters. However, a better understanding of the relationship between nanofilm parameters and growth factor release kinetics is still needed to guide the construction of an appropriate LbL nanofilm for affording an ideal growth factor release rate according to the dynamic cellular activity.

6. Control of Tissue Reaction by LbL Nanofilm for Regeneration Medicine

The LbL assembly approach has been demonstrated to be suitable for constructing nanofilms onto substrates or nanocoatings on cells to control cell functions because of its ability to easily regulate mechanical, biochemical and topographical properties. It also provides an available strategy to construct nanostructured coatings onto implants for tissue or organ repairs. In this section, we will briefly review recent and creative applications of multilayers for tissue engineering. Hammond and co-workers described 3D multilayered electrospun constructs for wound healing combined with cutting-edge electrospinning techniques and the LbL assembly technique.[56] An ultrathin coating of CS/HA onto a scaffold ensured the continuous release of active agents and provided a different swelling ratio of the functional surface avoiding cell adhesion for painless removal of surgical dressings. Compared with the 70±2% wound closure of uncoated constructs, dressings treated with the LbL ultrathin coating achieved 90±0.5% of wound closure. Meanwhile, they also created a degradable multilayered coating around implants using the LbL assembly technique which could sequentially release antibiotic and BMP-2 in a time-staggered manner from the nanocoating, firstly allowing for infection elimination and followed by complete, rapid bone tissue repair.[101] Hong[58] *et al*. also developed biomolecule-containing patchable and free-standing multilayered nanofilms using inkjet printing for human skin. This high-throughput assembly allowed for the fabrication of versatile desired shapes onto various substrates.

These examples demonstrate the significant potential of the LbL assembly technique for improving the functionality and bioactivity of implants, making it easy for the transplantation process of biomaterials and tissue repair. LbL assembly technology may also be adapted to produce analogues for other kinds of tissues, such as the pancreas and heart, improving the development of tissue engineering.

7. Conclusions

In this review, controllability of cell functions by LbL multilayered nanofilms through various strategies was discussed. The LbL assembly method is a well-established molecular-assembly technique. It has attracted much attention in tissue engineering, regenerative medicine and elsewhere, mainly due to its applicability to various materials, its simple and flexible manufacturing method and its ability to form highly tuned functional films.

In this review, we firstly compared and analyzed the various basic factors for the construction of LbL multilayers, including components, driving forces and especially new technologies for high throughput LbL assembly. Cell functions are inherently regulated and coordinated by ECM via mechanical and biological signals during all biological processes. Thus, ECM proteins, biocompatible polypeptides and polysaccharides are the major components as building blocks for LbL films used for the control of cell functions. Although the majority of reported LbL multilayers relied on electrostatic interaction biological recognition and supramolecular interaction have also attracted increasing attention in the fabrication of cell function controllable films. Meanwhile, liquid handling robots, inkjet printing and capillary flow all provide promising approaches for high throughput LbL assembly, which is very useful to fabricate a multilayers library for drug screening, and can also save biomaterials at the same time. Moreover, we systematically summarized the applications of LbL assembly to design and fabricate nanofilms on a substrate surface for 2D cell culture and nanocoating around cells, cell aggregates or implants for 3D tissue construction. In this review, cell adhesion, morphology, proliferation and differentiation were reported to be greatly affected by properties of the LbL multilayer, such as wettability, biological sites, stiffness, topological structure and so on, which are easily regulated by assembly approach, components and assembly conditions. Controllable release profiles of growth factors also

played an important role in cell proliferation and differentiation, which can be easily achieved by LbL assembly technique.

Remarkable advances in LbL nanofilms for controlling cell functions in tissue engineering have been made. However, some challenges still remain which require technological and methodological innovation. (i) Considering the value and variability of biomolecules, reducing material waste and improving deposition speed during the coating process still remain important. Combining with bioprinting for specific pattern at micro- or nanometer scale is likely to become a hot topic in the future. (ii) Despite the efforts made so far on advances in high throughput LbL assembly films for cell assay or drug screening, they are still in the early stages. Whether by capillary flow or printing, both rely on expensive instruments, skilled operators and strict requirements for sample and operating conditions. There are still significant technical challenges to be addressed for cell microarray to extend their applications and to be commercialized. Innovative methods and techniques with simple operation, wide applicability to materials and ability to accurately produce controllable homogenous films are required for further improvements. (iii) LbL assembly has led to considerable developments in the modulation of cell functions by adjusting the surface parameters of nanofilms. However, incorporation of several favorable biological factors in one LbL multilayer similar to natural ECM remains a major challenge. (iv) By adjusting the multilayer parameters, LbL assembly provides an efficient strategy for the prolonged storage of growth factors, allowing them to remain active and to sustain their release. However, a clear understanding of the relationship between nanofilm parameters and growth factor release kinetics is still needed to guide the construction of an appropriate LbL nanofilm for affording an ideal growth factor release rate according to the dynamic cellular activity.

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