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Advancing bone tissue engineering one layer at a time: a layer-by-layer assembly approach to 3D bone scaffold materials

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The layer-by-layer (LbL) assembly technique has shown excellent potential in tissue engineering applications. The technique is mainly based on electrostatic attraction and involves the sequential adsorption of oppositely charged electrolyte complexes onto a substrate, resulting in uniform single layers that can be rapidly deposited to form nanolayer films. LbL has attracted significant attention as a coating technique due to it being a convenient and affordable fabrication method capable of achieving a wide range of bio-material coatings while keeping the main biofunctionality of the substrate materials. One promising application is the use of nanolayer films fabricated by LbL assembly in the development of 3-dimensional (3D) bone scaffolds for bone repair and regeneration. Due to their versatility, nanoscale films offer an exciting opportunity for tailoring surface and bulk property modification of implants for osseous defect therapies. This review article discusses the state of the art of the LbL assembly technique, and the properties and functions of LbL-assembled films for engineered bone scaffold application, combination of multilayers for multifunctional coatings and recent advancements in the application of LbL assembly in bone tissue engineering. The recent decade has seen tremendous advances in the promising developments of LbL film systems and their impact on cell interaction and tissue repair. A deep understanding of the cell behaviour and biomaterial interaction for the further development of new generations of LbL films for tissue engineering are the most important targets for biomaterial research in the field. While there is still much to learn about the biological and physicochemical interactions at the interface of nano-surface coated scaffolds and biological systems, we provide a conceptual review to further progress in the LbL approach to 3D bone scaffold materials and inform the future of LbL development in bone tissue engineering.

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1 Introduction

Bone tissue has a complex, hierarchical, three dimensional (3D) porous structure and performs a range of unique mechanical, biological and chemical functions. Bone grafting material, the gold standard for the treatment of these injuries, has limitations relating to harvesting the tissues, lack of availability, etc. Thus, it is imperative to use synthetic bone grafting materials.^{1–3} The use of bone grafts and substitutes during orthopaedic surgery is increasing significantly, and the global market was valued at USD 2.98 billion in 2019 and is projected to reach USD 4.15 billion by 2026.⁴ Bone tissue engineering aims to replace injured bone tissue in order to restore functionality and promote healing. This engineering approach requires osteogenic cells, biochemical growth factors, and a porous, bioactive and biodegradable bone scaffold that is capable of accelerating bone formation and providing mechanical support during bone regeneration.^{5,6} The mechanical properties of an engineered bone scaffold should match those of



the host bone tissue, with appropriate strength to prevent failure and sufficient stiffness to avoid bone tissue resorption.^{7,8} Bone scaffolds should also have sufficient mechanical strength to withstand the hydrostatic pressures *in vivo* and to maintain the pore space required for cell and blood vessel in-growth.^{5,6,9,10} An interconnected, highly porous microarchitecture with suitable porosity (*i.e.*, 75–95 vol%) is a prominent feature in this hierarchy and plays an important role in cancellous bone scaffold design and for functional performance both *in vitro* and *in vivo*.¹¹ The degradation rate also must be closely controlled and matched with the growth rate of new bone (*e.g.* 9 months for spinal fusion scaffold or between 3 and 6 months for craniomaxillofacial applications) to gradually create space for new bone tissue formation and to eventually transfer the load to the new bone.^{6,12,13} By the time the injury site has regenerated, the scaffold should be completely degraded.¹⁴ The interface between the bone substitute material and the biological environment can trigger a wide variety of processes, from an initial inflammatory reaction to the ultimate bone tissue remodelling.¹⁵ The considerations for scaffold design are therefore numerous and complex, and furthermore are unique to individual sites of repair in patients.^{16,17}

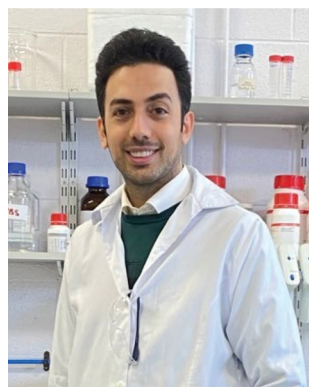
The application of surface modification treatments like using nanoscale coatings can enhance the performance of the scaffolds and improve the cellular responses for tissue repair.^{18,19} Nanoscale coatings offer an exciting opportunity for tailoring the surface and bulk property modification of bone tissue engineering scaffolds for specific applications.^{20,21} The biological properties, mechanical properties and degradation of bone scaffolds can be controlled by the application of nano- and microscale coatings using various deposition methods, such as atomic layer deposition, layer-by-layer (LbL) assembly, plasma spraying, electrophoretic deposition or sol-gel processes.^{22–25} These coatings can traverse material scales by affecting macroscale properties through structural features that extend to the nanoscale. In particular, the LbL assembly technique shows promise in providing an environment that resembles native bone by mimicking the structures and func-

tions of the native ECM, and promoting cell adhesion, proliferation, and migration.^{26–28} This technique has been shown to be a very simple, efficient, and versatile technique for surface modification in fabricating tissue engineering scaffolds.²⁹

Numerous studies have reported the use of LbL assembly to incorporate proteins, growth factors, polysaccharides, nucleic acids, and functional peptides into biomaterials, and to deposit coatings with controlled degradation rates.^{30–34} In addition, the physicochemical properties of multilayer films such as topography, stiffness and strength, and hydrophobicity can be optimized by altering the coating composition and processing parameters, which in turn enables control over biological cellular responses.^{35,36}

The bone tissue engineering field faces numerous challenges including difficulties of *in vitro* cell culture, mimicking osteogenesis in native tissue, the cost and complexity of manufacturing, and achieving biomaterial scaffolds that satisfy the diverse structural, mechanical and biological properties required for bone tissue engineering applications.^{37–39} Deposition of multifunctional coatings using LbL assembly is a potential route to addressing these challenges due to the low cost and simplicity of the technique, the fine control over composition and structure, the ability to achieve a tailored hierarchical structure and the potential for the high loading capability of bioactive molecules.²⁹ Furthermore, the fabrication of coatings using LbL assembly is of interest in the development of biocompatible implants for bone tissue repair and replacement applications.^{40,41} Over the last decade, research studies conducted on the LbL assembly technique applied to bone tissue engineering and bone regeneration have significantly increased by nearly five times in the number of publications, as shown by a keyword search on the ISI Web of Knowledge database of topic terms “layer-by-layer assembly for bone regeneration” that reveals an increase from 100 papers in 2010 to more than 500 papers in 2020.

Herein, a brief overview of LbL-assembled multifunctional films is presented with a focus on coatings with tailored mechanical and bulk properties, coatings on porous 3D templates, biocompatible coatings that promote cell and protein



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adhesion, biodegradable coatings, and coatings containing bioactive molecules and drug delivery systems. An overview of the LbL assembly fabrication technique is followed by a review of the properties and functionalities of LbL-assembled coatings that are relevant for producing 3D constructs that satisfy the diverse requirements for bone tissue engineering. Finally, a perspective on the broad potential afforded by combinations of LbL-assembled coatings in bone repair and bone tissue engineering is given.

2 Overview of the LbL assembly technique

2.1 Deposition process and mechanism

LbL assembly is a form of template-assisted manufacturing that is used to fabricate thin film coating layers and functionalize surfaces.^{42,43} The LbL technique is a simple and effective surface modification strategy.⁴⁴ The technique is based mainly on electrostatic attraction and involves the adsorption of oppositely charged electrolyte complexes onto a substrate, resulting in uniform single layers that can be sequentially deposited to form multilayer films.²⁹ In addition to electrostatic attraction, the creation of multilayer films can also be driven by covalent bonding, van der Waals forces, biological recognition and hydrophobic interactions, or a combination of these forces.⁴⁵ As illustrated in Fig. 1a, during a typical deposition sequence, a substrate is subjected to a negatively charged electrolyte (*e.g.* polyanion, or micro-/nanoparticles) and a negatively charged monolayer adsorbs onto the substrate surface. The substrate is next immersed in a positively charged electrolyte (*e.g.* polycation); the charge of the adsorbed cations is sufficient to neutralise the previous layer, invert the surface charge and create a positively charged monolayer on the surface. In order to remove unattached or weakly attached electrolytes and to prevent the intermingling of oppositely charged electrolytes, the substrate is usually rinsed with water or a suitable solvent between deposition steps.⁴⁶

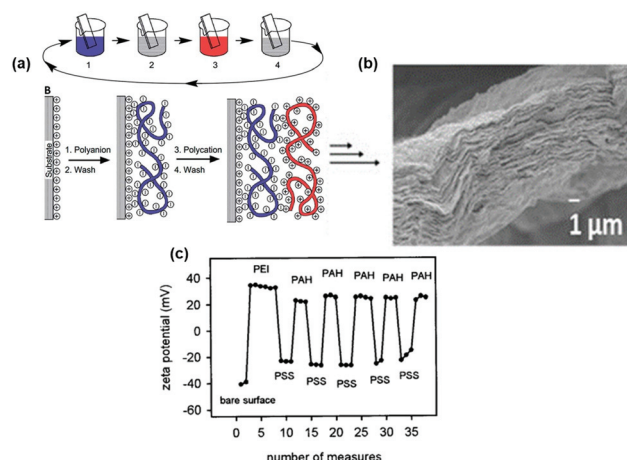


Fig. 1 (a) Schematic of LbL assembly using two oppositely charged polyelectrolytes where step 1 is a polyanion, step 2 and step 4 are water washes, and step 3 is a polycation. (b) Cross-sectional micrograph showing 60 quadrilayers of polyethyleneimine (PEI), polyacrylic acid, nanoclay (PEI/PAA/PEI/nanoclay) film fabricated with LbL assembly.⁴⁷ Reprinted with permission from *J. Mater. Chem.*, 16, 2006. Copyright 2006 Royal Society of Chemistry. (c) Typical change in zeta potential during the build-up of PSS/PAH bilayers using LbL assembly.⁴⁸ Reprinted with permission from *Langmuir*, 16, 2000. Copyright 2000 American Chemical Society.

As shown in Fig. 1a and b the deposition of electrolytes onto the surface does not result in the formation of 1 : 1 stoichiometric complexes, but rather there is an overcompensation of charge at the surface that allows deposition of further layers *via* electrostatic interactions.^{49,50} This process can be repeated until the desired thickness or material coating is obtained. Examples of a multilayer film fabricated in this way are shown in Fig. 1b. The LbL assembly process enables precise control (theoretically down to a single nanoscale layer) over the multilayered structure, composition, and thickness of the resulting films, as a wide range of species can be deposited in a pre-determined sequence.²¹ The potential of LbL assembly to deposit a wide range of functional constituents within multilayer-structured coatings makes the approach of LbL assembly



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a strong procedure for fabricating engineered tissue scaffolds that can be applied onto various porous template materials to achieve a wide range of properties, pore structures, and multifunctionality.⁵¹ The alternating change in surface charge after deposition of each layer is illustrated in Fig. 1c for a substrate coated with polycationic polystyrenesulfonate (PSS) and polyanionic polyallylamine (PAH) bilayers with an initial layer of PEI on a glass substrate.

2.2 Deposition methods

LbL-assembled films can be deposited by dipping a substrate into oppositely charged solutions, spraying solutions onto the substrate, or by spin-assisted LbL assembly, as illustrated in Fig. 2.^{52–54} There are advantages and disadvantages to all three methods of deposition. The traditional dipping assembly of multilayers is based on cyclic dipping of the substrate in solutions. It is the most studied and well-known and is a convenient and cost-effective technique. However, it can be time-consuming and can require long solution–substrate contact times.⁴⁰ The spray- and spin-assisted assembly methods were developed to accelerate the deposition process. In spray deposition, a multilayer film is created by spraying solutions perpendicular to the substrates and the removal of the excess solution is driven by gravity. The deposition process is faster, and the resulting films are smoother compared to the dipping method, however, films are often inhomogeneous due to incomplete wetting of the substrate edges. Spin-assisted deposition is also faster than the traditional dipping method, with the deposition of an individual layer taking seconds. This method can also be used with uncharged polymers, but the main limitation is that it is challenging to coat large areas or 3D structures.^{55,56}

Deposition of LbL multilayer films as coatings onto open-cell porous substrates for mechanical reinforcement, controlled porosity, or added functionality such as biocompatibility or bioactivity, is a potential route for addressing the design challenges associated with the currently available bone tissue scaffolds.^{58,59} The strategy is to use this simple, inexpensive and environmentally friendly method to produce a suitable

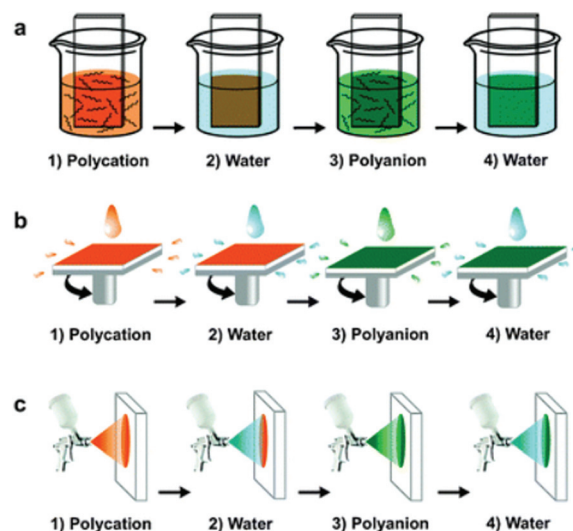


Fig. 2 Schematics of the LbL assembly fabrication process. From top: dipping method, spray-assisted method, and spin-assisted method.⁵⁷ Reprinted with permission from *Chem. Soc. Rev.*, 41, 2012. Copyright 2012 Royal Society of Chemistry.

coating for bone scaffolds with relevant characteristics including (a) high microscale thickness, (b) tailored mechanical properties, (c) controllable porosity, and (d) surface modification that allows for the desired physical, and (e) chemical and biological functionality.

The ability to fabricate LbL assembly coatings in a time- and cost-effective manner is a potential obstacle to the clinical translation of the technique. The processing times associated with LbL assembly are likely to be prohibitively slow for producing bone tissue scaffolds or customised porous materials for other applications. However, forced drying of specimens could significantly reduce the processing times to the required deposition time. Furthermore, the implementation of LbL assembly can be easily automated and improved for commercial production.^{60–62} Considerable work has focused on reducing the LbL assembly processing times by the incorporation of automated systems and faster deposition kinetics. For



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example, the utilisation of a rotating slide holder reduced the solution contact time up to 10-fold and facilitated the fabrication of coatings with increased thicknesses.⁶³ Electrophoretic deposition used to speed up the deposition of the LbL-assembled PEI/PAA/PEI/nanoclay coatings, resulted in nearly an order of magnitude increase in thickness (compared to coatings fabricated without an electrical current) and achieved controlled coating thicknesses in a much shorter time than the conventional LbL assembly dipping method.^{21,64}

2.3 Constituents and substrates

LbL assembly has been successfully implemented using a wide range of constituents including proteins, polymers, inorganic nanoparticles, clay nanosheets, organic dyes, dendrites, viruses and cells, nanotubes and nanofibers. These constituents can be deposited onto a wide range of substrates to form large area films and coatings on substrates of almost any shape.⁶⁵ Substrates reported in the literature include glass slides, silicon wafers, paper, quartz crystals, silicone rubber, titanium implants, 3D porous scaffolds, and biological cells.^{29,46,66} The thickness, roughness, and morphology of LbL-assembled films built from weak polyelectrolytes can be tailored through simple adjustments of assembly parameters such as the pH of solutions, salt concentration, the molecular weight of polyelectrolytes, time of rinsing, dipping times, and temperature.^{28,67,67,68} Due to this versatility, simplicity and variety of constituents, LbL assembly has been utilised in a wide-ranging number of biomedical applications, such as coating of implantable materials, tissue engineering, biosensors and drug delivery systems.^{41,52,69–71}

2.4 Film growth and thickness

The total thickness of a multilayer film is determined by the number of times a deposition cycle is repeated. Typically, there is a linear relationship between the number of deposition cycles and total thickness. The slope of this linear relationship is the average thickness of the repeating multilayer unit and the film build-up is usually proportional to the dimensions of the constituents deposited.^{57,68} Linear growth occurs when strong polyelectrolytes are used. Alternatively, some pairs of weak polyelectrolytes, such as poly-L-lysine (PLL)/polyglutamic acid (PGA) or polyacrylic acid (PAA)/PEI, exhibit a more rapid

“exponential” growth of thickness. The thickness increase of these exponential films is significantly faster compared to the linearly growing films and is not limited to the dimensions of the incorporated polymers or particles. The thickness and growth rate typically accelerate with the number of layers previously deposited and can reach several microns after fewer than 10 deposition cycles, whereas linearly growing films are typically on the order of several nanometres thick per deposition cycle.^{41,52,72}

In addition to the differences in growth and thickness, linearly and exponentially grown films exhibit different characteristics and properties. Typically, exponentially growing films are softer, have higher water content (up to 80% of volume) and are permeable to water, and large ions and molecules compared to films grown linearly.²⁹ Some exponentially grown polymer–nanoclay films (*e.g.* films consisting of PEI/PAA/PEI/montmorillonite (MTM) nanoclay) are unusual in that they exhibit a higher stiffness and hardness compared to films grown in a linear manner. This has been attributed to the dynamic structure of exponential films that is enabled by electrolyte mobility and allows polyelectrolytes to adjust and more closely interface with the nanoscale topography of clay sheets.^{73,74} The high modulus of exponentially grown films without nanoclays (PEI/PAA) has also been attributed to assembly conditions that result in highly cross-linked, interpenetrating polymer networks.^{44,75}

The exponential growth rate, permeability, stability and thickness per multilayer can be controlled and enhanced by altering the ionic strength or the pH of the polymer solutions.^{76,77} Adding salt to a weak polyelectrolyte results in film swelling, softening and smoothing as the amount of salt added is increased. By changing the pH of weak polyelectrolytes the charge on the functional groups is reduced and as a result, a higher growth rate and thicker films are achieved.^{73,78} The characteristics of films grown exponentially are contrasted with films grown linearly in Table 1. A key limitation of LbL assembly is that the nano- and microscale thicknesses, together with the slow rate of deposition,^{21,79} can restrict macroscale applications requiring thicker films.^{11,73,80} Fabrication of thick films using exponentially grown films can help increase the deposition rate, but typically remains limited to the order of ~100 μm total thickness in most practical cases.

Table 1 Exponential vs. linear growth

Film properties	Linear growth	Exponential growth	Ref.
Film thickness	Mass and thickness increase linearly as the number of deposited bilayers increases.	Thickness of dried films increased exponentially with the number of deposited layers	81
Polyelectrolytes during deposition	Usually observed in highly charged polyelectrolytes, or polyelectrolytes dissolved in solutions of low ionic strength	Caused by “in-and-out” diffusion of particular combinations of polyelectrolytes	68
Permeability	H ₂ O, small ions, small molecules	H ₂ O, large ions and large molecules	52
Mechanical properties	Elastic solids	Liquids or weak gels	52
Water content	Low (30% of volume)	High (up to 80% of volume)	
Ions from electrolyte	Very small	Usually high	52
Drug delivery	Limited	Can be loaded with biological molecules and act as reservoirs to deliver such molecules to cells	81



3 Properties and functions of LbL-assembled films for bone biomaterials

A fundamental factor in the design of an engineered bone scaffold is the choice of suitable materials, which depends on the specific scaffold application and plays a key role in the overall performance of the implant. The design and manufacture of multi-scale scaffold materials that satisfy the biological requirements (biocompatibility, biodegradability and promotion of cell and protein attachment) while combining the required mechanical properties and porosity is a key objective to their successful implementation in bone tissue engineering.^{6,27,51} A high level of interconnected porosity is required to stimulate bone growth into the scaffold, however, high porosity reduces the mechanical properties. One of the possible novel approaches to this ongoing challenge is to deposit a stiff and strong polymer-nanocomposite coating onto porous template scaffolds using LbL assembly.^{40,51,82} Deposition of nanocomposite coatings using LbL onto resorbable scaffolds would allow for a scaffold material with tailored biomechanical properties, which will be absorbed by the body over time.^{53,68,82,83}

Deposition of multifunctional thin films as coatings using LbL assembly for mechanical reinforcement, controlled porosity, and added functionality such as bioactivity is a potential route for addressing these complex design requirements. In this section, an overview of the properties and functions of LbL-assembled films are presented based on their architecture and performance with an emphasis on tailoring surface and bulk mechanical properties, adjusting biodegradability, achieving biocompatibility and the delivery of bioactive molecules and therapeutic drugs, and promoting cell and protein adhesion for bone tissue engineering applications.

3.1 Bulk mechanical properties

A successful bone scaffold should maintain mechanical stability under variable loading conditions while transferring sufficient force to newly formed tissue in order to stimulate

osteoblasts.^{11,84,85} In order to achieve this, it is widely accepted that the mechanical properties of a bone scaffold should ideally match that of the host bone tissue.⁸ Compared with conventionally fabricated composite coatings (e.g., *via in situ* polymerisation, melt intercalation, sintering, and vacuum evaporation)^{10,86–88} films prepared by LbL assembly have the advantages of improved mechanical properties owing to the capacity for high loadings of well-dispersed nanofillers, as well as the ability to tailor material structure and composition at the nano- and micro-scales and to easily add multifunctionality.^{57,73,89} Polymer nanocomposites with a hard, reinforcing phase of carbon nanotubes, carbon nanofibers, synthetic oxide nanoparticles, and natural nanoclays have been produced *via* LbL assembly. Several studies have investigated the utilisation of negatively charged MTM nanoclay for mechanical reinforcement of polymer films fabricated *via* LbL assembly.^{51,73,90}

Although a range of LbL-assembled films with good mechanical properties have been reported, the micro-scale thickness of these films is a challenge to macroscale applications requiring bulk materials, such as bone tissue scaffolds. The LbL assembly of conformal coatings deposited onto highly porous 3D templates can overcome this dimensional limitation and has been reported as a strategy for translating the mechanical properties of microscale coatings to the bulk scale.⁴⁷ Open cell porous foam templates are shown before and after coating with PEI/PAA/PEI/nanoclay quadlayers in Fig. 3a and b. As the thickness of the coatings increased, the bulk compressive modulus and density increased and the porosity of coated foams decreased. The compressive modulus is plotted as a function of density for these coated foams in Fig. 3c (black/white markers), and the predicted properties (dashed lines) were calculated using scaling laws for coated porous materials with varying coating thicknesses (increases along the dashed lines) and coating materials with varying elastic moduli (increases across the dashed lines). As shown in Fig. 3c, the predicted range of achievable moduli for these coated foams overlaps the range for cancellous bone.

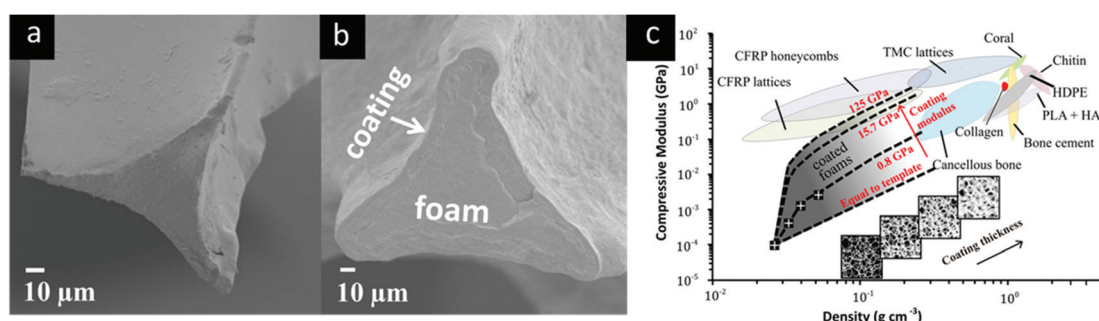


Fig. 3 (a) Cross-section of uncoated polyurethane foam strut and (b) nanocomposite-coated polyurethane foam strut⁴⁷ (c) Ashby plot of compressive modulus and density, including ranges for common engineering materials, bone scaffold materials, and cancellous bone. Experimental data (markers) and theoretical predictions (dashed lines) for nanocomposite-coated foams are included for coatings with elastic modulus ranging from 0.8 to 125 GPa.⁴⁷ Inserted images illustrate the change in foam architecture with increasing coating thickness. Reprinted with permission from ACS *Appl. Mater. Interfaces*, 8, 2016. Copyright 2016 American Chemical Society.



3.2 Local surface stiffness

Bone healing is highly sensitive to mechanical loading since mechanical stimulus is a critical regulator of bone structure and function.^{95,96} Many bone tissue engineering strategies rely upon seeding mesenchymal stem cells (MSCs), which have the potential to differentiate into various cell types.⁹⁷ MSCs are sensitive to the composition and structure of the materials onto which they are seeded.⁹⁸ The mechanical properties of 2D surfaces and 3D scaffolds are known to regulate the behaviour of attached cells,⁹⁹ influence the lineage specification of MSCs, and stimulate osteogenesis of MSCs both *in vitro* and *in vivo*.^{100,101} The stiffness of the microenvironment of pre-calcified collagenous bone tissue is above 30 kPa,¹⁰² and matrices with this approximate stiffness have been shown to be osteogenic. For example, Engler *et al.* demonstrated that MSCs can commit to osteoblasts when cultured on collagen-coated polyacrylamide (PAM) hydrogels with a stiffness of 25–40 kPa.¹⁰¹ Regulating differentiation of bone progenitor cells by mechanical stimuli and committing MSCs to an osteogenic lineage by using scaffold materials with a suitable stiffness *in vitro* has the potential to reduce the culture time required to pre-differentiate cells and eliminate the need for osteogenic induction supplements.^{3,103–105} The mechanical properties of the LbL-assembled films can be controlled in several ways: by tailoring

assembly conditions (*e.g.* pH, ionic strength, incorporation of reinforcing phase) or by using covalent crosslinking agents (*e.g.* glutaraldehyde (GA), genipin).^{51,106,107} The multilayered coatings are therefore a promising route for the regulation of MSC osteointegration *via* mechanical cues and studying cell behaviour on substrates with a wide range of mechanical properties achieved by simply varying the assembly conditions or the concentration of the crosslinkers. Examples of how the incorporation of the reinforcing phase results in mechanical reinforcement of multi-layered films and coatings are shown in Table 2. The remaining part of this section will focus on summarising how the cell behaviour can be controlled by modulating the assembly conditions or incorporating crosslinking methods.

3.2.1 Tailoring deposition conditions. The thickness, roughness, and morphology of LbL-assembled films can be tailored through simple adjustments of assembly parameters such as the pH of solutions, salt concentration, the molecular weight of polyelectrolytes, time of rinsing, frequency of intermittent drying steps, dipping times, and temperature.

Weak polyelectrolytes in an aqueous solution exhibit a pH-dependant average charge density per unit of functional groups along the polymer chain and thus the extent of interaction between charged polymers can be controlled, whereas in the strong polyelectrolytes the charge density is constant.

Table 2 Examples of mechanical reinforced LbL assembly films

LbL assembly components	Substrate	Mechanical properties	Thickness	Additional notes	Ref.
Polydiallyldimethylammonium chloride (PDAA)/MTM	Free-standing film	Strength 109 MPa Elastic modulus 13 GPa (200 bilayers)	4.9 μm for 200 bilayers	N/A	90
PVA/MTM (GA crosslinked)	Free-standing film	GA crosslinked: Strength 400 MPa Elastic modulus 125 GPa Non-crosslinked: Strength 150 MPa Elastic modulus 13 GPa	1.5 μm for 300 bilayers	GA crosslinking improved load transfer between polymer and nanoclay.	91
PEI/PAA/PEI/MTM	Glass slide	Elastic modulus 15.7 GPa (100 quadlayers)	100 μm for 100 quadlayers	Exhibited exponential growth during LbL assembly.	92
PEI/PAA/PEI/MTM covalently cross-linked	Free-standing film	Elastic modulus 45 GPa (20 quadlayers), 25 GPa (100 quadlayers)	920 nm for 10 quadlayers	Electrophoretic deposition used to enhance deposition rate during LbL assembly.	64
PEI/PAA/PEI/single wall carbon nanotubes (SWNT)	Free-standing film	Elastic modulus 15 GPa for 8 quadlayers	1 μm for 8 quadlayers	N/A	93
PDAA/MTM	Silicon wafers	Elastic modulus 9.5 GPa	528 nm for 100 bilayers	N/A	94
PEI/PAA ionically cross-linked	Porous stainless steel	Elastic modulus 23.4 GPa for 10 bilayers	1 μm for 10 bilayers	High stiffness was attributed to alternating high/low pH of the solutions, which resulted in high ionic cross-linking and inter-diffusion of PEI and PAA.	75
PEI/PAA	Free-standing film	Elastic modulus 0.225 GPa for 40 bilayers	N/A	N/A	93
PEI/PAA/PEI/nanoclay	Silicone substrate or free-standing film	Elastic modulus 0.601 $\pm$ 0.518 GPa (flexible substrate), 1.667 $\pm$ 1.057 GPa (free-standing) for 60 quadlayers	16.07 for 60 quadlayers	N/A	47



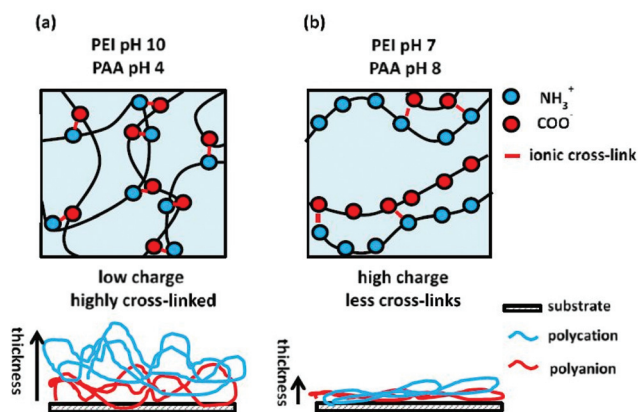


Fig. 4 Schematic representations of the interactions between PEI and PAA at pH that allows for (a) high degree of ionisation and (b) low degree of ionisation in both polyelectrolytes. Below, the film thickness affected by a change in pH is shown.⁷⁵ Reprinted with permission from *Adv. Mater.* 2014.²⁶ Copyright 2014 Advanced Materials.

Yang *et al.* reported the thickness of 30 bilayers of PEI/PAA bilayers to be 4740, 890, 350 or 90 nm depending on the combinations of pH values of solutions (10/4, 8/6, 4/4 and 7/7, respectively for PEI/PAA).^{108,109} Kim *et al.* reported that the thickest PAA/PEI films with the highest elastic modulus were assembled when the pH of PAA was 4, and the pH of PEI was 10, after considering pH values of 6, 8 and 10 (Fig. 4). The 10 bilayers of PEI/PAA were 1 μm thick and had an elastic modulus of 23.4 GPa.⁷⁵

The influence of the salt ions on the structural properties of polyelectrolyte multilayers has been reported previously, although there is a discrepancy between the results. The thickness of PAH/PAA films increased with an increase in the concentration of NaCl in an aqueous solution of PAH and PAA.¹¹⁰ In PEI/nanoclay bilayers, increasing salt concentrations (1 mM, 10 mM, 100 mM, 1 M) in the PEI solution with a pH of 8 also resulted in the formation of thicker films, whereas adding salt to unadjusted PEI (pH 10) and clay solution (pH 10.5) did not affect the thickness.¹⁰⁹ However, Mjahed *et al.* observed that when the salt concentration was above 0.48 M, PLL/HA film thickness and mass decreased.¹¹¹ A similar effect was observed in PAH/PAA films where the thickness decreased with an increased salt concentration.¹¹⁰

3.2.2 Addition of reinforcing layers. The stiffness of LbL coatings can also be increased through the deposition of a high stiffness reinforcing layer. Biocompatible MTM nanoclay platelets have been successfully incorporated as mechanical reinforcement in PDDA/MTM bilayer coatings and applied onto PAM hydrogels to increase surface roughness and stiffness.¹¹² The compressive modulus of the hydrogel coated with 10 bilayers of PDDA/MTM increased to 190 kPa, resulting in a cell adhesive surface that promoted epithelial cell adhesion and cell migration into the scaffold.⁹¹ Various open cell polyurethane foams have been used as models for cancellous bone. Polyurethane foam has a similar morphology to cancellous bone and many bone tissue scaffold like materials.

Because of this, polyurethane foam was used as a template to examine the possibilities of tailoring the mechanical properties of highly porous bone tissue scaffolds by the coating LbL process. The LbL assembly of conformal coatings deposited onto porous 3D templates is another example of this strategy using stiff coatings to reinforce softer substrates. Uncoated open-cell polyurethane foam samples with a compressive elastic modulus of 95.33 ± 9.8 kPa increased by up to 1100% to 882 ± 178.1 kPa after deposition of 15 PEI/PAA/PEI/nanoclay multilayers.^{51,113}

3.2.3 Crosslinking. Crosslinking provides a useful way to modify the mechanical properties of LbL coated substrates. Various chemical (e.g. EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and genipin) and physical (e.g. photo-crosslinking) crosslinking techniques have been employed to improve the stiffness of LbL-assembled films. EDC is a water-based crosslinker that can be applied after the assembly of polyelectrolytes containing amine and/or carboxyl groups, and leads to the formation of amide cross-linking bonds.^{106,107} A study by Ren *et al.* showed that the stiffness of PLL/hyaluronic acid coatings on glass slide substrates was directly proportional to the concentration of EDC used in post-assembly crosslinking. The adhesion of skeletal muscle cells (C2C12) increased as the stiffness increased. Cell differentiation was also found to be dependent on the film stiffness with the stiffest films (>320 kPa) promoting cell proliferation.¹¹⁴ In another study of EDC crosslinking of PLL/hyaluronic acid multilayers, a range of stiffness gradients from 200 to 600 kPa was reported.¹¹⁵ PAA/PAH films have also been crosslinked using EDC, exhibiting stiffness values ranging from 0.5 to 100 MPa.¹¹⁶ In both of these PLL/hyaluronic acid and PAA/PAH material systems, the cells preferentially attached to the stiffer sections of the film and exhibited increased proliferation and cell spreading.¹¹⁷

LbL-assembled PVA/MTM films crosslinked with GA displayed a brick-and-mortar arrangement of organic and inorganic constituents resembling those found in nacre and bone, and exhibited exceptionally high stiffness and strength (tensile modulus of up to 125 GPa and tensile strength up to 480 MPa). Such a highly controlled, layered structure is a unique advantage of the LbL assembly method.^{90,118} The mechanical properties of the selected films deposited by LbL assembly with and without inorganic reinforcing phases are summarised in Table 2, and demonstrate that several LbL-assembled material systems exhibit mechanical properties comparable to cortical bone (elastic modulus in the range of 7 to 30 GPa (ref. 13) and compressive strength of 100–230 MPa).

Genipin is a natural crosslinking agent derived from the gardenia fruit that has been used to modify the stiffness of bilayers of chondroitin sulfate A (CSA) and PLL deposited onto glass substrates. The resulting crosslinked films had elastic modulus between 170 and 280 kPa, a relevant range for the control of cells, which are capable of detecting small variations in stiffness.¹¹⁹ The LbL assembly films were biocompatible and promoted adhesion, proliferation, and early and late osteogenic differentiation of osteoblast precursor (MC3T3-E1) cells.



Because the stiffness and nano-topographical characteristics of the films influence physiological processes in cell behaviour such as adhesion and proliferation. Also, the stiffness and topography of the films were controlled by the amount of the cross-linking agent.^{83,117,119} The response of local surface stiffness to the cell state has significant implications for cell adhesion and differentiation as well as for tissue repair and regeneration. These results demonstrated the osseointegration of engineered bone substitutes and created multifunctional films capable of finely controlling the morphology and, in turn, influencing the subsequent behaviour of cells.^{120,121} Overall, the results show that with an increase in substrate stiffness controlled by cross-linking agents, the cells usually exhibit enhanced cell adhesion, enlarged cell spreading with defined actin organization, increased cellular contractility, decreased migration speed, and increased proliferation.^{117,122,123}

In a similar approach, photo-crosslinking of PAA/PAH films was achieved by grafting PAH with a photosensitive benzophenone. LbL-assembled films with a range of uniform stiffness and stiffness gradients were fabricated by varying the time of ultraviolet light exposure and by utilization of gradient density filters which regulated UV light exposure. Films with a stiffness gradient ranging from 55 to 140 MPa were obtained with both steep and shallow gradient slopes. Osteoblast-like cells (U-2 OS) spread and adhered better to the stiffer regions of the films.¹²⁴

3.3 Biodegradation

Biodegradation of scaffolds for bone tissue engineering applications should be tailored to achieve a gradual transfer of mechanical loads to the newly formed and surrounding bone tissue.^{13,127,128} In cell-seeded scaffolds, the degradation rate should be adjusted to avoid high levels of scaffold degradation during the initial incubation stage *in vitro*. The degradation rate of LbL-assembled films can be controlled depending on the type of polyelectrolytes used.^{27,129} Multi-layered films fabricated with natural polyelectrolytes such as polysaccharides can be degraded by various enzymes like lysozyme and the degradation rate can be tailored *via* the degree of crosslinking. Schneider *et al.* demonstrated that *in vitro* biodegradation of chitosan (CH)/hyaluronic acid and PLL/hyaluronic acid films in contact with hyaluronidase and macrophages can be controlled by altering the concentration of EDC during cross-linking treatments.¹²⁹ Compared to untreated films, cross-linked films had increased surface roughness, were 10-fold stiffer, and exhibited a slower degradation rate. Garza *et al.* demonstrated that the degradation rate could also be controlled *via* the thickness of biodegradable layers. The films were composed of exponentially growing PLL/hyaluronic acid bilayers that acted as reservoir layers and were alternated with biodegradable PLGA barrier layers that were incorporated by spray-casting. Films composed of two PLL/hyaluronan bilayers and one PLGA barrier were rapidly degraded by murine bone marrow cells (within 1 h).¹³⁰

LbL-assembled coatings can also be used to tailor the degradation rate of the underlying substrate material when de-

posited onto a biodegradable substrate.^{61,131} Magnesium is biodegradable, osteoconductive, biocompatible, and has an elastic modulus and compressive strength similar to that of bone and thus is an attractive material for the fabrication of implants and scaffolds for bone repair applications. However, its rapid degradation rate causes the release of high volumes of hydrogen, magnesium and other ions, and the limited bioactivity of magnesium implants *in vivo* limits their widespread applications.¹³² Surface modification of magnesium alloys by metal-organic framework-chitosan coatings has recently been presented as a new approach to control the degradation rate of magnesium implants for bone regeneration.¹³³ PLL/alginate multilayer coatings with fibronectin immobilised *via* EDC crosslinking have been deposited onto magnesium substrates in an effort to increase bioactivity. The coating reduced cytotoxicity in MC3T3-E1 osteoblast cell lines and an *in vitro* biodegradation study concluded that coated substrates were more resistant to corrosion, however, it did not alter the degradation kinetics of the magnesium alloy substrates.¹³⁴ LbL-assembled coatings have also been deposited onto AZ91D¹³⁵ and WE43¹³⁶ magnesium alloys for improved corrosion resistance. In both studies, the LbL-assembled coating temporarily reduced the hydrogen evolution of the magnesium substrates and enhanced the corrosion resistance and tailored the degradation rate.

3.4 Electroactive coatings

In addition to mechanical stimuli, the differentiation of MSCs along an osteogenic route can also be achieved with electrical stimulation, which has been reported to determine cell behaviour and help induce bone healing.¹³⁷ Pulsed electromagnetic fields, capacitive coupling, and direct current have each been used to promote bone regeneration.^{138,139} Several electroactive components (*e.g.* metal, carbon nanotube, graphene and conductive polymers) have been utilised in LbL assembly to fabricate functional coatings that can deliver such electrical stimuli.¹⁴⁰ Multilayer materials produced by the LbL assembly of PEI and the conductive polymer polyammonium-3-thienylethoxypropanesulfonate (STP) have been reported to improve osteoblast adhesion and proliferation and promote osteogenesis.¹⁴¹ The conducting polymeric system was electroactive, fully erodible, and suitable for adhesion and growth of cells derived from mouse fibroblast (L-929) and skeletal muscle (C2C12).¹⁴² Electroactive tetraaniline and degradable multilayered films consisting of PGA-graft-tetraaniline and PLL-graft-tetraaniline have been reported to support osteoblast function and promote osteogenic differentiation. Using this material system, the electrical stimulation of mouse-derived pre-osteoblast MC3T3-E1 cells *in vitro* resulted in increased expression of osteopontin and runt-related transcription factor 2 (RUNX2), which are markers of osteogenesis.^{143,144}

3.5 Cell interactions and protein adhesion

The surface properties of implants influence tissue and cellular events, including protein adsorption and cell behaviour, both of which are important to bone tissue remodelling.^{145,146}



For this reason, considerable efforts have been devoted towards the surface modification of bone scaffolds for added functionality and the creation of a suitable environment for cell growth and tissue formation.^{38,147} LbL-assembled multilayer coatings are a promising tool for controlling the attachment behaviour of cells onto surfaces because of the importance of surface properties in cell function.¹³⁰ Recently, significant efforts have been devoted to optimizing the surface properties of LbL assembly films, such as surface bioactivity, roughness, hydrophilicity, *etc.*¹⁴⁸ In addition, polyelectrolyte multilayer films have been shown to be a powerful tool for the immobilization of biomolecules with preserved bioactivity.

The electrostatic charge of the final layer at the surface of a multilayer film can have a dominant effect on cell attachment; cells may prefer positively or negatively charged terminating layers depending on the cell line.¹⁴⁹ Because the ability of the LbL deposition technique in 3D mimetic architectures will be an original means of controlling supra-cellular organization, the reciprocal interactions between active cells and active polyelectrolyte multilayer surfaces offer enormous potential to control cell behaviour.³⁸ Tryoen-Toth *et al.* observed higher viability and adhesion of SaOS-2 osteoblast-like cells when PEI-(PSS/PAH) coatings on glass slides were terminated with PAH, PGA and PLL layers, whereas the number of attached cells decreased on PSS and PEI terminating layers. A significant downregulation of alkaline phosphatase (ALP, a phenotypic marker for the early stage biomineralisation) was observed in SaOS-2 cells seeded on PEI-terminated layers after 24 h of contact, whereas only a small downregulation was measured for SaOS-2 cells in contact with negatively charged PSS- or PGA-terminated films. Osteocalcin expression (a marker for the mature osteoblast phenotype) in SaOS-2 cells at 24 h was not influenced by any of the polyelectrolyte films. This study concluded that the electrostatic charge of the outer layer of the LbL-assembled films played a major role in early osteoblast phenotype regulation. The LbL-assembled films terminated with PSS, PGA and PLL layers showed good biocompatibility towards SaOS-2 cells and were deemed as biologically inert for bone tissue engineering applications.¹⁵⁰

The several concepts for adjusting the chemical, physical, and mechanical properties of PEM films have fostered studies on the influence of these factors on cellular interactions. Adhesion of cells to the scaffold material is an important prerequisite for the successful colonisation of engineered bone substitutes.^{38,151} To improve the adhesion of pre-osteoblast MC3T3-E1 cells onto nanofibre poly-L-lactic acid (PLLA) scaffolds Liu *et al.* coated the scaffold surface with gelatin and poly(diallyldimethylammonium chloride) (PDAC). The amount of gelatin was controlled *via* the number of layers deposited, and the wettability of the scaffold was controlled by changing the final layer. The number of MC3T3-E1 cells on coated scaffolds was significantly higher compared to uncoated scaffolds at 4 h and 24 h time points after cell seeding, and the number of cells increased as the number of PDAC/gelatin bilayers increased. Furthermore, the cells proliferated at a higher rate and were more evenly distributed on coated scaffolds compared to the uncoated scaffolds.¹⁵²

Recent research has significantly broadened the understanding of the effects of nanocoatings in controlling cell behaviour because the 3D nanostructured surfaces can directly affect intracellular signalling pathways.^{83,89,153} The LbL assembly of layers or sheets of cells may be utilized to fabricate scaffolds with cell colonization and proliferation extending throughout the scaffold. LbL cell sheets developed for bone regeneration based on interactions between hydroxyapatite (HA) microparticles and pre-osteoblast MC3T3-E1 cells were fabricated by Hu *et al.* The LbL-assembled film mimicked the natural combination of the inorganic/organic bone matrix. The morphology of the cells in deeper layers was comparable to that of the top layer. The pre-osteoblast cells within the film maintained high osteogenic capacity.¹⁵⁴ Therefore, the LbL assembly could be a suitable tool to incorporate cells directly into the porous scaffolds for homogeneous cell distribution and colonisation inside the bone scaffold.

Having robust protein adhesion and absorption into the surface of biomaterial plays a crucial role in stronger cell adhesion for tissue regeneration. Protein adhesion is necessary for the formation of a bio-interface between implanted materials and native bone.^{155,156} Adsorption of proteins onto LbL-assembled films has been studied widely, and a kinetic model of protein adsorption has been described.^{157,158} PLL/DNA bilayers have been used to enhance protein adsorption and biomimetic mineralisation on the surface of titanium implants for osteoblast cell culture experiments. PLL is a positively charged polyelectrolyte that is biocompatible and can be easily conjugated with bioactive molecules. DNA has also been used for LbL assembly with layers of PEI, PDAA and PAH.^{159–161} The high-phosphate content in DNA was found to be beneficial for the deposition of calcium during bone formation due to the high affinity for calcium ions. The LbL assembly of PLL and DNA has also been suggested as a way to improve the bioactivity of titanium for bone tissue repair applications. *In vitro* investigation has confirmed that osteoblasts isolated from neonatal rat calvarias adhered to coated titanium surfaces and resulted in improved cell/cell and cell/substrate interactions compared to uncoated titanium control samples. Surfaces coated with PLL/DNA/PLL showed a higher number of overlapping and interconnected osteoblasts. The surface roughness of PLL/DNA coatings depended on the terminating layer and was higher with PLL as the last layer. Cell viability, assessed with the Alamar blue assay, demonstrated that tailored surface roughness improved the biocompatibility of titanium implants and cell adhesion.¹⁵⁶

To attract osteoblasts, titanium implants have been coated with calcium phosphate (CaP)-containing films. CaPs are bioactive and possess well-known osteoinductive properties that stimulate bone formation.¹⁶² Incorporation of biocompatible and antibacterial chitosan (CH) into the multifunctional LbL-assembled film prevented peri-implant infection, and HA particles induced bone mineralisation. The amount of mineralisation increased with increasing numbers of CH/HA bilayers. The coatings were biocompatible with pre-osteoblast MC3T3 cells and effective against infection by *Streptococcus gordonii*.¹⁶³



3.6 Drug and growth factor delivery

The functionality of bone scaffold materials can be enhanced by loading biomolecules (*i.e.*, growth factors and/or drugs) to treat bone disorders or promote bone regeneration by stimulating cell adhesion, proliferation and differentiation.^{164–166} Incorporated drugs should act at the desired location, at an adequate concentration and for a specific period of time.¹⁶⁷ Additional surface modification using the LbL approach allowed the fabrication of effective drug delivery carriers with improved targeting effects. LbL-assembled films can help achieve these requirements by providing spatial confinement of the drug, localised delivery to the tissue, and protection from exposure to physiological media.^{26,29,61}

Controlling the number of growth factors delivered to healing sites can help to maximise their efficacy and avoid side effects such as osteolysis, immunological reaction, and tumorigenesis (all of which are associated with the supraphysiological dosage of bone morphogenetic proteins (BMPs)).^{168,169} Growth factors can be deposited as layers within a multilayer film using electrostatic LbL assembly, or they can be loaded after LbL assembly in crosslinked films that serve as reservoirs for the proteins.^{170,171} Poly- β -aminoester 2 and PAA coatings have been utilised as delivery systems with tunable control over the delivery of growth factors including vascular endothelial growth factor (VEGF) and BMP-2. BMP-2, an osteoinductive agent, has been widely used to enhance the bioactivity of engineered bone scaffolds or implants and is known to stimulate the proliferation, migration and differentiation of MSCs. In a study by Shah *et al.*, LbL-assembled coatings of poly- β -aminoester 2-PAA/BMP-2/PAA and poly- β -aminoester 2-PAA/VEGF/PAA were deposited onto 3D osteoconductive scaffolds of macroporous PCL/ β -tricalcium phosphate (TCP). The delivery of the two growth factors was triggered by a change in pH from 5.0 to 7.4, causing destabilisation and charge imbalance in the coating, and from hydrolytic degradation of the poly- β -aminoester 2. BMP-2 eluted from the coating over 2 weeks, whereas VEGF was released within the first 8 days. Both growth factors retained their efficacy and induced ectopic bone formation in the intramuscular region of a rat femur.¹⁶⁸ Different studies have delivered BMP-2 through the use of various alternative LbL-assembled material systems, including employing a chemically crosslinked PLL/hyaluronic acid film deposited onto TCP/HA porous ceramics to deliver rhBMP (recombinant BMP-2);¹⁷² poly- β -aminoester 2/BMP-2 deposited onto 3D printed β -TCP/PCL;¹⁷³ and graphene oxide deposited onto titanium implants, which were evaluated *in vivo* in a mouse calvarial defect model.¹⁷⁴ Deposition of BMP-containing LbL-assembled films onto a substrate allows for a local administration and controlled delivery;¹⁶⁹ and has the potential to mitigate the main limitation associated with the current method for clinical delivery of BMP-2 through collagen sponge, *i.e.* rapid release without spatial control.^{172,175}

The application of the LbL assembly technique to deposit antibiotic coatings onto orthopaedic implants has the poten-

tial to reduce implant-related infections and accelerate osteogenesis.^{176,177} The main advantages of using such a technological approach are its relatively simple procedure and requirement of no special equipment when compared to other coating methods.^{41,178} Recent studies have focussed on the application of LbL assembly to coat titanium implants in an effort to prevent implant-related infection and increase the bone cell interactions at the implant surface. Multilayered hyaluronic acid-dopamine/CH deposited onto the surface of titanium alloy implants (Ti-Nb-Zr) have shown improved anti-infection properties, as well as accelerated osteoblast cell adhesion and differentiation when compared to non-coated titanium implants.¹⁷⁹ Tannic acid-alendronate (TA-ALN) nano-complexes have also been applied using the LbL assembly technique in order to augment the antioxidant, anti-inflammatory and osteogenic potentials. In this study also the final result showed that the TA-ALNs have great potential for anti-inflammation, and osteogenic acceleration and can be used to bone repair and regeneration in bone defects. Therefore, using LbL assembly or self-assembly could have significant potential in controlling antioxidant, anti-microbial, and osteogenic potency.¹⁸⁰

LbL-assembled films of poly- β -amino ester/PEI/gentamicin sulfate were deposited onto titanium implants to treat an existing infection in a rabbit bone model. The coating released 70% of the gentamicin *in vitro* during the initial three days through diffusion and film erosion because of the hydrolytic degradation of the poly- β -amino ester when placed in an aqueous environment, and continued to release the gentamicin over the next four weeks. The degradation products were not cytotoxic towards pre-osteoblast MC3T3-E1 cells. *In vivo*, the coated 3D titanium implants significantly decreased the viable bacterial count compared to the uncoated implant in a simulated one-stage re-implantation model to treat implant-related *Staphylococcus aureus* infection in rabbits.¹⁸¹

Systems for delivering both antibiotics and growth factors were combined by coating the surface of silicon substrates with a poly- β -aminoester 1 and 2, PAA loaded with gentamicin, bone growth factor BMP-2 with PDDA/chitosan and clay barrier layers for compartmentalised hybrid coatings with controlled release. The multidrug coating aimed to accelerate the bone healing process while preventing implant failure due to post-operative infection. The clay barrier blocked interlayer diffusion, leading to a 10-fold increase in the release rate of the growth factor and enabling customized release behaviour. These tunable LbL assembly coatings were implemented on planar silicon, but can potentially be translated into a variety of implants with complex geometries, including scaffold materials.¹⁸²

Another example of a dual system for the delivery of a cell adhesion peptide and growth factors was reported by Wang *et al.* Negatively charged oxidised alginate (OAlg) was deposited with positively charged chitosan (CH) and bovine-serum albumin (BSA)-based nanoparticles were loaded with BMP-2, resulting in positively charged BSA-BMP-2 nanoparticles. These nanoparticles were incorporated into films *via* LbL



assembly together with negatively charged peptide layers. The cell adhesion and proliferation properties of peptide-glycine-arginine-glycine-aspartate-serine (GRGDS) grafted OAlg/CH films were also explored. When deposited onto porous titanium scaffolds, GRGD and BMP-2 provided a favourable micro-environment for bone tissue ingrowth. The resulting coatings achieved sustained release of BMP-2 over 28 days. This long release period was attributed to bovine-serum albumin, which impeded the diffusion of BMPs. Both the biomolecules, BMP and GRGDS, promoted MSC attachment, proliferation and differentiation, and enhanced bone tissue formation.²⁸

Mertz *et al.* fabricated nanoscale barriers by alternately depositing linearly growing PDDA/PSS and exponentially growing PLL/hyaluronic acid. The barriers acted as mechanically active nanovalves: when a mechanical stimulus was applied, nanopores in the exponentially growing film opened and allowed the diffusion of PLL through the barrier. The opening and closing of the pores were reversible and could be controlled by the magnitude of mechanical deformation.⁸¹ Drug release can also be stimulated by pH and salt concentration. For example, PAA/PAH bilayers assembled at a pH of 2.5 have been loaded with methylene blue dye (as a model drug) within the permeable structure of the PAA. The dye was anchored to the PAA by interactions with the carboxylate groups, which were disrupted by a lower pH (2.5) in the environment, thereby triggering the release of the dye.¹⁸³

The addition of exosomes and osteogenic growth factors to LbL-assembled films has also been investigated for bone tissue regeneration applications. In a recent research study, Cheng *et al.* developed a core-shell silk fibroin (SF)/polycaprolactone (PCL)/polyvinyl alcohol (PVA) nanofibrous film using co-axial electrospinning and LbL assembly as part of a dual growth factor-release system. BMP2 was incorporated within the nanofibers and the connective tissue growth factor (CTGF) was attached to the surface by a LbL-assembled film. The combination of electrospinning and LbL approaches provided an effective platform for BMP-2 and CTGF delivery, and *in vitro* and *in vivo* studies showed improved angiogenesis and osteogenesis.¹⁷⁰ The LbL-assembled film fabricated from polycationic poly L-lysine (PLL) and glycosaminoglycan hyaluronic acid (HA) has also been shown to mimic the interactions between exosomes, cells, and the ECM, which dramatically increased the affinity to cells from different lineages.¹⁸⁴ Therefore, according to the recent studies, the incorporation of functional exosomes and osteogenic growth factors into LbL-assembled films is a promising approach to better mimic the host structure, improve the delivery of growth factors and augment biological functions.¹⁸⁵

3.7 Hierarchical natural structure in micro and nanoscale environments

Hierarchical nano- and micro-scale architecture and arrangement play important roles in cell performance and it has been postulated that bone scaffolds should be designed to mimic the hierarchical structure of the targeted bone.¹⁸⁷ Scaffold architecture plays a vital role in guiding cell interactions *via*

direct contact. An interconnected porous architecture is a crucial requirement for a functional bone tissue scaffold to promote vascularization, enable cell migration and facilitate the supply of nutrients and removal of waste products.^{188–190} The use of the LbL assembly technique to apply coatings on porous foam substrates has been successfully demonstrated. The LbL assembly of nanocomposite-coated foams shown in Fig. 4 resulted in materials with porosity ranging from 98.8% for uncoated foams to 96.6% for foams with the thickest coatings applied.^{47,51} In a similar approach, the LbL assembly of coatings onto colloidal crystals and subsequent removal of the colloidal crystal has been utilised to produce inverted colloidal crystal (ICC) multilayer materials. The LbL assembly of ICCs has been used to fabricate porous cellular environments targeting various tissues other than bone (*i.e.* connective tissue,¹⁹¹ cartilage,¹⁹² bone marrow¹⁹³).

It is well proven that microscale structures ensure the primary attachment in the bio-interface of the scaffolds and promote cell interaction while nanoscale organization has more significant effects on the adhesion and differentiation of cells.^{194–196} Bone consists of a mineral phase in the form of apatite nanocrystals embedded in a collagen matrix.¹⁹⁷ The nanometre-sized HA crystals play a key role in the high mechanical properties exhibited by bone.¹⁹⁸ Nanostructured surfaces have widely been reported to promote osteoblast adhesion and cell growth has been enhanced by the introduction of a randomly distributed nano-topographic surface at a scale of approximately 10 nm.^{199,200} Cell behaviour can also be influenced by nano- and microscale interactions with ECM components since stem cells are sensitive to the topographical features of the ECM.^{201,202} The ECM spans several length scales, is composed of fibronectin, collagen and laminin, acts as a storage of growth factors and cytokines, provides complex biochemical and physical signals, and induces cell-cell and cell-matrix interactions.^{186,203} Hence new strategies in bone tissue engineering include bioinspired 3D printed bone scaffolds with nano- and microscale features mimicking ECM structures.²⁰⁴ Nanostructured bone tissue scaffolds fabricated with nanoparticles, nanofibers, and hydrogels aim to mimic the ECM and provide structural support similar to that of the bone tissue architecture. However, combining current bone scaffold fabrication techniques with nanomaterials is challenging.^{203,205} Due to their large specific surface area, HA nanocrystals tend to agglomerate in polymer matrices, which differs from the homogeneous dispersion of HA in bone.^{198,206} LbL assembly is capable of producing multilayer structures with a broad range of potential compositions and functional biomolecules with a finely tuned dosage.¹⁸⁶ Fundamental studies have shown that LbL assembly can be successfully implemented using ECM components, including proteins, polysaccharides, growth factors, and cells,^{91,109,186} and the bioactivity of these components is preserved by the mild deposition conditions.

The LbL assembly of ECM-like biomolecules including hyaluronic acid and collagen has been reported as a strategy for mimicking the organic components of natural bone in syn-



thetic polymer scaffolds of PLLA and PLGA, which are commonly used in the regeneration of bone tissue due to their biocompatibility, biodegradability and suitable mechanical properties, but lack bioactivity and appropriate physiological activity.^{207,208} After the LbL assembly of ECM-mimicking collagen I coatings on PLLA substrates, the *in vitro* viability and proliferation of osteoblasts and ALP expression were dramatically improved, and cell extension was directed by contact guidance of the aligned collagen I fibrils.²⁰⁸ Collagen type I and HA nanoparticles have also been deposited onto microstructured PCL scaffolds fabricated by direct polymer melt deposition. The composition and density of the HA nanoparticles and collagen in the multilayer coating were tailored by changing the number of layers deposited by a continuous flow of solutions controlled with a peristaltic pump. Osteogenic differentiation was monitored for human MSCs seeded on these coated PCL scaffolds, and an increase in the activity of ALP confirmed the beneficial effect of the coating. Osteogenic differentiation was also confirmed through the analysis of osteoblast-specific bone sialo protein-II, BMP-2, osteopontin and osteocalcin expression.²⁰⁹

3.8 Combination multilayers for multifunctional coatings

The development of multifunctional bone scaffolds that combine improved mechanical properties with enhanced biofunctionality is a key objective of bone tissue engineering.^{167,210} Tables 1–8 present numerous examples of multilayered coatings that exhibit properties and functions relevant to bone tissue engineering and provide an indication of the large number of multilayers that could potentially be combined into a single LbL-assembled coating in order to obtain scaffold materials with very diverse compositions and capabilities.

In an example of combining numerous multilayers to produce a multifunctional coating, Shah *et al.*²¹¹ reported a coating system consisting of two different multilayer materials systems that were deposited onto titanium or PEEK bone implants. The first multilayer system consisted of chitosan/hydroxyapatite complexes (CH–HA) and PAA bilayers. These CH–HA/PAA layers were osteoconductive and had an average stiffness of ~11 GPa (within the typical range for cortical bone¹³), which mitigated the mechanical mismatch between implants and native bone and provided a permanent bone-implant interface. The CH–HA/PAA multilayers were combined with degradable layers of poly- β -amino ester 2/PAA/BMP-2/PAA containing the osteoinductive growth factor BMP-2. These quadlayers containing BMP-2 were deposited on top of the CH–HA/PAA layers, and enabled gradual and tunable release of therapeutic levels of growth factor without sudden burst-release. The loading of BMP-2 was controlled by the number of quadlayers deposited. The LbL-assembled coating resulted in a long-term stable fixation of the implant *in vivo* with rodent host tissue, indicating that the mechanical properties of the initial CH–HA/PAA base layers can be usefully combined with the biofunctionalities of the top layers in LbL-assembled coatings.

Mechanically reinforcing and biocompatible layers have also been combined to produce coating systems consisting of mechanically reinforcing PEI/PAA/PEI/nanoclay quadlayers capped with a final layer of thermally cross-linked PAA.⁵¹ These coatings were applied onto open-cell foam templates, resulting in coated scaffold materials with a wide range of tailorable porosity (72.3%–95.2%) within the suitable range for bone scaffolds^{212,213} and with a range of stiffness values (0.604–16.64 MPa) approaching the lower range of cancellous bone. Despite the presence of cytotoxic PEI within the coating structure, no cytotoxic response was observed in MSCs exposed to coated scaffolds with the cross-linked PAA final layers.

LbL-assembled coatings can also be combined with materials derived from other fabrication routes to produce novel and complex material systems. Romanelli *et al.* utilised organic nanofibers (fluorenylmethyloxycarbonyl protected valyl-cetylamine) as substrates that were coated with collagen type I, an HA binding peptide, and BMP-4 using LbL assembly.²¹⁴ These multilayered coatings were then incubated with HA nanocrystals and doped with TiO₂ nanoparticles. The coated nanofibers were embedded into an alginate hydrogel that is biodegradable under physiological conditions. The coated 3D scaffolds were biocompatible, enabled adhesion of pre-osteoblast MC3T3-E1 cells, and promoted osteogenic differentiation as indicated by ALP assay. Furthermore, the coated 3D scaffolds were biodegradable and demonstrated inherent antibacterial activity.

The LbL concept can also be integrated with other modern additive manufacturing technologies, *e.g.* 3D printing. Combining the LbL assembly technique with 3D printing platforms offers many advantages for the fabrication of functional LbL nanocomposites targeted towards regenerative medicine and pharmaceutical applications.^{215,216} Guduric *et al.*²¹⁷ fabricated PLA/chloroform solution as printing inks to create PLA membranes using extrusion-based 3D printing with human bone marrow MSCs and endothelial progenitor cells by the LbL bioassembly of cellularised sheets. Assessment of MSC viability, phenotype, and proliferation showed osteoblastic differentiation after 7 days in culture media, which indicated the potential of the LbL assembly approach for the promotion of homogeneous cell distribution within the scaffold. In another study by Chen *et al.*⁵⁸ fabricated hydroxyapatite–gelatin scaffolds using extrusion-based 3D printing for bone tissue engineering applications. Thereafter, LbL-assembled coatings of chitosan and sodium hyaluronate were deposited onto the scaffolds. The application of the LbL coating to the hydroxyapatite–gelatin scaffolds reduced the swelling ratio, increased the compressive strength and slowed down the degradation rate. In addition, there was no significant cytotoxicity effect on MC-3T3E1 cells and the scaffolds provided appropriate conditions for cell adhesion and proliferation, indicating that combining 3D printing with LbL assembly technologies offers great potential in bone tissue engineering as a sustained delivery system.

A plethora of potential combination multilayer coatings, which may include the material systems listed in Table 9 or



Table 3 Examples of crosslinked LbL-assembled films with controlled local stiffness

LbL assembly components	Substrate	Treatment	Functionality	Cell type	Ref.
DAR/PAA	Silicon wafer	Crosslinked with UV irradiation using a photo-mask	Tailored surface biological properties and selective absorption of PS nanoparticles	N/A	125
PAA/PAM conjugated with 4-azidoaniline	Glass slide	Crosslinked with UV irradiation using a photo-mask	Micropatterning and spatial of LbL multilayers film control over cell adhesion	L929	126
PAA/PAH grafted with benzophenone	Silicon wafer	Crosslinked with UV irradiation	Biocompatible surface with uniform stiffness gradient directing vascular and osteoblast cell behaviour	MG63 A7r5	124
PLL/hyaluronan	Glass slide	Crosslinked with EDC	Tunable stiffness of LbL assembly films. Cellular adhesion without adhesive protein coatings	U-2 OS	114
PLL/hyaluronan	Glass slide	Crosslinked with EDC	Cells adhere and spread along the stiffness gradient (200–600 kPa)	C2C12	115
PAA/PAH	Glass slides	Crosslinked with EDC	Controlled with time-dependent immersion in a solution of EDC, gradients of surface elastic moduli (0.5–110 MPa in the hydrated state)	MC3T3-E1 Human dermal fibroblasts	116
CSA/PLL	Glass slides	Crosslinked with genipin	Stiffness (170–280 kPa) and topography of the coating controlled by changing the concentration of genipin; stiffened surface films promoted cell adhesion, proliferation, and early and late osteogenic differentiation	MC3T3-E1	119
PDDA/MTM	3D PAM hydrogel scaffold	N/A	LbL assembly film modified the surface of the hydrogel, increased stiffness, and enhanced cell adhesion	CRL-7163 CCL-240	112

Table 4 Examples of biodegradable LbL assembled films

LbL assembly components	Substrate	Treatment	Functionality	Cell type	Ref.
CH/hyaluronic acid and PLL/hyaluronic acid	Glass slides	Crosslinked with EDC and <i>N</i> -hydrosulfosuccinimide (NHS)	Controlled the degradation by altering the concentration of EDC during treatments, crosslinking increased stiffness and surface roughness of biodegradable and bioactive films	HT29	129
PLL/hyaluronic acid (reservoirs) PLGA (barrier)	Silica slides	PLGA solution sprayed over the hydrated bilayers PLL/HA film for controlling the biodegradation	Multicompartment films degraded by seeded bone marrow cells	Murine bone marrow cells	130
PLL/alginate	AZ31 substrates (one of the commercial Mg alloy systems)	EDC crosslinking fibronectin immobilized	LbL assembly films improved the bioactivity of the scaffold; <i>in vitro</i> degradation of layers did not affect the degradation kinetics of the substrates	MC3T3-E1	134

Table 5 Examples of electroactive LbL-assembled films

LbL assembly components	Substrate	Treatment	Functionality	Cell type	Ref.
STP/PEI	Glass slides	Low voltage electrical stimulus (ES) applied to electroactive films	Erodible, electroactive LbL material system that promoted cell adhesion and growth	L-929	142
PGA-tetraaniline/PLL-tetraaniline	Glass quartz or silicon slides	Low voltage ES applied to electroactive films	Increase the level of biocompatibility because of using PLL and PGA, electroactive and degradable film enhanced the commitment of pre-osteoblast cells to osteogenic lineage	C2C12 MC3T3-E1	143



Table 6 Examples of LbL-assembled films promoting cell or protein adhesion

LbL assembly components	Substrate	Treatment	Functionality	Cell type	Ref.
PEI-(PSS/PAH) and terminating layers of PGA, (PGA/PLL), PSS, or (PSS/PEI)	Glass slides	N/A	Good biocompatibility of PSS-, PGA-, and PLL-terminated films for SaOS-2 cells	SaOS-2, PDL	150
Gelatin/PDAC	PLLA nanofibre scaffold	LbL-assembled coating was crosslinked with EDC and NHS	The amount of gelatin was controlled by number of bilayers deposited; coating improved hydrophilicity; coating improved proliferation and homogeneous distribution of osteoprogenitor cells	MC3T3-E1	152
PLL/DNA	Titanium films	Both layers of PLL and DNA deposited	Bioactive coating enhanced proliferation of osteoblasts	Osteoblasts	156
CH/HA	Titanium discs	N/A	Coatings increased biomineralisation, exhibited biocompatibility with pre-osteoblast cells, and significant anti-bacterial activity against infection by <i>Streptococcus gordonii</i>	MC3T3-E1	163

Table 7 Examples of LbL-assembled films for drug delivery

LbL assembly components	Substrate	Treatment	Functionality	Cell type	Ref.
PDDA/PSS (barrier)	Silicone sheet	Fluorescein isothiocyanate labelled PLL and rhodamine Red TM -X, succinimidyl ester used to monitor diffusion	Barriers responded to mechanical stimuli; diffusion of PLL through the barriers can be switched on/off by tuning the mechanical stretching	N/A	81
PLL/hyaluronic acid (reservoir) PAA/PAH	Glass and silicon slides	Assembled films loaded with methylene blue dye	The release of methylene blue dye was pH-sensitive (lower pH increased the release rate). Release was controlled by the incorporation of capping layers	N/A	183
Poly-β-amino ester/ PEI/gentamicin sulfate	3D titanium Implant	Solutions prepared in sodium acetate buffer for drug (gentamicin) release experiments	Coated titanium implant locally delivered hydrophilic antibiotics (gentamicin sulfate) for treatment of bone infection <i>in vivo</i>	MC3T3-E1	181
Poly-β-aminoester 2-PAA	Macroporous PCL/ β-TCP cylindrical scaffold	Solutions prepared in sodium acetate buffer	BMP-2 eluted from the coating for 2 weeks; release of VEGF was released in the first 8 days; both growth factors retained efficacy	MC3T3-E1	168
BMP-2-PAA Poly-β-aminoester 2-PAA rhVEGF165-2-PAA PLL/hyaluronic acid	TCP/HA porous scaffolds	Assembled films were crosslinked with EDC and NHS; rhBMP-2 was loaded in crosslinked films	Coating acted as a delivery reservoir of rhBMP-2; could be stored and maintained bioactivity for 3 weeks; degree of crosslinking influenced the loading of BMP-2	C2C12	172
Poly-β-aminoester 2/ chondroitin sulfate	PCL-β-TCP scaffolds	Terminating bilayer of BMP-2/ chondroitin sulfate	Coatings facilitated the microgram-scale release of active BMP-2; 80% released during the initial 2 days and the remaining 20% released over the following 2 weeks	MC3T3-E1	173
Graphene oxide (GO)-NH ³⁺ / GO-COO-	Planar titanium	Assembled films were crosslinked with NHS, loaded with BMP-2, and freeze-dried	Coating increased adsorption and sustained release of BMP-2 and improved <i>in vitro</i> osteogenic differentiation; <i>in vivo</i> more robust bone formation observed in coated implants	MSCs	174
Poly-β-amino esters 1/PAA/gentamicin sulfate Poly-β-amino esters 2/PAA/BMP-2 CH/OAlg /GRGDS	Planar silicon wafer	Barrier layers of CH/PDDA introduced between drug and protein-containing quadlayers	Coating acted as a delivery system of antibiotics and growth factors; clay barrier slowed diffusion-based release	MC3T3-E1	182
CH/OAlg/ BSA-BMP-2/OAlg Hyaluronic acid- dopamine/CH	Titanium discs and porous titanium scaffolds	Substrates treated with polydopamine	Sustained release of BMP-2 over 28 days; ECM-mimicking coatings with BMP-2 promoted MSC functions and new bone formation	MSCs	186
BMP2/CTGF	Ti-Nb-Zr alloys	Dopamine-modified HA (DHA)/CH multilayers were deposited on the surface of Ti alloys	Coating improved anti-infection properties and accelerated osteoblast cell adhesion and differentiation	MC3T3-E1	179
	SF/PCL/PVA nanofibrous film	Separation of the labelled proteins in the nanofibers performed to monitor the distribution	Sustained release of BMP2/CTGF promoted MSC functions for angiogenesis and osteogenesis	MSCs	170



Table 8 Examples of LbL-assembled films with hierarchical structures

LbL assembly components	Substrate	Treatment	Functionality	Cell type	Ref.
Collagen I/hyaluronic acid	PLLA discs	Substrate surface was modified by covalently bonded PEI	Collagen improved the biocompatibility of substrates (<i>i.e.</i> cell viability, cell proliferation and ALP expression. Osteoblast extensions were directed by contact guidance of the aligned collagen fibrils)	Osteoblasts	208
HA/collagen	PCL microfibrinous scaffold	HA was modified with dopamine-conjugated hyaluronic acid	LbL-assembled films showed enhanced osteogenic activities	MSCs	209

Table 9 Examples of multifunctional LbL assembled films

LbL assembly components	Substrate	Treatment	Functionality	Cell type	Ref
MSCs/endothelial progenitor cells (EPCs)	3D printed PLA membranes	3D constructs were prepared by assembling four PLA membranes seeded with MSC alone or co-culture of MSCs and EPCs	Coatings promoted a homogeneous distribution of cells throughout the scaffold and osteoblastic differentiation was evident	MSCs	217
CH-HA/PAA (base)	PEEK and titanium rods and sheets	N/A	CH-HA/PAA eased mechanical mismatch and encouraged deposition of cohesive trabecular bone on the implant surface	EPCs MSCs	211
Poly- β -amino ester 2/PAA/BMP-2/PAA (drug delivery system) PEI/PAA/PEI/nanoclay	Polyurethane open cell foam	Altered assembly conditions: pH of solutions, salt concentration, contact time between solution and template, number of layers deposited and cyclic loading crosslinked at 180 °C	Tuned release of BMP-2 controlled by hydrolytic degradation of poly- β -amino ester 2 Tunable mechanical (compressive strength and stiffness) and physical (density and porosity) properties of coated foams. Crosslinked coatings biocompatible with MSCs	MSCs	51
Thermally crosslinked PAA capping layers Collagen/HA binding peptide/BMP-4	Fluorenylmethyloxycarbonyl protected valyl-cetylamine nanofibers	Assembled template was incubated with HA nanocrystals blended with TiO ₂ nanoparticles and coated with alginate to form a 3D scaffold	Coated scaffolds were biodegradable, biocompatible, allowed for adhesion of pre-osteoblast cells, promoted osteogenic differentiation and demonstrated inherent antibacterial activity	MC3T3	214
Chitosan/sodium hyaluronate	3D-printed hydroxyapatite-gelatin scaffolds	Scaffolds were coated with 10 and 20 bilayers of the coating and then freeze-dried	Swelling, mechanical and degradation properties were improved following the application of the LbL-assembled coating. Scaffolds provided appropriate conditions for cell adhesion and proliferation of MC3T3-E1 cells	MC3T3-E1	58

others, remain unexplored and provide rich opportunities for multifunctional material systems for bone scaffolds and wider biomedical applications. In addition to the wide range of constituents that may be utilised in LbL assembly, the thickness, roughness, and morphology of coatings may be tailored through adjustment of assembly parameters (*e.g.* the number of depositions, pH values of solutions and salt concentrations, intermittent drying steps, and dipping times).^{67,81,218} Tuning

the physical and mechanical properties of the porous structures by depositing suitable LbL-assembled coatings and tailoring the assembly conditions could lead to an appropriate balance of sufficiently high mechanical properties and high levels of porosity required for engineered bone tissue applications, whereas the biological requirements could be satisfied by the incorporation of biomolecules and surface modifications.^{51,215}



4 Concluding remarks and future perspective

An ongoing challenge for biomaterials remains to satisfy the very diverse requirements for engineered tissue within a single material system. LbL assembly offers the advantage of a large variety of constituents that can be combined into a single multilayered material system, and is a relatively simple and low-cost coating technique to implement onto a wide variety of substrate materials. In recent years a range of new methods and materials have been explored for the development of LbL-assembled materials. These studies have reported the capability to provide controlled thickness, mechanical reinforcement, local control of mechanical stiffness, biodegradability, biologically useful electroactivity, enhanced cell and protein adhesion, and drug delivery, and to enable hierarchical microstructural features. LbL assembly has been implemented for deposition onto planar 2D substrates and 3D porous substrates, resulting in larger-scale bulk materials. Several examples of LbL-assembled material systems that satisfy one or more of the key requirements of biomaterials for bone tissue engineering, including tailored mechanical properties on bulk and local scales, a porous micro-architecture, biodegradability, biocompatibility and bioactivity, and the delivery of drugs and growth factors have been developed. More recently, combinations of different multilayers have been reported to produce LbL-assembled coatings with multifunctional capabilities derived from combining coatings that satisfy different requirements (e.g. mechanically reinforcing layers combined with drug delivery layers).

The application of the LbL approach for bone scaffold materials in bone repair and regeneration is full of opportunities and challenges. Despite the development of many new technologies and approaches for bone tissue repair and regeneration, enormous scope for further progress remains. Going forward, LbL assembly presents a novel approach for the further development of synergistic combinations of multilayer material systems that individually satisfy one or more requirements and that together can potentially meet all of the diverse needs within a combined coating material system for bone tissue engineering applications. Progress toward this goal has the strong potential for exciting technological advances and high impact in the repair of bone defects and a wider range of biomedical applications. In the past decade, the development in LbL assembly in the biomedical and tissue engineering fields has been growing rapidly, and it is expected that a dramatic increase in demand for academic and industrial applications will be witnessed in the design and fabrication of multifunctional coatings using this LbL approach.

LbL assembly is a highly versatile and simple approach for multilayer coatings in order to enhance the mechanical, biological, chemical and physical properties of biomaterials. This new technique has attracted biomaterials researchers in the last decade and probably in the next few coming years has great ability to be considered more than ever because it has

excellent capability to fabricate mechanically robust multilayer coatings with controlled micro and nanostructures, controlled degradation rate, and above all tunable local surface stiffness from extensive choices of usable materials for a wide range of biomedical applications. The LbL assembly approach, because of the versatility and tunability to fabricate multilayer coatings with controllable structures and mechanical properties, will be considered as a promising technique in the field of biomaterials for tissue repair. LbL has also strong potential for application as a method for cell encapsulation in cell-based biosensors, cell transplantation, cell/molecule delivery, and tissue engineering in the near future. The LbL assembly technique not only provides advances for surface modification with nanoscale control but also opens the new door of biomaterials for the fabrication of novel deposited scaffolds for tissue engineering applications. In conclusion, the application of the LbL approach for bone scaffold materials in bone repair and regeneration is full of opportunities and challenges. In the future, further studies on the effects of coating mechanisms using loading conditions and perfusion on the scaffold materials coated on bone cell behaviour and bone repair under hydrated conditions will be developed in bone tissue engineering. The development of the LbL technique occurs, mainly in the biomedical and tissue engineering field, and it is expected that a dramatic increase in demand for academic and industrial applications will be witnessed in the design and fabrication of multifunctional coatings by the LbL approach.

Conflicts of interest

There are no conflicts to declare.

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