Perspective: AIChE 2014 Alpha Chi Sigma Award

Layer-by-Layer Approaches to Staging Medicine from Surfaces

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Introduction

An important new direction for medicine will be the ability to create therapeutic regimens that can be tailored to specific conditions or indications. The traditional medication or treatment routine today involves standardized quantities of drugs, often delivered separately, each with its own unique mode of distribution within the body. Medical symptoms may rise and fall according to the changes in drug concentration over time, and side effects are often substantive because of the need to introduce excessive amounts of drug due to clearance or lack of targeting. Instead of relying on more traditional approaches to medical therapies, it would be ideal to create modes of delivery that release only the desired amount of drug over the appropriate time frame. Furthermore, key therapeutics often must be delivered in combination; for example, treatments following cataract surgery require the administration of both an anti-inflammatory and an antibiotic drug, and cartilage injury can be treated with steroids and growth factors. When there are multiple drugs involved in a treatment, there is usually a different desired rate and amount of release for each compound, depending on the function of the therapeutic and its half-life in the body. There are several situations in which the relative amounts of drug would ideally vary with time or for which drugs would be more synergistic if released in a specific order. In other cases, the key to the transformational therapeutic impact is simply the accomplishment of long-term sustained near-linear release of effective localized doses of a drug in a compact form that allows compliance to the contours of the body and/or an implant. Both of these challenges require the control of the spatiotemporal release of single or multiple agents; this can be achieved from device or scaffold surfaces with true modularity and adaptability of design.

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We have investigated means of incorporating therapeutics directly into thin films with alternating adsorptions on the basis of charge, hydrogen bonding, or other complementary interactions (Figure 1). This approach, known as the *layer-by-layer (LbL) technique,* was first introduced with polyelectrolytes as a means of constructing highly controlled thin films by Decher and coworkers[1,2] and has since become an extremely versatile and universal tool that can be used to construct a broad range of thin film material systems for energy, membrane, optical, and electroactive materials systems[3–6] and a range of biomaterials.[7–10] The deposition method can be adapted toward different modes of application, including spray methods[11–15] (Spray-LbL) that use convection to greatly shorten assembly times, spin-casting approaches[16,17] (spin-assisted LbL) that are very rapid and lead to highly ordered thin films on planar surfaces, and microfluidic[18–20] and other methods[21,22] that take advantage of the laminar flow of differently charged species in microdevices. Several aspects of the method lend itself to the generation of thin-film drug-delivery systems, including the fact that the process is entirely water based and relies on deposition from dilute aqueous solutions. Furthermore, the resulting thin film materials are intrinsically ionically crosslinked and can be exposed to in vitro or in vivo conditions without a compromise in film quality. The capability of generating thin films from water directly, without the need for additional chemical modifications of the film components, enables the direct incorporation of growth factors, DNA, antibodies, and other biologic drugs via electrostatic or hydrogen bonds. The films can be designed to undergo degradation or release by the incorporation of hydrolytically[23–25] or enzymatically[26,27] degradable polymers or by the use of polyelectrolyte pairings that will become unstable when exposed to physiologically relevant pHs and ionic strengths.[28] These properties have led to a huge expansion in important advances in delivery from LbL thin-film coatings for biomaterials or biomedical applications.[7,29–32] In this Alpha Chi Sigma Award Perspective, an overview is provided that focuses on recent work from our laboratory using the LbL approach to generate controlled

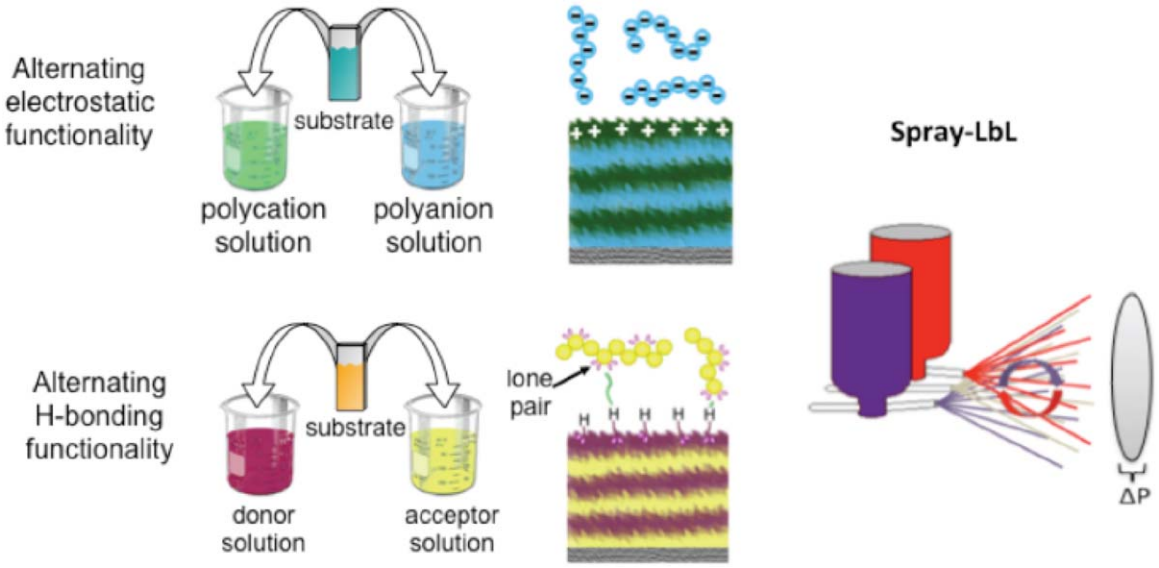

Figure 1. Schematic of the alternating adsorption LbL assembly.

The concept is based on the adsorption of multivalent charged species in alternation to generate a thin film that can differ in composition along the *z* direction of the film. Typical adsorbed single layers range from a few to a few tens of nanometers in thickness. Blurred lines in the schematic indicate that polyelectrolytes are highly interpenetrated across layers. Different components can be introduced in the adsorption step, and other complementary interactions, such as hydrogen bonding, can be used to build thin films. Spray-LbL is one of a few processes that can speed assembly of the LbL thin films.

release, with examples that demonstrate the range of release applications for which this approach can be applied for localized and systemic release. These range from coatings of implants to tumor-targeting nanoparticles. A much broader perspective of the field was written previously,[6] and there are several recent reviews and references to this rapidly growing field of biomedical applications for LbL systems that present the work of many outstanding research labs that are doing pioneering work in this area.[31,33–39]

Because assembly is based on ionic interactions or similar complementary secondary interactions, thermodynamics favors charge or interaction compensation with each step, and this method can be used to enable high loadings of the drug into submicrometer- to micrometer-scale coatings that typically contain 10–40 wt % of the therapeutic. These numbers are considerably higher than those achieved with direct loading in more commonly used bulk polyesters, polyketals, or other common degradable polymer backbones because of the limits of phase separation.

The LbL method provides a means to create films nanolayers at a time; this enables the construction of highly conformal films in which the composition can be finely tuned.[40] The ability to achieve high loadings while enabling very controlled release patterns is a true enabler in the delivery of both small-molecule and biologic drugs, which often suffer from a much more limited range of release behaviors. Each adsorbed layer varies in thickness from 1–2 nm to as thick as a few hundred nanometers, depending on the material system adsorbed and its relative charge density.[41] During assembly, there is usually considerable interpenetration of polymeric species above and below the point of deposition, and under certain film construction conditions, interdiffusion may also occur.[42,43] However, despite this tendency to generate nanoscale blends of materials with LbL, separate regions can be generated by the deposition of several sets of layers of a given composition along the thickness of the film followed by the subsequent deposition of additional multilayer systems. Through the placement of groups of drug-containing layers in the film in a systematic fashion, it is possible to design LbL film architectures that each have their own relative rates of release and unique release profiles. The beauty of this approach is its simplicity—a film can be constructed from the bottom up to create controlled corelease or sequential release patterns that are consistent with the desired therapeutic treatment.

In the case of the release of singular drugs, the use of this approach enables stable, uniform coatings that can release in long or short profiles or with a combination of release profiles (e.g. a burst release followed by sustained release profile). For the release of two or more drugs, LbL coatings can be used to incorporate dissimilar drugs into one uniform thin film that independently controls the release of each therapeutic. The manipulation of these release profiles can lead to much more synergistic drug-release profiles informed by native biological processes.

Bone Regeneration: Orthopedic and Craniofacial Applications

An outstanding example of why we would like to use ultrathin film coatings as a means of delivery is provided in the areas of orthopedic implants, bone regeneration, and craniofacial reconstruction. A number of biologic therapies have been introduced that can stimulate and promote bone regeneration, including growth factor therapies that in theory provide great promise for a range of conditions, from whole-joint implant integration to fracture repair and even the treatment of early stage osteoarthritis. These growth factors

include bone morphogenetic growth factor 2 (BMP-2), which recruits adult stem cells from the marrow to the site of injury or repair and induces their differentiation into bone cells or osteoblasts, basic fibroblast growth factor, which induces the proliferation of cells for tissue regeneration, platelet-derived growth factor (PDGF), which is mitogenic and supports cell proliferation and the generation of angiogenic cells, and vascular endothelial growth factor (VEGF), which is an angiogenic factor that induces the formation of blood vessels and, ultimately, vasculature in tissues. These and other molecules, such as extracellular matrix biomolecules hyaluronan, collagen, and fibronectin, support an environment of tissue formation. Unfortunately, the mode of delivery of growth factors that has been commercially available involves the incorporation of proteins in a collagen gel that acts as a depot. Products such as Infuse gel yield rapid bolus release of factors that reside only for a brief period near the site of repair before being rapidly cleared.[44] To achieve appropriate amounts to gain effect, doses that are orders of magnitude higher than the physiologically relevant amounts must be applied. Such high dose levels have led to the induction of latent cancer cells and other undesired side effects that have led to setbacks in the development of growth factor therapeutics for these applications. Other solutions, such as traditional polymer encapsulation, lead to issues with solvent and temperature treatments that deactivate proteins or more complex systems that cannot be easily incorporated into orthopedic devices.

The LbL coating of devices enables the unique advantage of the direct incorporation of a protein drug from aqueous solution at conditions under which the protein maintains its active form. Furthermore, the film formed can contain high weight fractions of protein incorporated with each adsorbed layer, in contrast to most polymeric delivery systems; this leads to loadings from several to a few hundred micrograms per square centimeter, which is sufficient for many therapeutic applications.[45–47]

We have demonstrated the usefulness of this approach by developing orthopedic implant coatings that can generate new bone tissue around a whole joint implant and improve bone integration to achieve a high-strength material interface with the native bone. In this study, we took advantage of the stacked architecture of LbL films to generate a "permanent" base layer consisting of positively charged chitosan decorated hydroxyapatite nanoparticles adsorbed in alternation with negatively charged poly(acrylic acid),; these were designed to provide an osteoconductive underlayer into which bone could integrate and form a strong bond in the presence of the hydroxyapatite calcium phosphate nanoparticles. Atop the osteoconductive layer, we generated alternating layers containing BMP-2 and a degradable polycation that could release the growth factor in a consistent and controlled near-linear fashion.[48] When this dual thin film was used to coat titanium or plastic [poly(ether ether ketone)] orthopedic implants in a rat tibia bone implant model, we found significant increases in the amount of bone deposited around the implant and true adhesion and integration of bone cells to the implant because of the combination of the hydroxyapatite osteoconductive layer and the ability to induce bone-cell recruitment and differentiation at the localized site of the implant from the very gradual release of BMP-2, with rates on the range of nanograms per hour to maintain tissue concentrations in the physiologically relevant range of 50–100 ng/mL for up to 30 days.[49] Because nanoscale features were coated by the LbL films, a microtextured implant surface could be maintained, and the increased surface area yielded additional depots of film that were released in a more extended fashion. The result was a bone-material interface with dramatically increased pullout strength and high adhesion that exceeded that of bone cement by a factor of 3–4 times. That enhancement of osteointegration led to native bone-material interfaces; in fact, the pullout experiments indicated failure within the native bone itself rather than at the interface with the implant. The compatibility of delivery of growth factors with LbL systems was also demonstrated by other groups[30,50–53] with both degradable and nondegradable platforms and either direct incorporation or absorption of factors in preassembled films to illicit a range of desired cellular responses.

The ability to stage release also plays a key role in the delivery of two very different types of molecular systems. For example, it is possible to directly incorporate small charged molecules into an LbL film if they are alternated with a polyelectrolyte to achieve high loadings of drug, including antibiotics[54–56] and anti-inflammatory drugs.[57–59] We can adapt these LbL formulations to generate gentamicin-containing layers atop a series of BMP-2 containing growth factor release layers to achieve staged delivery. If these two different drug components are introduced in sets of layers in sequence and separated by a barrier layer that blocks interdiffusion between layers, it is possible to achieve a staggered release.[23] Such barriers can be achieved with chemical or physical means. When nanoscale clay sheets of laponite are layered with a nondegradable polyion to construct barrier layers between drug systems, it is possible to control the relative rate of release for each set of therapeutics in a controlled fashion by changing the thickness of the barrier,[60] as shown in Figure 2. In these thin-film systems, therapeutic amounts of gentamicin sulfate are released first with a rapid release profile to eliminate existing infection within the implant. This is followed by a slower continuous and more prolonged release of gentamicin to prevent infection long term. The release half-life of BMP-2 can be adjusted by a factor of 10 with the introduction of a barrier layer between gentamicin and the growth factor film components. Through further manipulation of the amount and the location of these ultrathin film interlayers, it was possible to show modular adjustments in the rates of release of both drugs and a separation between the two drugs that would be desirable in treatment of infected orthopedic joint implants as a means of first eliminating infection, followed by the recruiting of bone cell progenitors to the site for bone regeneration and repair. Other nanomaterials may also be used to control the release of proteins in staggered fashion; for example, graphene oxide sheets can be layered to produce delays in sequence for as long as several weeks.[61]

The coating of porous scaffolds with LbL growth factors enables stem-cell recruitment and the formation of new, fully remodeled bone. We initially demonstrated this concept by coating tricalcium phosphate scaffolds with growth factors to generate coated thin films with micrometer-scale thickness that can recruit MSCs from the bone marrow to ectopic

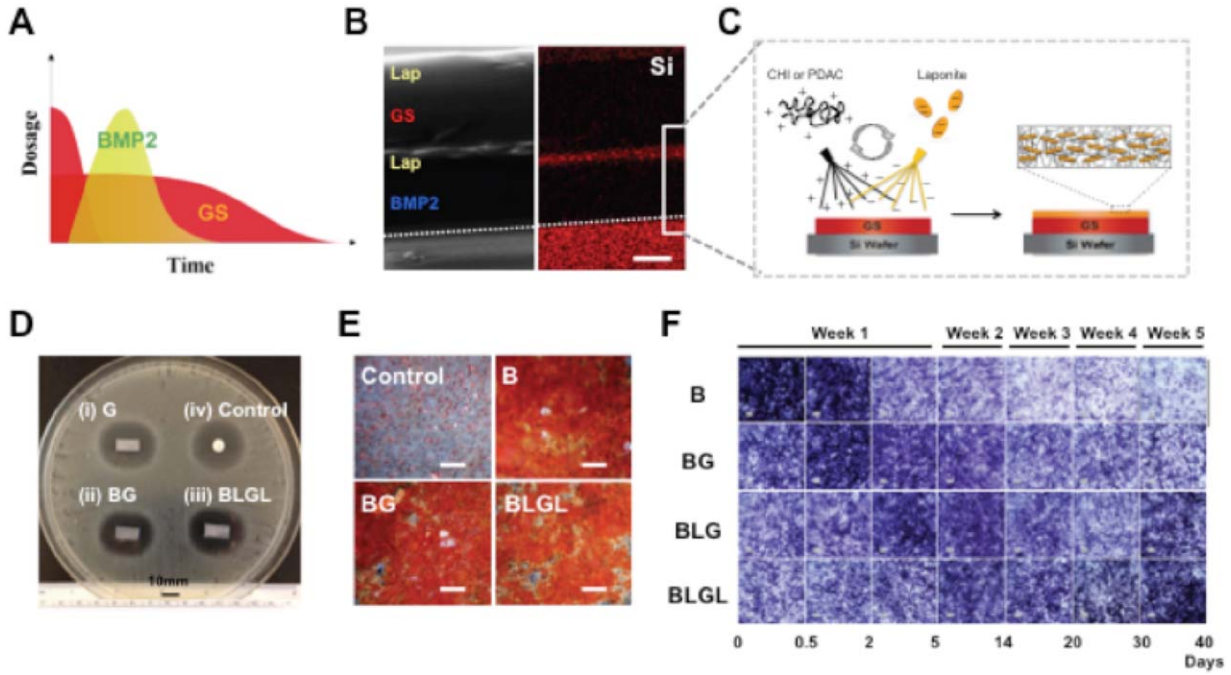

Figure 2. Design and characterization of the composite multilayer coating with laponite barrier layers for the tunable staged release of drugs.

(A) Desired release profiles of BMP-2 and gentamicin for accelerated bone healing and infection treatment. (B) Cross-sectional SEM image (left) of a composite film $B_{40}L_{15}G_{40}L_{15}$ and its corresponding EDS mapping of element Si (right). The data confirmed the compositional distribution of laponite in the film. (C) Schematic of the spray LbL assembly of the barrier layers. (D) Comparison of the antibacterial activity of the LbL films with a commercially available gentamicin product. The composite films were highly antimicrobial and effective against a common source of infection, *S. aureus*. (E-F) Preosteoblast differentiation assay. The Alizarin Red quantification at day 21 on cells differentiated with different release formulations as depicted in part E confirmed the dose-dependent presence of calcium deposits. The Alkaline phosphatase data in part F showed that the sustained release of BMP-2 from the composite films and the effect of barrier layers on the modulation of rhBMP-2 release. Reproduced with permission from Min J, Braatz RD, Hammond PT. Tunable staged release of therapeutics from layer-by-layer coatings with clay interlayer barrier. *Biomaterials*. 2014;35:2507. Copyright 2014 Elsevier. GS = Gentamicin; Lap = Laponite clay; CHI = chitosan; PDAC = poly(diallyl dimethyl ammonium chloride. Composite Film architectures in E–F represented as B = BMP2 tetralayers only; BG = BMP2 tetralayers + Gentamicin bilayers; BLG = BMP2 tetralayers + Laponite barrier layers + Gentamicin bilayers; BLGL = BMP2 tetralayers + Laponite barrier layers + Gentamicin bilayers + Laponite barrier layers.

sites.[45] Tricalcium phosphate supplies calcium in place of large amounts of native bone. We demonstrated that the co-release of BMP-2 with VEGF is beneficial to bone development across the entire millimeter-scale scaffold.[62] VEGF was deposited as the top layer and was, therefore, naturally released more rapidly because of surface-based erosion; this approach enables the formation of blood vessels and provides vascularized tissue to support further infiltration of stem cells, pre-osteoblasts, and osteoclasts. In earlier studies, we showed that by combining VEGF and BMP-2 in this kind of sequence, it was possible to much more completely generate bone in an ectopic site, such that the entire porous scaffold was filled with highly vascularized and well-developed mature bone; this was in contrast to systems that contained only BMP-2, which only enabled the formation of a thin shell of bone around the implant area.

Dual-factor release for healing large cranial bone defects

We extrapolated these approaches toward the practical application of craniofacial and bone defect repair. Here, it is highly desirable to be able to design a construct that is easily conformable with facial features. We have used this approach to create a composite device consisting of a biodegradable porous ultrathin multilayer polymer "skin" to repair a craniomaxillofacial bone defect.[63] Here, we used a simple and well-characterized polymer scaffold formed from the phase-inversion processing of poly(lactic-*co*-glycolic acid). The membranes could be generated with defined physical properties, such as thickness, porosity, and surface roughness [Figure 3(A)], and then cut into customized shapes based on the desired application. For applications of bone replacement, one may desire stiffer scaffolds that are shaped or molded to the facial shape, or flexible, adaptable membranes that may be applied to a large but fairly uniform area. When LbL films containing the appropriate growth factors are incorporated onto the membrane, the film coats the interior of the pores and all available surface area, and these interconnected pore surfaces then act as long-term depots of release. The intentional release of a factor that supports angiogenic activity can be followed by a slower release of a bone inductive factor such as BMP-2. Here, we took advantage of the modular and scalable nature of the LbL platform

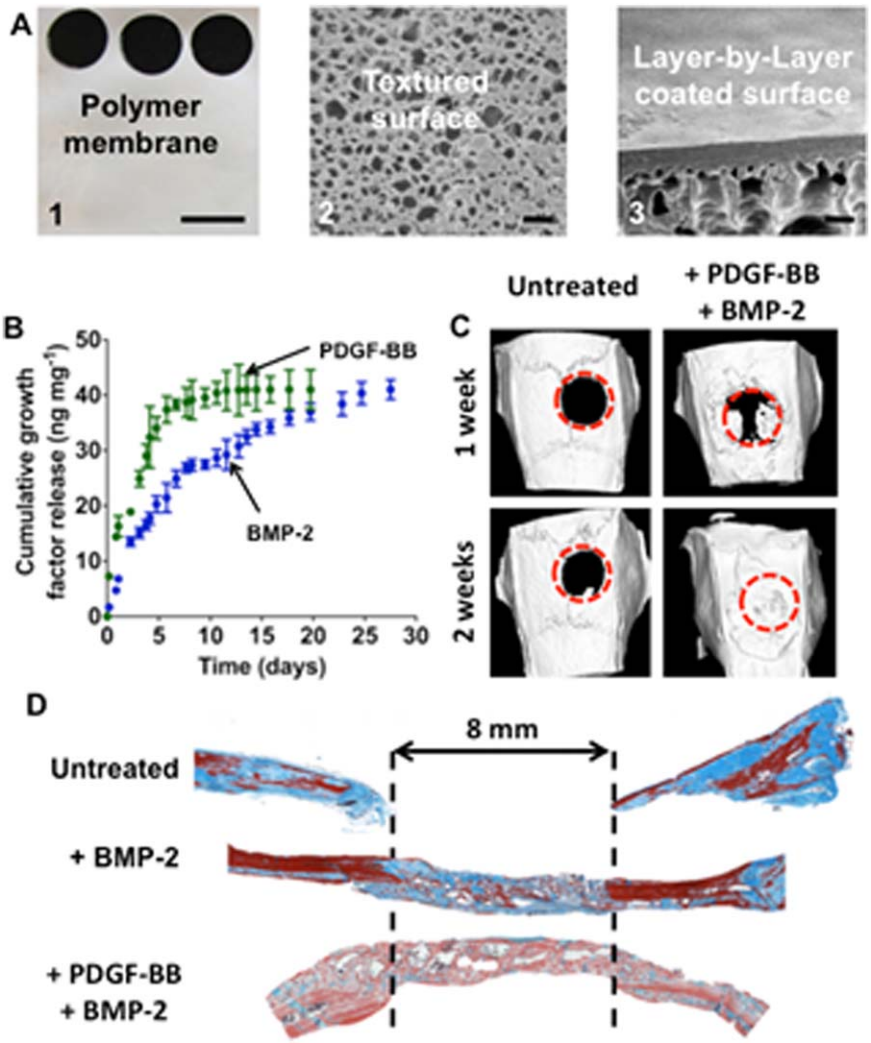

Figure 3. Building a poly(lactic-*co*-glycolic acid) membrane with bioactive interfacial properties.

(A) A photograph of the polymer membrane, with holes punched out that are the size of a bone defect. The surface of the membrane contained interconnected pores that could be coated in a conformal manner [scale bars = 2 μm (2, 3) and 8 mm (1)]. (B) Tunable release of bioactive growth factors. Mitogenic PDGF was released faster than BMP-2 when released in combination. The dose of growth factor could be tuned. (C) X-ray images demonstrating that the membrane resulted in the rapid closure of a large area bone defect in a rat calvaria. The untreated control did not heal. (D) Massons trichrome histology stain on a cross section of the rat calvaria at 4 weeks postimplant. No bone was observed in the untreated defect, whereas immature bone was observed when the single growth factor BMP-2 was delivered. Dual-growth-factor delivery resulted in the formation of mature bone, which bridged the 8-mm defect. Reprinted with permission from Shah NJ, Hong J, Hyder MN, Hammond PT. Osteophilic multilayer coatings for accelerated bone tissue growth. *Adv Mater.* 2012;24:1445. Copyright 2014 National Academy of Sciences.

to stack films with PDGF (a mitogenic agent that supports angiogenesis) atop films containing BMP-2 (induction of bone tissue) to support active cell proliferation for vascular and bone tissue. The controlled growth factor release, as pictured in Figure 3(B), was able to replicate many of the key cellular regenerative processes that substantially enhance bone formation by inducing first angiogenesis and then osteogenesis. The use of this porous and flexible membrane as a kind of tissue repair skin enabled the generation of vascularity within the developing bone matrix; this led to a mature and well-developed bone. The rapid repair rate of the bone is notable in a critically sized rat calvaria defect. When compared with treatments containing no PDGF and only BMP-2, regardless of dose levels, the dual-growth-factor system yielded much more mature bone that looked very similar histologically to the native bone; the presence of the vascular system in this bone enabled a much more complete remodeling of bone and thus a smoother and more uniform regenerated tissue.

Controlling and fine-tuning staggered and extended release

As demonstrated in the examples of regulating two very different small molecules, such as an antibiotic and a growth factor, or the staged release of two different proteins with

similar composition, such as the growth factors VEGF and BMP-2, there are strong motivations for creating highly controlled release platforms that enable preprogrammed modes of release. In many cases, it is desirable to delay the release of one therapeutic when another is released or to heighten efficacy by optimizing the release profiles of two different drugs from the same surface. Even though LbL approaches can yield the generation of different architectures, if polyelectrolytes or drug molecules have a tendency to diffuse between layers, the achievement of a true stagger with marked delays in release can be challenging. A second challenge is the achievement of long-term release that may extend beyond a few weeks, particularly for small-molecule drugs. There are few delivery platforms, beyond mechanical devices, that can exhibit high loadings and enable extended release. Significant advances in treatments can be made with formulations that can be used to coat biomedical devices. One way of achieving control within polyelectrolyte multilayer films is to use covalent chemistry to manipulate release behavior.

By incorporating known biologically compatible quantitative click chemistries, we have been able to demonstrate the formation of *in situ* crosslinks with direct adsorption from aqueous solutions.[64] For improved biocompatibility and bioorthogonality, we most recently investigated the use of the poly(β-L-malic acid) (PMLA) backbone, which is a negatively charged polyanion that undergoes hydrolysis along its ester backbone and contains a carboxylic side group that can be used as a handle for functionalization and a source of high charge density.[65] A version of these PMLA polymers was synthesized to contain a fraction of azide-functionalized side groups (PMLA-az) and a separate set of polyions was designed to present dibenzylcyclooctyne groups (PMLA-DBCO), which can undergo spontaneous reaction with the azides under aqueous buffer conditions. We assembled films composed of an alternating tetralayer of (chitosan/PMLA-az/lysozyme/PMLA-DBCO)$_n$ that underwent crosslinking during the construction of the film. We found that the inclusion of these covalent crosslinks during film assembly limited the interdiffusion that causes the mixing of components.[64] It was possible to effectively partition the model protein, lysozyme, within a defined compartmental region; this resulted in the complete delay of release of the highly interdiffusive protein for 24 h. The length of time of drug delay depended on the number of layers introduced. The additional deposition of a protein-containing film atop barrier film and the lysozyme film architecture generated sequential release behavior with discrete and temporally separated protein delivery. This method of blocking or delaying release can be thought of as a complement or alternative to the physical barrier approaches that use nanoclays or graphene oxide.

Polymer-drug conjugation

Polymer-drug conjugates have provided promising opportunities in the area of drug delivery since they were first introduced; however, these systems are generally used as soluble prodrugs that are directly administered. It is possible to significantly enhance and broaden their range of delivery time frames and their net loading with the adsorption of drug-modified polyelectrolytes within LbL films. A prodrug formulation of diclofenac, a small-molecule nonsteroidal anti-inflammatory drug, was generated by its direct conjugation with the poly(L-glutamic acid) backbone through triethylene glycol and hydrolysable ester linkages (PGA-TriEG-Diclof). When the polymer-drug conjugates were assembled into LbL films composed of (chitosan/PGA-TriEG-Diclof)$_n$ and poly-L-lysine (PLL)/PGA-TriEG-Diclof)$_n$, they were capable of substantial drug loading (>25 wt %) because of the covalent immobilization of the small molecule to a large polyacid; these numbers were much higher than the more standard 1–3% loadings characteristic of hydrophobic small-molecule drugs in bulk polymer blends. The release kinetics from the PLL-based film architecture exhibited the release of active diclofenac for more than 16 months, this potentiates this method as an interesting strategy for long-term small-molecule release from an entirely biodegradable thin film.[66] Possible applications include the important issue of chronic pain; the delivery of diclofenac or similar compounds might be adapted in such coatings to administer pain relief for extended time periods from orthopedic implants, coated injectable microparticles for cartilage injection in osteoarthritis, or in the long-term release of appropriate antibiotics to prevent infection from catheter or stent surfaces.

Wound Healing: Regulating Native Healing Processes with LbL Delivery

The regeneration of hard bone tissue is a promising area, but the LbL platform is also amenable to the coating of a range of materials appropriate for soft tissue wound healing. It sis possible to fully coat degradable gelatin or collagen sponges with LbL films, as demonstrated in work that incorporated hemostasis proteins such as thrombin[67] or the antibiotic, vancomycin,[68] onto these surfaces. It is also possible to coat a broad range of cotton, nylon, or silk wound and suture materials that are currently used by surgeons to address soft tissue wounds and incisions. In many applications, the same kinds of therapeutics discussed for orthopedic applications are relevant here, for example, the delivery of PDGF and VEGF to enhance wound closure and the formation of the granulation tissue needed to establish the deposition of collagen and matrix materials within the wound.

A second approach is the release of small-interfering RNA (siRNA), which corrects a genetic dysregulation in wounds that do not heal correctly. In such cases, there are often proteins overexpressed by cells in the wound bed that somehow delay or disrupt the natural healing process. Two particular areas of interest for siRNA applications are diabetic ulcers and *hypertrophic scarring,* which is the formation of excess scar tissue, or fibrotic tissue, during the healing of a large, soft tissue wound. In the first case, Type II diabetes tends to cause neuropathy and the formation of foot ulcers that do not close due to the physiology of glycemic patients. It has been demonstrated that the gelatinase, matrix metalloproteinase 9 (MMP-9), is highly overexpressed in diabetic ulcers, and thus, it constitutes a meaningful target for addressing this condition.[69] Furthermore, there are a number of additionally known dysregulated gene pathways impacted by diabetes; this makes this methodology a promising one for the long-term healing and closure of debilitating ulcers and the

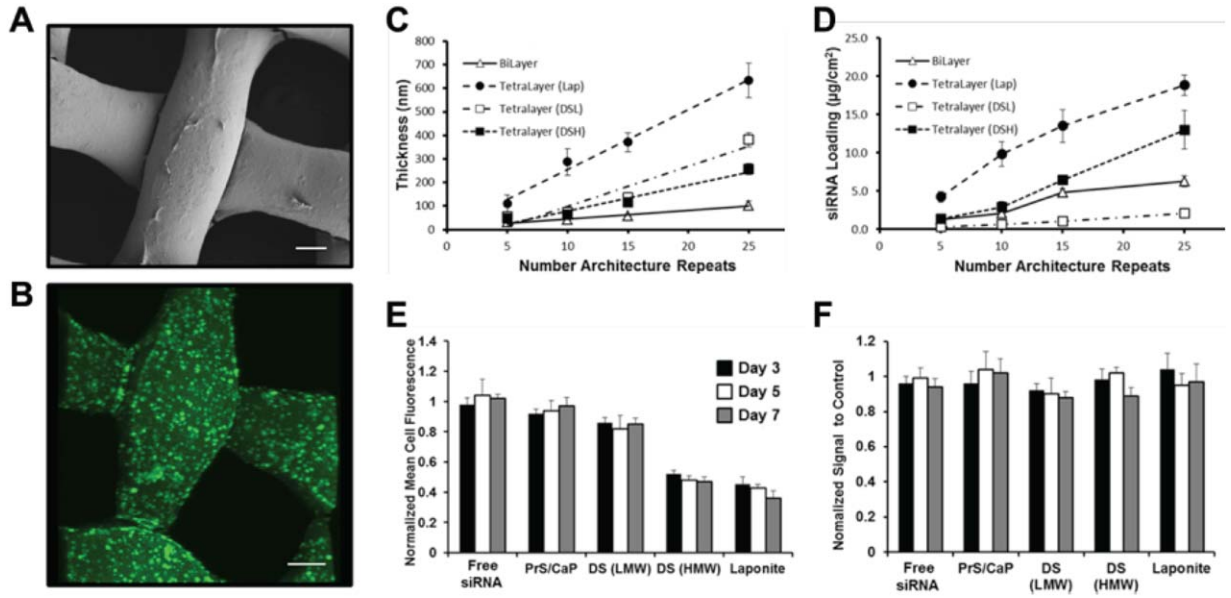

Figure 4. LbL thin films for the local sustained delivery of siRNA.

(A) SEM image of coated Tegaderm woven nylon bandage. (B) Three-dimensional projection of the confocal imaging study of the uniform incorporation of fluorescently labeled siRNA in calcium phosphate nanoparticle containing films. (C) Film growth as determined by the repeated deposition of LbL film layers for four distinct film architectures investigated. (D) Incorporation of siRNA into film-coated bandages per square centimeter. (E) Relative mean cell fluorescence of Green Fluorescent Protein (GFP) expressing the NIH-3T3 cells treated with siGFP-containing LbL-coated sutures over 1 week in vitro. (F) Impact of the treatment group on cell viability. Reproduced with permission from Castleberry S, Wang M, Hammond PT. Nanolayered siRNA dressing for sustained localized knockdown. *ACS Nano*. 2013;7:5251. Copyright 2013 American Chemical Society.

prevention of foot loss and overall mobility that may occur under extreme conditions.

Despite the promise, the delivery of siRNA directly to a localized region of the body is particularly challenging. siRNA must be introduced in a controlled fashion that maintains its activity while preventing the rapid clearance or degradation by RNases found in the application site or in the bloodstream. The water-based nature of LbL and the charge density of siRNA allow for its ready inclusion in LbL films. Nucleic acids in general can be readily incorporated into LbL films, from plasmid DNA[70–74] to short oligonucleotide and RNA sequences.[75,76] We examined a range of formulations with different polycations, LbL architectures, and both pre-encapsulated and free forms of siRNA to determine optimal formulations with high loadings of siRNA.[77] Through the incorporation of siRNA first into calcium phosphate nanoparticles followed by the incorporation of these charged particles into an LbL film structure, we ensure the packaging of siRNA for delivery and its subsequent protection from RNase degradation.

Highly conformal films containing siRNA can be formed on bandage surfaces, as shown in Figure 4. Early formulations of these systems were designed to release siRNA for the knockdown of green fluorescent protein (GFP) in vitro; extended gene suppression was observed for a period of 5–7 days, appropriate for the 1–2–week time period desired for the intended application of these bandages before change. Notably, the films were incubated by floating above two-dimensional cell cultures; in this configuration, knockdown was significant. In moving forward, siRNA against MMP-9 is being examined in vivo with diabetic mouse models, and new work in our lab involves the extension of this work to address extensive scarring and fibrosis.

Moving to the Nano Level: LbL Nanoparticles for Cancer Applications

The concepts of tunable release and adaptive release profiles of multiple drugs is also highly compelling for the systemic delivery of drugs with nanoparticle approaches.[78–83] LbL provides a platform by which one can consider core-shell architectures in which the shell consists of LbL films loaded with drug molecules of differing size and charge.[9] Nanoparticle systems are typically prepared by alternating adsorption and centrifugation processes of charged colloidal suspensions; new approaches are under investigation to speed this process, and this makes it a very promising approach for nanoparticle formulations.[84,85] The water-based encapsulation process enables the incorporation of siRNA, peptides, and proteins; certain inhibitors and other small-molecule therapeutics may also be incorporated with charge in conjunction with hydrophobic interactions or other secondary interactions; this would make the use of LbL nanoparticles a universal and modular platform for constructing effective combination therapies. An area of particular relevance is the targeting of tumors for the delivery of cancer drugs, in which it is desirable to create a nanoparticle that can release a drug in a controlled manner, exhibit tumor responsiveness for delivery, and effectively target and localize the delivery vehicle.

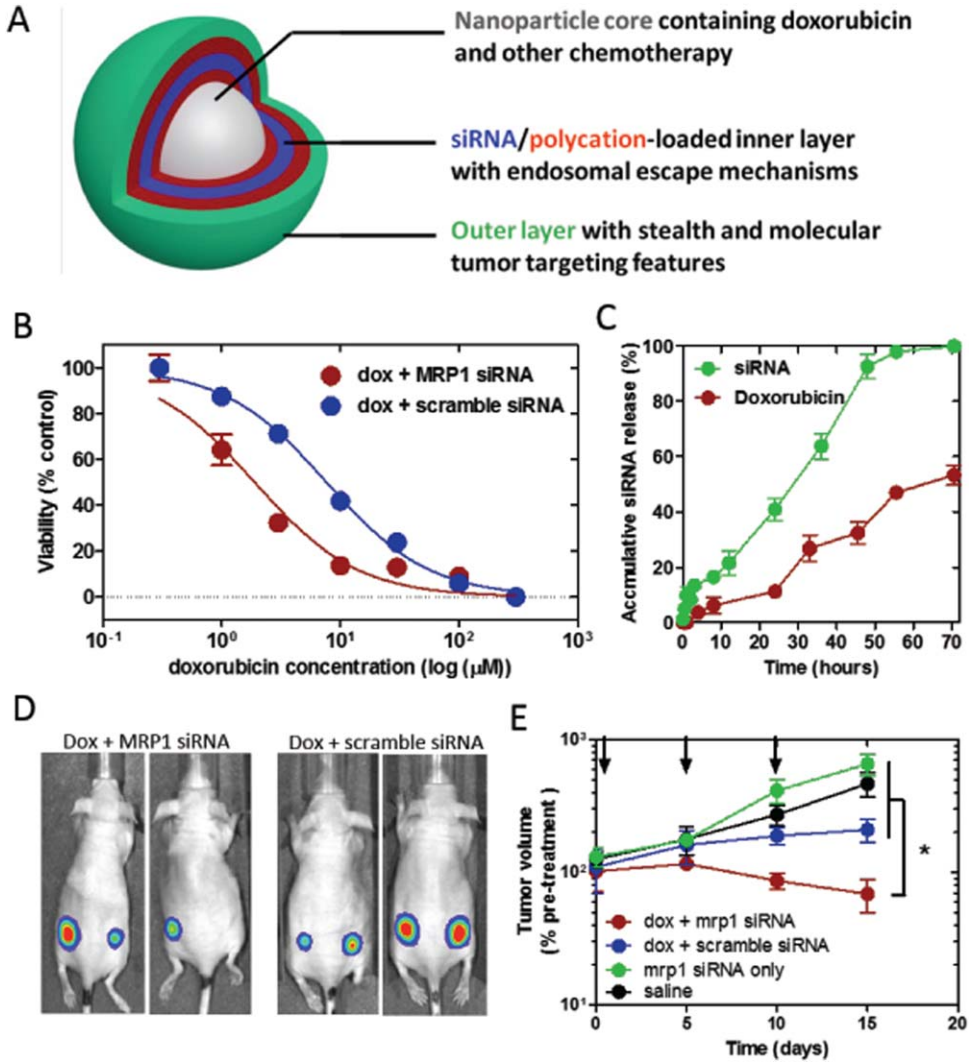

Figure 5. LbL nanoparticles for cancer combination therapies.

(A) Schematic of modular combination drug-delivery platform based on LbL nanoparticles. (B) Examination of the siRNA-enhanced cytotoxicity of the combo therapy in MDA-MB-468 cells. The results represent the mean plus or minus the standard deviation ($n = 3$, $p < 0.05$). (C) Release profile of the two therapeutics components: siRNA and doxorubicin (dox) from the LbL nanoparticles in tissue culture medium over 72 h ($n = 3$). (D, E) Assessment of the efficacy of the mrp1 siRNA doxorubicin LbL liposomes to subcutaneous MDA-MB-468 xenografts in nude mice, compared to the single-component-therapy samples and untreated controls. The mice were treated through repeated tail vein injection at 1 mg/kg siRNA and/or doxorubicin on days 0, 5, and 10 (marked by arrows). The tumor volume was monitored by luciferase bioluminescence ($n = 6–8$, $*p < 0.05$). Reproduced with permission from Deng ZJ, Morton SW, Ben-Akiva E, Dreaden EC, Shopsowitz KE, Hammond PT. Layer-by-layer nanoparticles for systemic codelivery of an anticancer drug and siRNA for potential triple-negative breast cancer treatment. *ACS Nano*. 2013;7:9571. Copyright 2013 American Chemical Society.

The use of LbL approaches yields several unique advantages, including the ability to design a nanoparticle structured to release two or more agents in a controlled manner. The most aggressive cancers typically exhibit genetic mutations that can act to enhance the survivability of tumor cells when under attack from chemotherapy drugs. If some of these genetic pathways can be blocked, standard chemotherapy drugs could have a much greater impact on resistant tumors, and highly effective personalized treatments could be constructed for patients with known genetic characteristics. As shown in the schematic in Figure 5, one could design a system with a chemotherapy drug encapsulated in a nanocarrier such as a polymeric nanoparticle or liposome. The core would then be used as the basis for LbL assembly, and alternating layers could be used to incorporate a second therapeutic agent, for example, siRNA or an inhibitor to block the genetic pathways relevant to a specific tumor type. Furthermore, additional layers could be used to introduce components that regulate endosomal escape or cell uptake. An outermost set of layers could provide stealth capabilities to prevent the particle from being taken up by immune cells in the blood stream. What is exciting about this approach is that each of the layered components could be independently varied without directly impacting the other components. Very few nanoparticle formulation approaches enable the level of modularity and work with the range of drug

components that can be incorporated into LbL nanoparticle systems.

The outermost layers of the LbL system are key, as they determine the cell-material interactions that govern nanoparticle fates. We have found that the use of weak polyacids and bases can yield useful pH-responsive behavior in these outer layers, unique to the LbL complex, that undergo environmentally triggered transformations. Hyaluronic acid (HA), when layered with PLL, exhibits a dense and brushy outer layer of highly charged acid groups in the bloodstream at a pH of 7.4; under these conditions, the PLL/HA final layers are effective stealth systems that prevent protein adsorption from the environment and do not engage with surrounding cells in the bloodstream. When these nanoparticles accumulate in tumors, the more hypoxic environment typical of solid tumors yields a lower pH of approximately 6.5- 7.0. Under these conditions, the PLL/HA system loses charge rapidly within this window and becomes near neutral in charge under hypoxic conditions. These changes in charge, along with swelling and potential changes in the mechanical properties of the nanoparticles, lead to a near doubling in the uptake of nanoparticles by the surrounding cells.[86] Thus, the simple combination of two polyelectrolytes with LbL yields environmentally responsive behavior that can induce uptake only when the nanoparticle has colocalized with the tumor. Because the HA outer layer also binds the CD44 receptor, which is commonly overexpressed on a range of tumor cells (breast, ovarian, colon), it is possible to take advantage of this system to gain a nanoparticle that provides three routes to tumor targeting: the nanoparticle size enables tumor accumulation because of the leaky vasculature of tumors, the hypoxic tumor response transforms the stealth layer into a cell-uptake layer, and the CD44 has a targeting mechanism for molecular affinity to specific tumor types. The results[86] indicate that this kind of fine-tuning of the film can yield a very effective targeting system because of this multimode means of enhancing the uptake.

To demonstrate the potential of the LbL nanoparticle approach for the optimized release of dual drugs, we examined delivery to triple-negative breast cancer with an siRNA against mrp1 protein, a protein that acts as a pump to remove doxorubicin and other DNA-damaging drugs from the cytosol.[87] We generated a nanoparticle core from negatively charged lipids that readily formed liposomes that could encapsulate the water-soluble drug. The nanoparticles were then coated with alternating layers of siRNA against mrp1 and poly-L-arginine, which can regulate endosomal escape after cell uptake. Because the loading of siRNA with poly-L-arginine was quite high, only a single layer was needed to obtain an estimated 3500 copies of siRNA per single carrier. These first two layers were followed by PLL/HA as the outer stealth layer, and thus, the simple design of these systems was complete. The release of the siRNA was relatively rapid, with complete release taking place over a period of 40 h. The doxorubicin was released for a much longer time period, with a half-life of 70 h.

The nanoparticles were tested against controls that included untreated mice, mice treated with doxorubicin alone, or doxorubicin plus an siRNA that did not code against any specific gene (Figure 5). The results indicate that although doxorubicin administered by itself had a minimal response in which the rate of growth of the tumor was somewhat slowed, with the siRNA combination treatment of mrp1 siRNA with doxorubicin, the rate of tumor growth not only significantly decreased, but there was a decrease in tumor volume. Under typical conditions, doxorubicin is not generally a useful therapy for advanced triple-negative breast cancer. These stark differences in the treatment in the presence of siRNA speak to the potential of the use of LbL approaches to package therapeutics together while achieving tailored release profiles.

Conclusions and Reflections on Translation

On the basis of these examples and many others, the LbL delivery approach is adaptable and modular and can provide a controlled platform for a broad range of therapeutics. Key advantages include the ability to package drug molecules at a high density on a variety of surfaces and the ability to control release over a very broad range of time periods, from a minutes to months. The ability to manipulate the architecture of these thin films enables staged and sequential release approaches and finely tuned release profiles for each drug component. The translation of these technologies is eminent and facilitated by approaches that enable the rapid screening of LbL technologies, including a recently developed capillary flow approach that uses very small quantities of sensitive or expensive biologic and nucleic acid drugs to generate libraries of formulations for in vitro optimization.[88] Furthermore, recent demonstrations of the commercialization of spray-LbL approaches[89] and the adaptability of spray[15,85] and other LbL methods[21,22] that have been recently introduced for the coating of particles at rapid speed provide commercial routes for the coating of systems from larger scale implants and devices down to nanoscale particles for a broad range of biomedical applications. This enabling water-based assembly technology promises to enable practical applications and to support advances toward personalized medicine and self-scheduled combination therapeutic approaches.

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