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Engineering nanolayered particles for modular drug delivery☆

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ABSTRACT

Layer-by-layer (LbL) based self-assembly of nanoparticles is an emerging and powerful method to develop multifunctional and tissue responsive nanomedicines for a broad range of diseases. This unique assembly technique is able to confer a high degree of modularity, versatility, and compositional heterogeneity to nanoparticles via the sequential deposition of alternately charged polyelectrolytes onto a colloidal template. LbL assembly can provide added functionality by directly incorporating a range of functional materials within the multilayers including nucleic acids, synthetic polymers, polypeptides, polysaccharides, and functional proteins. These materials can be used to generate hierarchically complex, heterogeneous thin films on an extensive range of both traditional and novel nanoscale colloidal templates, providing the opportunity to engineer highly precise systems capable of performing the numerous tasks required for systemic drug delivery. In this review, we will discuss the recent advancements towards the development of LbL nanoparticles for drug delivery and diagnostic applications, with a special emphasis on the incorporation of biostability, active targeting, desirable drug release kinetics, and combination therapies into LbL nanomaterials. In addition to these topics, we will touch upon the next steps for the translation of these systems towards the clinic.

1. Introduction — building nanocarriers layers at a time

One of the rapidly growing areas of drug delivery is the use of electrostatic assembly methods based on the alternating adsorption of multivalent systems with complementary interactions, including charge and hydrogen bond association, to create highly controlled drug release systems. This approach, also known as layer-by-layer (LbL) assembly [1–3], is grounded in the idea that any number of materials systems can be incorporated into a thin film through the use of alternating charge or other interactions [4–6]; such versatility provides a means of designing ultrathin drug carrier coatings [7–12] and capsules [13–16] with customized content and release characteristics from the surfaces of a range of different substrates. These systems have been successfully developed for release from both macroscopic and microscopic surfaces such as biomedical implants and microneedles. Over the past decade, as many labs have investigated the ability to apply the concepts of LbL assembly to nanoparticles and nanometer scale colloids [17–22], new and exciting possibilities have enabled the combination of unique features of polyelectrolyte complexes with the control of the nanolayered assembly method. In this review article, we address the many advantages of LbL assembly, and its versatility in providing a broad range of properties and function for passive and active targeted, environmentally responsive, and combination delivery systems that would be difficult to produce using the more traditional bulk polymeric nanoparticle. The possibilities provided by these unique delivery systems, and the recent advances from several research labs worldwide, will be discussed, including the unique ability to design these systems from the inside out. For example, the spatial control over the constituents of an LbL nanoparticle allows researchers to manipulate and engineer systems that exhibit precise functionalities both in the exterior and interior layers of the LbL film.

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The modular nature of the LbL nanoparticle is one of the key advantages to its use as a multifunctional drug delivery nanocarrier, as shown in Fig. 1. First, the assembled films can be adsorbed onto a number of core nanomaterials systems. The inner core can be any particle system that can be manipulated to exhibit charge. Potential drug-loaded core particles include degradable polymeric nanoparticles such as poly-L-lactic/glycolic acid (PLGA) [23], poly(D,L-lactic acid) [24] or other well-established polymeric materials, which can be optimized to encapsulate hydrophobic drugs. Liposomes are common drug carriers that can contain hydrophilic drugs in the core and hydrophobic drug in the bilayer, and are readily coated with LbL layers [25,26]. Metal and metal oxide cores, including solid and hollow nanoparticle structures down to as low as 10 nm in size can be used as LbL nanoparticle cores [18], as well as quantum dots [27], carbon nanotubes [28], halloysites [29], and mesoporous silica [30]. This versatility enables the potential to address a full range of nanomedicine applications [31].

A range of different polyelectrolytes can be introduced onto the surface of the core nanoparticle using LbL. Simple charged polypeptides and/or polysaccharides or synthetic polyions can be introduced in alternating assembly to create an ionically crosslinked thin film membrane which acts as a gate for regulating drug release from the core [32]. The simplicity of the LbL process, as well as the aqueous nature of the assembly process, enables incorporation of biologic drugs such as proteins [33], peptides [34], and nucleic acids (including RNAi [35], DNA [36], and oligonucleotide sequences) that regulate immune response [37], gene transcription [38], or gene editing. This capability enables the intermediate LbL bilayers to serve not only as regulators of drug release, but also as drug carriers that can accomodate high loadings of drug — ranging from 10 to 20% by weight of the LbL film, to potentially as high as 50% or more based on the assembly process [5,39–41]. This enables LbL films to approach near-stoichiometric amounts of material for charge compensation, and thus enables an ultrathin film coating that is only a few tens of nanometers thick [36,42,43] to contain significant amounts of therapeutic wrapped around the nanoparticle core. Notably, these loadings contrast significantly with the more typical 1–10% weight loadings observed in many solid polymer films and coatings [44].

Finally, the outer bilayer of an LbL nanoparticle system plays a critical role in the interactions of the nanoparticle with cells, and ultimately in the routing of nanoparticles within the body. The layer-by-layer approach involves the adsorption of polyelectrolyte chains onto an oppositely charged polyion layer, and it is known that depending on the degree of ionization of the polyion adsorbed and the underlying polymer, one can achieve dense, brush-like layers that can present relatively high charge density [6]. These dense, brush-like or loopy chain conformations can yield hydrated and highly charged particle systems that regulate protein opsonization and monocyte uptake in the bloodstream, providing key "stealth" properties for systemic delivery in the blood stream [27]. Careful choice of the final polyanion/polycation pair yields an outer layer with a characteristic isoelectric point and highly tunable outer shell chain density. These characteristics are unique to the LbL approach, and can provide intrinsic molecular targeting, or the option to control targeting of the nanoparticle by taking advantage of the inherent properties of the tumor microenvironment, as will be described in the following sections. Furthermore, the free functional groups on the polyelectrolyte outer layer provide opportunities for further chemical functionalization [45–47], and the attachment of ligands including small molecules, peptides, glycans, antibodies, and antibody fragments, all of which may be used for molecular targeting of specific organs and cell types. Alternatively, the use of polycations as the final layer of a nanoparticle may be used to engage a number of non-specific mechanisms of cell uptake.

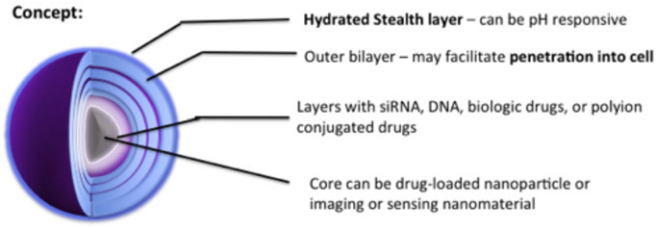

Fig. 1. Schematic of modular platform technology of an LbL nanoparticle delivery system for theranostic or combination drug therapies.

The unique characteristics of LbL films include the ability to manipulate ionizable groups such as amines or acid groups so that the films buffer when exposed to the pH of the tumor microenvironment or the endosomal compartment, leading to film swelling or even film destabilization. With this capability, the LbL nanoparticle approach can provide a means of controlling or gating drug release from a pre-loaded nanoparticle core in a stimuli-responsive fashion, as is discussed in later sections. LbL coatings can also transform an imaging or diagnostic agent that serves as the core particle into a theranostic system via the incorporation of drugs in the intermediate layers. The broad range of new therapies that can be introduced using this nanoparticle technology provides an exciting route to cancer therapy and tumor targeting, targeted therapies for infectious disease, vaccines for immunotherapy, gene therapy, and other areas in which systemic delivery in nanomedicine is highly relevant. The ability to change both the core and the inner and outer layers of the multilayered system enables incorporation of additional functionality such as imaging or light-activated therapies or incorporation of additional drugs.

The scope of this review is to cover the surge of interest and research in designer LbL nanoparticle systems towards biomedical applications, including some of the in vitro research that forms the underpinnings of understanding of how these nanoparticle systems interact with cells. It will not attempt to cover the large body of work involving layer-by-layer hollow microcapsule systems, which are well captured in other recent reviews [9,10,17,48–52]; rather, it will focus, with a few exceptions, on systems containing nanoparticle cores within the range of 10 to 200 nm, typically thought to be relevant for targeted nanoparticles in systemic delivery applications.

The key advantages of these systems are:

- Tunable exterior layers that can enable highly effective stealth properties
- Implementation of active targeting, stealth properties, environmental responsiveness
- A modular platform enabling combinations of different drug types, including hydrophobic drugs, hydrophilic and biologic drugs and nucleic acids
- The potential to stage timing of release of different drugs
- A means to manipulate release kinetics independent of nanoparticle core composition

Here we will cover each of these advantages in detail, and provide examples of how LbL systems can be manipulated to exploit the nature of the charged polyplexes that constitute these systems. The focus will be on the biomedical applications that these LbL nanoparticles enable, and how these modular systems can be adapted or utilized for systemic drug delivery of nanoparticles for therapeutic, diagnostic, and theranostic applications, as well as potential additional routes for targeted delivery. Finally, we investigate the translational potential of this technology, and advances towards bringing these highly promising nanoparticle systems to market and ultimately to the clinic.

2. LbL nanolayers as biofunctional nanoparticle surfaces

2.1. Physiological interactions of LbL nanoparticles

The modular nature of LbL nanoparticles [18,20,27,42,53–58] allows their cellular and physiological interactions to be chemically tuned for a

range of diagnostic and therapeutic applications. Following administration, these biological interactions occur first with the outermost layers of the particles; thus, rationally engineering the interactions of these terminal LbL layers (or the lack thereof) is of critical importance. In the following section, we review how the various physiochemical properties of LbL nanoparticles (e.g. size, charge, compressibility, etc.) dictate their interactions with cells in the body and, in Section 2.2, how these surface properties can be chemically modulated to direct outcomes and achieve targeting to specific tissues or cell types in vivo.

2.2. Size, surface charge, and structure

A key issue that may be considered in the design of LbL nanoparticle systems is the effect of an additive process on particle size. In applications where prolonged circulation is desired, for example in tissue-targeting or drug delivery, LbL nanoparticles should be somewhat larger than the renal clearance threshold (<6 nm), but smaller than 200–250 nm in hydrodynamic diameter (HD) in order to avoid efficient splenic clearance. Particles are able to maintain longer plasma half-lives and more effectively accumulate in tumors when they are under 100 nm [59], which fits the NCI definition of a nanoparticle [60]. Once extravasated from circulation, optimal sizes can range widely depending on anatomical site and application. However, smaller particles are often preferred in tumor-targeting applications where desmoplasia (stromal compaction) is often a significant barrier to efficient transport. Other important size-dependent barriers, reviewed elsewhere [61–63], include the blood–brain-barrier (ca.15 nm), the nuclear pore complex (ca. 20–40 nm), the lung periciliary layer (20–40 nm), and the transmucosal mesh (ca. 340 nm).

LbL assembly onto nanoparticles can be achieved using a range of approaches (refer to Section 6) with subsequent sizes largely dictated by the nanoparticle core itself and the thickness of the surrounding polymer layers. For centrifugal-based LbL colloid assembly with polymers lacking significant secondary structure, individual layers typically add 4–5 nm HD to the total particle diameter (2–2.5 nm radial thickness) [27,35,64]. Spray-based methods [65] often yield nominally thicker shell layers, adding 5–8 nm HD to particle diameter per adsorbed layer. Biopolymers such as hyaluronic acid (HA), which present complex secondary structure, can often adsorb with layer thicknesses [36,42,43] many times greater than polyelectrolytes such as poly(L-lysine) (PLL) or dextran sulfate (DXS) [66,67]. Charge density on the initial particle core can further dictate subsequent layer thickness, with denser adsorbed layers required for efficient charge reversal. Thus, depending on the end-application, LbL systems with multiple layers can be utilized without compromising function. In applications such as imaging in which much smaller nanoparticles are coated, much can be achieved with just 2 to 4 layers in an LbL coating, as will be discussed in upcoming sections.

Surface charge is another key design parameter for LbL nanoparticles used in biomedical applications. As with other nanoscale diagnostics and therapeutics, positively charged constructs should be avoided due to attraction to the negatively charged luminal surface of blood vessels [68], the kidney globular basement membrane [69], and epithelial cell membrane surface [70], as well as their tendency for erythrolysis of sialic acid-rich red blood cell surfaces [71]. Electrostatically stabilized (polyanionic) LbL nanoparticles, while capable of relatively long circulation, can also interact with various extracellular matrix (ECM) components including collagen type I. Wilson et al. [72] for example, found that polyanion-terminated gold nanoparticles could reduce the lag (nucleation) phase of collagen self-assembly and increase the dynamic shear modulus of polymerized collagen gels (Fig. 2.1a). In contrast, polycation-terminated LbL nanoparticles had no effect on the mechanical properties of the collagen. Interestingly, both polyanion- and polycation-terminated nanoparticles inhibited gel contraction by cardiac fibroblasts, suggesting that these LbL nanoparticle architectures influence cell behavior via multiple mechanisms. As demonstrated above, the biophysical interactions of LbL nanoparticles are highly dependent on their physiochemical properties and while, size- and charge-dependent interactions are well-described in many cases, others such as shape [65,73–75], surface topography (roughness) [76], and mechanical stiffness [77–79] – recently explored with micron and submicron LbL nanoparticles – have yet to be fully described with nanoscale LbL particles. Future research in these areas will no-doubt expand the diversity of potential biomedical applications utilizing these novel structures.

2.3. Chemically tailoring the biological interactions of LbL nanoparticles

2.3.1. Surface modification for steric stability

Steric stability – from both synthetic and native biopolymers – can greatly improve the in vivo stability of nanoparticles. This phenomenon is attributed, in part, to decreased serum protein adsorption (i.e., protein corona [80,81] formation) and opsonin binding, the latter of which can contribute to efficient clearance by the mononuclear phagocyte system. For this reason, LbL nanoparticles allow the advantageous use of highly negatively charged polyelectrolytes as the final outer layer of nanoparticles designed for systemic delivery. A dense negative charge yields significant repulsion between the nanoparticle and many of the negatively charged serum proteins, as well as cells that the particle may encounter, which have net negative outer membranes due to the charged polysaccharides bound to most mammalian cells. Furthermore, polyanion functional groups that introduce a number of bound water molecules at physiological conditions may provide additional energetic barriers that enable longer plasma half-lives. Antifouling biopolymers such as polyanionic hyaluronic acid [36,42,43] and alginate [64] for example, allow LbL nanoparticles to circulate for significantly longer residence times – in some cases as long as 24 to 28 h elimination half-lives [35,58] – than their non-sterically shielding anionic counterparts. The density of the macromolecular brushes generated on the nanoparticle surface are a function of the underlying charge of the positively charged layer as well as the charge density and size of the polyanion adsorbed as the final layer — both of these parameters are readily adapted with pH, ionic strength and choice of polyion in generating the LbL film. The resulting outer layer may in some cases present a much denser, and therefore more effective, brush layer than those generated using other means such as covalent chain grafting to particle surfaces. Because a number of native extracellular matrix molecules may be used in this process, it may also be possible to optimize these systems to achieve lowered recognition by the immune system.

Along with the use of intrinsically charged polyions, it is possible to incorporate neutral stabilizing polymer layers. Due to their intrinsic ability to block adsorption [82], such steric stabilizers are typically appended to already-adsorbed polymer layers in a “grafting-to” type fashion. As with other biomedical colloids, poly(ethylene glycol) (PEG) is the most common steric polymer employed with LbL nanoparticles, however poly(2-ethyl-2-oxazoline) (PEOx), poly(vinyl alcohol) (PVA), and other water-borne polymers provide opportunities for further research. In its simplest form, a neutral steric stabilizing layer this can be achieved using a polyelectrolyte-containing block copolymer such as PLA-b-PEG [83], many of which are commercially available.

Kim and coworkers [83] have similarly demonstrated the use of a PEG block copolymer to sterically stabilize LbL-functionalized liposomes, here, with the block copolymer incorporated as both the terminal and intermediate polyanion layers. Doxorubicin-containing cationic liposomes were layered with 5.5 bilayers of poly(L-aspartic acid)-block-PEG and PLL, resulting in a neutral (approximately −5 mV) PEG-stabilized nanoparticle. In this block copolymer multilayer format, layer thicknesses were relatively large, adding ca. 15 nm HD to particle diameter per adsorbed layer (7.7 nm radial thickness) (Fig. 2.1b).

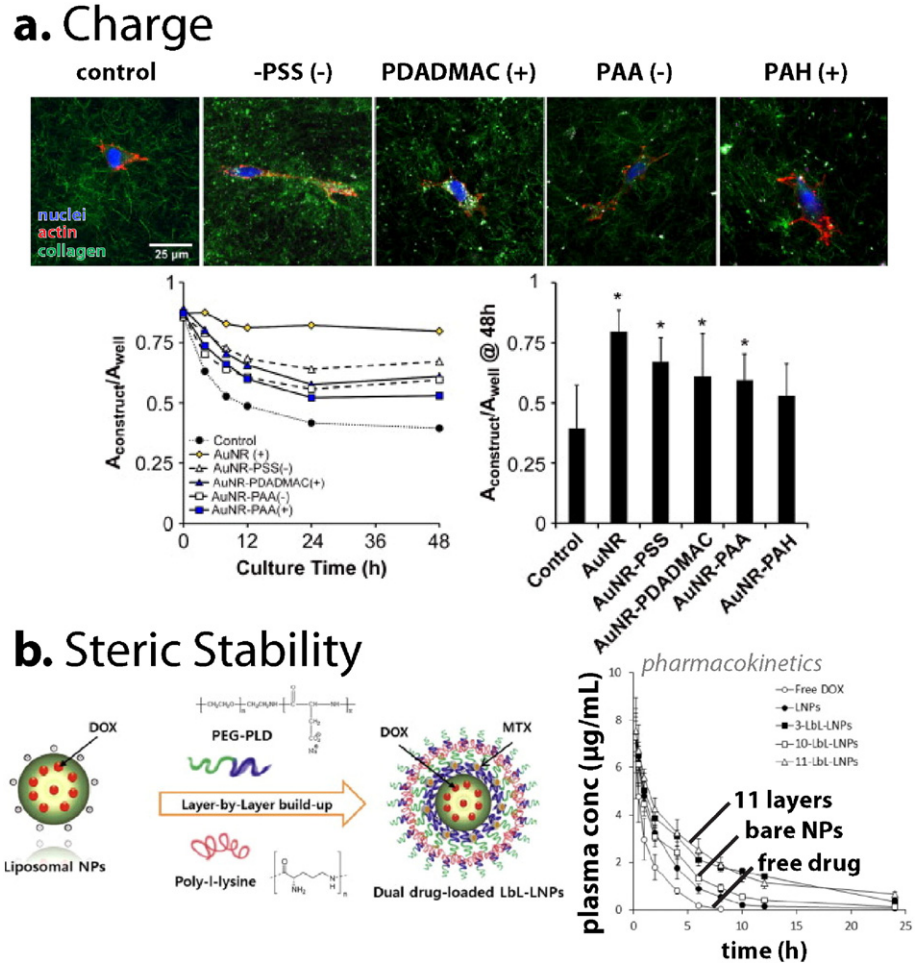

Fig. 2.1. Modular LbL nanoparticle architecture provide diverse biophysical interactions. a) Collagen interactions from LbL nanoparticles of varying charge (top) and particle-inhibited gel contraction by cardiac fibroblasts (bottom). b) Poly(ethylene glycol)-block-poly(L-aspartic acid) stabilized liposomal doxorubicin (left) and LbL stabilization-dependent circulation kinetics in Sprague–Dawley rats.
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Interestingly, drug release kinetics were significantly slowed for nanoparticles of identical terminal composition but increased multilayer numbers; circulation half-lives in mice, however, were identical for compositionally related LbL nanoparticles (ca. 5.8 h, first order).

Click-type and carbodiimide coupling chemistries are also highly amenable to the installation of steric polymers onto LbL nanoparticle surfaces. Polyelectrolytes containing biorthogonal azide and alkyne moieties, or transcyclooctene (TCO) and tetrazine groups, are also widely available or obtained in high yield and allow for the facile conjugation of end-functional linear polymers such as PEG-azide or PEG-tetrazine, respectively. Carbodiimide coupling is an attractive option for LbL nanoparticles terminated with weak polyelectrolytes such as polyacids and polyamines and allows for the addition of a wide variety of linear and/or branched stabilizers. For example, Shen and coworkers [84] used carbodiimide coupling to covalently conjugate PEG to the surfaces of PLGA submicron particles coated with PEI/PAA multilayers. The authors demonstrated significant reduction in the magnitude of anionic surface charge (approximately −37 to −12 mV), interestingly, in the absence of interparticle crosslinking as indicated by dynamic light scattering (DLS) measurements. Lastly, although less-explored with nanoscale LbL particles, surface stabilization can also be achieved via living polymerization (e.g. atom-transfer radical-polymerization, ATRP [79]) off of particles bearing surface-bound initiator groups, allowing for radially heterogeneous multilayered coating with antifouling terminal groups such as hyaluronic acid [42,43].

2.3.2. Microenvironment-responsive LbL nanoparticles

The outer layers can serve as outstanding passive targeting agents; however, LbL systems can be tuned to be responsive to microenvironments that are medically relevant. In prior work [58,63], we demonstrated a biotin–avidin conjugation approach to prepare pH-responsive multilayered nanoparticles via a biotinylated cationic terminal layer. Using neutravidin as an intermediate linker, iminobiotin-labeled PEG was installed on the outer particle surface, shielding an underlying cell-penetrating polycation surface that was revealed at hypoxic tumor pH conditions [42,43]. While particles displaying both iminobiotin-PEG and pH-nonresponsive biotin-PEG freely circulated for >24 h, only pH-sheddable LbL nanoparticles efficiently targeted breast tumor xenografts for extended periods in vivo, colocalizing with hypoxia inducible factor 1 α (HIF1α) and decreasing relative hepatic accumulation.

On the other hand, it is also possible to take advantage of the physicochemical nature of an LbL film to devise a bilayer of two weak polyelectrolytes (e.g. polyacid and polybase) that exhibit a shift in charge at a given pH. A pH responsive outer layer LbL film of (PLL/HA) is a strongly negatively charged particle at bloodstream pH that swells, increases in surface roughness, and loses cell-repulsive anionic surface charge at hypoxic tumor pH [42,43], thus causing significant uptake in hypoxic tumor regions; the same HA terminated film also targets the CD44 receptor, which is overexpressed highly in many aggressive cancer types including ovarian and lung cancer, thus imparting multiple

targeting mechanisms within a singular bilayer thin film. Another means of enabling cell-responsive behavior is to utilize other aspects of the cellular microenvironment, such as the more highly reducing cytoplasmic environment. Yang et al. [85] have developed methods for the efficient cellular delivery of LbL nanoparticles by making use of a pH responsive, 'bioreducible' polymer layer consisting of a disulfide crosslinked low molecular weight BPEI obtained by reaction with dithiobis(succinimidyl propionate) (DTSSP, Lomat's reagent). The thiol-modified polyamine was used to condense siRNA and was subsequently shielded using a polycation-b-PEG copolymer containing poly(2-(2-aminoethyoxy)ethoxy) phosphazene (PAEP). At pH 6.8, cell internalization and in vitro cell killing was augmented ca. 4- and 2.8-fold, respectively, compared with physiologic pH (7.4). In vivo, the pH-responsive LbL nanoparticles targeted subcutaneous tumor xenografts >2-fold more efficiently than non-pH responsive constructs, with tumor-specific mRNA silencing improved ca. 2.4-fold.

2.3.3. Affinity-targeting of LbL nanoparticles

Another powerful aspect of LbL nanoparticle modularity is the ability to further decorate particle surfaces with affinity ligands that can bind or associate with a given cell type, organ, or tissue of interest. With LbL nanoparticles, this targeting can be directed towards surface proteins on diseased or peripheral cells and often initiates ATP-dependent cell uptake through various mechanisms including phagocytosis, caveolin- or clathrin-mediated endocytosis, macropinocytosis, and other pathways. Common LbL nanoparticle affinity ligands include small molecules [86], peptides [86], glycopolymers [87], aptamers [88,89], and antibodies [45]. For modifications with poorly water soluble targeting ligands such as folic acid, carboxamide coupling [90] is an attractive option for the amidation of polyacids that can proceed in both aqueous and methanol solvents. For example, Morton et al. previously used carboxamide coupling to install hydroxyapatite-binding bisphosphonate alendronate ligands onto PAA which was used to terminally layer doxorubicin-containing LbL nanoparticles [91]. 143B osteosarcoma xenograft-bearing mice intravenously treated with the particles exhibited progressive disease from the untargeted constructs, but significant disease stabilization from the targeted nanoparticles, reflecting the altered biodistribution profiles of actively targeted LbL nanoparticles. Lin and coworkers [86] have also explored the use of affinity targeting to develop an LbL nanoparticle-based magnetic resonance imaging (MRI) contrast agent based on Gd^{3+}-conjugated silica nanoparticles. By electrostatically adsorbing a cationic, integrin-binding K_7RDG peptide onto the PSS-terminal LbL nanoparticles, in vitro targeting and *T*1-weighted MRI contrast from HT-29 colon cancer cells was markedly improved. Boyer et al. [87] employed a related approach whereby a copolymer synthesized by reversible addition-fragmentation (RAFT) polymerization was side chain modified with glucose and galactose, the latter of which can specifically bind with hepatic galactose/N-acetylgalactosamine receptors. The particles underwent crosslinking in the presence of the glucose-binding lectin, concavalin A, and were competitively displaced by free sugar, demonstrating target-specific binding by the LbL nanoparticles.

2.3.4. Exogenous-targeting of LbL nanoparticles

In addition to ligand (affinity) based targeting, LbL nanoparticle activity and tissue accumulation can be directed via external actuation. This exogenous trigger can take many forms including, photothermal conversion [92–94], photoisomerization, photocleavage, and magnetic enrichment [33,95,96]. Zebli et al. [97] for example, demonstrated that magnetic fields could be used to exogenously enrich circulating LbL nanoparticles in a tissue specific manner (Fig. 2.2a). Using PSS/PAH-coated iron oxide nanoparticles and a microfluidic flow chamber mimicking vascular circulation, the authors showed that magnetic fields could be used to selectively augment nanoparticle accumulation in MDA-MB-435s breast cancer cells in a spatially selective manner. Further, Richtering and coworkers [95] demonstrated that magnetic nanoparticles can also be incorporated into submicron LbL particles consisting of a thermoresponsive poly(N-isopropylacrylamide) (PNiPAM) core surrounded by positive or negative iron oxide nanoparticles, and interlayers of PSS/PDADMAC (Fig. 2.2b). At physiologic temperatures, the particles were ca. 240 nm HD, swelling to 340 nm HD between 20 and 30 °C. At both temperatures, the particles could be directed by an applied magnetic field, providing opportunities for magnetic field-based tissue enrichment or hyperthermia-modulated tissue transport.

3. Drug delivery with LbL nanoparticles: regulation of release kinetics

While the outer layers of LbL-modified drug carriers can provide favorable in vivo stability and active targeting properties, the rest of the film is capable of additional functionalities useful for drug delivery. In this section we address the ability to regulate the release of therapeutics encapsulated in the core template through the generation of an LbL membrane around the core, which acts as a nano-scale drug reservoir. This useful feature is tunable through the careful selection of LbL film constituents, which can work in concert to provide a desirable release profile for numerous applications ranging from cancer therapy to oral insulin administration. This section is organized by the identity of the core template for various LbL formulations, but it should be noted that any of the LbL films discussed below could be formed on any suitable template as needed.

3.1. Responsive LbL films for controlling delivery from loaded cores

The LbL platform has broad applicability towards modifying conventional nanoparticles that have already been extensively characterized. This includes important drug-carriers such as poly(lactide-co-glycolide) (PLGA) [23,84,98], mesoporous silica [30,86], and liposomal [25,26] colloidal systems. Each of these vectors has attractive properties for drug delivery, but each also has weaknesses that have hindered their clinical translation. Here we review how recent work in the LbL community is addressing some of these weaknesses through the introduction of multifunctional LbL films.

3.1.1. LbL modification of PLGA cores

PLGA particles are well studied materials that can encapsulate a variety of cargo, including small molecule drugs, proteins, and nucleic acids [98], commonly through emulsification techniques [99]. These templates benefit from LbL modification in several ways, the first being increased specific targeting and avoidance of RES clearance, as discussed in Section 2. Another major benefit, and what will be focused on in this section, is greater control over drug release by minimizing the initial burst-release of cargo, as well as by extending the duration of release. For example, Venkatraman and coworkers [100] characterized the effects of LbL-modification on submicrometer sized PLGA particles, and found that LbL coatings of either PAH/PSS or PLL/DXS were able to suppress burst release of FITC-dextran (4 kDa) and extend its release over 4 days under physiological conditions in vitro (Fig. 3.1a-b). While both PAH/PSS and PLL/DXS films slowed release of the model drug in these systems, only PAH/PSS films benefited from increased layer depositions. This is likely due to differences in the binding affinity that holds these films together, and hydrophobicity of the polymer backbones involved, leading to a more swollen and permeable film in the case of PLL/DXS. Once crosslinks were introduced however, both films efficiently reduced burst release (ca. 3 fold) and extended the duration of release.

Morton at el. [64] described the in vitro and in vivo regulation of small molecule drug release from several LbL-modified PLGA nanoparticles. This work demonstrated that three different LbL formulations (*PLGA*-(*PLL/DXS*)$_3$, *PLGA*-([*PLL/DXS*]$_2$/*PLL/Alginate*), and *PLGA*-([*PLL/DXS*]$_2$/*PLL/HA*)) could reduce initial burst-release from 35%

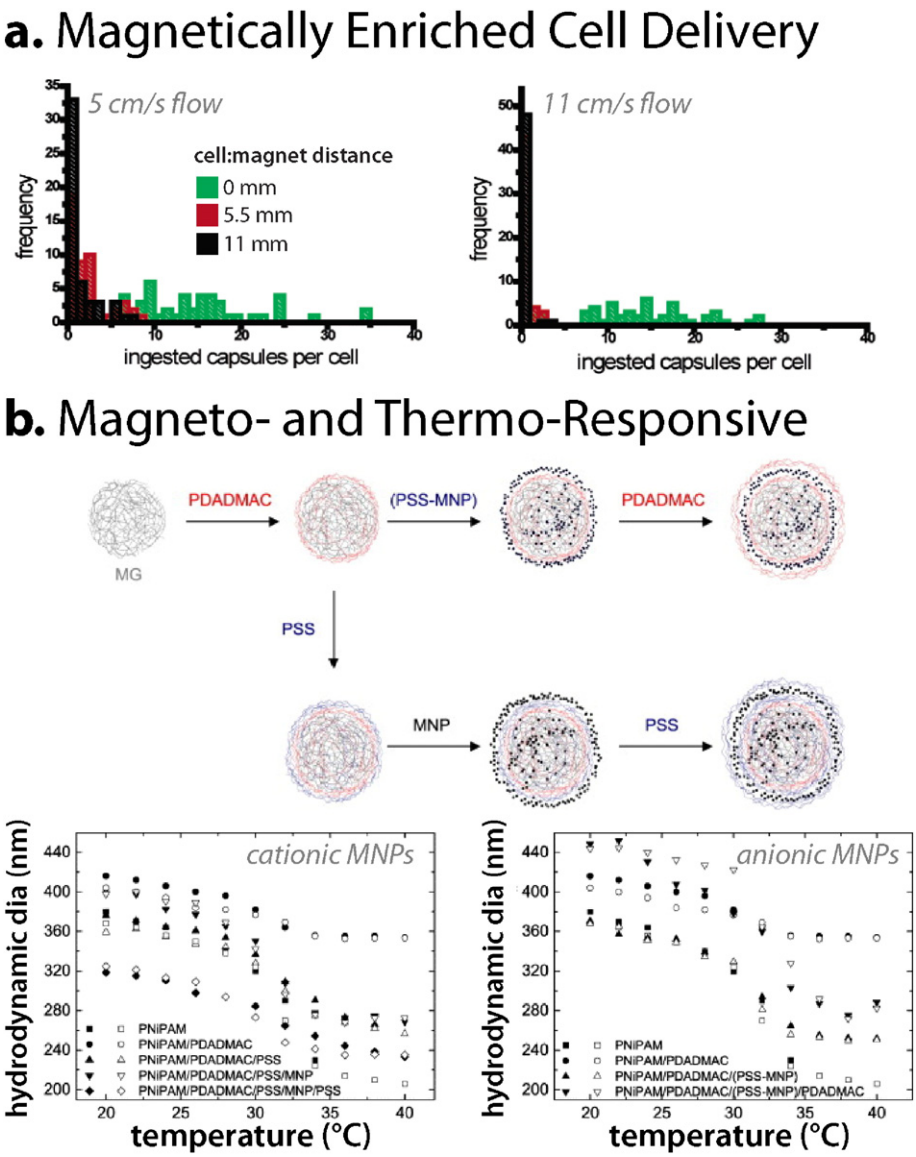

Fig. 2.2. LbL nanoparticles can be engineered to respond to exogenous triggers that can direct their accumulation. a) Magnetic field-directed enrichment of PSS/PAH-coated iron oxide nanoparticles in microfluidic flow cultures of MDA-MB-435s breast cancer cells. b) LbL nanoparticles constructed from a thermoresponsive poly(N-isopropylacrylamide) (PNiPAM) core and a PSS/PDADMAC multilayered shell containing magnetic iron oxide nanoparticles. The particles respond to magnetic field actuation and, at physiologic temperatures, shrink from ca. 340 to 240 nm HD.
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(uncoated PLGA) to less than 20% of the entrapped doxorubicin under physiological conditions in vitro, and extended drug release over the course of 5 days (Fig. 3.1c). This work also demonstrated that alginate and HA-terminated formulations could sustain the delivery of the model drug cardiogreen (indocyanine green dye) in vivo for up to 24 h, in stark contrast to bare PLGA cores that release their full cargo within 8 h (Fig. 3.1d).

3.1.2. LbL modification of mesoporous silica cores

Another excellent template for LbL assembly is colloidal mesoporous silica (MS), a highly characterized material system with great potential for multifunctionality [101]. Sub-100 nm MS nanoparticles can be prepared at large scales [102], and with extensive control over their porosity [103], which subsequently dictates their drug loading and release behaviors. The versatility of MS drug-carriers is highlighted by their capacity to carry either hydrophilic [104] or hydrophobic cargo [105], depending on the loading technique employed. MS particles are naturally amenable to LbL-modification due to their innate negative charge, but can be chemically modified to yield cationic particles as well [106]. Due to their stability in organic solvents, these materials can also be functionalized through hydrogen-bonding driven LbL assembly [14]. While MS drug-carriers provide several distinct advantages, their clinical translatability has been hindered by toxicity concerns [107,108]. However, in LbL-modified MS systems, it appears that problems with toxicity can be resolved either by core-dissolution to remove silica from the final carrier [106,109] or by introduction of biocompatible surfaces [30].

Several stimuli-responsive LbL formulations have been prepared from silica templates, which highlight the capacity for LbL films to be more than passive diffusion barriers. By exploiting the well-characterized pH-dependent swelling of LbL films [110], Chen and coworkers [111] were able to design LbL drug carriers templated from MS nanotubes that exhibited pH-specific drug release in vitro depending on the choice of film constituents. This approach takes advantage of the pH-dependency of LbL assembly, where it is generally found that the ideal pH is that which maximizes the charges of the

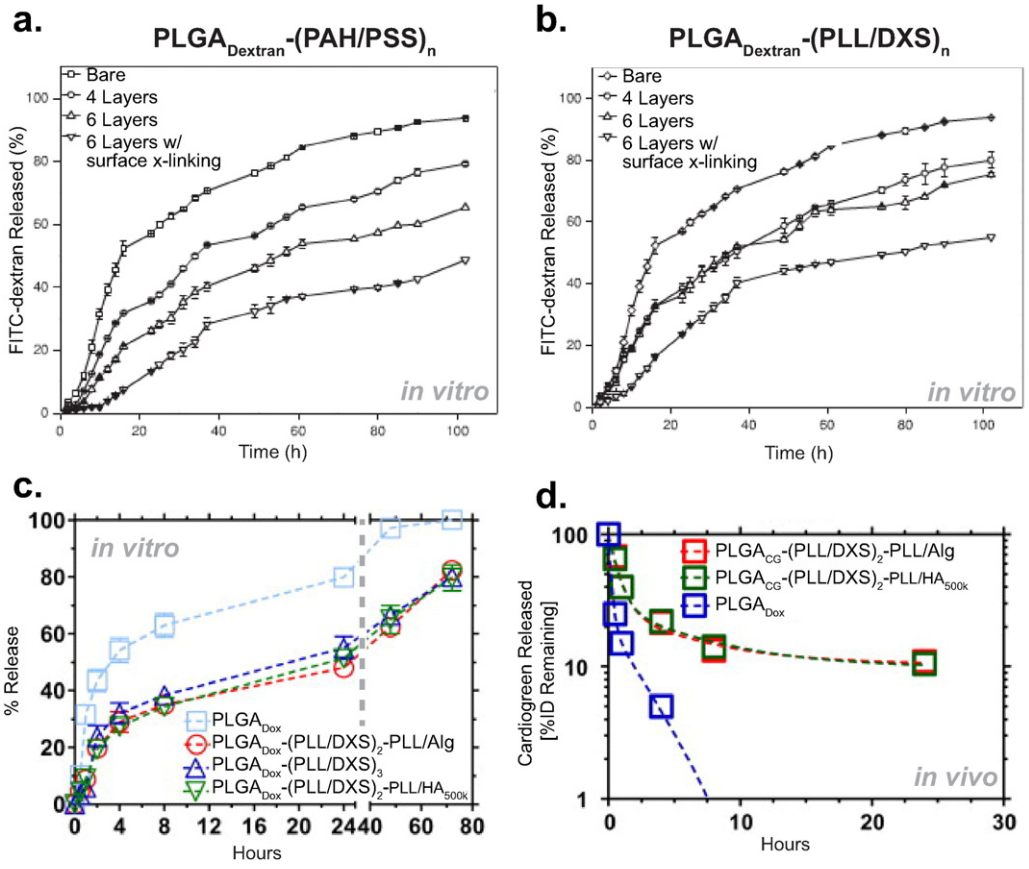

Fig. 3.1. LbL films regulate the release of drugs encapsulated in the core template, allowing for extended release and reduced burst-release both in vitro and in vivo. a) Submicrometer PLGA particles can be coated with poly(allylamine hydrochloride) (PAH) and poly(styrene sulfonate) (PSS) to slow the release of the model drug FITC-dextran (4 kDa) from the core. Additional layers further slow release, and crosslinking of the amines in the film (via glutaraldehyde) provides the slowest release profile. b) Similarly, coating these particles with poly(L-lysine) (PLL) and dextran sulfate (DXS) slowed release, but differ from PAH/PSS films in that additional layers do not slow release, highlighting the differences in permeability between different LbL films. c) PLGA nanoparticles also benefit from LbL modification, and can be coated with PLL, DXS, alginate (Alg), and hyaluronic acid (HA) to significantly slow the release of doxorubicin from the PLGA core. d) These observations carry over to in vivo experiments, where PLGA nanoparticles coated with biocompatible LbL films prolong the release of a model drug, cardiogreen (CG), over 24 h.
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precursor polyelectrolytes. By titrating away from this ideal pH value, the electrostatic forces that hold the LbL film together weaken and allow for swelling, becoming more permeable to the diffusion of cargo. Therefore, by carefully choosing the polyelectrolytes for a given application, an LbL nanoparticle can selectively respond to pH of the target tissue to initiate drug release. This effect is demonstrated nicely by

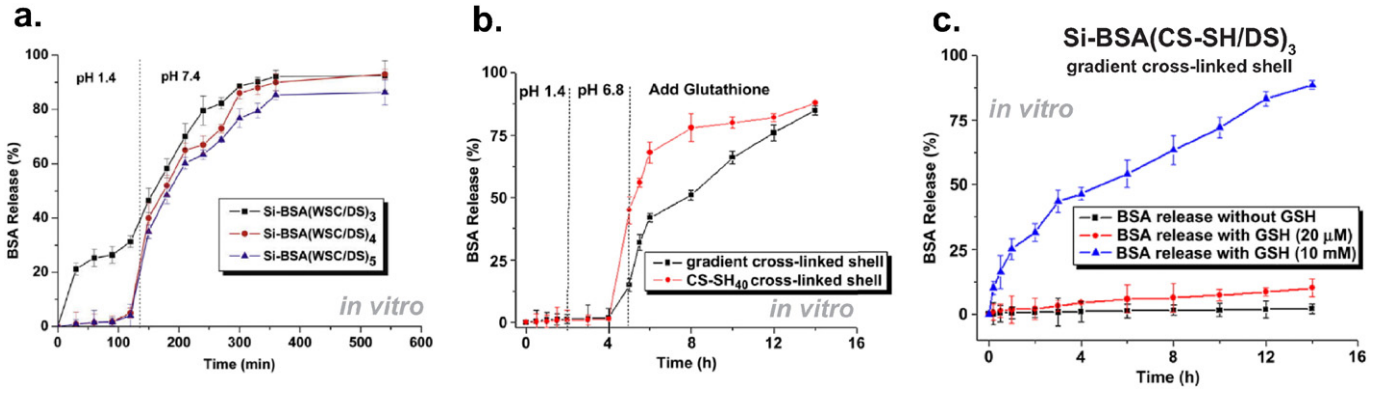

Fig. 3.2. Careful selection of LbL film constituents generates smart, responsive films that regulate the release of drugs from the core template. Such films can significantly reduce payload leakage outside of the targeted environment through several means. a) LbL films can be triggered to degrade and release drugs by changes in pH, and in this case a film constructed from water-soluble chitosan (WSC) and dextran sulfate (DS) exhibits stability at acidic pH (WSC protonated) but begins to disassemble at neutral pH (WSC deprotonated). [Si = silica nanoparticle, BSA = bovine serum albumin] b) Introduction of bio-responsive elements adds further control to such films, and in this case silica nanoparticles were coated with cysteine-conjugated chitosan (CS–SH) in order to form disulfide crosslinks throughout the film. These crosslinks prevent release of cargo until exposure to intracellular, disulfide-breaking enzymes (glutathione, GSH). Additional control over release is introduced by spatially controlling crosslinking density through the film by using CS–SH with differing degrees of cysteine conjugation. c) Disulfide-crosslinked LbL films are stable at extracellular GSH concentration but slowly release cargo over 14 h at intracellular concentrations of GSH.
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Shu et al. [109], who designed smart LbL carriers suitable for oral delivery of an encapsulated protein. In this system, BSA-loaded MS nanoparticles were coated with low molecular weight (6 kDa) water-soluble chitosan (WSC) and DXS. By using WSC, which is only cationic under acidic conditions, the nanoparticles were highly stable in conditions similar to the stomach environment (pH 1.4, 37 °C, and agitation) and prevented release (<10%) of the encapsulated protein (Fig. 3.2a). However, at the neutral pH found in the intestinal lumen, WSC loses its cationic charge and the capsules readily release their cargo. This platform was later modified by replacing WSC with cysteine-conjugated chitosan (CS–SH) in order to generate disulfide crosslinks within the LbL film [106], which prevented drug release at neutral pH until exposure to intracellular levels of glutathione (Fig. 3.2b-c). By generating CS–SH with different degrees of cysteine conjugation, the authors were able to generate a crosslinking gradient within the film, which they demonstrate is more effective in preventing burst-release after GSH addition. Importantly, this approach highlights the broad range of release profiles achievable with the use of an LbL system, and the ability to introduce orthogonal responsive elements in a hierarchical manner to specifically control not only when release occurs, but also the kinetics once release is initiated.

The pH-responsive functionality of LbL films is not limited to electrostatically stabilized formulations, as was recently demonstrated by Li et al. [112] with a biomolecular LbL particle templated from MS nanoparticles. In this system, MS nanoparticles were electrostatically coated with a layer of PEI and Concanavalin A (Con-A). Con-A is a carbohydrate-binding protein that can be adsorbed onto surfaces via hydrophobic attraction, and allows for subsequent bio-templated LbL assembly with the neutral polysaccharide glycogen or galactomannan [113]. Con-A loses its carbohydrate-binding ability at acidic pH, causing the LbL film to rapidly disassemble and release doxorubicin encapsulated in the core. These types of films are also sensitive to degradation via competitive binding [110], wherein Con-A dissociates from glycogen in favor of a carbohydrate to which it has greater binding affinity towards, such as glucose.

3.1.3. LbL modification of liposomal cores

Highly biocompatible liposomal drug carriers are among the most promising templates for LbL modification. Liposomes are of particular interest due to their versatility and use in some of the most well known and readily produced nanomaterials for therapeutic applications. Like other traditional nanoparticle cores, liposomal drug carriers can be modified via LbL assembly to improve stability and drug release characteristics while simultaneously providing new stimuli-responsive behavior and targeted biological interactions. Work by Fukui and Fujimoto [26] demonstrated that chitosan and dextran sulfate-coated liposomes exhibited increased stability against surfactants and improved drug-retention. This work tested the retention of several model drugs (1-hydroxy pyrene-3,6,8-trisulfonic acid (HPTS), alendronate, and glucose) and observed prolonged in vitro cargo-retention over 60, 85, and 144 h, respectively. Interestingly, if the films were built using DNA instead of DXS, then heating the liposomes to 60C could trigger release, likely via denaturation of the adsorbed DNA (Fig. 3.3a). Along these lines, Kim and colleagues [83] described an LbL-modified liposome loaded with doxorubicin, which exhibits a pH dependent release profile under physiological conditions in vitro (Fig. 3.3b). This formulation leveraged the pH-sensitivity of LbL films composed of weak polyelectrolytes, in this case PLL and the block co-polymer poly(L-aspartic acid)-b-PEG. At slightly acidic pH, such as in the tumor microenvironment, protonation of aspartic acid's carboxylic acid side chain initiates film breakdown down and release of cargo. However, at pH 7.4 the films are stable, and improve drug retention significantly relative to bare liposomes after deposition of only 3 layers.

Liposomal cores present a special advantage due to their ability to easily co-encapsulate drugs, including the simultaneous loading of hydrophilic and hydrophobic payloads [114,115]. Combining this capability with responsive LbL films can drastically improve the efficacy of liposomal combination therapies, as was demonstrated by Dreaden et al. [43] with a nanoparticle formulation consisting of dual-inhibitor loaded liposomes functionalized with a bilayer of PLL and HA. With the aid of the pH-responsive and CD44 receptor-targeted functionality of the LbL film, this nanoparticle successfully and safely delivered a synergistic inhibitor pair in a murine model of triple-negative breast cancer, leading to disease stabilization and a 4-fold decrease in tumor size compared to controls (Fig. 3.3c). Notably, use of a non-responsive and non-targeting drug-loaded liposome (coated with PLL/DXS) exhibited less therapeutic efficacy, whereas drug-free vehicles of the PLL/HA formulation exhibited a small but significant (ca. 1.6 fold) reduction in tumor size. This intriguing result may indicate an antiproliferative role of the LbL film itself, though further work is required to fully characterize the possible mechanisms at play. Importantly, this approach overcame the profound hepatotoxicity associated with free administration of this combination therapy [116].

3.2. Direct LbL modification of therapeutics

3.2.1. Stabilizing insoluble drug crystals

In addition to functionalizing traditional colloidal systems, the LbL technique has been applied towards generating soluble nano-drug carriers directly from insoluble drugs. Use of a sonication-assisted LbL assembly technique developed by Agarwal et al. [57] allows for the functionalization of drug crystals that, while under ultrasonication, are nanoscopic in size. Subsequent introduction of appropriate polyelectrolytes can stabilize these nanocolloids in solution and provide a template for additional LbL assembly. Notably, this approach achieves very high (ca. 90 wt.%) drug-carriers in the 100–200 nm size range relevant for systemic delivery. This approach has been applied towards the generation of LbL nanoparticles from important hydrophobic drugs including tamoxifen [57], paclitaxel [117], curcumin [118], resveratrol [119], camptothecin [120,121], and ibuprofen [122]. Importantly, as indicated by Lvov and coworkers, this approach allows for extended release profiles of hydrophobic drugs, generally on the order of 6–8 h, though longer durations are possible with additional layers [119]. However, burst-release kinetics continues to dominate in these systems, although introduction of crosslinks within the LbL film may be able to overcome this issue. Overall, LbL functionalization of insoluble drug crystals is a promising technique to improve the efficacy of a variety of drugs whose insolubility is a major limiting factor for clinical efficacy.

3.2.2. Stabilizing polyplexes

Along the lines of applying LbL assembly directly to therapeutics, interesting LbL formulations have emerged that use polymer and nucleic acid complexes as a core template [123]. Generally, the mixture of highly cationic polymers with nucleic acids leads to a cationic nanoparticle and excess free polymer, which can be useful for mediating in vitro transfection experiments. However, in vivo, these polymer–DNA complexes are often unstable, toxic, and mediate weak transfection [124], likely due to the rapid clearance typical for cationic nanoparticles and free polycations. Fortunately, by introducing additional functionality through LbL assembly, these materials become more stable and exhibit improved transfection efficiencies. In addition, the removal of free polymer during the preparation of multilayered nanoparticles can reduce in vivo toxicity without compromising gene transfection efficiencies. For example, Saul et al. [22] demonstrated improved in vitro efficacy of PEI–DNA complexes through the addition of PEI/PAA multilayers on the complexes, thus eliminating the presence of free polymer.

Harris et al. [125] further demonstrated the efficacy of this approach by electrostatically adsorbing a coating of an anionic designer peptide onto a cationic polyplex composed of a custom poly(beta-amino-ester) (C32–117) and plasmid DNA. Two designer peptides were used, each consisting of a long stretch of glutamic acid residues (poly-E) but one of the peptides contained a short cationic insert (poly-E-cat).

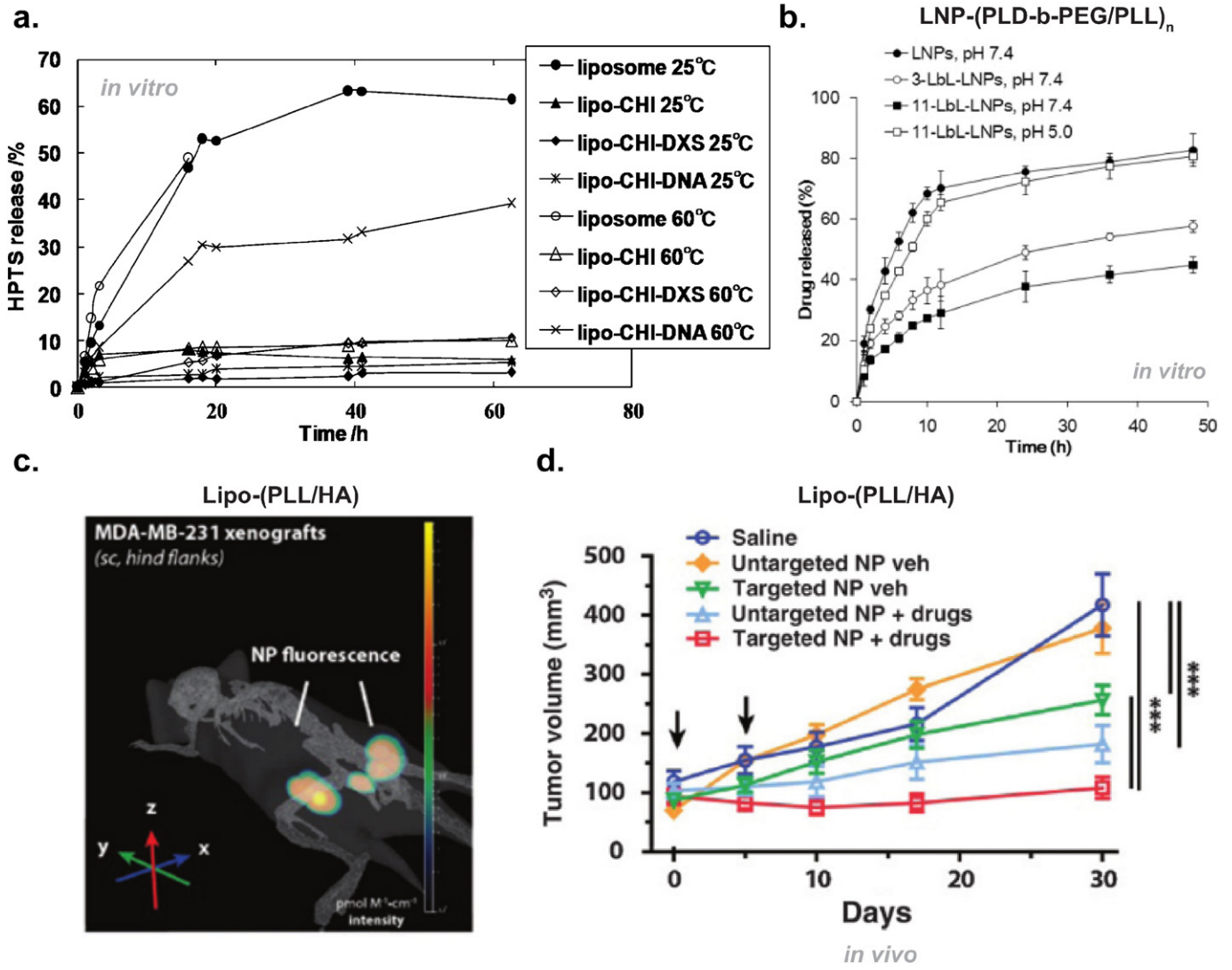

Fig. 3.3. LbL films introduce multiple avenues of control over the delivery and release of drugs from clinically-approved drug carriers like liposomes. a) Temperature-responsive film constituents can allow a thermally-triggered release profile, as is achieved in this nanoparticle formulation where 1-hydroxy pyrene-3,6,8-trisulfonic acid (HPTS)-loaded liposomes were coated with chitosan (CHI) and DNA. Heating the particles to the denaturation point of DNA disrupts the film and allows slow leakage of the cargo over 60 h. b) Use of weak polyelectrolytes such as poly(L-aspartic acid)-b-polyethylene glycol (PLD-b-PEG) and poly(L-lysine) to generate LbL films produces nanoparticles that begin to disassemble at acidic pH, such as in the hypoxic tumor microenvironment. [LNP = liposomes]. c) Films composed of PLL and hyaluronic acid (HA) combine the pH sensitivity of weak polyelectrolyte-based LbL films with receptor–ligand targeting via HA-CD44 binding. This bimodal targeting allows for favorable accumulation of liposomes into tumors in vivo, and d) maximizes the effect of the drug cargo to stabilize disease progression in a model of triple-negative breast cancer.
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Interestingly, peptide-coated polyplexes exhibited nonlethality in mice, in contrast to equivalent dosages of uncoated polyplex. Furthermore, the in vivo biodistribution and transfection efficiency of these nanoparticles was shown to be dependent on both the peptide sequence and the degree of surface functionalization with the peptide. By optimizing these parameters, efficient gene delivery could be targeted to one of two tissues: the liver, or the bone marrow and spleen (Fig. 3.4a-b). Other recent work with LbL-polyplexes has yielded interesting formulations that combine both electrostatic and hydrophobic interactions to coat and stabilize DNA [126] and siRNA [127] polyplexes.

LbL modification of polyplexes provides biostability and control over biodistribution, but another important advantage is the condensation of large, loose polyplexes into a nanoparticle suitable for systemic delivery. This effect is most profound with LbL modification of RNAi- and antisense oligonucleotides (ODN)-microsponges [36, 128], a novel class of materials composed of polymers of repeating antisense oligonucleotides that self-assemble with the inorganic byproducts of their synthesis to form micrometer scale particles [129]. These particles can mediate potent gene knockdown if internalized by cells, where the necessary machinery exists to break them down into their functional units. While these are promising vectors of gene delivery, their systemic delivery is hindered by their large size. Fortunately, this obstacle can be overcome by applying LbL assembly techniques, which condenses microsponges down to ~200 nm (Fig. 3.4c), and stabilizes them for effective delivery both in vitro and in vivo (Fig. 3.4d-e) [36].

Overall, responsive LbL films highlight the versatile and tunable multifunctionality offered by LbL nanomedicines. Importantly, the functionality provided by the LbL film is independent of the identity of the core template, introducing the possibility to develop modular therapies that can be adapted to different templates as needed. By careful selection of the individual components of the film, as well as clever use of chemical modifications after film assembly, researchers can develop highly specialized materials to control the release of therapeutics from a diverse range of nanoparticle drug-carriers.

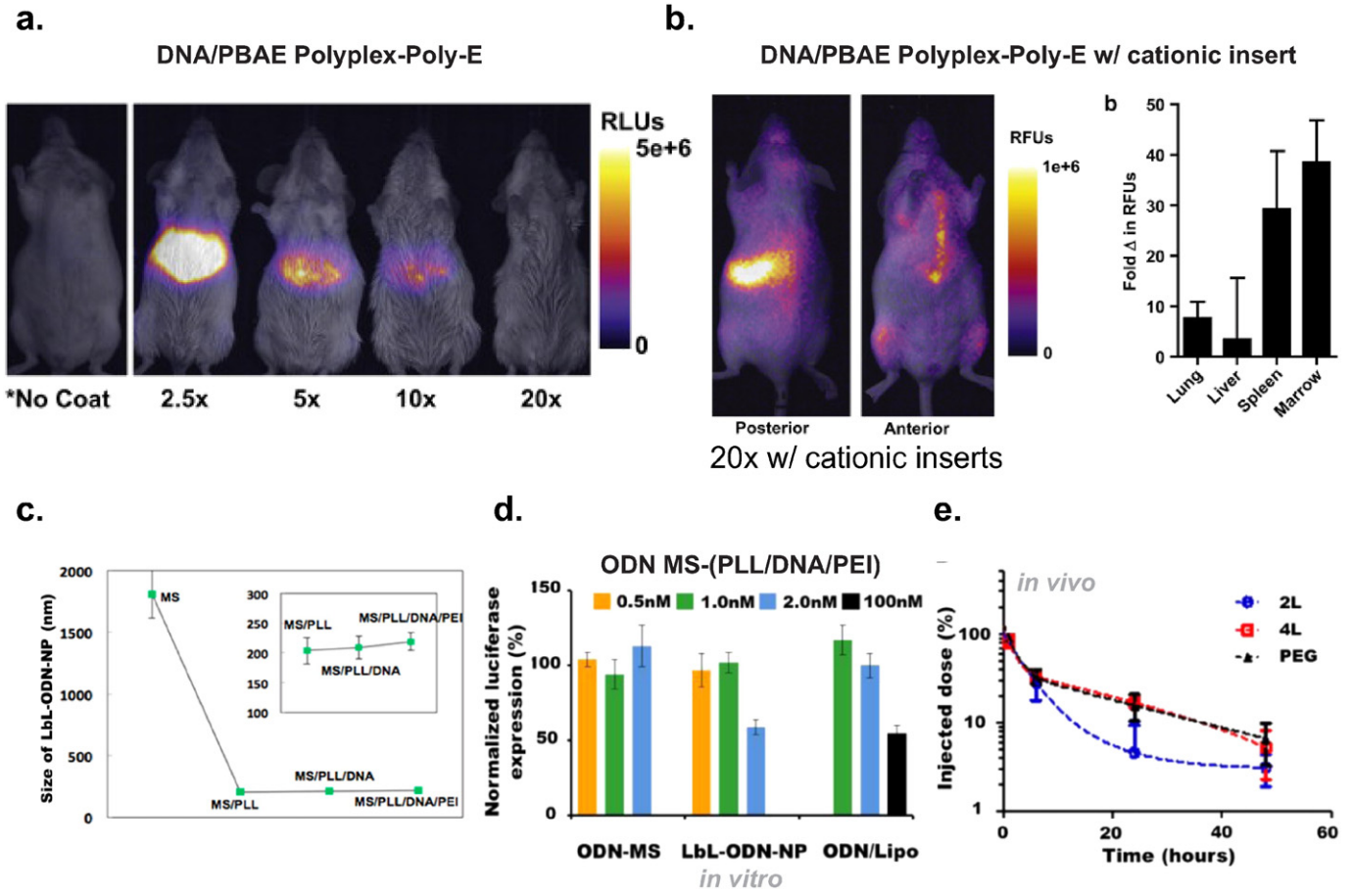

Fig. 3.4. LbL modification improves the stability, transfection efficiency, and size of fragile template cores like nucleic acid polyplexes and microsponges, making them suitable for in vivo gene delivery. a) Coating DNA/poly(beta-amino-ester) (PBAE) polyplexes with peptides consisting of glutamic acid (Poly-E) allows for in vivo stability and high luciferase transfection rates in the liver. The extent of outer layer surface functionalization can be controlled by the degree of excess poly-E used during assembly (denoted in the figure as 2.5×, 5×, 10×, and 20× fold-excess of poly-E to core), and plays an important role in transfection efficiency. b) By changing the outer coating to a glutamic acid peptide containing cationic inserts, the same nanoparticles were redirected to the marrow and spleen, highlighting the role the outer layer plays in biodistribution. c) Antisense oligonucleotide microsponges (ODN-MS) become highly condensed through LbL-assembly, achieving a submicrometer size (~200 nm) suitable for systemic delivery. d) LbL-modified ODN-MS can more efficiently silence genes and e) exhibit extended blood circulation times in vivo. [2 L = (PLL/DNA), 4 L = (PLL/DNA/PEI/DNA), PEG = (PLL/DNA/PEI/poly-(L-glutamic acid)-b-polyethylene glycol)]. Reprinted with permission from (a–b) [110], (c–e) [44]. Copyright (a–b) 2010 Elsevier, (c–e) 2014 American Chemical Society.

4. The LbL film as a vector for drugs and combination therapy

A key advantage to the LbL platform is the capacity to incorporate therapeutics directly into the LbL film itself. This can be achieved through a variety of means, including direct adsorption of charged biopolymers including proteins [33], peptides [34], and nucleic acids [24,130,131], as well as through the incorporation of synthetic drug-conjugated or drug-loaded polymers [132]. Because the drug is adsorbed into the nanocomposite, and deposition steps can be repeated, very high weight percent loading is possible [5,39–41]. This section discusses how researchers have leveraged this capability to successfully deliver therapies without a drug-loadable template. Additionally, this section covers formulations that combine drug-loaded templates with therapeutic-containing films to create sophisticated combination therapy vectors.

4.1. Functional protein and peptide delivery from LbL films

It has been well established for some time that incorporation of proteins into the LbL film can be achieved without destabilizing structural or functional features, making the approach amenable towards therapeutic protein delivery [9,12,40,133]. Of note, Tseng and colleagues [33] demonstrated that functional protein encapsulation can take place in nanoscale LbL colloidal systems when they sequentially adsorbed glycol chitosan (GC) and bovine serum albumin (BSA) onto 10 nm magnetic iron oxide nanoparticles (Fig. 4.1a). Characterization of the nanoparticles using circular dichroism (CD) indicated that the adsorbed BSA retains its native structural conformation (Fig. 4.1b). Subsequent probing of active binding sites with several small molecules further corroborated that BSA was functionally active within the LbL film.

Researchers studying the incorporation of the peptide hormone insulin into LbL films have similarly confirmed the retention of structure and function with electrostatically adsorbed biomolecules [34,134]. Recent work by Choi and colleagues [135] extends this principle to LbL films containing basic fibroblast growth factor (bFGF) assembled onto 30 nm iron oxide magnetic nanoparticles by leveraging the role of another film constituent, heparin sulfate (HS). HS is a highly anionic biopolymer, which makes it easy to work within the context of LbL assembly. Notably, HS also binds to various growth factors, including bFGF, in a process that stabilizes and aids in docking with relevant cell-surface receptors [136,137]. In this system, Choi et al. take advantage of both the electrostatic attraction and structural affinity between HS and bFGF to mediate LbL assembly. Once adsorbed onto a heparin-sulfate layer, the bFGF layer, which has a net positive charge at pH 7.4, was a suitable foundation for the adsorption of anionic graphene oxide. Although no structural analysis was done in this work, retention of biological activity is suggested by the induction of fibroblast proliferation using bFGF-containing nanoparticles.

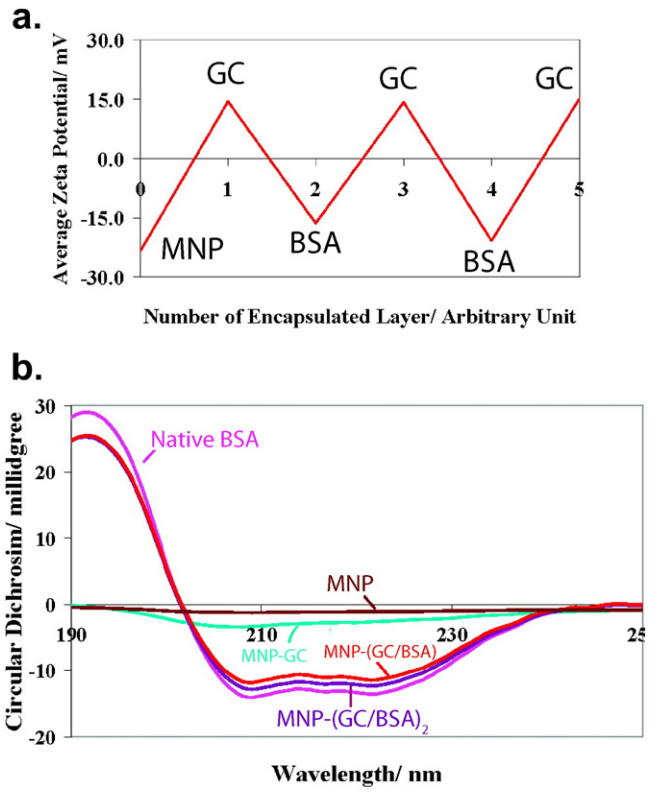

Fig. 4.1. Proteins can be incorporated into an LbL film in nanoparticle systems, where they retain their structure and function. a) The construction of protein-containing LbL films on a 10 nm magnetic nanoparticle (MNP) is demonstrated by the sequential reversal of surface charge after the deposition of glycol–chitosan (GC) and bovine serum albumin (BSA). b) The structural properties of BSA are preserved in the resulting LbL film, as determined through circular dichroism experiments.
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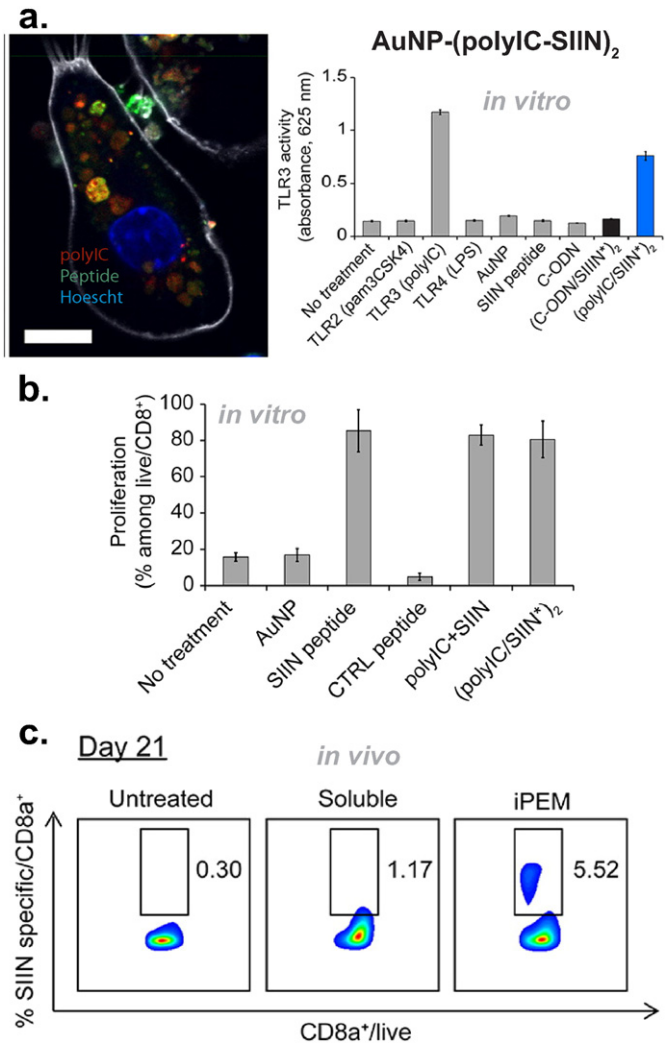

Fig. 4.2. LbL films can be composed entirely of immunomodulatory polymers to build novel nanoparticle vaccines. In this case, gold nanoparticles (AuNP) are coated with the adjuvant polyinosinic–polycytidylic acid (polyIC) and a cationic version of the SIINFEKL peptide antigen (SIIN*). a) Dendritic cells internalize the resulting nanoparticles, which specifically induce toll-like receptor 3 (TLR3) activation (right panel). b) In coculture experiments, the LbL nanoparticles promote proliferation of antigen-specific CD8 + T cells. c) Mice immunized with the LbL vaccine show significantly higher CD8 + T cell responses relative to immunization with free adjuvant and antigen.
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4.2. Immunotherapy from LbL films

The incorporation of functional biopolymers into LbL films presents an opportunity to develop novel immunotherapies. Indeed, this concept has already been applied on the macroscale for the development of efficacious and pain-free LbL-microneedle mediated vaccination [138,139]. Interest for colloidal LbL vaccines has previously focused on the stabilization and coating of antigen-salt precipitates [140–142] or polyelectrolyte–antigen complexes [143], but recent work [144,145] describing the direct incorporation of antigen peptides into LbL films on nano- and submicrometer templates is yielding interesting results. For example, an LbL nanoparticle vaccine was recently described by Zhang et al. [146], where the LbL film was constructed entirely from immunologically active polymers in order to simultaneously deliver both adjuvant and antigen. In this work, gold nanoparticles were functionalized through the sequential deposition of the anionic adjuvant polyinosinic–polycytidylic acid (polyIC) and a cationic version of the SIINFEKL peptide antigen. In vitro, this vaccine was able to induce dendritic cell maturation and antigen-specific CD8 + T cell proliferation in a co-culture experiment, matching the activity of the free adjuvant/antigen pair (Fig. 4.2a-b). However, intradermal injection of this vaccine into mice was able to promote antigen-specific CD8 + T cell proliferation, yielding a significantly stronger response (ca. 5-fold) than that of freely administered polyIC and antigen (Fig. 4.2c).

These early reports of LbL nanoparticle vaccines are promising, though more work is required to bring reported responses inline with leading immunotherapies like amphiphile-based [147] immunizations. In particular, further optimization of antigen peptides that are suitably charged for incorporation into an LbL assembly should be explored. Special attention should be given to the incorporation of promising but systemically toxic adjuvant molecules, given the LbL platform's capacity to safely deliver cargo that might otherwise pose safety concerns. Lastly, efforts to include active targeting outer layers in these systems could help potentiate vaccine responses by specifically trafficking cargo to professional antigen-presenting cells. Overall, this work represents an exciting new direction for LbL-based nanomedicine, where the highly modular and versatile nature of LbL assembly may allow for the development of sophisticated nanoparticle vaccines.

4.3. Gene delivery from responsive LbL films

One of the most promising applications of therapeutic LbL films is gene therapy. Incorporation of DNA into planar LbL films was first described in 1993 by Lvov and colleagues [148], and subsequent work in the early 2000s extended this to colloidal templates [16,28,149–151]. Soon after, efforts with DNA-loaded LbL microcapsules demonstrated the ability to successfully transfect cells in vitro [152]. By 2008, Fuller et al. demonstrated the in vitro transfection capability of silica “C dot”

nanoparticles coated with PEI and DNA. Shortly thereafter, Breunig and colleagues [153] fabricated LbL particles composed of gold nanoparticles coated with PEI/siRNA/PEI. This formulation demonstrated significantly reduced polymer-mediated cytotoxicity compared to free PEI and mediated gene-specific knockdown in CHO-K1 cells in vitro. Notably, this platform was capable of effective siRNA delivery even in the presence of serum. Follow-up work by this group [154] demonstrated that the same platform could deliver plasmid DNA to cells in vitro, though expression of exogenous genes was not reported. Subsequent research focused on developing robust gene delivery vectors capable of both in vitro and in vivo gene delivery by optimizing the parameters that influence nucleic acid stabilization in vivo [155], efficient uptake into cells [35], desirable sub-cellular trafficking via endosomal escape [156], and efficient cytosolic release of the nucleic acid cargo [157]. In particular, clever selection of LbL film constituents, particularly cationic elements, has shown promise in mediating endosomal escape and cytosolic cargo release.

Previous work found that the choice of the cationic polymer can strongly influence transfection efficiency with polyplexes in vitro [158]. Green and colleagues [159] recently demonstrated that this principle holds for LbL nanoparticles as well by demonstrating how different combinations of PEI and custom biodegradable polycations (SS37 and 447) impacted nucleic acid loading and transfection efficiency. Notably, non-degradable PEI exhibited significantly higher loading (ca. 5-fold) of nucleic acid into the LbL film relative to the biodegradable polycations (Fig. 4.3a). The authors also found that the degree of terminal layer saturation played an important role in final in vitro transfection efficiency, implying the need to explore this parameter more closely (Fig. 4.3b). Although not all permutations of the LbL film were fully explored, this study touches on critical parameters for the design of LbL films for gene therapy, namely the balancing between nucleic acid loading efficiency, polycation toxicity, and endosomal escape capability. Important future work should systematically compare the transfection efficiency, toxicity, and sub-cellular localization of nanoparticles composed with different polycations, including more detailed trafficking studies that can examine the mechanism of endosomal escape and release in these unique systems in comparison to more typical synthetic gene vectors.

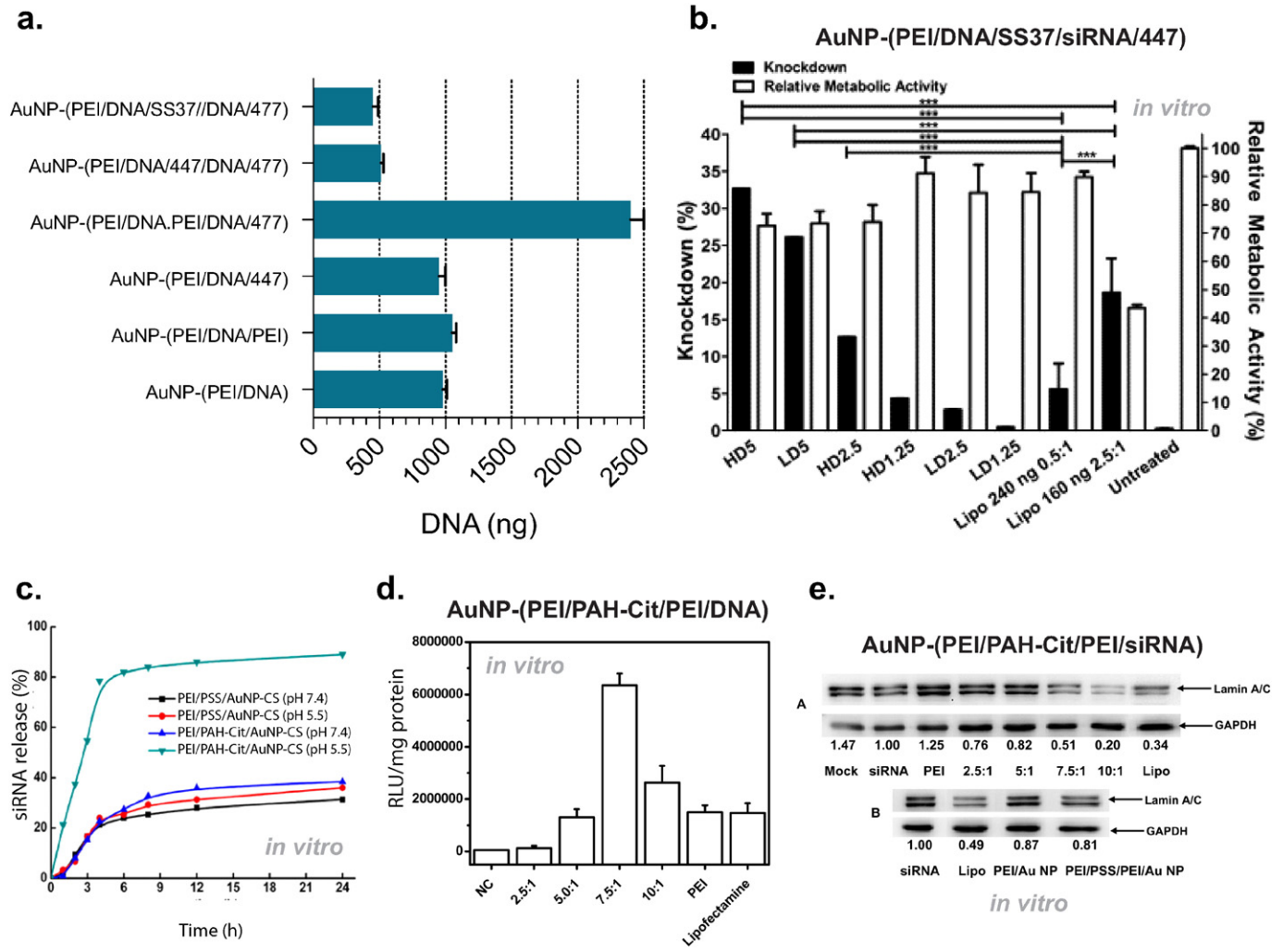

Fig. 4.3. Gene delivery through LbL films presents the opportunity to build sophisticated nanostructures to optimize transfection efficiency. Multiple variables are involved in the development of successful LbL gene carriers, and among the most important to optimize is the cationic elements of the LbL film. a) Different nucleic acid loading efficiencies have been observed between nondegradable polyethylenimine (PEI) and biodegradable poly(beta-amino-esters) like SS37 and 447. Chart generated from raw data from [145]. b) Additional consideration should be given to the degree of functionalization of outer cationic layers, and in this case improved biocompatibility and transfection occurred if the surface was less saturated. [LD = low dose, HD = high dose, Lipo = lipofectamine; the fold excess of polycation to core during assembly is given by the number following LD or HD]. c) Responsive polymers, such as charge-reversing poly(allylamine hydrochloride)-citraconic anhydride (PAH-Cit), facilitate the release of nucleic acid cargo at lysosomal pH where they become cationic and trigger film disassembly. In contrast, LbL nanoparticles built from nonresponsive poly(styrene sulfonate) (PSS) films retained their cargo. d) Charge-reversing elements within the film improved gene delivery significantly, and e) similarly aided in gene silencing via siRNA delivery.
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Responsive LbL films show promise for mediating endosomal escape and subsequent release of nucleic acid cargo. In particular, implementation of charge-reversing polymers for LbL assembly appears to be an effective strategy for triggering disassembly of the nanoparticle within an acidifying endosome. Guo et al. [157] demonstrated this approach by using poly(allylamine hydrochloride)-citraconic anhydride (PAH-Cit), nucleic acids, and PEI in an LbL assembly templated from gold nanoparticles. PAH-Cit exhibits a negative charge at neutral pH, but at pH 5 becomes cationic and rapidly destabilizes the LbL film through electrostatic repulsion (Fig. 4.3c). Nanoparticles consisting of gold core-(PEI/PAH-Cit/PEI/Nucleic acid) exhibited improved in vitro gene transfection and silencing, approximately 3 and 2 fold, respectively, compared to free PEI and lipofectamine (Fig. 4.3d-e). Subsequent work by this group extended this platform towards therapeutic gene delivery [160] and silencing [161] in several drug-resistant cancer cell lines in vitro.

In a similar approach, Chen at al. [162] designed charge-reversing LbL carriers of plasmid DNA encoding small hairpin RNA (shRNA) for gene silencing in vivo. This work used chitosan-aconitic anhydride (CS-Aco) as an anionic constituent in a gold-(PEI/CS-A/PEI/shRNA) formulation. In the acidic lysosome, the CS-Aco polymer is hydrolyzed into cationic chitosan to trigger film disassembly and cargo release. Importantly, this formulation was stable and nontoxic in vivo, mediating efficient silencing of the drug-resistance protein ABCG2 in HepG2 liver cancer cells. Mice harboring HepG2 xenografts exhibited reduced tumor burden using the charge-reversing formulation alongside freely administered doxorubicin. These results are interesting given that the nanoparticle's outermost layer is composed of the nucleic acid cargo, which could be damaged by nucleases during delivery. Other reports [93,163] also demonstrate that nucleic acids are resistant to degradation after adsorption onto an LbL particle in vitro, but further documentation of the limitations of this stability should be explored. Nonetheless, additional outer layers can provide critical benefits to LbL nanoparticles that include extended blood circulation times and active targeting properties, as discussed in Section 2.

4.4. Combination therapies from therapeutic LbL films and functional cores

One of the key strengths of the LbL nanoparticle approach is the ability to introduce two or more drug types within the layers and the core of the nanoparticle. Combining therapeutic-loaded LbL films with functional core templates is a powerful means to deliver combination therapies that can attack disease from multiple angles. Multipronged therapeutics are gaining a great deal of interest, particularly for the treatment of drug-resistant cancer with molecules that can engage or silence genes to enhance the effects of chemotherapy. Because of the modular nature of the LbL platform, nanomedicines can be designed that combine various therapies into a single particle, thereby guaranteeing the co-delivery of a specific drug ratio to target cells. Moreover, recent efforts [83,164] to modulate the temporal release from these systems indicate the potential to stage drug release in such a way as to maximize the effects of each drug.

Deng et al. [35] have recently demonstrated the therapeutic efficacy of a targeted LbL combination therapy platform that co-delivers siRNA and chemotherapy to a subcutaneous murine model of triple-negative breast cancer. This approach functionalizes a doxorubicin-loaded liposome with a functional, therapeutic-containing LbL film composed of PLA/siRNA/PLA/HA. In this formulation, the highly hydrated outer HA coating promotes biostability by reducing opsonization and nonspecific uptake, while simultaneously mediating receptor-mediated endocytosis via the CD44 receptor commonly overexpressed in breast cancer. Meanwhile, the biodegradable cationic PLA layers help to mediate endosomal escape and siRNA release into the cytoplasm. More specifically, this work aimed to overcome drug-resistance towards doxorubicin in triple-negative breast cancer, which is often mediated by enhanced drug efflux via upregulation of transmembrane drug efflux pumps, like the multidrug resistance protein 1 (MRP1). To that end, the applied therapy introduced siRNA against MRP1 (siMRP1) to break down the cancer's defense mechanisms and thereby maximize the effect of the co-delivered cytotoxic agent. The combination of siMRP1 with doxorubicin led to both efficient in vivo gene silencing and synergistic therapeutic efficacy when compared to the effects of monotherapy (Fig. 4.4). Notably, this work reported a differential

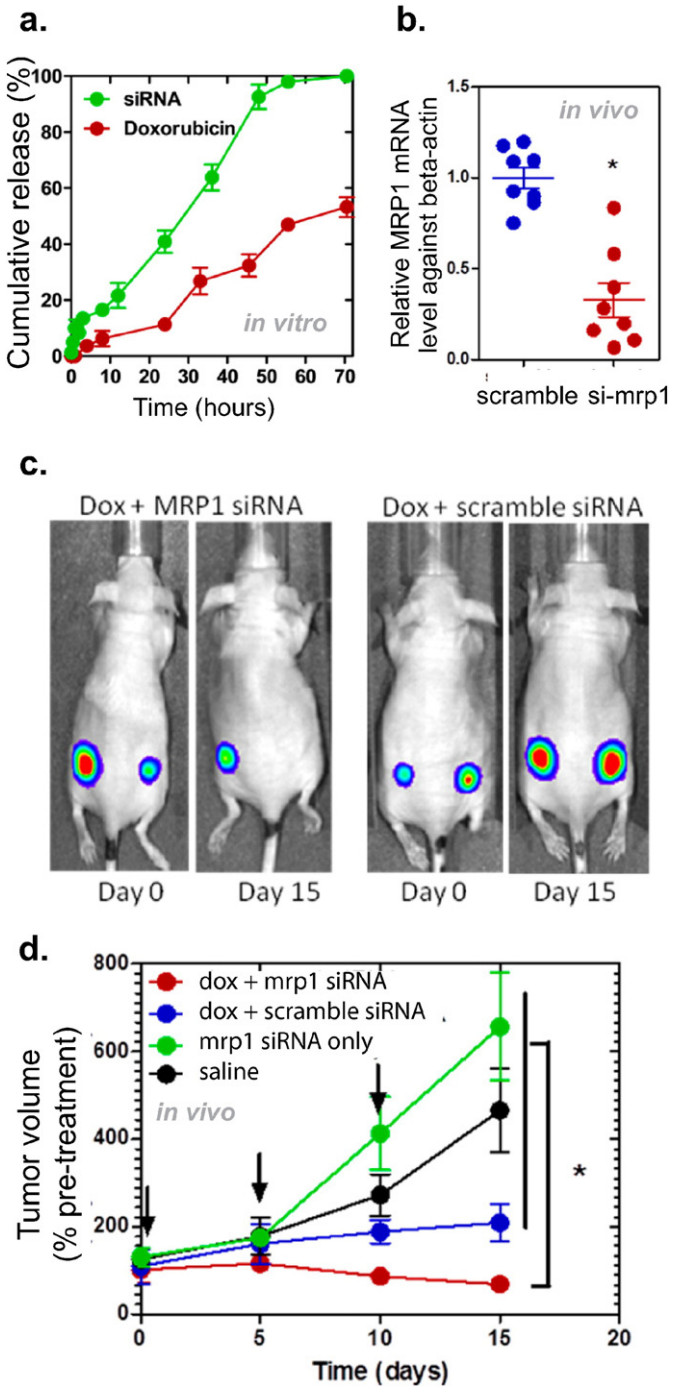

Fig. 4.4. Assembly of a therapeutic LbL film onto a drug-loaded core can generate sophisticated carriers of synergistic combination therapies. In this case, a doxorubicin-loaded liposome is coated with a gene-delivering LbL film composed of poly(L-arginine), siRNA against MRP1, and hyaluronic acid (PLA/siRNA/PLA/HA). a) This approach allows for differential release profiles of the therapeutics, with siRNA being released prior to the cytotoxic agent. b) The resulting nanoparticles efficiently silence expression of MRP1, a drug-resistance protein, within triple-negative breast cancer xenografts. c) The combination of gene therapy with chemotherapy provides significant improvements in therapeutic efficacy over monotherapy.
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release profile of the siRNA and chemotherapeutic, demonstrating the potential for staggered release from an LbL nanoparticle platform. This provides a key advantage, as it allows the siRNA time to sensitize the cell before the cytotoxic agent is released.

Overall, leveraging LbL technology opens the door towards the development of multifunctional vectors that can control the delivery of synergistic combinations of drugs. The approach allows researchers to carefully select different therapeutics and incorporate them into the carrier in such a way as to individually regulate their release. Simultaneously, careful selection of film constituents can provide further control over the biodistribution, pharmacokinetics, and stimuli-responsive drug release of these systems.

5. Diagnostic and theranostic applications of LbL nanoparticles

The LbL approach also lends itself towards other important biomedical applications such as imaging-based diagnostics through the incorporation of an imaging core, or by the conjugation of appropriate dye molecules to one of the thin film components. By using the techniques discussed previously, it is possible to develop sophisticated diagnostic and theranostic LbL nanoparticle systems. Here we will touch on several modes of imaging can be implemented into LbL systems using variations on known assembly methods, enabling the use of different imaging modalities that include paramagnetic, fluorescent, and near-infrared techniques, sometimes in combination for dual or multi-mode imaging technologies.

5.1. Magnetic resonance imaging with LbL nanoparticles

Magnetic resonance imaging (MRI) is a powerful imaging modality which has been broadly used for early tumor diagnosis, vascular imaging, neuroimaging, and functional imaging [48]. Current MRI contrast agents include paramagnetic small molecule agents (e.g. Gd^{3+}, Mn^{2+}) and superparamagnetic iron oxide (SPIO) nanoparticles [48]. Lin and colleagues [86] demonstrated the preparation of multifunctional MRI and luminescent LbL nanoparticles through the adsorption of a Gd-containing polyelectrolyte onto a luminescent core (Fig. 5.1). The authors studied the effects of Gd-polyelectrolyte deposition, and observed that additional layers granted greater MRI contrast. Notably, the small size of the formulation (~50 nm) is promising for use as a systemically administered MRI contrast agent.

SPIO Fe_3O_4 based nanoparticles are desirable for MRI imaging due to their strong contrast (i.e. high T_2 relaxivity), high magnetization, and excellent biosafety [165,166]. SPIO nanoparticles can be incorporated into LbL nanoparticles by constructing a polyelectrolyte film directly onto a suitably charged SPIO core. Shi et al. [167] use this approach to coat a 20 nm SPIO core with poly(glutamic acid) (PGA) and PLL. The resulting nanoparticle could then be terminated with a positively charged dendrimer, which could be further crosslinked via amidation with acetic anhydride and functionalized with a cancer targeting ligand (Fig. 5.3). Notably, these nanoparticles show specific targeting to a murine xenograft tumor model with enhanced MRI contrast. The extended time period of retention of the nanoparticles of a few days in the tumor is likely a consequence of targeting in combination with the small size of the LbL nanoparticle.

5.2. Fluorescence imaging with LbL nanoparticles

One main challenge of fluorescent imaging is quenching, which leads to diminished fluorescent signals, often due to energy transfer upon close contact between fluorophores [168,169]. LbL assembly provides precise control over the distances between nanoparticle core and adsorbed film components, thereby allowing the study of distance-dependent fluorescent quenching in imaging applications. For example, Decher and coworkers [170] used FITC- or rhodamine B-conjugated PAH as the fluorophore and unmodified PAH/PAA bilayer pairs as spacer layers to control the distance between fluorophore and core. It was found that increasing the distance between the fluorophore and core (~8 nm, 10 layers of PAH/PAA) significantly reduced fluorescence quenching by 5-fold, compared to 1 layer of PAH/PAA. Importantly, this study provides insights on how to reduce quenching when designing multiple fluorophore loaded LbL nanoparticles.

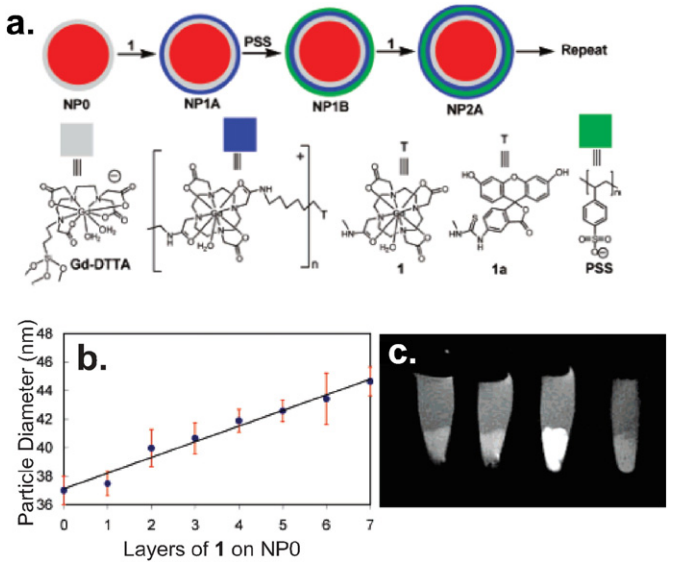

Fig. 5.1. LbL technology can be leveraged to create multimodal imaging nanoparticles, such as dual MRI and luminescent systems. In this system, hybrid silica nanoparticles (NP0) were loaded with luminescent probes and coated with the MRI contrast agent Gd-(siloxylpropyl)-diethylenetriamine tetraacetate (Gd-DTTA). This core could be LbL-modified using PSS and a cationic Gd-polyelectrolyte, Gd(III)-DOTA oligomer 1. (a) Schematic illustration of LbL assembly for hybrid nanoparticles with Gd-DOTA oligomer. (b) Dependence of LbL nanoparticles size on the number of deposited layers demonstrates the retention of very small sizes compatible with systemic administration. (c) T1-weighted MR images of cells that have been incubated with various nanoparticles. From left to right: control cells without nanoparticles, cells with MRI nanoparticles without targeting ligand, cells with MRI nanoparticles with RGD targeting ligand, cells with MRI nanoparticles with fewer Gd-containing layers.
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5.3. Near-infrared imaging with LbL nanoparticles

Lanthanide-doped rare-earth upconversion nanoparticles (UCNPs), which can be excited by near-infrared (NIR) light and emit high-energy photons, possess significant biomedical applications [171, 172]. Compared to traditional fluorescence imaging, UCNPs exhibit several advantages including enhanced tissue penetration, improved photostability, and reduced tissue autofluorescence [173–175]. Li and colleagues [176] demonstrated a proof-of-concept LbL formulation using a charged UCNP core coated with PAH and PSS. In this system, the LbL modified UCNP is functionalized with biotin and used as the fluorescent resonance energy transfer (FRET) donor, while biotin functionalized gold nanoparticles act as the FRET acceptor. Once in the presence of tetravalent avidin, the gold nanoparticle and UCNP become linked together, yielding a FRET signal.

Near-infrared probes, emitting in the NIR region (700–900 nm), can provide fluorescence imaging with higher resolution, lower background noise, and reduced light scattering [177,178]. Typical NIR dyes include inorganic quantum dots and organic fluorophores such as indocyanine green, the only FDA approved NIR dye for direct administration in biomedical diagnostics [179]. NIR quantum dots are core-shell structured nanoparticles composed of semiconductor materials such as CdSe, CdTe, PbS, and related alloys. Poon et al. [27] have established electrostatically assembled LbL nanoparticles for systemic delivery of QDs. Notably, LbL modification significantly increased the stability of the QDs in circulation, enhancing their circulation time.

5.4. Theranostic applications of LbL nanoparticles

Theranostic nanoparticles provide a single nanoplatform which integrates diagnostics and therapy for use in the new era of personalized medicine [180]. Although still in its early stages, this approach is already generating interesting results. For example, Cheng el al. [181] have used LbL assembly to coat SPIO and gold nanoparticles onto UCNP cores, yielding multifunctional theranostic nanoparticles. Here, the diagnostic functionality is provided by the luminescent UCNP core and the MRI-compatible SPIO nanoparticles within the film. Simultaneously, the SPIO nanoparticles also help to guide nanoparticles to the tumor via exogenous magnetic cues, and the gold nanoparticles provide a photothermal therapeutic effect upon light activation (Fig. 5.2). By using both magnetic guidance and light activated photothermal therapy, these nanoparticles successfully suppressed tumor growth and prolonged survival. These promising initial results suggest the potential to provide effective therapeutic treatments alongside multimodal diagnostics (Fig. 5.2d-e).

LbL theranostics can leverage the photoresponsive nature of certain core templates, like gold or up-conversion nanoparticles (UCNPs), to initiate phototherapy in addition to triggering drug release from therapeutic-loaded films. For example, Wang and colleagues [182] described the assembly of a therapeutic LbL film onto a NIR-responsive UCNP. In this system the UCNP is coated with PAA and PEI, loaded with the photosensitizing molecule chlorin e6, and finally terminated with siRNA against the oncogene Plk1. This nanoparticle could silence

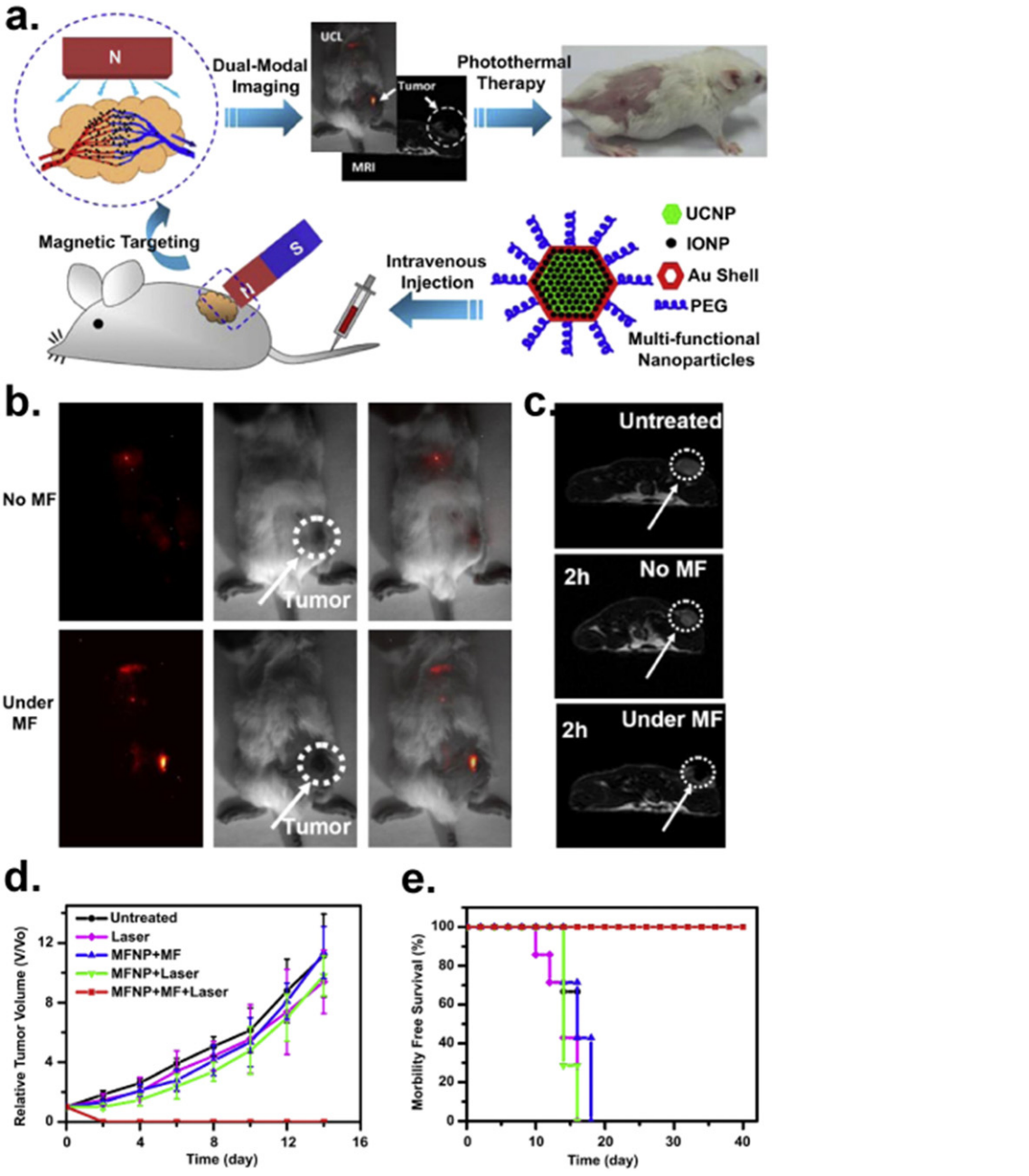

Fig. 5.2. LbL modification allows the preparation of potent theranostic nanoparticles. (a) Schematic illustration showing the composition of LbL assembled theranostic nanoparticles and the concept of in vivo imaging-guided magnetically targeted PTT. (b) Representative in vivo upconversion luminescent images of tumor-bearing mice with or without magnetic field (MF). (c) Representative T2 weighted images of tumor-bearing mice. (d) The tumor growth curve under different treatment groups. (e) Survival curve of tumor-bearing mice after different treatment groups.
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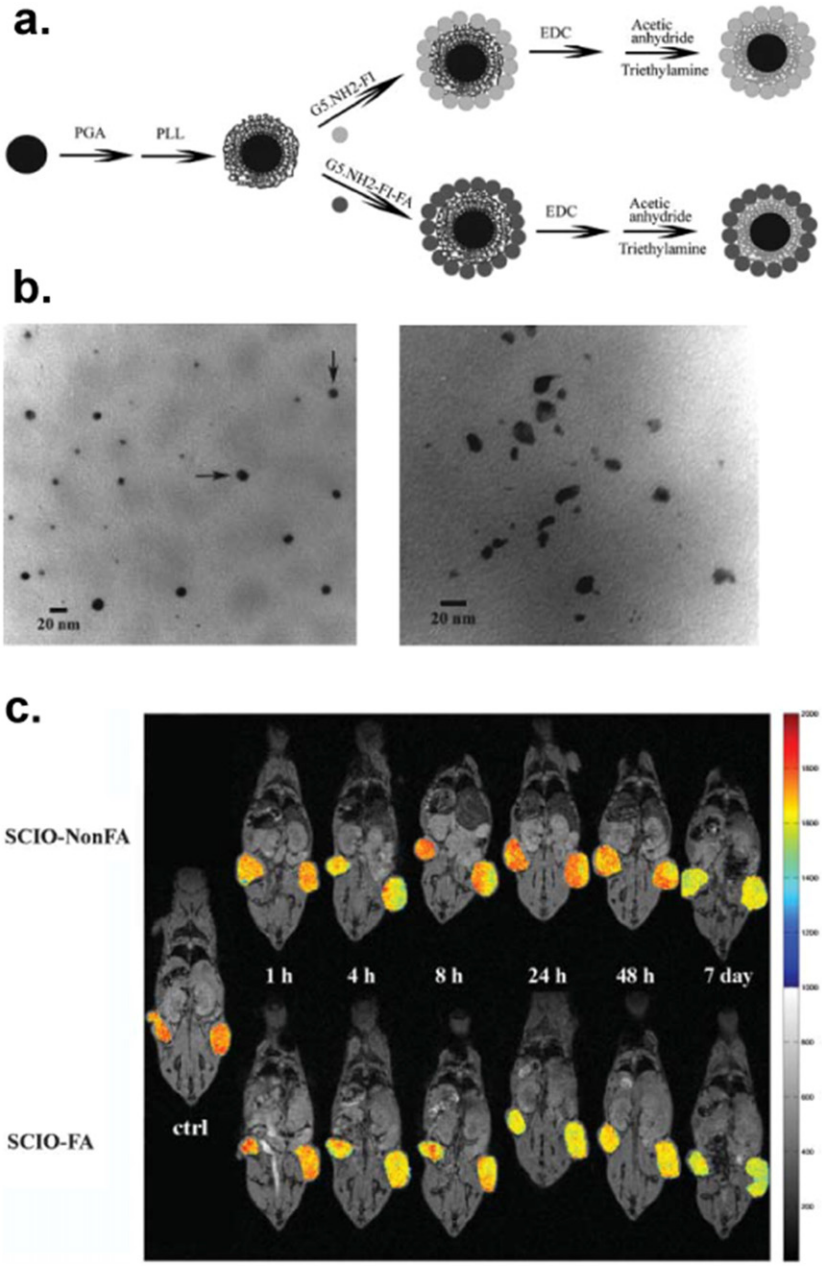

Fig. 5.3. LbL functionalization can improve the in vivo efficacy of MRI contrast agents to provide prolonged imaging of tumors. (a) LbL fabrication of targeted iron oxide NPs using PGA/PLL polyelectrolyte pair with a cationic dendrimer as the outermost layer. The outer layer can be further crosslinked and functionalization with targeting ligand (folic acid, FA). (b) TEM images of LbL nanoparticles. Left: before crosslinking and functionalization. Right: after crosslinking. (c) In vivo MR imaging of tumor: T2-weighted MR images of a murine xenograft model of epithelial carcinoma, at different time points after tail vein injection of LbL nanoparticles.
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Plk1 in HeLa cells in vitro, and interestingly exhibited better performance in serum-containing media relative to serum-free media. Excitation of the UCNP by NIR light also caused entrapped chlorin e6 molecules to generate cytotoxic singlet oxygen, and combination of this photodynamic therapy with delivery of functional siRNA exhibited a stronger antiproliferative effect in vitro than either treatment by itself. Shen et al. [93] have employed a related approach, using PEI-terminal LbL gold nanorods to complex (anionic) small interfering RNA (siRNA). These multifunctional nucleic acid carriers silenced their target protein, pyruvate kinase isoenzyme type M2 (PKM2), in vitro and provided spatially-resolved near-infrared laser photothermal cell killing in triple-negative breast cancer monolayer cultures (808 nm, 500 mW, 7 min).

Chen et al. [92] evaluated the in vivo efficacy of a similar approach, using noble metal-based photothermal contrast agents [62,183–189] to fabricate polyelectrolyte- (PDADMAC/PSS) coated gold nanorods that could be post-synthetically loaded with doxorubicin chemotherapeutic. Following tissue-penetrant near-infrared laser exposure of immunocompetent mice bearing S180 murine sarcoma xenografts (765 nm, 1.8 W cm^{-2}, 5 min), tumor progression was markedly slowed compared with equivalent doses of free doxorubicin.

5.5. Future directions for LbL diagnostic and theranostic nanoparticles

In current theranostic platforms, diagnostic tools can be a limiting factor for promising disease detection. For example, MRI is of high cost and requires sophisticated magnetic tuning and prolonged imaging processing incompatible with real-time tracking. Current fluorescence imaging tools, including UCNP, QD, and organic dyes, are within the

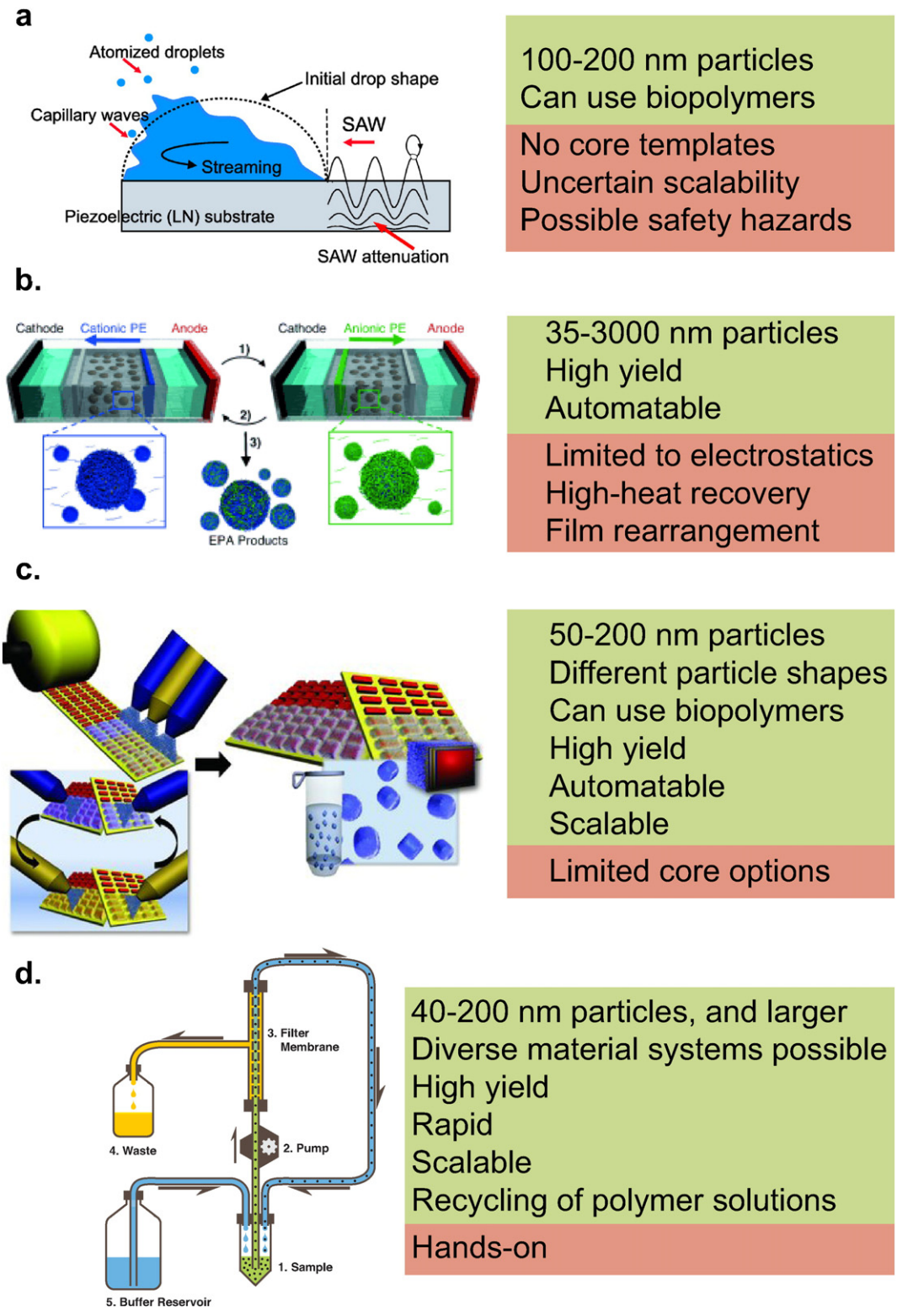

Fig. 6.1. This figure summarizes the major advances in LbL nanoparticle fabrication and highlights each method's key strengths and weaknesses. For details on these methodologies, refer to the main text.
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visible or first window of NIR region, which has limitations in tissue penetration and imaging resolution. As a result, fluorescence imaging in the second window of the NIR region (NIR-II, 1000 nm–1700 nm) has attracted much attention as it can enhance the penetration and provide high-resolution images in a real-time scale and low cost [190–195]. A variety of systems such as polymeric micelles [193], lipids [191,192], and bacteria phages [196] have been used for NIR-II imaging; however, these systems lack the modularity to package multiple functionalities such as stealth, targeting, and therapeutics. LbL-based NIR-II imaging is a promising future approach towards addressing these concerns,

and possesses significant potential towards the next generation of theranostics.

6. Translational steps towards LbL nanomedicine

The LbL platform is a promising means to develop modular and multifunctional nanoparticles with important biomedical applications that span drug delivery and diagnostics. However, before this technology can be successfully translated to the clinic, the field must focus on resolving persistent barriers limiting the preparation of these materials. In this section we discuss efforts to develop automatable fabrication processes for LbL nanoparticles that can be scaled to meet the needs of clinical and industrial implementation. Additionally, efforts to characterize the long-term stability of LbL nanoparticles, both in aqueous and lyophilized states, will be discussed.

6.1. Improving the fabrication of LbL nanoparticles

LbL assembly is a facile technique that generally only involves repeating two key steps: (1) exposing the substrate to a solution of adsorbable polymer, and (2) washing of the substrate to remove excess polymer. This simplicity is fully realized for the LbL functionalization of macroscopic materials, which can make use of hands-off automatable devices such as dipping robots. In contrast, colloidal LbL assembly lacks such an automatable and robust synthetic approach because the washing step becomes highly nontrivial at the submicro- and nanometer scales. The necessity for efficient purification between layer depositions has been well documented through systematic experiments by Decher and colleagues [197], who demonstrate that LbL nanoparticle assembly requires a significant excess of polyelectrolytes relative to the core template to fully saturate the surface and avoid particle flocculation. Excess polymer must then be removed prior to the addition of the subsequent layer, otherwise flocculation and the generation of a polyplex byproduct will occur. Traditionally, this has been carried out through extensive centrifugal wash steps following each layer deposition. Centrifugation is time intensive, requires manual handling of materials at every step, necessitates extensive optimization, and is notorious for losing significant product by driving the formation of insoluble aggregated pellets [13].

Soon after colloidal LbL assembly was first described, efforts were underway to solve the problem of fabrication. Reports spanning the past 16 years have yielded several interesting options for automating the production of large (>5 μm) particles [198–202]. Several promising options have also been presented for preparing LbL nanoparticles, such as an innovative template-free approach to LbL assembly described by Yeo and colleagues [203]. In this work, LbL was carried out through a series of atomization and suspension steps, and produced particles on the order of 100–200 nm composed entirely of assembled polymers (Fig. 6.1a). This approach successfully creates nanoscale LbL nanoparticles, but at the expense of being able to functionalize a core nanoparticle template. As such, it limits the ability to incorporate promising functionalities such as imaging cores and drug-loaded cores. Furthermore, it is unclear whether the atomization technique could limit the types of polyelectrolytes introduced into this system. The potential to aerosolize biologically active nanoparticles into the workspace also necessitates serious investigation of potential safety hazards associated with this technique.

In 2013, Richardson et al. [204] described a novel LbL assembly method in which core templates could be immobilized in agarose gel, and then coated through the systematic electrophoresis of charges species through the gel (Fig. 6.1b). This approach generated both micro and nanometer scale LbL colloidal systems (3 μm to 35 nm) at high yields (85%). The main drawback of this approach is the need to melt the agarose to recover the LbL nanoparticles, which could damage sensitive formulations. Indeed, this work observed that regardless of the charge of the terminal layer, all formulations exhibited a negative charge. This was attributed to potential re-arrangement of the films during the agarose melting step. Such rearrangement could initiate premature release of cargo located in the film, and could conceal elements of the outer layer that may be needed for biostability and active targeting.

A novel synthetic approach described by Morton et al. [65] leverages PRINT (particle replication in non-wetting templates) technology to facilitate the high-scale fabrication of LbL nanoparticles (Fig. 6.1c). PRINT technology allows for the production of highly uniform PLGA nanoparticle arrays onto planar surfaces. Notably, these particles can be produced in a variety of shapes and sizes ranging from 50 to 200 nm, and can be functionalized via aqueous spray-assisted LbL assembly while still immobilized on the planar array, eliminating the tedious centrifugal washing step. Subsequent recovery of the NPs requires only sonication of the macroscopic array to release the particles into solution, providing high yields even for highly layered formulations. This approach has significant potential to be automated to develop in-line manufacturing of LbL nanoparticles in a high-throughput, roll-to-roll fashion, and may well prove to be a solution for polymeric or biomacromolecular core particles that can be manipulated in PRINT. However, this technique is unable to use alternative core materials, such as liposomes and quantum dots.

Recent work has reported promising results for preparing colloidal systems using tangential flow filtration (TFF) technology, which can mediate highly efficient purification through a diafiltration process (Fig. 6.1d). Initial reports by Lohse et al. [205] demonstrated TFF-based millifluidic reactors could be used to prepare and functionalize gold nanoparticles. In particular, successful LbL functionalization of these materials was possible, but faced difficulties with efficient purification. Subsequent efforts by Correa et al. [32] resolved these issues by implementation of next-generation filtration membranes, composed of numerous porous, hollow fibers. This approach introduces solutions to very high surface areas of porous material to mediate the rapid purification of colloidal systems at high yields (ca. 96% per layer). Notably, this technique was able to generate biocompatible nanoparticles ranging from 40 to 150 nm composed of polystyrene, liposomal, or silica core templates functionalized with over a dozen biomedically relevant polyelectrolytes. Recent work by Björnmalm et al. [206] has also demonstrated the use of TFF technology for the preparation of LbL micro- and submicrometer scale particles, indicating the broad applicability of this technology for the scale-up of LbL colloidal preparations. However, this TFF technique is still a hands-on process, and while it represents a highly scalable flow-based approach, further work will be required to develop the degree of automation enjoyed for macroscopic LbL preparations.

6.2. Long-term stability of LbL nanomedicines

With the growing reports of clinically relevant LbL nanomedicines, it is becoming increasingly important to satisfy important questions about the long-term stability of LbL therapeutics. Unfortunately, little documentation exists on the shelf lives of different LbL formulations, particularly those that are drug-loaded and may experience leakage during storage. Furthermore, despite the widespread use of hydrolytically degradable polymers in the inner layers of these systems, there is little documentation on the limitations that hydrolytically degradable constituents place on the stability and subsequent in vivo stability of such systems after storage. These questions are vital for the eventual commercial and clinical success of these materials, both in terms of stockpiling, shipping, and storage.

Full reports on the matter of long-term stability are lacking, but important information can be inferred from the characterizations of various drug-loaded systems. For example, during characterization of their surface-enhanced Raman spectroscopy LbL nanoprobes, DeVetter et al. [207] observed temperature-dependent encapsulation stability. In these studies a Raman-active small molecule, methylene blue, differentially diffused out from an LbL film composed of PAA and PAH over the

course of 5 weeks, depending on the storage temperature. Samples stored at 22 °C and 37 °C exhibit a notable decay in spontaneous Raman signal over 5 weeks, suggesting outward diffusion of methylene blue away from the gold core. Samples stored at 4 °C provided more stable Raman spectra over 5 weeks, but still displayed some loss of signal. The authors then explored the effect of cross-linking on the stability of these probes, and found that crosslinking the amines on the outer PAH layer could significantly improve signal retention at 4 °C over the course of 5 weeks. These results are interesting, and provide insight on how drug-loaded LbL nanoparticles may behave under prolonged aqueous storage conditions, as well as means to extend their shelf lives.

Correa et al. [32] recently evaluated the stability of a small library of LbL nanoparticles formulated from carboxylate-modified latex cores coated with PLA and terminated with one of ten different polyanions. These particles were stored at 4 °C in distilled water for over 3 months, and broadly maintained their size, uniformity, and surface charge characteristics. However, some formulations did begin to increase in size, which indicates the need to test the long-term stability of new LbL formulations. This work also characterized the capacity for doxorubicin-loaded LbL liposomes to be lyophilized and stored at room temperature using a variety of different cryoprotectants, with or without a polyvinvly alcohol (PVA) based stabilizers. The results indicated that LbL formulations required a cryoprotectant to survive lyophilization and rehydration, but do not benefit from inclusion of PVA. LbL-modified liposomes were adequately protected by disaccharide cryoprotectants, and were able to maintain drug encapsulation, size, uniformity, and charge more reliably than bare liposomes. In particular, trehalose demonstrated the most consistent protection for these particles. The fact that these systems seem to be readily stored and lyophilized in a manner consistent with pharmaceutical manufacturing practices is quite promising for translation to commercial product.

7. Conclusions

The increasingly rapid pace of research in the field of electrostatically assembled drug delivery systems is revealing the promise of LbL nanoparticles for biomedical applications. The LbL platform has proven ideal for generating exciting new drug formulations that encompass a broad range of materials including small molecules, peptides, proteins, and the full range of nucleic acids, from siRNA to plasmid DNA. Moreover, the LbL approach allows for high drug loadings in nano-scale thin film coatings, thus enabling the delivery of therapeutic quantities of drug. Furthermore, the ability to select from a variety of functional nanoparticle cores, ranging from imaging to drug encapsulating colloidal templates, highlights the flexibility of this approach. A major advantage unique to the LbL platform arises from the stepwise assembly of these systems, which allows researchers to spatially separate encapsulated drugs and functional constituents, around or in the core. The exquisite spatial control within these systems not only provides a means of controlling the rate of release of individual drugs, but also provides the staged or staggered release of multiple drugs from a singular nanoparticle platform. This capability allows the biomedical and biomaterials community to design delivery systems capable of controlled, pre-programmed release of a series of therapeutics with efficacy and precision, and is already beginning to address the challenges facing the effective implementation of synergistic combination therapies.

Ultimately, the nature of these hierarchically structured nanolayer coatings enables the use of ionic and secondary interactions to program pH, oxidation, and cellular responses to enact a variety of functional responses. These responses can allow the destabilization and release of encapsulated cargo in the target tissue, as well as the transformation of the outer layers to enable active targeting capabilities such as molecular ligand binding. What is perhaps most fascinating about these LbL systems is the ability to achieve gating, targeting, and stealth properties from as little as a single bilayer film on a nanoparticle core. Furthermore, it has been shown that therapeutic dual delivery systems can be comprised of as few as four layers.

The future of these systems is promising in light of new developments in the manufacture and rapid assembly of LbL nanoparticles, as well as from demonstration of their stability using common storage methods. With current developments, we have a number of approaches available that establish the basis for scalable processing approaches, and further engineering of processes along these lines will help to establish the production of these systems with great fidelity and at high volume. To achieve the next steps in the translation of LbL nanoparticle systems, we must look towards further investigations using more meaningful *in vivo* models, and the partnering of these approaches with a clinical understanding of the best therapeutic and diagnostic approaches that take advantage of the unique capabilities described above for these systems. Ultimately, through more extensive collaboration and engagement of the biomedical and clinical community, we should see the extension of these approaches from key frontier areas, such as cancer and vaccines for immunotherapy, to an even broader range of common and important biomedical applications, including the treatment of osteoarthritis and the development of therapies for the eye. The LbL nanoparticle approaches described here simply mark the beginning of an exciting evolution of the field from the inventive and developmental phase to the realm of true clinical application.

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