Published in final edited form as:
Adv Drug Deliv Rev. 2008 June 10; 60(9): 979–999.

Multilayered Polyelectrolyte Assemblies as Platforms for the Delivery of DNA and Other Nucleic Acid-Based Therapeutics

Christopher M. Jewell and **David M. Lynn***
Department of Chemical and Biological Engineering, University of Wisconsin – Madison, 1415 Engineering Drive, Madison, WI 53706, USA

Abstract

Materials that provide spatial and temporal control over the delivery of DNA and other nucleic acid-based agents from surfaces play important roles in the development of localized gene-based therapies. This review focuses on a relatively new approach to the immobilization and release of DNA from surfaces: methods based on the layer-by-layer assembly of thin multilayered films (or polyelectrolyte multilayers, PEMs). Layer-by-layer methods provide convenient, nanometer-scale control over the incorporation of DNA, RNA, and oligonucleotide constructs into thin polyelectrolyte films. Provided that these assemblies can be designed in ways that permit controlled film disassembly under physiological conditions, this approach can contribute new methods for spatial and/or temporal control over the delivery of nucleic acid-based therapeutics *in vitro* and *in vivo*. We describe applications of layer-by-layer assembly to the fabrication of DNA-containing films that can be used to provide control over the release of plasmid DNA from the surfaces of macroscopic objects and promote surface-mediated cell transfection. We also highlight the application of these methods to the coating of colloidal substrates and the fabrication of hollow micrometer-scale capsules that can be used to encapsulate and control the release or delivery of DNA and oligonucleotides. Current challenges, gaps in knowledge, and new opportunities for the development of these methods in the general area of gene delivery are discussed.

Keywords

Gene delivery; Polyelectrolyte; Multilayered Films; Layer-by-layer; DNA; Transfection; Capsules

1. Introduction

Materials and methods that provide spatial and temporal control over the localized release of therapeutic agents play significant roles in the development of localized therapies [1]. Methods for the delivery of drugs from the surfaces of intravascular stents [2,3], for example, have had an enormous clinical impact and have firmly established both the feasibility and importance of methods for localized drug delivery. Despite these significant advances, however, progress toward the development of localized gene-based therapies remains limited. This is due, at least in part, to the lack of materials and approaches that can be used to provide spatial and temporal control over the release and delivery of DNA and other nucleic acid-based therapeutics from surfaces.

*Corresponding author Tel.: 011-608-262-1086 Fax: 011-608-262-5434, *E-mail address:* dlynn@engr.wisc.edu.

Spatial and temporal control over the administration of DNA and soluble DNA/vector complexes can be achieved *in vivo* using a variety of methods, including delivery through catheters or by direct tissue injection [4–14]. For systemic administration, the modification of these complexes with receptor-specific ligands also permits the targeting of these complexes to specific cells or tissues [15–19]. Numerous additional strategies have been developed for the controlled, localized, sustained, and triggered release of DNA and soluble DNA complexes *in vitro* and *in vivo* [20–26]. From the standpoint of the delivery of DNA from surfaces, however, several fundamental questions remain. How, for example, does one develop effective methods for the release of large, polyanionic macromolecules such as DNA from the surface of a piece of stainless steel?

Several reports demonstrate that the immobilization of DNA and DNA/vector complexes on surfaces can be used to increase the internalization of DNA by cells and promote surface-mediated transfection *in vitro* [27–33]. In addition, several reports have demonstrated that it is possible to provide localized control over the delivery of DNA *in vivo* by encapsulating DNA in thin films of degradable polymers that can be deposited readily onto the surfaces of interventional devices, such as intravascular stents [34–41]. These approaches have established the feasibility of surface-mediated DNA delivery and will play significant roles in the development of new gene-based therapies.

This review focuses on a relatively new materials-based approach to the release of DNA from surfaces and the design of macromolecular assemblies for the delivery of nucleic acid-based constructs: methods based on the layer-by-layer assembly [42,43] of thin multilayered films. Layer-by-layer methods of assembly provide convenient – and often nanometerscale – control over the incorporation of DNA and other nucleic acid-based materials into multilayered polyelectrolyte assemblies. Provided that these materials can be designed in ways that permit controlled disassembly under physiological conditions, this approach has the potential to provide spatial and/or temporal control over the release of nucleic acid-based therapeutics and could lead to more effective methods of delivery.

The application of multilayered polyelectrolyte films and layer-by-layer methods of assembly to problems in the general areas of biology, medicine, and biotechnology continues to advance rapidly [44–50]. The focus of this review is fixed specifically on reports demonstrating the incorporation of nucleic acid-based materials into multilayered films in ways that provide opportunities for subsequent release and advances toward therapeutic applications. It is not our intention to provide a comprehensive overview of other applications of these exciting new methods in the broader context of drug delivery (for example, application to the controlled release of small molecules, proteins, or other agents). However, where appropriate, we do provide leading references and citations of other comprehensive reviews that will provide interested readers with additional background and information on emerging applications or related concepts that connect with many of the motivations, opportunities, and examples discussed below.

The remainder of this review is organized as follows. In the section below, we provide a brief introduction to methods for the layer-by-layer assembly of multilayered polyelectrolyte thin films, as well as an overview of specific ways in which these processes and materials appear well suited for the incorporation and subsequent release of DNA. Following this overview, we describe applications of these methods to the fabrication of DNA-containing films that can be used to (i) provide control over the release of DNA from surfaces and (ii) promote the surface-mediated transfection of cells. We then highlight literature reports that describe the application of layer-by-layer methods to the fabrication of micrometer-scale capsules that can be used to encapsulate and control the release of DNA. The review concludes with consideration of recent

literature describing approaches to the delivery of other nucleic acid-based materials and new, non-traditional methods of film assembly.

2. Multilayered Polyelectrolyte Films: Background, Structure, and Application to Controlled Release

2.1 Layer-by-Layer Assembly of Multilayered Polyelectrolyte Thin Films

The iterative, layer-by-layer adsorption of oppositely charged polyelectrolytes on surfaces is well established as a method for the bottom-up assembly of multilayered polymer films [42, 43,45,51]. The technique takes advantage of attractive electrostatic forces between charged polymers and oppositely charged surfaces, and film growth is achieved stepwise by the repetitive exposure of substrates to dilute polycation and polyanion solutions. As depicted in Scheme 1, the iterative dipping of a substrate (e.g., a glass microscope slide) into solutions of oppositely charged polyelectrolytes yields multilayered films composed of alternating layers of cationic and anionic polymers. The thicknesses of these films typically range from tens or hundreds of nanometers to up to several micrometers, depending on the number of layers deposited and the solution conditions (e.g., pH, ionic strength, etc.) used during fabrication. Additional details regarding the fabrication, internal structures, and applications of multilayered polyelectrolyte films can be found in several recent reviews and are not discussed in greater detail here [42–46,51,52].

In general, layer-by-layer methods of fabrication offer precise, and often nanometer-scale, control over the compositions and thicknesses of composite films (e.g., by control over the number and orders of layers of polymer deposited). This process is also entirely aqueous-based, and can thus be used to fabricate films using a broad range of synthetic and natural polyelectrolytes (such as proteins and DNA). Finally, these methods are well suited to the deposition of films on objects having complex and irregular shapes and can be used to fabricate thin films on a wide variety of macroscopic, microscopic, and nanoscopic substrates.

The attributes described above, when combined, form an attractive platform for the assembly of nanostructured films of interest in the contexts of biology and medicine [44–50]. Below, we describe several aspects of layer-by-layer assembly and multilayered polyelectrolyte films that have the potential to address critical challenges and create new opportunities in the general area of controlled release and, more specifically, the controlled or localized delivery of DNA or other nucleic acid-based materials.

2.2 Advantages of Multilayered Polyelectrolyte Films for Controlled Release and DNA Delivery

Multilayered polyelectrolyte films provide unique and attractive thin-film platforms for the controlled release of both small molecule drugs and macromolecular therapeutics. Several past reports have demonstrated, for example, that these assemblies can behave as thin-film reservoirs for the diffusion-controlled release of small-molecule drugs [53,54]. The layer-by-layer assembly process can also be used to fabricate thin films on the surfaces of micrometer- or nanometer-scale drug crystals to slow the dissolution of drugs or other compounds small enough to diffuse through these materials [55–57].

The ability to incorporate biomacromolecules such as DNA and proteins into the structures of multilayered polyelectrolyte films also provides a platform for the release of macromolecular therapeutics. These biological polyelectrolytes, however, are large molecules and, as a result, they do not diffuse readily through multilayered polyelectrolyte assemblies in the manner of small molecules. Approaches to the release of DNA and proteins from multilayered films have therefore not focused on diffusion-based strategies (as described above), but instead fall into

two general categories: (i) methods for the encapsulation of these species within hollow multilayered film capsules (coupled with methods for triggered capsule destruction) [45,49, 58], and (ii) approaches that involve the incorporation of these species directly into the structures of the films themselves (coupled with the introduction of chemical functionality that permits the disruption or disassembly of the films under physiological conditions) [47,50]. Applications of these two approaches to the delivery of DNA and other nucleic acid-based structures are discussed in additional detail in subsequent sections of this review.

Layer-by-layer assembly offers numerous potential advantages relative to conventional methods for the incorporation and release of DNA and other nucleic materials. Because DNA can be incorporated directly within these films as an anionic layer, layer-by-layer methods allow precise control over the loading (or dose) of DNA simply by controlling film thickness or the number of layers of DNA deposited during fabrication (e.g., Scheme 1). In addition, these methods are entirely aqueous and, in contrast to conventional methods for the encapsulation of DNA in thin polymer films, do not require the use of organic solvents that could remain in these materials post-assembly.

Layer-by-layer methods also permit spatial control over the absolute positions and relative locations of multiple different DNA constructs within a film (e.g., by depositing multiple layers of one type of DNA, followed by several layers of a different DNA construct encoding a different gene product). Provided that film disassembly can be made to occur in a manner that does not disturb the internal organization of individual layers, films having such structure provide opportunities to design films that could provide sophisticated control over the timing and the order with which multiple different DNA constructs are released (e.g., simultaneous release, sequential release, pulsatile release, etc.).

A final point that deserves mention is that multilayered polyelectrolyte films are inherently multicomponent – in general, one layer of a cationic polymer must typically be deposited for each layer of anionic polymer during fabrication (e.g., Scheme 1). In the context of DNA delivery, this inherent juxtaposition of DNA with alternating layers of cationic polymers (a class of materials used broadly for the delivery of DNA to cells [59–61]) creates new opportunities to design films that promote the efficient internalization and trafficking of DNA by cells.

2.3 Incorporation of DNA and Brief Overview of Methods for Controlled Film Disassembly

The first example of the incorporation of DNA into a multilayered polyelectrolyte thin film was reported by Lvov *et al.* in 1993 [62]. These investigators demonstrated that it was possible to fabricate films using sturgeon sperm DNA and synthetic cationic polymers such as poly (allylamine). In the time since that initial report, over 100 additional reports have been published describing the application of layer-by-layer techniques to the fabrication of multilayered films using DNA and other nucleic acid constructs. The overwhelming majority of these reports has made use of model (i.e., non-functional) DNA constructs or oligonucleotide fragments and has been motivated by an extraordinarily broad range of fundamental and applied interests. Although these past studies lie outside our current focus, this large body of past work has contributed new principles and important physical understandings that have formed a basis for more recent work on the incorporation and release of plasmid DNA and functional oligonucleotide constructs. These more recent approaches are discussed in additional detail below.

As described above, DNA-containing multilayered films can only be useful in the context of controlled release if they can be fabricated or assembled in ways that ultimately permit them to be disassembled under physiologically relevant conditions [47,50]. Multilayered polyelectrolyte assemblies are often structurally stable in physiologically relevant

environments owing to the polyvalent nature of electrostatic interactions between layers [42, 43,45,48,51]. The last several years, however, have seen a dramatic increase in the number of reports describing strategies that can be used to disassemble multilayered films or destabilize capsules fabricated from these materials [45,47,49,50,58]. For example, several reports have demonstrated that films can be disrupted using strategies based on (i) changes in environmental variables such as pH or ionic strength that disrupt ionic interactions in these assemblies [63–68], (ii) incorporation of polyelectrolytes with functionality that can be chemically or enzymatically cleaved (reviewed in [47,50]), (iii) fabrication of films based on receptor–ligand interactions (as opposed to electrostatic interactions) that can be disrupted in response to specific small molecules [69–71], and (iv) application of light, ultrasound, electrical potentials, and other external stimuli [49,58,72–74].

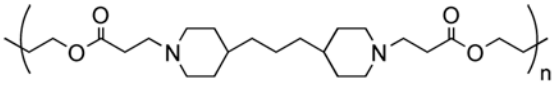
These and many other important approaches to the disruption of multilayered films and capsules have been reviewed extensively elsewhere [45,47,49,50,58]; the underpinning science and broader scope of these approaches are thus not discussed in further detail here. In principle, any of these approaches could be used to design systems that promote the release or delivery of DNA. Approaches that have been applied specifically to the fabrication of films that can be used to release nucleic acid-based materials and promote cell transfection are discussed in the following sections.

3. Controlled Release of DNA from Surfaces

3.1 Incorporation of DNA into Hydrolytically Degradable Multilayered Films

3.1.1 Films Fabricated from Plasmid DNA and Degradable Poly(β-amino ester)s

—Vázquez *et al*. demonstrated in 2002 that it is possible to design multilayered films that erode gradually in physiologically relevant media by incorporating hydrolytically degradable polymer **1** as a cationic component during assembly [75]. Polymer **1** belongs to a large class of polyamines known as poly(β-amino ester)s that have been used in several past contexts to deliver DNA to cells [76–85]. This polymer can be rendered cationic by protonation and it is hydrolytically degradable by virtue of the esters located in the polymer backbone [77]. As such, this polymer provides a means of promoting both layer-by-layer assembly with anionic polymers such as DNA (i.e., through electrostatic interactions) and a means of promoting film disassembly in aqueous media (e.g., through degradation of the polymer).



Polymer **1**

Multilayered films 100 nm thick fabricated from alternating layers of sodium poly(styrene sulfonate) (SPS) and polymer **1** eroded and released SPS into solution over a period of ~40 hours when incubated in phosphate-buffered saline (PBS) at 37 °C [75]. Subsequent experiments demonstrated that this layer-by-layer approach could also be extended to the incorporation and release of calf thymus DNA and other model anionic polymers.

Zhang *et al.* extended the use of polymer **1** to fabricate multilayered films that permit control over the release of transcriptionally active plasmid DNA [86]. In these experiments, films were fabricated on planar silicon and quartz substrates by depositing alternating layers of polymer **1** and a supercoiled plasmid DNA construct encoding enhanced green fluorescent protein (EGFP). The deposition of 8 layers of polymer **1** and DNA resulted in multilayered films ~100 nm thick. Subsequent experiments demonstrated that films ~100 nm thick contain approximately 2.7 (± 0.8) μg of DNA/cm^2 [87], and that the amount of DNA in these materials

can be increased or decreased in a straightforward manner by increasing or decreasing the number of layers of polymer and DNA deposited [86,88].

The incubation of polymer **1**/DNA films in PBS at 37 °C resulted in the sustained release of plasmid DNA into solution over a period of ~30 hours (as determined by characterization of solution absorbance at 260 nm; Figure 1) [86]. Characterization of the film erosion process by UV/visible spectrophotometry and ellipsometry demonstrated that both film thickness and the amount of DNA in these materials decreased over this same time period (Figure 1).

Further physical characterization of film erosion processes using atomic force microscopy (AFM) and scanning electron microscopy (SEM) revealed that these DNA-containing films undergo complex, nanometer-scale changes in surface structure upon incubation in PBS [87, 89,90]. Although the erosion of films fabricated from polymer **1** is clearly complex and deserves additional study, recent work suggests that ester hydrolysis plays an important role in governing the erosion of these films [91,92] and provides a framework from which to fabricate films that provide greater control over the release profiles of these materials (as discussed below).

Characterization of plasmid DNA recovered from the erosion of polymer **1**/DNA films using agarose gel electrophoresis demonstrated that it was released in an open-supercoiled form [86,88]. Additional cell-based experiments conducted using recovered DNA demonstrated that the DNA was released in a form capable of mediating high levels of EGFP expression in COS-7 cells. These results demonstrate that plasmid DNA can be incorporated into erodible multilayered films and that it can be released and recovered without loss of biological function.

3.1.2 Surface-Mediated Cell Transfection—Jewell *et al.* expanded upon the observations above to demonstrate that macroscopic objects coated with films fabricated from polymer **1** and plasmid DNA could be used to promote the localized and surface-mediated delivery of DNA to cells *in vitro* [87]. For example, when film-coated quartz slides were placed in contact with COS-7 cells in the presence of serum, expression of EGFP was observed in cells after 48 hours (as determined by fluorescence microscopy; Figure 2A). When the slides were prepared appropriately (e.g., by coating them with multilayered films on only one side) cell transfection was localized largely to cells growing under or in contact with the film-coated portions of the slides (Figure 2B). In these experiments, an average of 18% of cells were observed to express EGFP. Finally, films fabricated using different numbers of layers of two different plasmids (encoding either EGFP or red fluorescent plasmid, RFP) promoted the contact-mediated co-expression of both EGFP and RFP when placed in contact with cells [87].

In the examples above, the surface-mediated delivery of DNA to cells is likely aided, at least in part, by the creation of high local concentrations of DNA around cells growing in contact with or in the vicinity of film-coated slides. Regardless, the levels of surface-mediated transfection in the study reported above are much lower than those reported using conventional solution-based methods of transfection (e.g., using cationic lipid formulations or pre-formed polymer/DNA particles, as described above [59,60]). Additional experiments will be required to understand the extents to which changes in film properties such as thickness (and, thus, the amount of DNA released) and polymer structure correlate to differences in surface-mediated transfection, and whether changes in these parameters can be used to optimize levels of transfection further.

Initial characterization of film incubation media by dynamic light scattering suggests the possibility that polymer **1** may play a role in promoting the internalization and processing of DNA by cells (e.g., by remaining bound to DNA upon release) [88]. Although additional

analytical work will be required to establish this potential role of the polyamines in these materials more firmly, it bears noting again that the inherent commingling of DNA and cationic polymers in these multilayered assemblies does provide opportunities to optimize the performance of these materials further. It could prove possible, for example, to enhance transfection by fabricating films using degradable polyamines that bind DNA more strongly than polymer **1** (to promote the release of condensed DNA), or by incorporating additional layers of conventional gene delivery polymers, such as poly(ethylene imine), that address specific intracellular barriers to the processing of DNA (such as endosomal escape). Approaches to the incorporation of auxiliary transfection agents or pre-formed polymer/DNA polyplexes are discussed in further detail below in our discussion of multilayered assemblies fabricated using enzymatically degradable polyelectrolytes.

3.1.3 Release of DNA from the Surfaces of Implantable Devices—As described above, an important benefit of the layer-by-layer process used to fabricate multilayered polyelectrolyte assemblies is that it can be used to fabricate uniform and conformal thin films on the surfaces of topographically and topologically complex objects. Jewell *et al.* demonstrated that the methods described above for the fabrication of multilayered polymer **1**/DNA films on planar substrates could be extended to the deposition of thin films onto stainless steel intravascular stents [88]. Figure 3 shows SEM images of stents coated with films ~120 nm thick fabricated from eight layers of polymer **1** and plasmid DNA encoding EGFP.

Figures 3A–C show images of an unexpanded stent as-coated on a polymer balloon assembly; Figures 3D–F show images of a film-coated stent after balloon expansion. When combined, these images demonstrate that it is possible to fabricate uniform thin films that conform faithfully to the contours of stents and that these films do not crack, peel, or delaminate upon stent expansion [88]. These coating methods could provide a potential alternative to conventional approaches that have been used to coat intravascular stents with DNA-containing polymer films [35,36,38–40], several of which require the use of organic solvents or can result in non-uniform coatings or webs of polymer film that span the spaces between stent struts.

The film-coated stents described above released transcriptionally active DNA when incubated in PBS or cell culture media and could be used to direct the transfection of COS-7 cells *in vitro* [88]. Additional physical and biological characterization of these materials will clearly be required before it is possible to determine whether this approach will ultimately be useful for stent-mediated delivery of DNA *in vivo.* However, the results above illustrate the potential of this new approach and provide a basis for the further evaluation of these new materials in animal models. With further development, the layer-by-layer approach outlined above could also prove useful for the localized release of DNA from a wide variety of other implantable materials and indwelling devices.

3.1.4 Toward Tunable and Extended Release—The examples above demonstrate that it is possible to sustain the release of plasmid DNA from surfaces by fabricating multilayered films from hydrolytically degradable polyamines. However, in all of the examples above, films fabricated using polymer **1** erode and release DNA relatively rapidly (e.g., over one to several days) [86,88]. Ultimately, films that provide for release over extended periods or that permit broad and tunable control over film erosion could lead to more effective therapies.

Zhang *et al.* demonstrated recently that it is possible to extend and tune film erosion and the release of model anionic polymers by synthesizing and incorporating analogs of polymer **1** with increased hydrophobicity [91–93]. In principle, modification of the hydrophobicity, charge density, and side chain structures of hydrolytically degradable polyamines could also be used to design films that prolong the release of DNA or permit the tunable erosion of DNA-containing films. Below, we describe a recent approach to the fabrication of thin multilayered

films that can be used to release plasmid DNA from surfaces over a time period of several months.

Several groups have reported recently that it is possible to control both the assembly and disassembly of cationic polymers with DNA by using cationic polymers with side chains that can be hydrolyzed to either (i) introduce negative charge [94] or (ii) remove a pendant cationic group [95–97]. In contrast to hydrolytically degradable cationic polymers (which undergo changes in molecular weight that lead to changes in the strength of interactions with DNA) these approaches lead to polymers that undergo time-dependent shifts in net charge (e.g., from a polymer that is substantially cationic to a polymer that is either less cationic or anionic upon side chain hydrolysis; see polymer **2** in Scheme 2 [98]). These 'charge-shifting' polymers present new opportunities to design multilayered films that erode under physiological conditions [99].

Zhang *et al.* demonstrated recently that 'charge-shifting' cationic polymer **2** can be used to fabricate thin multilayered films that erode slowly and release plasmid DNA over prolonged periods [98]. Figure 4 shows the release of DNA from a polymer **2**/DNA film ~80 nm thick incubated in PBS at 37 °C. These data and other experiments demonstrated that DNA was released for up to 90 days (three months) under these conditions. Characterization of released DNA demonstrated that it was structurally intact and capable of mediating transgene expression in COS-7 cells over the entire 90 day period of these experiments. These data demonstrate that the incorporation of 'charge-shifting' cationic polymers presents a route to the design of assemblies that erode very slowly and release DNA over periods much longer (e.g., ~55 times longer) than have been reported for the release of DNA from films fabricated using hydrolytically degradable polyamines such as polymer **1** [86,88]. Future modifications to the structures of polymer **2** or 'charge-shifting' polymers of similar design [94–97] should lead to opportunities to tune film erosion or prolong the release of DNA further.

3.2 Incorporation of DNA into Enzymatically Degradable Multilayered Films

3.2.1 Films Fabricated from Poly(L-Lysine)—Ren *et al*. demonstrated recently that it is possible to design films that degrade and release DNA in the presence of enzymes by fabricating films using poly(L-lysine) (PLL) as a cationic film component [100]. Films fabricated from PLL and salmon sperm DNA were found to be relatively stable upon incubation in PBS. However, film thickness decreased significantly over a period of 35 hours when PLL/DNA films were incubated in solutions of PBS containing α-chymotrypsin (which can degrade PLL). Characterization of film erosion solutions using an ethidium bromide fluorescence assay demonstrated that DNA was released into solution over this same 35 hour time period.

Additional physical characterization of film erosion solutions using transmission electron microscopy (TEM) and measurements of zeta potential revealed the presence of aggregates with sizes ranging up to several hundred nanometers and slightly positive surface charges [100]. Although additional characterization will be required to determine the compositions of these aggregates, these results provide additional support for the view that the polyamines in these multilayered materials could serve as a basis for the design of films that release DNA in a form (e.g., a condensed polyplex) that promotes the internalization and processing of DNA by cells. Additional work by this group demonstrated that PLL/DNA films could also be disrupted or deconstructed and made to release DNA upon exposure to solutions of high ionic strength [101].

The work reported above demonstrates the basis of an approach that could, with further development, lead to films that erode only in the presence of specific enzymes. Subsequent work by Ren *et al.* has also demonstrated the basis of an approach that can be used to tune the rates at which these enzymatically degradable films erode. For example, the crosslinking of

PLL/DNA assemblies using glutaraldehyde was reported to enhance the stabilities of these films in the presence of trypsin [102]. The relative stabilities of crosslinked PLL/DNA films correlated to the degree of film crosslinking, with higher levels of crosslinking leading to more stable films (Figure 5). Varying the extent of crosslinking could also be used to influence the rates at which DNA was released into solution, although DNA release was only monitored over a short 24-hour period in this study. More research will be required to determine whether glutaraldehyde-mediated crosslinking or other methods of crosslinking can be conducted in a manner that does not damage incorporated DNA. However, the extension of this basic approach to films fabricated from plasmid DNA or other functional nucleic acid constructs could prove useful for the design of materials that release DNA only in environments in which specific enzymes are present.

3.2.2 Toward Sophisticated Control: Time-Scheduled Expression of Two Different Plasmids—As mentioned above, one potential advantage of stepwise, layer-by-layer methods of assembly with respect to other methods for the fabrication of DNA-containing thin films is the ability to control with precision the absolute and relative locations of individual layers within a film. Films fabricated from multiple different layers of different DNA constructs provide, in principle, new approaches to the design of materials that permit sophisticated levels of temporal control over the release and expression of multiple genes.

Jessel *et al.* demonstrated recently that imbedding layers of two different plasmids at different depths in films fabricated from PLL and poly(glutamic acid) (PGA) can be used to control the timing with which two plasmids are expressed by attached cells [103]. These investigators first demonstrated that imbedding a single layer of plasmid DNA and a cationic cyclodextrin agent deep in multilayered PLL/PGA films resulted in films that were able to able to support the attachment, growth, and transfection of several different cell types. Characterization of transgene expression by indirect immunofluorescence staining demonstrated that nearly quantitative transfection efficiencies could be achieved using this approach. Similar experiments conducted by imbedding a single layer of DNA in the absence of the cationic cyclodextrin agent did not result in transfection. This result demonstrates again the potential importance of approaches to film fabrication that either exploit or incorporate cationic transfection agents into the structures of multilayered films designed to transfect cells.

Subsequent experiments demonstrated that imbedding two different layers of two different plasmid constructs (i.e., encoding two different gene products) resulted in measures of control over the timing with which these plasmid constructs were expressed in cells [103]. For these experiments, plasmids encoding EGFP or a nuclear transcription factor were deposited at different depths within a film by separating each layer with additional PLL/PGA layers. When cells were grown on these films, the expression of the plasmid located in the lower layers of the films could be delayed by ~4 hours relative to the expression of the plasmid located in the topmost portion of the films (Figure 6). Reversing the order in which the two plasmid constructs were imbedded was reported to reverse the order in which the two plasmids were expressed [103].

The results discussed above demonstrate a basis for control over the rates at which two different plasmids are expressed by attached cells and, thus, illustrate one way in which layer-by-layer methods could prove useful for the design of scaffolds for applications such as tissue engineering (for which precise control over the orders in which growth factors or other agents are administered is often desired [24,104]). Although the delays in the expression of the two DNA constructs reported above were relatively short (~4 hours) [103], it will likely prove possible to manipulate expression profiles further by manipulating the distances between the DNA layers or by depositing intermediate layers of natural or synthetic materials that are more difficult for cells to degrade rapidly. For example, Wood *et al.* recently demonstrated that

intermediate layers of crosslinked polyelectrolytes could be used to control the rates at which two different polysaccharides were released from films fabricated using polymer **1** [105]. The extension of this general approach to the fabrication of DNA-containing films could permit the design of films that provide greater levels of control over the release of multiple different DNA constructs.

3.2.3 Incorporation of Polyplexes and Viral Vectors—The discussion of several of the approaches above has brought to light potential opportunities to leverage the cationic layers of multilayered films to promote cell transfection. As an alternative to leveraging the inherent availability of cationic polymers in these assemblies, Meyer *et al.* have recently reported an approach to the imbedding of preformed polyamine/DNA complexes into multilayered films [106].

As described above, polyamines are widely used as non-viral agents for the delivery of DNA to cells, largely because they condense DNA into nanometer-scale polyplexes that are positively charged and sufficiently small (e.g., ~200 nm) to be readily internalized by cells [59–61]. In addition, functional polyamines such as poly(ethylene imine) (PEI) have the ability to address specific intracellular barriers to transfection (such as escape from acidic intracellular vesicles) and can thus significantly increase levels of gene expression.

Meyer *et al*. demonstrated that positively-charged polyplexes formulated using plasmid DNA and linear PEI could be adsorbed onto multilayered films terminated with a final layer of anionic polymer [106]. Following the deposition of the polyplexes, continued deposition of polycation and polyanion layers resulted in further film growth. Characterization of the resulting assemblies by infrared spectroscopy and AFM confirmed that DNA was not displaced from the films upon the deposition of these additional layers and suggested that the DNA was likely still complexed to some extent with linear PEI. Although the structures of the polyamine/DNA complexes upon incorporation into films will ultimately need to be characterized more completely, this approach offers potential opportunities to further optimize the delivery of DNA to cells. Cell-based transfection experiments demonstrated that these films were capable of promoting transgene expression in attached cells [106].

In a more recent report, Dimitrova *et al.* have foregone the use of auxiliary cationic polymers and plasmid DNA altogether, and demonstrated that the potential advantages of layer-by-layer assembly can be combined with the well known advantages of virus-based methods of cell transfection [107]. In this work, a functional adenoviral gene delivery vector was either adsorbed onto or imbedded within films fabricated from a range of synthetic (nondegradable) or natural (enzymaticaly degradable) polyelectrolytes. Cell-based experiments demonstrated that these materials can promote high levels of transgene expression in several different cell types and represent an exciting new avenue of research in this area.

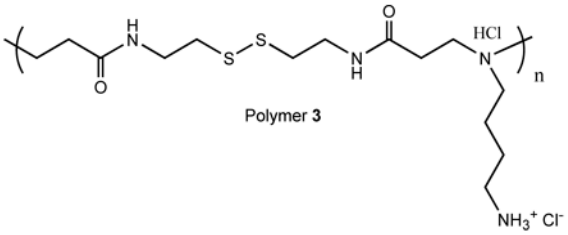
3.3 Triggered Release of DNA Under Reducing Conditions: Disulfide-Containing Films

For certain applications, it could prove desirable to trigger the disassembly of an otherwise stable multilayered film and localize the delivery of DNA at precisely defined times. Blacklock *et al.* have demonstrated the basis of a chemical approach to the triggered disassembly of DNA-containing multilayered films by exploiting the reversibility of the thiol-disulfide bond [108]. The basic concept behind this work is that disulfide bond-containing assemblies that are stable under non-reducing conditions can be destabilized by exposure to reducing environments that cleave disulfide bonds, such as those found in intracellular environments and in certain extracellular environments.

Blacklock *et al.* assembled multilayered films using plasmid DNA and a high molecular weight cationic polypeptide synthesized by the oxidative coupling of a short peptide sequence derived

from the HIV-1 TAT protein (Scheme 3) [108]. This polypeptide is stable in non-reducing media, but degrades in the presence of chemical reducing agents that cleave disulfide bonds in the backbone of the polymer. As a result, films fabricated from this polymer and DNA were generally stable in physiological media, but disassembled and released plasmid DNA over a period of ~24 hours upon exposure to the reducing agent dithiothreitol (DTT).

A similar approach was recently reported by Chen *et al.* [109]. These investigators synthesized a reductively degradable cationic poly(amidoamine) (polymer **3**) by the conjugate addition of a primary amine to a disulfide-containing bisacrylamide. Similar to the work described above, fabrication of multilayered films using this polymer and plasmid DNA resulted in films that were stable in PBS, but which disassembled and released DNA upon exposure to DTT. Rates of film erosion and the release of DNA were found to correlate to the concentration of DTT in solution; DNA was released rapidly (e.g., in 30 minutes) in 10 mM solutions of DTT, and more slowly (e.g., ~9 days) in 1 mM solutions of DTT [109]. These results provide a basis for the redox-based tuning of film erosion and the release of DNA.



Polymer **3**

The redox-active materials described above have not yet been evaluated in *in vitro* cell transfection assays. However, approaches based on thiol-disulfide chemistry appear well suited for methods of DNA delivery designed to exploit the reducing potentials of certain physiological environments. Blacklock *et al.* have suggested that the microenvironments surrounding cell membranes may be sufficient to promote the disassembly of these films [108]. In general, approaches based on thiol-disulfide chemistry may also be particularly well suited to the design of films that can be disassembled upon exposure to the reducing environments inside cells. Additional work describing the use of thiol-disulfide chemistry and layer-by-layer procedures to fabricate multilayered film capsules for the intracellular delivery of oligonucleotides is discussed separately, below.

3.4 Other Approaches

Yamauchi *et al.* recently demonstrated an approach that provides spatial, temporal, and active control over the transfection of cells based on the application of electric pulses to multilayered films deposited onto electrodes [110]. In this work, films composed of PEI and plasmid DNA encoding EGFP were fabricated layer-by-layer onto transparent indium-tin oxide (ITO) glass. These films were demonstrated to be stable in physiologically relevant media and did not transfect attached cells. However, when electric pulses were applied to these electrodes (at field strengths ranging from ~100–200 V/cm), DNA was released into solution. Moreover, when electric pulses were applied to film-coated electrodes on which either HEK 293 or hippocampal neuronal cells (Figure 7) were growing, high levels of expression of EGFP were observed. Cells growing on films incorporating layers of two different plasmids (encoding either EGFP or RFP) could be induced to express both EGFP and RFP upon the application of an electric pulse. In general, levels of transfection and cell viability were found to be dependent upon the field strength applied and the number of PEI/DNA layers deposited [110].

Although the specific mechanism through which the application of electric pulses leads to the release of DNA from these films is not yet clear, the results and field strengths used in this

study are consistent with electroporation-based mechanisms for the transfer of DNA into cells [110]. The ability to apply electric pulses to electrodes with both spatial and temporal control presents future opportunities to extend this approach to the use of film-coated micropatterned electrodes to generate arrays of transfected cells with temporal control and high levels of spatial resolution.

Yamauchi *et al.* have also reported a layer-by-layer approach to the fabrication of DNA-containing films that makes use of cationic lipid-DNA complexes [111]. In this approach, films were deposited onto functionalized gold surfaces by alternately exposing these substrates to solutions of positively charged lipoplexes (formed from plasmid DNA and a commercially available cationic lipid formulation) and naked plasmid DNA (as a negatively charged film component). Although this study did not determine whether incorporated lipoplexes remained structurally intact upon deposition, this basic approach has the potential to combine many of the benefits of layer-by-layer assembly with several of the known benefits of lipid-mediated methods of DNA delivery. Gold substrates coated with these cationic lipid/DNA assemblies were able to promote localized and surface-mediated transfection when placed in contact with HUVEC and HEK 293 cells *in vitro* [111]. This approach could thus prove useful for the localized release of DNA from the surfaces of a wide range of implantable devices.

4. Coated Colloids and Fabrication of Hollow Microcapsules

While the focus of this review thus far has emphasized methods for the fabrication of multilayered films that can be used to promote the release or delivery of DNA from the surfaces of macroscopic substrates, substantial research efforts have also been directed toward the development of layer-by-layer approaches to the delivery of DNA and oligonucleotides from microscale objects.

As described above, layer-by layer methods of assembly permit the deposition of thin films on a wide variety of macroscopic, microscopic, and nanoscopic objects [43,45,48,49,51,58]. As shown schematically in Scheme 1, fabrication on the surfaces of macroscopic objects can be achieved readily by iteratively dipping these substrates into solutions of oppositely charged polyelectrolytes (or by using other methods, such as spin-coating, spray-coating, etc.). For deposition onto the surfaces of smaller objects, such as microparticles and nanoparticles, however, approaches based on dipping are less useful. As a result, the fabrication of film-coated microparticles and nanoparticles is most often performed by successive cycles of suspension, centrifugation, and resuspension in polyelectrolyte solutions [45,49,58], as illustrated schematically in Scheme 4. Provided that successive layers of polyelectrolyte can be deposited without a significant amount of particle aggregation, this method permits the coating of particles and also provides an approach to the fabrication of hollow multilayered film capsules (e.g., by fabrication onto sacrificial template cores that can be removed after fabrication, as discussed below).

These methods have been used by several groups to fabricate microscale and nanoscale systems for the encapsulation, presentation, or controlled release of a broad range of different species [45,49,58]. Below, we review applications of this approach that have been used to fabricate microscale assemblies composed of DNA or designed to encapsulate DNA and oligonucleotide constructs. In contrast to approaches to the release of DNA from macroscale objects, approaches to the coating of microscale and nanoscale objects should prove useful for the design of multilayered systems that can be injected, are able to pass unhindered through the circulatory system, and can be internalized by cells.

4.1 Fabrication of DNA-Containing Films on the Surfaces of Micrometer-Scale Particles

Reibetanz *et al.* used template-assisted layer-by-layer assembly to deposit multilayered films fabricated from protamine sulfate and dextran sulfate on the surfaces of 3 μm silica spheres [112]. Imbedding a single layer of plasmid DNA encoding EGFP within these films yielded particles that were capable of promoting transgene expression when administered to HEK 293T cells (Figure 8). These investigators also reported that silica particles coated with multilayered films containing imbedded layers of two different plasmids (one encoding EGFP and one encoding RFP) mediated the co-expression of both EGFP and RFP [112].

Levels of transfection observed in these experiments were low, ranging from 3–5% of the sub-population of cells that had internalized film-coated particles [112]. Although this work did not incorporate specific structural elements designed to promote film disassembly or the intracellular release of DNA, the observation of EGFP expression demonstrates that the imbedded DNA was, to some functional extent, made accessible to cells in these experiments. It is likely that the incorporation of mechanisms designed to promote the intracellular disassembly of these films (as described below) would lead to higher levels of transfection. Additional experiments by these investigators also suggest that the overwhelming majority of multilayer-coated particles remains sequestered in endosomes after internalization by cells [113]. As noted above under Section 3, the incorporation of additional cationic polymers such as PEI into the structures of these multilayered films could thus be useful in addressing this important intracellular barrier and could increase the levels of transfection that can be achieved using this approach.

The results above demonstrate proof-of-concept and the potential of layer-by-layer methods to contribute to the development of new particle-based approaches to DNA delivery. In this context, however, several additional points deserve consideration. For example, in the experiments described above, only a small number of cells were reported to internalize film-coated particles [112]. The template particles used in this study were ~3 μm in diameter and are much larger than the range of sizes that are generally considered to be optimal for internalization by endocytosis (e.g., from ~50–200 nm) [114]. The extension of this basic approach to the deposition of films on nanoparticles would likely improve transfection efficiencies in cells that internalize particles by endocytosis. However, the deposition of films on micrometer-scale particles, as demonstrated here, could prove useful for the design of novel delivery systems for DNA vaccines, for which size-based targeting of particles to phagocytocic cells of the immune system (e.g., macrophages) is desired [83,115–117]. Ultimately, the coupling of this general approach with new methods for the biofunctionalization of layer-by-layer capsules [118,119], the conjugation of targeting agents [120,121], and the incorporation of design elements that address specific intracellular barriers to cell transfection could be used to improve transfection efficiencies and target these particles to specific types of cells.

The layer-by-layer approach can also be used to deposit films on microparticle and nanoparticle cores fabricated from a wide range of different materials [45,49,58]. The deposition of films onto particles that are degradable could lead to enhanced biocompatibility and expand the functionality of this approach (for example, by providing a mechanism for the sustained delivery of other encapsulated agents). Trimaille *et al*. have demonstrated the basis of such an approach by adsorbing PEI onto particles composed of the degradable polymer poly(lactic acid) (PLA), followed by adsorption of plasmid DNA [122]. Although this report did not describe the deposition of additional layers of polymer or DNA, this approach could clearly be extended to fabricate PLA particles coated with multilayered films.

4.2 Hollow Multilayered Film Capsules

Numerous groups have demonstrated that it is possible to use template-assisted layer-by-layer assembly to fabricate hollow multilayered capsules by depositing polyelectrolytes onto cores that can be dissolved, degraded, or otherwise removed after film formation [45,49,58]. This approach has been used widely to develop approaches to either encapsulate or deliver a wide range of macromolecular agents. Applications of this approach to DNA-containing systems have followed one of two approaches: (i) the fabrication of DNA-containing films on removable cores (resulting in hollow capsules containing DNA in the capsule walls), or (ii) the deposition of multilayered films onto template cores coated with DNA or oligonucleotides (which, after removal of the core, results in the encapsulation of these materials inside hollow capsules). Below, we review examples of each approach.

4.2.1 Fabrication Using DNA as a Building Block—Schüler *et al.* reported the fabrication of multilayered films by depositing alternating layers of herring sperm DNA and spermidine on the surfaces of positively charged melamine formaldehyde particles (1.8 μm or 5.7 μm in diameter) [66]. Removal of the particle templates after fabrication by treatment with acid resulted in hollow multilayered capsules, as determined by AFM and TEM. These hollow DNA/spermidine microcapsules were demonstrated to be stable in water. However, the capsules decomposed (and, by inference, released DNA) over 12 hours when exposed to solutions of high ionic strength.

The values of ionic strength investigated in this study were significantly high (e.g., solutions of NaCl ranging from 1.0 M to 5.0 M); the behavior of these capsules at physiological ionic strengths was not reported. It is likely, however, that capsules that disintegrate at physiological ionic strength could be fabricated by manipulating the conditions under which the films were assembled. In addition, films that release DNA at higher ionic strengths could possibly be useful for the delivery of DNA in physiological environments with elevated ionic strength [101].

The incorporation of other chemical mechanisms designed to promote the disassembly of DNA-containing films (as described above and, in additional detail, below) could also significantly expand the potential of this approach. In addition to chemical approaches to disruption, several groups have used physical methods to trigger the disruption of hollow multilayered film capsules [49,58,72–74]. Borden *et al.* recently described an approach to the fabrication of PLL/DNA capsules on lipid-coated microbubbles that can be disrupted or ruptured by ultrasound insonification [74]. This approach could thus prove useful for the targeted and remotely triggered release of DNA from microbubbles circulating in the vasculature or disseminated into tissues. A more complete understanding of the mechanical properties of hollow capsules fabricated from DNA [123,124] will be important to identifying the range of applications for which approaches based on the administration of hollow polymer/DNA microcapsules are best suited.

4.2.2 Encapsulation of Nucleic Acid-Based Cargo—Shchukin *et al.* reported a template-assisted approach to the fabrication of hollow multilayered film capsules containing DNA in their interiors (rather than within the structure of the films, as described above) [125]. In this work, calf thymus DNA was precipitated as a complex with spermidine onto the surfaces of 4 μm manganese carbonate ($MnCO_3$) template particles, followed by the deposition of alternating layers of chondroitin sulfate (CS) and poly(arginine) (PA; Scheme 5). Dissolution of the $MnCO_3$ template cores using dilute acid (0.01 M HCl) resulted in hollow multilayered CS/PA capsules with walls ~40 nm thick encapsulating DNA/spermidine complexes.

Subsequent experiments using fluorescently labeled DNA and confocal microscopy demonstrated that the majority of encapsulated DNA/spermidine complexes were located in the vicinity of capsule walls (Figure 9A) [125]. However, further treatment of these capsules with acid (0.1 M HCl for 10 minutes) resulted in the observation of fluorescence distributed throughout the aqueous interiors of the capsules (Figure 9B). The average concentration of DNA encapsulated using this approach was estimated to 0.4 mg/mL of capsule volume, and exposure of these capsules to pH values lower than 3.0 was reported to result in the release of encapsulated DNA [125]. This approach thus holds promise as a method for the loading of DNA into hollow capsules for a broad range of delivery applications. Additional experiments will be required to determine the maximum loading of DNA that can be achieved using this approach, as well as the loading levels that are optimal or most appropriate for specific gene delivery applications. As noted above, the extension of this approach to the encapsulation of plasmid DNA in smaller (i.e., nanometer-scale) capsules will also provide a basis for the design of capsules capable of targeting the delivery of DNA to a broader range of cells. In addition, the incorporation of chemical functionality that allows for the controlled disruption of capsule walls in intracellular environments (as described below) will also contribute significantly to the development and successful application of this approach.

Zelikin *et al.* have described a polycation-free approach to the encapsulation and release of short oligonucleotide constructs that makes use of hydrogen-bonded multilayered films that disintegrate in reducing environments [126]. As described in Section 3, this approach also exploits the well-known reversibility of the thiol-disulfide bond. However, rather than fabricating assemblies using reductively degradable polyelectrolytes [108,109] these investigators used polyelectrolytes with thiol-functionalized side chains to reversibly stabilize multilayered films after assembly (Scheme 6) [126,127].

In this approach, short oligonucleotide sequences (~30 bases) were adsorbed to the surfaces of amine-functionalized silica template particles [126]. Following adsorption, hydrogen-bonded multilayered films were assembled using poly(vinylpryrolidone) (PVPON) and a thiol-functionalized poly(methacrylic acid) derivative (PMA_{SH}). Subsequent treatment of these assemblies with an oxidizing agent (to crosslink the chains of PMA_{SH} in these assemblies) followed by treatment with hydrofluoric acid and ammonium fluoride (to remove silica template particles) resulted in hollow capsules containing oligonucleotides (as determined by fluorescence confocal microscopy). These multilayered film capsules retained encapsulated oligonucleotides for at least 72 hours at physiological pH. However, exposure of these films to a thiol-disulfide exchange reagent resulted in rapid film disintegration and the immediate release of oligonucleotide [126].

The approach described above has not yet been extended to the delivery of oligonucleotides to cells. As described above, however, methods for promoting film disassembly that are based on thiol-disulfide chemistry appear well suited for the fabrication of micrometer- or nanometer-scale capsules designed to release nucleic acid-based payloads rapidly upon exposure to reducing environments inside cells.

Finally, Kreft *et al.* have reported an approach to the loading of hollow multilayered film capsules *after* capsule formation [128]. In this study, fixed human red blood cells (erythrocytes) were used as templates for the deposition of multilayered films. Subsequent removal of the cell cores by incubation in a strongly oxidizing solution resulted in hollow capsules ~5 μm in diameter. The walls of these capsules were impermeable to calf thymus DNA, as determined using confocal microscopy and a fluorescent DNA-intercalating dye (Figure 10A). However, capsules that were incubated with DNA, dried, and then subsequently resuspended were found to contain DNA in the interiors of the capsules (Figure 10B). While the mechanism through which the loading of DNA in these capsules occurs is currently unclear, these results

demonstrate that the multilayered film walls of these capsules are somehow made permeable to DNA during the drying process [128]. With further development, this approach could provide promising methods for the post-fabrication loading of hollow polyelectrolyte microcapsules with functional DNA and other agents for a variety of different controlled release applications.

5. Other Approaches

5.1 Layer-by-Layer Deposition onto Nanometer-Scale Polycation/DNA Polyplexes

As described above, polycations are used widely as agents for the non-viral delivery of DNA because they can condense DNA into positively charged, nanometer-scale particles (polyplexes) that are small enough to be internalized by cells [59,60]. Provided that additional anionic polymers can be adsorbed onto positively charged polyplexes without disassembling the original core complex, this strategy provides an approach to the layer-by-layer coating – and subsequent stabilization or further functionalization – of polyplexes for the delivery of DNA to cells and tissues.

Trubetskoy *et al.* demonstrated that positively charged polyplexes formed from plasmid DNA and PLL could serve as templates for the subsequent adsorption of the anionic polymer succinylated PLL (SPLL; prepared by addition of succinic anhydride to PLL) [129]. Measurement of zeta-potential (surface charge) demonstrated that the addition of SPLL to DNA/PLL polyplexes reversed the charges of these complexes to yield negatively charged, colloidally stable ternary DNA/PLL/SPLL complexes. Subsequent experiments demonstrated that it was possible to deposit an additional layer of PLL on these ternary complexes to yield positively charged polyplexes. Characterization of the sizes of these complexes using dynamic light scattering demonstrated that the diameters of these ‘recharged’ particles increased by an average of ~10 nm upon the addition of each additional polyelectrolyte layer [129].

The ‘recharging’ of polyplexes by the deposition of additional anionic polyelectrolytes provides opportunities to address issues related to the non-specific internalization, toxicity, and colloidal instability of positively charged polyplexes in physiological media, all of which can influence the effectiveness of polycation-based approaches to gene delivery [129]. Subsequent work by Trubetskoy *et al.* demonstrated that the recharging of DNA/PEI polyplexes using poly(acrylic acid) could be used to increase levels of cell transfection *in vitro* and increase levels of gene expression in the lung *in vivo* [130]. Zaitsev *et al.* have also employed this approach by using transfer RNA (tRNA) and poly(vinyl sulfate) to recharge polyplexes for vascular gene delivery [131].

5.2 Incorporation and Release of RNA

Recksiedler *et al.* recently described an approach to the fabrication of electrically conducting multilayered films that can be used to promote the release of RNA under physiologically relevant conditions. In this work, films were fabricated using high molecular weight commercially available RNA and poly(anilineboronic acid) (PABA) [132]. PABA was chosen for this study because it is a redox-active polymer and because it can interact with biomacromolecules such as RNA through the formation of reversible dative bonds. The iterative dipping of substrates into appropriately prepared solutions of RNA and PABA resulted in layer-by-layer film growth, as determined by UV/visible spectrophotometry and ellipsometry. Subsequent characterization of the resulting films by reflective infrared spectroscopy suggested that the RNA and PABA in these assemblies interact through dative bonds, as well as through electrostatic interactions [132].

The preparation of multilayered films using a redox-active polymer presents opportunities to design films that respond to the application of electrical potentials and promote the release of

incorporated RNA. These investigators demonstrated that the repeated cycling of electrical potentials (from −0.2 V to +1.4 V, versus a Ag/AgCl reference electrode) to films fabricated on indium-tin oxide (ITO) glass resulted in a loss of electroactivity and a reduction in film absorbance that was consistent with a decrease in the amount of RNA in the film (direct characterization of RNA release was not reported in this study) [132]. Additional work will clearly be required to establish the general feasibility of the above approach and to characterize the physical and structural integrity of released RNA.

In principle, any of the broad range of layer-by-layer approaches described above that have been used to incorporate and release DNA and oligonucleotides could also be used to design multilayered materials for the delivery of RNA. In view of the recent explosion of interest in RNA interference (RNAi) and the development of methods for the effective delivery of small interfering RNA (siRNA) [133–137], it seems very likely that many additional examples of layer-by-layer approaches to the assembly of RNA-containing films will appear in the literature soon.

5.3 Multilayered Films Composed Entirely of DNA

As discussed above, the layer-by-layer assembly of multilayered films is driven by polyvalent interactions (electrostatic interactions, hydrogen bonding, etc.) between appropriately designed and oppositely functionalized polymers [42,43,45,51]. In most of the examples above, film growth is governed primarily by electrostatic interactions between the negatively charged backbone of DNA and positively charged polymers. Johnston *et al.*, however, have demonstrated in a series of recent reports that it is possible to use layer-by-layer methods of assembly to fabricate films composed entirely of negatively charged DNA by taking advantage of the hybridization of appropriately designed complementary strands of single-stranded DNA [138–140].

In one example, these investigators designed two 40-mer single-stranded diblock DNA oligomers, poly$A_{20}G_{20}$ and poly$T_{20}C_{20}$ (A = adenosine, G = guanine, T = thymidine, C = cytosine). In this scheme, one block of each oligomer was designed such that it is able to hybridize to a complementary block of the second oligomer (i.e., A with T, and C with G; see Scheme 7) [138]. The alternating exposure of substrates to solutions of these two diblock oligomers resulted in layer-by-layer growth, as characterized by quartz crystal microgravimetry.

Subsequent experiments demonstrated that the compositions and sequences of these constructs play important roles in governing film assembly as well as the structures and stabilities of these materials in aqueous media [138–140]. For example, the exposure of these films to solutions of reduced ionic strength, (e.g., between 0 and 100 mM NaCl) resulted in partial or substantial film disassembly, depending upon the structure of the oligonucleotides used to fabricate the films [138–140]. Additional studies will be required to understand the mechanism through which changes in ionic strength influence the stability of these assemblies. However, these investigators suggest an interplay between cohesive interactions generated by complementary base pairing and repulsive electrostatic interactions between backbone phosphate groups, which become stronger as ionic strength is reduced and the negative charges of the phosphate groups become less shielded [138–140]. Regardless, these current results suggest a platform for the design of DNA/DNA films that could be used to release designed or sequence-specific oligonucleotides under physiological conditions.

5.4 Characterization of Film Permeability and Oligonucleotide Diffusion

As described above, DNA is a large, polyanionic molecule and, as a result, it cannot diffuse through ionically-crosslinked multilayered films in the manner of a small molecule. As a result,

the approaches described above have focused largely on methods of delivery that involve film or capsule disruption rather than the diffusion of DNA or oligonucleotides through multilayered films. Caruso and coworkers demonstrated recently, however, that multilayered films fabricated from poly(styrene sulfonate) (PSS) and poly(allylamine) (PAH) are, in fact, permeable to small, single-stranded oligonucleotides. These investigators have also reported on methods for the quantification of film permeability and the measurement of oligonucleotide diffusion constants based on a fluorescence-based molecular beacon approach (Scheme 8) [141,142].

In this work, DNA-based molecular beacons were immobilized in the pores of mesoporous silica particles, and these functionalized beads were subsequently coated with PSS/PAH multilayered films [141,142]. Incubation of film-coated beads in solutions of oligonucleotides targeted to disrupt the stem-loop structure of the molecular beacons resulted in increases in fluorescence in ways that (i) varied according to the length of the target DNA sequence, the number of layers of the film, and other factors, and (ii) could be interpreted in terms of differences in film permeability. For example, experiments using four model target DNA sequences (from 15 to 60 bases long) demonstrated that shorter oligonucleotides diffuse through films more rapidly than larger oligonucleotides and that, in general, thicker films are less permeable than thinner films [141,142].

While these size-based correlations are not completely unexpected, the broader significance of these reports in the context of oligonucleotide delivery is the introduction of straightforward, modular, and high-throughput fluorescence-based methods that permit quantitative measurements of film permeability and oligonucleotide diffusion. A more complete and quantitative understanding of the diffusion of oligonucleotides across thin multilayered films will help guide future efforts to develop diffusion-based approaches to the release of oligonucleotides from multilayered film capsules.

6. Summary and Outlook

Layer-by-layer methods of assembly provide convenient, nanometer-scale control over the incorporation of nucleic acid-based constructs into thin polyelectrolyte films. Provided that these assemblies can be designed in ways that permit controlled film disassembly under physiological conditions, this approach also has the potential to contribute new methods for spatial and/or temporal control over the delivery of nucleic acid-based therapeutics. In the sections above, we have described applications of layer-by-layer assembly to the fabrication of multilayered films that can be used to sustain or trigger the release of plasmid DNA from surfaces and promote surface-mediated cell transfection. We have also highlighted the application of these methods to the coating of colloidal substrates and the fabrication of hollow micrometer-scale capsules that can be used to encapsulate and release DNA and oligonucleotide constructs.

Although the potential of these new approaches seems apparent, it is important to keep in mind that these methods are, indeed, new. As a result, it is too early to determine the full range of biological and gene delivery applications for which these methods and materials might be best suited. It is also important to realize that the approaches described above have emerged largely from fundamental research by investigators in the materials science community – while the incorporation of DNA into multilayered films was first reported in 1993, the first applications of this approach to the release and delivery of functional DNA have emerged only recently. As this work has evolved in a biological direction, it has also become clear that these new approaches will be subject to many of the same barriers, limitations, and general concerns encountered and, to some extent, addressed previously by other researchers developing new materials for the delivery of DNA. This new area of research is thus now poised to benefit

tremendously from the new perspectives and interdisciplinary work of researchers in biology, medicine, and the pharmaceutical sciences. It is our hope that this review, and the work described herein, will serve as a catalyst for the development of these methods for a broad range of delivery applications.

7. Acknowledgments

We thank Eric M. Saurer for providing the illustration in Scheme 4. Work from the authors' own laboratory was supported by the National Institutes of Health and the Arnold and Mabel Beckman Foundation. D. M. L. is a Research Fellow of the Alfred P. Sloan Foundation.

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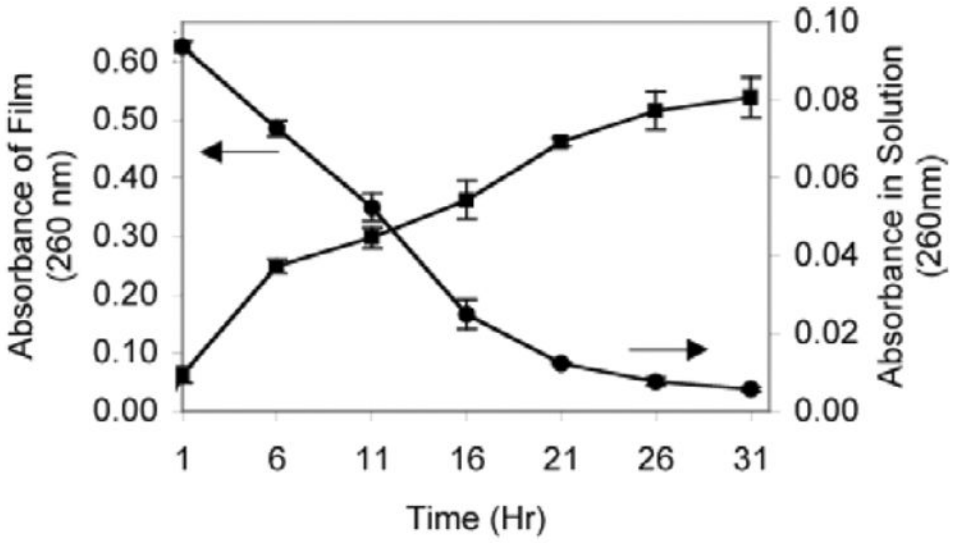


Figure 1.
Plot of absorbance vs. time for a 100 nm thick film fabricated from polymer **1** and plasmid DNA incubated in PBS at 37 °C. Closed circles (●) correspond to the amount of DNA in the film; closed squares (■) correspond to the amount of DNA released into the incubation buffer. Reproduced with permission from reference [86].

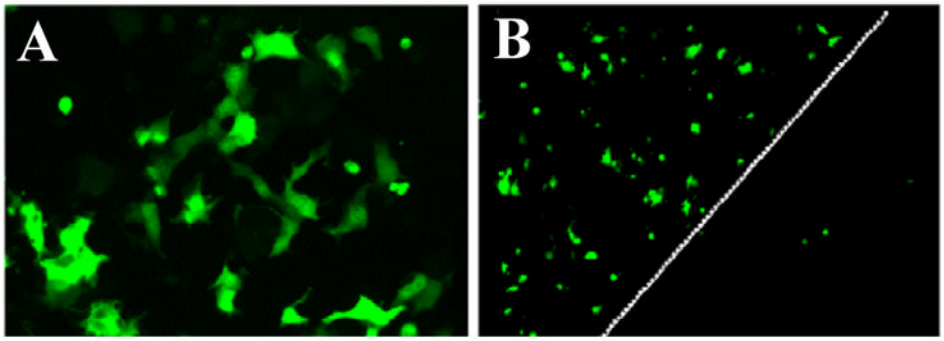


Figure 2.
Fluorescence microscopy images showing the surface-mediated transfection of COS-7 cells promoted by contact with glass slides coated with multilayered films fabricated from plasmid DNA and polymer **1**. A) Image showing transfected cells located under a film-coated slide. B) Lower magnification image showing the edge of a film-coated slide (indicated by white dotted line) showing spatially localized cell transfection. Reproduced with permission from reference [87].

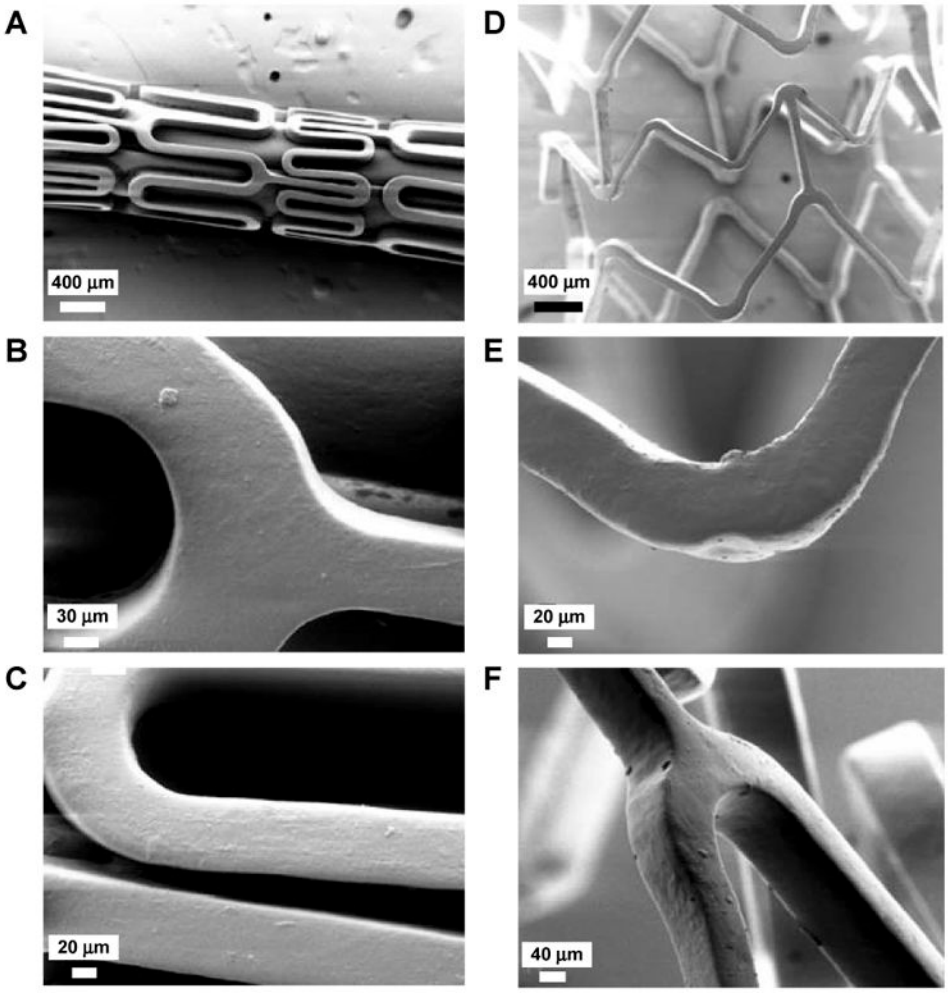


Figure 3.
Scanning electron microscopy images of stainless steel intravascular stents coated with multilayered films fabricated from polymer **1** and plasmid DNA. Images correspond to different magnifications and perspectives of a coated stent as-coated on a balloon assembly (A–C) and after balloon expansion (D–F). Reproduced with permission from reference [88].

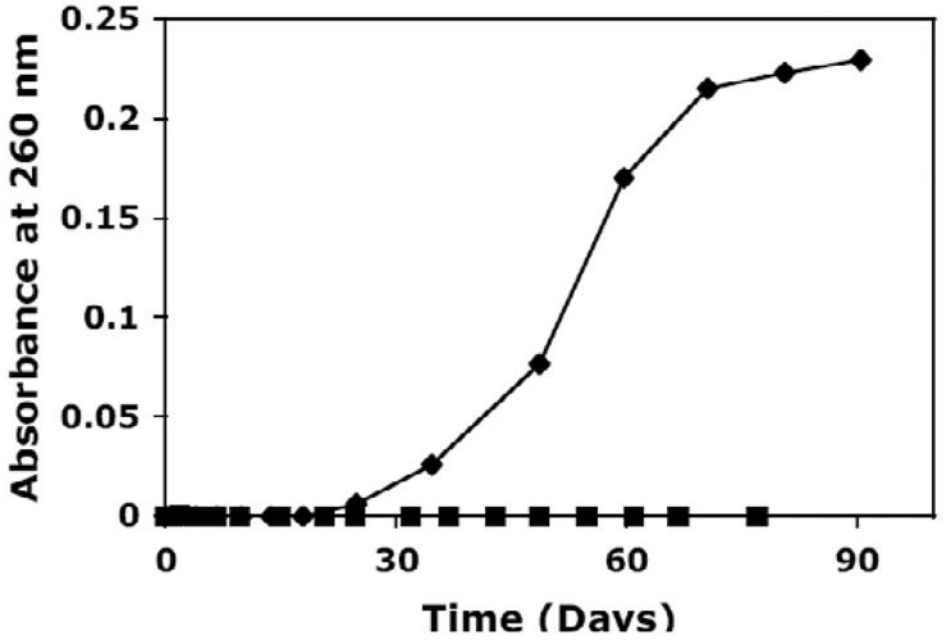


Figure 4.
Plot of absorbance vs. time showing the release of DNA from films fabricated from plasmid DNA and 'charge-shifting' polymer **2** (◆) or an analogue containing side chain amide bonds (■; see reference [98]). Release of DNA occurs over a period of three months upon incubation in PBS at 37 °C. Reproduced with permission from reference [98].

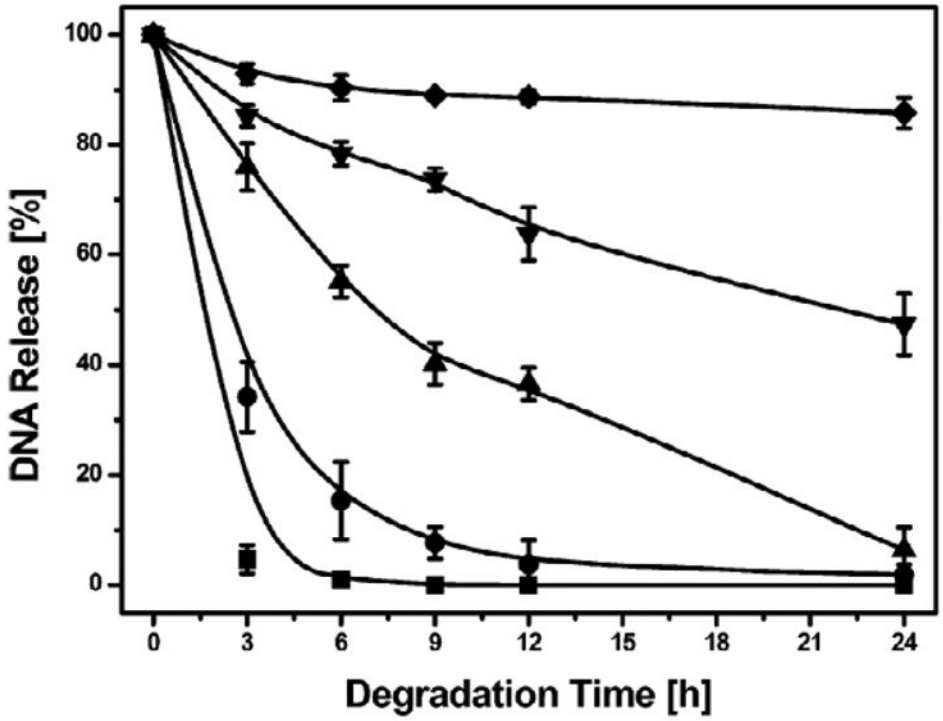


Figure 5.

Release of DNA from PLL/DNA films treated with a glutaraldehyde cross-linking agent for varying times and subsequently incubated in a trypsin solution for 24 hours. Symbols correspond to data obtained from films treated with glutaraldehyde for: (■) 0 min (original film); (●) 10 min; (▲) 30 min; (▼) 60 min; (◆) 180 min. A relative absorbance of 100% represents the absorbance of the original PLL/DNA film, while 0% designates the absorbance of bare quartz substrates. Reproduced with permission from reference [102].

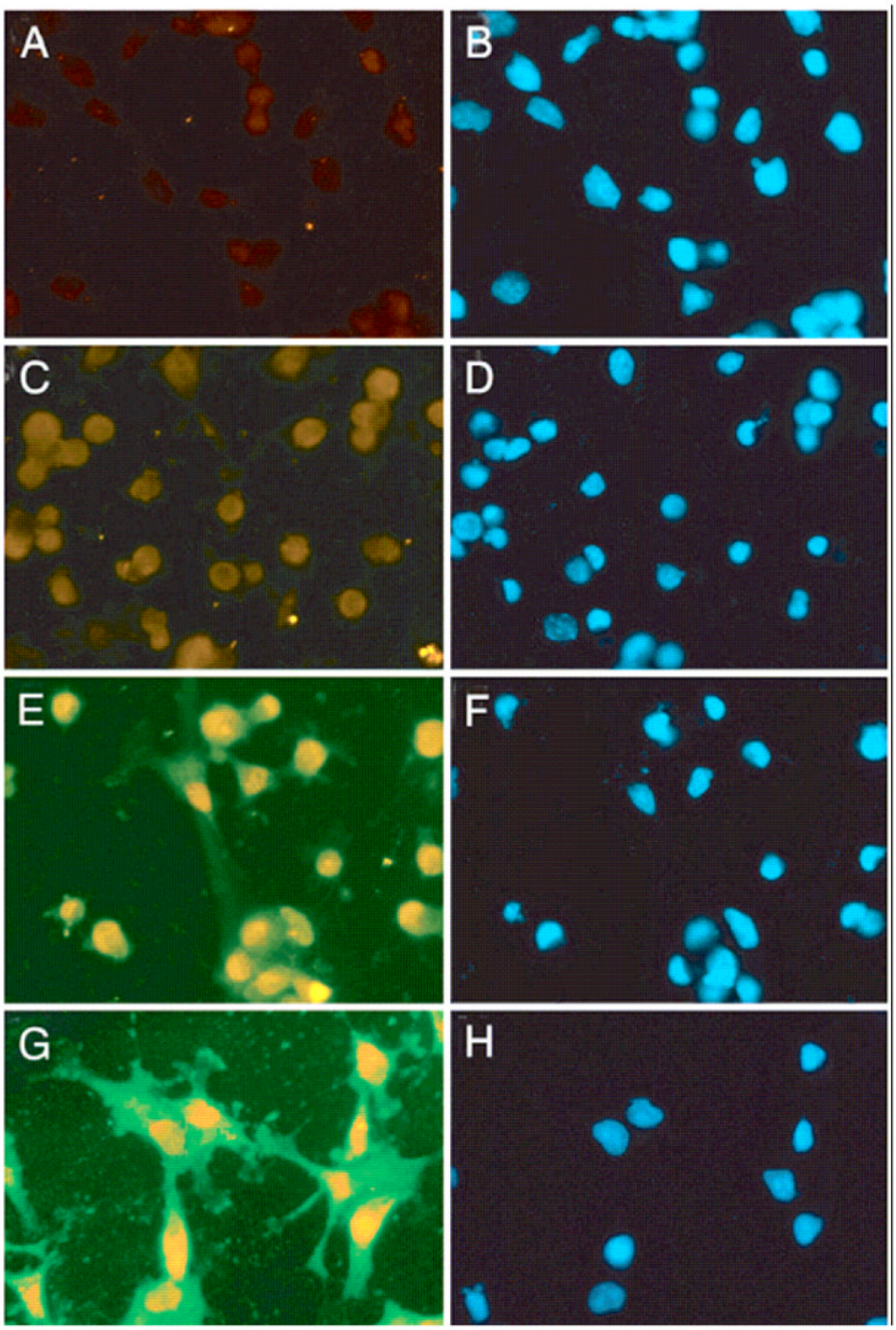


Figure 6.
Expression of a nuclear transcription factor (SPT7, red stain) and EGFP (green stain) in COS cells grown on the surfaces of films fabricated from PLL and PGA containing a layer of plasmid encoding SPT7 imbedded in the upper portion of the film and a layer of plasmid encoding EGFP imbedded in the lower layers of the film. Images correspond to (A and B) cells growing on control films (no imbedded plasmid), and cells growing on plasmid-containing films for 2 h (C and D), 4 h (E and F), and 8 h (G and H). The expression of SPT7 (red) and EGFP (green) was detected by indirect immunofluorescence staining; the blue color in B, D, F, and H corresponds to cell nuclei stained by Hoechst 33285. Reproduced with permission from reference [103].

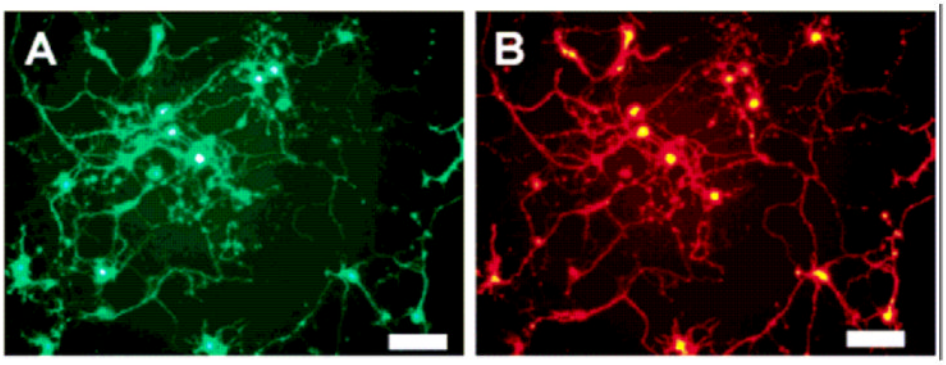


Figure 7.
Electric-pulse-triggered gene transfer into hippocampal neurons growing on ITO glass electrodes coated with multilayered films fabricated from poly(ethylene imine) and either a plasmid encoding EGFP (A) or RFP (B). An electric pulse (200 V/cm, 10 ms) was applied 72 h after cell seeding to promote transfection. Adapted with permission from reference [110].

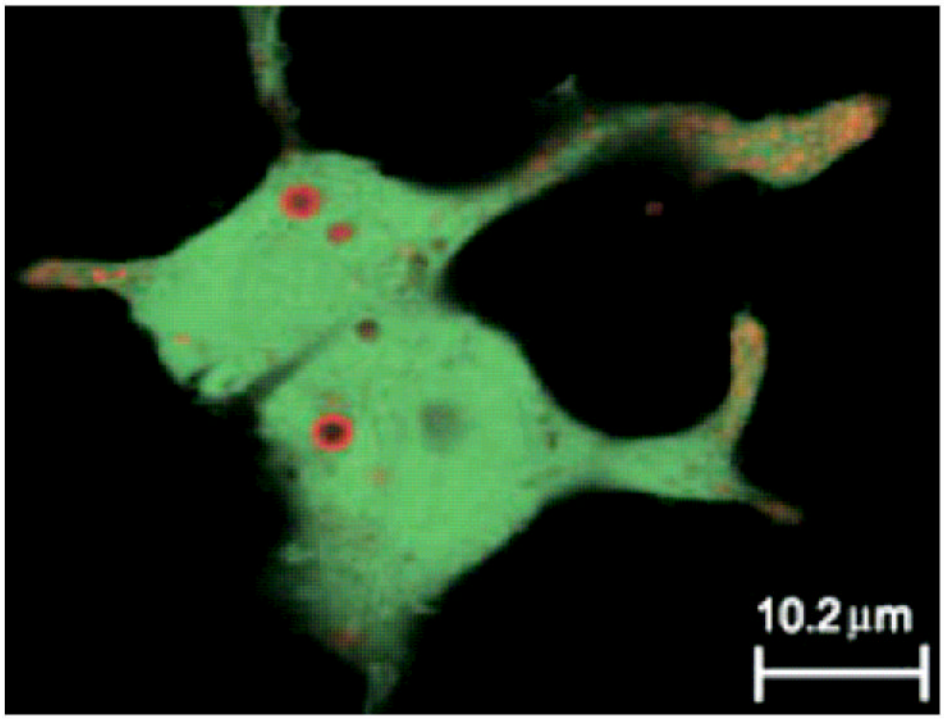


Figure 8.
Confocal microscopy image of HEK 293T cells expressing EGFP (green) after internalizing silica spheres (3 µm) coated with a multilayered film containing an imbedded layer of plasmid DNA encoding EGFP. Red corresponds to fluorescently labeled protamine used to fabricate the multilayered films. Reproduced with permission from reference [112].

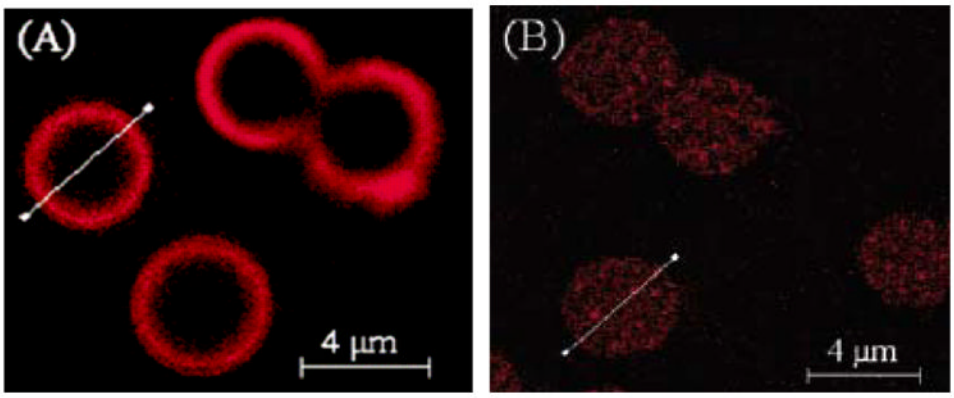


Figure 9.
Fluorescence confocal microscopy images of DNA-containing capsules (A) just after decomposition of the template core (see Scheme 5) and (B) after dissolution of inner DNA/spermidine complexes. Reproduced with permission from reference [121].

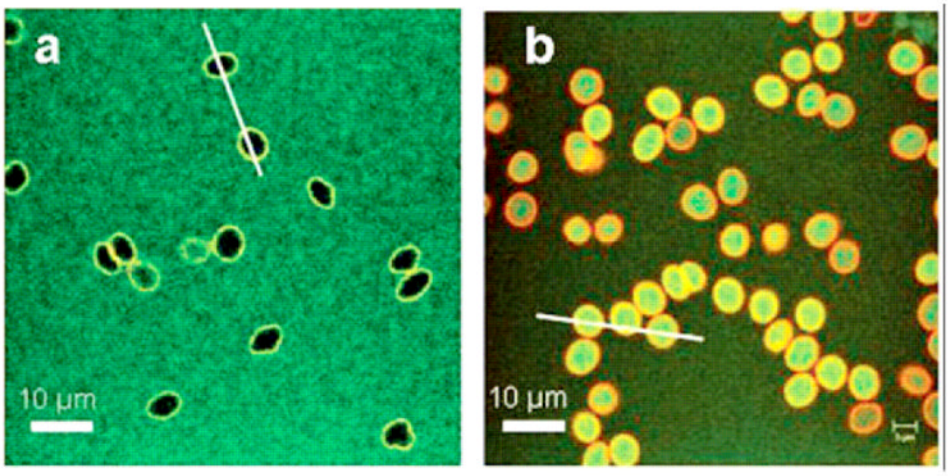
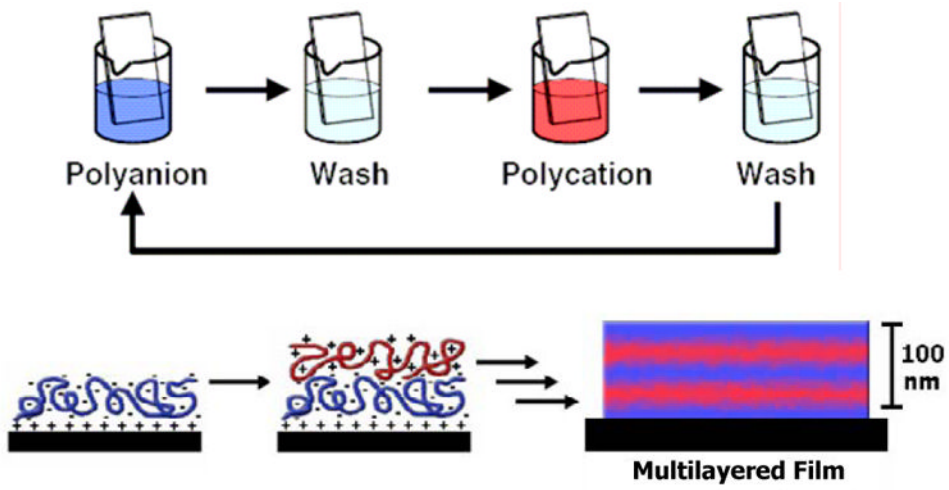
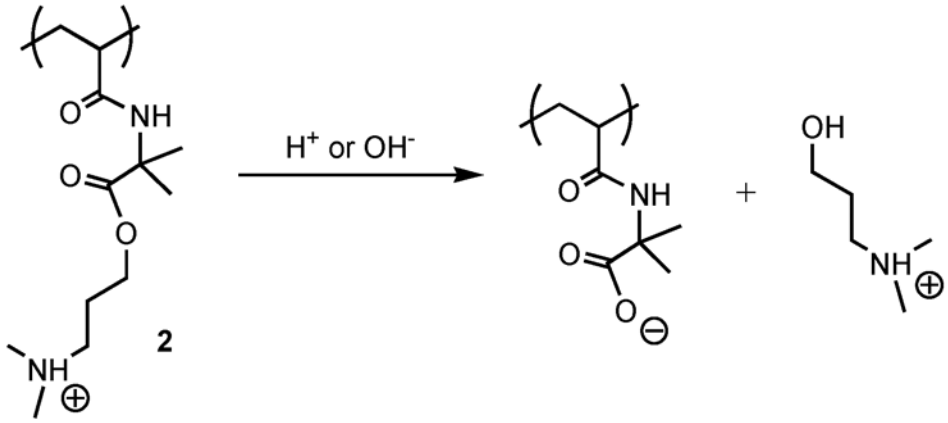


Figure 10.
Post-fabrication loading of DNA into hollow polyelectrolyte multilayer capsules fabricated using removable human red blood cell cores. A) Fluorescence confocal microscopy images showing hollow capsules suspended in a DNA solution (green) showing impermeability of the capsules to DNA. B) Images of capsules after drying in the presence of DNA followed by resuspension showing DNA-filled capsules. Reproduced with permission from reference [124].

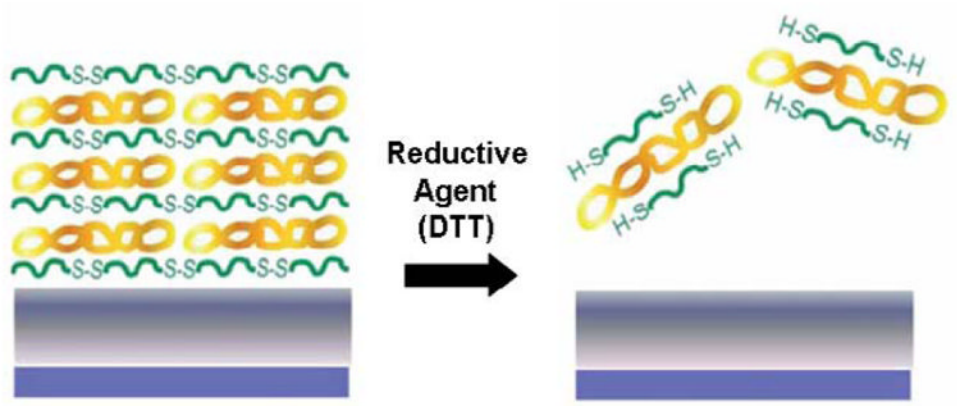


Scheme 1.

The layer-by-layer deposition of oppositely charged polyelectrolytes on surfaces is a well-established method for the fabrication of thin multilayered films. Top: Fabrication proceeds by iterative dipping of a substrate into dilute aqueous solutions of cationic and anionic polymers. Bottom: Electrostatic interactions guide the assembly of multilayered polyelectrolyte assemblies.

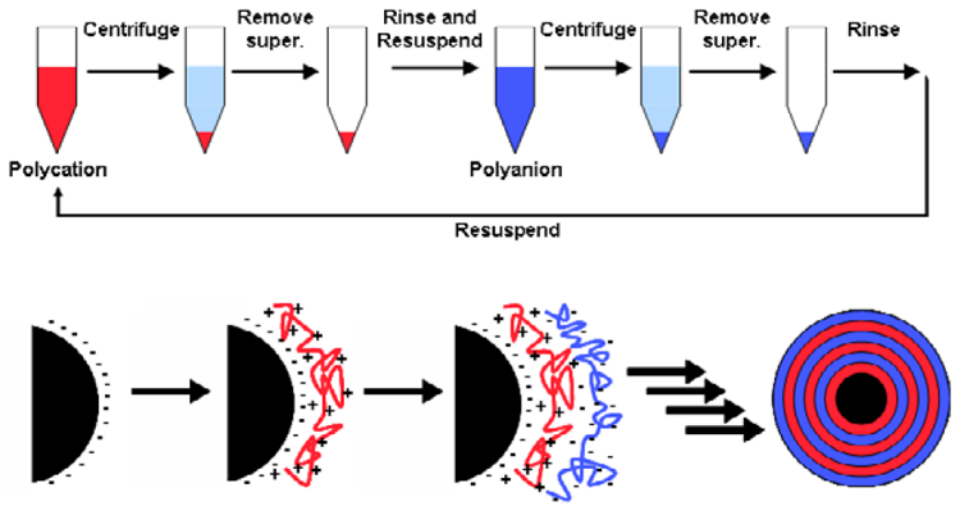


Scheme 2.
Structure of a side-chain functionalized ‘charge-shifting’ cationic polymer. Side chain hydrolysis results in a shift of the net charge of the polymer from cationic to less cationic or anionic. Reproduced with permission from reference [98].



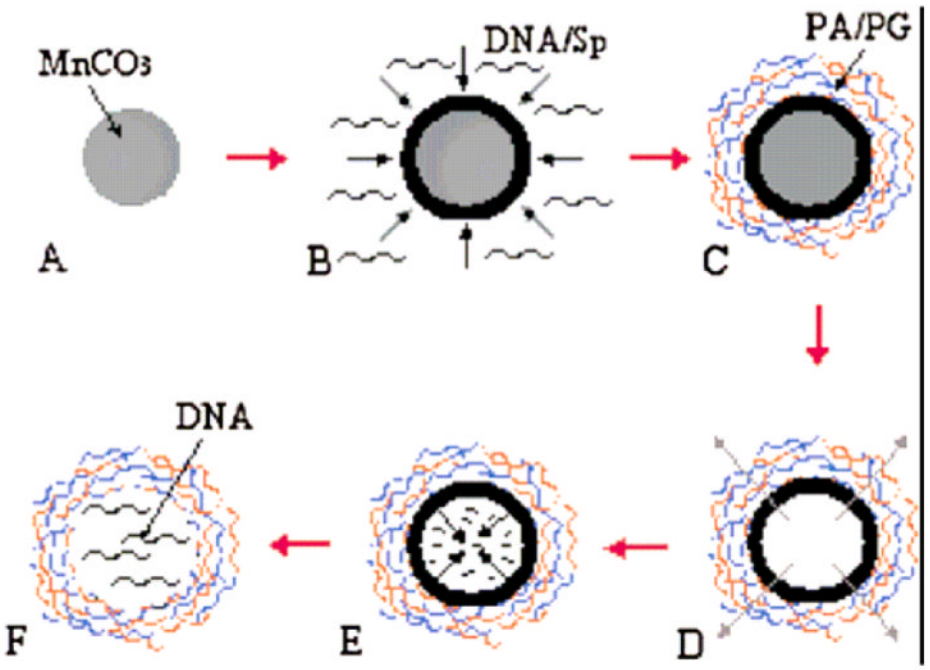
Scheme 3.

Schematic illustration showing the disassembly of multilayered films fabricated from plasmid DNA and a reductively degradable peptide-based cationic polymer. Stable films disassemble upon exposure to a chemical reducing agent. Adapted with permission from reference [108].



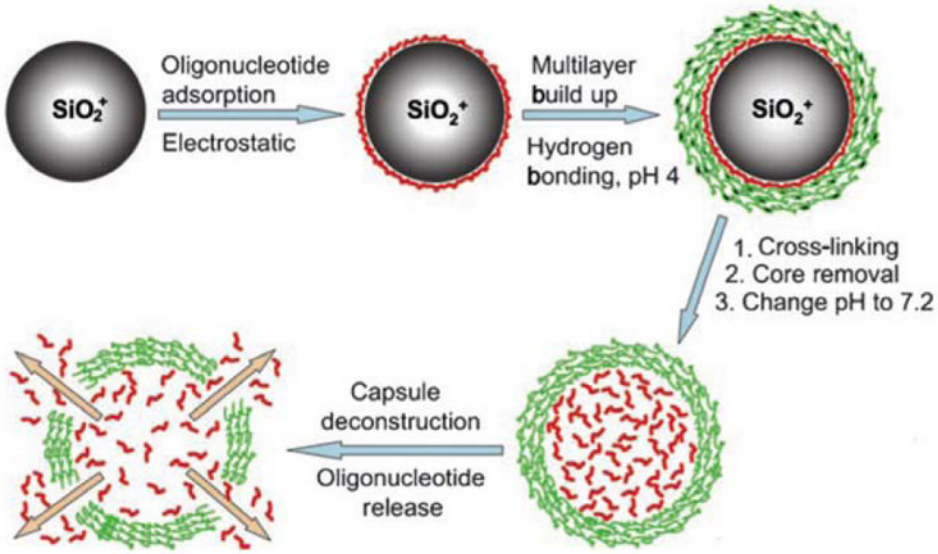
Scheme 4.

Assembly of multilayered polyelectrolyte films on colloidal substrates by sequential exposure to oppositely charged polyelectrolyte solutions. In contrast to dipping-based fabrication procedures illustrated in Scheme 1, fabrication is conducted by repeated cycles of centrifugation and resuspension to yield colloids coated with thin multilayered films.



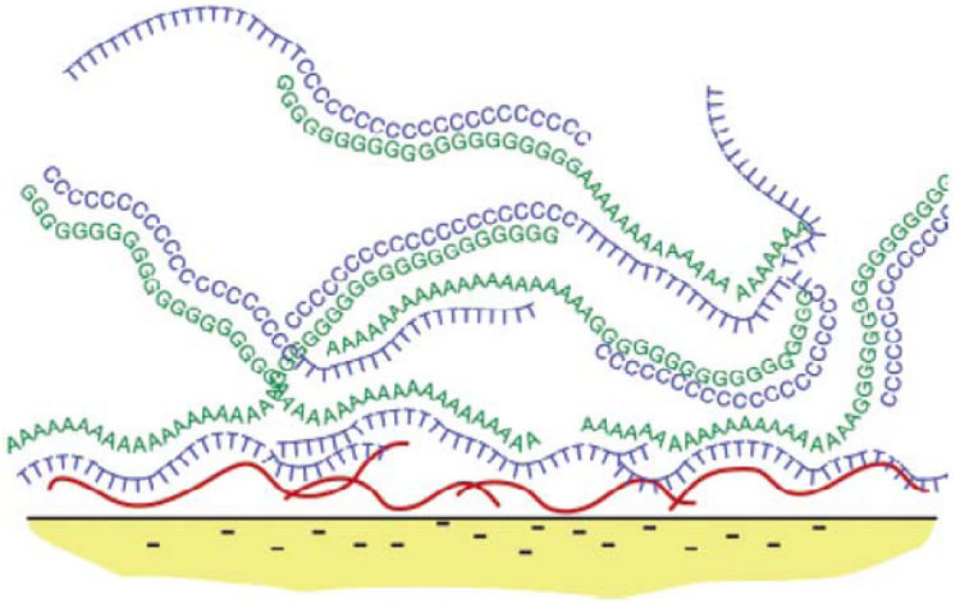
Scheme 5.

Schematic illustration of encapsulation of DNA in a hollow multilayered film capsule. (A,B) Controlled precipitation of DNA/spermidine complexes onto the surfaces of template particles. (B,C) Layer-by-layer assembly of a multilayered film on the surfaces of the particles. (C,D) Dissolution and removal of the particle template cores. (D,E) Dissolution of DNA/spermidine complexes. Reproduced with permission from reference [121].



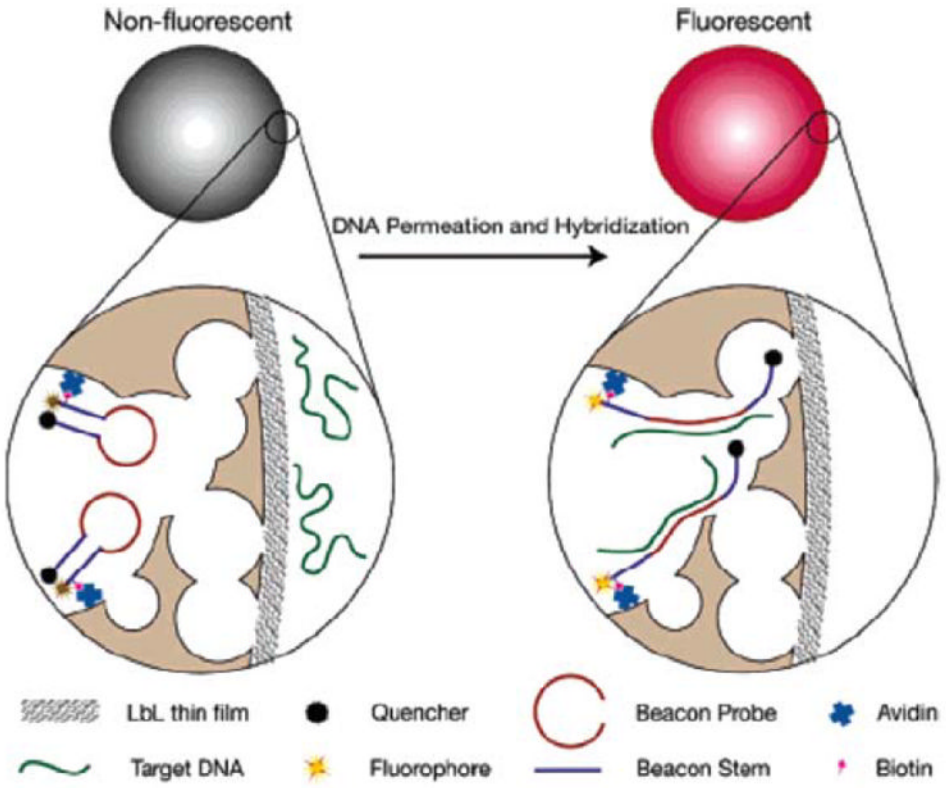
Scheme 6.

Schematic representation of the template-assisted encapsulation of short oligonucleotides in disulfide bond-stabilized multilayered capsules. Treatment of stabilized capsules with chemical reducing agents results in triggered film disruption and release of encapsulated oligonucleotides. Reproduced with permission from references [122].



Scheme 7.

Schematic illustration showing the basis of an approach to the layer-by-layer assembly of multilayered films composed entirely of DNA. Repeated exposure of substrates to solutions of specifically designed block oligonucleotides with complementary sequences leads to the growth of multilayered films guided by complementary base pairing. Reproduced with permission from reference [134].



Scheme 8.

Schematic illustration showing a molecular beacon-based approach to measuring the permeability of multilayered polyelectrolyte films to oligonucleotides. Left) Biotin functionalized molecular beacons are immobilized inside avidin-modfied porous particles, and the particles are subsequently coated by fabrication of a multilayered film on the surfaces of the particles. Right) Diffusion of an oligonucleotide with a sequence targeted to the molecular beacon through the multilayered film activates fluorescence. Reproduced with permission from references [137].