Construction of Biodegradable Multilayer Films via Layer-by-Layer Self-Assembly as Gene Delivery System

Kefeng Ren, Jian Ji*, and Jia-cong Shen

Department of Polymer Science and Engineering, Zhejiang University, Hangzhou 310027, China
Tel: +86-571-87951108, Fax: +86-571-87951948, email: jianji@mail.hz.zj.cn

Keywords: layer-by-layer, protamine, gene delivery, biodegradation.

Abstract. Films composed of alternating layers of protamine and DNA were constructed using the layer-by-layer method on quartz and subsequently studied the enzymic degradation in vitro. UV-visible spectrometry measurement indicated the uniform assembly of Protamine/DNA multilayer films. UV-visible spectrometry and fluorescence spectrometry results revealed that the Protamine/DNA multilayer films were in vitro enzymic biodegradable. The novel biodegradable multilayer of Protamine/DNA may have great potential for gene therapy applications in tissue engineering, medical implant etc.

Introduction

Localizing and sustaining the presence of therapeutic gene at targets are very important for gene delivery and gene therapy. The development of new methods for the fabrication of thin films that provide precise control over gene release profiles could lead to significant advances in the fields of gene delivery and gene therapy. The layer-by-layer (LbL) assembly of different components, on a separate front, has shown to be an attractive avenue for fabrication of thin multilayer films of ordered structures containing various functional supramolecular moieties. [1] A common approach based on the LbL method is via electrostatic attraction between oppositely charged polyelectrolytes, permitting sequential deposition of these species from diluted solutions onto a surface. The advantage of the LbL method is that the layer thickness and composition can be precisely tailored by varying the number of adsorption cycles and the type of charged species. [1,2] Recently, several laboratories [3-6] are involved in the development of the multilayer films as functional delivery system includes the incorporation of new functionality into LbL films and then design to release incorporated material.

In this study, alternate layer-by-layer self-assembly of protamine with DNA was explored (Scheme 1). Protamine is a basic peptide that contains 75 % of arginine and assumed to be degraded under physiological conditions by enzyme in the living body. Protamine is expected to play a dual role, serving as polycations of the
films as well as a biodegradable element designed to control release; similarly, DNA serves as polyanions and as a functional material. We hypothesized here that the enzymatic degradation of polyelectrolytes in multilayer films could serve as a strategy to release therapeutic gene in vivo. The current objective of this paper is to assess the possibility of construction of biodegradable polyelectrolytes/DNA multilayer films via LbL method and control release of DNA in an in vitro enzymic system.

**Experimental Section**

**Materials.** Protamine sulfate and ethidium bromide (EtBr) were purchased from BBI. Deoxyribonucleic acid (DNA, Fish Sperm, Sodium Salt), N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPEs, free acid, High Purity Grade) was purchased from AMRESCO. α-Chymotrypsin (from Bovine pancreas, 74 U/mg Protein) was purchased from Worthington. Water used was distilled three times. All reagents were used as received.

**Multilayer Films Fabrication.** Protamine/DNA films were LbL assembled onto quartz (10 × 20 mm). The first layer of protamine was adsorbed onto the quartz surface by immersing the quartz substrate in 2 mg/mL Protamine/HEPEs buffer (20 mM HEPEs, pH 7.4, with 75 mM NaCl) for 30 min. The Protamine-covered substrates were then dipped in water five times, followed by drying under a stream of N₂. DNA was then attached to the Protamine layer by immersing the quartz substrate sequentially in 1 mg/mL DNA/HEPEs buffer (20 mM HEPEs, pH 7.4, with 75 mM NaCl) for 30 min. Multilayer composite films composed of alternating Protamine and DNA layers were fabricated via repeating the above procedure. Before the measurements, all samples were dried at room temperature in vacuum drier for at least 12 hours.

**Characterization of Multilayer Films.** A UV-visible spectrum was measured on a UV-visible spectrophotometer (CARY 100 BIO, America). All measurements were carried out at room temperature.

**Degradation of Multilayer Films.** An enzymic degradation study in vitro was carried out and α-chymotrypsin was selected as model protease to evaluate the biodegradability of (Protamine/DNA)ₙ films. (Protamine/DNA)₁₀ films were selected as the degradation samples. Multilayer films were exposed to a system containing 5 U/ml α-chymotrypsin in PBS pH 7.4 at 37 °C. The samples were removed from the incubation solution at appropriate time intervals, washed, and then vacuum dried at room temperature in vacuum drier for at least 12 hours.

**Characterization of Multilayer Films Degradation.** A UV-visible absorption spectrum change of multilayer films on quartz substrates was measured on a UV-visible spectrophotometer (CARY 100 BIO, America). The incubation solution that contained released DNA was added (100:1, vol %) EtBr solution (50 µg/ml) and then measured on a spectrofluorophotometer (RF-5301PC, SHIMADZU, Japan) with an excitation wavelength of 530 nm, a 10 nm slit, and an emission wavelength of 590 nm with a 10 nm slit. All measurements were carried out at room temperature.

**Results and Discussion**

**Monitoring the LbL Deposition Process.** Multilayer films growth on quartz slides can be easily monitored by UV-visible absorption spectroscopy. The UV-visible absorption spectra changes of Protamine/DNA multilayers 1, 2, 3, 4, 5, 6, and 10 bilayers are shown in Fig 1. DNA contributes a characteristic absorption band at 260 nm. The linear increase of the absorbance as a function of the number of layers suggests that the amount of DNA deposited each time is constant, as can be seen in the inset of Fig. 1.
**Deconstruction of Multilayer Films.** The in vitro enzymatic degradation of \((\text{Protamine/DNA})_{10}\) was investigated to study the deconstruction of the enzymic biodegradable multilayer and its application potential as gene delivery system.

UV-visible spectrometry was used to monitor the decrease of DNA incorporated into the films. The UV-visible absorption of multilayer films on quartz substrates at 260 nm, which is the characteristic absorption band of DNA, was measured at appropriate degradation time intervals. As can be seen in Fig 2, the relative UV-visible absorbance of \((\text{Protamine/DNA})_{10}\) films decreased as a function of degradation time. The control curve of degradation in PBS without enzyme shows a decrease in absorbance, while the curve of degradation in PBS with 5 U/ml α-chymotrypsin shows a more significant decrease.

**Fig 1.** UV-vis absorption spectra of Protamine/DNA films as a function of layer number. The inset shows a plot of DNA absorption at 260 nm as a function of bilayer number. The outmost layer was DNA layer.

**Fig 2.** Relative UV-visible absorbance spectra of film at 260 nm as a function of degradation time. A relative absorbance of 100% represents the absorbance of undegraded Protamine/DNA multilayer films, while 0% designates the absorbance of bare quartz substrates. □: Control curve of degradation in PBS without enzyme; ■: Curve of degradation in PBS with 5 U/ml α-chymotrypsin.

**Fig 3.** Fluorescence spectra of EtBr added to incubation solution as a function of degradation time. □: Control curve of incubation solution without enzyme; ■: Curve of incubation solution with 5 U/ml α-chymotrypsin.
function of degradation time in the PBS containing 5 U/ml α-chymotrypsin while only little amount of DNA was lost under the same conditions without enzyme. For the most part of DNA within the films was released under the enzymic conditions in 9 hours, these data proved that the Protamine/DNA multilayer films are biodegradable and DNA within the films can be released.

Measurement of amount of DNA contained in the incubation solution can also probe the degradation of the multilayer films. The fluorescence enhancement of ethidium bromide (EtBr), which can produce a strong fluorescence signal upon intercalation with DNA, has been used to qualitative analysis of DNA. [8,9] Here, we chose an EtBr fluorescence assay to measure qualitatively the amount of DNA contained in the incubation solution. As can be shown in Fig 3, the fluorescence intensity of the incubation solution increased with time in 9 hours when multilayer film were exposed to enzyme in PBS buffer while the fluorescence intensity changed little when the multilayer films exposed to PBS buffer without enzyme. These data suggest that the multilayer films were deconstructed and simultaneously DNA was released. The observation is consistent with the conclusion drawn from UV-visible spectrometry experiments.

**Conclusion**

Biodegradable multilayer films composed of alternating layers of protamine and DNA were constructed using the layer-by-layer method. UV-visible spectrometry measurement indicated the uniform assembly of Protamine/DNA multilayer films. UV-visible spectrometry and fluorescence spectrometry results revealed that the Protamine/DNA multilayer films were in vitro enzymatic biodegradable. The novel biodegradable multilayer of Protamine/DNA might guarantee the sustaining and stabilizing the presence of therapeutic gene at targets, which may have great potential for gene therapy applications in tissue engineering, medical implant etc.

**Acknowledgment:** This research work is supported by the Natural Science Foundation of China (50373036).

**References**