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Multilayered thin films from poly(amido amine)s and DNA

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ABSTRACT

Dip-coated multilayered thin films of poly(amido amine)s (PAAs) and DNA have been developed to provide surfaces with cell-transfecting capabilities. Three types of PAAs, differing in side chain functional groups, were synthesized and characterized for their properties in forming multilayered structures with ultrasonicated calf thymus DNA (CTDNA) as model DNA. All three polymers display a multilayer build-up in linear profiles as demonstrated by UV spectroscopy. More highly charged side chains were found to provide the lowest deposition of DNA. Surface profiles of the obtained films were investigated by atomic force microscopy (AFM) and static water contact angle measurements to reveal complete surface coverage after at least four layer pair depositions, where alternating patterns of surface profiles were observed depending on whether the cationic polymer or the anionic DNA layer was on top. The stability of the formed surfaces was investigated *in vitro* under physiological and reductive conditions. Owing to the presence of disulfide bonds in the PAA main chain, the films were readily degraded in the presence of 1 mM of DTT *in vitro*. Under non-reductive physiological conditions, two of the thicker films underwent thermodynamic rearrangement, which resulted in release of approximately half of the incorporated material within 1 h, which was caused by the physiological salt concentration. Further, this unpacking phenomenon proved useful in transfecting COS-7 cells seeded on top of these multilayers containing functional plasmid DNA encoding for green fluorescence protein (GFP). Two out of the three different multilayers facilitated good COS-7 cell attachment, proliferation, and transfection *in vitro* within 2 days of culture. Fluorescence staining further revealed the presence of DNA-containing released film material among cultured cells. The present work demonstrates the possibility of coating surfaces with thin films that are conveniently adjustable in thickness and amount of active agent to provide cell-transfecting functionality. In this manner transfection can be achieved by simply culturing cells on a multilayer-coated surface in their optimal culture condition (in the presence of serum) and without the need of removing the transfection agent to avoid cytotoxicity.

1. Introduction

Multilayered thin films containing functional DNA have been of great interest during the past two decades in parallel to the ever-growing interest in the layer-by-layer (LbL) assembly technique in various fields [1]. The possibility to fabricate intricate multilayered structures within nanometer range precision, combined with the versatility of the fabrication technique, have made LbL assembly in the biomedical field an interesting option to easily modify the surface of biomedical devices [2], not only to enhance their performance but also to add additional functionality for therapeutic effect.

Due to the inherent properties of DNA as a natural biological compound, multilayers containing DNA have been investigated for their possibility of providing relatively low-immunogenic coatings [3] and surface-mediated cell transfection on various substrates [4–7]. Multilayers containing a top DNA layer have shown a significant increase in primary rat dermal fibroblast proliferation as compared to non-coated glass surfaces *in vitro*, while *in vivo* tissue response studies also showed that the DNA-coatings are histocompatible without showing signs of inflammation or adverse reactions [3]. The current study does not concern the CpG oligodeoxynucleotides (CpG ODN) known to be immunostimulants [8,9]. The DNA used for *in vitro* viability and transfection efficiency experiments is of plasmidic nature.

Another key parameter in multilayered systems containing DNA is the polyelectrolyte counterpart. Natural polyelectrolytes such as chitosan [10] as well as synthetic polymers such as polyethyleneimine (PEI) [11] and polyallylamine hydrochloride (PAH) [12] have been successfully incorporated into multilayered systems complementing also their widely studied potency in forming polyplexes

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with DNA. Multilayers composed of DNA with more than a single type of polyelectrolyte have also been developed where highly-studied polyelectrolytes such as PEI, PAH, and polystyrene sulfonate (PSS) are used mostly for their well-known ability to form stable precursor layers [13,14] or to provide further distinction in the multilayer architecture and/or enhanced stability [15]. In addition to directly depositing DNA from a DNA solution, optimized cellular uptake of functional DNA has also been achieved by packing DNA in a liposome system [16,17] or precomplexing DNA with known polyelectrolytes such as PEI and chitosan to form a polyplex prior to the deposition step [18,19].

Poly(amido amine)s (PAA) denote a class of biodegradable polymers, which has been shown to be able to complex biotherapeutics such as proteins [20], plasmid DNA (pDNA) [21–23], and small interfering RNA (siRNA) [24] with relatively lower toxicity as compared to the standard PEI. The inherent peptidomimetic structure provides these polymers with biocompatibility and degradability while the presence of amines provides the necessary positive charges for the interaction with DNA. Furthermore, the versatility of the synthesis and possibilities to incorporate a wide variety of functionalities make this class of polyelectrolyte an interesting choice to be used in LbL assembly with DNA.

A special property of bioreducible PAAs in relation to the intended applications as bioactive surfaces, relies on the finding that cell membranes have reductive properties owing to the presence of reducing enzymes and thiol groups in proteins present in the cell membrane [25,26]. This has triggered attempts to achieve a localized controlled release of active agents from bioreducible multilayered thin films. Delivery of DNA in this respect is particularly interesting as there have been many attempts at surface-mediated cell transfection even without incorporation of a multilayer structure [27–29] as pioneered by the group of Oupický [30,31].

On another note, multilayered coatings may also be viewed as a delivery system that releases therapeutic agents, in the sense similar to a hydrogel, for example. Therapeutic agents to be released are incorporated during multilayer assembly and are subsequently released either via simple diffusion [32], biodegradation of the polyelectrolyte building block [33], or induced by internal [34] or external [35] triggers. In view of cell transfecting surfaces, Lynn and Bechler reported that the two multilayer components (i.e., hydrolysable poly(β-amino ester) and DNA) were found to co-localize within transfected cells cultured in the vicinity of the surface, indicating that either the polymer–DNA complex is released, or the separate components are released and later form a complex in cell culture medium before being internalized by cells [36].

Here we present the syntheses of three PAAs and the characterization of their properties in forming multilayered thin films with ultrasonicated calf thymus DNA (CTDNA) as model DNA. The effects of structural differences among the three polymers have been evaluated for their properties to form multilayers that could act as bioactive surfaces. Release profiles were studied based on the relative DNA content within the constructs under physiological conditions and in reducing environment. Finally the ensembles containing reporter plasmid pCMV-GFP (i.e., pDNA encoding green fluorescence protein (GFP)) were assessed for their efficacy in surface-mediated cell transfection of COS-7 cells.

2. Materials and methods

N,N′-cystaminebisacrylamide (CBA, 99.9%) was purchased from Polysciences (Eppelheim, Germany). 4-amino-1-butanol (ABOL, 98.0%), *N*-Boc-1,4-diaminobutane (NBDAB, ⩾97.0%), histamine dihydrochloride (HIS·2HCl, ⩾99.0%), calcium chloride ($CaCl_2$, ⩾93.0%), triethylamine (TEA, ⩾99.0%), *tert*-butylamine (*t*BA, ⩾99.5%), trifluoroacetic acid (TFA, ⩾99.0%), sodium chloride (NaCl, ⩾99.5%), calf thymus DNA sodium salt (CTDNA, type 1, fibers, 16.7 A260 unit/mg solid, 6.0% sodium), dithiothreitol (DTT, ⩾99.0%), sulfuric acid (H_2SO_4, 95–98%), and hydrogen peroxide (H_2O_2, 30 wt.% in H_2O) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$, 99.0–102.0%) and disodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$, 99.5%) were purchased from Merck (Darmstadt, Germany). All reagents and solvents were purchased at the highest purity available and used as received. Milli-Q water (18.2 MΩ cm at 25 °C) was obtained from a Synergy® water purification system (Millipore).

PBS buffer was prepared by dissolving 1.54 g of $Na_2HPO_4{\cdot}2H_2O$, 0.30 g of $NaH_2PO_4{\cdot}H_2O$, and 8.20 g of NaCl into 1.00 L of Milli-Q water and adjusting the pH to 7.4.

CTDNA solution in water was prepared by dissolving shredded CTDNA into Milli-Q water to the final concentration of 1.0 mg/mL. The solution was placed in an ice bath and then sonicated (Sonoplus HD2070, Bandelin, Berlin, Germany) for 20 min at 10% power, 20 kHz frequency, and 7 cycles (i.e., 0.7 s of active interval followed by 0.3 s of passive interval).

^{1}H NMR spectra were recorded on an AVANCE III-400 MHz NMR (Bruker, Wormer, The Netherlands) spectrometer. Gel permeation chromatograms were recorded on a Polymer Labs GPC 220 in 0.1 M NaOAc buffer pH 4 with 25% methanol as eluent and 0.7 mL/min flow rate against poly(ethylene glycol) (PEG) standards.

Ultraviolet (UV) characterization of multilayered thin films was performed in the dry state using a UV-2401 PC (Shimadzu, 's-Hertogenbosch, The Netherlands). Each film fabricated on UV-transparent 7.5 × 37 × 1 mm quartz glass (Ted Pella, Redding, USA) was measured in three different arbitrary positions. Absorbance scan was carried out in the 200–400 nm wavelength range. All data points were then corrected for baseline offset by subtracting the absorbance value at 400 nm from each data point. Relative absorbance values were obtained by normalizing each data point with the respective value at time 0.

AFM characterization was performed on a Multimode AFM (Bruker, Wormer, The Netherlands) with Nanoscope IV controller in contact mode using an MSCT cantilever with moderate spring constant of 0.5 N/m. Multilayered thin film samples were fabricated on 100 mm single side polished silicon wafer (n-type, 525 μm thick, MESA+NanoLab, Enschede, The Netherlands) diced into 7.5 × 32 mm pieces.

Contact angle measurements were performed on a Krüss G10 (KRÜSS, Hamburg, Germany) contact angle measuring instrument.

Poly-D-lysine-coated 96 well plates (PDL-TCPS) for multilayer build-up for cell culture and transfection experiments were purchased from Greiner (Alphen aan den Rijn, The Netherlands).pCMV-GFP pDNA reporter gene (3487 bp) was purchased from PlasmidFactory (Bielefeld, Germany) at 1.0 mg/mL concentration in water for injection (WFI).

COS-7 cells (European Collection of Animal Cell Cultures (ECACC) Catalog No. 87021302) were grown in DMEM containing 4.5 g/L glucose and GlutaMAX™ (Invitrogen, Breda, The Netherlands) supplemented with 2% (v/v) PennStrepp (Lonza, Breda, The Netherlands) and 10% (v/v) fetal bovine serum (Lonza, Breda, The Netherlands).

Cell imaging was performed at 4×, 10×, 20×, and/or 40× objectives using an EVOS digital inverted microscope (EMS, Wageningen, The Netherlands) equipped with GFP, RFP and DAPI light cubes for expressed GFP, EthD-1, and Hoechst 33258 fluorescence imaging, respectively. EthD-1 and Hoechst 33258 were purchased from Invitrogen (Breda, The Netherlands) and Aldrich, respectively.

Fluorescence intensity measurement was carried out in an Infinite M200 PRO plate reader (Tecan, Giessen, The Netherlands). AlamarBlue for cell viability measurements was purchased from Invitrogen (Breda, The Netherlands).