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Poly(amido amine)-based multilayered thin films on 2D and 3D supports for surface-mediated cell transfection

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ABSTRACT

Two linear poly(amido amine)s, pCABOL and pCHIS, prepared by polyaddition of cystamine bisacrylamide (C) with 4-aminobutanol (ABOL) or histamine (HIS), were explored to form alternating multilayer thin films with DNA to obtain functionalized materials with transfection capacity in 2D and 3D. Therefore, COS-7 cells were cultured on top of multilayer films formed by layer-by-layer dipcoating of these polymers with GFP-encoded pDNA, and the effect of the number of layers and cell seeding density on the transfection efficiency was evaluated. Multilayer films with pCABOL were found to be superior to pCHIS in facilitating transfection, which was attributed to higher incorporation of pDNA and release of the transfection agent. High amounts of transfected cells were obtained on pCABOL films, correlating proportionally over a wide range with seeding density. Optimal transfection efficiency was obtained with pCABOL films composed of 10 bilayers. Further increase in the number of bilayers only marginally increased transfection efficiency.

Using the optimal multilayer and cell seeding conditions, pCABOL multilayers were fabricated on poly(ε-caprolactone) (PCL), heparinized PCL (PCL-HEP), and poly(lactic acid) (PLA) disks as examples of common biomedical supports. The multilayers were found to completely mask the properties of the original substrates, with significant improvement in cell adhesion, which is especially pronounced for PCL and PLA disks. With all these substrates, transfection efficiency was found to be in the range of 25–50% transfected cells. The pCABOL/pDNA multilayer films can also conveniently add transfection capability to 3D scaffolds. Significant improvement in cell adhesion was observed after multilayer coating of 3D-plotted fibers of PCL (with and without an additional covalent heparin layer), especially for the PCL scaffold without heparin layer and transfection was observed on both 3D PCL and PCL-HEP scaffolds. These results show that layer-by-layer dip-coating of pCABOL with functional DNA is an easy and inexpensive method to introduce transfection capability to biomaterials of any nature and shape, which can be beneficially used in various biomedical and tissue engineering applications.

1. Introduction

The layer-by-layer (LbL) fabrication technique has emerged as a versatile way not only to modify or improve surface properties, but also to add functionalities to the surface of various biomedical materials [1–4]. For example, a substantial development in tissue engineering relies on scaffolds for mechanical cell support and many medical operations involve mechanical support devices such as stents, prostheses, and other implants that have specific shapes and sizes. Often these devices are made of specific materials chosen for their mechanical strength and biodegradability, but do not really provide optimal performance in their interactions with cells [5]. LbL assembly offers an excellent option to add functionality to biomedical materials by coating various substrates with thin multilayers of functional macromolecules simply by dipping the substrates alternately into two aqueous solutions containing the desired macromolecules. The resulting surface can promote or reduce cell adhesion [6–8], deliver small drugs [9–11] or therapeutic proteins [12–15], induce differentiation [16–19] and cell transfection [20–22]. Moreover, the concentration and variety of material incorporation and release can be adjusted simply by adding or reducing the deposition cycles.

A promising application for LbL assembly that has recently received increasing attention is surface-mediated cell transfection [23]. As early as 1993, Lvov et al. reported the preparation of a multilayer using DNA as a building block [24]. Since then, several research groups, notably the groups of Lynn and Hammond, have studied the potential of LbL-based multilayers to provide localized delivery of transcriptionally

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active DNA [22,23,25–28]. The DNA may be deposited between the layers [20,25], or pre-complexed with polymers into polyplexes [29,30]. Notably, surface-mediated cell transfection has been attempted on intravascular stents [26,27], flexible stainless steel [20], poly(D-lactic acid) film [31], and micro-needles [28] aimed for vaccination application [32,33].

Multilayer coatings for surface-mediated transfection purposes on 3D scaffolds for tissue engineering have not been extensively reported. Attempts to introduce transfection capability to 3D scaffolds are mostly carried out before [34,35] or after [36–38] seeding into the scaffolds, by loading the plasmid or polyplex into the matrix of the scaffolds [39,40] or adsorbing on the surface [41–43]. Mineral coatings have also been reported, most notably CaP [44], which was incubated with lipoplexes to induce transfection upon cell seeding [45]. Compared to these techniques, LbL dipping technique offers the possibility to design more intricate multilayer design for prolonged or scheduled release [46] of multiple components. Moreover, the aqueous conditions for formation of the LbL films enable to preserve native structures of functional macromolecules. As a recent example, Holmes et al. reported coating of 3D scaffolds with multilayers of glycol-chitosan and hyaluronic acid. The multilayer-coated scaffolds were found to enhance tissue growth relative to non-coated scaffolds, and additional transfection capability could be observed by depositing a layer of DNA-containing lipoplexes [47]. Hammond and co-workers have reported the multilayer assembly on poly(lactic-co-glycolic acid) molded into microneedle arrays for transcutaneous delivery of plasmid DNA [28]. The multilayer-coated microneedles successfully induced transfection on mice in vivo.

To enable multilayer formation, a pair of macromolecules is needed that interact with each other. For negatively-charged DNA, a positively-charged biocompatible polymer may serve as a good counterpart. Poly(β-amino ester) (PBAE) introduced by the group of Lynn, is one of the most extensively studied polymer for this purpose [22,25–28]. In this paper we report on the preparation and properties of multilayers of DNA with bioreducible linear poly(amido amine)s (PAA). Poly(amido amine)s are a class of peptidomimetic polymers synthesized via Michael-type addition polymerization of amines and bisacrylamides. Through the presence of amide bonds, PAAs are inherently biodegradable through hydrolysis. Moreover, through the availability of various building blocks, these polymers can also be designed to incorporate various moieties for added functionality such as charge-shift, bioreducibility, 'stealth' properties and targeting moieties [48–50].

Here, we report the fabrication, characterization and cell transfection properties of multilayered thin films of DNA with pCABOL and pCHIS, respectively. These linear, bioreducible PAAs were selected because these polymers have previously shown to be two of the best performing PAAs in polyplex systems for cell transfection [51]. Cell transfection efficiency of the multilayers was optimized using flow cytometry as an analytical tool to determine transfection efficiency as a function of the PAA type, cell seeding density, and layer number. Finally, with the optimized conditions, surface-mediated cell transfection was accomplished on poly(ε-caprolactone) (PCL), heparinized PCL (PCL-HEP), and poly(lactic acid) (PLA) substrates, both in 2D (disk shape) and 3D (fiber deposited PCL and PCL-HEP).

2. Materials and methods

2.1. Chemicals, syntheses and characterization

N,*N*′-Cystamine bisacrylamide (CBA, Polysciences), 4-amino-1-butanol (ABOL, Sigma-Aldrich, Zwijndrecht, The Netherlands), *N*-Boc-1,4-diaminobutane (NBDAB, Sigma-Aldrich, Zwijndrecht, The Netherlands), histamine dihydrochloride (HIS·2HCl, Sigma-Aldrich, Zwijndrecht, The Netherlands), calcium chloride ($CaCl_2$, Sigma-Aldrich, Zwijndrecht, The Netherlands), triethylamine (TEA, Sigma-Aldrich, Zwijndrecht, The Netherlands), *tert*-butylamine (*t*BA, Sigma-Aldrich, Zwijndrecht, The Netherlands), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$, Merck, Darmstadt, Germany), disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$, Merck, Darmstadt, Germany), sodium chloride (NaCl, Sigma-Aldrich, Zwijndrecht, The Netherlands), dithiothreitol (DTT, Sigma-Aldrich, Zwijndrecht, The Netherlands), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, Sigma-Aldrich, Zwijndrecht, The Netherlands) and glucose (Sigma-Aldrich, Zwijndrecht, The Netherlands) were purchased in the highest purity available and used as received. Solvents were of reagent grade and used without further purification unless otherwise noted. Milli-Q water was obtained from a Synergy® water purification system (Millipore). PBS buffer was prepared by dissolving 1.54 g of $Na_2HPO_4 \cdot 2H_2O$, 0.3 g of $NaH_2PO_4 \cdot H_2O$, and 8.2 g of NaCl into 1 L of Milli-Q water and adjusting the pH to 7.4. HEPES buffered glucose (HBG) was prepared by dissolving 4.79 g of HEPES, and 50 g of glucose into 1 L of Milli-Q water and adjusting the pH to 7.4.

2.2. Polymer synthesis

The PAA polymers pCABOL and pCHIS were synthesized by polyaddition of cystamine bisacrylamide (C) with 4-aminobutanol (ABOL) or histamine (HIS) according to modified literature procedures [51]. Therefore, in a brown polymerization flask containing 2 mL methanol/water 3/1 and 200 mM $CaCl_2$ as catalyst, *N*,*N*′-cystamine bisacrylamide (1.04 g; 4.0 mmol) was mixed with an equimolar amount of 4-amino-1-butanol (0.37 g) or histamine dihydrochloride (0.74 g), respectively [52]. Polymerization was carried out under N_2 atmosphere for two days at 70 °C during which a gradual viscosity increase was observed. The polymerization was terminated by adding excess *tert*-butylamine into the mixture and stirring at 70 °C for two or three more days. After bringing the flask to room temperature, the solution was diluted and acidified to pH ~5 by addition of 4 M HCl and purified by ultrafiltration using a 1000 Da MWCO membrane. The purified polymer solution was then freeze-dried leaving white transparent solid as the final product in its HCl-salt form at ~50% recovery. ^{1}H NMR spectroscopy confirmed complete termination and allowed determination of the number-average MW based on the *tert*-butylamine end-group. ^{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. pCABOL ^{1}H NMR (D_2O) δ (ppm) = 1.35 (s, 9H, $(CH_3)_3R$); 1.60 (m, 2H, CH_2CH_2NR); 1.77 (m, 2H, CH_2CH_2OH); 2.74 (t, 4H, $CH_2CONHRNHCOCH_2$); 2.85 (t, 4H, CH_2SSCH_2); 3.22 (t, 2H, $HO(CH_2)_3CH2NR$); 3.44 (t, 4H, $NCOCH_2CH_2NRCH_2$); 3.53 (t, 4H, $CH_2CH_2SSCH_2CH_2$); and 3.62 (t, 2H, CH_2OH). ^{1}H NMR end group analysis M_w = 9 kg/mol. GPC M_w = 3.8 kg/mol (M_w/M_n = 1.18). pCHIS ^{1}H NMR (D_2O) δ (ppm) = 1.39 (s, 9H, $(CH_3)_3R$); 2.78–2.95 (m, 8H, $CH_2CONHRNHCOCH_2$ & CH_2SSCH_2); 3.23 (t, 2H, CCH_2); 3.48–3.80 (m, 10H, $(CH_2)_3$ N & $CH_2CH_2SSCH_2CH_2$); 7.45 (s, 1H, NC = C*H*); and 8.71 (s, 1H, N = C*H*). ^{1}H NMR end group analysis M_w = 5.5 kg/mol. GPC M_w = 4.6 kg/mol (M_w/M_n = 1.26).

2.3. Substrate preparation

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).

Poly(lactic acid) (PLA) sheets with a thickness of 120 μm were kindly provided by Sidaplax (Ghent, Belgium) and cut in 6 mm diameter disks with a biopsy puncher (Miltex, Rietheim — Weilheim, Germany).

Poly(ε-caprolactone) (PCL) M_w 45,000 was purchased from Sigma, (Zwijndrecht, The Netherlands). 2D disks were fabricated by placing the PCL grains in between two hydrophobic silica wafers and heating the PCL grains in a press at 100 °C. Pressure was applied to the molten polymer. After cooling, the polymer sheet was removed from the press and