Cell Recognition

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Cell-Specific Gene Transfection from a Gene-Functionalized Poly(D,L-lactic acid) Substrate Fabricated by the Layer-by-Layer Assembly Technique**

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Controlled and efficient gene delivery/transfection is essential in many important biomedical applications. This process is highly regulated by the cellular microenvironment.[1] Various strategies[2,3] have previously been employed to deliver genes, but these achieved only limited success.[4] However, this might be improved with gene delivery/transfection induced by a material's surface.[4,5–7] For example, van den Beucken et al. demonstrated that a DNA–polymer layered film can be used for biomedical applications.[7] Nevertheless, none of these reports established both cell-specific recognition and gene transfection on the surface of a material.

Herein, we present a method to fabricate gene-tagged poly(D,L-lactic acid) (PDLLA) substrates that show potential for both cell-specific recognition and continuous interfacial delivery of plasmid DNA by a material's surface. Our approach involves the formation of nanostructured, paired multilayers of galactosylated chitosan and plasmid DNA on PDLLA films by using the layer-by-layer (LbL) assembly technique. This technique is based on the sequential adsorption of polyanions and polycations through electrostatic interactions. It allows the functionalization of biomaterials both to control cell activity and to deliver drugs locally.[8,9] The results we present herein may have wide applications in tissue engineering,[10] the development of cell-/gene-stimulating biomaterials,[11] gene therapy,[3,9] and implant technology.[12]

Previously, we hypothesized that chitosan/DNA complexes might be formed during the degradation of the multilayer structure, thereby leading to local gene transfection.[13] To test this hypothesis, we fabricated galactosylated chitosan/plasmid DNA (GC/pDNA) multilayered materials and investigated their in vitro degradation behavior, as well as the mechanism of in situ gene transfection. GC was chosen as the polycation and pSV-β-galactosidase plasmid DNA, the reporter gene in this study, was selected as the polyanion. GC was synthesized by the conjugation of galactose groups bearing lactobionic acid (LA) with chitosan molecules (for characterization, see the Supporting Information). The galactose group is a specific ligand for the asialoglycoprotein receptor (ASGP-R) of hepatocytes.[14] Therefore, GC simultaneously plays a role as both a polycation and a cell-specific ligand.

PDLLA surfaces coated with poly(ethylene imine) (PEI)/$(pDNA/GC)_5$ or PEI/$(pDNA/GC)_5$/pDNA were imaged by atomic force microscopy (Figure 1a, left and right, respectively). With GC as the outermost layer, a rough surface morphology with granular structure is displayed. The boundaries are clearly visible between granules (Figure 1a, left), probably due to the rigid features of the chitosan molecules resulting in weak diffusion.[15] By contrast, with pDNA as the outermost layer, star-like structures are displayed, which seemingly correspond with GC granules underneath (Figure 1a, right). The root-mean-square (RMS) roughness values for surfaces coated with PEI/$(pDNA/GC)_5$ or PEI/$(pDNA/GC)_5$/pDNA layers were (410 ± 12.4) nm and (63.4 ± 6.8) nm ($n = 4$), respectively. The water contact angle gradually decreased for the initial four layers but, from the fifth layer onwards, began to alternate between around 60° for GC layers and 39° for pDNA layers (Figure 1b, top); this indicates full coating of the GC or pDNA layers with their distinct physical properties. This alternating contact angle between different layers was consistent with the results of other studies.[8,12,16]

The buildup of the GC/pDNA paired layers was also verified by UV/Vis spectroscopy. The absorbance intensity at 260 nm (a DNA indicator) increased as the GC/pDNA pair number increased (Figure 1b, bottom). Furthermore, we observed exponential growth behavior for GC/pDNA, consistent with previous results.[16] These results imply that pDNA was well incorporated within the films.

The hypothesis that the GC/pDNA complex forms along with the degradation of the multilayered film was verified by transmission electron microscopy. The TEM image displays uniform, highly condensed globules of GC/pDNA complexes with a size of around 0.5–1 μm after 24 h of incubation, in contrast to those with loose boundaries and a smaller size (around 0.2–0.4 μm) that are visible after incubation for 48 h (Figure 2a, left and right, respectively). The decrease in

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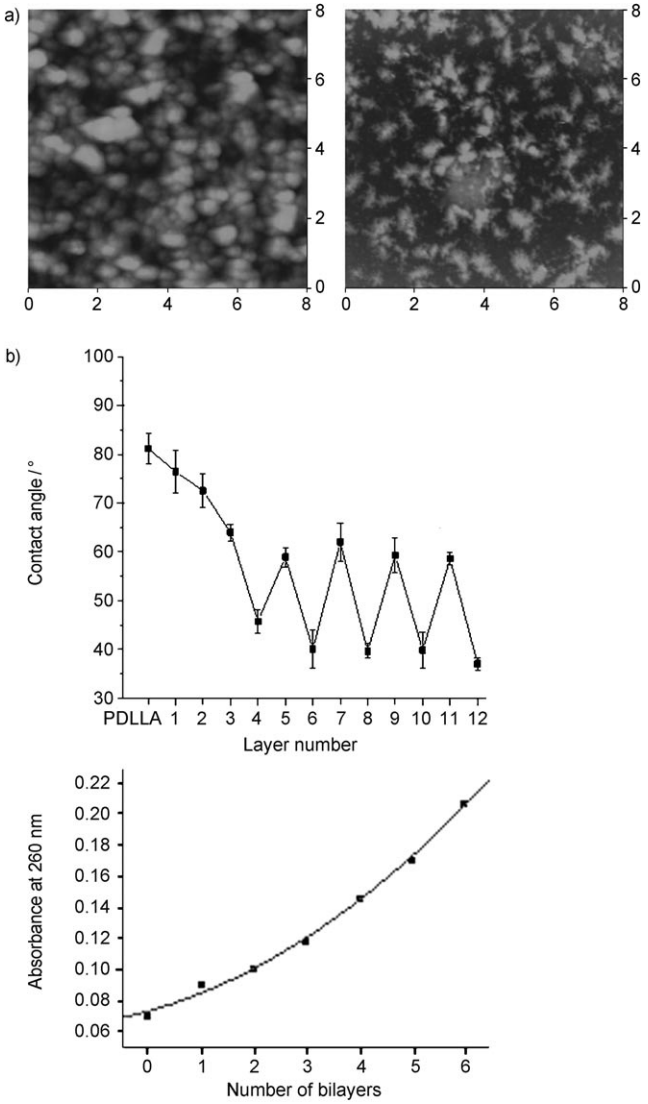

Figure 1. Multilayer growth characterization. a) AFM height images of LbL-coated PDLLA films with different layers: PEI/(pDNA/GC)$_5$-coated PDLLA film (left) and PEI/(pDNA/GC)$_5$/pDNA-coated film (right). Film morphologies were observed in air after drying. Scale in μm. The maximum *Z* ranges are 700 nm (left) and 100 nm (right). b) Water contact angles as a function of the number of coating layers. The even layer numbers correspond to pDNA layers; the odd layer numbers correspond to GC layers, except for the first layer, which was coated with PEI ($n=6$).

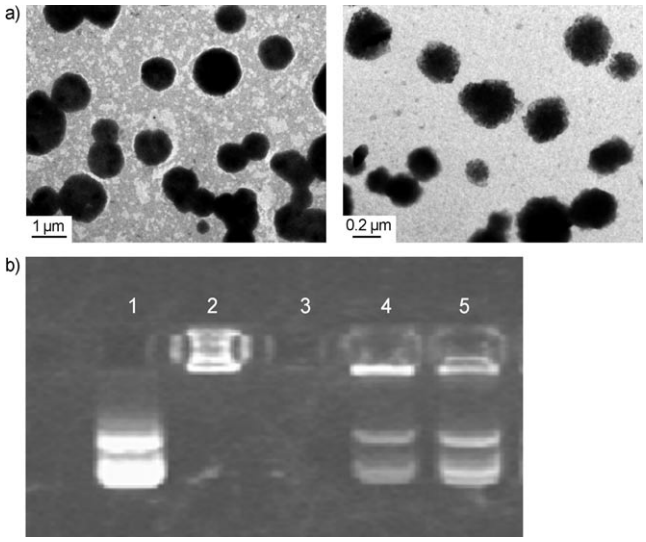

Figure 2. In vitro degradation characterization. a) TEM images of GC/DNA complexes released from the PDLLA/PEI/(DNA/GC)$_5$/DNA film after 24 h (left; scale bar: 1 μm) and 48 h of incubation (right; scale bar: 0.2 μm), respectively. b) Agarose gel electrophoresis for GC/DNA complexes released from PDLLA/PEI/(DNA/GC)$_5$/DNA films after 24 h of incubation. Lane 1: plasmid DNA alone (2 μg); lane 2: GC/DNA complex after DNase digestion for 30 min with no release of pDNA; lane 3: free plasmid DNA incubated with DNase, which results in complete pDNA digestion; lanes 4 and 5: GC/DNA complex with addition of 0.4 and 0.5% sodium dodecylsulfate, respectively, after DNase I (4 U) treatment.

particle size and the architecture loosening could be attributed to the degradation of the GC, which leads to breakdown of the GC/pDNA complexes with time. It is worth noting that such a change in either particle size or structure is beneficial for gene uptake and transfection by cells.[17] In addition, continuous release of pDNA from the multilayer was observed (see the Supporting Information).

The formation and stability of the GC/pDNA complexes from degraded fragments were further investigated by gel electrophoresis assays. Compared to that of naked plasmid DNA, no mobility of pDNA was observed for the GC/DNA complexes, even after DNase I treatment (Figure 2b, lane 1 versus lane 2). This reveals that the pDNA was largely protected against DNase I digestion by the GC. While naked pDNA was completely degraded by DNase I treatment, the pDNA extracted from the GC/pDNA complexes by different doses of surfactant treatment remained nearly intact (Figure 2b, lane 3 versus lanes 4 and 5). It is important to maintain intact GC/pDNA complexes for gene transfection.[18]

GC displays less toxicity to cells than the commonly used poly(ethylene imine), PEI, and chitosan. Hepatoma G2 (HepG2) cells cultured on GC/pDNA-multilayer-coated PDLLA films appeared as spindle shapes, in contrast to the round shapes of those cultured on native PDLLA films. This indicates greater cell spreading/migration potential on the multilayer-coated PDLLA films (see the Supporting Information).

To investigate the local cell-specific recognition and in situ gene-transfection performance of multilayered PDLLA films, we measured the *o*-nitrophenol production level. When the pDNA is transfected in cells, β-galactosidase is expressed to catalyze the conversion of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) into *o*-nitrophenol. Therefore, the more β-galactosidase expressed, the greater the *o*-nitrophenol production. The product can be detected with a characteristic absorbance peak at 420 nm. Our studies by using conventional methods with GC/pDNA complexes in solution indicated that gene-transfection efficiency is selectively dependent on cell type and the LA substitution degree of the GC. With an increase in the galactose substitution degree of the

GC, the gene-transfection efficiency was increased in HepG2 but not in human embryonic kidney (HEK293) cells. This result is related to the lack of ASGP-R receptors in HepG2 cells (see the Supporting Information).

The gene transfection to cells from multilayered structures of GC/pDNA with either 10.9% (GC 1) or 19.4% (GC 2) LA-substituted GCs, together with that from chitosan/pDNA as a control, was evaluated from the expression level of *o*-nitrophenol with HEK293 and HepG2 cells cultured for 2 days. In all cases, β-galactosidase expression increased with time. However, when normalized to the gene transfection from chitosan/pDNA in the cell culture, the influence of the material surface on the gene transfection was also selectively dependent on cell type (Figure 3). With an increase in the LA

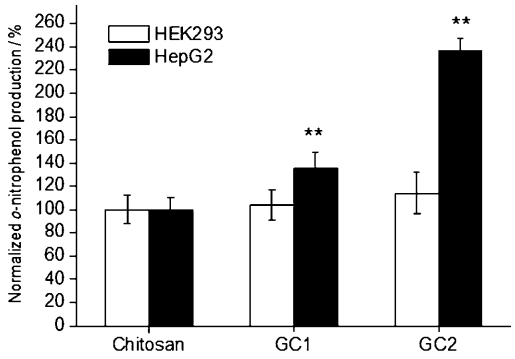

Figure 3. Gene transfection of HEK293 and HepG2 cells from the multilayer-modified PDLLA films with cell-specific and cell-nonspecific properties ($n=6$). **: $p<0.01$ (compared to the control).

substitution of the GC, the gene-transfection efficiency in HepG2 cells increased, whereas there was no effect in HEK293 cells. The expression of β-galactosidase from GC/pDNA multilayered structures was 1.35 ± 0.14 (GC 1, $p<0.01$, $n=6$) or 2.37 ± 0.11 (GC 2, $p<0.01$, $n=6$) times that of the control. This is consistent with in vitro studies with GC/pDNA complexes in solution (see the Supporting Information). Taken together, these results demonstrate that, in vitro at least, cell-specific recognition from the material surface of the multilayered structure increases the in situ transfection efficiency.

In conclusion, gene-stimulating PDLLA substrates with cell-specific recognition and in situ gene-transfection properties can be fabricated by the LbL technique. Such multilayered structures can form GC/pDNA complexes during degradation and, by doing so, improve the efficiency of in situ gene transfection. More importantly, the ability of this material's surface to target only specific cell-surface receptors provides great potential to enable efficient in situ gene transfection specifically for various cell types. If this finding is proved by further studies to be true in vivo, it will be very useful in the development of gene-stimulating biomaterials, gene therapy, tissue engineering, and biosensors for biomedical applications.[3,9–11,19]

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