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Rational design of degradable polyanion for layer-by-layer assembly for encapsulation and release of cationic functional biomolecules

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A novel degradable polyanion, poly(phthalic ethylene glycol ester), was synthesized in one pot in a single step. The degradable polyanion assembles with various polycations to form layer-by-layer films that can encapsulate physiologically active biomolecules. Polyanion degradation can induce film disassembly and release of the encapsulated functional protein.

There is a high demand in the biomedical and bioengineering fields for the development of new materials and methods that control drug release. Controlled release of drugs and biological agents has been explored using various approaches involving synthetic and natural polymers. Layer-by-layer (LbL) assembly of oppositely charged polymers is a powerful method for readily imparting various functional properties to the surfaces of solid and soft materials. This enables chemical functionalization of the surfaces of implantable devices and scaffolds for tissue engineering. For example, LbL enables the encapsulation of a broad range of small and large functional molecules in polyelectrolyte multilayered films. The encapsulated substances can be released from the films depending on the polyelectrolyte molecule and the conditions.

Lynn et al. developed a degradable polycation, poly(β-amino ester) (PBAE), and achieved controlled release under physiological conditions of encapsulated DNA (polyanion) from a multilayered film of the PBAE. They also reported localized delivery of plasmid DNA and its transfection of cells using LbL assembly. PBAEs are designed for assembly with polyanions, but various bioactive polycations (e.g., polycationic proteins and peptides) can not be encapsulated in the LbL assembly of PBAEs. Hammond et al. used a naturally derived degradable polyanion, poly(β-L-malic acid), which was extracted from eukaryotic microbes and purified, to encapsulate polycationic proteins in LbL assembly, and achieved controlled release of proteins based on hydrolytic degradation of poly(β-L-malic acid). However, to our knowledge, there is no report of degradable synthetic polyanion that can be available for LbL assembly.

In the present study, we synthesized a novel degradable polyanion that can assemble with polycations (especially polycationic enzymes) to form LbL films, and showed that the LbL film can encapsulate and release a polycationic antibacterial enzyme. Synthetic polymers are preferred in therapeutic applications because they minimize the risk of contamination by pathogenic agents (viruses, pathogenic proteins, etc.). A degradable synthetic polyanion for LbL assembly must meet the following design criteria: (i) it can be synthesized via a simple procedure in considerable yields, (ii) it has high solubility in water and forms a polyelectrolyte complex with polycations, (iii) the degradation rate is suitable for LbL assembly in aqueous solution (minimizing degradation), and (iv) degradation in aqueous solution induces disassembly of the LbL film. To satisfy these criteria, we designed a degradable polyanion, poly(phthalic ethylene glycol ester)
(PPEGE), which has ester bonds as the degradable part and carboxy groups as the anionic part (Fig. 1a). PPEGE was synthesized via ring-opening polymerization between 4,4'-oxydipthalic anhydride (ODPA) and poly(ethylene glycol) 200 (PEG 200). This ring-opening polymerization enables simultaneous introduction of ester bonds and carboxy groups into the polymer backbone. This is a one-pot, single-step reaction, without any protection (or deprotection) of monomer functional groups.

We investigated the synthesis of PPEGE under various conditions (Table S2). First, the effect of the PEG 200 concentration was examined. Although the reaction theoretically progresses via ring-opening polymerization with an ODPA/PEG 200 molar ratio of 1:1, a molar ratio of 0.50:0.48 gave the largest weight-average molecular weight ($M_w = 1.56 \times 10^4$), with a yield of 57%. This could be related to the ODPA purity. Carboxylic acid anhydrides are sensitive to small amounts of water; therefore a small portion of ODPA could have been hydrolyzed prior to the reaction, although special care was taken to avoid water contamination of the experimental setup. The reaction time also affected the $M_w$. 3 h gave the largest $M_w$ under the present conditions. A reaction time longer than 3 h resulted in a low $M_w$, possibly because of hydrolysis of the produced PPEGE.

ODPA was not soluble in N,N-dimethylformamide (DMF) at the target concentration (0.48 m) in the absence of N,N-diisopropylethylamine (DIEA); therefore DIEA was added to the ODPA/DMF solution. The addition of DIEA increased the $M_w$ and a DIEA concentration of 0.9 resulted in the largest $M_w$ (2.01 $\times 10^4$), in a yield of 53%. There are two isomeric forms of the ester produced from ODPA and PEG 200 (Fig. S1). $^1$H NMR spectroscopy indicated that the ratio of form 1 to form 2 was 3:1. PPEGE with the largest $M_w$ (i.e., 2.01 $\times 10^4$) was used in the following experiments.

The solubility of PPEGE in phosphate buffer (0.1 m, pH 6.0) was more than 10 g L$^{-1}$ at 25 °C. We observed that a polyelectrolyte complex was formed when equivalent volumes of 1 wt% PPEGE and 1 wt% chitosan solutions were mixed (Fig. S2).

The degradation of PPEGE in aqueous solution was investigated (Fig. 1b). At 37 °C and pH 7.4 (physiological conditions), the $M_w$ of PPEGE decreased to 34% relative to the initial $M_w$ after 7 d. At a lower temperature (25 °C), the $M_w$ of PPEGE after 7 d was 73% relative to that of the initial PPEGE. Matrix-assisted laser desorption ionization time-of-flight mass spectrometric (MALDI-TOF/MS) analysis of the degraded PPEGE showed the presence of low-molecular weight PPEGE (875–1650), indicating hydrolysis of ester bonds in PPEGE (Fig. S3). The m/z values of the degradation products indicate the presence of the short oligomers comprising ODPA and PEG 200. Under the conditions for LbL assembly (25 °C and pH 6.0, see later), the degradation rate was slower than that under physiological conditions. These results show that PPEGE degradation is based on ester hydrolysis in the polymer backbone. It should be noted that degradation of dry PPEGE can be prevented at −20 °C for more than 6 months.

The use of PPEGE as a polyanion in an LbL film was shown by deposition of PPEGE/polycationic protein on a planar silicon substrate. We used lysozyme (pl of 11), which is an antibacterial enzyme, as a model polycationic protein. Prior to preparation of LbL films, lysozyme was conjugated with fluorescein isothiocyanate. Initially, 10 bilayers of poly(sodium 4-styrenesulfonate)/polyethyleneimine ([PSS/PEI]$_{10}$) were deposited on surface-aminated silicon substrates, using a dipping method, to prepare a charged base surface suitable for PPEGE adsorption. In Fig. 2a, 0 bilayer (ca. 8 nm) indicates the (PSS/PEI)$_{10}$ film in dry form; this layer helped deposition of PPEGE/lysozyme films on the silicon substrate.

The LbL assembly of PPEGE/lysozyme on the (PSS/PEI)$_{10}$ bilayers was performed at pH 6.0 and 25 °C. Under these conditions, the PPEGE degradation rate was slow enough to allow assembly (half-life $t_{1/2} > 8$ d). The thickness of the (PPEGE/lysozyme)$_{30}$ film increased with increasing numbers of bilayers; the (PPEGE/lysozyme)$_{30}$ film had a thickness of about 86 nm (Fig. 2a). Film growth was exponential with the number of bilayers at the beginning of the film preparation; this has also been observed for LbL films composed of other weakly charged polyelectrolytes. The sequential growth of the film thickness suggests successful preparation of LbL films composed of PPEGE and lysozyme. The surface morphology of the dried (PPEGE/lysozyme)$_{30}$ film was observed using an atomic force microscope (AFM). The AFM observation reveals the relatively smooth surface of the film (Fig. S4). Particulates observed on the surface would be crystalline salt.

We used two other polycationic proteins (ribonuclease A and cytochrome c) to explore the versatility of (PPEGE/polycationic protein)$_{30}$ films. The pl values of ribonuclease A (from bovine pancreas) and cytochrome c (from horse heart) are 9.6 and 10.2–10.4, respectively. The thicknesses of the
and reported the significance for controlled release.
was almost complete within 4 h. Hammond et al. described (PPEGE/lysozyme) of encapsulated lysozyme from the film. The film thickness gradually decreased to 14 nm in 16 h (buffered saline (PBS) at 37 °C). The film thickness was 75% of the intrinsic activity. This indicates that lysozyme was encapsulated in the LbL film and lysozyme release were faster than PPEGE degradation without significant loss of enzymatic activity. Erosion of the degradable bilayers, (PSS/PEI) remained on the silicon substrate. Lysozyme was simultaneously released into the solution (Fig. 2b and c). The total amount of lysozyme released at 16 h was 118 µg cm⁻², and it had an enzymatic activity of 220 units cm⁻², which was 75% of the intrinsic activity. This indicates that lysozyme was encapsulated in the LbL film without significant loss of enzymatic activity. Erosion of the film and lysozyme release were faster than PPEGE degradation (Fig. 1b), implying that partial degradation of PPEGE accelerated disassembly of the LbL film.

In the investigations, the release of lysozyme from the film was almost complete within 4 h. Hammond et al. described the burst release of encapsulated substrates from LbL films and reported the significance for controlled release. We introduced several PPEGE/chitosan bilayers into the PPEGE/lysozyme LbL films to delay film erosion and lysozyme release (Fig. 3a). Fig. 3b shows that film erosion was changed by the introduction of PPEGE/chitosan bilayers, although the initial film thicknesses were almost the same. Lysozyme release was also delayed by increasing the number of PPEGE/chitosan bilayers (Fig. 3c). These results indicate that the introduction of relatively stable bilayers into the films can control lysozyme release from degradable LbL films.

The biocompatibility of PPEGE is important in biomedical applications of PPEGE/polycation LbL films. We evaluated the cytotoxicity of PPEGE to HeLa cells. HeLa cells were cultured for 16 h in Dulbecco’s modified Eagle’s medium containing PPEGE at various concentrations. The PPEGE toxicity was negligible at PPEGE concentrations less than 0.1 wt% (Fig. 4a).

One possible application of PPEGE/lysozyme LbL films is as implantable biomaterials that release antibacterial activity. The antibacterial activity of the PPEGE/lysozyme LbL film was evaluated by placing a Bacillus subtilis suspension on the film and incubating for 5 h. PBAE, which is a degradable and non-toxic polycationic material, was used to prepare a PPEGE/polycation LbL film without an antibacterial agent, for use as a positive control. Formation of the PPEGE/PBAE LbL film was confirmed using ellipsometry (Fig. 5). B. subtilis grew on a bare silicon substrate and on the PPEGE/PBAE film (the initial optical density at 660 nm (OD₆₆₀) of the B. subtilis suspension was 0.08). In contrast, B. subtilis growth on the PPEGE/lysozyme film was significantly inhibited (Fig. 4b). These results show that the PPEGE/lysozyme LbL film had antibacterial activity derived from the released lysozyme.

In conclusion, we designed and synthesized a novel hydrolytically degradable polyanion, PPEGE. PPEGE was easily synthesized in a single step via ring-opening polymerization between ODPA and PEG 200. PPEGE was degraded under physiological conditions, and had low cytotoxicity to mammalian cells. PPEGE can assemble with polycationic proteins, and with chitosan and PBAE, to form thin LbL films. The LbL film was eroded in aqueous solution and released the encapsulated protein, with maintenance of the enzymatic activity. LbL assembly is based on positively and negatively charged polyelectrolytes. A degradable synthetic polycationic material, PBAE, has already been reported to be useful in LbL films that can encapsulate and release polyanionic substances, and the development of a degradable, low-toxicity, synthetic polyanion will increase potential applications of LbL assembly with degradable synthetic polycationic materials.

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