Stimulated release of small molecules from polyelectrolyte multilayer nanocoatings†

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Free thiol-containing polyelectrolytes serve simultaneously as a material for self-assembly of a multilayer nanocoating and as a carrier of small molecules for release from the coating in response to an environmental cue.

Layer-by-layer self-assembly (LBL) is a convenient method for making polyelectrolyte multilayer coatings with thickness control on the nanometre scale.[1,2] Repetitive alternating immersion of a suitable substrate in a solution of oppositely-charged polyelectrolyte molecules leads to build up of a multilayer coating by electrostatic attraction and counterion release. Polyelectrolyte LBL has been used to modify surfaces for a broad range of applications, from anisotropic coatings to tissue culture coatings.

A polypeptide multilayer nanocoating is simply a nanometre-thick multilayer coating made of polypeptides.[3,4] Key features of such coatings are biocompatibility, biodegradability, and environmental benignity. The physical, chemical, and biological properties of a polypeptide multilayer nanocoating are functions of polypeptide design, coating architecture, the coating fabrication process, and post-fabrication treatment. For example, the amino acid cysteine (Cys), which is distinguished from the other usual amino acids by a free thiol group in the side chain, is incorporated in a polypeptide chain by design of molecular structure. Following coating preparation by LBL, intra- and interlayer disulfide bonds are formed between Cys residues by exposure of the coating to an oxidizing agent.[5–9] Such treatment alters coating viscoelasticity and stability. Other physical properties of polypeptide multilayer coatings, *e.g.*, thickness, refractive index, and surface morphology, are controlled by the degree of polymerization of the polypeptides and the ionic strength and pH of the polypeptide adsorption solution.[10–12]

Polyelectrolyte multilayer coatings could be useful for drug delivery, for example, from an implant device such as a stent.[13,14] In one approach, model drugs are loaded into the coating after coating preparation, and the small molecule is released by diffusion.[15,16] In another approach, model drug microcrystals are encapsulated within a multilayer coating; again, release is governed by diffusion.[17–19] Here, model thiol-bearing molecules, namely, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), have been "loaded" onto Cys-containing 32-mer polypeptides; these labeled peptides have been incorporated into multilayer films by LBL, and 2-nitro-5-thiobenzoate dianions (TNB) have been released from the coatings by a change in the redox potential of the surrounding liquid medium. DTNB, also known as Ellman's reagent,[20] can be used to quantify free sulfhydryl groups and disulfides in peptides and proteins.[21–24] In earlier work, we used DTNB to show formation of disulfide bonds in multilayer films made of Cys-containing peptides.[5] Here, an increase in the reducing potential of the solution models the passage of a coated particle from outside a living biological cell to inside.

Peptides $(KVKGKCKV)_3KVKGKCKY$ ("P1") and $(EVEGECEV)_3EVEGECEY$ ("N1") (Genscript, Inc., USA) are oppositely charged at neutral pH due to protonation of lysine (K) and deprotonation of glutamic acid (E). The other amino acid residues are valine (V), glycine (G), and Cys (C). 4–15 kDa poly(L-lysine) ("PLL"), 13 kDa poly(L-glutamic acid) ("PLGA"), and DTNB were from Sigma (USA). DL-Dithiothreitol (DTT), a reducing agent, was from Gold Biotechnology, Inc. (USA). All other reagents were from Sigma. P1, N1, PLL and PLGA were dissolved in TA buffer (10 mM tris(hydroxymethyl)aminomethane, 10 mM sodium acetate, 20 mM NaCl, 0.1% NaN_3, pH 7.4) to concentrations of 1 mg mL^{-1}.

Peptides P1 and N1 contain Cys residues. A free thiol group on a peptide will react with a DTNB molecule under oxidizing conditions (Fig. 1a). In the process, a TNB molecule forms a mixed disulfide with a Cys side chain and a TNB molecule is released to the surroundings. An aqueous solution of free TNB is

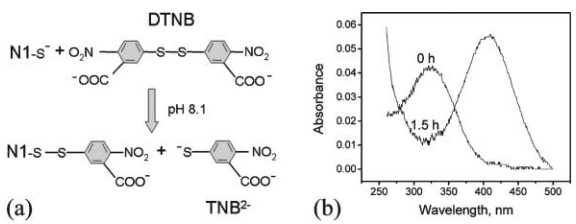

Fig. 1 Loading of the model drug onto peptide N1 for subsequent multilayer film assembly. (a) DTNB reacts with Cys side chains in N1. A TNB group becomes attached to a peptide thiol group by formation of a disulfide bond. (b) Absorbance spectra of LN1 solution after extensive dialysis to remove unreacted DTNB but before (0 h) and 1.5 h after adding DTT. The peak at 412 nm in the 1.5 h spectrum is due to TNB dianions, while that at 328 nm (0 h) is due to TNB–thiol mixed disulfide.[27] The sharp increase in absorbance below 300 nm is due to DTT. The shoulder evident around 275 nm at later time points (not shown) is due to oxidized DTT. See ESI.†

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yellow and has an absorbance peak at 412 nm at mildly basic pH.[25,26] The extinction coefficient of TNB ranges from 11 400 to 14 150 M^{-1} cm^{-1} depending on conditions.[25]

Lyophilized N1 was prepared for loading by dissolution of peptide in "reducing TA buffer" (TA buffer, 10 mM DTT, pH 8.1) and incubation at ambient temperature for 24 h. 2 mL of N1 solution was introduced into 1000 MWCO dialysis tubing (SpectroPor 7, Spectrum Laboratories, Inc., USA) and dialyzed against 200 mL of "DTNB solution" (TA buffer, 10 mM DTNB, pH 8.1) with continuous stirring. The DTNB solution was changed after 1, 2, 4, and 17 h. The dialysis bag containing labeled N1 (LN1) was then immersed in pH 7.4 TA buffer to remove excess DTNB and to shift the pH from 8.1 to 7.4. TA buffer was changed after 1, 2, 4, and 17 h. The final concentration of LN1 was adjusted to 1 mg mL^{-1} with pH 7.4 TA buffer. UV absorbance spectra of LN1 before and after treatment with DTT are shown in Fig. 1b.

Polypeptide multilayer coatings were assembled on quartz microscope slides (Electron Microscopy Sciences, USA) after substrates were cleaned as described previously.[11] A substrate was repetitively immersed in a positively-charged polypeptide solution (P1 or PLL) and in a negatively-charged polypeptide solution (N1, LN1, or PLGA) for 15 min per peptide adsorption step. Each peptide deposition step was followed by rinsing of the coated slide in separate deionized water baths for 2 min, 1 min, and 1 min. 30 layers of polypeptide were assembled in this way. In some cases, as indicated below, two "capping" bilayers of P1 and N1, symbolized as $(P1/N1)_2$, were assembled on top of the 30-layer P1/LN1 coatings.

To release TNB from polypeptide multilayer nanocoatings, a pH 7.4 LN1 solution was diluted 4-fold with deionized water, and DTT was added to a final concentration of 2 mM (Fig. 1b). The mixture was gently agitated on a rocking platform throughout the TNB release process. Solution absorbance spectra were recorded with a Shimadzu UV mini 1240 UV-Vis spectrophotometer (Japan). The coating covered about 2 cm^2 of substrate in each case. The TNB absorbance peak was brought into the detectable range by immersing P1/LN1 coatings on 10 separate quartz plates in 3 mL of release medium (pH 7.4 TA buffer diluted 4-fold with deionized water; 0, 0.1, or 1 mM DTT). The absorbance of the release medium was recorded by scanning spectrophotometry at defined time points. Each coating was immersed for 5 min for the first absorbance measurement and 15 min for subsequent measurements. The beaker containing the film sample and release medium was gently rocked throughout the release process.

Polyelectrolyte multilayer nanocoatings are promising for numerous areas of technology development, for instance, surface modification and device fabrication. Some envisaged applications have no direct relationship to medicine or biology; others are biomedical in nature. Small molecule model drugs, for example, methylene blue,[15] rhodamine B,[28] and carboxyfluorescein,[29] have been "loaded" into polyelectrolyte multilayer coatings after fabrication. Polynucleic acids too have been used to fabricate multilayer films for subsequent release.[30–33] Redox-sensitive multilayer films have been made from poly(anilineboronic acid) (PABA) and ribonucleic acid (RNA) by the formation of a boronate ester, a boron–nitrogen dative bond, and electrostatic interactions.[32] When the covalent bonds between PABA and RNA are broken, RNA is released.

Disulfide bonds play an important role in maintaining the structure and function of numerous secreted proteins, for instance, immunoglobulin G, insulin, and lysozyme. The intracellular environment is reducing, the extracellular environment oxidizing. In previous work, we have investigated the impact of disulfide bond formation on the stability of polypeptide multilayer coatings and capsules.[5,7–9,34] Recently, Zelikin *et al.*[35] and Blacklock *et al.*[33] have studied disulfide bond stabilization of other kinds of polymeric multilayer film. In the present work, TNB was "loaded" onto Cys-containing polypeptides by disulfide bond formation, the loaded peptides were then incorporated into multilayer coatings by LBL, and model drug release was stimulated by a change in the redox potential of the surrounding environment of the coating.

DTT can maintain monothiols completely in the reduced state and reduce disulfides quantitatively.[36] Polypeptide N1 was treated with DTT to break possible inter- and intramolecular disulfide bonds and to protect the free thiol groups of Cys residue side chains. The resulting N1 molecules, which have several free thiol groups each, were used for TNB loading in mildly basic solution (Fig. 1a). The absorbance peak at 328 nm in Fig. 1b, which is due to TNB and thiol mixed disulfides,[27] shows that N1 molecules were successfully loaded with TNB. Tyrosine absorbance at 275 nm is more than 10-fold smaller than that of TNB in the near UV.[25,37]

30-Layer P1/N1, P1/LN1, P1/PLGA, PLL/N1, and PLL/PLGA coatings were assembled on quartz slides by LBL. Fig. 2 presents the build up in film thickness with number of absorption steps. P1/N1 showed the largest amount of material deposited. The difference in optical mass between P1/N1 and PLL/PLGA suggests the importance of linear charge density, amino acid sequence, and degree of polymerization in multilayer film buildup, consistent with earlier work.[7,10,38,39] Strong coulombic forces will both attract oppositely-charged species and repel like-charged ones, limiting film thickness increment.[39,40] The assembly of P1/LN1 resembles that of PLL/N1 and P1/PLGA. Each "loaded" TNB molecule increased peptide hydrophobicity and added a single negative charge in these experiments. Both electrostatic interactions and hydrophobic interactions influence peptide assembly behavior and film stability.[39,41] The difference in assembly behavior between P1/LN1 and P1/N1 is indirect evidence of loading of N1 with TNB, consistent with the data in Fig. 1b.

Polypeptide design plays an important role in the drug loading and release process described here. We have found that TNB molecules can be loaded onto P1 and onto hen egg white lysozyme (HEWL), and that TNB can be released from the labeled molecules in solution on addition of DTT (data not shown). But

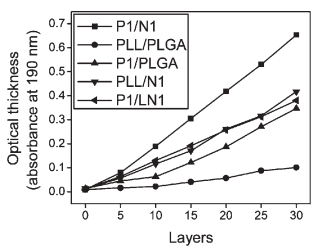

Fig. 2 Multilayer film absorbance (optical thickness) at 190 nm *vs.* number of layers. Assembly behavior falls into three categories: P1/N1; PLL/N1, P1/LN1, and P1/PLGA; and PLL/PLGA. From previous work it is known that each layer of dry film is a few nm thick.[7,11]

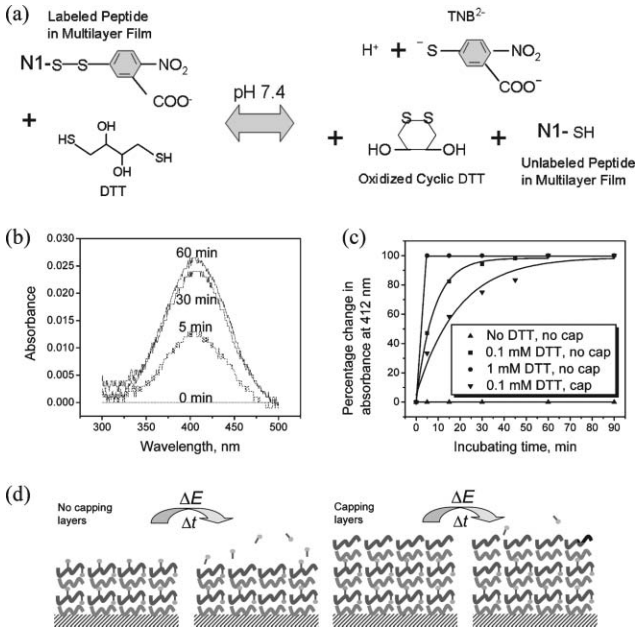

Fig. 3 Release of TNB from multilayer nanocoatings. (a) TNB dissociates from peptide LN1 on the inward diffusion of DTT. (b) Absorbance spectra of liquid medium surrounding of 10 P1/LN1 nanocoatings, recorded 0, 5, 30, or 60 min after immersion in 0.1 mM DTT solution. TNB absorbance increases with time. (c) Absorbance at 412 nm of release media for 10 P1/LN1 films *vs.* incubation time. "Capped" films had $(P1/N1)_2$ on the outer surface (see panel d). TNB release kinetics depend on redox potential and the physical behavior presented by the capping layers. (d) Schematic diagram of redox-stimulated release of TNB from polypeptide multilayer nanocoatings. DTT molecules are omitted for simplicity. ΔE signifies the change in reducing potential, Δt time.

neither labeled P1 nor labeled HEWL proved useful for multilayer film assembly by LBL (data not shown). P1 and HEWL are positively charged at pH 7.4, while TNB groups are negatively charged. The combination of different signs of charge in a single molecule will decrease average linear charge density and suitability for electrostatic LBL. Charge distribution in HEWL is complex, and some of the numerous hydrophobic groups present form a "hydrophobic core" in the intact native enzyme. LN1, by contrast, is useful for fabricating multilayer films by electrostatic LBL.

Similar to its behavior in solution (Fig. 1b), TNB was released from LN1 in multilayer coatings on immersion in an aqueous reducing environment (Fig. 3a). The absorbance peak at 412 nm (Fig. 3b) shows the change in TNB concentration as a function of coating incubation time in 0.1 mM DTT solution. Fig. 3c compares the kinetics of TNB release from coatings with and without $(P1/N1)_2$ "capping" layers. The first-order time constant for 0.1 mM DTT was 8 min. The higher the DTT concentration, the higher the probability of a collision of DTT with TNB in the coating in a given amount of time, and the faster the release of TNB from the coating. TNB was not released to a detectable level under mild oxidizing conditions (0 mM DTT). The concentration of TNB in 0.1 mM DTT solution after 1 h was 2.2 μM. Addition of capping bilayers to coatings containing LN1 reduced the rate of release of TNB. The time constant for capped films was 19 min, about twice as large as for non-capped films. The capping layers contained no TNB; they could inhibit inward diffusion of DTT and outward diffusion of TNB, and the free thiol groups could bind TNB and DTT (Fig. 3d).

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