Supporting Information

A Polysaccharide-Based Antibacterial Coating with Improved Durability for Clear Overlay Appliances

Sohyeon Park[†], *Hyun-hye Kim*[‡], *Seok Bin Yang*[§], *Ji-Hoi Moon*[§∥], *Hyo-Won Ahn*[*⊥], *Jinkee Hong*[*†]

[†]Department of Chemical and Biomolecular Engineering, Yonsei University, 50 Yonsei-ro, Seodaemun-gu, Seoul, Republic of Korea

[‡]Graduate Student, Department of Dentistry, Graduate School, Kyung Hee University, Seoul, Korea, Republic of Korea

[§]Department of Maxillofacial Biomedical Engineering, School of Dentistry, Kyung Hee University, Seoul, Republic of Korea

[∥]Department of Life and Nanopharmaceutical Sciences, Kyung Hee University, Seoul, Republic of Korea

[⊥]Department of Orthodontics, Kyung Hee University School of Dentistry, Seoul, Republic of Korea

*E-mail: hyowon@khu.ac.kr

*E-mail: jinkee.hong@yonsei.ac.kr

S1. Materials and Methods

S1.1 Preparation of polysaccharide-based multilayer films.

Si wafers and PETG thermoplastic polymer sheets (Thermoforming Foil Track A; Forestadent, Pforzheim, Germany), with a thickness of 1.0 mm, were used as the substrates. The substrates were sonicated in deionized (DI) water for 5 min, and cleaned using oxygen plasma (CUTE-1B; Femto Science, Yongin, Korea) for 2 min. The oxygen plasma-treated substrates were incubated in 5 mM of an aqueous solution of branched polyethyleneimine (BPEI, molecular weight (Mw) ≈ 25,000, Sigma-Aldrich, St. Louis, USA) for 6 h, and subsequently rinsed twice with DI water for 4 min. Next, fresh polysaccharide solutions, with opposite charges, at 1 mg/mL, were prepared via the dissolution of sodium carboxymethyl cellulose (CMC, Mw ≈ 250,000, Sigma-Aldrich, St. Louis, USA) and Chitosan (CHI, medium Mw, degree of deacetylation = 75~85%, Sigma-Aldrich, St. Louis, USA) in DI water; the pH values of the prepared solutions were adjusted to 4. The surface-treated substrates were successively immersed in the CMC and CHI solutions for 10 min at room temperature, and washed with DI water twice. By repeating this cycle *n* times, where the substrate was immersed alternately in the CHI and CMC solutions, a (CMC/CHI)*n* (*n* = number of bilayers) film was obtained.

S1.2 Crosslinking techniques used for the multilayer films.

First, crosslinking of the multilayer films was performed using 1-Ethyl-3-(3-Dimethylaminopropyl) Carbodiimide hydrochloride (EDC, Mw ≈ 191.71, Daejung, Seoul, Korea) and N-hydroxysulfosuccinimide (NHS, Mw ≈ 115.09, Sigma-Aldrich, St. Louis, USA). The substrates (Si wafer and PETG) with the LbL-assembled CMC/CHI multilayer films were immersed in 0.05 M 2-(N-Morpholino) ethane sulfonic acid hydrate (MES buffer, Mw ≈ 195.2,

Sigma-Aldrich, St. Louis, USA) containing 0.2 M EDC and 5 mM of NHS for 20 min; they were then immersed in a phosphate buffered saline (PBS, 1X, Gibco® Life Technologies, Gaithersburg, USA) for 10 min to remove any unreacted residue. Subsequently, to achieve additional crosslinking, the substrates were immersed in 2.5% glutaraldehyde solution (Mw ≈ 25,000, Sigma-Aldrich, St. Louis, USA) for 40 min, and rinsed with DI-water for 5 min.[1]

S1.3 Characterization of the multilayer films.

All the surface analyses were performed using the multilayer film-coated Si wafers. A profilometer (Dektak 150; Veeco Instrument Inc., Oyster Bay, USA) was used to measure the thickness of the LbL-assembled multilayer films on the Si wafer. The surface morphologies of the films, prior to and following crosslinking, were observed using an atomic force microscopy (AFM; NX10, Park Systems) and a field-emission scanning electron microscope (FE-SEM, LIBRA 120 microscope; Carl Zeiss, Oberkochen, Germany). In the case of each sample, to assess the wettability of the films with the changes in their surface morphologies, we measured the static contact angles of the multilayer films three times, using a contact angle goniometer (Smart Drop Standard., Femtobiomed, Korea). The volume of each water droplet was 4 μL.

S1.4 Cell viability assay.

In the case of the cell viability testing, the crosslinked $(CMC/CHI)_{20}$ film-coated Si wafers were used as an experimental group. The cytotoxicity of each sample was evaluated using C2C12 which is an immortalized mouse myoblast cell line. The cells were maintained at 37 °C in a humidified incubator under 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco® Life Technologies, Gaithersburg, USA) and 1% penicillin/streptomycin antibiotics (Gibco® Life Technologies,

Gaithersburg, USA). Subsequently, the cells were seeded in a 12-well plate at 1 mL per well, with a density of 1×10^4 cells/mL. Following an incubation period of 24 h, the cultured medium was replaced with the fresh medium, and then dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, USA) or the crosslinked film-coated Si wafers ($1\ cm \times 2\ cm$), which were sterilized with UV radiation for 15 min, were placed in each well, except in the case of the wells with negative groups. Here, a pure cell-growth medium and DMSO-added growth medium were used for the negative and positive control groups, respectively.

Following 24 h of treatment, the Si wafers and cultured medium were removed from the well plates; subsequently, the cells were incubated with 1 mL of a growth medium containing 10% 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, (MTT, M2128, Sigma-Aldrich, St. Louis, USA) solution for 2 h. Following the MTT treatment, the cultured medium was removed from each well and 150 μL of DMSO was added to dissolve the MTT formazan. The relative amounts of viable cells were compared by determining the optical density at a wavelength of 540 nm.

S1.5 Antibacterial testing.

(1) Bacterial strains and media.

Streptococcus mutans (*S. mutans*) GS-5 was anaerobically grown at 37 °C (85% N_2, 10% H_2, and 5% CO_2) in a brain–heart infusion broth (BHI, Difco Laboratories, Detroit, MI, USA).

(2) Observation of bacterial biofilm formation using scanning electron microscopy (SEM).

During the antibacterial testing, the bare PETG sheets were used as a control group. However, the crosslinked $(CMC/CHI)_{20}$ film-coated PETG sheets were used as an experimental group. The biofilm formation on the samples was investigated, as described elsewhere. Briefly, bacterial strains were grown to exponential phase, and then, the bacterial

culture was adjusted to 1×10^8 colony forming units/mL using McFarland standards. The bacterial suspension was added to a polystyrene 24-well plate (1 ml per well) containing the samples, and the plate was anaerobically incubated at 37 °C.

Following 24 h, the samples were removed using sterile forceps and placed into a fresh 24well plate. The planktonic and loosely adhered bacteria were removed by aspirating the spent media; this was followed by washing twice with physiological saline. The remaining biofilm cells on the samples were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h; they were then post-fixed with 1% OsO_4 in 0.1 M phosphate buffer for 1 h at 4 °C, and dehydrated through a graded ethanol series. The samples were dried via critical-point drying, coated with gold using a sputter-coater (IB-3, Eiko, Tokyo, Japan), and observed using a S4700 FE-SEM (Hitachi High Technologies America, Inc., Pleasanton, CA, USA). In the case of each strain tested, SEM experiments were performed in duplicate, and representative images of the biofilms were selected.[2]

(3) Use of adenosine triphosphate (ATP) bioluminescence to evaluate the bacteria viability.

The samples were incubated with the bacterial suspensions for 24 h, as described above. To measure the amount of adenosine triphosphate (ATP) present in the biofilm cells, a BacTiterGlo viability assay kit® (Promega, Madison, WI, USA) was utilized according to the manufacturer´s instructions. The luminescent signal was recorded using a Dynex Triad multimode plate reader (Dynex Technologies, Chantilly, VA, USA) and is expressed in the form of relative luminescence units (RLU) normalized to the surface area of the sample (RLU/cm^2).

S1.6 Testing of chemical resistance.

To test the chemical resistance of the materials, the thickness of the film-coated Si wafers was measured, at nanoscale, because the chemical stability of such materials is determined by changes in the nano-film thickness. The film-coated Si wafers were divided into two groups. Group 1 was the control group, in which non-crosslinked $(CMC/CHI)_{20}$ films were coated on Si wafers, and Group 2 was the experimental group, in which crosslinked $(CMC/CHI)_{20}$ films were coated on Si wafers.

The test was performed, under simulated intraoral conditions with artificial saliva (Taliva, Hanlim Pharmaceutical, Korea), in an incubator (MI-20A, SNT) at 37 °C (mouth temperature). This was to evaluate the chemical stability of the sample in an oral cavity. Taliva is composed of 1.2 mg/mL potassium chloride, 0.15 mg/mL calcium chloride, 0.34 mg/mL potassium hydrogen phosphate, 10 mg/mL sodium CMC, 30 mg/mL D-sorbitol, 5 mg/mL magnesium chloride, and 0.84 mg/mL sodium chloride.[3]

To evaluate the stability of the coating for acidity and salivary enzymes in oral cavity, five different saliva solutions were prepared; bare artificial saliva of pH 6.7, acidic artificial saliva of pH 4.7 and basic artificial saliva of pH 8.7, 700 U/mL of lysozyme (from chicken egg white, Sigma Aldrich) added artificial saliva of pH 6.7, and 300 U/ mL of alpha-amylase (from human saliva, Sigma-Aldrich) added artificial saliva of pH 6.7. Subsequently, 10 mL of each artificial saliva was placed in each vial, and then, three Si wafers from each group were placed in each

vial.[4-5]

The Si wafers were removed from the vials kept in the incubator and their thicknesses were measured using a profilometer over a period of seven days. To compare the degree of thickness reduction of the films under the various conditions, the normalized mean thickness (thickness/initial thickness × 100, %) was calculated for each group and condition. A repeated measure two-way analysis of variance (ANOVA) was performed to determine the relationship between the normalized mean film thickness and the crosslinking of the nanofilms. P-values of <0.05 were considered statistically significant.

S1.7 Testing of mechanical properties.

When evaluating the mechanical properties, the specimens were divided into four groups, namely Groups A-1, A-2, B-1, and B-2. Here, Group A consists of PETG sheets that have not undergone a thermoforming process, and Group B consists of PETG sheets that have been subjected to a thermoforming treatment. The PETG sheets in Group B were vacuum-formed on a wood reference block (20 × 60 × 20 mm) using a thermoforming machine (Track V; Forestadent, Pforzheim, Germany) according to the manufacturer's instructions (rated temperature 160 °C, cooling period 45 s). Groups A and B were subdivided into Groups 1 and 2 depending on the presence or absence of the crosslinked $(CMC/CHI)_{20}$ film coating. Groups A-1 and B-1 represented the experimental groups in which the crosslinked $(CMC/CHI)_{20}$ nanofilms were coated onto the PETG sheets. Group A-2 and B-2 were the control groups that consisted of the bare PETG sheets without a coating.

(1) Tensile tests

In the case of the tensile testing, ten dumbbell-shaped specimens (width of 10 mm, length of 40 mm) were prepared for each group (Figure S2a). The test was performed using a universal

mechanical testing instrument (Instron 3367, Instron Co., Canton, MA, USA), with a load cell of 3 kN. Using a loading rate of 1.5 mm/min, the maximum tensile load was measured at room temperature. The data obtained from each mechanical test were analyzed using Bluehill® Lite version 2.0 software (Instron Co., Canton, MA, USA). The obtained data were expressed as the mean ±standard deviation.

The data did not satisfy the normality assumption; the Mann–Whitney U-test was performed to analyze the tensile-strength datasets with regard to the crosslinked $(CMC/CHI)_{20}$ nano-film coating present in each group. In addition, ANOVA was performed to assess the interaction between the independent variables, namely the thermoforming process and coating. P-values of <0.05 were considered statistically significant.

(2) Fracture-resistance testing

For the fracture-resistance tests, 15 rectangular shaped specimens (width of 20 mm, length of

55 mm) were prepared for each group (Figure S2b). Rectangles were notched at the center of each length of the specimen using disks (Gebr. Brasseler GmbH & Co. KG, Lemgo, Germany), producing double-edge-notched-tension specimens. In the case of each group, 15 specimens were constructed, with various lengths between the notch tips (L; L = 6, 8, 10, 12, and 14 mm); three specimens were produced for each of these lengths.

Using an Instron 3367 with a load-cell of 3 kN, each specimen was loaded in tension, at a crosshead speed of 1.5 mm/min, until fracture occurred. To determine the relative total work of fracture (W_f), the areas under the load–elongation curves were normalized using the Origin version 8.0 software (OriginLab Co., Northampton, MA, USA). In the case of each group, least square-regressions were performed to determine the line of best fit for five pairs of W_f vs. L data points. The data points were obtained by plotting the intercept and slope with the

W_f values determined for the various specimens (with L = 6, 8, 10, 12, and 14 mm), to estimate the essential work of fracture (EWF) and plastic work of fracture (PWF), respectively.

The EWF is the energy that is essential for the generation of new fracture surfaces, which solely depends on the fracture surface area. It is an intrinsic material property, used for the evaluation of crack-initiation resistance. The PWF refers to nonessential work, consisting of the energy that is plastically dissipated throughout the zone surrounding the fracture. The PWF depends on the specimen volume, geometry, and loading configuration; therefore, it is not an intrinsic material property. It is regarded as a measure of resistance to crack propagation, and is an indicator of ductility. Therefore, the EWF value was used as a criterion to evaluate the fracture resistance properties.[6-7]

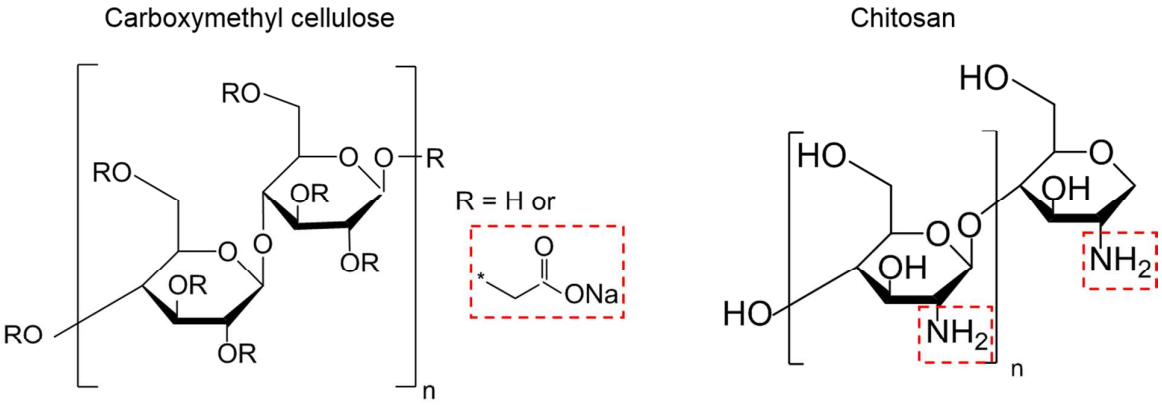

Figure S1. Chemical structure of CMC and chitosan showing the principal functional groups for preparing multilayer films.

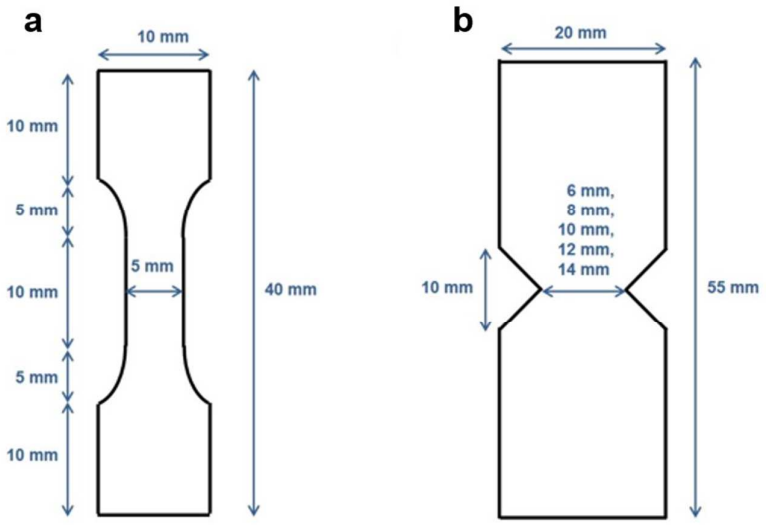

Figure S2. Design of the PETG sheets used for mechanical testing. (a) Dumbbell-shaped specimen used for tensile testing. (b) Rectangular specimen notched at the center of each long edge, used for fracture-resistance testing.

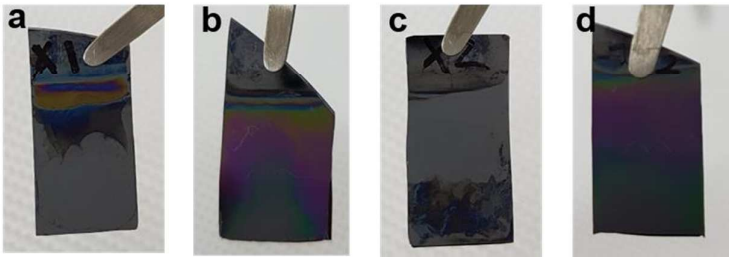

Figure S3. Appearance of each film following the stability tests under various enzyme conditions. (a) Non-crosslinked $(CMC/CHI)_{20}$ film and (b) crosslinked $(CMC/CHI)_{20}$ film

under α-amylase-added saliva. (c) Non-crosslinked $(CMC/CHI)_{20}$ film, and (b) crosslinked $(CMC/CHI)_{20}$ film under lysozyme-added saliva.

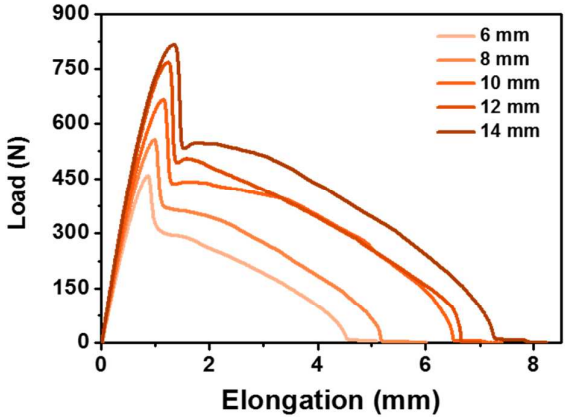

Figure S4. Representative load–elongation curves obtained for specimens with a series of various notch lengths (L); L = 6, 8, 10, 12, and 14 mm.

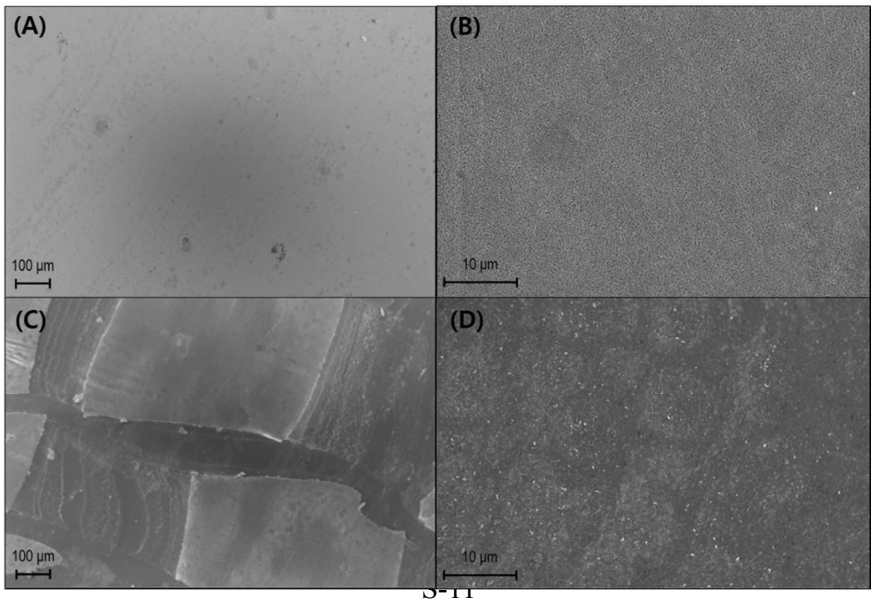

Figure S5. Field-emission scanning electron microscopy (FE-SEM) images showing the surface morphology of $(CMC/CHI)_{20}$ films on PETG sheets. (A) Image at 226× magnification, obtained before the thermoforming process. (B) Image at 5,000× magnification, obtained before the thermoforming process. (C) Image at 226× magnification, obtained after the thermoforming process. (D) Image at 5,000× magnification, obtained after the thermoforming process.

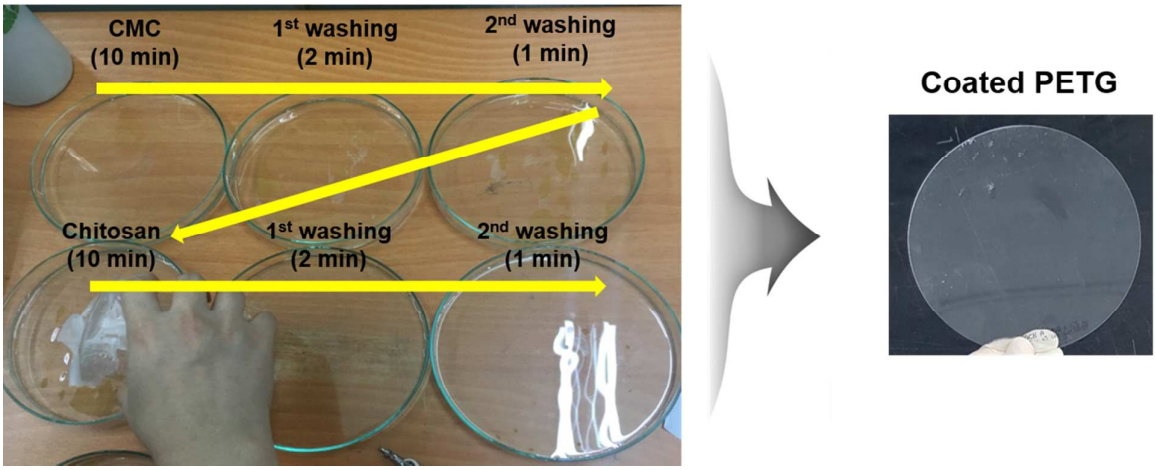

Figure S6. Layer-by-layer assembly process using PETG as substrate and completed coated PETG.

REFERENCES

1. Nuraje, N.; Asmatulu, R.; Cohen, R. E.; Rubner, M. F., Durable antifog films from layer-bylayer molecularly blended hydrophilic polysaccharides. *Langmuir* **2010,** *27* (2), 782-791.
2. Jang, E.-Y.; Kim, M.; Noh, M. H.; Moon, J.-H.; Lee, J.-Y., In vitro effects of polyphosphate against Prevotella intermedia in planktonic phase and biofilm. *Antimicrobial agents and chemotherapy* **2016,** *60* (2), 818-826.
3. Jeong, H.; Cho, Y.-A.; Cho, Y.; Kang, E.; Ahn, H.-w.; Hong, J., Durable urushiol-based nanofilm with water repellency for clear overlay appliances in dentistry. *ACS Biomaterials Science & Engineering* **2016,** *2* (3), 344-348.
4. Gandara, B. K.; Izutsu, K.; Truelove, E. L.; Mandel, I.; Sommers, E. E.; Ensign, W., Sialochemistry of whole, parotid, and labial minor gland saliva in patients with oral lichen planus. *Journal of dental research* **1987,** *66* (11), 1619-1622.
5. Lee, J. Y.; Chung, J. W.; Kim, Y. K.; Chung, S. C.; Kho, H. S., Comparison of the composition of oral mucosal residual saliva with whole saliva. *Oral diseases* **2007,** *13* (6), 550554.
6. Karger-Kocsis, J., Toward understanding the morphology-related crack initiation and propagation behavior in polypropylene systems as assessed by the essential work of fracture approach. *Journal of Macromolecular Science—Physics* **1999,** *38* (5-6), 635-646.
7. Peres, F. M.; Schön, C. G., Application of the essential work of fracture method in ranking the performance in service of high-density polyethylene resins employed in pressure pipes. *Journal of Materials Science* **2008,** *43* (6), 1844-1850.