Integrated Catalytic Activity of Patterned Multilayer Films Based on pH-Induced Electrostatic Properties of Enzymes**

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The fabrication of layer-by-layer (LbL) assembled multi-layered structures has attracted widespread interest since the first introduction of this approach by Decher and co-workers in the early 1990s.[1] Multilayered films prepared by LbL assembly are promising candidates for a wide variety of applications, including electronic and optical devices, super-hydrophobic surfaces, and biochemical sensors.[2–13] Patterned multilayer structures have been fabricated via the selective growth of multilayers on hydrophilic surfaces based on the microcontact printing (μCP) of a hydrophobic alka-nethiol;[14–17] such structures have also been prepared using lift-off methods based on conventional photolithography.[18–20] Another alternative approach involves the use of a random graft copolymer of oligoethyleneoxide allylether and maleic anhydride as a surface modifier for multilayer patterning since the deposition of this polymer prevents the non-specific adsorption of polyelectrolytes (PE) on surfaces.[21] However, none of these methods are adequately biocompatible for the fabrication of patterned structures on bioactive substrates such as enzymes. For this purpose, the multilayer-patterning method has to be a biocompatible process that does not cause any physical or chemical damage to the biomaterial substrate. Examples of possible damage that may lead to a partial or complete loss of enzymatic activity include degradation as a result of thermal treatment, UV light, and harsh solvents, conditions that are ubiquitous in conventional patterning methods. Recently, Hammond and co-workers have reported that multilayers deposited onto hydrophobic poly(dimethylsi-loxane) (PDMS) can be transferred onto different substrates, yielding patterned multilayer structures.[22] This biocompati-ble process based on multilayer transfer requires that the hydrophobic interactions between the PE base layer and the PDMS surface are weaker than the electrostatic interactions between the top surface layer and the transfer substrates.

For the preparation of enzyme-based electrochemical sensors sensitive to different analytes, a variety of catalytic components need to be inserted within electroactive films.[23,24] Furthermore, direct contact between probe molecules and the sensing components is desirable to facilitate high sensitivity.[25] In light of these requirements, the patterning of metal nanoparticle (NP) layers with catalytic properties on enzyme surfaces offers diverse possibilities for preparing electroactive films with integrated catalytic properties.

Herein, we introduce a novel, robust patterning method for the selective deposition of organic/organic and organic/inorganic multilayers using an enzymatic surface that offers electrostatic charge reversal via pH control. Furthermore, we demonstrate that highly efficient electrochemical sensors with integrated catalytic properties can be prepared based on this novel patterning method. The presence of a hydrophilic enzymatic layer with electrostatic charges in unpatterned areas effectively screens the deposition of negatively and positively charged PE layers such as hydrophobic surface modifiers by inducing electrostatic repulsion between the enzyme and the next PE layers; this repulsion can be engineered via pH control of the enzymes. Therefore, the selective growth of multilayers via electrostatic repulsion can be exclusively achieved on enzyme-coated substrates because of the unique pH-dependent electrostatic properties of enzymes. That is, for substrates coated with conventional PEs such as cationic poly(allylamine hydrochloride) (PAH) or anionic poly(styrene sulfonate) (PSS) instead of enzymes, selective deposition is restricted by the electrostatic interaction between both the PE coated on the unpatterned area and PE layers on the patterned area. It is worth noting that our approach is based on fundamentally different chemistry as compared to the multi-layer transfer processes or conventional multilayer patterning methods. Importantly, this patterning method may be useful for a wide variety of potential applications since the enzymes

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can be readily adsorbed onto a variety of hydrophilic substrates without the need for any harsh surface treatment such as the gold coating step necessary for hydrophobic alkanethiol μCP. We have also found that electrochemical sensors prepared from patterned multilayers are more effective in terms of integrated catalytic (i.e., metal and biocatalytic) properties as compared to unpatterned electrochemical sensors. This contrast has been demonstrated using the selective deposition of platinum NP (Pt_{NP})/PE multilayers on catalase (CAT)/PE multilayers. This work is significant because the preparation of multilayer patterns has been demonstrated using the pH-induced properties of an enzymatic surface without the incorporation of any hydrophobic components or the involvement of photolithographic processes that may degrade enzymatic activity. Additionally, the electrochemical activity has been significantly increased as a result of the direct contact between the probe molecules and the respective catalytic components in patterned and unpatterned areas. For improved pattern quality on polar materials, hydrophilic composite stamps have been used instead of PDMS. These stamps have been used without any additional chemical treatment.[26] The

patterned multilayer films with integrated catalytic properties demonstrated here provide the basis for constructing biosensors and other biomedical devices.

The ζ-potential of CAT has been examined as a function of pH, as shown in Figure 1a, for multilayer-coated silica particles. The measured pI (i.e., isoelectric point) is 5.6. Therefore, CAT bears an overall positive charge at pH < 5.6 and a negative charge at pH > 5.6. (CAT/PSS)$_5$ multilayers have been prepared on a PAH-coated quartz crystal microbalance (QCM) electrode by utilizing electrostatic interactions at pH 3. Under these conditions, the adsorbed amounts of CAT and PSS per bilayer are 755 ng cm^{-2} (frequency change, $\Delta F \approx 42.7$ Hz) and 132 ng cm^{-2} ($\Delta F \approx 7.5$ Hz), respectively (Fig. S1, Supporting Information). PSS layers have been deposited onto multilayer surfaces that have a cationic CAT layer as the top surface using the μCP method. A hydrophilic composite stamp has been used and the deposited layers are characterized by a high quality of patterning over large areas. Indeed, this method achieves selective and uniform deposition of PSS on the CAT layer (Fig. S2, Supporting Information). Upon dipping these multilayer-coated substrates into a
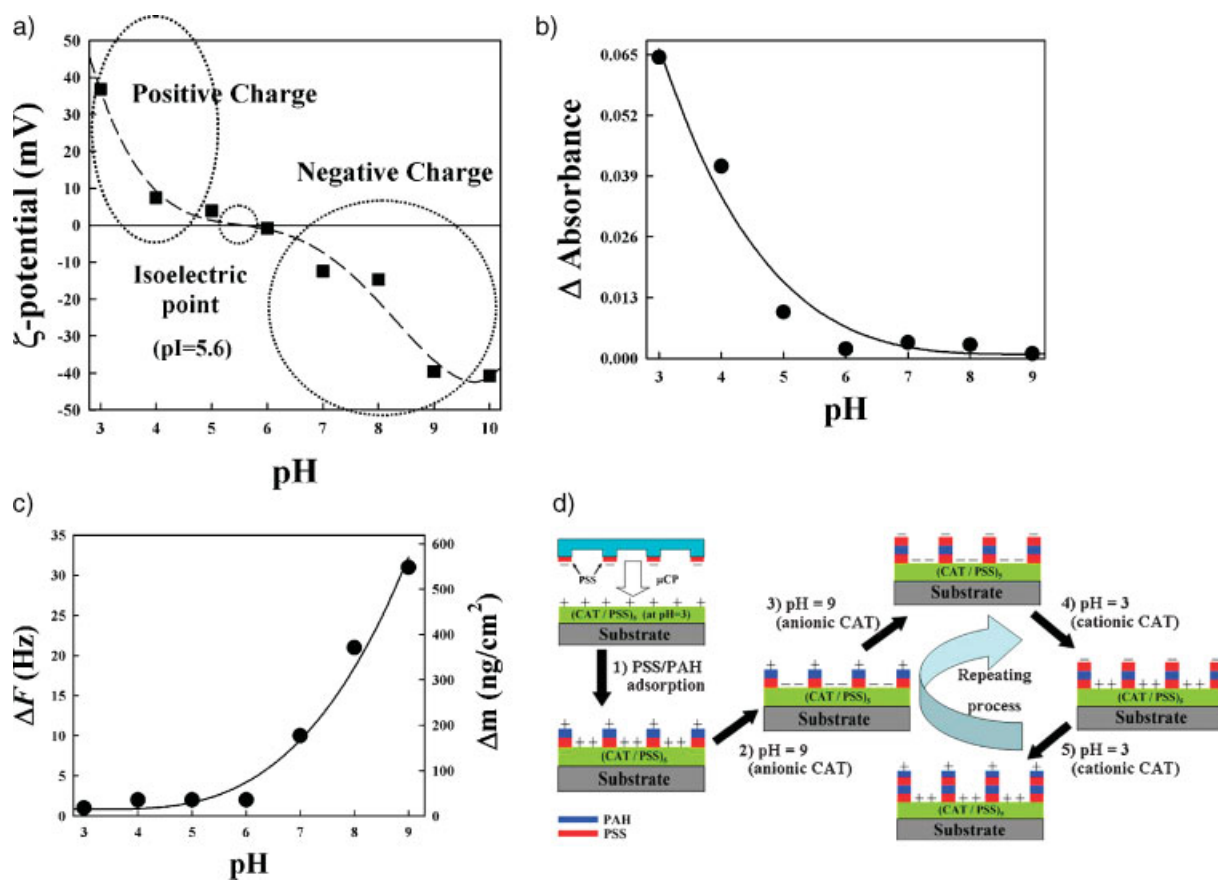

Figure 1. a) ζ-potential of (CAT/PSS)$_2$/CAT-coated silica colloids measured as a function of increasing pH from pH 3 to 10. b) Absorbance and c) frequency changes as a function of CAT solution pH during the assembly of PAH/CAT on a quartz glass and CAT/PSS on a quartz crystal microbalance electrode, respectively. The absorbance of PAH/CAT has been measured at 225 nm. d) Schematic depiction of the formation of patterned (PAH/PSS)$_n$ multilayers using pH-induced electrostatic properties of CAT.

cationic PAH deposition solution at pH 3, PAH is selectively adsorbed onto the patterned PSS surface because of the electrostatic repulsions between PAH and CAT at this pH value. To deposit the next PSS layer, the pH of the PSS solution has been adjusted to >5.6 to convert the positively charged (on average) unpatterned CAT surface into a negatively charged surface. After charge reversal of the CAT, the next PSS layer is selectively deposited onto the PAH patterns. These results indicate that the overall charge of the enzymes governs the selectivity of the deposition process. Therefore, the pH of the enzyme has been repeatedly and successively adjusted to 3 (positive charge) and 9 (negative charge) to maintain a high charge density and facilitate sequential deposition. The electrostatic charge reversal of CAT is strongly supported

by the observation that the amount of PSS adsorbed onto the CAT-coated quartz glass substrate significantly decreases upon increasing the pH of the deposition solution from 3 to 6 (Fig. 1b). On the other hand, the adsorbed amount of PAH increases above pH 6 (Fig. 1c). This protocol — involving control of the electrostatic properties of CAT, followed by the deposition of PAH or PSS — constitutes the key step in the preparation of sensors and can be continuously repeated. As a consequence, the patterned PSS/PAH multilayers can be successfully built up onto PSS/CAT multilayers, as illustrated in Figure 1d.

Figure 2 shows that the thickness of the patterned PSS/PAH multilayers increases from about 3.5 nm for one bilayer to 19 nm for seven bilayers. These results strongly support our hypothesis that an enzymatic surface with electrostatic charge
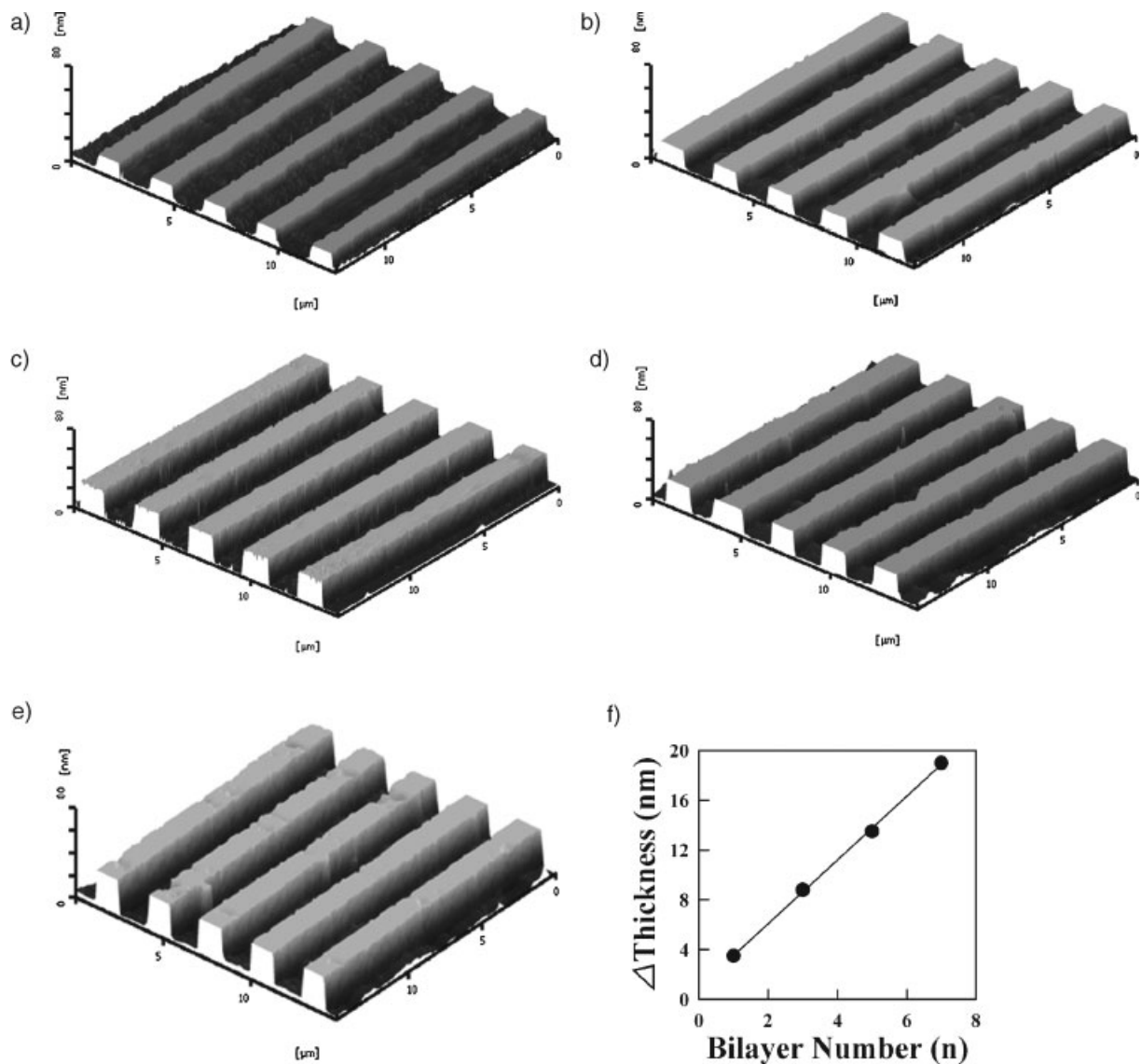

Figure 2. Atomic force microscopy (AFM) images of patterned PSS/(PAH/PS)$_n$ multilayers formed on multilayered (CAT/PSS)$_5$ films: a) $n = 0$, b) $n = 1$, c) $n = 3$, d) $n = 5$, and e) $n = 7$. f) Change in the thickness of patterned multilayers with increasing bilayer number (n) from 1 to 7.

reversal induced by varying the solution pH can be effectively used for the selective deposition of multilayers.

The electrostatic patterning method has been used to fabricate hybrid electrochemical sensors composed of metal NPs and biocatalysts, as shown in Figure 3a. First, citrate-ion-stabilized Pt NPs (Pt_{NP}) with a diameter of ca. 5.8 nm have been synthesized (Fig. S3, Supporting Information). In this case, the patterned $PSS/(PAH/Pt_{NP})_5$ (M_{Pt}) multilayers have been selectively deposited onto $(PSS/CAT)_5$ (M_{CAT}) multilayer-coated electrodes. Since CAT and Pt_{NP} are known to be efficient catalysts for NO[23,24,27,28] and H_2O_2,[29–32] it is envisioned that sensors integrating these hybrid multilayer films will exhibit greater sensitivity than those exclusively based on M_{Pt} or M_{CAT} multilayers. The patterned M_{Pt} multilayers have been selectively deposited onto M_{CAT} multilayers using the electrostatic patterning method (Fig. 3a). In this case, the adsorbed amount of Pt_{NP} per bilayer is 3074 ng cm^{-2} at pH 9. Although this amount can be increased up to 5180 ng cm^{-2} at pH 7 because of the decrease in charge repulsion between groups having the same charge (Fig. S4, Supporting Information),[11,12] the deposition solution has been adjusted to pH 9 to achieve the selective deposition of Pt_{NP} onto PAH layers.

The plots denoted B and C in Figure 3b and c show the cyclic voltammograms (CVs) of M_{CAT} and M_{CAT}/patterned-M_{Pt} multilayers for sensing H_2O_2 and NO gas at 50 mV s^{-1}, respectively. As shown in the curves denoted A in Figure 3b and c, relatively weak electrocatalytic behavior towards H_2O_2 is observed for the bare Au electrode. In contrast, the electrode modified with M_{CAT} multilayers displays a pair of redox peaks with an oxidation peak potential at 0.1 V and a reduction peak at −0.37 V, implying enhanced catalytic activity of CAT towards H_2O_2. Furthermore, the electrode modified with M_{CAT}/patterned-M_{Pt} multilayers exhibits a more sensitive catalytic response with significantly higher redox peaks as compared to M_{CAT}-multilayer-modified electrodes. This behavior has also been confirmed for the electrochemical activity of NO (Fig. 3c). Electrodes modified with patterned M_{Pt}/M_{CAT} multilayers give rise to the strongest oxidation peak around 1.4 V with a significantly more dramatic increase in peak current as compared to the response for M_{CAT} multilayers. Although the redox behavior has been measured at a fixed scan rate of 50 mV s^{-1}, an increase in the scan rate may result in the enhanced growth of redox peaks, as demonstrated by several research groups.[33,34] Recently, we reported that the sensitivity of electrochemical sensors based on $(Au_{NP}/PE)_n$ multilayers can be significantly improved by increasing the number of bilayers, and more specifically by increasing the Au_{NP} content in the films.[6] This strongly supports the possibility that our approach may also be useful to control the sensitivity of sensors via careful design of the multilayers (involving control of the solution pH and bilayer number for adsorbed Pt_{NP} and CAT in the hybrid films). Furthermore, we expected that sensors using patterned hybrid multilayer films would exhibit greater electrochemical activity than those prepared from unpatterned hybrid multilayer films. That is, the electrochemical reaction of H_2O_2 and NO gas in the patterned
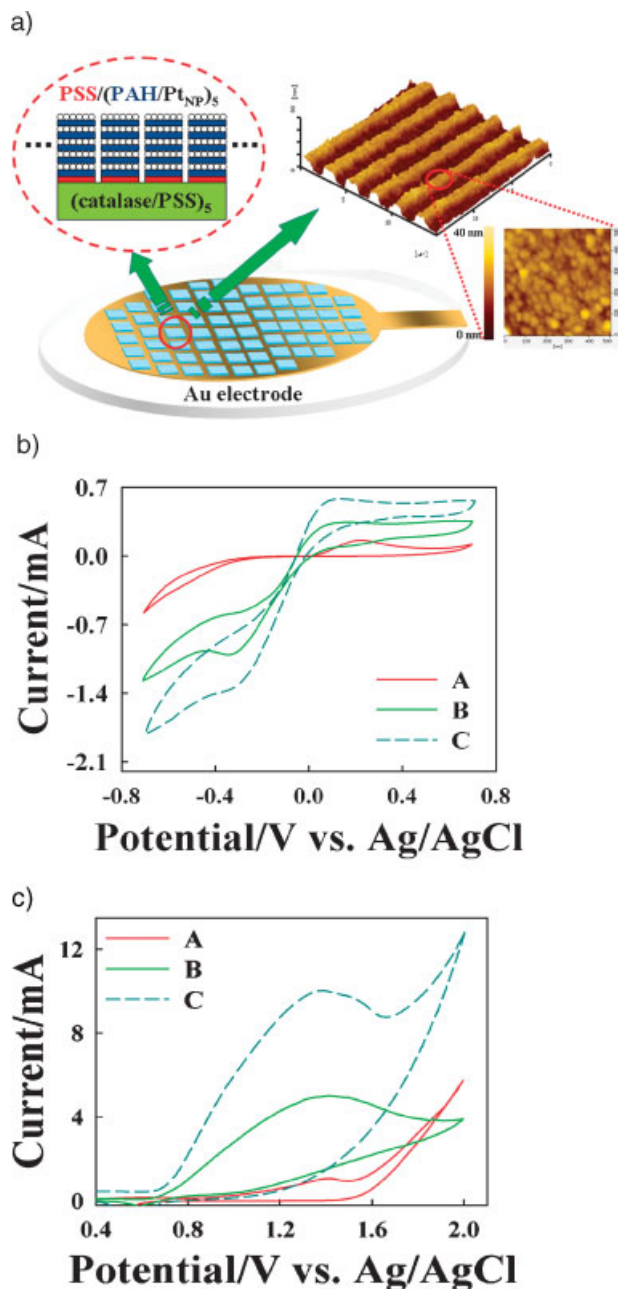

Figure 3. a) Schematic depiction and AFM images of $(PAH/Pt_{NP})_5$ multilayers patterned onto a $(CAT/PSS)_5$ multilayer-coated QCM electrode by taking advantage of the pH-induced electrostatic properties of CAT. Cyclic voltammogram measurements of the bare electrode (curve A), M_{Pt}/M_{CAT} (curve B), and M_{CAT}/patterned-M_{Pt}-modified electrodes (curve C). The measurements have been made using b) pH 7.0 phosphate buffered saline (PBS) solution containing 21 mM H_2O_2 and c) pH 3.0 PBS containing 21 mM $NaNO_2$.

films effectively occurs via direct contact with both the M_{CAT} and the M_{Pt} multilayers, leading to a much improved sensitivity. To investigate this possibility, the CVs of the system electrode/$(PAH/Pt_{NP})_5$/$(CAT/PSS)_4$/CAT without patterning have been measured (Fig. S5, Supporting Information). The electrochemical activity of the unpatterned films has been

observed to be significantly lower than for patterned films, although the adsorbed amount of Pt_{NP} is almost the same as the amount of Pt_{NP} in the patterned M_{Pt} system (achieved by halving the concentration of the initial Pt_{NP} solution). Additionally, the electrochemical activity of the $(PAH/Pt_{NP})_5$ multilayers is slightly higher than that of $(CAT/PSS)_4/CAT$ for H_2O_2 and NO sensing. Based on these observations, the decrease in electrochemical activity observed for the electrode/ patterned $(PAH/Pt_{NP})_5/(CAT/PSS)_4/CAT$ sensor can be attributed to the M_{CAT} layer partially screening the direct contact between $(PAH/Pt_{NP})_5$ and H_2O_2 (or NO gas).

Continuous cycling tests have been used to show that $M_{CAT}/$ patterned-M_{Pt} multilayers prepared by taking advantage of the pH-dependent electrostatic properties of CAT are adequately stable. That is, very negligible changes in redox peaks are observed during the cycling test, indicating that these films are electrochemically and structurally stable (Fig. S6, Supporting Information).

In conclusion, we have demonstrated that novel patterned multilayers can be prepared using the pH-induced electrostatic properties of enzymes in a mild pH range. Furthermore, electrochemical sensors based on this patterning method show higher electrochemical activity than those based on simple multilayered architectures. We anticipate that this approach will boost the performance of biosensors in fields such as biocatalysis and diagnostics.

Experimental

Materials: Anionic PSS (molecular weight (M_w) = 70 000), cationic PAH (M_w = 70 000), and CAT were purchased from Aldrich. Anionic Pt NPs (Pt_{NP}) were synthesized by the citrate reduction method. Briefly, 250 mL of 1.79 mM $H_2PtCl_6 \cdot 6H_2O$ was maintained at room temperature with vigorous stirring. Subsequently, 20 mL of 68 mM sodium citrate was rapidly added to solution with mixing. The successive addition of 70 mM $NaBH_4$ (1 mL) resulted in a color change of the solution from dark yellow to dark brown. The synthesized Pt nanoparticles were about 5.8 ± 1 nm in diameter (100 particles were sampled) as corroborated by transmission electron microscopy (TEM) images. These nanoparticles were dispersed in aqueous solutions at pH 7 and 9.

Preparation of LbL Multilayer Films: The concentration of PAH and PSS solutions used in all of the experiments was 1 mg mL^{-1}. Si substrates with an anionic surface were prepared by heating at 65 °C for 5 s in a 5:1:1 (v/v/v) mixture of $H_2O/H_2O_2/29\%$ NH_3 solution (RCA solution). These substrates were first dipped for 10 min in a cationic CAT solution at a pH of 3, followed by two washing cycles involving dipping in pH 3 water for 1 min, and air-drying under a gentle stream of nitrogen. Anionic PSS was subsequently deposited onto the CAT-coated substrates by using the same adsorption, washing, and drying procedures described above. This process was repeated until the desired number of layers was deposited.

Fabrication of Polymer Mold: The microscale line patterns formed on the Si wafer were replicated on an urethane-related acrylate prepolymer coated onto a polyester film (SK Chemicals) and cured for 30 min using a 365 nm, 135 mW cm^{-2} UV light source. After release of the pattern from the Si wafer, the mold was cured for 12 h. Next, the prepared thin polymeric mold was placed inside a PDMS frame, and a mixture of Norland Optical Adhesives (NOA) 63 prepolymer and poly(ethylene glycol) diacrylate (PEGDA) hydrogel precursor solution was poured on this patterned polymeric mold and cured under UV

light ($\lambda = 365$ nm) for 30 min to obtain negative relief patterns of the polymeric mold. The composite mold was prepared by blending the NOA 63 prepolymer and a PEGDA hydrogel precursor solution in a 6:4 (w/w) ratio [26]. The PEGDA hydrogel precursor solution was prepared by mixing PEGDA and the photoinitiator, 2-hydroxy-2-methylpropiophenone (HOMPP), in a 95:5 (v/v) ratio. This ratio of PEGDA and HOMPP was selected to endow the composite mold with high transparency after UV polymerization.

QCM Measurements: A QCM device (QCM200, SRS) was used to investigate the mass of material deposited after each adsorption step. The resonance frequency of the QCM electrodes was ca. 5 MHz. The adsorbed mass of PEs and Pt_{NP}, Δm, was calculated from the change in QCM frequency, ΔF, according to the Sauerbrey equation [35]: ΔF (Hz) $= -56.6 \times \Delta m_A$, where Δm_A is the mass change per unit quartz crystal area in $\mu g \ cm^{-2}$.

CV Measurements: The electrochemical activity of multilayers adsorbed onto QCM Au electrodes was investigated by measuring CVs (compactstat, Ivium). To sense H_2O_2 and NO, stock solutions of H_2O_2 and $NaNO_2$ were adjusted to a concentration of 21 mM. $NaNO_2$ is known to generate free NO by the following reaction:

$$3HNONO \rightarrow H^+ + NO_3^- + 2NO + H_2O \tag{1}$$

Surface Morphology of Patterned Multilayers: The line patterns of PSS formed on $(CAT/PSS)_5$ multilayers were investigated by optical microscopy. The surface morphology and pattern height of patterned $(PAH/PSS)_5$ and $(PAH/Pt_{NP})_5$ adsorbed onto Si substrates were studied by atomic force microscopy (AFM) in tapping mode (SPA400, Seiko).

ζ-Potential Measurements: The ζ-potentials of CAT were measured using an electrophoretic light scattering spectrophotometer (ELS-8000) after the alternate adsorption of CAT and PSS onto 600 nm diameter silica particles. $(CAT/PSS)_2/CAT$-coated silica particles were prepared as follows: 100 μL of a concentrated dispersion (6.4 wt %) of negatively charged 600 nm silica particles was diluted to 0.5 mL with deionized water. To this dispersion, 0.5 mL of CAT (1 mg mL^{-1} at pH 3) was added, and after 20 min the excess CAT was removed by three repeated centrifugation (7000 g, 5 min)/washing cycles. PAH (1 mg mL^{-1} at pH 3) with added 0.5 M NaCl was then deposited onto the CAT-coated polystyrene particles under the same conditions. The above process was repeated until five layers were deposited. Subsequently, the ζ-potentials of the colloids were measured with increasing pH from 3 to 10.

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