Direct Visualization of Reversible Switching of Micropatterned Polyelectrolyte Brushes on Gold Surfaces Using Laser Scanning Confocal Microscopy

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We apply confocal fluorescence microscopy for real time studies of reversible conformational changes of poly(methacryloyloxyethyl phosphate) (PMEP) brushes chemically grafted onto gold substrates. Oregon green 488 fluorophores chemically attached onto the PMEP polymers were used as reporters for probing the conformational changes. Use of a specially designed liquid flow microchamber allowed dynamic imaging of the brushes under varying environmental conditions. The fluorescence intensities exhibited fully reversible brightness changes on alternation of the solution in the chamber between water and KCl. This reversible quenching behavior is consistent with a conformational change between an extended and a collapsed brush configuration. The fluorescence quenching behavior of the brushes was found to be dependent on ion concentration as well as polymer grafting density and was caused by nonradiative energy transfer to the polymer scaffold and the gold substrate.

Introduction

The chemical attachment of one end of polymers onto solid substrates, forming so-called "polymer brushes", represents an effective and versatile approach for the creation of functional surfaces.[1–6] In particular, polyelectrolyte brushes, anchoring ionic groups within the polymer chains, are capable of responding to a wide range of external stimuli, such as pH, temperature, light, and ionic strength.[7] Subtle environmental changes give rise to distinct conformational changes of individual polymer chains, leading to an amplified response of the brushes. The unique properties of polyelectrolyte brushes have led to the creation of "smart" surfaces for numerous potential applications in microcantilever sensing and actuation, controlled delivery of drugs, bioseparations, etc.[7–9]

Although a number of sensitive techniques have been reported to characterize the conformational change of brushes on surfaces, including atomic force microscopy (AFM),[10] ellipsometry,[11] and neutron scattering,[12] these are limited in their ability to monitor real-time dynamic events. Their operation is furthermore not compatible with complex biological environments (i.e., living cells), limiting their usefulness in biomedical settings. Laser scanning confocal microscopy (LSCM) does not suffer from these disadvantages and is a versatile technology for measurement of macromolecules. Steady state fluorescence measurement has been used to probe conformational changes of macromolecules in free solution.[13,14] We have previously reported on the use of optical techniques for the characterization of the conformational changes of pH-responsive polymers in solutions.[15] In this paper, we report on the use of LSCM for dynamic studies of fluorophore-labeled polyelectrolyte brushes, and for the first time are able to directly observe reversible conformational changes of polymer brushes on surfaces.

Phosphate-containing poly(methacryloyloxyethyl phosphate) (PMEP) brushes tethered to gold surfaces were chosen as model systems for this study. These brushes have been previously characterized by AFM[10] and micromechanical sensors.[16] The PMEP molecules were first grafted onto a gold film to form a micropatterned polymer brush surface before labeling of the polymer chains with Oregon green 488 fluorophores. We show here that the dye-labeled polyelectrotelyte brushes produce "on–off" optical signals as brushes switch between conformational states on the surface in response to environmental variations.

Experimental Section

Materials. All chemicals were analytical reagent grade and were used as received. 2-(Methacryloyloxy)ethyl phosphate (MEP) was

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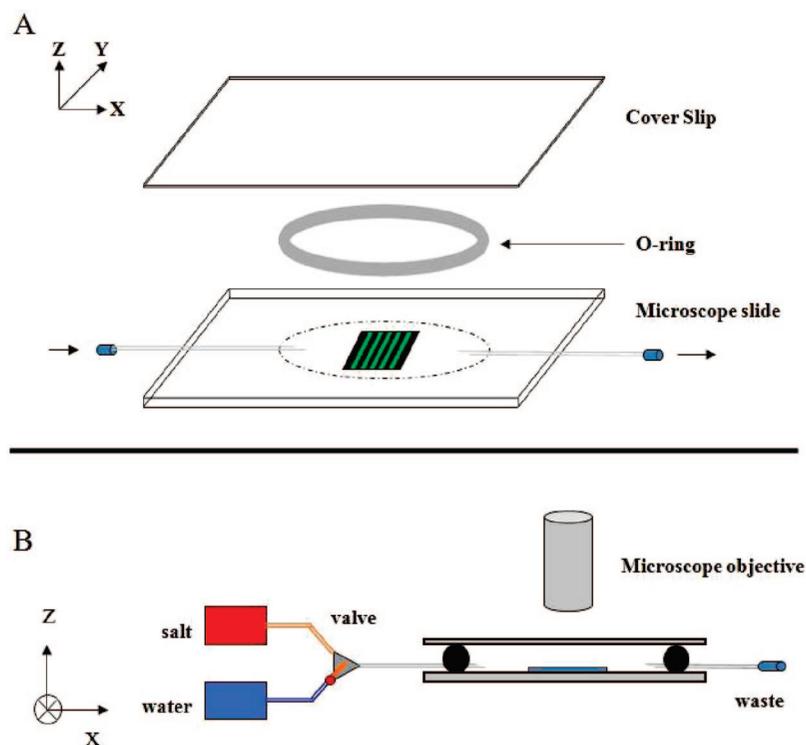
Figure 1. Schematic representation of (A) the flow microchamber and (B) experimental setup.

obtained from Aldrich. It was neutralized to pH 7 with NaOH solution at a concentration of 3 mol L^{-1} and then stored in a refridgerator until use. Ultrapure water was generated with a Millipore Simplicity 185 system. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), dimethylaminopyridine (DMAP), copper(II) bromide (99%) (CuBr$_2$) and 2,2′-dipyridyl (99%) (bipy) were all obtained from Aldrich. CuCl was stored under vacuum until needed. Gold film was prepared via thermal evaporation of a 20 nm gold layer on glass coverslips (thickness, 0.1 mm) with 2 nm Cr as the adhesive layer. Oregon green 488 was purchased from Molecular Probes.

Preparation of Polyelectrolyte Brushes. A patterned monolayer of BrC(CH$_3$)$_2$COO(CH$_2$)$_6$SH was self-assembled on a vapor-deposited gold film via microcontact printing and used as the initiator for the surface polymerization reaction. An inert monolayer was then formed on the exposed gold areas by exposing the chip to a 2 nM solution of hexadecanethiol (HDT) in ethanol to protect uncovered surface area. The chemically tethered polymer brushes were produced by atomic transfer radical polymerization (ATRP) from the initiator-modified gold surface. The grafting density of the polymer brushes on the gold surface could be changed by the addition of inert thiol to the bulk assembly solution for the microcontact printing step. Through variation of their relative concentration, grafting densities were adjusted to 10%, 20%, and 100%, forming the brush configurations reported here. The polymerization solution contained 20 mL of 3 mol L^{-1} MEP aqueous solution (60 mmol monomer), CuBr (0.144 g, 1 mmol), CuBr$_2$ (0.011 g, 0.05 mmol), and bipyridine (0.39 g, 2.5 mmol) (all from Sigma Aldrich). The polymerization was performed at 60 °C for a range of reaction times. PMEP-modified substrate was soaked in a 10 mL of water solution containing 0.0575 g of EDC in H$_2$O for 15 min, and transferred to an Oregon green 488 solution (0.1 mg of dye and 16 mM imidazole in 10 mL of H$_2$O) for 30 min. Dye-labeled brushes were extensively rinsed with pH 9 solution in deionized water to remove physically adsorbed dyes.

Design of Flow Microchamber. A reaction flow microchamber was designed to permit in situ imaging of polymer brush dynamics via LSCM. The flow microchamber was mounted on an inverted microscope with the patterned polymer brush slide positioned in the center of the chamber. A thin rubber O-ring (thickness 1.0 mm, inner diameter 16.0 mm) was sandwiched between a thin coverslip

(22 × 22 mm, Menzel-Glaser) and a microscope slide (76 × 26 mm, Menzel-Glaser) to form the microchamber, with a volume of 200 μL. The small volume improves the efficiency with which solution can be exchanged and reduces reagent consumption. Use of a coverslip to serve as the bottom wall of the chamber permits imaging at short working distance from the objective lens. Two syringe needles were connected to flexible polyethylene tubings and inserted into the chamber through the O-ring seal to form a flow inlet and outlet, respectively (see in Figure 1). Solutions were pumped through the chamber at a constant flow rate of approximately 1.0 mL min^{-1} by a 1.0 mL syringe (BD Plastipak), and fluorescence from the labeled brushes was monitored in real time.

Experimental Setup and Calculation of Quenching Efficiency. Measurements were performed using a Leica TCS SP5 confocal microscope with laser illumination at 488 nm. The emission of Oregon green 488, peaking at 525 nm, was collected through a 10× objective (Leica, HC PL APO CS 0.40) over an emission band pass from 500 to 580 nm. Aqueous KCl solutions at different concentrations were slowly injected into the flow microchamber using a 1.0 mL syringe, alternating with injections of deionized water. Fluorescence images were taken 5 min after each injection. A quantitative comparison of the change in fluorescence emission was made by comparison with experiments performed in pure water. A quenching efficiency (Q) was calculated according to eq 1:

$$Q = \frac{I(\text{water}) - I(\text{salt})}{I(\text{water})} \times 100\% \qquad (1)$$

Intensities of the brushes were normalized with background intensities obtained from areas covered with inert thiol. All quenching data presented correspond to averages of five measurements.

Results and Discussion

Figure 2A illustrates the chemistry involved in the labeling of polymer brushes with dyes. The reaction occurs at random sites along the polymer chains. As the conformation of the PMEP changes in response to environmental stimuli such as ion concentration and pH,[7] the ensemble behavior of the dye emission is modulated because the immediate environment of the dyes is
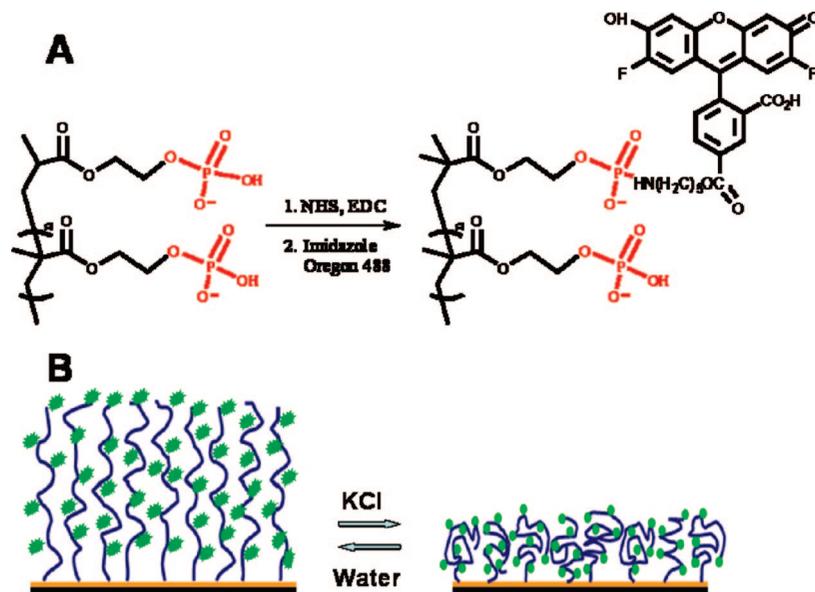
Figure 2. (A) Labeling of Oregon green 488 dye on PMEP brush. (B) Schematic representation of reversible conformational change in dye-labeled PMEP brushes.

changed. We have verified that the fluorescence behavior of free Oregon green 488 dye is independent of KCl concentration over the range brushes investigated (0.1−1.0 M), and therefore, any observed changes in the quenching efficiencies of polymer attached dyes report directly on the conformational changes of the PMEP brushes. The dye molecules located deep within the brushes and close to the gold substrate are most heavily quenched by nonradiative energy transfer to the substrate and the polymer scaffold. For molecules exposed to solvent (i.e., near the top of the brushes), conformational changes also affect the exposure of dyes to dissolved molecular quenchers such as oxygen.

In deionized water at pH 7, the brushes are monoprotonated and bear one negative charge on each phosphate group within the polymer chains. As a result, the polymers adopt an extended conformation, resulting in a "swelling" of the brushes due to interchain/intrachain electrostatic repulsion. In concentrated salt solutions, in contrast, the charges are screened by the salt ions and the repulsive forces vanish, resulting in the "collapse" of the brushes (Figure 2B). The swelling/collapse states of the brushes impact on the distance between the dyes within polymer chains and between different chains, as well as the average distance between polymer segments and the substrate. When two identical fluorophores are within close proximity, the fluorescence diminishes due to self-quenching.[17,18] As the distance increases, self-quenching is reduced with a corresponding increase in fluorescence emission levels. At close distance from the gold substrate, the fluorescence can be quenched by the gold. Each confocal fluorescence measurement took less than 10 s to perform. When a new solution is injected into the flow chamber, a 5-min interval was taken for the solution to reach equilibrium before a fluorescence image was taken.

Figure 3A,B shows the fluorescence images when the same polymer brushes are sequentially exposed to deionized water and 1.0 M KCl solution. The fluorescence intensity is drastically reduced upon the collapse of the brushes, corresponding to a quenching efficiency of $Q = 47\%$ for the 1.0 M KCl solution. Thus, the fluorescence is nearly halved upon exchange of water by the KCl solution, but it is fully recovered as water is readmitted
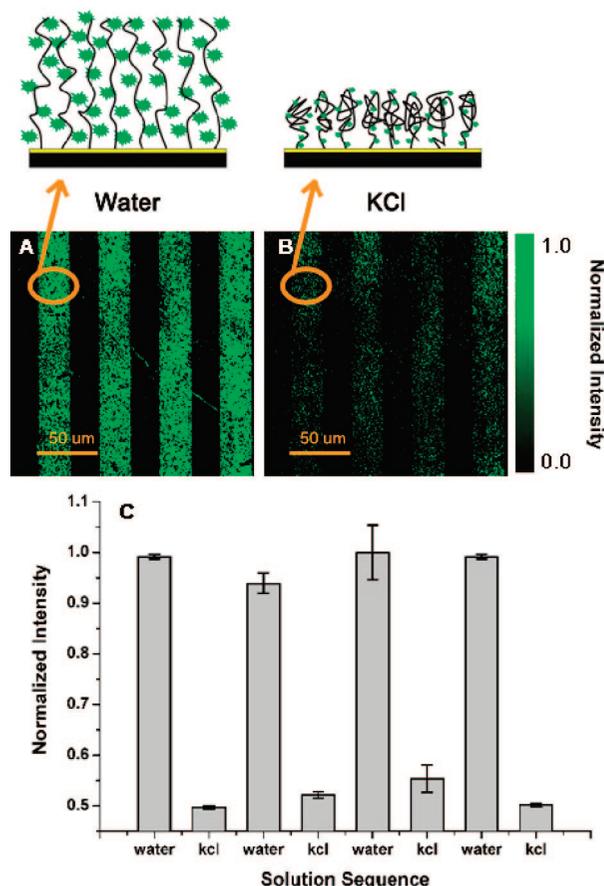
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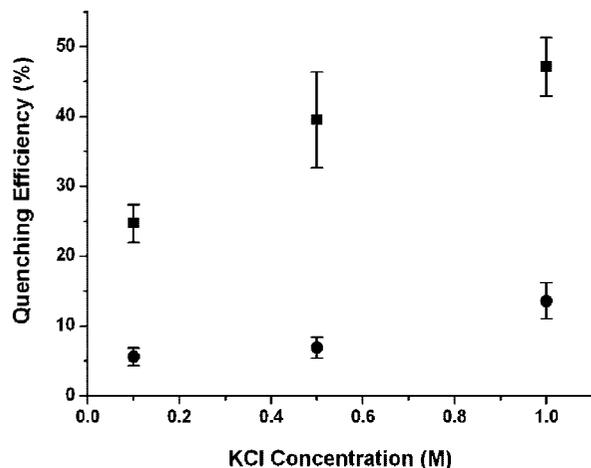(18) Song, X.; Swanson, B. I. *Anal. Chim. Acta* **2001**, *442*, 79–87.

Figure 3. Reversible conformational change of polymer brushes between water and 1.0 M KCl solution as observed by confocal fluorescence imaging. The images were taken at the same sample surface region in (A) water and (B) 1.0 M KCl solution using a confocal microscope. (C) Normalized fluorescence intensities from the polymer brush as the solution was cycled four times between H₂O and KCl solutions, respectively.

into the flow chamber (Figure 3C). This behavior is fully consistent with a picture where brushes switch between an extended, "upright" configuration and a fully collapsed configuration and is in support of previous observations on the reversible actuation

Figure 4. Variation in quenching efficiency of PMEP brushes on gold substrate (■) and on glass substrate (●) as a function of KCl concentration.

Figure 5. Variation of fluorophore quenching efficiency in polymer brushes as a function of grafting density in 0.1 M (■) and 1.0 M (○) KCl solutions.

of microcantilevers by the PMEP brushes, which we suggested was induced by the conformational changes.[16]

We investigated the dependence of the conformational dynamics of PMEP with salt concentration using brushes formed from initiators deposited at 20% grafting density (Figure 4). The quenching efficiency increased from 25% to 47% with KCl concentrations changing from 0.1 to 1.0 M, as the phosphate groups on the polymer brushes get more effectively screened by the salt ions. To establish the contribution of the gold surface to the overall quenching efficiency a control experiment was performed with dye-labeled brushes on glass substrates. One end of the polymer brushes was directly grafted onto glass; therefore, quenching by gold was excluded in this case. As shown in Figure 4, the quenching efficiency increases from 5.8% to 14% on variation of KCl concentration from 0.1 to 1.0 M, approximately 3.8 times less than when gold is present. This demonstrates the dominating effect of gold on the overall quenching behavior.

Figure 5 shows the effect of brush grafting density on quenching efficiency. The quenching efficiency increases from 22% to 44% as the grafting density is increased from 10% to 100% in the 0.1 M KCl solution. For the corresponding situation in the 1.0 M KCl solution, the quenching efficiency increased from 41% to 56%. Ellipsometry measurements (see supporting materials) revealed that the thickness of the polymer brushes increases with grafting density. At low densities (e.g., at 10%), the polymer chains are effectively isolated from one another and act independently to form random coil conformations or so-called "mushrooms" on the surface.[7] In this regime, the interchain repulsive interaction is insufficient for brushes to fully "stand up" in a neutral environment, which results in a certain degree of quenching of polymer dyes by the metal surface already before any salt solution is added. As a result, the effect of salt addition is relatively low at low grafting density. As the grafting density increases, interchain interactions play a dominant role on the
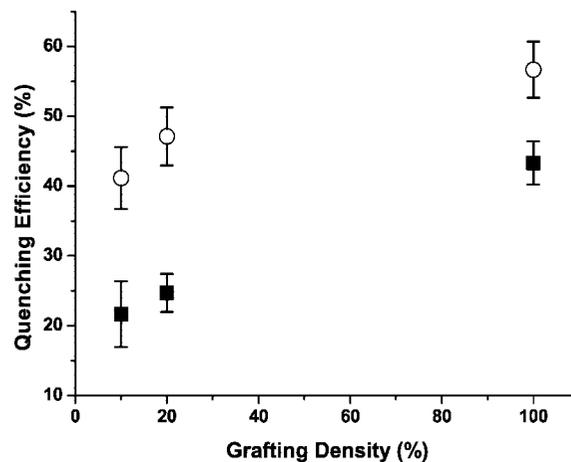
conformation the brushes adopt and the brushes fully swell in neutral solution and collapse only upon addition of salt. Therefore, the quenching efficiency increases at 20% and 100% coverage.

Conclusions

In summary, we have presented a method for in situ visualization of reversible conformational changes of fluorophore-labeled polyelectrolyte brushes grafted onto gold substrates via confocal fluorescence imaging. The brushes emit strong fluorescence at a swelled conformation. The addition of salt ions to the solution increases charge screening effects along the polymer backbone, leading to their partial collapse, which was observable via severe quenching of the observed fluorescence. The quenching efficiency Q was measured and found to correlate with the extent of conformational change in the polyelectrolyte brushes. Q was amplified several fold in the presence of gold and was found to be a function of grafting density. Confocal imaging of the polymer brushes in the designed flow chamber offers good temporal resolution and the fluorescence profile of the brushes could be studied in different solutions over many cycles.

Supporting Information Available: Variation in the dry thickness and the static contact angle of the brushes at various grafting densities (see S1), and AFM morphology of PMEP brush at various grafting densities (see S2). This information is available free of charge via the Internet at http://pubs.acs.org.

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