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Probing the recognition specificity of a protein molecularly imprinted polymer using force spectroscopy

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ABSTRACT

Molecularly imprinted polymers (MIPs) are synthetic receptors obtained by polymerization using molecular templates. We have synthesized MIP films (co-polymers of acrylamide and different acrylic acid-based cross-linkers) with specific binding sites for cytochrome c, which were imprinted in the bulk or in the surface. The binding specificity of the polymers was studied at the macroscale by equilibrium binding experiments with fluorescein-labeled cytochrome c. Imprinting factors of up to 4.1 were obtained that were a function of the cross-linker used and the degree of cross-linking. We have then employed, for the first time, AFM force spectroscopy to directly measure the force of interaction of the protein with the synthetic receptor sites obtained by molecular imprinting. The polymer surfaces were scanned with AFM cantilevers carrying covalently attached cytochrome c molecules, giving raise to specific binding events with binding forces between 85 and 95 pN. Control cantilevers without cytochrome c or with covalently attached bovine serum albumin, as well as non-imprinted control polymers, did not yield specific binding events. We believe that these results demonstrate the great potential of force spectroscopy for the characterization of molecularly imprinted polymers.

1. Introduction

The design and synthesis of biomimetic materials capable of binding a target molecule with similar affinity and specificity to antibodies has been a long-term goal of bioorganic chemistry. One approach to the generation of artificial macromolecular receptors is molecular imprinting of synthetic polymers (Arshady and Mosbach, 1981; Wulff and Sarhan, 1972; Zimmerman and Lemcoff, 2004). This is a process where binding monomers and cross-linking monomers are co-polymerized in the presence of a target molecule (the imprint molecule), which acts as a molecular template. The binding monomers initially form a complex with the imprint molecule, and following polymerization, their functional groups are held in position by the highly cross-linked polymeric network. Subsequent removal of the imprint molecule reveals binding sites complementary in size, shape, and chemical functionality. In that way, a molecular memory is introduced into the polymer that is now capable of selectively binding the target. Molecularly imprinted polymers (MIPs) have a higher physical and chemical stability than biomacromolecules, and can more easily be integrated in industrial fabrication processes. These properties make MIPs potentially very suitable as recognition elements for chemical sensors, biosensors or biochips (Haupt, 2003).

The existence of imprinted binding sites has so far been proven merely indirectly, for example, via the specific binding of target molecules to the polymer in equilibrium binding assays (Vlatakis et al., 1993; Yilmaz et al., 1999), using chemical sensors (Ayela et al., 2007), or by affinity chromatography (Fischer et al., 1991). In some cases, a more direct demonstration was possible, for example with larger imprint molecules like proteins when the imprinted sites could be imaged by atomic force microscopy (Shi et al., 1999).

Atomic force microscopy (AFM) is a versatile tool that has provided new avenues for microscopists to address the structure, properties and functions of biological specimen under physiological conditions and with unprecedented (sub)nanometer resolution. Indeed, AFM already permitted to study many biological systems: biomolecules (Jiang et al., 2007; Lohr et al., 2007), lipid films (El Kirat and Morandat, 2007; Johnston, 2007; Morandat and El Kirat, 2007a,b), 2D-protein crystals (Scheuring, 2006) and living cells (Gaboriaud and Dufrene, 2007; Verbelen et al., 2007).

Moreover, AFM-based force spectroscopy allows the direct measurement of forces with remarkable sensitivity and positional precision. In this mode, the cantilever deflection is recorded as the AFM tip is pushed towards the sample and retracted from it.

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The force–distance curves can then be analyzed to probe quantitatively the physical properties such as local elasticity, surface forces, surface charges and hydrophobicity. Furthermore, with AFM tips bearing active biomolecules, force spectroscopy allows to measure intermolecular and intramolecular interactions, thus providing new insights into the molecular bases of biological processes such as protein folding (Muller et al., 2006) and receptor–ligand interactions (Dupres et al., 2007; Hinterdorfer and Dufrene, 2006).

In this study, we use, for the first time, AFM force spectroscopy to provide a direct proof of the existence of molecularly imprinted sites. We measure the specific binding forces between cytochrome c (cyt c) and receptor sites created at the surface of polymer films by molecular imprinting. We first demonstrate that these sites are specific for cyt c using fluorescein-labeled cyt c and equilibrium binding assays. Then, we probe the specificity of the imprinted polymers at the nanometer scale with AFM tips bearing covalently attached cyt c.

2. Materials and methods

2.1. Reagents

Cytochrome c from horse heart, trypsin from porcine pancreas, avidin from egg white, albumin from bovine serum (BSA), (3-aminopropyl)triethoxysilane (APTES), 11-mercaptoundecanoic acid (MUA), 11-mercaptoundecane-1-ol (MUOH), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), ammonium persulfate (AP), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), acrylamide (AAm), *N*,*N*′-methylenebisacrylamide (MBA), *N*,*N*′-ethylenebis(acrylamide) (EBA), 1,4-bis(acryloyl) piperazine (PDA), poly(ethylene glycol)dimethacrylate (PEGDM), sodium dodecyl sulfate (SDS), propylamine, 1-hydroxybenzotriazole hydrate (HOBt), *N*,*N*′-diisopropylcarbodiimide (DIC), *N*,*N*-dimethylformamide (DMF), methacrylic anhydride and fluorescein 5-isothiocyanate (FITC) were from Sigma (St. Louis, MO, USA) and used without further purification. The photosensitive polymerization initiator 4,4′-azobis(4-cyanovaleric acid) (Vazo-68) was from DuPont Chemicals (Wilmington, DE, USA).

2.2. Activation of glass substrates

Freshly cleaned glass substrates were incubated for 30 min in a 0.1 vol.% propylamine solution in toluene. The surfaces were then rinsed with toluene and incubated overnight in a 1 vol.% APTES solution in toluene. After silanization, the surfaces were rinsed with toluene followed by acetone and dried under a stream of nitrogen. The polymerization initiator Vazo-68 was grafted on the surface by immersing the glass substrates in a solution containing 1 mmol of Vazo-68, 1 mmol of HOBt and 1 mmol of DIC in 45 mL DMF for 20 h in the dark. Unreacted aminopropyl groups were converted into amides by incubating the glass surfaces in a solution of DMF/methacrylic anhydride 5:1 (v/v). Finally, the activated glass was rinsed with DMF and ethanol and dried under a stream of nitrogen.

2.3. Preparation of MIPs and non-imprinted control polymers

As a typical example, a MIP cross-linked with 3.3% MBA was prepared as follows. AAm (120 mg), MBA (8.7 mg), AP (1 mg), TEMED (0.005 vol.%) were solubilized in ultrapure water to a final volume of 1 mL. To this mixture was added 400 μL Tris-buffered saline (TBS) pH 7.2 for the 2D-MIP, or 400 μL of TBS pH 7.2 containing cyt c at 1.25 mg/mL for the 3D-MIP. The MIP precursor solution was deposited on a mica surface with grafted cyt c (2D-MIP) or on bare mica (3D-MIP) and covered with an activated glass slide (1 cm × 1 cm). Chemical grafting of cyt c to mica for the preparation of 2D-MIPs was done using the bifunctional coupling agent bis(sulfosuccinimidylsuberate) (BS^3). Briefly, the mica was first silanized in the gas phase with APTES by placing the mica substrates together with a small vial containing 100 μL APTES in a 7 dm^3 closed recipient under vacuum for 30 min at RT, followed by curing for 1 h at 100 °C. Then, the substrates were incubated for 30 min in a 10 mM solution of BS^3 in ultrapure water. After rinsing with water, the surface was incubated in a 1 mg/mL solution of cyt c in ultrapure water for 30 min at room temperature. The surface was then rinsed with water and unreacted active esters were neutralized by incubation in TBS.

The polymerization was performed by heating at 35 °C for 90 min. The mica was separated from the polymers attached to the glass slides by incubation for 3 h in TBS pH 9.2. Remaining protein was eliminated from the polymers by digestion with trypsin (20 μg/mL in TBS pH 8.0) for 1 h, followed by incubation for 1 h in a solution of 10 wt.% SDS in water. Finally, the polymers were rinsed three times with ultrapure water and stored at 4 °C. Template removal was verified by staining MIP and NIP films with Coomassie Blue, and recording the UV–vis absorption spectra of the gels. No difference in the spectra was observed between MIP and NIP. Non-imprinted control polymers were prepared in the same way as the 3D-MIP but without the addition of cyt c to the precursor solution.

2.4. Preparation of fluorescein-labeled cyt c (fluo-cyt c)

10 μL of FITC solution (10 mg/mL in DMSO) were added to 1 mL of cyt c (1 mg/mL in 0.1 M sodium bicarbonate buffer pH 9.0) and incubated at 37 °C for 1 h. The fluorescein-labeled cyt c was separated from unconjugated probe by gel filtration on a Sephadex™ G-25 column (PD-10, GE Healthcare Europe GmbH, Orsay, France) equilibrated with TBS pH 7.2. The fractions containing fluo-cyt c were collected and stored at −20 °C. To determine the fluorescein/cyt c ratio, the cyt c concentration was measured with the Bradford method (Bradford, 1976) and the bound-fluorescein via its absorption at 494 nm, which yielded 2.8 probes/protein molecule.

2.5. Binding assays with fluo-cyt c

For binding experiments, fluo-cyt c was incubated at 500 ng/mL with the MIPs and the corresponding non-imprinted control polymers in TBS pH 7.2 for 3 h at 37 °C. The surfaces were then rinsed three times with TBS pH 7.2. The adsorbed fluo-cyt c was digested by treatment with trypsin (20 μg/mL final concentration in TBS pH 8.0) for 2 h at 37 °C. The fluorescence released into the supernatant was then measured (Cary Eclipse Fluorescence Spectrophotometer, Varian Inc., Palo Alto, CA, USA) with the excitation and emission wavelengths set at 485 and 535 nm, respectively. The amount of fluo-cyt c desorbed from the surfaces was determined by comparison with a standard curve obtained by trypsic digestion of fluo-cyt c solutions ranging from 25 to 1000 ng/mL.

2.6. Atomic force microscopy

2.6.1. Topography analysis

All surfaces were investigated using a Nanoscope III Multimode AFM (Veeco Metrology LLC, Santa Barbara, CA, USA) equipped with a 125 μm × 125 μm × 5 μm scanner (J-scanner). A quartz fluid cell was used without the O-ring. Topographic images were recorded in contact mode using oxide sharpened microfabricated Si_3N_4 cantilevers (Microlevers, Veeco Metrology LLC, Santa Barbara, CA, USA), with a minimal applied force (<500 pN) and at a scan rate of 5–6 Hz. The spring constants of the cantilevers were measured using the thermal noise method (Picoforce; Veeco Metrology Group), yielding a mean value of 0.0106 ± 0.001 N/m. The curvature radius of