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Covalent Attachment of Bacteriorhodopsin Monolayer to Bromo-terminated Solid Supports: Preparation, Characterization, and Protein Stability

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Abstract: The interfacing of functional proteins with solid supports and the study of related protein-adsorption behavior are promising and important for potential device applications. In this study, we describe the preparation of bacteriorhodopsin (bR) monolayers on Br-terminated solid supports through covalent attachment. The bonding, by chemical reaction of the exposed free amine groups of bR with the pendant Br group of the chemically modified solid surface, was confirmed both by negative AFM results obtained when acetylated bR (instead of native bR) was used as a control and by weak bands observed at around 1610 cm^{-1} in the FTIR spectrum. The coverage of the resultant bR monolayer was significantly increased by changing the pH of the purple-membrane suspension from 9.2 to 6.8. Although bR, which is an exceptionally stable protein, showed a pronounced loss of its photoactivity in these bR monolayers, it retained full photoactivity after covalent binding to Br-terminated alkyls in solution. Several characterization methods, including atomic force microscopy (AFM), contact potential difference (CPD) measurements, and UV/Vis and Fourier transform infrared (FTIR) spectroscopy, verified that these bR monolayers behaved significantly different from native bR. Current–voltage (*I–V*) measurements (and optical absorption spectroscopy) suggest that the retinal chromophore is probably still present in the protein, whereas the UV/Vis spectrum suggests that it lacks the characteristic covalent protonated Schiff base linkage. This finding sheds light on the unique interactions of biomolecules with solid surfaces and may be significant for the design of protein-containing device structures.

Keywords: adsorption · monolayers · photoactivity · retinal proteins · Schiff bases

Introduction

The interfacing of functional proteins with solid supports is a promising application for the development of devices for bioelectronics, biosensors, and biooptics.[1] Bacteriorhodopsin (bR) is a transmembrane retinal protein that serves as a photochemical proton pump. It is found in the cell membrane of *Halobacterium salinarum*, where it forms natural two-dimensional crystalline arrays in the purple membrane (PM).[2] The purple membrane is usually called the bacteriorhodopsin membrane, or the bR membrane in short. Herein we shall refer to the bR membrane as bR. Long-term stability against thermal, chemical, and photochemical degradation, together with desirable photoelectric and photochromic properties, has made bR a promising candidate for biological device applications. Recently, a few studies were reported that aimed at integrating a bR monolayer into semiconductor nanobioelectronic devices[3] and current-carrying junction devices.[4]

To achieve the aim of device application, the preparation of high-quality PM monolayers on solid supports is of crucial importance. Attempts so far at immobilizing and processing PM fragments onto solid supports were mostly based on physical/chemical (noncovalent) attachment, including the conventional Langmuir–Blodgett (LB) method,[5] electrical sedimentation,[6] chemisorption,[7] and layer-by-layer electrostatic deposition.[8–10] Physical charac-

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terization and photoelectric measurements have indicated that PM films formed by many of the above methods are oriented to some degree, that is, the orientation of the sheet-like PM fragments, in terms of which of its sides faces the support, is nonrandom.[8–9] Additionally, a few attempts toward the preparation of oriented PM monolayers through covalent attachment by, for example, immunochemical molecular-recognition methods[11–12] or the Au–S bonding of genetic bR mutants,[13] have been described in the literature. For fundamental studies, simpler techniques for the efficient preparation of oriented PM films (especially monolayers) are needed.

The immobilization of proteins such as antibodies or enzymes and the conservation of their activity may yield functional surfaces suitable for use in many fields, including biotechnology and materials science. However, some proteins may undergo (partial) denaturation upon immobilization onto solid supports. Whereas the protein–surface interactions that govern the behavior of immobilized protein molecules are not well-established, surface chemistry has been shown to play a fundamental role in protein adsorption and conformational changes for many types of proteins.[14–15] As for bR, although it is an exceptionally stable protein, and our physical characterization as well as photoelectric measurements have indicated that dry bR monolayers formed by electrostatic interaction retain their photoactivity,[4,8–9] relatively little is known about the stability/activity of bR after it has bound covalently to solid surfaces. The structural stability of bR depends on many factors,[16] including intramolecular and intermolecular interactions, protein–lipid interactions, protein–retinal interactions, and even protein–surface interactions, upon immobilization on solid surfaces.

Recent work by our group led to the preparation and characterization of high-quality bR monolayers suitable for solid-state devices and current transport.[4,8–9] In the present study, we report a new way of preparing PM monolayers on bromo (Br)-terminated solid supports through covalent binding. Bonding occurs upon chemical reaction of the exposed free amine groups of bR with the pendant Br groups of the chemically modified solid surface. The involvement of an amine group in the binding process is supported by experiments with modified bR, the amine groups of which were acetylated to prevent covalent protein–surface bonding. Unexpectedly, these bR monolayers lost (most of) their photoactivity. To our knowledge, this is the first report that clearly shows that bR, which is considered to be an exceptionally stable protein, can lose its photoactivity upon chemical immobilization on a solid support. Several characterization methods—atomic force microscopy (AFM), contact potential difference (CPD) measurements, and UV/Vis and Fourier transform infrared (FTIR) spectroscopy—indicate conformational alterations of the bR in the monolayer upon reaction with the brominated surface. We suggest that these changes led to the observed changes in optical absorption and loss of photoactivity. Interestingly, current–voltage (*I*–*V*) measurements indicate that the retinal chromophore probably still occupies the binding site, but lacks the characteristic protonated Schiff base linkage. This finding is significant for the further understanding of protein–surface interactions and their remarkable effect on bioactivity, an issue that has considerable relevance for potential device applications of proteins, especially those that require integration with existing or future (opto)electronics.

Abstract in Chinese:

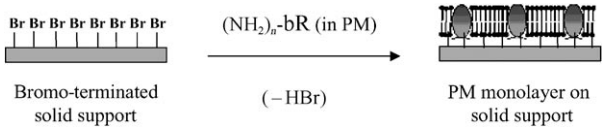
[中文摘要]:

细菌视紫红质(bR)单层膜可以通过蛋白质表面的氨基与固体表面修饰的溴(Br)基团之间的化学反应而定向固定在固体表面。然而，原子力显微镜，接触电势差，紫外可见吸收光谱，傅立叶红外光谱，以及电流-电压曲线表征共同证明bR-Br相互作用使得细菌视紫红质蛋白质分子严重失去光反应活性。

Results and Discussion

Binding and Orientation

Bromo (Br)-terminated solid supports were chosen for the present studies because they provide groups that can react with exposed protein amine groups. The bR protein has six lysine residues located close to the protein surface, five of which are on the cytoplasmic side. We used primarily a short-chain Br-propyl trichlorosilane (C3-Br) molecule to prepare self-assembled monolayers (SAMs) on solid supports, which are expected to bind the bR protein covalently (Scheme 1). Figure 1a shows a representative atomic force microscopy (AFM) image of the resultant bR monolayer, prepared with 20 mM Tris buffer (pH ≈ 9.2) on the Br-terminated silicon surface. The roughly circular features, 0.5–1 μm in diameter and 5 nm thick, are the PM fragments. Typical surface coverage was 20–30%, similar to what was reported for thiolated PMs on Au.[13] The coverage did not change after further thorough washing with 1 M aqueous NaCl, which would have washed off the physically adsorbed proteins (e.g., proteins strongly attached to the surface by electrostatic interaction). This observation strongly supports the idea that the attached bR fragments are chemically bound.



Scheme 1. Schematic representation of covalent attachment of a bR monolayer on a bromo-terminated solid support by reaction of the exposed free amine groups of bR with the pendant Br group of the monolayers.

To confirm the formation of a protein–surface covalent bond, we washed the samples with ethanol. Figure 1b shows a representative AFM image of the structured nanoporous bR patches after washing with ethanol, a treatment that would have removed physically adsorbed bR completely. A relatively clean AFM picture was obtained, which we inter-

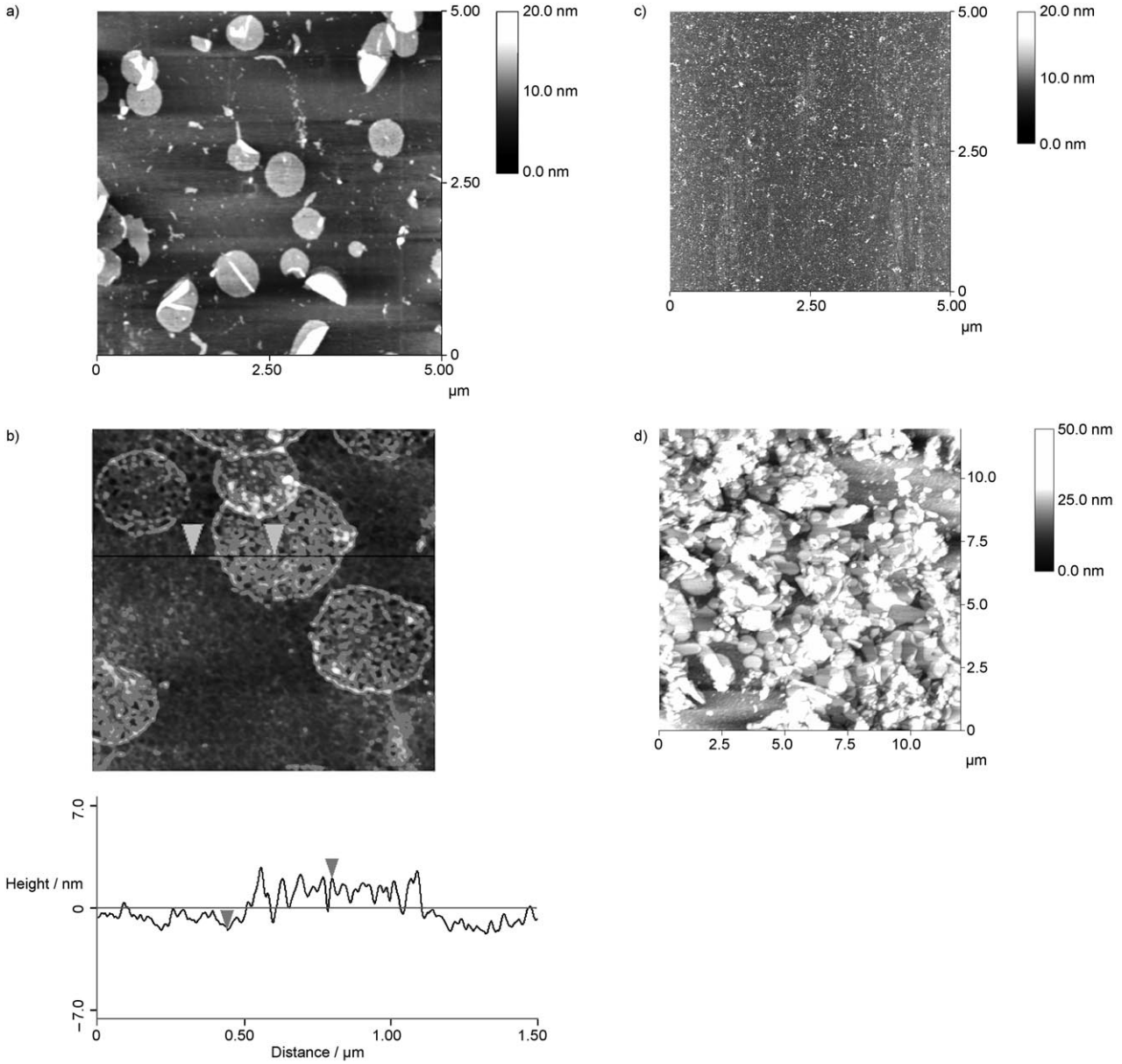


Figure 1. a) Typical AFM topographical image of a PM monolayer on the C3-Br- terminated silicon surface, prepared from a pH 9.2 suspension of PM fragments. b) Typical AFM image and line scan of a PM monolayer on the C3-Br-terminated Si surface, prepared from a pH 9.2 suspension of PM fragments, after thorough rinsing with pH 9.2 Tris buffer, 1 M aqueous NaCl, and ethanol. The line scan shows that the average height of the features is about 3.5 nm (between the two markers). c) Typical AFM image of the C3-Br-terminated Si surface after deposition of an acetylated PM and thorough rinsing with pH 9.2 Tris buffer and 1 M aqueous NaCl. d) Typical AFM image of PM multilayers on the C16-Br-terminated Si surface, prepared from a suspension of pH 9.2 PM fragments, after thorough rinsing with pH 9.2 Tris buffer and 1 M aqueous NaCl. The result was similar when the sample was prepared from a pH 6.8 PM suspension.

pret as arising only from (presumably covalently) attached bR and some captured embedded lipids (light dots in the figure) that remained bound. The nanopores inside the PM patches resulted from the removal of physically adsorbed bR fragments. It is known that ethanol treatment of bR films causes bR denaturation, leading to a net angle between the seven helical segments of the bR polypeptide and the normal to the membrane plane of around 54.7°, as compared to 0° for a native bR film.[17] The thickness that we measured for our ethanol-treated monolayer by section analysis of the AFM picture, 3–4 nm (compare section analysis of AFM in Figure 1b), agrees well with this angle.

In principle, FTIR spectroscopy should be able to provide direct experimental evidence of chemical bonding upon immobilization of bR monolayers onto a Br-terminated surface. However, in practice, the signal is too small to be discerned from the much stronger signal that originates from the bulk of the protein polypeptide chain. Strong experi-

mental support for covalent bonding between the surface Br group and bR amine residues was obtained by carrying out a control experiment with acetylated instead of native bR. Lysine residues with amine groups that are exposed on the bR surface can be protected by acetylation.[18] In such acetylated bR samples, there would be no free amine groups available at the protein surface; therefore, there should not be covalent bonds formed with the Br surface. Indeed, we found that such samples could be washed off almost completely from the Br-terminated Si surface with pH 9 Tris buffer or 1 M aqueous NaCl (Figure 1c) after overnight incubation.

As five of the six free lysine residues are located on the cytoplasmic (CP) side, with the remaining one on the extracellular (EC) side,[9,19] it is possible that these resultant bR monolayers, prepared on Br-terminated surfaces, are at least partially oriented, because the CP side of bR is the one that is more liable to react with the Br-terminated support surface. This possibility is supported by our AFM observations. Previously, we found that a monolayer of PM fragments (0.5–1 μm diameter, in a suspension of pH≈9) with approximately 70% coverage can be prepared by electrostatic interaction.[8–9] Higher yields were not obtained probably due to steric hindrance and interfragmental electrostatic repulsion. In the present case of non-electrostatic immobilization, it is reasonable to assume that about half of the bR fragments settle from the aqueous suspension onto the surface with their cytoplasmic side towards the surface, and about half do the opposite, that is, settle with the extracellular face towards the substrate surface. Furthermore, we can assume that a major fraction of the extracellularly oriented fragments would not bind to the surface (i.e., this fraction would be washed off), as most of the lysine residues are located on the cytoplasmic side. Therefore, the surface-coverage yield would be slightly over 25–30% (≈50% of ≈70%), which agrees with the AFM observations. A similar AFM observation was reported (and a similar explanation was given) for Au–S bound bR mutants.[13] Although complete (100%) orientation is difficult to achieve, and we cannot provide direct microscopic evidence for the degree of orientation, macroscopic contact potential difference (CPD) measurements (see below) confirm that these monolayers have an overall net orientation.

In contrast to the above results, preparations of monolayers of bR on Br-terminated solid supports, modified with much longer molecules such as 1-bromo-16-trichlorosilyl (hexadecane) (C16-Br), gave much poorer coverage at both pH 9.2 and 6.8, as can be seen from the AFM images (Figure 1d). In this case, there was a tendency for PM multilayers and overlayers to form. A possible explanation is that the pendent Br group may react with additional reactive groups within the protein. After washing of these samples with ethanol to remove bR that was not bound covalently, the surface coverage of the resultant monolayer decreased significantly (to <20%). This is in contrast to the case with the shorter chains (C3-Br) at pH 6.8, as described below. Therefore, we suggest that the observed PM multilayers and overlayers formed because they were caught between/on surface-bound PM patches upon sample drying.

Surface Coverage

The coverage of bR monolayers is important not only for maximizing protein signals at the monolayer level, but also for more practical applications, such as protein-based sensors and, as in the present case, the ability to prepare structures that are suitable for current-transport measurements. We found that the coverage of bR monolayers increased significantly from 25–30 to 80–90% by lowering the pH of the incubated PM suspension to around 6.8 (with deionized water) instead of using a suspension of pH≈9.2 with 20 mM Tris buffer. Figure 2 shows a representative AFM image of a much denser, continuous bR monolayer on the C3-Br-terminated Si surface. Line scan analysis indicates that the thickness of the membrane is 5–6 nm, which is characteristic of monolayer formation. Because of the improved coverage, we used these monolayers for further characterization in this study, unless otherwise stated.

This result is chemically counterintuitive because the amine group is expected to react more efficiently with the Br group at pH 9.2 than at pH 6.8, because at pH 6.8, the amine group would be mainly protonated, which would prevent nucleophilic attack on the alkyl bromide.[20] However, we can explain this behavior tentatively by realizing that coulombic interactions between molecules, and between molecules and the surface, compete with the desired covalent-binding process. At high pH (9.2), if the negative charge density of the PM is large enough, the electrostatic interaction becomes the dominant driving force. This phenomenon, along with steric hindrance due to the lateral size of the PM fragments (0.5–1 μm) at this pH value, makes it difficult to obtain a good PM monolayer with a surface coverage higher than around 70% by electrostatic deposition.[8–9] In a neutral medium (pH≈6.8), the charge density of the bR is too low for efficient electrostatic coverage, which is driven by the coulombic attraction between the fragments and the oppositely charged supporting surface. Thus, for the same reason that neutral pH is not so effective for electrostatic deposition,[10] it may still be good enough for chemical bonding, because there is less competition from electrostatic deposition and less repulsion between PM fragments than in a more basic medium. Furthermore, the PM fragments in a neutral medium are smaller (as shown in the AFM images in Figure 2), thus decreasing the steric-hindrance effect and further improving PM monolayer formation. Also, one should take into account that the pK_a of the amine group can be modified significantly if the group is near the solid support in the vicinity of the Br group, which may thus allow reaction even at pH 6.8.

Photoactivity of Surface-Bound bR

BR, in spite of its high stability, is known to denature (to some extent) if spread over an air/water interface.[17] Al-

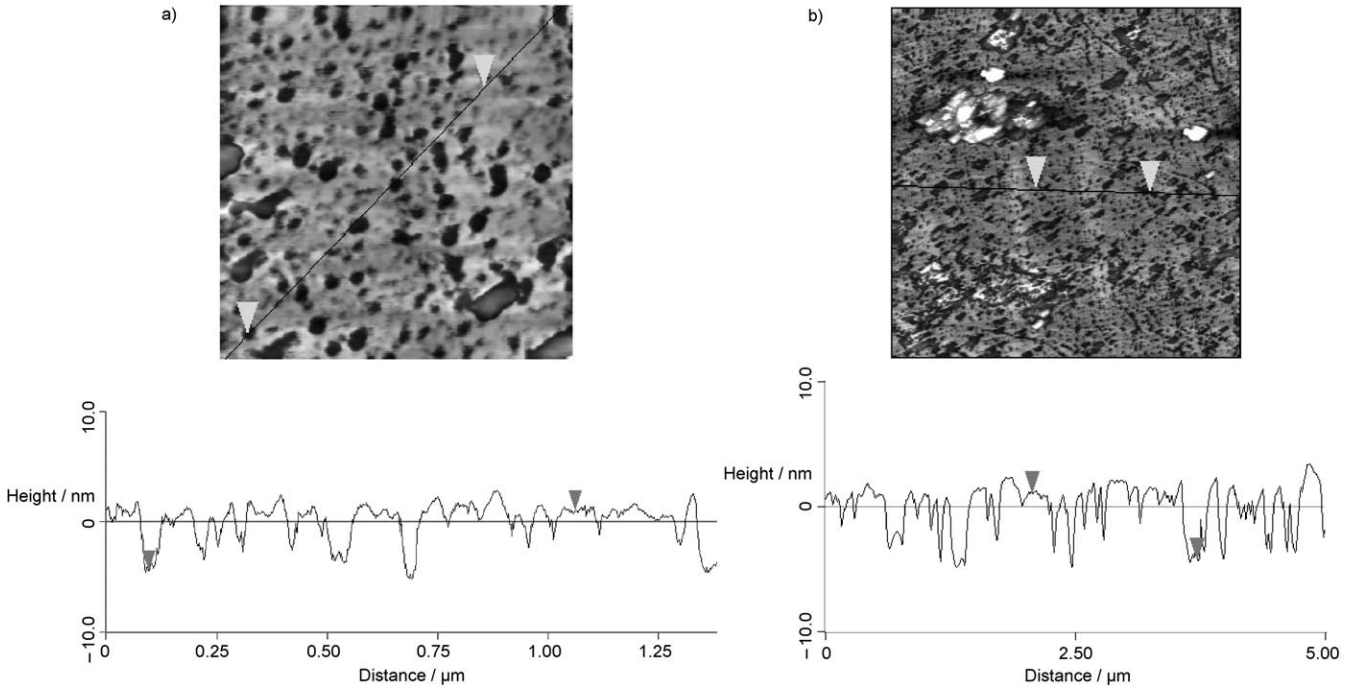


Figure 2. Typical AFM images and line scans of a PM monolayer on the C3-Br-terminated Si surface, prepared from a suspension of pH 6.8 PM fragments, after thorough rinsing with pH 9.2 Tris buffer. a) Scan range = 1 μm. b) Scan range = 5 μm.

though it was previously confirmed that a dry bR monolayer, formed by electrostatic interaction, retains its photoactivity,[4,8–9] the stability/activity of bR, if covalently bound to Br-terminated solid surfaces, is not known. We used several characterization methods, including AFM, CPD measurements, and UV/Vis and FTIR spectroscopy, and verified that these bR monolayers have different absorption maxima to native bR and that they have lost their protein photoactivity.

CPD Measurements

CPD measurements, in the dark and under illumination, were used to evaluate the photoresponse of the as-prepared oriented bR monolayers, because we found that this method can probe the net orientation of bR in solid-state monolayer preparations.[8–9] The changes in CPD between an Au reference and a sample surface, prepared on a degenerate n-Si wafer, before and after illumination are shown in Figure 3a. After protein attachment, the CPD values in the dark for Si/C3-Br and Si/C3-BR monolayer samples were measured to be (-0.35 ± 0.05) and (-0.50 ± 0.10) V, respectively, with the variation due to the coverage. These results show that bR attachment increases the work function, a finding that is consistent with the PM layer adding an additional negative surface dipole (dipole with its negative pole pointing towards and its positive pole pointing away from the surface). This fits with what is expected if the cytoplasmic side faces the substrate.[8,21] However, illumination with a green light ($28\ mW\,cm^{-2}$) caused no detectable change in the sample surface photovoltage (SPV; the change in CPD upon illumi-

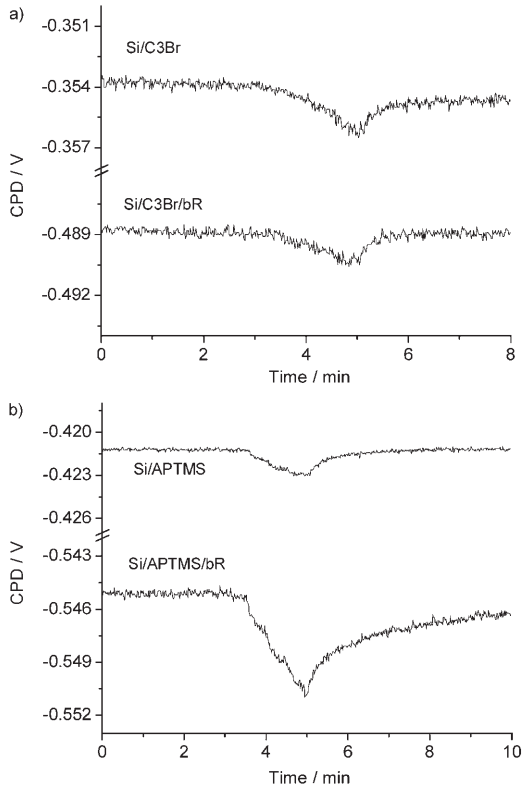


Figure 3. Surface photovoltage (SPV) responses of monolayers of PM and control silane on a) C3-Br-terminated and b) APTMS-modified degenerate Si/SiO_2 substrate, after illumination (after 3 min in the dark) with green light for 2 min. The light intensity was increased gradually from 1 to $28\ mW\,cm^{-2}$ between 3 (3.5 min for b)) and 5 min.

nation), compared with the response of a control Br-terminated surface without bR (there would be a small SPV due to the Si substrate in both cases). In contrast, we previously found that the response magnitude of the SPV of a native bR monolayer, prepared by electrostatic deposition, increased gradually and saturated at around 6 mV upon continuous illumination with green light (28 mW cm^{-2}).[9]

As the bR monolayers have a net orientation with the cytoplasmic side facing the substrate, on the basis of the CPD values in the dark, the lack of CPD photoresponse can be ascribed to the effects of covalent binding and/or the substrate on the photoactivity of bR. To clarify this possibility, PM fragment monolayers were prepared by electrostatic deposition onto (3-aminopropyl)trimethoxysilane (APTMS) monolayer-modified[22] degenerate n-Si substrates, and CPD measurements were performed on these samples. As shown in Figure 3b, upon illumination with green light (28 mW cm^{-2}), the SPV increased gradually with light intensity and saturated at 4–5 mV (28 mW cm^{-2}; after subtraction of the control response). This effect is similar to that of PM monolayers prepared by electrostatic deposition on Al/AlO_x substrates.[9] Therefore, we conclude that a bR monolayer, prepared by electrostatic deposition on Si, retains its photoactivity, and the substrate effect on bR photoactivity can be ruled out.

UV/Vis Spectroscopy

Our earlier work also showed that the UV/Vis spectra confirm that bR absorption and photoactivity are maintained in monolayers prepared by electrostatic deposition.[8] We summarize those results as follows: upon green light (>495 nm) illumination, the characteristic absorption of ground-state bR, with a maximum at around 560 nm, disappears, and a maximum at around 420 nm appears, thus indicating the formation of a photochemically induced intermediate. The system decays thermally to the original ground state over a few minutes, as shown by the reappearance of the 560-nm band and the disappearance of the 420-nm band.[8]

Figure 4 shows a representative UV/Vis spectrum of a sample with two bR monolayers, one on each side of a C3-Br-terminated quartz substrate (80–90% monolayer coverage on each side). The characteristic absorption of native bR at around 560 nm was not observed, and the spectrum did not change regardless of whether it was recorded in the dark or upon illumination with green light. However, even though the spectrum is noisy, a feature can be seen at short wavelengths, including the 280-nm region, which originates from the protein polypeptide backbone and is suggestive of the presence of bR. After smoothing (Figure 4, inset), a band centered around 380 nm was noted. This band can be assigned to the formation of free retinal,[23] although its assignment to the retinal Schiff base cannot be completely excluded. These spectra, especially if compared with the spectrum of a noncovalently attached bR monolayer with similar OD (optical density),[8] are consistent with the idea that the bR experienced a major conformational change or even denaturation.[24]

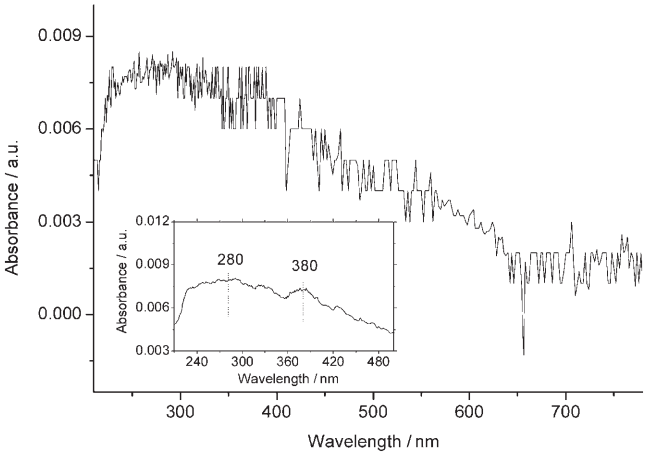


Figure 4. Raw UV/Vis spectrum of two PM monolayers on both sides of the C3-Br-terminated quartz. Inset: spectrum after data smoothing. No detectable change was observed upon illumination with green light or in the dark.

To shed further light on the cause for such conformational change after the reaction with the pendant Br group of the chemically modified solid surface, we treated bR with alkyl bromide in solution. This reaction did not affect the bR absorption maximum (568 nm in the light-adapted form). Thus, we conclude that the reaction with the Br group itself did not affect the protein conformation and stability; rather, it is the covalent attachment to the solid support that affects the protein conformation and stability.

AFM Measurements

AFM section analysis was performed to check for a possible thickness difference that might be induced by bR conformational changes or partial denaturation. PM fragment monolayers with low coverage on C3-Br-terminated Si were selected for characterization because the remaining bare areas of the substrate can provide clear contrast. As a control, electrostatically deposited PM fragment monolayers on APTMS (C3-NH_2)-modified Si, at low coverage, were checked as well. Both types of samples were cleaned by rinsing with pH 9.2 Tris buffer and deionized water (without rinsing with ethanol). AFM section analysis (Figure 5) shows that the typical thickness of single PM patches is around (5.0 ± 0.5) and (5.5 ± 0.5) nm for electrostatically deposited (photoactive) and covalently bound (photoinactive) samples, respectively. Although the roughly 0.5-nm increase in thickness of the PM patches is within the experimental error, it may indicate that the protein conformation was altered, which would fit the idea of some kind of conformational change in bR due to covalent bR binding to the Br-terminated surface.

FTIR Spectroscopy

FTIR spectroscopy was previously used to characterize the thermal stability and conformational changes of bR.[25–26] Here, we used attenuated total reflectance (ATR) FTIR spectroscopy to analyze the adsorbed protein conformation.

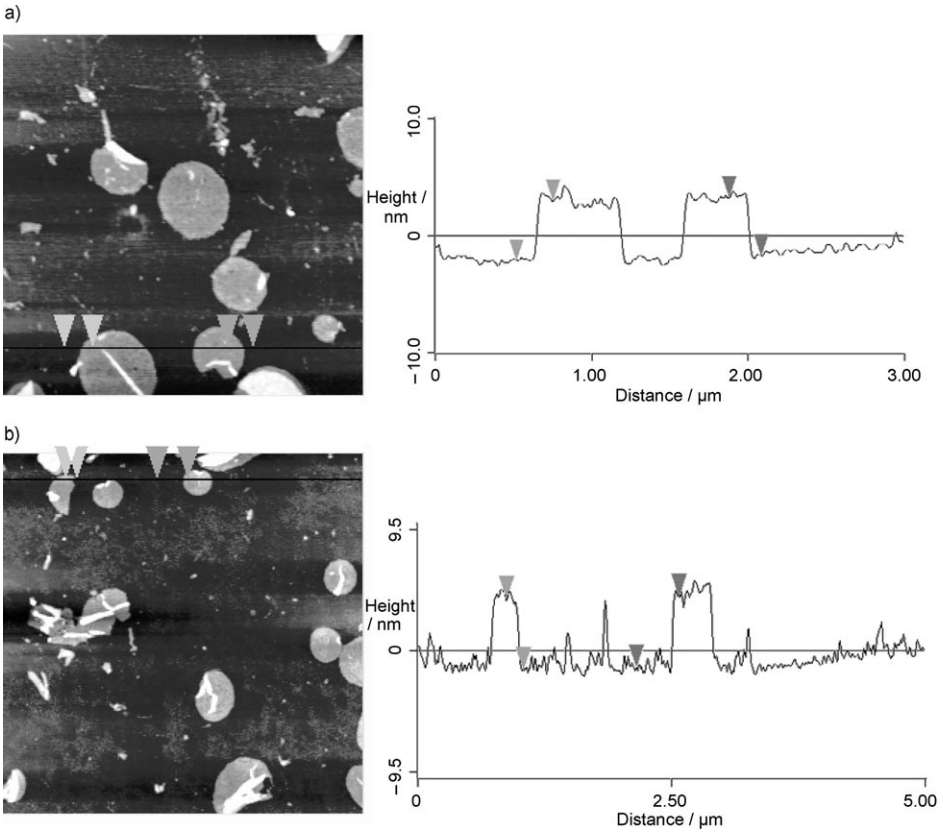


Figure 5. Typical AFM images and line scans of PM patches deposited on a) the APTMS (C3-NH_2)-terminated and b) the C3-Br-terminated silicon surface.

The protein IR spectra show peaks arising mainly from amide I/II bond vibrations. The amide I band (centered at 1700–1600 cm^{-1}) and the amide II band (centered at 1500–1600 cm^{-1}) are known to be sensitive to the conformation adopted by the protein backbone and can, thus, be very useful for determining the protein secondary structure.[14,27] Previous FTIR spectroscopic results of electrostatically deposited (photoactive) bR monolayers on Si showed the amide I (1659 cm^{-1}) and amide II (1546 cm^{-1}) signatures of bR,[8] which indicates that the PM monolayers comprise a large fraction of the α-helix structure, and that the secondary structure of bR is preserved at the interface. A similar FTIR spectrum was reported for monolayers prepared by the Langmuir–Blodgett method.[28]

Upon covalent attachment of bR monolayers onto a Br-terminated Si surface, the amide I and II bands of the protein were centered at around 1650 and 1538 cm^{-1}, respectively (Figure 6). The frequencies of the amide I and II bands underwent a blue shift by approximately 9 and 8 cm^{-1}, respectively, relative to those of the PM monolayers, prepared by electrostatic deposition. The observed blue shift of the amide I and II bands indicates conformational changes of the protein. Previous studies assigned the (amide I) bands with maxima at 1655–1650 cm^{-1} to α-helices, those at 1648–1644 cm^{-1} to random chains, the features at 1639–1635 cm^{-1} to extended chains, and those at 1632–1621 cm^{-1} to extended chains or β-sheets.[14] These component bands largely overlap and contribute to the characteristic broad amide I band observed in our IR spectrum (Figure 6). Furthermore, shoulders at 1633 and 1688 cm^{-1} were observed on both sides of the amide I band. These weak features were reported to arise from a small fraction of β-sheet or β-turn structure (1633 and 1688 cm^{-1}) and from the C=N vibration of the retinal Schiff base (1633 cm^{-1}).[28] Bands observed at lower wavenumbers ($\approx$1610 cm^{-1}) are often considered to arise from intermolecular bonding.[14,29] These spectra, therefore, provide further evidence for conformational alterations of bR upon binding to the Br-modified substrate.

Current–Voltage (I–V) Measurements

Previously, we found[4] that current transport through a bR monolayer is affected considerably by the presence of the retinal chromophore. Only if retinal or an analogue was present in the protein could we measure current (a few nA with a nominal contact area of $\approx$0.002 cm^2), and only noise (pA level) was measured if retinal was not present in the protein. Therefore, *I–V* measurements provide an indication for the presence of retinal (or an analogue) in the protein.

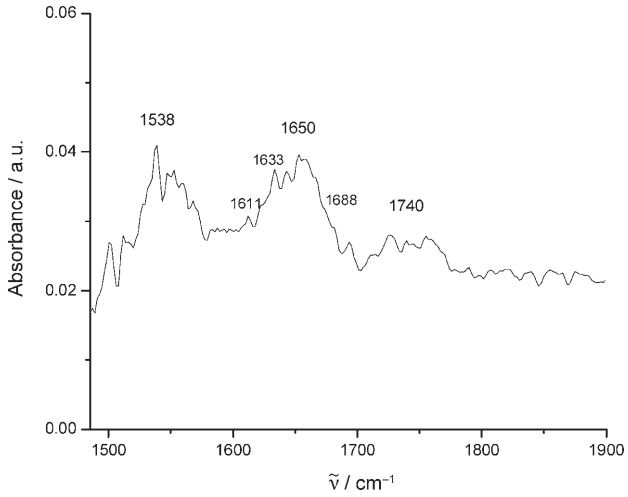


Figure 6. FTIR spectrum of a PM monolayer on the bromo-terminated silicon surface.

I–V measurements were carried out with the high-coverage samples (80–90%; Figure 2) sandwiched between the C3-Br-terminated substrate and an Au top electrode. On each sample, several 0.5-mm diameter Au pads were deposited by the soft "LOFO" (lift-off float-on) technique.[30] Figure 7a shows a typical *I–V* curve for the control C3-Br monolayer measured at 293 K. A current of around −3 μA at −1.0 V applied bias was observed, which is similar to the current flowing through a monolayer of molecules with similar chain length, 3-mercaptopropyltrimethoxysilane (MPTMS).[31] The *I–V* curves are asymmetric, which is probably due to the asymmetric contacts with the molecules.

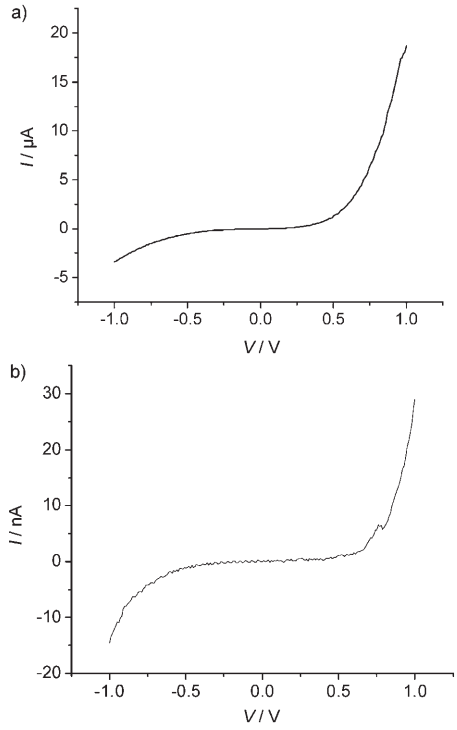


Figure 7. Typical room-temperature *I–V* curves of a) a degenerate Si/SiO_2 (≈2 nm)/C3-Br monolayer/Au planar junction and b) a degenerate Si/SiO_2 (≈2 nm)/C3-Br monolayer/PM monolayer/Au planar junction.

Typical currents through the covalently bound PM monolayer were 10–30 nA at −1.0 V, and the current asymmetry with respect to the voltage became less pronounced than that obtained without the bR monolayers (Figure 7b). The junctions were less stable with respect to successive potential cycling than the previously reported bR monolayer junctions[4] that were prepared by vesicle fusion. One plausible cause is coverage, as the present samples have only 80–90% coverage, compared to >90% for those prepared by vesicle fusion. However, the current magnitudes are comparable to those measured for bR monolayer junctions, in which retinal or retinal analogues are present in the protein.[4] Therefore, on the basis of our previous results,[4,32] which showed that retinal or an analogue has to be present in the protein to obtain such a current magnitude, we conclude that retinal is still present in the protein. This result, in combination with the structural information obtained from UV/Vis and FTIR spectroscopy, indicates that, upon covalent attachment of bR to the Br-terminated solid supports, the retinal–protein linkage is altered due to protein conformational alterations to some extent. These changes are sufficient for the protonated Schiff base connection between retinal and the protein to hydrolyze, although mere Schiff base deprotonation cannot be completely excluded. In both cases, the protein loses its photoactivity, but the retinal still remains within the protein.

The exact reasons for the loss of protein photoactivity are not yet clear. Notably, chemical attachment of the bR lysines leads to the generation of HBr, which may be trapped under or in the PM patch. The cytoplasmic half-channel is wide enough to allow HBr to diffuse into. Due to its high reactivity, the retinylidene Schiff base, as well as other functionally important amino acids, may react and be irreversibly modified, thus resulting in loss of protein photoactivity. However, this mechanism is unlikely because it is known[33,34] that in solution, bR retains its photoactivity even at very low pH (pH<1). Furthermore, the concentration of HBr would be very low under the reaction conditions. Some other possibilities are that the bR in the present monolayers is (partially) denatured, or that it is trapped in one conformation. Such situations can lead to loss of protein photoactivity if several Br groups that are bound to the surface react with several lysine residues of one protein, because these covalent bonds may distort the protein conformation sufficiently to break the protein–retinal bond. If this explanation is correct, decreasing the surface density of C3-Br should have an effect. More studies, which include lowering the C3-Br surface density, are needed to test these hypotheses.

Conclusions

BR monolayers can be prepared on Br-terminated solid supports through covalent attachment by reaction of the exposed free amine groups of bR with the pendant Br group of the chemically modified solid surface. The bonding is consistent with the results of AFM studies on surfaces obtained with either native or acetylated bR, as well as with the results of FTIR spectroscopy. The coverage of the resultant bR (sub)monolayers depends on the pH of the PM suspension, increasing as the pH changes from 9.2 to 6.8.

These as-prepared resultant bR monolayers lose their photoactivity, as confirmed by several characterization methods, including AFM, CPD measurements, and UV/Vis and FTIR spectroscopy. Current–voltage measurements indicated, however, that the retinal moiety is still present in the protein but lacks its regular protonated Schiff base linkage to the protein.

Similar reactions of bR in solution with alkyl bromide do not lead to loss of photoactivity and, thus, do not appear to affect the bR conformation and structure significantly. At present, it is difficult to pinpoint the exact cause for the dif-

ference in behavior in solution and on the solid surface. In any case, the fact that the loss of protein photoactivity is induced by the surface-confined chemical attachment of bR on Br-terminated surfaces is clear. Because of this, it is also tempting to suggest that several Br groups bound to the surface in a condensed manner react with more than one lysine residue located in one protein molecule. These covalent bonds impose severe restrictions on the protein conformation, thus resulting in significant conformational alterations. If this suggestion is confirmed by further studies, then it has implications for the incorporation of proteins in semiconductor-based technology, because it can provide guidelines as to how to incorporate proteins in electronic devices through tailored chemical bonding while maintaining their biological functions completely or to a large extent.

Experimental Section

Preparation of Br-Terminated Self-Assembling Monolayers on Solid Supports

Bromopropyl trichlorosilane (C3-Br) was purchased from Aldrich and was distilled before use by kugelrohr distillation. 1-Bromo-16-trichlorosilyl(hexadecane) (C16-Br) was prepared as reported in the literature.[35] The material obtained herein was identical to the known material, as determined by ^{1}H NMR spectroscopy.

For the preparation of self-assembling monolayers (SAMs) of trichlorosilanes, different substrates were used: quartz slides (QSIL Quarzschmelze Ilmenau GmbH, microscope slides made of synthetic QUARZGLASS, class A acc. to DIN 58297, both sides polished) for characterization by UV/Vis spectroscopy, n-Si wafers (both sides polished) for ATR FTIR spectroscopy (Wafer World, Inc., prime grade, $<100>$, 2–10 Ω cm resistivity), n-Si wafers (one side polished) for ellipsometry and wettability studies,; and degenerate n-Si wafers (one side polished, $\approx 10^{-3}$ Ω cm resistivity) for electrical measurements.

Quartz slides and Si wafers were cut, rinsed in hexane, acetone, and ethanol for 30 s each, and dried in a filtered nitrogen stream. Samples were pretreated in ozone (UVOCS apparatus) for 20 min. They were subsequently immersed in piranha solution (concentrated $H_2SO_4/H_2O_2 = 70{:}30$ (30% *v/v*)) at 80 °C for 20 min. The samples were then washed three times with deionized water and dried in a filtered nitrogen stream. Piranha treatment yielded an oxide layer, which was measured by ellipsometry to be (1.7 ± 0.3) nm thick, and a surface that was totally wetted by water. All the substrates were used within 0.5 h.

Cleaned Si wafers (or quartz slides) were coated in a nitrogen-purged glove box, in which ambient humidity was 24–28%, with a solution (0.022 mM) of trichlorosilane (100 μL) in DCH (dicyclohexylphosphine) (10 mL). Samples were withdrawn from the silane solutions after the indicated deposition time (1 h), cleaned by sonication for 15 min in hexane, mechanically wiped and washed with ethanol to remove physisorbed material, and dried under a filtered nitrogen stream.

Preparation of PM Monolayer on Br-Terminated Solid Supports

A bulk suspension of PM fragments containing wild-type bR was prepared as described elsewhere.[36] Two types of suspensions of PM fragments (0.5 OD) with different pH values were used for monolayer preparation: one with Tris buffer (20 mM, pH ≈ 9.2), the other with only deionized water (pH ≈ 6.8). A suspension of PM fragments with acetylated bR[9] (pH ≈ 9.2) was used as a control to check the role of the free amine groups of bR in chemical bonding. The solid supports, modified with Br-terminated SAMs, were, after being cleaned with deionized water and dried in a flow of nitrogen, immersed in a suspension of PM fragments for >3 h at room temperature. The solid supports were then thoroughly rinsed with pH 9.2 Tris buffer and Milli-Q water, after which they were ready for characterization. Samples used for checking chemical bonding were further rinsed thoroughly with 1 M aqueous NaCl and ethanol.

Monolayers of the PM fragments were prepared on the following solid supports for detailed characterization: quartz slides for UV/Vis spectroscopy, double-sided polished Si wafers for ATR FTIR spectroscopy, and degenerate n-Si wafers (resistivity $\approx 10^{-3}$ Ω cm) for AFM and CPD and *I–V* measurements.

For comparison and to check if there were any substrate effects on bR bioactivity, PM fragment monolayers were also prepared by electrostatic deposition onto a Si substrate modified by a monolayer of APTMS.[22]

Reaction of bR with Alkyl Bromide in Solution

bR (1.6×10^{-5} M) was dissolved in phosphate buffer (1 mL, 20 mM, pH 7), and a solution of ethyl bromide (40 equiv) in ethanol (20 μL) was added. The reaction was allowed to proceed for 12 h, after which the solution was centrifuged, and the resulting pellet was washed three times with water and dissolved in phosphate buffer.

Instruments

The thickness of the monolayers was measured by using a variable-angle spectroscopic ellipsometer Model M44 (J. A. Woollam Co.) with an Xe source and a spot diameter of 1 mm. Ellipsometric measurements were made after the equipment was calibrated against a 25.0-nm SiO_2 layer on Si. Data were collected at take-off angles of 68–70° and at 44 wavelengths between 624 and 1109 nm. The thickness, deduced from the ellipsometry measurements, was compared to a theoretical thickness estimated from the length of the fully extended chain of the SAM-forming molecule, calculated by using PCMODEL (Serena Software). The thickness used for comparison was from the Si atom of the silane to the most remote atom on the chain.

AFM topographic images were acquired in tapping mode under ambient conditions (Nanoscope IIIa; Digital Instruments, Inc.) with a standard silicon nitride cantilever. The UV/Vis spectra of bR monolayer membrane on quartz glass were recorded in the dark or under green-light illumination with an 8540 diode-array spectrophotometer (Hewlett Packard Co., USA). CPD measurements were performed in the dark and under green-light illumination by using a commercial (Besocke) Kelvin probe, as part of a home-built setup, inside an N_2-filled glove box (relative humidity ≤ 20%). In this work, the green light was obtained with a tungsten halogen light source and the combination of a cut-off filter ($\lambda > 550$ nm) and a heat filter.

ATR FTIR spectra were recorded on a Bruker Vector 22 spectrometer equipped with an MCT detector. Spectra of the as-deposited films were collected with a $60 \times 10 \times 0.45$ mm^3 Si parallelogram prism prepared in-house by polishing the two short edges of a freshly cut double-sided polished Si wafer to a 45° angle. The background spectrum was collected after piranha treatment of the cleaned ATR prism, and sample collection was done after monolayer film deposition. The background spectrum of the clean ATR prism was subtracted from each sample spectrum. Typically, we collected 1000 scans at a nominal spectral resolution of 4 cm^{-1}.

Current–Voltage Measurements

I–V measurements were carried out in a class 10000 clean room at 293 K and 40% relative humidity. *I–V* characteristics were measured by using a W needle, connected to a micromanipulator to contact the Au pad (an InGa drop on the Au minimizes mechanical (pressure) damage to the film), and an HP 4155 semiconductor parameter analyzer, in the voltage scan mode; these measurements were made under ambient conditions.

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