



Published in final edited form as:

Langmuir. 2008 January 15; 24(2): 345–349. doi:10.1021/la7030973.

Improved Surface Modification Approach for Micromechanical Biosensors

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Abstract

We have investigated the sensing performance of protein-based microcantilever biosensors prepared from multiple surface conjugation chemistries. The 11-mercaptoundecanoic acid monolayers were prepared according to both traditional and modified processes. In three protein-based biosensors, the modified process improved microcantilever sensing performance by increasing the bending amplitude, a critical step toward developing a cost-effective microcantilever-based sensor platform for medical diagnostics and environmental and drug screening applications. Scanning electron microscopy (SEM) images demonstrated that proteins immobilized on the microcantilever surface using the modified chemistry approach formed a compact layer.

Microcantilevers hold the position of a cost-effective and highly sensitive sensor platform for medical diagnostics, environmental testing, and fast-throughput analysis.^{1–3} A unique property of microcantilevers is that they undergo bending due to molecular adsorption or absorption by confining the adsorption and absorption to one side of the cantilever. The bending of the microcantilever results from analyte-induced changes in the surface characteristics or film volume of the microcantilever.

Microcantilevers typically are thought to bend as a result of interactions of molecules on the cantilever surface. Any change in these interactions will produce changes in the bending of the microcantilever. From a molecular point of view, the binding results in electrostatic attraction,⁴ repulsion,⁵ steric effects,¹ and intermolecular interactions. One or more of these effects produce changes in surface stresses on the microcantilever. By monitoring changes in the bending response of a cantilever, surface stress changes induced by adsorption or molecular recognition can be precisely and accurately recorded.

In most biosensors, bioreceptors are chemically linked on the sensor surfaces by using a variety of surface conjugation chemistries. It is often assumed that surface conjugation chemistries utilized by other chip-based microsensors can be transferred to microcantilever devices; however, the mechanism of surface stress-induced bending is substantially different from the mechanisms of other sensor platforms. In many cases, the signal/noise (S/N) ratio of microcantilever sensors prepared from traditional surface conjugation chemistries is too small to ensure a reliable and user-friendly sensor. We have identified several examples of

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the importance of monolayer formation and conjugation chemistry on microcantilever sensitivity. The improvements of these sensor are dependent on the reaction time,⁴ coadsorbents,⁵ selection of conjugation chemistries,⁶ and enhanced compactness and order of the self-assembled monolayer (SAM) film. For microcantilever biosensors, it is believed that the packing density on the surface is critical for the large bending amplitude, stability, sensitivity, and reproducibility of microcantilever sensors.^{7,8}

The most commonly used functional groups for the attachment of bioreceptors are carboxyl and amino moieties.^{9,10} These are usually introduced onto the gold surface by exposure to corresponding amino or carboxyl groups containing thiol compounds. The thiols form a monolayer on the gold surface through a well-known self-assembly process. After monolayer formation, the bioreceptors are conjugated on the surfaces through cross linkers. A widely used cross-linking scheme for the conjugation of bioreceptors utilizes 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) (Scheme 1). Although this approach has been widely used for multiple sensor devices, it has often been observed⁶ that the microcantilevers modified by a typical EDC/NHS process either did not deflect upon exposure to the analytes or did not generate reproducible results, possibly because of low packing density or defective surface coatings. Recently,¹¹ Jiang et al. investigated the surface characteristics of the amino- and carboxyl-topped monolayers generated from thiol compounds and observed unwanted particles on the surfaces. They further suggested that the co-addition of CF₃COOH and thiols during the SAM process would produce a smoother and cleaner surface. These observations suggest that unwanted particles inhibit the EDC/NHS cross-linking process, leading to unsuccessful microcantilever sensor development. Thus, by using a modified self-assembly process that removes unwanted particles from the surface, it may be possible to develop microcantilever sensors with better sensing performance. Experiments described here demonstrate the effectiveness of CF₃COOH treatment in enhancing microcantilever sensing performance. We have focused on protein-based sensors because of their broad applicability and the active interest in this area of microcantilever biosensing.

In our experiments, we used commercially available silicon microcantilevers (Veeco Instruments). The dimensions of the V-shaped silicon microcantilevers were 180 μm in length, 25 μm in leg width, and 1 μm in thickness. One side of each cantilever was covered with a thin film of chromium (3 nm), followed by a 20 nm layer of gold, both deposited by e-beam evaporation. On the uncoated side of the commercial microcantilever was a 12–19- \AA -thick, naturally grown SiO₂ layer (native oxide layer). All chemicals were used as received from Sigma-Aldrich.

Proteins were immobilized on the gold side of the microcantilevers. A typical EDC/NHS surface procedure⁸ (method A) for protein immobilization was used. For method A, the microcantilevers were thoroughly cleaned with piranha solution and rinsed with water. (**Caution!** Piranha solution reacts violently with many organic materials and should be handled with care.) The microcantilevers were then immersed for 15 h in a 1 mM solution of 11-mercaptoundecanoic acid (MUA) in ethyl alcohol and rinsed in EtOH. Next, the microcantilevers were immersed in a 0.05 M 4-morpholinepropanesulfonic acid (MES) buffer solution containing 100 mg/mL EDC and 100 mg/mL NHS (pH 6.8) for 30 min at room temperature and then immersed in a solution containing 2 mg/mL protein in MES solution for 3 h. The microcantilevers were then rinsed with MES buffer solution.

In the modified approach (method B), the first monolayer-formation process was altered by adding 0.2 mL of CF₃COOH to the 1 mM solution of MUA in EtOH. After 15 h of immersion in this solution, the microcantilevers were washed with EtOH, 10% (v/v) NH₃–

H₂O/EtOH, and then EtOH. The remainder of the procedure was identical to that described in method A.

The deflection experiments were performed in a flow-through glass cell (Digital Instruments) similar to those used in atomic force microscopy (AFM). The microcantilever was immersed in a buffer solution. For continuous flow-through experiments, the buffer solution was initially circulated through the cell using a syringe pump. A schematic diagram of the apparatus used in this study was previously published.⁴ A constant flow rate was maintained during each experiment. Experimental solutions containing different concentrations of analytes were injected directly into the flowing fluid stream via a low-pressure injection port/sample loop arrangement with a loop volume of 2.0 mL. This arrangement allowed for continuous exposure of the cantilever to the desired solution without disturbing the flow cell or changing the flow rate. Because the volume of the glass cell, including the tubing, was only 0.3 mL, relatively fast replacement of the liquid in contact with the cantilever was achieved. Microcantilever deflection measurements were determined using the optical beam deflection method. Bending of the cantilever was measured by monitoring the position of a laser beam reflected from the gold-coated side of the cantilever onto a four-quadrant AFM photodiode. We define bending toward the gold side as “bending up”; “bending down” refers to bending toward the silicon side. When adsorption occurs on the gold surface, the downward bending is generally caused by the repulsion or expansion of molecules on the gold surface (compressive stress). Upward bending is caused by the attraction or contraction of molecules on the gold surface (tensile surface stress). The cantilever was immersed in the buffer solution until a baseline was obtained, and the voltage of the position-sensitive detector was set as background corresponding to 0 nm.

Our initial test compared the responses of the MUA-modified microcantilevers prepared from the two SAM processes upon exposure to hexylamines. In one of the earliest reports on microcantilever chemical sensors, Fritz, etc. reported an occurrence of tensile surface stress¹ on microcantilever surfaces due to the complexation of amines with carboxyl groups on the top monolayer surface. We observed the same phenomenon for microcantilevers modified using a typical MUA monolayer-formation process (i.e., the SAM process in method A) (Figure 1). Microcantilever bending reached equilibrium after approximately 20 s. The tensile surface stress changes on microcantilevers prepared using method A are not yet fully understood. One possibility is that each hexylamine forms hydrogen bonds with two carboxylic acids, thus bringing two MUA molecules closer and increasing the tensile surface stress on the SAM surface. In contrast to surfaces prepared using method A, microcantilevers modified by the improved approach (i.e., the SAM step in method B) showed the expected compressive surface stress changes (Figure 1). The compressive surface stress change on these microcantilevers is due to the repulsion of complexed hexylamines. This is expected because SAM monolayers formed from longer-chained thiols generate more significant compressive surface stress changes than those from shorter-chained thiol compounds. The repulsive forces did not occur on the microcantilevers prepared from the SAM process using method A. This is consistent with Jiang's work,¹¹ suggesting that the blocking particles prevent the formation of a compact hexylamine layer on top of the carboxyl monolayer surface.

We selected three proteins for validation of the microcantilever biosensors: horseradish peroxidase (HRP), acetylcholine binding protein (AChBP), and calmodulin (CaM). These proteins were selected to represent three different categories: enzyme, proteins that change conformation in response to complexation with agonists or antagonists, and proteins that change conformation in response to metal ions, respectively. It has been reported that the

conformational changes of a protein can result in surface stress changes that can be detected from the consequent bending response of the microcantilevers.^{12,13}

Horseradish peroxidase is a heme-containing peroxidase that reduces hydrogen peroxide through a three-step reaction.¹⁴ Recently, we developed an HRP-modified microcantilever biosensor for hydrogen peroxide (H_2O_2) sensing.¹⁵ HRP was immobilized on the cantilever surface through a layer-by-layer multilayer approach. It is expected that a microcantilever covered with a layer of HRP through surface conjugation would also deflect upon exposure to hydrogen peroxide. However, the microcantilever prepared via method A did not deflect in response to hydrogen peroxide, indicating the difficulty of producing a defect-free, compact structure in the protein film using method A (Figure 2). Microcantilevers prepared with method B, as expected, showed deflection on exposure to hydrogen peroxide (Figure 2). These results suggested that the HRP coating was more compact on microcantilevers prepared using method B. Scanning electron microscopy (SEM) images (Figure 3) confirm this hypothesis. Particles were observed on the microcantilever surface at low power amplification (Figure 3b) for the protein films prepared using method A. These particles are believed to be protein aggregates. At high power amplification (Figure 3c), gold grains were still seen, and the protein was scattered on the gold surface. For protein films prepared from method B, the surface was uniformly covered by a layer of HRP enzyme, and the gold grains were obscured by the protein layer (Figure 3e,f).

We also investigated calmodulin (CaM) and acetylcholine binding protein (AChBP)-based microcantilever biosensors. Calmodulin, a small (17 kD) heat-stable, acidic protein, is an abundant and ubiquitous calcium-binding protein that serves as an activator of numerous cellular enzymes.¹⁶ In the absence of Ca^{2+} , CaM consists of a short dumbbell structure with two globular domains connected by a helical linker. Upon binding four Ca^{2+} ions, CaM undergoes a large conformational change and becomes more elongated. The acetylcholine binding protein (AChBP) is a homolog of the nicotinic acetylcholine receptor amino terminal and is potentially useful in monitoring ligands that interact with these receptors.¹⁷ Because of its close structural similarity to ligand gated ion channel receptors (LGICs), AChBP has been used as a template for the computer modeling of ligand-gated ion channel receptors.^{18–21} Like CaM, AChBP undergoes a conformational change on the binding of ligands.^{22–23} AChBP sensors could be used to monitor acetylcholine or nicotine or in the high-throughput screening of potentially useful therapeutic drugs.

CaM-aggregated particles and pinholes were found on surfaces prepared using method A but were not seen on surfaces prepared using method B (Figure 4). In contrast, the SEM images of AChBP on surfaces prepared using the two methods did not show significant differences, and no AChBP particles were seen on either surface. Thus, the surface-treatment procedure appears to have a larger impact on some proteins than on others. The precise reason for these differences remains unclear, although it may be due to different protein structural characteristics or to different protein/protein or protein/surface interactions. However, surface preparation using methods A and B did produce a differences in microcantilever performance for CaM- and AchBP-modified microcantilevers. AChBP-modified microcantilevers prepared using method B exhibited a larger deflection amplitude when compared to those prepared using method A (Figure 5). The deflection amplitude increased by nearly 50% with improved response times. The Langmuir adsorption model also produced a better fit of the deflection–time profile of microcantilevers prepared by method B compared to that for method A (Figure 5, dashed lines). The bending of CaM-modified microcantilevers improved significantly when prepared using method B versus method A (Figure 6). Response times and amplitudes were significantly improved using method B, and the Langmuir adsorption model produced a better fit when method B was used. The improved bending response afforded by method B is likely the result of the tighter packing

of proteins on the microcantilever surface. Although it is possible that this close packing of an allosteric protein such as CaM and AChBP might interfere with protein function, our data demonstrate that these proteins continue to bind ligands under these conditions.

In summary, we compared the effects of the special treatment of carboxyl SAM monolayers using method B to those of non-treated monolayers (method A) using three different protein surfaces. Three different protein classes were evaluated as sensor molecules: an enzyme (HRP), an ion-binding, regulatory protein (CaM), and an allosteric ligand-binding protein (AChBP). All three protein-based microcantilever biosensors showed improved response characteristics when using the modified method (method B). SEM showed increased surface densities on microcantilevers prepared with method B, supporting the hypothesis that unwanted particles inhibit cross linking via EDC/NHS. The resultant increased surface packing and subsequent improved response amplitudes increase the effective sensitivity of protein-based microcantilevers and facilitate the development of cost-effective microcantilever-based sensors platforms for medical diagnostics and environmental and fast-throughput analysis.

Acknowledgments

This work was supported by the NSF Sensor and Sensor Network (ECS-0428263), the Chinese Scholarship Council, and the National Center for Research Resources (NCRR, grant no. 5P20RR016466), a component of the National Institutes of Health (NIH). The content of this letter is solely the responsibility of the authors and do not necessarily represent the official views of the agencies funding this work.

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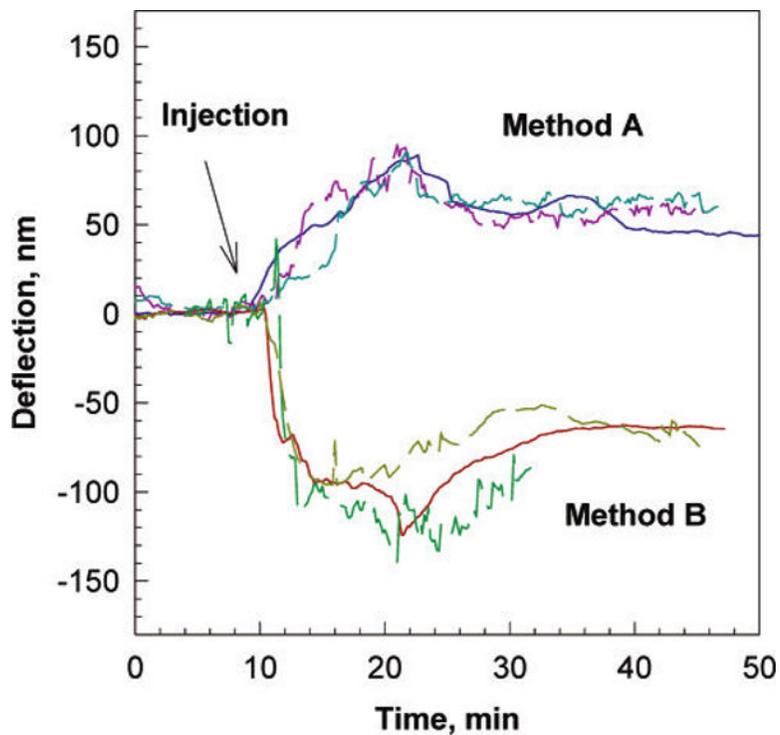


Figure 1. Bending responses as a function of time for microcantilevers coated with a monolayer of 1-MUA prepared using the SAM processes described for methods A and B upon exposure to saturated hexylamines in air. For each method, three independent microcantilevers were tested. The standard error was within 10%.

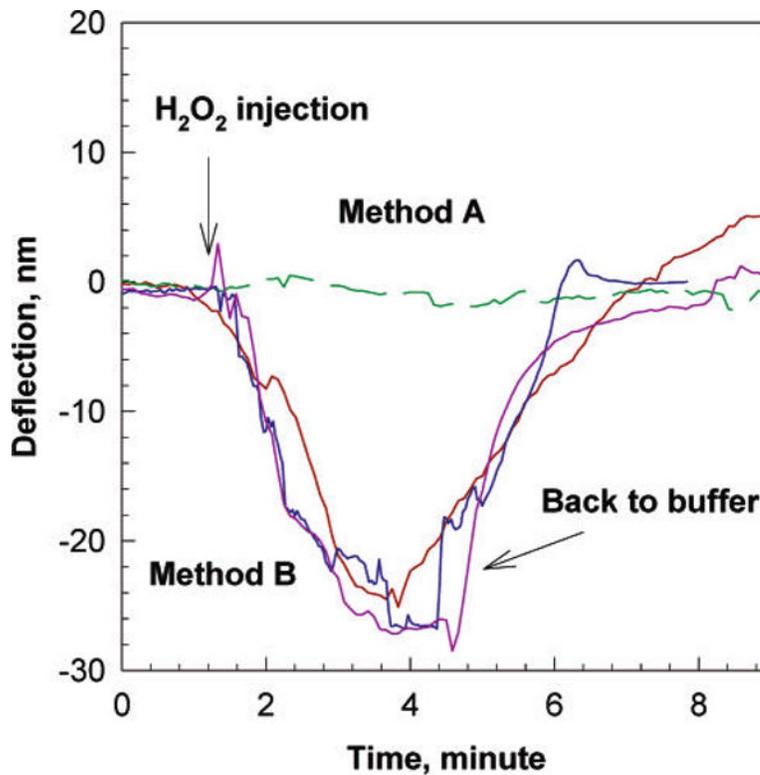


Figure 2. Bending responses as a function of time for microcantilevers coated by HRP prepared using methods A and B on exposure to 1×10^{-5} M H_2O_2 solution. For each method, three independent microcantilevers were tested. Only one test from method A was shown for clarity. The standard error was within 10%.

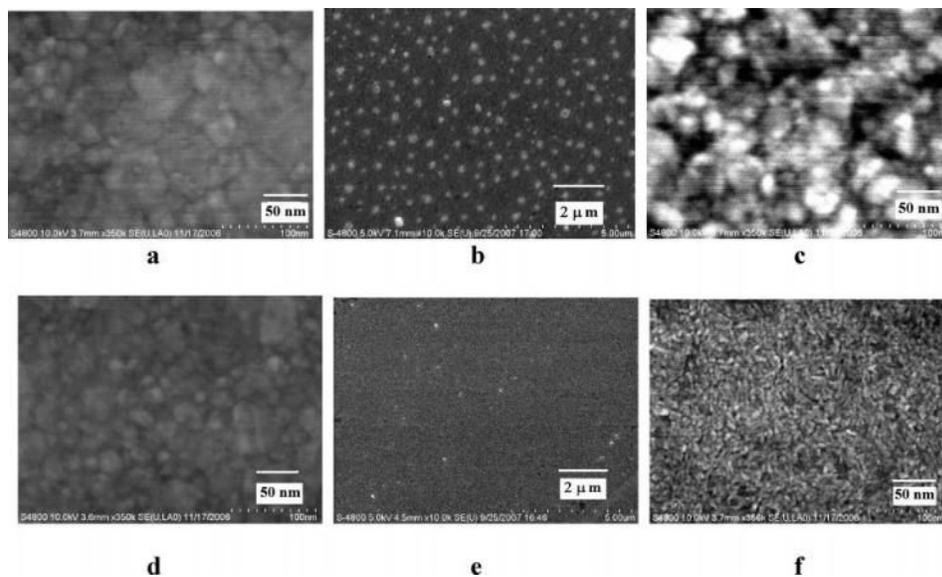


Figure 3. SEM images of microcantilever surfaces. (a) The gold surface after the EDC/NHS cross-linker step using method A, (b and c) HRP surface prepared using method A, (d) gold surface after the EDC/NHS cross-linker step using method B, (e) and f) HRP surface prepared using method B. Scanning electron micrograph (SEM) measurements were conducted on a Hitachi S4800 SEM.

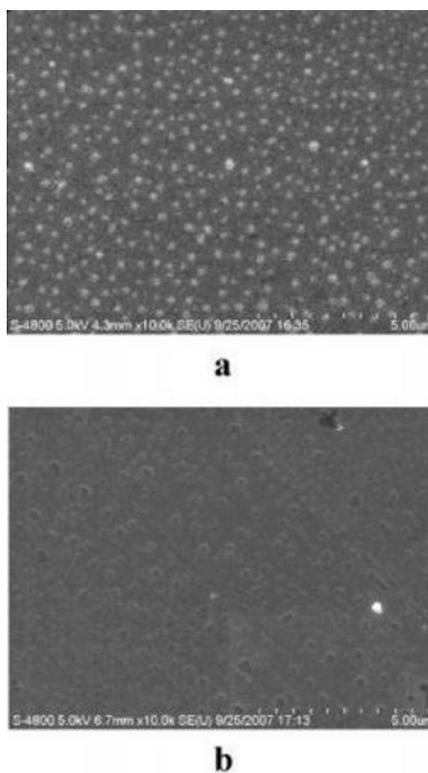


Figure 4. SEM image of (top) a CaM surface prepared using method A and (b) a CaM surface prepared using method B.

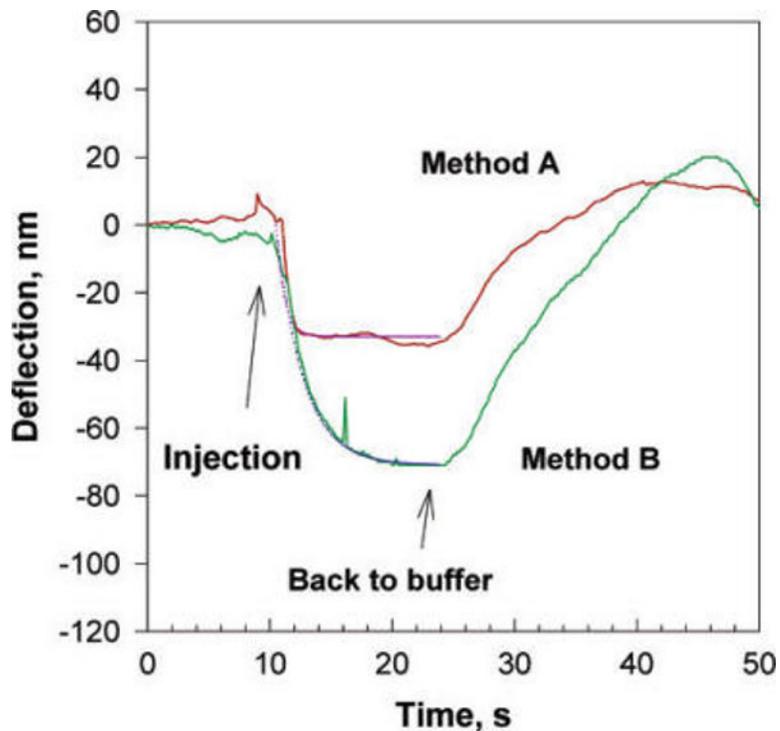


Figure 5.

Bending response as a function of time for AChBP-coated microcantilevers prepared using methods A and B on exposure to 1×10^{-5} M acetylcholine solution. The Langmuir adsorption model was used to describe the absorption of acetylcholine on the protein-covered surface. The rate of formation of a fraction of a monolayer, θ , is proportional to the concentration of acetylcholine in solution and to the fraction of the surface remaining free of sorbant, $1 - \theta$. Thus, the cantilever bending vs time is described by $\delta \propto 1 - \exp(-kt)$, where δ is the surface stress, k is the reaction rate, and t is the time. k was calculated to be 1.5 and 0.45 min^{-1} for methods A and B, respectively, using a nonlinear curve-fitting method to fit the observed experimental data. The fitted curve was shown by the dashed line.

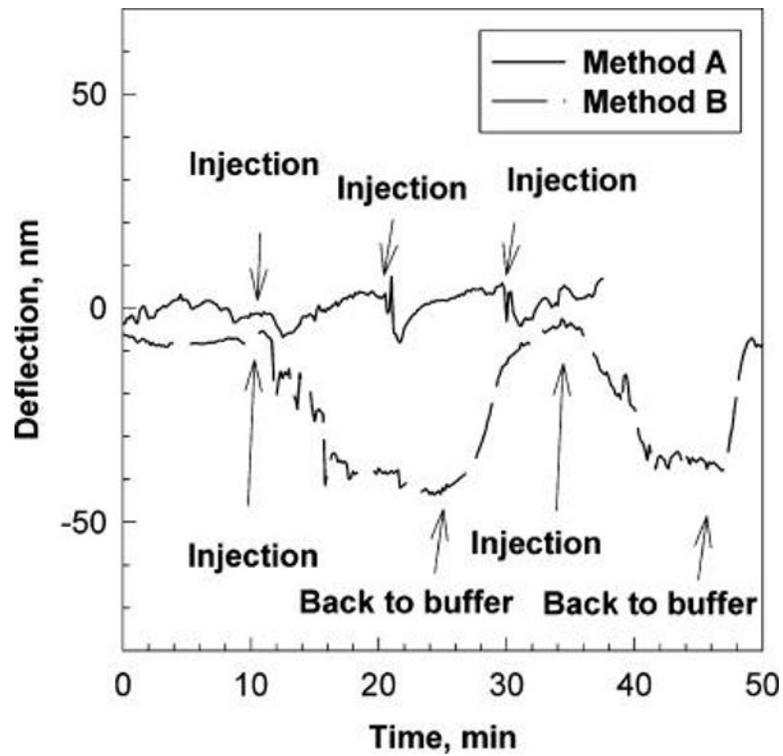
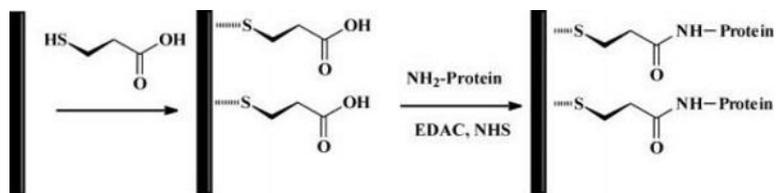


Figure 6. Bending response as a function of time for CaM-coated microcantilevers using methods A and B on multiple exposure to 1×10^{-5} M Ca^{2+} solution.



Scheme 1. Surface Conjugation Chemistry of Proteins on a Gold Surface Using the EDC/NHS Pair