Nano-Cantilevers for an Ultra-Sensitive Bio-Assay

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ABSTRACT

E-beam lithography and lift-off processes have been used to fabricate nano-cantilever arrays. The nano-cantilever arrays can be coated with specific reagents to detect and measure the presence of particular antigens and/or complementary DNA sequences with a smaller sample size and at much earlier stages of disease progression compared to current medical diagnostic technologies. Using the cantilever material stack (Au/Cr/Si), we have assessed the binding affinity of Au, Cr, and Si with Protein G, and antibodies for Prostate Specific Antigen (PSA) and Cancer Antigen 125 (CA125), an ovarian cancer-associated antigen. Based on our experiments, we see that the thin gold layer of the Au/Cr/Si samples, provides increased bio-material binding affinity, and the chromium layer has a similar, if not less, binding affinity compared to the silicon chip alone.

Keywords: cantilever array, bioassay, resonance, nano-cantilever, cancer diagnosis

1 INTRODUCTION

With advances in nanotechnology, there exist unique opportunities to probe and detect various biomolecules in serum at a reduced concentration compared to what is possible using current "macro" technologies or various evolving "micro" technologies. Recent papers have reported the application of micro-cantilevers as bio-assay tools [1-5]. It is found that when specific bimolecular interactions occur on one surface of the micro-cantilever beam, the cantilever bends. The cantilever deflection can then be measured, and the presence of particular antigens and/or complementary DNA sequences can be detected in a given serum. Two most often used methods to measure the deflection of the cantilever are laser beam deflection and piezoresistance change embedded in the cantilever. Although laser beam can be used for micro-scale cantilevers, they cannot be used for nano-cantilevers. Similarly, piezoresisitve materials are limited by thickness for potential use on nano-cantilevers. On the other hand, the change in resonant frequency can be used to determine biomolecule binding on nano-cantilevers.

In this paper, we report the design and fabrication of nano-cantilevers and coating of nano-cantilevers with antibodies for various bioconjugate material detection.

2 PRINCIPLE AND DESIGN

The resonance frequency, f, of an oscillating cantilever can be expressed as [5]

$$f = \frac{1}{2\pi}\sqrt{\frac{k}{nm}} \tag{1}$$

Where k is the spring constant and m is the mass of the cantilever. For a rectangular cantilever, n is 0.24. The change in mass due to a change in resonant frequency an be given as [5]

$$\Delta m = \frac{k}{4\pi^2 n}\left(\frac{1}{f_2^2} - \frac{1}{f_1^2}\right) \approx \frac{k}{4\pi^2 n}\frac{2\Delta f}{f_1^3} \tag{2}$$

Where f_1 and f_2 are the resonance frequencies before and after the addition of the mass, respectively. n=1 when the added mass is at the tip of the cantilever, and n=0.24 when the additional mass is uniformly distributed over the length of the cantilever – on one side of the cantilever or both sides of the cantilever. The assumption is made that the spring constant does not change after the mass addition.

For a simple tip-loaded cantilever beam of length L, width W, and thickness t, the spring constant is given by

$$k = \frac{EWt^3}{4L^3} \tag{3}$$

where E is the effective young's modulus of the beam material. In Table 1, the theoretical resonant frequency and mass resolution are listed for different cantilever dimensions, assuming a minimum detectable resonant frequency change of 1 Hz [6]. The first set of dimensions corresponds to the dimensions of the nano-cantilevers fabricated in this study. The last set of listed dimensions corresponds to typical dimensions for an AFM cantilever. If the minimum detectable resonant frequency change is

10Hz, the mass resolution will be decreased 10 times correspondingly.

As shown in Table 1, 0.002 attogram mass change could be detected by the nano-cantilever. With 10Hz detectable frequency change, the nano-cantilever can still detect 0.02 attogram mass change, which is the same order of magnitude as the human protein molecule mass.

t(μm)	W(μm)	L (μm)	K (N/m)	*f* (kHz)	Δm (ag)
0.025	0.1	1	0.055	17939	0.002
0.100	1	10	0.035	718	20
0.200	5	50	0.011	57	12510
1.500	50	200	0.738	26	8006154

Table 1: Mass sensitivity for four different cantilever dimensions. (E= 140GPa, density is 7174 kg/m^3)

3 FABRICATION

We have used electron-beam lithography and lift-off processes to fabricate the nano-cantilever arrays. Lift-off processes have the capability to provide high-fidelity patterns with very fine features. These processes work well with unidirectional deposition methods that do not provide significant sidewall coverage, such as filament or e-beam evaporation. Sputter deposition, which is multi-directional, provides sidewall coverage, and therefore, complicates the lift-off processes. The sidewall coverage in the sputter deposition process is due to the fact that the sputtered atoms deposit randomly at various angles on the rotating substrate.

Two liftoff methods are being pursued for the fabrication of nano-cantilevers: a single-layer resist approach and a bi-layer resist approach, as illustrated in Figure 1. Prior to spinning the resist a sacrificial metal layer is deposited, that will later be selectively etched to release the patterned cantilever. The bi-layer process uses a thin layer of PMMA (Polymethylmethacrylate) resist spun over a thicker base layer of MMA (methylmethacrylate) resist. The single layer process uses only a positive resist, e.g., PMMA. In both processes, the total resist height to deposited metal height is desired to be 7:1 or greater; a ratio less than 7:1 makes the liftoff process difficult.

As illustrated in Figure 1, on a bare wafer (1a), a thin layer of release metal is first deposited (1b). In the single layer process, PMMA is spun on the wafer, patterned, and developed. In the bi-layer process, a thick layer of MMA is spun on the wafer, followed by a thinner layer of PMMA (1c). After patterning of the bi-layer with e-beam lithography tool, the MMA base layer is undercut during developing, due to greater exposure sensitivity as compared with the PMMA top layer (1d). In both the single-layer and bi-layer resist cases, the cantilever metal film, Cr, is deposited (1e). Liftoff is performed using acetone in an ultrasonic bath. For smaller aspect ratios, a mechanical polish, e.g., a gentle wipe with an acetone soaked wiper, is needed to complete liftoff (1f). Once the cantilever metal deposition is complete, a second layer of resist is spun and patterned to define a release window, which allows the selective etching the release layer (1g). By selectively etching the release layer a free-standing cantilever can be obtained (1h).

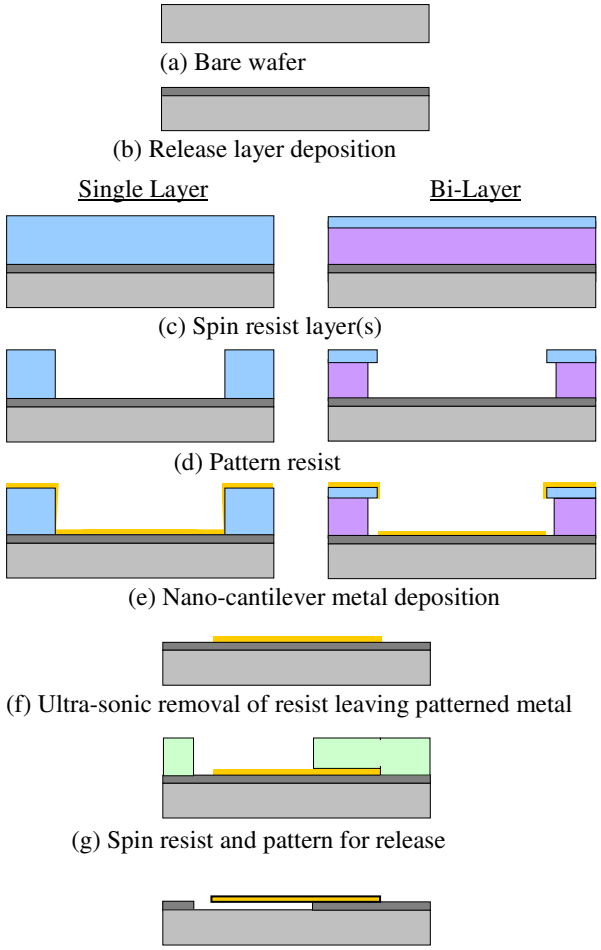


Figure 1: Fabrication steps for nano-cantilevers

Figure 2 provides an SEM image of the fabricated Cr/Au nano-cantilevers on a Si wafer. These cantilevers are 100nm wide, 25nm thick and 1000nm long. Figure 3 shows some cantilevers with different shapes. The leg width of the triangular cantilever is 100 nm, while the leg width of the rectangular cantilever is 200 nm. Both shapes are 25 nm thick and 1000 nm long.

4 PROTEIN AND ANTIBODY BINDING

Tumor-associated circulating antigens are being extensively used to determine diagnosis and prognosis of tumors, to predict response to therapy, and to predict relapse. The current nano-cantilever approach will offer an opportunity to improve the sensitivity of detection of tumor-associated antigens by several orders of magnitude and thus improve cancer patient management. We have

performed experiments to assess the binding affinity of Au, Cr, and Si with a recombinant Protein G, a mouse monoclonal (PS2) Immunoglobulin G1 (IgG1) PSA antibody associated with Prostate Specific Antigen (PSA) as well as a mouse monoclonal (1F6) IgG1 CA125 antibody associated with ovarian Cancer Antigen 125 (CA125). We have also tested the selective binding of antigen (secondary anti-mouse IgG1 antibody) to the primary monoclonal antibody, and not to the "bare" surface of the metal by blocking using bovine serum albumin (BSA) protein, a protein that will bind up any available protein binding sites on the test surface but does not interact with the secondary antibody. In addition to blocking, we have used a phosphate buffered saline (PBS)-TWEEN® detergent solution during the wash steps and in the application of the secondary antibody to reduce any unwanted binding, i.e., binding that would bias or add noise to the measurement.

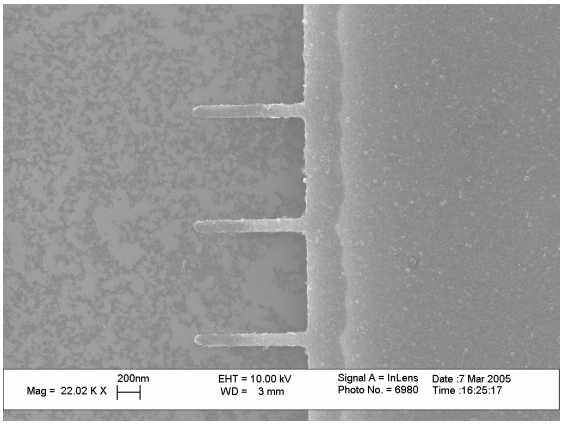


Figure 2: Nano-cantilever array (100x25x1000nm)

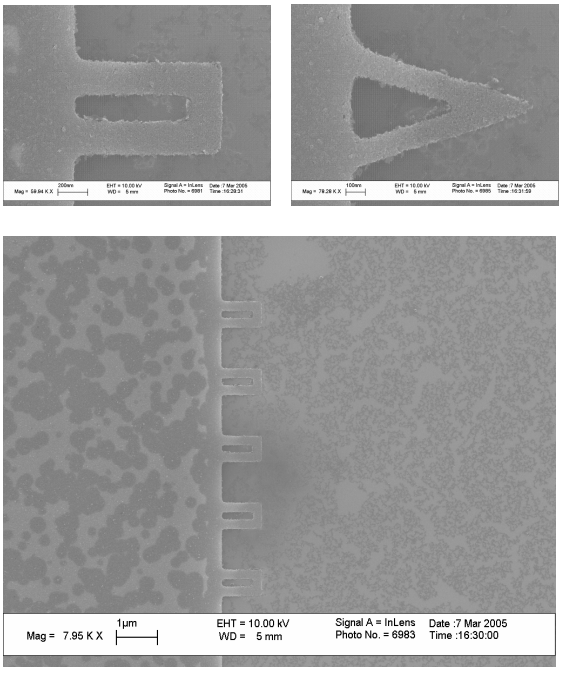


Figure 3: Nano-cantilever array with different shapes

The relative binding affinity of each sample type was determined by applying the primary, PSA antibody or CA125 antibody or Protein G, for an incubated period and then testing for its presence on the surface through the use of secondary anti-mouse IgG1, HRP-linked antibody. The secondary antibody binds mouse IgG1 antibodies, as well as Protein G, with high affinity. The presence of the Horseradish Peroxidase (HRP) label can be measured by applying a substrate, namely, tetramethylbenzidine (TMB). The HRP catalyzes in the presence of the substrate, resulting in blue color where the blueness is relative to the concentration of the HRP labels, and thus the relative presence of the secondary antibody. The concentration of the secondary antibody, in turn, is relative to the concentration of the primary, PSA antibody, CA125 antibody or Protein G. Thus, from the blueness of the reaction, a relative measurement can be made of the presence of the primary and thus, the affinity of binding between the primary and Si, Cr/Si, and Au/Cr/Si samples.

The columns of Table 2 and Table 3 correspond to the primary biomaterial tested for binding, while the rows correspond to the sample chip, the binding surface, used, i.e., Si, Cr/Si or Au/Cr/Si. The columns labeled "control" represent experiments used to gauge the noise of the testing procedure. For these control tests, no primary biomaterial was used, rather only PBS was added during the primary incubation stage. All other testing steps remained the same.

The TMB substrate is slightly light sensitive and has a default level of "blueness" associated with it. To gauge this bias, the absorbance level of only the TMB substrate was measured for each test, and its corresponding value is listed in the "TMB control" column. The results from Table 2 and Table 3 should not be compared in terms of a strict absolute. Rather, the values of an experiment within a given test should be compared to those of other experiments within that same test, e.g., the PSA antibody results for Si and Cr/Si. Thus, a conclusion of the *relative* binding affinity for a particular biomaterial-surface combination can be made among the different material surfaces, Si, Cr/Au, and Au/Cr/Si, for a given primary biomaterial, PSA antibody, CA124 antibody or Protein G.

	control	control	PSA	PSA	PG	PG	TMB control
a	0.086	0.107	0.337	0.385	0.417	0.336	
b	0.091	0.079	0.266	0.301	0.287	0.255	
c	0.103	0.143	0.491	0.551	0.545	0.508	0.029

Table 2: Absorbance results for samples (a: Si, b: Cr/Si, c: Au/Cr/Si)

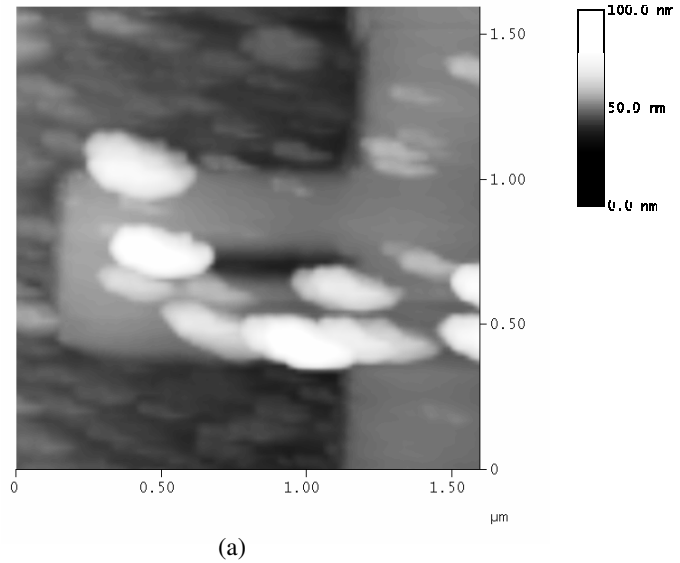
	control	control	PSA	PSA	PG	PG	TMB
a	0.147	0.149	0.424	0.424	0.580	0.456	
b	0.122	0.093	0.474	0.474	0.358	0.426	
c	0.108	0.120	0.586	0.586	0.971	0.860	0.033

Table 3: Absorbance results for samples

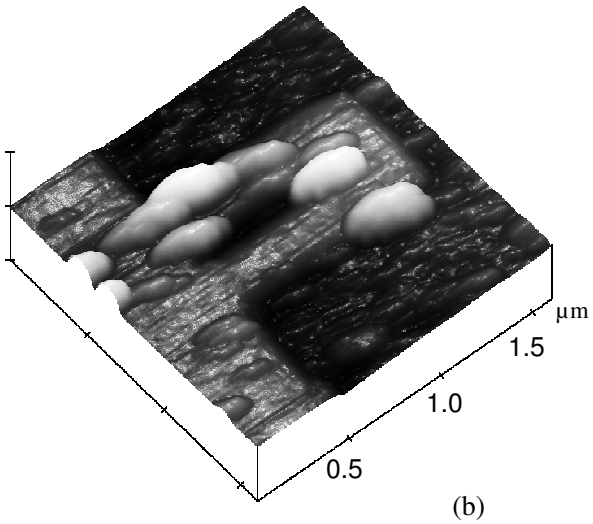
The results shown in Table 2 and Table 3 signify that the thin gold layer of the Au/Cr/Si samples, provides an increased binding affinity for each of the primary biomaterials tested. The chromium layer has a similar, if not less, binding affinity compared to the Silicon chip alone. The highest binding affinities were measured for the CA125 antibody on Au/Cr/Si stack.

5 AFM IMAGING OF BINDING

An additional binding experiment was performed to allow the imaging of bio-materials bonded to the surface of the cantilevers, using an anti-flagellin polyclonal antibody and randomly sheared flagella fragments. The fragments were produced by randomly shearing a flagella solution using a pipette. Non-fragmented flagella, a polymer of approximately 70,000 sub units of flagellin, can have a size ranging form 5-10 µm in length. The larger size of the flagella fragments make them easier to image than the antibodies, or an antigen, themselves. Nano-cantilevers with a 0.5 nm Au layer, atop a 25nm Cr base, were used in this experiment. Samples containing the nano-cantilevers where incubated with the polyclonal anit-flagellin antibody for 1.5 hours, after which a PBS wash and a 30 minute BSA blocking step was implemented, as discussed in the previous section. The flagella fragments were applied in a buffered solution for a duration of an hour. The samples were rinsed and allowed to dry before Atomic Force Microscopy (AFM) was used to image the binding surface. Flagella fragments, which are 200-300 nm in size, are shown bonded to the cantilever surface, in Figure 4. Figure 4a is the top view of the rectangular nano-cantilever with flagella fragments, while Figure 4b is an isometric view. These results demonstrate a relatively high binding avidity for the test procedure.



(a)



(b)

Figure 4: (a) Top view (b) isometric view of flagella fragments bonded to a nano-cantilever

6 SUMMARY

Through e-beam lithography and lift-off processes, arrays of nano-cantilevers have been fabricated. These arrays can be used as non-invasive and ultra-sensitive bio-assays, and provide an opportunity to increase the sensitivity of detection of tumor-associated antigens with a smaller sample size and at much earlier stages of disease progression compared to current medical diagnostic technologies.

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