Cell Adhesion

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DNA-Coated AFM Cantilevers for the Investigation of Cell Adhesion and the Patterning of Live Cells**

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The forces governing cell–cell adhesion are vitally important to many biological processes, including cell differentiation, tissue growth,[1,2] tumorigenesis,[1,3] and proper functioning of the vertebrate immune response.[4,5] The strengths of these interactions are typically characterized through the attachment of single living cells to probes that are capable of force measurement, such as suction micropipettes.[6,7] More recently, optical tweezers[8,9] have been applied to capture single cells and to measure these forces with high accuracy, but this technique is limited to applying forces in the piconewton range.[10] Atomic force microscopy (AFM)[11] provides an attractive alternative to these methods, because it is capable of quantifying forces in the piconewton to nanonewton range, and this technique has indeed been used to measure the mechanical properties of live single cells[12] and to study adhesion forces at the single-cell level.[13–19] Several fundamental adhesion measurements have been achieved by coating AFM cantilevers with fibronectin[19] or lectins[14,16–18] that bind to carbohydrate moieties on the cell surface, but especially in the latter case the cell-binding molecules themselves have been reported to have a degree of cytotoxicity that can influence the cellular properties being evaluated.[17,20–22] Thus, while these studies highlight the utility of AFM for the measurement of cell receptor–ligand interactions, an expanded set of cantilever attachment methods will be needed for the study of cell–cell interactions over widely varying time scales.

To address this need, we have compared three biomolecule-mediated methods for the attachment of live cells to AFM cantilevers, with an emphasis on the cell viability, adhesion strength, and probe reuse that each technique can achieve. These studies have indicated that cell attachment through the use of complementary DNA strands has the least influence on viability and does not appear to activate cell signaling pathways. This method also offers overall superior adhesion strength, but this parameter can be attenuated to allow cells to be transferred from one surface to another. We were able to demonstrate this concept by picking up free cells and placing them in exact positions on a substrate bearing DNA strands with longer complementary regions. This "dip-pen"[23–25] live-cell patterning demonstrates the reusability of the DNA-mediated cell adhesion method and could prove useful for the construction of complex mixtures of cells with well-defined spatial relationships.

To allow the comparison of several attachment strategies, three different biomolecules (DNA, concanavalin A (ConA), and an antibody) were attached to silicon nitride AFM cantilevers for cell anchoring. For all attachment methods, the thin layer of silicon oxide on the working surface was covered with aldehyde groups as outlined in Figure 1a. The surfaces produced using these steps were characterized by contact-angle measurements (see Figure S1 in the Supporting Information).

Amine-functionalized DNA was attached to the aldehyde groups through reductive amination (Figure 1b).[26,27] First, the aldehyde-coated cantilever was immersed in an amine-functionalized single-strand DNA (ssDNA) solution and then heated to promote imine formation. After cooling to room temperature, an aqueous solution of sodium borohydride was used to reduce the imines to nonhydrolyzable amine linkages. This step also served to reduce any unreacted aldehyde functional groups to alcohols. By coupling 5′-amine-functionalized DNA strands bearing fluorescein isothiocyanate (FITC) at the 3′ end, the presence of the strands could be

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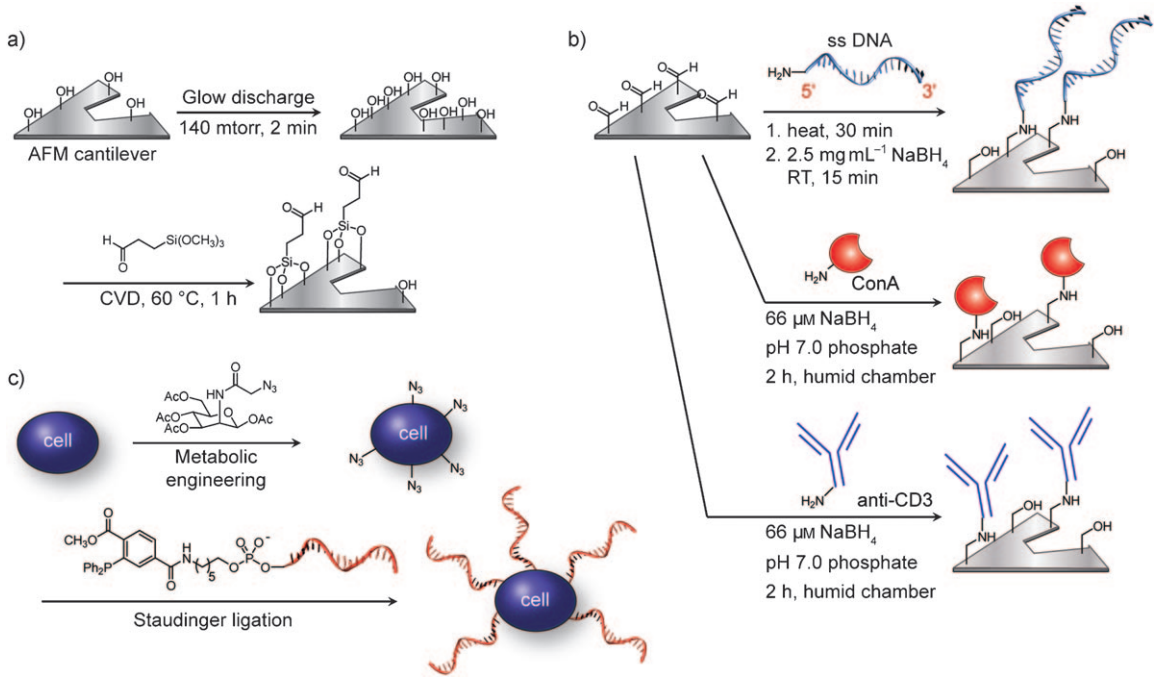

Figure 1. Covalent attachment of biomolecules to cantilevers and cell surfaces. a) After surface oxidation using an oxygen plasma, aldehyde functional groups were introduced onto silicon nitride cantilevers using chemical vapor deposition (CVD). b) Solutions of anti-CD3 IgG or ConA containing sodium borohydride were introduced onto aldehyde-coated cantilever surfaces in a humid chamber (IgG = immunoglobulin G). DNA modification was achieved by immersing cantilevers in an amine-functionalized ssDNA solution at 100 °C for 30 min and subsequent exposure to a sodium borohydride solution. c) Metabolic engineering was used to introduce azide groups onto cell surfaces by treatment with peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz). Phosphine-functionalized ssDNAs were synthesized and covalently attached to the exterior of cells by Staudinger ligation.

verified by fluorescence imaging (see Figure S2 in the Supporting Information).

In previous efforts, proteins have been attached to AFM tips through nonspecific adsorption and through glutaraldehyde crosslinking to amine groups introduced on the tip surface.[28] To afford more well-defined linkages (and thus realize more homogeneous cell attachment), we chose instead to use the simple reductive amination strategy that was used for the amino-DNA strands. Surface lysine residues on ConA and anti-human CD3 antibodies (anti-CD3) were reacted with the aldehyde functional groups on the cantilever surfaces (Figure 1 b),[29] but a lower concentration of reducing agent (66 μM) was used to minimize the reduction of disulfide bonds that are required to maintain protein tertiary structure.[30–32] The concentrations of the proteins (20 μM ConA and 6 μM anti-CD3) used in the reactions are easily achieved using commercially available samples. As described above for DNA, FTIC-labeled ConA and anti-CD3 samples were used in some experiments to verify biomolecule attachment using fluorescence microscopy (see Figure S3 in the Supporting Information). Similar levels of fluorescence were detected for each.

To prepare live cells bearing ssDNA on their surfaces, we first introduced azide functional groups into glycoproteins embedded in the plasma membrane, as previously described.[33] Peracetylated *N*-α-azidoacetylmannosamine (Ac_4ManNAz) was added to cells, which then metabolized and displayed the azide on their surfaces (Figure 1 c).[34] Triarylphosphine-modified ssDNA was prepared through the reaction of 5′-amine-modified ssDNA with a phosphine pentafluorophenyl (PFP) ester. This reagent was then used to label the cell-surface azide groups through Staudinger ligation,[35] yielding stable amide linkages. Flow cytometry experiments have previously verified the ability of phosphine–DNA conjugates to undergo ligation to azide-modified cell surfaces.[33] Although many cell types would be expected to be compatible with this system (and have been explored previously using the DNA-based adhesion method),[33] nonadherent Jurkat cells were chosen for these studies, because they do not secrete their own extracellular matrix. Thus, all cell adhesion events arise solely from the biomolecules on their surfaces.

The effects of the adhesion molecules on the viability of the cells were assessed using two different methods. First, suspensions of unmodified Jurkat cells were supplemented with ConA or anti-CD3 antibodies, and the solutions of DNA-coated cells were supplemented with the complementary sequence. Figure 2 a shows the growth curves of the resulting cells over a three-day period. The propagation of the DNA-modified cells was the same as that of unmodified cells, but the anti-CD3-treated cells showed delayed growth. ConA-coated cells aggregated and were no longer alive after 12 h. The morphologies of the cells after the addition of the reagents are shown in Figure S5 in the Supporting Information.

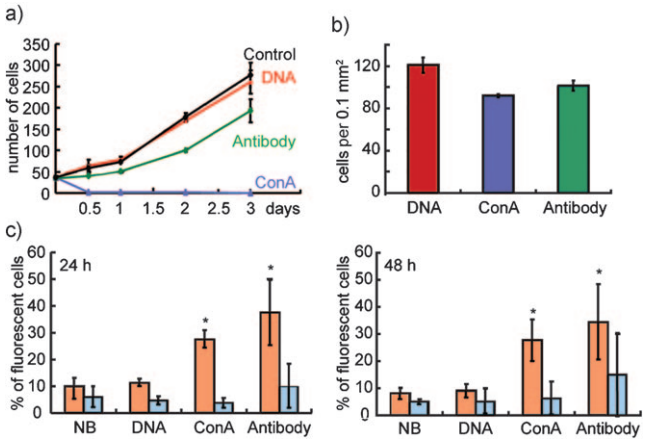

Figure 2. Comparison of biomolecule-based adhesion methods. a) Bulk cell growth rates were first determined in the presence of the adhesion molecules. A suspension of Jurkat cells was combined with ConA or anti-CD3 IgG, and a solution of DNA-coated cells was combined with the complementary DNA strands. At various time points the total number of cells was counted. The control sample was grown in the absence of any adhesion molecules. b) To evaluate cell capture efficiency, solutions of 20 μM FITC-labeled ssDNA, 20 μM FITC-labeled ConA, and 6 μM FITC-labeled anti-CD3 IgG were applied to aldehyde-coated glass slides, and the biomolecules were attached by reductive amination. Solutions containing 1×10^7 Jurkat cell mL^{-1} were then introduced onto the resulting slides. The samples were incubated for 10 min at room temperature and then washed with two portions of phosphate-buffered saline (PBS) before evaluation. c) To evaluate cell viability, cells were immobilized on DNA, ConA, and anti-CD3 IgG coated aldehyde slides. After immobilization for 24 and 48 h, the cells were incubated with a solution of annexin V–FITC (black bars) and PI (gray bars). The cells were evaluated within 1 h by fluorescence microscopy. * ConA and antibody immobilized cells that were partially stained by annexin were counted as cells undergoing apoptosis. NB represents control samples that were not bound to the surfaces. Error bars represent one standard deviation.

As a second comparison method, the three cell-adhesion molecules were coated onto commercially available aldehyde-coated glass slides using the same reductive amination procedures outlined above. By visual inspection, all three surfaces were able to achieve efficient cell binding (Figure 2b), but only the DNA-conjugated cells appeared morphologically unchanged after 48 h. The ConA- and anti-CD3-immobilized cells exhibited significant changes during this time period, likely owing to crosslinking of their surface receptors.[36–38] The viability of the surface-immobilized cells was determined after 24 and 48 h using annexin V and propidium iodide (PI) staining.[39] For the DNA-immobilized cells, the low percentage of apoptotic and necrotic cells was similar to that of unmodified cells (Figure 2c). However, the ConA and anti-CD3 immobilized cells showed significantly higher numbers of apoptotic cells compared to the control samples. Thus, the DNA molecules appear only to hybridize with their complementary partners and should have much less potential to disturb the overall physiology of the cells in force measurement experiments.

Live cells were readily captured by AFM tips bearing all three of the biomolecules. This capture was accomplished simply by touching the cell membrane with the cantilevers, with contact times as short as five seconds resulting in the transfer of the cells to the AFM tips (see Supporting Information Movie 1). No cells were captured by tips lacking the appropriate biomolecules.

Our assay to determine the strength of cantilever attachment was designed such that cell–cantilever adhesions were fewer in number, and therefore weaker overall, than DNA-based adhesions between a cell and the complementarily functionalized glass slide. Owing to this arrangement the cell–cantilever interaction would be expected to rupture first, yielding the strength of the interaction that a relatively low concentration of biomolecules can achieve. Rupture of the cell–cantilever interaction before the cell–surface interaction was verified by visual observation during experiments. The force of de-adhesion was measured for each attachment method using two different retraction rates and two different contact forces (Figure 3a). The measured force of de-

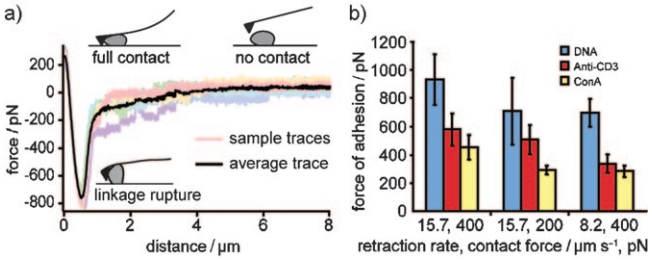

Figure 3. AFM measurement of de-adhesion force. a) Six sample traces for a single cell are shown in shades of gray, with the average trace shown in black. At zero distance, the cell is in full contact with the cantilever, which is applying a positive force. As distance increases, the cantilever is pulled away from the glass slide surface, causing the cell–cantilever linkage to rupture and result in the zero-force, no-contact region. The force of de-adhesion was calculated as the difference between the curve minimum and the horizontal no-contact region. b) Adhesion forces were measured under different retraction rates (15.7 and 8.2 μm s^{-1}) and contact forces (400 and 200 pN) for the DNA, ConA, and antibody systems. Data were obtained by measuring six de-adhesion events on more than four different cells. Error bars represent one standard deviation.

adhesion increased with contact force and retraction rate across all attachment methods, as predicted by the Bell model.[40] The ConA attachment method yielded zero-force attachment events in 12% of the de-adhesion measurements. Such events were not observed in the DNA and antibody cases.

A significant spread of forces was observed for all three attachment methods; however, under all experimental parameters, the DNA method displayed the strongest average adhesion, followed by antibody attachment, then ConA (Figure 3b). As a control experiment, we also demonstrated that the capture efficiency of ConA and anti-CD3 is not affected by the presence of DNA strands introduced on the cell surface (see Figure S6 in the Supporting Information). It should be noted that the overall de-adhesion forces determined for each attachment strategy depend on both the number of linkages and the retraction rates[40] and therefore do not reflect the absolute strengths of the individual

biomolecular interactions. For comparison, the force required to separate a typical 20 bp DNA duplex has been previously determined to be 38–50 pN,[41] suggesting that in our experiments roughly 20–25 individual linkages are made between the cell and the cantilever if the interaction strengths are assumed to be simply additive, though multiple parallel bonds can exhibit more complicated scaling behavior.[44] Similar reasoning would suggest that about ten ConA–mannose interactions (at 47 pN each[42]) and 12 antibody–antigen interactions (at 49 pN each[43]) are involved. Experiments to determine the number of linkages involved in each adhesion event are in progress to determine these effects more accurately. Nevertheless, our current results show that the DNA hybridization method leads to the most robust attachment under typical preparation conditions, even though the strength of each individual linkage is likely to be less than that of the other biomolecules.

The strength of the cell–cantilever interaction can be tuned by varying the number of interacting strands and the length of the complementary regions, and the reversibility of DNA hybridization also allows the tips to be used many times. Both of these advantages allowed us to use AFM tips to arrange cells one at a time into patterns. In a recent report,[45] it was shown that individual DNA strands could be moved from one location to another on a printed substrate, allowing small-molecule dyes to be printed in a similar fashion.

To do this, a 5 μM solution of a shorter DNA strand (13 bases) was applied to the cantilever, and an 80 μM solution of a longer strand (20 bases) was coupled to the glass slide. DNA-coated Jurkat cells were incubated in CO_2-independent media and applied to the uncoated side of glass slide under an AFM instrument. The cantilever was then lowered into contact with the cell for ten seconds with a contact force of 400 pN. The cantilever was then retracted, and cell attachment to the cantilever was confirmed visually (see Supporting Information Movie 1). The attached cells were then moved to the DNA-coated side with maximum rate of 1 mm s^{-1}. The cantilever was lowered into contact with the slide, and the cell was allowed to interact with the substrate for ten seconds with a 400 pN contact force. The cantilever was then retracted, whereupon the cell remained attached to the glass slide. By applying this printing method, cells can be given an (x,y) coordinate to position them precisely on a 2D substrate (Figure 4). The cells were found to remain viable after patterning (Figure S7 in the Supporting Information).

In summary, we have described the development of a versatile DNA-based adhesion method for the study of cell–cell interactions by AFM. The key advantages of this platform include the reusability of the tip, the tunability of the interaction strength, and the use of well-defined chemical linkages. Of the three biomolecule-based attachment strategies that were used, the DNA method proved superior in terms of cell viability after attachment. The use of AFM to form accurate and programmable patterns of individual cells provides a useful tool to understand the influence of neighboring interactions on cell differentiation and regulation. In a previous report, we have shown that complex patterns can be prepared through the self-assembly of DNA-coated cells on surfaces printed with complementary oligonucleotides.[46] The AFM dip-pen method described herein provides a useful complement to this technique that can achieve the higher resolution needed to create and interrogate clusters consisting of multiple cell types. We are currently using this method to elucidate fundamental adhesion mechanisms involved in cancer metastasis, immune synapse formation, and cell–cell communication.

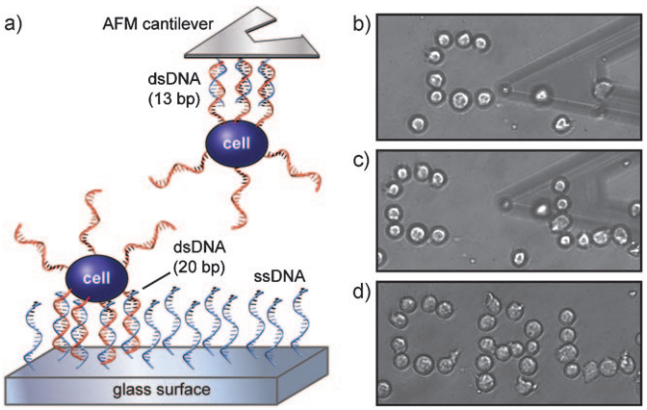

Figure 4. Dip-pen patterning of live cells. a) By attaching a shorter DNA strand (13 bases) to a cantilever and a longer strand (20 bases) to the glass slide, a single living cell can be transported by the AFM instrument and directly printed at a desired location on the glass slide. b–d) This process shown stepwise for the formation of a single pattern of cells.

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