

Transport and Detection of Unlabeled Nucleotide Targets by Microtubules Functionalized With Molecular Beacons

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ABSTRACT: Shrinking biosensors down to microscale dimensions enables increases in sensitivity and the ability to analyze minute samples such as the contents of individual cells. The goal of the present study is to create mobile microscale biosensors by attaching molecular beacons to microtubules and using kinesin molecular motors to transport these functionalized microtubules across two-dimensional surfaces. Previous work has shown that microfluidic channels can be functionalized with kinesin motors such that microtubules can be transported and directed through these channels without the need for external power or pressure-driven pumping. In this work, we show that molecular beacons can be attached to microtubules such that both the fluorescence reporting capability of the beacon and the motility of the microtubules are retained. These molecular beacon-functionalized microtubules were able to bind ssDNA target sequences, transport them across surfaces, and report their presence by an increase in fluorescence that was detected by fluorescence microscopy. This work is an important step toward creating hybrid microdevices for sensitive virus detection or analyzing mRNA profiles of individual cells.

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Introduction

In eukaryotic cells, kinesin motor proteins move along microtubule filaments, transporting intracellular vesicles

to the cell periphery and powering the movements of chromosomes during cell division. Because of this robust microscale transport ability, there is considerable interest in integrating this biological transport system into engineered microdevices. Microtubule-kinesin based systems have been shown to transport biological cargo such as viruses and antibodies in vitro (Bachand et al., 2006; Ramachandran et al., 2006), and microfabricated channels functionalized with kinesin motors have been used for accumulating large populations of microtubules and controlling their direction of transport (Cheng et al., 2005; Hiratsuka et al., 2001; Huang et al., 2007; Jia et al., 2004; Kim et al., 2007; van den Heuvel et al., 2006). Achieving controlled transport and rapid detection of ssDNA and RNA molecules is important for a range of potential microscale biosensors and lab-on-a-chip applications, and the kinesin-microtubule system is a promising mechanism for this. Molecular beacons (MBs) are single strands of labeled DNA that increase their fluorescence upon hybridizing RNA or DNA complementary to the beacon's 15–25 base probe sequence (Tyagi and Kramer, 1996). Our goal is to bring together the transport capabilities of the kinesin-microtubule system with the detection abilities of molecular beacons to develop a novel microscale approach for transporting and detecting minute quantities of ssDNA or RNA.

Developing systems that transport and detect ssDNA and mRNA at the microscale are of significant interest because they have the potential for increasing the sensitivity of detection and the specificity of separation. DNA microarrays and Northern blots are very useful for profiling gene expression levels, but they generally require large input samples, which are often achieved through PCR amplifica-

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tion. Developing methods to quantify mRNA expression in single cells would be an important advance because it would eliminate the inherent signal averaging that occurs when analyzing a population of heterogeneous cells taken from, for instance, a tissue biopsy (Levsky et al., 2002; Sims and Allbritton, 2007). A highly sensitive system for detecting ssDNA or RNA could be used for virus detection in clinical as well as food safety and homeland security applications. Because kinesin motors in microchannels can transport microtubules up concentration gradients and against fluid flow (Huang et al., 2005; Huang et al., 2007), this system can be used to concentrate analytes and transport them away from interfering background signals, resulting in increased signal-to-noise ratios for detection. An envisioned micro-engineered device (Jia et al., 2004) would include a chamber for introducing a sample such as the lysate from an individual cell, kinesin functionalized channels through which the microtubule-bound DNA or RNA would be transported and detected, and a reservoir for collecting and concentrating the analyte.

Past work has shown that kinesin-driven microtubules can be functionalized with cargo such as nanoparticles (Bachand et al., 2004), fluorescently labeled DNA oligonucleotides (Taira et al., 2006), and RNA aptamers (Hirabayashi et al., 2006). However, to date no groups have been able to functionalize microtubules so that they both detect and transport unlabeled DNA or RNA targets; our work here is a step toward this goal. As a target, we have chosen Simian virus 5 (SV5), a member of the Paramyxoviridae family, which encompasses viruses such as the measles and mumps (Lin et al., 2005). SV5 is believed to be capable of infecting humans (Cohn et al., 1996), but there is no association of SV5 with diseases or symptoms (Hsiung et al., 1965), making SV5 an excellent model virus to work with.

In this report, we demonstrate that molecular beacons can be attached to microtubules so as to retain both the fluorescent beacon function and the kinesins' ability to transport microtubules along a surface (Fig. 1). We show that, although secondary structure complicates detection of viral mRNA, moving microtubules are able to fluorescently report the presence of ssDNA targets, validating this approach as a strategy for sensitive detection in future hybrid microdevices.

Materials and Methods

Kinesin and Microtubules

Bovine brain tubulin was isolated and purified as previously described (Williams and Lee, 1982), and a subset was labeled with either Alexa Fluor 647 carboxylic acid succinimidyl ester (Molecular Probes, Invitrogen, Eugene, OR) or biotin, using a standard protocol (Hyman et al., 1991). Microtubules were polymerized with a 10:9:1 ratio of biotinylated tubulin/unlabeled tubulin/Alexa 647 labeled tubulin, as previously described (Muthukrishnan et al., 2006), and

stabilized with 10 μ M paclitaxel (Sigma-Aldrich Co., St. Louis, MO). Unless otherwise noted, experiments were performed in BRB80 buffer (80 mM PIPES, 1 mM MgCl₂, 1 mM EGTA, pH to 6.9 with KOH). *Drosophila* conventional kinesin was bacterially expressed and purified as previously described (Hancock and Howard, 1998).

Molecular Beacons and Target DNA Oligonucleotides

Two molecular beacons were designed with target sequences complementary to the V/P gene of SV5 (GenBank Accession # AF052755). Molecular beacon 1 (MB1), purchased from Integrated DNA Technologies (Coralville, IA), had the sequence 5'(6-FAM) **AGGCAGTTGACTCTTGATCTT-TGCCT** (Dabcyl)3', where bold indicates bases in the self-hybridizing stem. The loop and the 5' stem were complementary to bases 227–248 of the SV5 V/P gene. Based on results from MB1, an mRNA folding program (RNAstructure 4.0, University of Rochester) was used to identify regions of the SV5 V/P mRNA that are less likely to contain secondary structure that could inhibit hybridization to the molecular beacon. The sequence of Molecular Beacon 2 (MB2) was 5'(Alexa 546) **CGAGGTTTGGTCGCGGTTT-CTTTGGTCC-dT-CG** (BHQ1)3', where the bold-face nucleotides are in the self-hybridizing stem, and the dT indicates the biotinylated thymine base. MB2, purchased from TriLink Biotechnologies, Inc. (San Diego, CA), was complementary to bases 285–305; unless otherwise stated, all experiments described below were performed with MB2. An identical DNA oligonucleotide, complementary to the entire probing sequence of the MB2, was also synthesized and used as a target ssDNA sequence. The sequence of this DNA oligo is 5'-ACCAAAGAAACCGCGACCAAA-3'.

In Vitro Viral RNA Synthesis

A plasmid containing several SV5 genes, pBH311, was provided by Dr. Biao He's lab (Penn State University). PCR was used to amplify cDNA for the V/P gene, and a T7 MAXIscript in vitro transcription kit (Ambion, Inc., Foster City, CA) was used to transcribe mRNA from the cDNA. The transcription reaction was incubated for 1 h at 37°C. RNA was purified by phenol–chloroform extraction followed by ethanol precipitation, and the concentration was measured in a spectrophotometer before use in the hybridization reactions.

Functionalizing Microtubules With Molecular Beacons

To attach molecular beacons, 0.7 μ M neutravidin (Pierce Biotechnology, Inc., Rockford, IL) was added to a solution of biotinylated microtubules (0.43 μ M total tubulin concentration) in BRB80 plus 10 μ M paclitaxel, and the solution was gently vortexed for 20 s. Molecular beacons were then added to a concentration of 1 μ M and briefly

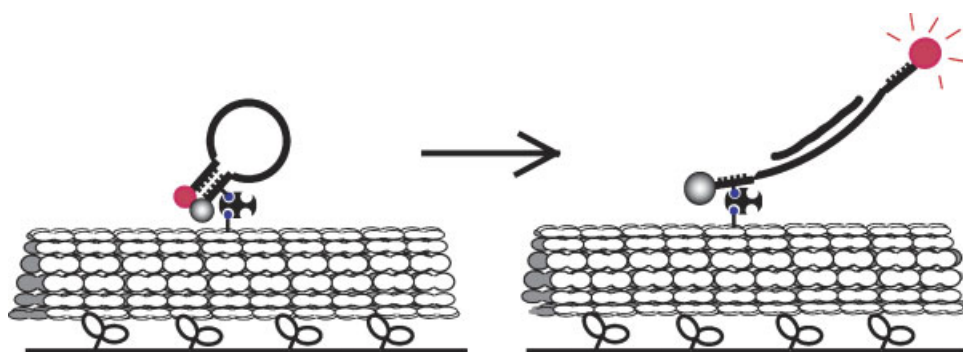


Figure 1. Schematic of molecular beacons on microtubules. Biotinylated molecular beacons are attached to biotinylated microtubules through a neutravidin bridge. An unlabeled complementary DNA or RNA target hybridizes to the molecular beacon in a quenched state, opening the beacon and enhancing the beacon fluorescence. Kinesins immobilized on the surface transport these molecular beacon-functionalized microtubules. The molecular beacon is enlarged for clarity—the actual dimensions are as follows: microtubules are 25 nm diameter, neutravidin is ~5 nm in diameter, the beacon stem is 1.7 nm long, and the beacon loop has a diameter of 2.3 nm. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

vortexed. This solution was incubated at room temperature for 25 min, and the microtubules were then pelleted by centrifugation at 30 psi (~95,000 rpm or 178,000 g) for 10 min in a Beckman Airfuge to remove any unbound neutravidin and beacons. Pelleted microtubules were resuspended in BRB80 containing 0.2 mg/mL casein and 10 μ M paclitaxel.

Microtubule Gliding Assay

Flow cells with volumes of 10–15 μ L were constructed using a microscope slide (Fisher Scientific, Pittsburgh, PA), coverglass (Corning #1.5, 18 mm square), and double-sided tape as a ~100 μ m thick spacer. The glass was pretreated with casein by adding 0.5 mg/mL casein in BRB80 and incubating for 5 min. Kinesins were immobilized on the glass by introducing a solution of 0.5 μ g/mL kinesin in BRB80 supplemented with 0.2 mg/mL casein and 1 mM ATP, and incubating for 5 min. Finally, the beacon-functionalized microtubules in motility solution (BRB80 with 0.2 mg/mL casein, 10 μ M paclitaxel, 1 mM ATP, 20 mM D-Glucose, 0.02 mg/mL glucose oxidase, 8 μ g/mL catalase, 0.5% 2-mercaptoethanol) were introduced. For quantifying fluorescence intensity, some experiments replaced the ATP in the motility solution with the nonhydrolyzable ATP analog AMP-PNP (1 mM) to immobilize the microtubules on the kinesin-functionalized surface.

Characterizing MB2 Fluorescence in Solution With Neutravidin and a ssDNA Target

A spectrofluorometer (Photon Technology International, Lawrenceville, NJ) with Felix 1.42 software (Photon Technologies International) was used to quantify the fluorescence of a 100 μ L sample of 0.1 μ M beacons in BRB80 buffer in a 45 μ L cuvette (Starna Cells, Inc., Atascadero, CA). The sample was excited at 556 nm and the

peak fluorescence at 574 nm was recorded. Neutravidin was added to a concentration of 1 μ M, and then complementary DNA oligo was added to 4.5 μ M. The fluorescence was measured after each addition, and the fluorescence of BRB80 buffer alone was measured and this background subtracted from all measurements. Volume changes were accounted for in the data presented.

Quantifying the Fluorescence of Molecular Beacons on Microtubules by TIRFM

Microtubules were visualized by Total Internal Reflection Fluorescence Microscopy (TIRFM) using a Nikon TE2000 inverted microscope (60 \times , 1.45 NA, CFI Plan Apo TIRF oil objective), and imaged using a Photometrics Cascade 512B CCD camera (Photometrics, Roper Scientific, Tucson, AZ). MetaVue software (Molecular Devices Corp., Downers Grove, PA) was used to quantify fluorescence. Alexa 546 on the molecular beacons was excited at 543 nm and the emission observed in the range of 600 ± 37 nm. Alexa 647 on the tubulin was excited at 633 nm and the emission observed in the range of 685 ± 37 nm. To quantify beacon fluorescence, line scans were taken along the lengths of a number of microtubules to obtain a fluorescence value for each microtubule. Background fluorescence was calculated by measuring the mean fluorescence in microtubule-free areas of the screen. The data are presented as fluorescence values after subtracting the background.

Molecular Beacon Hybridization to Viral mRNA

To determine the fluorescence kinetics and amplitude upon RNA binding, MB1 was suspended in BRB80 to a concentration of 100 nM, and then a threefold excess of mRNA was added to the solution. The sample was excited at 491 nm in the spectrofluorometer, and the fluorescence intensity was measured once per second at 509 nm. For the faster hybridization experiment shown in Figure 6B, the

cuvette holder was heated to 40°C, MB2 was suspended to a concentration of 50 nM in BRB80 plus 150 mM NaCl, and then a fivefold excess of mRNA was added to the cuvette. Fluorescence intensity readings were taken at a rate of once per second by exciting at 556 nm and measuring the fluorescence at 574 nm. Fluorescence readings account for the dilution of the MBs due to volume changes.

Results

The goal of this study was to achieve capture and fluorescence detection of unlabeled nucleotide targets on microtubules moving across kinesin-coated surfaces. We first formulated a strategy using biotin–avidin to attach the beacons to microtubules and confirmed that these microtubules move across kinesin-coated surfaces. We next tested the fluorescence reporting of molecular beacons in the presence of a complementary ssDNA target both in solution and on the microtubules. Finally, we investigated the binding kinetics of viral mRNA to both MB1 and MB2 under conditions that are compatible with the kinesin-microtubule system. This viral mRNA target was used to explore the potential for integrating this novel transport and detection scheme into future viral biosensors.

Beacons on Microtubules

The first question we addressed is: can molecular beacons be attached to microtubules and visualized by fluorescence microscopy? For our experiments, it is important to be able to visualize both the microtubules (labeled with Alexa 647)

and the molecular beacons attached to them (labeled with Alexa 546). It is important to confirm that there is minimal bleedthrough of fluorophores to the complementary filter set, and that there are no complications from fluorescence energy transfer between the fluorophores. Motility assays were carried out containing mixtures of beacon-functionalized and control microtubules, and the microtubule and beacon fluorescence were imaged using TIRF microscopy. In Figure 2A, the red filter set was used to image the Alexa 647 labeled microtubules, while in Figure 2B the green filter set was used to image the Alexa 546 labeled beacons. To enhance the beacon fluorescence, the beacon-functionalized microtubules were incubated with an excess of unlabeled complementary DNA oligonucleotides prior to imaging. All microtubules could be observed with the red filter, while only microtubules with molecular beacons could be observed under the green filter. This result also shows that there is minimal fluorescence overlap between the two filters (the tubulin fluorescence does not leak through when the green filter cube is used). Hence, we are able to visualize the fluorescence from molecular beacons that are on microtubules, and we confirm that the fluorescence is indeed from the beacons and not from the labeled tubulin.

Beacon-Functionalized Microtubules Move Along a Kinesin-Coated Substrate

The second question we addressed is: do microtubules labeled with molecular beacons retain their ability to act as tracks for kinesin motor proteins? We approached this question by testing whether beacon-labeled microtubules could be transported along kinesin-functionalized

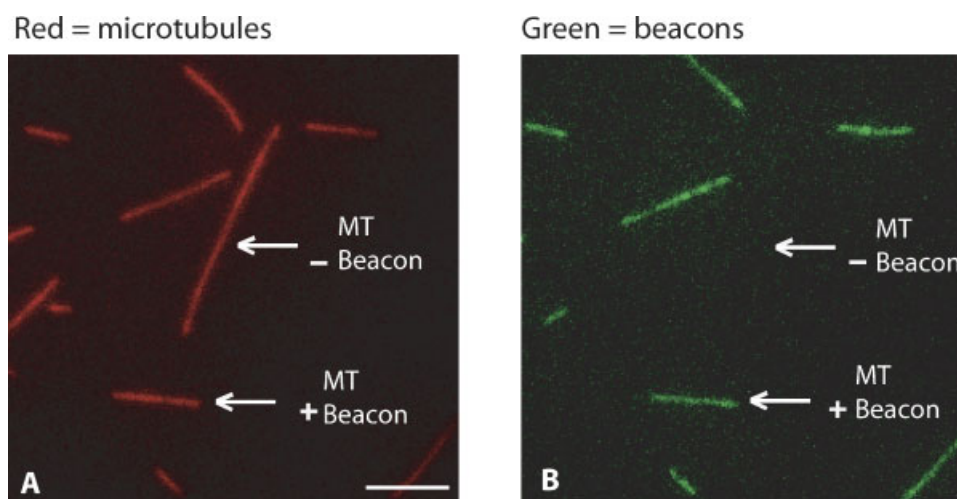


Figure 2. Molecular beacons on microtubules (MT) in the microtubule gliding assay. The assay contains microtubules with and without molecular beacons. **A:** All microtubules can be seen under the red filter because they all contain tubulin labeled with Alexa 647. **B:** Microtubules functionalized with molecular beacons (containing the Alexa 546 fluorophore) can be seen under the green filter, but beacon-free microtubules cannot, demonstrating that fluorescence is not from bleedthrough from the red fluorophore. One control microtubule (MT - Beacon) and one beacon-functionalized microtubule (MT + Beacon) are highlighted. Images were taken using a black and white camera and false colored for clarity. Complementary DNA oligonucleotide is present in this experiment. Scale bar is 5 μm . [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

surfaces. A standard microtubule gliding assay containing surface-adsorbed kinesin motors was set up, control or beacon-labeled microtubules were introduced into the flow cell in the presence of ATP, and microtubule gliding velocities were recorded. The motility assays contained a saturating level of unlabeled complementary DNA target to ensure that the beacons are fully functional. Figure 3 shows sequential images in 5-s intervals of several beacon-functionalized microtubules. Microtubules without beacons moved at an average velocity of 820 ± 53 nm/s ($N=9$), and microtubules labeled with beacons moved at an average velocity of 560 ± 91 nm/s ($N=14$). In a similar experiment, Taira et al. (2006) found that binding a linear DNA oligonucleotide to a microtubule through streptavidin caused a 35% decrease in the gliding velocity, similar to the 32% decrease observed here. Seitz and Surrey (2006) found that quantum dot-functionalized kinesins moved ~ 3 -fold slower along microtubules that had been densely covered with irreversibly bound kinesin heads, consistent with the idea that blocking the path that kinesins take along a microtubule causes the motor to slow somewhat. Because the reduction in speed here is less than two-fold and other transport properties were not noticeably affected, the ~ 3 min that a beacon-labeled microtubule would take to move $100 \mu\text{m}$ should be suitable for the envisioned microscale transport applications.

Fluorescent Characterization of Molecular Beacons in Solution

Before quantifying the fluorescence reporting characteristics of the molecular beacons bound to microtubules, we first characterized these beacons in solution in the absence of microtubules. The experimental goal was to test the fluorescence enhancement from the beacons when they hybridize to their complementary DNA target. A baseline beacon fluorescence was measured by quantifying the fluorescence of $0.1 \mu\text{M}$ of molecular beacon MB2 in BRB80 in a spectrofluorometer, and normalizing this value to 1.0. Because the beacons are attached to the microtubules through a bridging neutravidin molecule, we next tested the fluorescence of the beacon when bound to neutravidin in solution. A 10-fold excess of neutravidin ($1 \mu\text{M}$ neu) was added to the beacon solution; this excess was chosen to reduce the likelihood of having multiple biotinylated beacons bound to any neutravidin molecule, thus minimizing any interactions between beacons. In the presence of neutravidin, the fluorescence increased to a value of 2.4 times that of the beacons alone (Fig. 4). This finding that neutravidin binding causes an increase in beacon fluorescence, even in the absence of a complementary DNA target, suggests that the neutravidin binding to the biotin on the beacons destabilizes the self-hybridization in the beacon stem, causing it to spend a greater fraction of the time in the open (fluorescing) conformation. Finally, a saturating concentration ($4.5 \mu\text{M}$) of complementary DNA

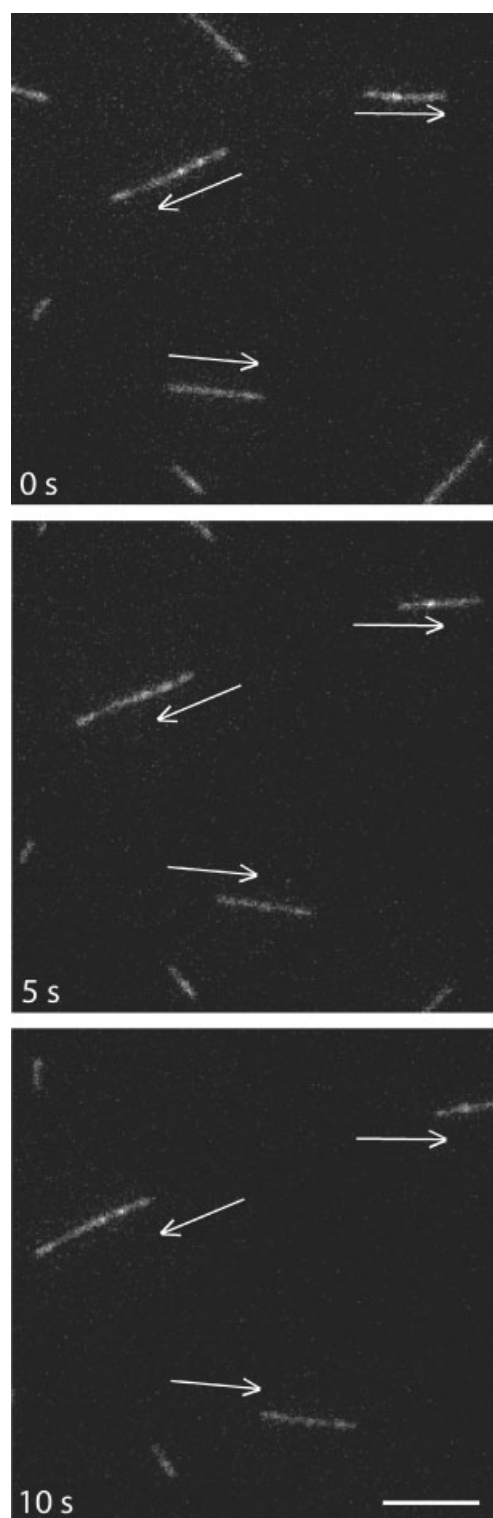


Figure 3. Immobilized kinesins transport molecular beacon-functionalized microtubules in the microtubule gliding assay. All microtubules in the images have molecular beacons attached to them. Images are taken at 5-s intervals, and the white arrows show the direction of the MT movement. Scale bar is $5 \mu\text{m}$.

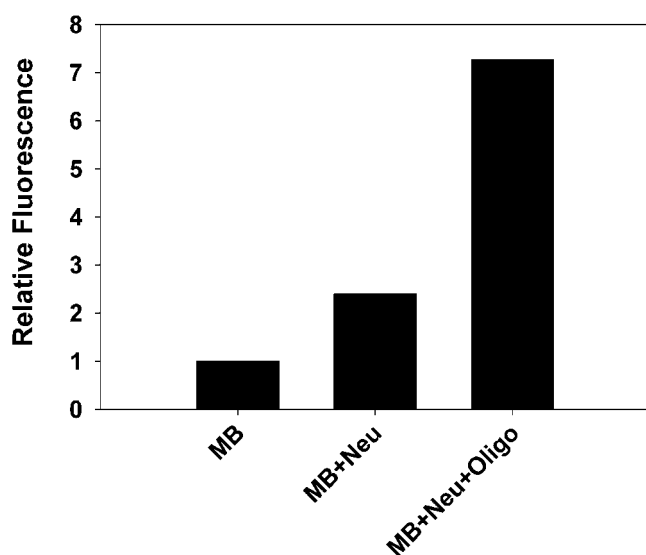


Figure 4. Characterization of molecular beacon fluorescence in solution. Molecular beacon MB2 was suspended in BRB80 buffer at a concentration of 0.1 μM , excited at 556 nm in a spectrofluorometer, and the signal at 574 nm recorded. The fluorescence intensity of beacons alone is normalized to 1. Neutravidin (Neu) was added to a concentration of 1 μM , complementary DNA oligonucleotide target (Oligo) was added to a concentration of 4.5 μM . Volume changes from the additions of neutravidin and the oligo were accounted for in the fluorescent values shown.

oligonucleotide was added to the solution to hybridize all of the beacons in solution (Fig. 4). Maximal fluorescence was reached after less than 1 min from the time of adding the complementary DNA oligo, indicating fast hybridization kinetics. DNA binding increased the fluorescence to a value 7.3 times that of the beacons alone, or threefold higher than the neutravidin-bound beacons in the absence of complementary target.

Fluorescence Reporting of Microtubule-Bound Molecular Beacons

After characterizing the molecular beacons in solution, we then quantified the fluorescence reporting of beacons bound to microtubules. Biotinylated microtubules were incubated first with neutravidin and then with molecular beacons, and the beacon-labeled microtubules were introduced into a kinesin-functionalized flow cell in the presence of AMP-PNP. In the presence of AMP-PNP, kinesin motors bind tightly to microtubules, immobilizing them on the surface (Romberg and Vale, 1993), enabling accurate quantification of the fluorescence by total internal reflection fluorescence microscopy. An image of the surface-immobilized microtubules was captured using a 16-bit Cascade 512B CCD camera, and the microtubule fluorescence intensities quantified using MetaVue software (Fig. 5A). Next, fresh motility solution containing 3.75 μM complementary DNA oligonucleotide was introduced into the flow cell. After a ~ 2 min incubation, images were captured using identical

camera settings and illumination, and the microtubule fluorescence intensities were quantified (Fig. 5B). Qualitatively, the microtubules appear brighter in the presence of the complementary target, and quantitative analysis confirms that the average fluorescent intensity increased by a factor of 2.2 from $3,765 \pm 1,240$ (mean \pm SD, $N = 40$) to $8,260 \pm 2,830$ ($N = 33$). A t -test for these two fluorescence intensity populations gives a t -value of 8.5 ($P < 0.0005$), confirming the statistical significance of this result. Consistent with this result, when a spectrofluorometer was used to measure the fluorescence of beacon-functionalized microtubules in solution, the addition of a complementary DNA oligonucleotide led to a 2.1-fold increase in fluorescence (data not shown). While this enhancement is less than the ~ 7 -fold observed in solution between beacons with complementary DNA and beacons alone, it agrees well with the 3-fold increase seen between beacons with complementary DNA and beacons bound to neutravidin in solution (Fig. 4).

Kinetics of SV5 Viral mRNA Binding to Molecular Beacons

After demonstrating that molecular beacon-functionalized microtubules can report the presence of target DNA oligonucleotides, we examined the beacons' ability to report the presence of SV5 viral mRNA. To extend the microtubule-molecular beacon system to mRNA targets, the molecular beacons must bind mRNA with reasonable kinetics, they must fluoresce with a sufficient amplitude, and they need to function in a buffer that is compatible with the kinesin-microtubule system. In preliminary experiments, we designed a molecular beacon (MB1) complementary to an arbitrary region of the SV5 V/P gene. We observed extremely slow kinetics of hybridization between MB1 and mRNA: after 20 min the fluorescence increased to only 1.4 \times , and even after 2 h, there was no sign of a plateau in the beacon fluorescence (Fig. 6A). We interpreted these slow kinetics to be caused by slow unfolding of the mRNA secondary structure in this region, and so to improve on this result we used the software package RNAstructure to identify regions of the mRNA that lack significant secondary structure. We then designed a molecular beacon (MB2) complementary to bases 285–305 of the SV5 V/P mRNA. Unless noted, all of the experiments described above used MB2. Despite this improved design, we found that the kinetics of hybridization between MB2 and mRNA at room temperature were still quite slow, which contrasts with the very fast kinetics ($\tau < 30$ s) of complementary DNA oligonucleotide binding to MB2 (data not shown).

To identify experimental conditions that maximize the RNA hybridization kinetics, we systematically altered the buffer composition and temperature and found that two important variables are the hybridization temperature and the NaCl concentration. Therefore, we increased the temperature to 40 $^{\circ}\text{C}$ and supplemented our buffer with

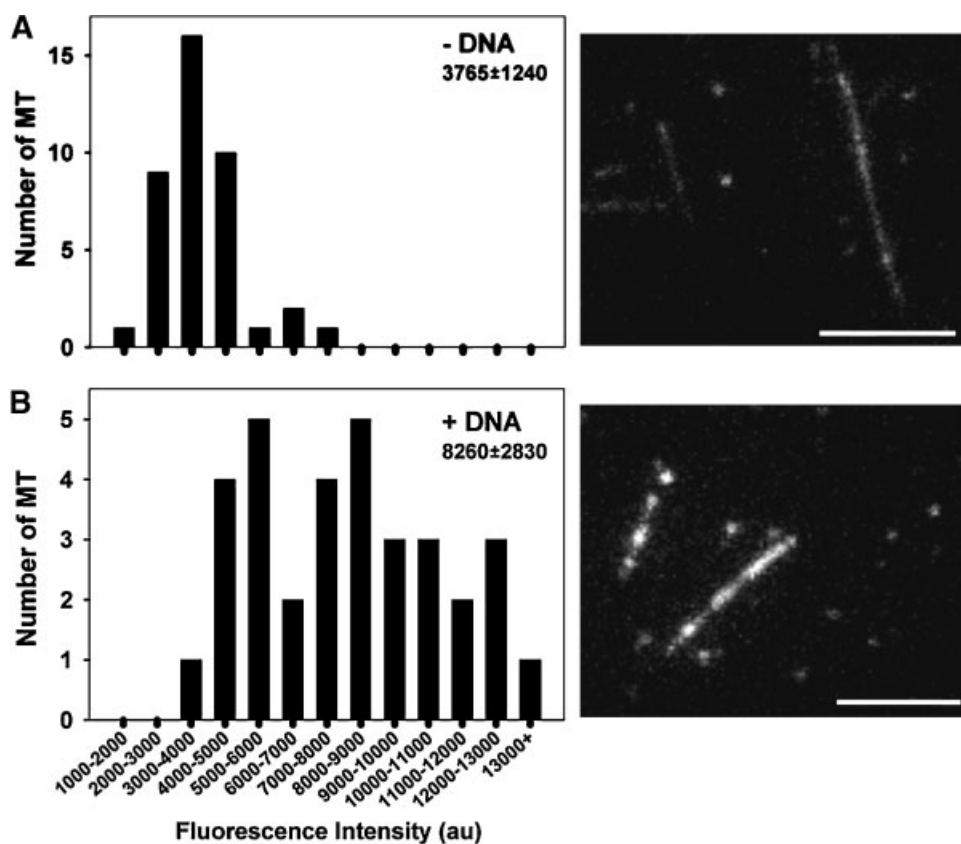


Figure 5. Molecular beacons bound to microtubules report the presence of complementary DNA targets. Microtubules were immobilized on kinesin-functionalized glass surfaces and the fluorescence intensity under total internal reflection fluorescence microscopy captured and quantified. **A:** Microtubules with molecular beacons before adding complementary DNA oligo, showing a histogram of fluorescence intensities on the left and a representative image on the right. **B:** Molecular beacon-functionalized microtubules in the presence of complementary DNA oligonucleotide. Fluorescence quantification revealed a 2.2-fold increase in fluorescence in the presence of the DNA target. Scale bars are 5 μm .

150 mM NaCl. Bohm et al. (2000) found that kinesins remain functional at 40°C, and in control experiments we confirmed that microtubule transport is not affected by the addition of 150 mM NaCl. MB2 (50 nM) was suspended in BRB80, and then a fivefold excess of mRNA was added to the solution. The faster kinetics ($\tau = 4.83$ min) of molecular beacon fluorescence upon binding to SV5 mRNA are shown in Figure 6B; the fluorescence increases roughly 1.8-fold and plateaus after approximately 15 min. To confirm that the beacons were maximally fluorescing and that hybridization was complete, an excess of DNA oligonucleotides was added, and the fluorescence did not change (data not shown). Under these improved conditions, the kinetics are faster, but the fluorescence increase is substantially less than the experiment with the DNA oligo in Figure 4 (~1.8-fold with RNA compared to 7.3-fold with DNA). We interpreted this reduced amplitude to the fact that, although the mRNA structure is destabilized at 40°C, the molecular beacons are also partially opened at this temperature. Hence, as with binding to neutravidin (Fig. 4), increasing temperature also appears to disturb the beacon conformation resulting in an

elevation in the off-state fluorescence. Because there was less than a twofold increase in fluorescence enhancement with the beacon free in solution, we anticipated that after binding to neutravidin and to the microtubule, the fluorescence reporting upon RNA binding would be negligible. Hence, further redesigning of the beacon is required for beacons to be able report RNA binding when bound to microtubules.

Discussion

Enhancing the ability to sensitively detect RNA or ssDNA in microengineered devices is an important step towards developing novel instrumentation for quantifying gene expression profiles in single cells or for detecting viruses from minute samples. Using microtubules as mobile bioprobes in such devices has the potential for increasing the sensitivity of detection by concentrating analytes and separating targets from undesired background signals. A number of groups have developed approaches for controlling kinesin-driven microtubule motility in engineered

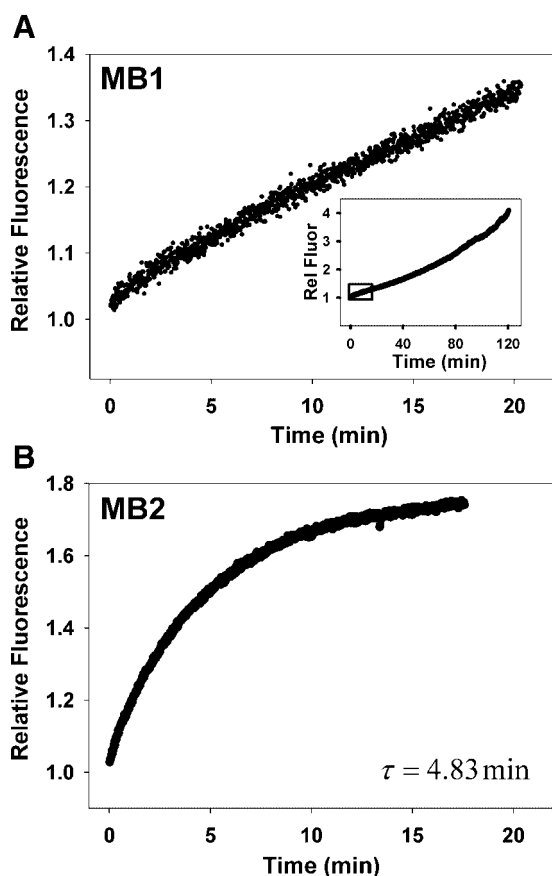


Figure 6. Molecular beacon fluorescence in the presence of viral mRNA. In contrast to ssDNA, which generally hybridized to the molecular beacons in under 1 min, viral mRNA hybridized slowly. **A:** Hybridization kinetics of SV5 V/P mRNA to beacon MB1 in BRB80 buffer at room temperature. The first 20 min are shown to provide a comparison to **panel B**. Note that even after 2 h, the fluorescence does not plateau (inset). **B:** The hybridization kinetics of SV5 V/P mRNA to MB2 was substantially faster due to targeting a different region of the mRNA, adding NaCl to the buffer, and increasing the temperature to 40 °C. The data were fit to a rising exponential with a time constant of 4.83 min. To confirm that the beacons in panel B are fluorescing maximally, excess DNA oligonucleotide complementary to the beacon was added at the end of the experiment and found to cause no change in beacon fluorescence.

microchannels (Cheng et al., 2005; Clemmens et al., 2003; Hess et al., 2002; Jia et al., 2004; Moorjani et al., 2003; van den Heuvel et al., 2006). Because these devices are fabricated using standard photolithography processes, integrating LEDs and photodiodes is an obvious next step that would enable sensitive fluorescence detection of analytes in these channels by bringing the fluorophores in close proximity with detectors.

An important step towards developing hybrid micro-devices for virus detection or mRNA analysis is demonstrating the ability to detect RNA or ssDNA on kinesin-transported microtubules. We chose molecular beacons for this study because they bind to specific ssDNA or RNA sequences and they report the interaction by fluorescence without the need for labeling the target sequence. For this microtubule-molecular beacon system to work optimally,

(a) the amplitude of beacon fluorescence enhancement must be measurable, (b) the kinetics of target binding must be sufficiently fast to enable rapid detection, and (c) the microtubules must retain their ability to move over a surface of immobilized kinesins. By polymerizing microtubules from a mixture of unlabeled tubulin, fluorescent tubulin and biotinylated tubulin, the microtubules retained proper interactions with immobilized kinesin motors, they could be observed by fluorescence microscopy, and they could be labeled with molecular beacons through a bridging neutravidin molecule. The level of molecular beacon labeling was sufficient for observation and quantification by fluorescence microscopy, and it is expected that because the beacons are concentrated on the microtubule, these fluorescent signals will be measurable by integrated detectors.

One interesting phenomenon we observed was that in the absence of target, binding neutravidin to the biotinylated molecular beacons increased the molecular beacon fluorescence, presumably by partially opening the stem. The result was that molecular beacons bound to neutravidin had a maximum of only a threefold fluorescence enhancement upon target binding (Fig. 4). Others have also observed that, compared to beacons free in solution, beacons immobilized on surfaces show smaller degrees of fluorescence enhancement upon target binding (Fang et al., 1999; Liu et al., 2000; Liu and Tan, 1999; Wang et al., 2002), but the mechanisms of the diminished fluorescence enhancement are not entirely clear. In some cases surface chemistry plays a role—Wang et al. (2002) found that beacons that displayed a 50-fold fluorescence enhancement upon DNA binding in solution had only 5-fold enhancement when covalently linked to agarose surfaces and <2-fold enhancement on glutaraldehyde-treated amino silane surfaces. Similarly, Li et al. (2001) found that attaching biotinylated beacons to a biotinylated surface through a bridging avidin molecule led to a maximal fluorescence enhancement of only 2.7-fold. In our work, where the microtubule and the beacon are joined through a bridging neutravidin molecule, the microtubule presents a very different surface than the glass or other materials normally employed for immobilizing beacons for sensor applications. Accordingly, the 2.2-fold fluorescence enhancement of beacons on microtubules was very similar to the 3-fold enhancement we observed for beacons bound to neutravidin in solution, strongly implying that neutravidin binding and not surface interactions was the reason that the fluorescence enhancement of beacons on microtubules was less than the value for beacons free in solution.

The molecular beacons used our experiments (MB2) were biotinylated on an internal thymine base on the quencher side of the beacon stem. This is the most popular approach for biotinylating molecular beacons and is used because it offers easy synthesis and, in theory, it avoids problems with fluorescence quenching (when internal fluorophores or quenchers are used) and target hybridization (if the biotinylated base is in the loop; Culha et al., 2004; Fang et al., 1999; Li et al., 2001; Liu and Tan, 1999; Liu et al., 2000;

Tan et al., 2000; Yao and Tan, 2004; Zhao et al., 2003). Yao and Tan (2004) suggested that, instead of biotinylating the beacon itself, a greater fluorescence enhancement can be achieved by adding an extension to one arm of the beacon stem and immobilizing the beacon through hybridization to a complementary biotinylated oligonucleotide that is bound to an avidin-coated surface. However, their results show that at pH 7, beacons immobilized in this manner showed only a threefold fluorescence enhancement upon DNA binding, similar to our present results.

While the amplitude and kinetics of molecular beacon fluorescence were adequate for the current study when using ssDNA oligo as the target, improving beacon performance is an area of future work for targeting RNA. In contrast to the rapid kinetics of ssDNA binding to the beacons, binding to RNA was considerably slower, which we attributed to slow unfolding of RNA secondary structure. While we attempted to detect the SV5 RNA in this work, our results suggest that ssDNA viruses could be more easily detected than RNA viruses. For instance, the Parvovirus B19 is a ssDNA virus that causes fifth disease in children and leads to infections in immunocompromised adults (Servey et al., 2007). A more rapid method for detection of Parvoviral DNA directly from patient samples would lead to faster diagnosis of the virus in immunocompromised patients. Despite redesigning the beacons and varying buffer composition and temperature, we were unable to attain time constants for RNA binding faster than roughly 5 min. Other workers have encountered similar difficulties in targeting mRNA with molecular beacons; for instance Liu et al. (2000) found that while beacons bound to surfaces through a biotin–avidin link were able to efficiently detect DNA oligos, they had problems targeting mRNA (Liu and Tan, 1999). RNA secondary structure has been shown to depend on magnesium concentration, and the rate of hybridization between molecular beacons and RNA can be altered by the magnesium concentration in the buffer (Hopkins and Woodson, 2005). However, because at least some magnesium is necessary to maintain microtubule stability (Menendez et al., 1998), it is not possible in our experiments to completely eliminate free magnesium in the solution.

There have been various efforts to overcome the difficulty in targeting mRNA with beacons. First, because the hybridization kinetics between beacons and mRNA is sensitive to the specific region being targeted, targeting different regions of a given RNA can result in much faster binding kinetics (Bratu et al., 2003). Second, altering the beacon chemistry can improve the RNA hybridization kinetics—beacons containing 2' O-methyl RNA bases or peptide nucleic acid (PNA) bases instead of standard deoxynucleotides have been shown to display faster hybridization kinetics (Majlessi et al., 1998; Tsourkas et al., 2003; Xi et al., 2005). It should be noted that standard molecular beacons have been used to detect cellular or viral mRNA in living cells (Peng et al., 2005), and to localize specific mRNAs in live cells by fluorescent microscopy (Cui et al., 2005).

In conclusion, we show here that molecular beacons can be combined with the kinesin–microtubule transport system to create mobile detectors that fluorescently report the presence of target nucleotide sequences. This system has the potential for both reporting the presence of an analyte and separating and concentrating specific analytes from a complex mixture. This work is an important step toward developing integrated microdevices that combine engineered materials with biological components to detect the presence of RNA or ssDNA viruses or to report mRNA expression levels in single cells.

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