Self-Assembling Holographic Biosensors and Biocomputers

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Abstract

We present concepts for self-assembly of diffractive optics with potential uses in biosensors and biocomputers. The simplest such optics, diffraction gratings, can potentially be made from chemically-stabilized microtubules migrating on nanopatterned tracks of the motor protein kinesin. We discuss the fabrication challenges involved in patterning sub-micron-scale structures with proteins that must be maintained in aqueous buffers to preserve their activity. A novel strategy is presented that employs dry contact printing onto glass-supported amino-silane monolayers of heterobifunctional cross-linkers, followed by solid-state reactions of these cross-linkers, to graft patterns of reactive groups onto the surface. Successive solution-phase addition of cysteine-mutant proteins and amine-reactive polyethylene glycol allows assembly of features onto the printed patterns. We present data from initial experiments showing successful micro- and nanopatterning of lines of single-cysteine mutants of kinesin interleaved with lines of polyethylene, indicating that this strategy can be employed to arrays of features with resolutions suitable for gratings.

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1. Introduction

This SAND report describes work carried out under a project titled "Self-Assembled Holographic Biosensors and Biocomputers". In this introductory section, we briefly describe the conceptual connections between biosensing, self-assembly, and biocomputing. We will then discuss the particulars of the type of system we propose, its biological and material components, and the chemistry and fabrication problems presented. We will then report the methods and results obtained in our preliminary investigation of how to fabricate self-assembling sensor components.

While the exact definition of a biosensor is unsettled, for our purposes biosensors are sensing devices that incorporate biological components, such as cells, proteins, or DNA. Biological materials are used because they may enable highly sensitive and specific sensors responses. For example, the most frequently encountered type of biosensor incorporates biomolecules, such as antibodies or enzymes, capable of highly-selective molecular recognition of a specific molecular analyte. Biological molecular-recognition elements (MREs) are used in such biosensors because they offer advantages over materials such as synthetic polymers, in terms of the strength of binding to analytes, and the ability to selectively bind to a particular analyte and not to other closely-related molecular structures. In principal, strong, selective binding to a molecular recognition element that is part of a sensor system can translate into sensitive, specific response of the sensor system.

In practice, sensitive and specific sensor system response requires several intermediate steps between entry of analyte into the sensor system and production of a useable signal. Sensitive and specific sensor system performance only results if the mass transfer of the analyte through the biosensor system is matched to the binding/unbinding properties of the primary sensor element (e.g., the MRE in the above example), and if the binding of the analyte to the sensor element can be made to produce a signal, such as an optical or electrochemical change, that can recorded as a macroscopic sensor response. Production of a macroscopic signal requires both transduction and amplification of a molecular binding event, where transduction is defined as a process by which energy is accepted in one form and released in another (for example, the conversion of chemical energy to mechanical energy).

Complex biological systems, such as cells, are capable of transduction, for example from chemical to mechanical energy or optical energy. They are also capable of amplifying chemical signals, either through chemical cascades, or by chemicals triggering self-assembly of molecular components into macroscopic structures. More complex biosensor designs may therefore borrow both MREs and signal transduction and amplification components from biological systems.

The work described in this report focuses on fabrication of sensor components, using biological and man-made materials, useful for transduction and amplification of chemical signals into macroscopic optical property changes. Specifically, we look at the intersection of chemical-to-mechanical energy transduction, self-assemble, and nanopatterning, in order to explore the possibility of chemically triggering the self-assembly of diffractive optical elements.

Biocomputing, like biosensing, lacks a crisp definition, but we take it to mean executing computational logic using systems that incorporate biological components. We also note the following tie of sensing to computing: successive stages of transduction and amplification can be used to implement feedback schemes, which may produce non-linear responses, which can in turn be used to represent logical functions. The most basic chemical sensor response is a "threshold" response, where the sensor returns a signal of "1" if the chemical is present above a certain threshold and a "0" otherwise. If signals from more than one sensor are somehow transduced into a form that another sensor system is capable of accepting, then the output of that system may be used to effect a logical operation. In the specific case we describe in this report, the most relevant feedback that could be established in the following order:

Optical -> chemical -> mechanical -> optical

In other words, if an optical energy pulse were to be introduced that released a chemical signal that triggered assembly of a diffractive optical element, then this element could be used to steer subsequent pulses, which using additional conventional optical components such as mirrors, could result in the development of a spatially distributed pattern of diffractive elements. If pairs of pulses were used, these could represent a series of logical inputs, and optical computing could be effected.

Before describing conceptual designs for biosensor and biocomputer architectures, we describe in the following section, biomolecular components that could be useful for building such devices.

2. Background

Microtubles (MTs) are essential components for cell division, neuronal structure, and transport along neuronal axons. MTs are polymeric structures, with dimers of α/β tubulin as the repeat units. The monomers form ribbon-like domains that self-assemble into linear polymeric rod-like structures (the MT proper) with a radius of about 11.2 nm, and lengths up to several microns (Li, DeRosier et al. 2002). Assembly requires the presence of guanosine triphosphate (GTP) and divalent cations. Polymerization is dynamic, in that, once nucleated, the MT tends to continually add tubulin dimers at one end of the rod (plus end), and lose units through de-polymerization at the other (minus) end. However, if the concentrations of tubulin units, GTP etc. are sufficient, then the plus end grows faster than the minus end loses units, leading to persistent structures.

MTs are particularly interesting as potential components for devices because of the combination of regular structural features, described above, and their ability to interact with in molecular motors. In the cell, motor proteins such as kinesin move along MTs in the presence of ATP and divalent cations. Kinesin can be purified and will form a monolayer on a clean glass surface. Although in the cell, kinesin moves on stationary MTs, when MTs are then deposited in the appropriate ionic buffer solution on a supported monolayer of kinesin, MTs will move along the surface if ATP is added, a so-called "inverted" motility assay. In principal, this means that MTs can be used in a variety of *in vitro* systems as molecular "mag-lev" trains, moving on a carpet of motor proteins.

Despite these attractive features, their dynamic structural nature would make MTs somewhat unsuitable for nanosystems, were it not that they can be stabilized using chemical cross-linking. These stabilized MTs can then be decorated, through additional conjugation reactions, with enzymes, fluorescent dyes or quantum dots, or gold nanoparticles (See Figure 1 and reference (Ramachandran, Ernst et al. 2006)). These materials then have enhanced optical or chemical properties. For example, MTs decorated with gold should be expected to diffract and scatter light much more strongly than native MTs, potentially making them useful in nanoscale optical systems, as we describe below.
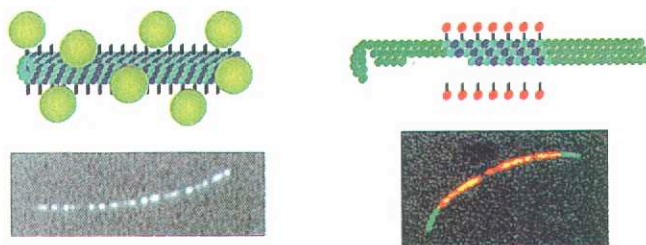

Figure 1: Schematic representation and fluorescence photomicrographs of (A) fluorescently labeled gold nanoparticles, and (B) nanocrystal quantum dots organized on microtubule filaments (after references (Ramachandran, Ernst et al. 2006) and (Bachand GD 2004)).

In general, stabilized and decorated MTs are of considerable interest for nanoscience and bio-nanomaterials studies. Kinesin and MTs have been studied as a means for directing the transport of nanoscale materials at synthetic interfaces, primarily in the inverted motility geometry in which MTs in a fluid layer are actively transport by surfaced tethered kinesin. The MTs represent "molecular shuttles" in this configuration, in that they are able to transport attached cargo in two-dimensions (within the plane of the kinesin monolayer). However, a requirement for useful devices is that it is possible to control and guide the motion of the MTs. At a bulk scale, motion can be activated or inactivated by the addition/removal of ATP or required ions, for example, through wash-in or wash-out methods, since the active MTs are adherent to the surface.

Guiding the motion of MTs at a microscopic scale has proven to be challenging. In the inverted motility configuration, MT motions motion is essentially isotropic in the plane of the kinesin monolayer, since the kinesin is not strongly ordered. The MTs are fairly rigid, and will not remain passively confined to "tracks" of micropatterned kinesin bounded by uncoated glass, but will deflect and then run across the tracks onto other tracks. A variety of methods have been used successfully to guide the kinesin-based transport of MT shuttles at synthetic interfaces (Clemmens, Hess et al. 2003) (Hiratsuka, Tada et al. 2001). In general, the MT must be mechanically confined to a microfabricated channel, with kinesin present on the bottom of the channel.

The major challenge addressed by the work presented in this report is the fabrication of nanoscale structures for the purpose of guiding MT transport. Since MTs are or interest precisely because they have regular nanometer-scale features and active motility, much is lost if they can only be manipulated in structures at the micron scale. In particular, the ability to guide MTs into regular arrays with submicron spacing would open up the possibility of using them to assemble diffraction gratings or other diffractive optical elements. Whereas there have been proposals to use DNA self-assembly to create diffractive elements (Levine 2002), and fabrication of fixed gratings using antibodies for biosensor applications has been reported(St John, Davis et al. 1998), no proposals for using MTs to make diffractive optics were found in the literature. However, MTs appear well suited, because it should be possible to enhance their index of refraction relative to glass and protein, using the demonstrated technology for decorating MTs with high-contrast materials such as nano-gold. As discussed in the next section, such systems would enable new biosensing and biocomputing approaches.

3. System Designs and Fabrication Requirements

Biosensor Systems

As discussed in the introduction, biosensing generally requires transduction between chemical signals and other kinds of signals. Because of their unique properties, stabilized MTs are potentially capable of the following types of interactions wherein different signal modalities (structural, electronic, chemical, optical) modulate each other in the following transduction schemes:

1. Chemical-> Structural: If ATP is added, MTs will move, and structures composed of them rearrange. MT motion can also be controlled by modulating ion concentrations or underlying kinesin activity.

2. Structural-> Structural: a decorated MT on a surface may block the transport of a MT trying to move perpendicular (native MTs have some ability to run across each other).

3. Structural-> Optical: MTs should be able to assemble into diffractive optical elements, or make such elements disassemble, if suitable guiding structures can be fabricated.

Simply based on these properties, a biosensor for ATP could be configured using transduction schemes 1) and 3) above:

A) On a suitable surface, an array of "MT-guiding" kinesin tracks with a suitable size and pitch for an optical grating (e.g., 100nm lines at 400nm spacing) would be fabricated, each track connected at one or both ends to adjacent to fields of uniformly-coated kinesin.

B) MTs would be prepared, stabilized by cross-linking, and decorated with nano-gold particles

C) A "reservoir" of MTs would sit on the uniformly-kinesin-coated region, which has connectivity to the tracks. The buffered solution coating the surface would contain all ions etc. required for MT motion, but not contain ATP, so the MTs would not be motile and would not migrate onto the tracks.

D) If a solution containing ATP were added, MTs would migrate about the surface, and some would migrate onto the array of tracks, and align into semi-regular array. (The efficiency of this might be improved by pre-aligning the MTs e.g., using channels).

E) When occupancy of the tracks by gold-decorated MTs was sufficient, the tracks would be observed to diffract an incident light beam onto a photosensor.

Such as sensor could, of course, also be configured to detect the ions required for MT/kinesin mobility, if ATP were present in the buffer and not these ions, though it should be pointed out that ATP detection is itself useful for viability assays, for example, as used to assay the presence of residual live cells following decontamination. Because ATP is ubiquitous in living cells, it is possible to use ATP detection as part of any assay involving live-cell lysis.

Biocomputing Systems

As described in the Introduction, moving from biosensing to biocomputing requires moving beyond transduction to produce feedback, or at least out put signals that can modulate downstream input signals. Additional transduction schemes (these are not properties of the biological components, but would be implemented using man-made components) can potentially enable the required kinds of feedback (the numbering below is continued from the list of transduction schemes given in the *Biosensors* section above):

1. Optical-> Chemical: It is possible to chemically "cage" certain compounds, including ATP, such that UV Light pulses can be used to photolyze the caging groups and release localized bursts of active compound (caged GTP and divalent ion chelators could also be used)

2. Electrical -> Chemical: Electrodes of Zn or Mg could be used to release high local concentrations of divalent cations that could modulate MT mobility.

Taken together, schemes 4), 3) and 1) imply that, it should be possible to set up the following feedback: Optical->Chemical->Structural->Optical by implementing a diffractive optical element that assembles in response to ATP addition, and thus in response to a UV light pulse that releases caged ATP. If the element itself were able to diffract the UV wavelengths required for uncaging ATP, then optical pulses could be used to assemble elements that could manipulate other pulses and determine whether and where they triggered other elements to assemble. While these cascades of transduction schemes are somewhat notional, we note in this context that control of MT motion using UV pulses to un-caged "caged" ATP has in fact been demonstrated (Hess, Clemmens et al. 2001). ATP-driven assembly of a diffraction grating must be demonstrated in order to make the overall feedback scheme practicable.

A more detailed design for an optical element that could be used on the optical feedback scheme just described is shown in Figure 2, which describes a four-state device. The gray lines are tracks of kinesin, and the MTs are arranged at the entrances to the tree structures on the left and right (MTs have a head and tail, and we are assuming that the MTs on the left are oriented so that they will move to the right or not at all, and symmetrically for the MTs on the right.) Starting with the upper left structure, if the left most MTs are stimulated or allowed to move (e.g., by a pulse of UV light releasing caged ATP or ions or removal of an obstructing MT) they will migrate onto the branches of the tree, forming a grating in the state shown on the upper right. However, if MTs come in

from both sides, in response to signals on both sides, then the pitch of the grating is doubled. If the grating is then illuminated by a light pulse coming toward the reader out of the plane of the page, the diffracted spots will be spaced (in this case vertically) inversely as the pitch, so that a line of regions on a plane above and parallel to the page that might be illuminated by a spot if only one or the other side were activated, would not be illuminated by a spot, forming a logic table (00->0, 01->1, 10->1,11->0).
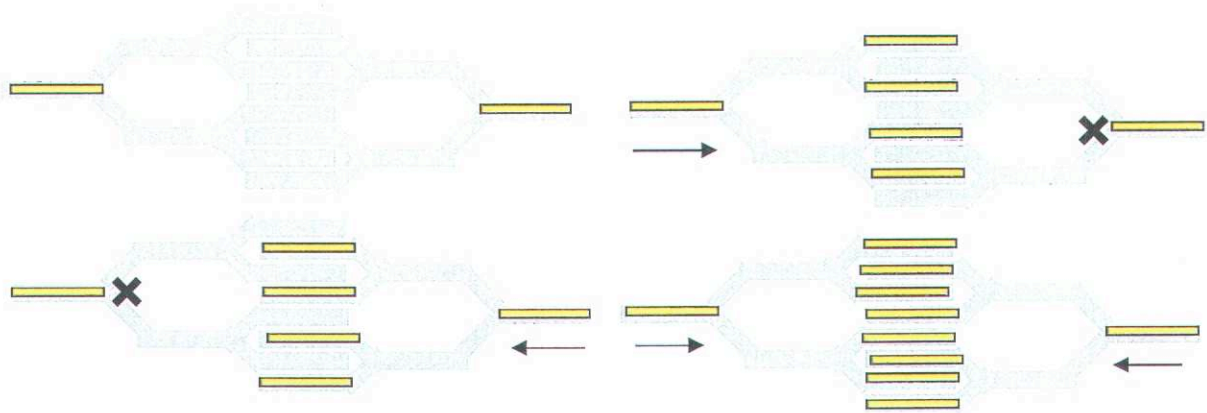
Figure 2: Pitch of grating formed by gold-decorated microtubules migrating on kinesin tracks can be varied depending on whether one or both inputs present.

An optical computing scheme could be implemented using a series of stacked planes, each covered with appropriately-positioned structures such as shown in figure 2. An initial spatially patterned light pulse would then set up a diffracting surface in each plane, and subsequent pulses (temporally spaced to allow for the response time of the rearrangements) would be modified by passing through the successive layers. After some number of pulses the output of spots from the last plane could be read. If reflective diffractive optics were employed as well, then feed back between the layers could be incorporated. It is clear that, at least in concept, optical computing could be performed in this way. (Note that these devices are at least trivially biosensors, in that they would require, and therefore sense the presence or absence of cationic species in the buffer).

Fabrication Requirements

Fundamental to the implementation of systems for optical biocomputing or biosensing using MTs, is the requirement for fabrication of structures capable of guiding the assembly of MTs into gratings. Other basic requirements such as MT stabilization, decoration of MTs with gold, kinesin immobilization, and initiation of MT motion using UV photolysis of caged ATP, have been demonstrated. Typical dimensions for gratings would be lines of 100-200 nm in width spaced 200-400nm apart.

As remarked above, MTs can be successfully guided by structures with walls. An array of individual fluidic channels of sub-micron width and height, branching from a main channel could be fabricated, for example, by embossing into a polymer substrate followed by bonding of a top-plate, and the walls coated with kinesin. In the presence of ATP, MTs would then migrate from the main channel into the array of channels. However, such a device would likely have substantial baseline diffraction, due to the large difference in diffractive index between the substrate and aqueous buffer, and would be challenging to fabricate.

We chose instead to fabricate arrays of kinesin lines using PDMS stamping methods. These methods are simple and inexpensive to carry out, and stamps of gratings are commercially available, or are easily cast using a ruled diffraction grating as a master. A considerable literature exists on stamping of proteins (Ramachandran, Ernst et al. 2006), (Renault, Bernard et al. 2003). We were also able to use a recently-developed kinesin with an engineered cysteine mutation at the C-terminal, offering the possibility oriented covalent immobilization by conjugation of the cysteine sulfhydryl to the surface. However, three fabrication hurdles presented themselves:

1. If proteins are stamped in a liquid "ink", then diffusion creates a fundamental restriction on the feature size. For certain very stable proteins such as antibodies, dry formulations may be
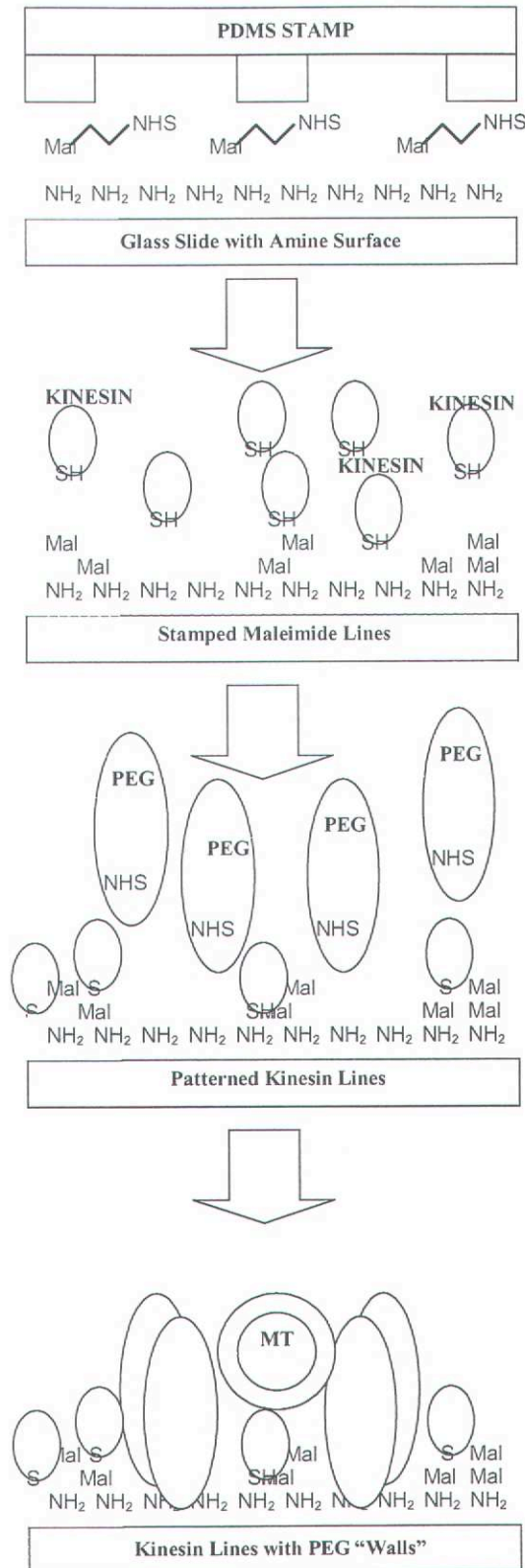

Figure 3: Stamping and Protein Attachment

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stamped, and small feature sizes obtained (St John, Davis et al. 1998). Because kinesin can not be reconstituted into and active form once it is dried out, we were presented with a significant fabrication problem: how to use PDMS stamping to pattern nanometer-scale features of a protein than must remain in aqueous buffer.

2. MTs need some degree of physical confinement (i.e., walls) to orient their motion. The implication is that the kinesin lines must be interleaved with lines of a material that does not bind MTs and is significantly "taller" than a MT would be adherent to an adjacent kinesin monolayer surface. (Given that the kinesin is ~5nm-10nm tall and the MT 11.2nm in radius, a wall height in excess of 30nm appears to be desirable.) As a result, two materials must be patterned, kinesin and a "bulky" material for confinement.

3. Considerable complexity and cost would be introduced by the requirement to register and accurately offset multiple stamping steps by less than a micron. Only one stamping step could be used.

In light of these issues, we developed the following fabrication strategy:

1. Two different materials would be immobilized using orthogonal attachment chemistries.

2. Glass slides were first uniformly coated with amino-silane layer to generate a monolayer of reactive primary amines.

3. Reactive groups were then nanopatterned by dry stamping of a cross-linker onto the amine surface. The cross-linker contained an amine-reactive N-hydroxysuccinimide (NHS) ester group and a sulfhydryl-reactive maleimide group. (Figure 3).

4. The NHS ester of the cross-linker was allowed to react in the *solid phase* with the surface amines, and then excess cross-linker removed by flooding with low-pH solution. This step leaves behind maleimide groups in the pattern of the stamp.

5. Lysine and N-terminal amines of proteins may react with maleimide at basic pH. Previous studies with peptides indicated that cys-maleimide conjugation occurs efficiently at pH near neutral. The kinesin can be patterned by incubation of cys-kinesin in aqueous buffer with the maleimide-patterned slide. (Figure 3, Second from top)

6. A large MW linear polyethylene glycol (PEG) with an NHS ester at its terminus was obtained in order to form the confining walls. (Figure 3, third from top)

7. The NHS-PEG will react with the regions on the amine-coated surface that have not been stamped with the cross-linker, and will project farther from the surface than the microtubules on the kinesin (Figure 3, bottom)

In the following sections, we describe the methods used and the initial results we obtained.

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4. Materials & Methods

Kinesin Production

Construction and expression of recombinant T. lanuginosus kinesin (TKIN) clones.

T. lanuginosus was obtained from the American Type Culture Collection (catalog # 36350), and grown at 55□C in potato dextrose broth. cDNA was generated from total RNA was isolated using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT) primers, and subsequently amplified using sequence specific primers (Forward: 5'-ATGTCGGGCGGTGGAAATATCAA-3'; Reverse: 5'-GATATCGAATTCCTGCTTCGCTG-3') based on the previously published sequence (22). The resulting 2,358-bp amplified product (TKIN) was cloned into the pET-Blue1 expression system (Novagen, Madison, WI). Site directed mutagenesis (SDM) was used to change an EcoRI restriction site in the base plasmid to a BamHI site. A 10x poly-histidine tag was inserted at the 3' end of the amplified TKIN sequence using the EcoRI/BamHI sites. A cysteine residue (TKIN-Cys) was introduced into the TKIN sequence, directly upstream of the 10x His tag, using SDM of phenylalanine784 to cysteine784. All TKIN constructs were sequenced to verify the sequence, mutation, and proper orientation in the plasmid.

Purification of TKIN

TKIN-Cys was expressed in E. coli strain Tuner(DE3)pLacI (Novagen, Madison, WI) grown in LB medium with 100 µg/ml ampicillin and 34 µg /ml chloramphenicol, at 30°C with shaking at 250 rpm. Protein expression was induced with 0.5mM isopropyl-β-D-thiogalactoside when the OD600 ~ 0.7, and cells were harvested by centrifugation 4 hr after induction. Cell pellets were resuspended in lysis buffer (50 mM phosphate buffer, pH 7.0, 500 mM NaCl, 30 mM imidazole, 5 mM β-mercaptoethanol (βME), 1 mM phenylmethylsulfonyl fluoride, and 100 µM ATP) and disrupted with Bugbuster Protein Extraction Reagent (Novagen, Madison, WI). The suspension was centrifuged at 40,000 x g for 20 min, and the supernatant was batch bound to pre-equilibrated Ni2+-NTA resin (Qiagen, Valencia, CA). The slurry was washed with a buffer consisting of 50 mM phosphate buffer, pH 7.0, 500 mM NaCl, 50 mM imidazole, 100 µM ATP and 5 mM βME, and protein was eluted with a buffer of 50 mM phosphate buffer, pH 7.0, 50 mM NaCl, 1 M imidazole, 100 µM ATP and 5 mM βME. Eluted fractions were analyzed by SDS-PAGE; fractions containing purified TKIN were combined, dialyzed against 20 mM phosphate buffer (pH 7.0), and precipitated with ammonium sulfate (0.57 g/ml). The pellet protein was resuspended in BRB80 (80 mM PIPES, 1 mM MgCl2, 1 mM ethylene glycol bis(β -aminoethyl ether) N, N'-tetaacetic acid), and exchanged into BRB80 or BRB80 with 0.1 – 10 mM ATP using P6-spin columns (BioRad Laboratories, Hercules, CA). Protein concentrations were determined by the Bradford method (25).

Thermomyces kinesin with Cys insertion and his tag (Size: 88.7 KDa, pI: 6.28) was shipped on dry ice from the Bachand Lab at Sandia/NM . (Concentration received: ~0.2 mg/ml, Amount received: ~750 ug, Buffer condition received: 30 mM sodium phosphate, 10mM ATP.)

PDMS Stamp

Stamps made from poly-dimethylsiloxane (PDMS) were ordered from Platypus Technologies (www.platypustech.com/). The nanopatterned stamp was 200nm lines with 60nm depth and 400 nm spacing. The PDMS micro-patterned stamps had a series of 100 μm X 10 mm lines.

Reagents

Cross-linkers N-[ß-Maleimidopropyloxy]-succinimide ester (BMPS) and (N-[g-Maleimidobutyryloxy] succinimide ester (GMBS) were obtained from Pierce Biotechnology. Amino-trimethoxypropylsilane (ATPS) was obtained from Sigma-Aldrich. Sulfhydryl terminated polyethylene glycols of 30kDa and 5kDa were obtained from Nektar (www.nektar.com).

Procedure for Immobilization of Thermomyces kinesin to silica beads

Preparation of the silica beads (7um-1000 angstrom porous)

Sonicate silica beads in methanol to disperse them, centrifuge, and wash pellet in de-ionized water for 1 hour over boiling water bath. Centrifuge, and suspend pellet in 10% hydrogen peroxide. Transfer to round-bottom flask and heat in boiling water bath for 1 hour on rotary evaporator. Centrifuge, and suspend pellet in methanol by vortexing, then transfer to vacuum flask, and evaporate methanol under low vacuum. Dry beads in vacuum oven (120C overnight), and store dried beads in dessicator.

1. Silanization of beads with ATPS

    a. add 10% 3-Aminopropyltriethoxysilane (pH 7) to the beads

    b. degas by sonicating under vacuum for 10 minutes

    c. incubate for 3hrs in 80 degC water bath with rotating flasks@150rpm

    d. wash x3 with PBS

2. Attachment of BMPS to the silanized beads

    a. dissolve ß -maleimidopropionic acid N-hydroxysuccinimide ester (BMPS) in DMF @ ethanol (8 mM)

    b. add 300 ul of BMPS to the bead solution suspended in 9 ml ethanol

    c. incubate exactly 1 hr @RT rotating@200 rpm

    d. wash x3 with ethanol, 1x with DI water and 1x with PBS

Fluorescein labeling of kinesin for attaching to silica beads

1. Concentrate protein to 5mg/ml in 50ul reaction volume in PBS/1mM NaN₃/10mM ATP

2. Add 1M sodium bicarbonate buffer to pH8.3

3. Dissolve NHS-fluorescein in DMF at 10 mg/ml

4. Add slowly 5ul of the NHS-fluorescein to protein

5. Incubate the reaction for 1hr @RT

6. Remove excess of dye and exchange buffer to PBS/1mM NaN₃/10mM ATP by gel filtration (Zeba desalt spin column)

Protein attachment

1. Prepare protein solutions (blank, and kinesin without and with attached fluorescein) in PBS/1mM NaN₃/10mM ATP at a protein concentration 0.15 mg/ml.

2. Add 2 ml protein solution per 63 mg of beads and incubate overnight at 4C on vertical rotator

3. Wash with PBS/1mM NaN₃/10mM ATP and store in this solution at 4C.

Activity Assay

ATPase activity of immobilized TKIN was determined based on the protocol given in reference (Huang and Hackney 1994).

Protocol for Assaying Amount of Fluorescein-labeled protein attached to silica beads using total hydrolysis

1. Add 500 ul 4N NaOH to 500ul (~50mg of beads) in 5 ml Teflon bottle and seal with stopper.

2. Incubate @ 95 degC overnight

3. After cooling, add 1000 μl 2N HCl and 500ul citrate carbonate buffer (pH 9.3)

4. Transfer to plastic tube and centrifuge @6000 rpm for 40 minutes to remove residual silica beads

5. Acquire fluorescein scan (scan speed 100, excitation (480nm) slit 2.5nm, Emission (518 nm) slit 10nm, 10 scans/spectrum).

For kinesin labeled with NHS fluorescein: Kinesin was first incubated with 100X NHS-Fluorescein in triethylammonium bicarbonate buffer (pH 8.3), and labeled protein purified by size-exclusion chromatography.

Protocol for PDMS stamp printing of kinesin on glass for nano-patterning

Preparation of Amino-Functionalized Glass Slides:

1. Clean plain glass slides in piranha solution (70:30 v/v mixture of concentrated H_2SO_4 to 30% H_2O_2) for at least 12 hours at room temperature. Remove from the piranha bath, and washed for at least 12 hours in ddH_2O. Store in ddH_2O until further use

2. Prepare 3:5:92 3-aminopropyltriethoxysilane:ddH_2O:ethanol solution and stir it for 10 minutes to allow for hydrolysis and formation of silanol

3. Pour 200 ml silanol solution to a glass slide tank containing a cleaned glass slide

4. Incubate the slide at RT with rotating (@100 rpm) the slide tank for 1hr

5. Remove the slide from the silanol solution, wash it for 30 seconds in 100% ethanol, and dry it by centrifugation to remove excess silanol from the surface

6. Cure the adsorbed silane layer at 115 °C for 1 hour

7. Cool to room temperature and wash the slide in 95% (v/v) ethanol for 30 minutes, repeat the wash four times

8. Store the amino slide under vacuum @ RT

This protocol gives an approximate density of 2-4 amino groups per nm^2

Printing BMPS/GMBS on Glass slides with PDMS Stamp:

1. Prepare 200 mM BMPS or GMBS solutions by dissolving these in DMF and then diluting 10-fold with 10 mM PBS (pH 7.5)

2. Rinse the PDMS stamps with ethanol and dry quickly and thoroughly with nitrogen

3. Apply the BMPS or GMBS solution and dry it quickly with nitrogen

4. Place treated PDMS stamp face down onto the Amino-functionalized glass slide. Make sure PDMS stamp is in full contact with the glass surface by resting thumb on back of PDMS stamp for 5 seconds

5. Gently peel PDMS stamp from the surface and left @ 37 °C for 5mins

6. Rinse quickly with low pH solution (KCl+HCl, pH2.2)

7. Wash four times with 10 mM PBS

Preparing NHS-ester PEG and attaching to Amino-functionalized glass:

1. Make NHS-ester by incubating PEG SH-PEG with 100X BMPS in phosphate buffer (20 mM KH2PO4, pH6) for 30 mins @RT

2. Remove free BMPS and exchange buffer to 10 mM PBS (pH7.5) by gel filtration

3. Incubate BMPS-printed glass with NHS-ester for 1hr @ RT

4. Wash four times with 10 mM PBS + 1mM ATP

Attachment of TKIN Single-Cysteine Mutant:

1. Prepare protein solution (1.5M fluorescein labeled kinesin in 10 mMPBS/1mM ATP)

2. Incubate the glass slide with the protein solution @4 °C for overnight

3. Wash with PBS, 100mM glycine/PBS, 0.05% tween-20/PBS, 0.5M NaCl/PBS, and 10mM PBS/1mM ATP

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5. Results & Discussion

Attachment of Thermomyces Kinesin to Silica Beads

Prior to attempting patterning of the TKIN cysteine mutant on glass surfaces, we established that it could be covalently immobilized to silica beads via its lone cysteine. It is challenging to determine the amount of protein immobilized per mg bead without using radioactive tracers, due to the minute amount of protein relative to silica, and the interference of the porous silica surface with methods such as fluorescence. Standard assays such as BCA are also too insensitive to bet useful for determining protein loads on planar substrates. Since the desired end product of this work is in fact protein patterned on a surface, it is desirable to develop an assay using non-radioactive tracers. Accurate fluorescence-based protein quantitation can potentially be performed by totally hydrolyzing immobilized fluorescently-labeled protein, liberating the fluorescent tracer, and eliminating surface effects.

Immobilization of fluorescein-labeled TKIN was accomplished by first forming an aminosilane monolayer on the surface beads. The primary amines in this monolayer will react with a variety of compounds. N-hydroxysuccinimide esters, in particular, are widely used, due to their commercial availability and reactivity at physiologic pH. We coupled the primary amines to maleimide groups using a heterobifunctional cross-linker with an NHS ester at one end and a maleimide group at the other. Based on the protein immobilization efficiency and patterning results (see below), this worked well, though we did not optimize factors such as linker length and stoichiometry.

Following immobilization of fluorescein-TKIN on beads, total hydrolysis and fluorescence measurements, using the procedures described in Materials and Methods above, showed a protein load of 4.3 mg/gram of beads, compared to 3.9 mg/g by BCA assay on a suspension of bead-immobilized TKIN. Minimal fluorescence was observed in solutions of NaOH-hydrolysate from unlabeled beads. These results indicate that there is rough agreement between the two measures. A geometric calculation of surface area gives 3.9×10^{-11} square meters per 5 micron diameter non-porous silica bead, or 0.55 square meters per gram. At a monolayer density of one protein per 100 square nanometers, full monolayer coverage would yield 10^{16} protein molecules per square meter or 16 nanomoles per square meter, or 8.8 nanomoles protein per gram of beads. These calculations predict for TKIN (88.7 kDa) a theoretical monolayer coverage of 0.78 mg/gram for non-porous beads. This number should increase by a factor of 10-100 for porous beads (which were the substrate actually used in these experiments) or 7.8 to 78 mg/gram beads, compared to a measured ~4 mg/g. Given the considerable uncertainty, due to the irregularity of the pore geometry, in the internal volume of the beads accessible to TKIN, as well as the correct theoretical monolayer density, the measured numbers indicate a satisfactory efficiency of attachment.

Unfortunately, it was not possible to establish whether the immobilized TKIN had ATPase activity. Initial measurements were performed on fluorescein-labeled TKIN immobilized on silica beads. Fluorescence measurements of hydrolyzed labeled TKIN indicated 4.8 dye molecules per protein, which level of labeling might affect activity.

However, subsequent ATPase measurements on unlabeled immobilized TKIN also did not show activity, indicating that the ATPase assay may not be sensitive enough, or that the immobilization and storage prior to the assay destroyed its activity. A full exploration of alternative activity assays and formulation of buffer conditions to stabilize the TKIN during and after immobilization was not within the scope of this project. Instead, we developed patterning methods using immobilization procedures that would allow patterning of TKIN in arbitrary buffers, as long as the buffer does not react with maleimides, primary amines, or sulfhydryls.

Stamping of Reactive Groups and attachment of Thermomyces Kinesin

When glass slides are treated with the amino-silane compound APTS, a self-assembled monolayer of aminopropyl groups is formed, as for beads. In order to attach TKIN via a cysteine sulfhydryl group, we used a cross-linker, the same chemical strategy as for bead attachment. However, we wanted to create spatial patterns, so we used PDMS stamping to apply cross-linker locally. Because we wanted to perform nano-patterning, we applied a moderate concentration of the cross-linker (20mM in PBS) to the stamp, and then dried the stamp under nitrogen. The cross-linker was applied and reacted *in the solid state*. To our knowledge, this is the first time such solid-state cross-linker coupling has been used with PDMS stamping, as most protein stamping applications use a specially formulated "ink" containing the protein to be immobilized and attach proteins directly.
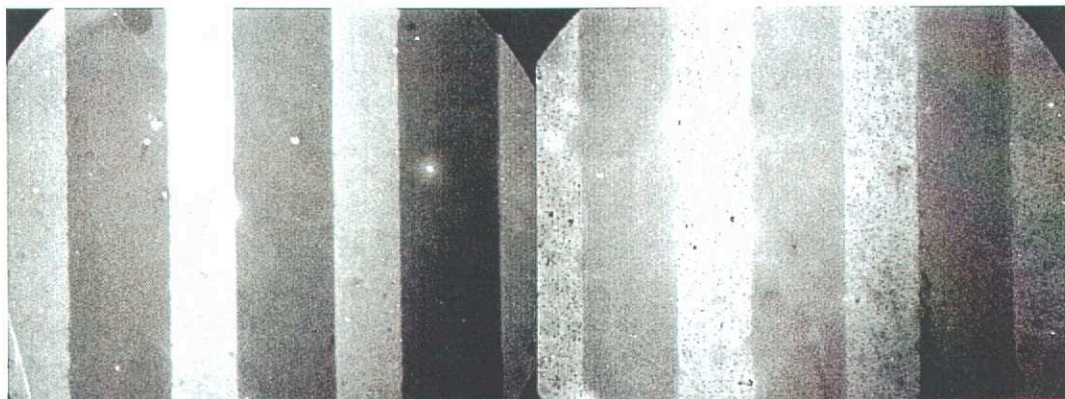

Figure 4: Fluorescence Micrographs of cys-TKIN and NHS-PEG (30kDa) immobilized onto amino-silane monolayers on glass microscope slides (Magnification 200x). Both slides were prepared identically and stamped with BMPS cross-linker using a PDMS stamp with 100 micron lines and 100 micron spacing. The slide on the left was incubated first with cys-TKIN, then washed and incubated with NHS-PEG. The slide on the right was first incubated with NHS-PEG, then washed and incubated with cys-TKIN. Images were acquired using a CCD camera mounted on an Olympus IX70 inverted fluorescence microscope with a standard fluorescein filter cube.

Results from stamping using a stamp with 100 micron lines are shown in Figure 4. Well-defined bands of fluorescence indicate selective localization of cys-TKIN to the regions where the cross-linker was stamped. When cys-TKIN was applied first, allowed to attach, and then NHS-PEG is applied, the bands formed are uniform in intensity. When the NHS-PEG was applied first, then the bands displayed many fluorescence lacunae, indicating that NHS-PEG is able to attach to the stamped regions. NHS-PEG may be attaching to residual amines in the stamped area that did not react with cross-linker, or, since the NHS-PEG was formed by reacting SH-PEG with cross-linker, there may be residual SH-PEG in the NHS-PEG. Together, the results in Figure 4 indicate that attachment of the cys-TKIN effectively blocks the NHS-PEG attachment sites, and should be done prior to NHS-PEG attachment.
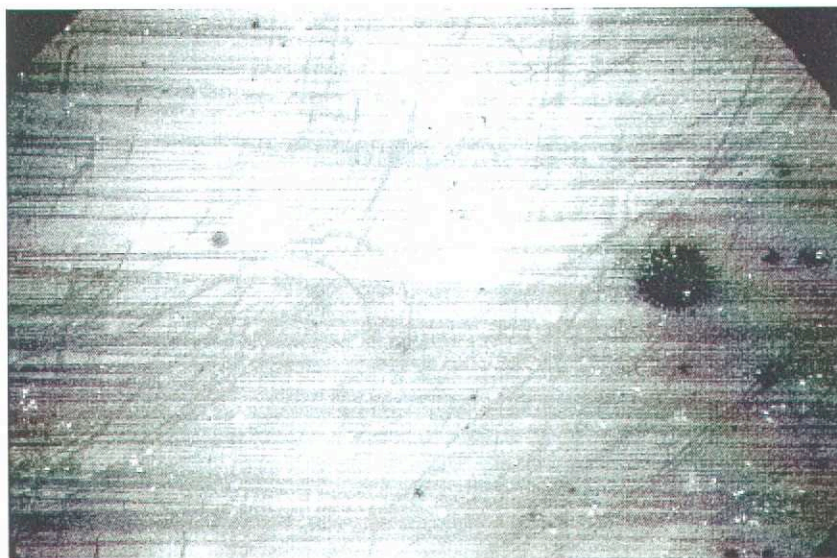

Figure 5: Fluorescence Micrograph of cys-TKIN and NHS-PEG (30kDa) immobilized onto amino-silane monolayers on glass microscope slides (Imaging method same as Fig. 4). A PDMS stamp with 200nm wide, 60nm tall lines (400nm pitch) was used.

Figure 5 shows the results of immobilization of cys-TKIN followed by NHS-PEG onto a slide that has been stamped with BMPS cross-linker using a PDMS stamp with 200nm lines and a 400nm pitch, a series of fine, roughly parallel lines, with some apparent scratching. While the lines size and regularity do not match the reported features of the stamp, the result is quite different in appearance than with the 100micron stamp (Figure 4). It appears that there are variable degrees of confluence between the lines, and that the efficiency of transfer/reactivity of the cross-linker with the slide surface varied. We have no independent data on the uniformity of the stamp surface. The stamp surface may have been damaged (there appear to be scratches), and manual stamping was not done in a clean room, so particles may have interfered with the uniformity of contact and pressure may have been uneven. Also, the amount of the cross-linker solution may have been such that the spaces between lines were filled once it was dried, and the drying it self may have caused ridges on the stamp to collapse together from surface tension effects. Despite all of these caveats, this result indicates that it is possible to pattern features at least on the order of a few microns with this technique.

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6. Conclusions

We have shown that Thermomyces kinesin with a single cysteine mutation can be patterned onto flat glass substrates into lines through a stamping and self-assembly process. Even with a process that has not been optimized, it is possible to make protein features only a few microns in size, interleaved with high-MW PEG features, without the requirement for formulating a viscous ink in which the protein is stable or stamping dry protein. The features are, for protein dissolved in aqueous buffer, far smaller than the diffusion length scale during a 5 minute immobilization reaction. Stamping of a cross-linker in the solid-state, rather than protein, followed by an ordered self-assemble process employing orthogonal attachment chemistries, makes the assembly of micron-scale interleaved features with different materials (protein and PEG) possible .

Proper diagnostic tools such as atomic force microscopy and confocal microscopy would be valuable in interpreting the results, and optimizing the attachment and fabrication methods, but were not available during the brief execution time of this exploratory project. In particular, it is necessary, for the purposes motivating the work presented here, to determine the surface topography of the interleaved patterned PEG and kinesin, in order to choose an appropriate molecular weight PEG to create features tall enough to be likely to confine microtubules. Following this, a matrix of immobilization conditions would need to be explored to allow maximal preservation of kinesin activity, as determined by reverse-mode microtubule motility tests. Although these studies were not in the scope of work presented here, our results do not indicate any fundamental barriers to the fabrication of kinesin structures that would enable self-assembly of MT gratings.

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