Enhancing the stability of kinesin motors for microscale transport applications†

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Biomolecular motors, such as kinesins, have great potential for micro-actuation and micro- or nanoscale active transport when integrated into microscale devices. However, the stability and limited shelf life of these motor proteins and their associated protein filaments is a barrier to their implementation. Here we demonstrate that freeze-drying or critical point-drying kinesins adsorbed to glass surfaces extends their lifetime from days to more than four months. Further, photoresist deposition and removal can be carried out on these motor-adsorbed surfaces without loss of motor function. The methods developed here are an important step towards realizing the integration of biological motors into practical devices, and these approaches can be extended to patterning and preserving other proteins immobilized on surfaces.

Introduction

In eukaryotic cells, kinesin motor proteins use the energy from ATP hydrolysis to transport intracellular cargo along protein filaments called microtubules. *In vitro*, kinesin-driven motion can be observed with purified components using the microtubule gliding assay, in which motors are adsorbed on a surface and microtubules are pushed along the surface by these motors.[1,2] These transport dynamics can be utilized for active transport in devices, with microtubules as carrier proteins, and there has been considerable interest in developing hybrid devices harnessing the mechanical motion provided by these motors. Recent progress towards developing kinesin-driven devices includes functionalization of microtubules with a variety of cargo and control of microtubule motion using microfabricated channels and electric fields.[3–14] However, a major issue in integrating these biological components into functional hybrid devices is the limited shelf-life of kinesin motors and microtubules. Motor proteins degrade rapidly (hours to days)[15] when the solution is stored at room temperature, which limits their utility from the standpoint of manufacturing devices. Hence, methods for improving the stability of conventional kinesin motor proteins for long term storage are needed.

One straightforward approach for storing such hybrid biological devices would be to freeze-dry (lyophilize) them to preserve protein function, and then reconstitute them just before use. By removing the water, which is the medium for most chemical degradation pathways, freeze-drying stabilizes proteins. However, protein structure and function can be compromised during both the freezing and the drying transitions, and several chemical degradation reactions, such as deamidation, peptide bond cleavage and oxidation, can occur in the solid state after freeze-drying.[16] In a recent report, Seetharam *et al.*[17] established that flow cells containing immobilized kinesins and microtubules can be lyophilized and reconstituted, but after four weeks of storage less than half of the microtubules were still motile, a significant degradation of activity.

Critical point drying is another method that is commonly used for drying biological specimens.[18] The critical point is the state of continuity between the liquid and vapor states where the surface tension is zero. By displacing the aqueous buffer sequentially with acetone and liquid CO_2 and then raising the temperature and pressure of the CO_2 to the critical point where the liquid state ceases to exist, the damaging effects of surface tension that occur in air drying are eliminated. There have been no reports to date using critical point drying for preserving motor proteins immobilized on surfaces. Here, we show that critical point drying is an effective and relatively simple method of stabilizing proteins immobilized on glass surfaces that does not require formulations of cryo- and lyoprotectants. Furthermore, this approach can be extended to patterning functional motors on surfaces using traditional photolithography approaches of photoresist deposition, patterning and removal, while preserving motor functionality.

Experimental

Microtubules and kinesin

Drosophila melanogaster conventional kinesin heavy chain was bacterially expressed and purified as previously described.[1] Bovine brain tubulin was purified and rhodamine labeled as previously described.[19] Microtubules were polymerized by mixing 32 μM rhodamine-labeled tubulin, 4 mM $MgCl_2$, 1 mM GTP and 5% DMSO in BRB80 buffer (80 mM PIPES, 1 mM

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EGTA, 1 mM $MgCl_2$, pH 6.9 with KOH) and incubating at 37 °C for 20 min. Polymerized microtubules were stabilized with 10 μM paclitaxel.

Freeze-drying

Flow cells were constructed by attaching a coverslip to a glass slide with double-stick tape and were sequentially incubated with casein solution (0.5 mg ml^{-1} casein in BRB80 buffer for 5 min) and kinesin solution (~5 μg ml^{-1} kinesin, 1 mM ATP, 0.2 mg ml^{-1} casein, 10% glycerol and 5% w/v sucrose in BRB80 buffer) for 5 min each. These flow cells were dried in a lyophilizing container surrounded by dry-ice, which was connected to a vacuum of 150–200 μm Hg and a trap temperature of −84 °C. The dry-ice was allowed to sublime and a vacuum was held for 8 h. As the dry-ice sublimed, the sample temperature increased, allowing mass transfer of water from sample to trap. These flow cells were stored at 4 °C in a box containing Drierite.

Critical point drying

Glass coverslips were treated with casein (0.5 mg ml^{-1} casein in BRB80) and kinesin (~5 μg ml^{-1} kinesin, 1 mM ATP, 0.2 mg ml^{-1} casein in BRB80 buffer) solutions for 5 min each. The coverslips were dehydrated by sequentially incubating the sample in 50%, 75% and 100% acetone in BRB80. The samples were then loaded in a closed drying chamber where the acetone was displaced by liquid CO_2. The temperature of the chamber was increased from 10 °C to the critical point of CO_2 (31.1 °C, 73 atm pressure), where the liquid vapor interface vanishes. These coverslips were stored at 4 °C in a box containing Drierite.

Patterning dried motors

Shipley 1811 photoresist (Shipley Corporation) was spin-coated on critical point-dried coverslips and the samples were dried overnight at room temperature. This method contrasts with the standard process where the photoresist is baked at 90 °C for 90 s to drive off excessive solvent, as these temperatures denature motor proteins. The photoresist was then patterned by UV exposure through a photo mask using a Karl Suss contact aligner. Exposed photoresist was removed by soaking the sample for 60 s in Microposit MF 351 developer diluted 1 : 4 in DI water, which created regions with no motor activity. Finally, the remaining photoresist was removed by flowing acetone over the sample to expose regions containing functional kinesin motors.

Microtubule motility

Freeze-dried flow cells and flow cells constructed with critical point-dried coverslips were reconstituted with motility solution (~32 nM microtubules, 1 mM ATP, 10 μM paclitaxel, 0.2 mg ml^{-1} casein, 20 mM D-glucose, 0.02 mg ml^{-1} glucose oxidase, 0.008 mg ml^{-1} catalase, 0.5% β-mercaptoethanol in BRB80 buffer). Microtubule movements were observed by fluorescence microscopy and videotaped for further analysis.[8] Microtubules shorter than 1 μm were excluded from analysis.

Results and discussion

Functionally, kinesin motor proteins have two modes of failure: they can denature completely and lose their ability to bind microtubules, or they can inactivate such that they bind microtubules irreversibly. While complete denaturation can sometimes be compensated for by starting with very high motor concentrations, inactivated motors stall microtubule movement and thereby reduce the functionality of microtubule-based devices. In order to compare the effectiveness of different drying methods in preserving the motor functionality, both the number of microtubules per given area and the fraction of microtubules moving were used as metrics for assessing motor function.

To assess motor survival after storage, critical point-dried coverslips were used to construct flow cells, motility solution was introduced, and the resulting microtubule movements analyzed. In critical point drying, the aqueous solution must first be replaced by a miscible solvent, which is then replaced by liquid CO_2. When ethanol was used as the dehydrant, very few microtubules were observed, consistent with ethanol denaturing or desorbing the surface-adsorbed kinesin motors. Similar results were obtained when the freezing step was omitted and the aqueous buffer was replaced by ethanol, followed by the introduction of microtubule-containing motility solution, indicating that it is most likely exposure to the ethanol that denatures the motors. In contrast, when acetone was used as a dehydrant, numerous microtubules bound to the surface upon reconstitution with motility solution, and a large fraction of them moved. To test the suitability of these critical point-dried samples for long term use, the samples were stored at 4 °C and tested at 3, 12 and 20 weeks. As seen in Fig. 1 and supplementary movie 1,† the number of microtubules bound to the motor-functionalized surface was roughly constant over the 20 week period, and the

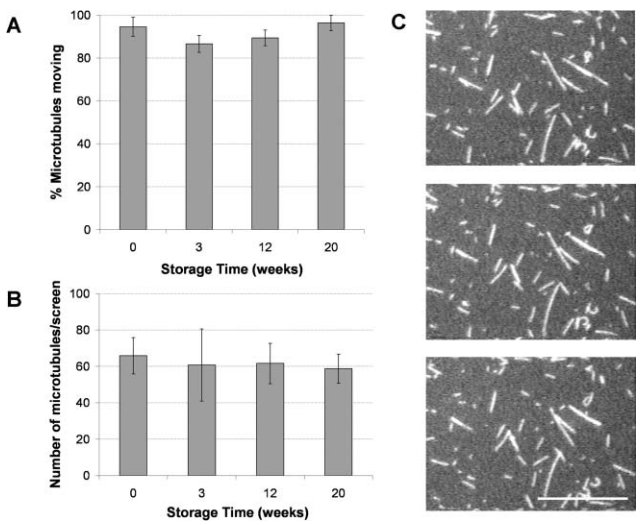

Fig. 1 Microtubule motility in critical point-dried samples. (A) The fraction of microtubules moving following different durations of storage at 4 °C. At each time point, 45 video screens [54 μm × 70 μm] were analyzed from a total of three flow cells. (B) The number of microtubules bound per video screen. (C) Screen captures showing microtubule movement following 5 weeks of storage. Frames are 4 s apart, scale bar is 15 μm.

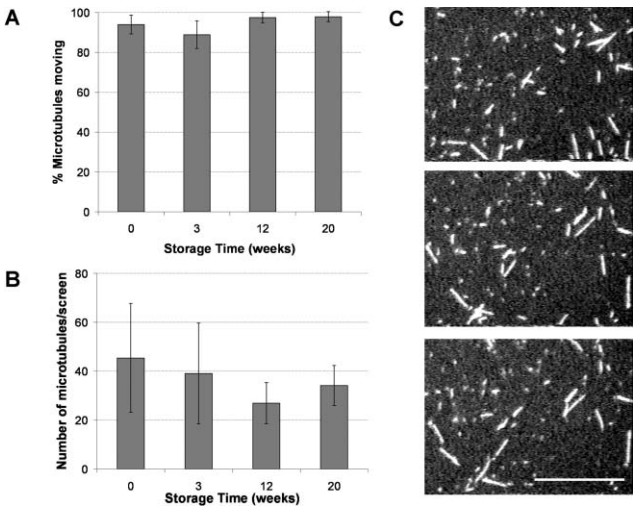

Fig. 2 Microtubule motility in freeze-dried samples. (A) The fraction of microtubules moving following different durations of storage at 4 °C. At each time point, 45 video screens [54 μm × 70 μm] were analyzed from a total of three flow cells. (B) The number of microtubules bound per video screen. (C) Screen captures showing microtubule movement following 5 weeks of storage. Frames are 4 s apart, scale bar is 15 μm.

fraction of moving microtubules remained near 100%. These data suggest that after critical point drying, storage for 20 weeks results in very little inactivation or denaturation of the surface-adsorbed kinesin motors.

As a comparison, kinesin-functionalized coverslips were freeze-dried and stored up to 20 weeks at 4 °C and then reconstituted with motility solution and analyzed. The movement of microtubules after storage for 20 weeks is shown in Fig. 2 and supplementary movie 2.† As seen in Fig. 2, the fraction of moving microtubules was high, indicating that few motors were inactivated. In both critical point-dried and freeze-dried samples, a subset of microtubules moved in a spiral pattern, presumably pivoting around one irreversibly bound motor at their leading end (supplementary movies 1 and 2†). However, the stalled and spiraling microtubules comprised only a small fraction of the bound filaments. The number of microtubules on the surface was somewhat lower for freeze-dried than for critical point-dried samples, indicating that a significant fraction of the motors were denatured by freeze-drying. With no storage time, the freeze-dried samples had roughly one-third fewer bound microtubules than the critical point-dried samples (this difference was statistically significant, unpaired *t*-test $P = 0.003$) and the number fell following storage. These freeze-drying results can be compared to the results from Seetharam *et al.*[17] who found that when flow cells containing both kinesin motors and microtubules were freeze-dried, stored at either room temperature or −80 °C and reconstituted, less than half of the microtubules moved after 24 days of storage. The high percentage of motile filaments observed here could be a result of introducing fresh microtubules into the flow cells, and because lyophilized microtubules are commercially available‡, one strategy would be to reconstitute the microtubules and the motor-functionalized surfaces separately and then combine them. Notably, in both the present experiments and those of Seetharam *et al.*, there was significant heterogeneity observed across different regions of the surface, which may indicate that by optimizing specific experimental variables, these freeze-drying results could be improved.

For designing hybrid microscale devices that use the kinesin-microtubule system for transport or actuation, it is important to be able to pattern kinesin motors in specific regions on a surface, for instance to guide microtubules in one direction. To date, it has been shown that by patterning photoresist[7,11,20] or by nanoimprinting a fluorinated polymer on a protein adsorbent surface such as glass,[3] functional kinesin proteins can be patterned on surfaces. However, all of these approaches lead to three dimensional surfaces, and it would be advantageous both to be able to pattern motors on flat two-dimensional surfaces and to use traditional photolithography processes on kinesin-functionalized surfaces. One advantage of critical point drying is that because the aqueous buffer is removed prior to drying, the salt residues that remain following freeze-drying are eliminated, resulting in a smooth surface that is amenable to photoresist deposition.

Using critical point-dried samples, we investigated whether it is possible to deposit photoresist on top of the preserved motors and pattern this photoresist to create patterns of functional surface-adsorbed kinesins. In initial studies, the motors did not survive treatment with Shipley 1811 developing solution, but we found that the motors were stable in acetone,[21] which is used to strip the photoresist. Hence, when a UV-exposed region of photoresist is developed, the underlying motors come into contact with the developing solution and either denature or are removed, while motors in the unexposed region are protected by the photoresist. After the remaining photoresist is removed using acetone, motility solution is introduced and the microtubules observed under the microscope. As seen in Fig. 3 and supplementary movie 3,† in the region where the photoresist was not exposed, microtubules were observed binding to the surface and moving across it, while no activity was observed in the exposed regions. The edges of the patterns were somewhat diffuse because the normal photoresist baking step was not used. However, the differences in the patterned and unpatterned regions were very clear. The number of microtubules bound per screen was 18.2 ± 4.1 (mean ± SD; $n = 6$), which is roughly 3-fold lower than unpatterned critical point-dried samples (Fig. 1), possibly due to incomplete stripping of the photoresist. Nevertheless, this result shows that a population of surface-adsorbed kinesin motors can be dried, covered with photoresist, and then exposed to acetone, and a significant fraction of the motors still retain their activity. This result expands the range of microfabrication techniques that can be used with kinesin motors, and suggests that similar approaches could be used to pattern antibodies or other functional proteins on surfaces.

Conclusion

Future microdevices that interface biological components with engineered materials have great promise, but the fragility of

‡ Cytoskeleton, Inc, Denver, CO, USA.

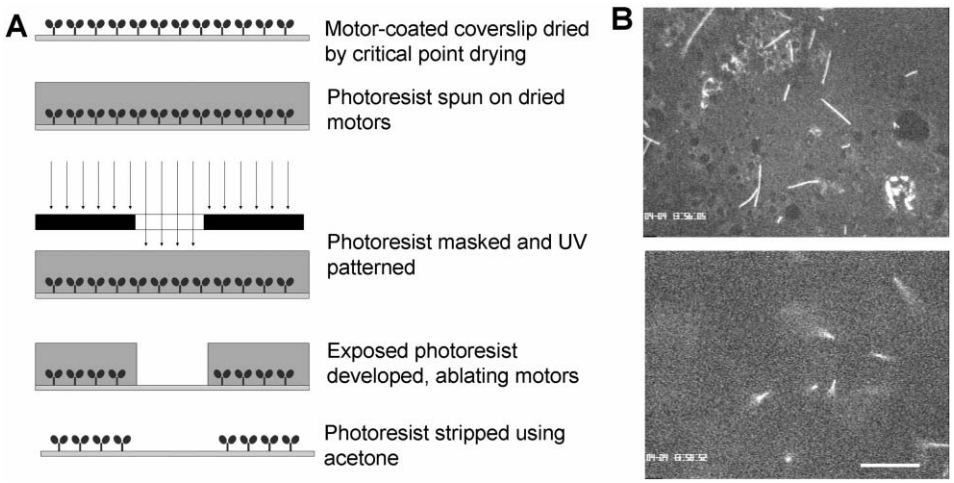

Fig. 3 Using photolithography to pattern critical point dried motors on glass substrates. (A) The patterning process. Photoresist is deposited on top of the motor-functionalized surface, patterened using contact lithography, developed to pattern motors in specific regions, and then stripped to expose functional motors. (B) Microtubule motility on a region of the surface protected by the photoresist (top) and a region of the surface where motors were exposed (bottom). See also supplementary movie 3† for microtubule activity on photoresist-protected surface (scale bar 15 μm).

the protein components often outweighs the improved functionality they provide. For these devices to become realistic, it is important to develop approaches for preserving protein function over time in novel device geometries. Because hybrid microdevices that incorporate the kinesin-microtubule system are at the forefront of this research area, methods for storing kinesin-functionalized samples long-term are needed. Here, we show that both critical point drying and freeze-drying kinesin-functionalized surfaces can extend the lifetime of the motors to months. Furthermore, following critical point drying, motors can be patterned using traditional photolithography approaches, which results in two-dimensional surfaces that contain both motor-functionalized and motor-free regions. These approaches extend the range of fabrication processes that can be used with kinesin motors, and they can be extended to other functional proteins immobilized on surfaces for sensors and other devices.

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References

1 W. O. Hancock and J. Howard, *J. Cell Biol.*, 1998, **140**, 1395–1405.
2 S. M. Rogers and J. M. Scholey, in *Encyclopedia of Life Sciences*, John Wiley & Sons Ltd, Chichester, 2004, DOI: 10.1038/npg.els.0002621, http://www.els.net/.
3 L. J. Cheng, M. T. Kao, E. Meyhöfer and L. J. Guo, *Small*, 2005, **1**, 409–414.
4 H. Hess, *Science*, 2006, **312**, 860–861.
5 H. Hess, G. D. Bachand and V. Vogel, *Chemistry*, 2004, **10**, 2110–2116.
6 M. Hirabayashi, S. Taira, S. Kobayashi, K. Konishi, K. Katoh, Y. Hiratsuka, M. Kodaka, T. Q. Uyeda, N. Yumoto and T. Kubo, *Biotechnol. Bioeng.*, 2006, **94**, 473–480.
7 Y. Hiratsuka, T. Tada, K. Oiwa, T. Kanayama and T. Q. Uyeda, *Biophys. J.*, 2001, **81**, 1555–1561.
8 Y.-M. Huang, M. Uppalapati, W. O. Hancock and T. N. Jackson, *Biomed. Microdev.*, 2007, **9**, 175.
9 L. Jia, S. G. Moorjani, T. N. Jackson and W. O. Hancock, *Biomed. Microdev.*, 2004, **6**, 67–74.
10 T. Kim, M. T. Kao, E. F. Hasselbrink and E. Meyhöfer, *Nano Lett.*, 2007, **7**, 211–217.
11 S. G. Moorjani, L. Jia, T. N. Jackson and W. O. Hancock, *Nano Lett.*, 2003, **3**, 633–637.
12 G. Muthukrishnan, B. M. Hutchins, M. E. Williams and W. O. Hancock, *Small*, 2006, **2**, 626–630.
13 G. Muthukrishnan, C. A. Roberts, Y. C. Chen, J. D. Zahn and W. O. Hancock, *Nano Lett.*, 2004, **4**, 2127–2132.
14 M. G. L. van den Heuvel, M. P. de Graaff and C. Dekker, *Science*, 2006, **312**, 910–914.
15 C. Brunner, K. Ernst, H. Hess and V. Vogel, *Nanotechnology*, 2004, **15**, S540–S548.
16 M. C. Lai and E. M. Topp, *J. Pharm. Sci.*, 1999, **88**, 489–500.
17 R. Seetharam, Y. Wada, S. Ramachandran, H. Hess and P. Satir, *Lab Chip*, 2006, **6**, 1239–1242.
18 H. P. Burstyn and A. A. Bartlett, *Am. J. Phys.*, 1975, **43**, 414–419.
19 R. C. Williams, Jr. and J. C. Lee, *Methods Enzymol.*, 1982, **85 Pt B**, 376–385.
20 H. Hess and V. Vogel, *J. Biotechnol.*, 2001, **82**, 67–85.
21 V. Verma, W. O. Hancock and J. M. Catchmark, *IEEE Trans. Adv. Packag.*, 2005, **28**, 584–593.