# Long-Term Storage of Surface-Adsorbed Protein Machines

Nuria Albet-Torres* and Alf Månsson

School of Natural Sciences, Linnaeus University, SE-39245 Kalmar, Sweden

**S** *Supporting Information*

**ABSTRACT:** The effective and simple long-term storage of complex functional proteins is critical in achieving commercially viable biosensors. This issue is particularly challenging in recently proposed types of nanobiosensors, where molecular-motor-driven transportation substitutes microfluidics and forms the basis for novel detection schemes. Importantly, therefore, we here describe that delicate heavy meromyosin (HMM)-based nanodevices (HMM motor fragments adsorbed to silanized surfaces and actin bound to HMM) fully maintain their function when stored at −20 °C for more than a month. The mechanisms for the excellent preservation of acto-HMM motor function upon repeated freeze—thaw cycles are discussed. The results are important to the future commercial implementation of motor-based nanodevices and are of more general value to the long-term storage of any protein-based bionanodevice.
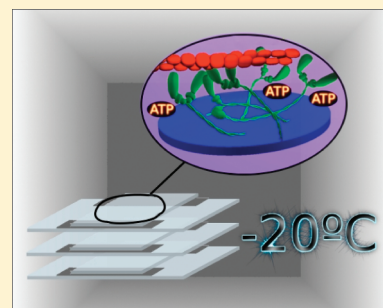


## ■ INTRODUCTION

The effective, but simultaneously cheap and simple, long-term storage of proteins with complex function is essential in nano-biotechnology (e.g., in the development of commercially viable biosensors). However, this is a challenging task with considerable variability among different proteins, both in stability and in the optimal storage method.[1] Moreover, the mechanisms for protein deterioration/preservation during storage under different conditions are not fully understood even though conformational instability, proteolytic degradation, and metal-ion-induced oxidation are of importance.[2]

The storage of complex protein nanomachines immobilized at artificial surfaces is of particular relevance to the biosensor field and surface immobilization might also increase protein stability during storage (ref 3, see further below), but this idea requires further exploration. The actomyosin motor system relies on intact enzymatic function, actin polymerization, and actomyosin bimolecular interactions, and it is sensitive to deterioration by several mechanisms.[4−6] It should therefore be a critical test system for investigations of functional changes during storage.

The actomyosin system is also of specific interest because recent investigations have identified potential molecular-motor-driven lab-on-a-chip applications (e.g., in diagnostics (cf. refs 7 and 8), periodic chemistry,[9] sorting,[10−12] and biosensing[13]). Such applications, where myosin- or kinesin-propelled cytoskeletal filaments (actin filaments or microtubules) act as shuttles for cargo transportation, have certain advantages compared to microfluidic-driven transport.[7,14] This includes a greater potential for miniaturization and a unique approach to the concentration of analyte molecules and the separation of analyte-binding and detection sites in diagnostics. In addition, molecular-motor devices are self-sustained by the transduction of the chemical energy of ATP directly into mechanical work, thereby circumventing the need for external power supplies (e.g., to drive pumps

for microfluidics). In this context, the actomyosin motor system (compared to kinesin microtubules) has advantages such as high speed (10 $\mu$m/s) and a greater potential for miniaturization.[7,15,16]

Previous studies on motor-driven nanodevices have focused on technically advanced developments (e.g., guided transport on nanostructured surfaces and approaches to cargo pick up and transport by motor-driven shuttles[14,17,18]). However, less attention has been devoted to the absolutely essential long-term storage of the devices. Whereas some studies of microtubule—kinesin motor systems have used lyophilization[19,20] or critical-point drying,[20] studies on actomyosin have been limited to the effects of storage at 4 °C, which was found to be possible for a little more than a week.[21,22]

On the basis of the idea that surface immobilization may have certain stabilizing effects during storage, we study here the effects of freeze—thaw cycles on the delicate motor function of actomyosin immobilized on silanized surfaces. The results show that complete in vitro motility assay systems with surface-adsorbed myosin motor fragments (heavy meromyosin, HMM), actin filaments, and ATP can be readily stored at −20 °C for at least a month without a loss of function. This was achieved without cumbersome procedures such as freezing in liquid nitrogen or the use of expensive equipment (e.g., −80 °C freezers or lyophilization equipment). The general mechanisms for the long-term functional preservation of surface-immobilized delicate protein nanomachines are discussed. Our findings unfold new possibilities for portable molecular-motor-based nanodevices as well as for new developments toward the optimized storage of other proteins with complex function.
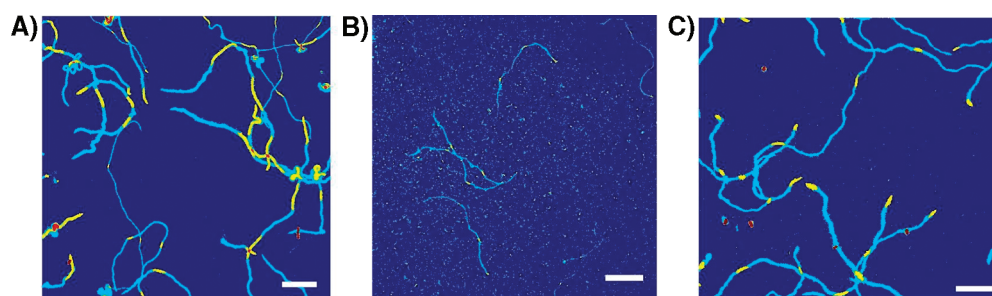
**Figure 1.** Actin filament path traces are integrated for 10 s: (A) the flow cell before freezing (same sequence as shown in Movie 1 in the Supporting Information), (B) the same flow cell after having been frozen for 17 days and thawed without adding any new solution (the same as in Movie 2), and (C) the same flow cell after adding new actin and ATP-containing solution (the same as in Movie 3). White bars, 10 $\mu$m. Yellow parts of the traces indicate the filament position 0, 3.2, and 6.8 s after the onset of recording.
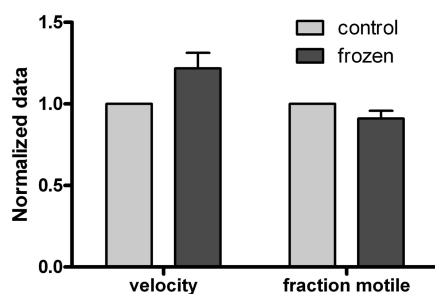


**Figure 2.** Effects of one freeze—thaw cycle on the quality of HMM-propelled actin filament motility. Data from five different experiments. The control velocity was 5.97 $\pm$ 0.63 $\mu$m/s, and the control fraction of motile filaments was 0.74 $\pm$ 0.04. The duration of the freezing period varied between 4 and 30 days (average 16.00 days) without an appreciable difference in the effect on motility over this period. Error bars: SEM.

## EXPERIMENTAL SECTION

The myosin motor fragment, heavy meromyosin (HMM, chymotryptically cleaved myosin), and actin filaments were obtained from rabbit fast skeletal muscle.[4,5] Actin was fluorescently labeled using tetramethylrhodamineisothiocyanate-phalloidin (TRITC-phalloidin).[5] Glass slides were silanized with trimethylchlorosilane (TMCS) and mounted in a flow cell for in vitro motility assay (IVMA) experiments[5,21] with incubation steps as follows: (1) HMM (120 $\mu$g/mL, 2 min), (2) bovine serum albumin (BSA, 1 mg/mL), (3) nonfluorescent blocking actin (1 $\mu$M, to block rigor heads), (4) TRITC-labeled actin filaments, and (5) assay solution (aMC130, ionic strength 130 mM, 1 mM MgATP, and 0.6% methylcellulose). Between steps 3 and 4, the flow cell was rinsed with solution a40 (1 mM ATP, ionic strength 40 mM) followed by solution r65 (no ATP, ionic strength 65 mM). Rinsing with solution r65 was employed between steps 4 and 5. (See the Supporting Information for a detailed composition of solutions.) The motility assays were performed at a temperature of 27.6 ($\pm$1.0) °C.

For the storage of motility assays by freezing, flow cells were placed in a regular −20 °C freezer directly after observation in the microscope without washing away the ATP-containing assay solution (except in some cases, as indicated below). After periods of freezing, flow cells were thawed at room temperature (approximately 20 °C) for a few minutes before the observation of in vitro motility. After a first observation, flow cells were rinsed with r65 solution and then incubated with new labeled actin, r65 solution and aMC130 assay solution.

The actin filaments were imaged using an inverted epifluorescence microscope (Nikon Eclipse TE300). Data was acquired using a CCD camera and analyzed using a MATLAB-based algorithm.[23] GraphPad Prism software was used for graphic representation and statistical

analysis by means of a paired or unpaired (two-tailed) $t$ test as appropriate. Data are given as the mean $\pm$ standard error of the mean (SEM).

## RESULTS AND DISCUSSION

The HMM-propelled actin filament motility was maintained after the freezing and thawing of whole flow cells that contained all of the components of the IVMA (Experimental Section). When the flow cell had been frozen with both actin filaments and an ATP-containing assay solution and stored at −20 °C for up to 30 days, motility was resumed after thawing without the addition of new assay solution. This is exemplified in Figure 1A—C (Supporting Information, Movie 2). However, for quantitative comparison to the prefreezing conditions, it was important to avoid complications attributed to photobleaching, the consumption of ATP, and the accumulation of possible toxic products (e.g., gluconic acid due to the antibleaching mixture) during the first observation in the fluorescence microscope (before freezing). We therefore added a new assay solution and new actin filaments prior to quantitative measurements (Figure 1C). It can be seen in Figure 2 that both the sliding velocity and the fraction of motile filaments were similar to the values before freezing (no significant difference; $p$ = 0.08 (velocity), $p$ = 0.13 (fraction motile)). Data from different experiments was pooled in a single diagram because there was no apparent difference between flow cells kept frozen for different time periods (up to 30 days). Thus, motility was maintained for more than 1 month of freezing without a noticeable decay over this time period (Figures 3 and 4). In contrast to what was reported for lyophilization and critical-point drying[20] and subsequent reconstitution of the kinesin—microtubule motility assays, we did not observe any appreciable freeze—thaw-induced variability in function between different regions of a flow cell. Because we did not test lyophilization or other drying methods, these approaches might not be ideal for actomyosin (cf. ref 24).

Surprisingly, a given surface with immobilized HMM could be taken through more than one freeze—thaw cycle (at least two) without substantial deterioration of the motility quality. Even after three cycles and altogether 60 days of storage at −20 °C, motility remained with 25% motile filaments (68% motile in control before freezing) and some filaments moving at a similar velocity as in the control experiments (Supporting Information, Movie 4). That there is progressive deterioration with repeated freeze—thaw cycles is not surprising because several stresses on the proteins are associated with the physical-chemical changes during the freezing and thawing processes per se,[25,26] suggesting
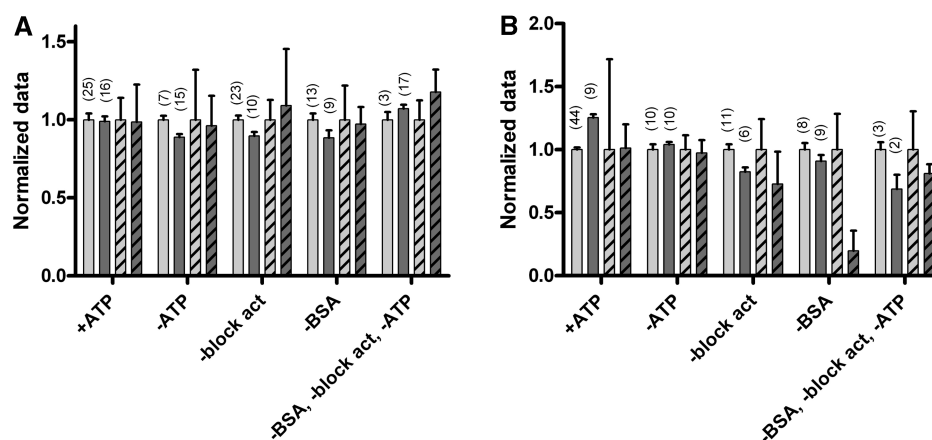
**Figure 3.** Effect of in vitro motility assay incubation conditions on motility quality after storage. (A) Flow cells frozen for 6 days. (B) Flow cells frozen for 30 days. In both A and B, the flow cells were subjected to only one freeze−thaw cycle. Bars show the velocity data (smooth bars) and the fraction of motile filaments (striped bars) of five different flow cells treated in different ways (shown in the x axes; see the text for a further explanation). Bright bars show data for the control flow cells (day 0), and dark bars show data after the freeze−thaw cycle. Error bars: SEM. Numbers in parentheses give the number of filaments from which the average sliding velocity was obtained by automatic tracking. One criterion for inclusion in the analysis was a coefficient of variation of the frame-to-frame velocity of <0.2. (See the text.) The fraction of motile filaments with error bars was obtained by observing three different areas of the flow cell.
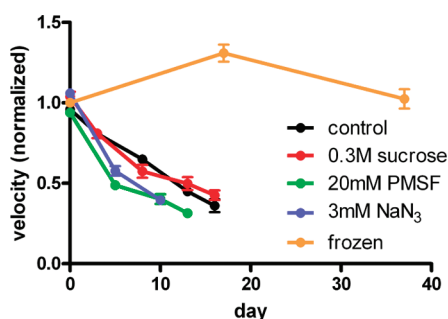


**Figure 4.** Time series of normalized velocity. Results for five different flow cells are shown, four of which were kept at 4 °C under different conditions: control (black), 0.3 M sucrose (red), 20 mM PMSF (green), or 3 mM $NaN_3$ (blue) and another flow cell (orange) was frozen at −20 °C and thawed for every observation.

that these, rather than the storage at −20 °C, are most detrimental to function. Therefore, it also seems likely that storage at −20 °C should be possible for a considerably longer time than that demonstrated here.

Experiments were performed (Figure 3) to investigate the importance of different components in the standard IVMA assay incubation for the maintenance of actin motility after a freeze−thaw cycle. Five different conditions were tested: Flow cells frozen (i) with ATP-containing assay solution and incubated with all other components (+ATP), (ii) with all components except BSA (-BSA), (iii) with all components except blocking actin (-block.act.), (iv) without ATP solution (rinsed after checking the control actin motility) but otherwise incubated with all components (-ATP), and finally (v) with all components except BSA and blocking actin (-ATP, -BSA, -block.act.). The results (Figure 3) show no clear loss of motility in any of these cases upon storage at −20 °C. (However, it should be noted that the omission of blocking actin led to a low fraction of smoothly moving filaments (cf. Kron et al.;[5] Homsher et al.[27]) both before freezing (i.e., under control conditions) and after freezing. This, in turn, led to very few filaments analyzed with respect to sliding

velocity in both cases as a result of the stringent criteria imposed by the automatic analysis system (e.g., with the requirement of a coefficient of variation (CV) of the frame-to-frame velocity of <0.20). We therefore also performed a manual analysis of the case without blocking actin and BSA in Figure 3B. With this approach, filaments were tracked using the computer mouse, and the inclusion of more erratically moving filaments (CV < 0.5) was possible. Importantly, this analysis gave results consistent with those based on the automatic analysis of smoothly moving filaments (CV < 0.2). Thus, data based on filament paths with CV < 0.50 gave a velocity of $7.86 \pm 0.27$ $\mu$m/s before freezing ($n = 18$) and $7.15 \pm 0.39$ $\mu$m/s after freezing ($n = 26$), a difference that was not statistically significant ($p = 0.158$)).

For comparison, we also investigated the storage of the motility assay flow cells (containing all of the components of the IVMA) in a cold room at 4 °C (Figure 4). Different conditions were tested: (1) control, (2) 0.3 M sucrose (to counteract unfolding), (3) 20 mM PMSF (protease inhibitor) and (4) 3 mM $NaN_3$ (antibacterial). No appreciable difference was observed among the different conditions (Figure 4), but in all flow cells, the actin filament velocity was reduced to half the control value after 5 to 14 days.

To summarize the above findings, HMM-propelled actin filament sliding was very well preserved when flow cells were subjected to up to two freeze−thaw cycles. This shows that the complex actomyosin motor system fully maintains its function under these conditions. This, in turn, relies on the maintenance of the structural integrity of the dynamic[28] and proteolysis-sensitive[4] actin filaments. It also relies on the maintained capability of the majority of the myosin motor domains to bind to actin and undergo complex sequences of structural changes that lead to the propulsion of actin filaments coupled to different steps in the turnover of ATP[29] on the myosin active site. Moreover, our results suggest that neither the surface immobilization of HMM nor the HMM surface density is appreciably altered by the freeze−thaw cycles (and several rinsing and incubation steps).[9,30−33] We can draw this conclusion because

the velocity of actin propulsion by low-duty-ratio myosin II motors (in contrast to microtubule propulsion by high-duty-ratio kinesin[34]) is known[33,35] to be quite sensitive to the density of appropriately oriented HMM molecules on the surface. It is also important to view the results in the context of evidence[5,36] that the mode of storage of HMM in solution prior to motility assays has critical effects on the velocity and/or the fraction of motile filaments. The latter variables are sensitive not only to the myosin head density but even more to the presence of damaged myosin heads.[37] Our results suggest that, in contrast to the freezing of HMM in solution, freezing after surface immobilization gives excellent preservation of actomyosin function upon storage at −20 °C and for up to two freeze−thaw cycles. Considering the complexity of the system, this degree of preservation might seem unexpected because proteins tend to degrade and lose activity as a result of freeze−thaw cycles for a variety of stresses.[1,2,38] These include cold denaturation, freeze concentration (with osmotic gradients, local changes in pH, and local crystallization of solutes), and finally the effects of ice formation, such as surface-induced denaturation.[38] With regard to HMM, in particular, it has been indicated that freezing in solution at −20 °C for later use in the IVMA is not advisable, even if glycerol is added as a cryoprotecting agent.[36,39] This may be understood because some regions of the HMM molecule exhibit conformational instability (cf.ref 6) whereas other regions are sensitive to oxidation. The good preservation of function in the present work may, to a substantial degree, be attributed to factors related to the surface immobilization of the proteins. One important factor may be the high local concentration at the surface because it has been shown that after freezing and thawing in solution a higher concentration of several proteins induces a higher recovery of activity.[40] Whereas HMM is adsorbed on TMCS surfaces in a distribution of different conformations,[30] the myosin heads will tend to oscillate thermally within 40 nm from the surface.[30] With the HMM incubation concentration of 120 $\mu$g/mL, as in the present work, one would expect the adsorption of >5000 HMM molecules/$\mu$m$^2$ corresponding to an HMM concentration of ∼0.2 mM (∼70 g/L) in a layer within 40 nm from the surface. This is similar to the protein concentration in blood plasma. In addition to the effect attributed to high protein concentration in the HMM layer, it is also likely that the mode of adsorption of HMM is important with the heads generally tens of nanometers from the TMCS surface.[30] Finally, an additional effect related to surface immobilization is the presence of unstirred layers at surfaces that will minimize shear stresses on the proteins. The dominating HMM head conformation (different in the presence and absence of ATP) and the presence and absence of actin did not seem to be critical to long-term storage. However, the data in Figure 3 indicate a tendency toward reduced motility quality after storage in the absence of incubation with BSA and blocking actin. The inclusion of these incubation steps may thus be advisable and consistent with expectations for enhanced protein stability with further increases in the protein concentration close to the surface. We performed the experiments in silanized flow cells with a local physical environment (with respect to solutions and surface substrates) that are suitable for future nanodevices (cf. refs 9 and 32). One may consider using silanes with longer alkyl chains to increase the surface hydrophobicity further, with the potential for additionally increased adsorption of proteins.[41] If such increased adsorption had been seen for HMM, this might further increase the stability during freezing and thawing. However, under the present HMM

incubation conditions, the HMM density on TMCS surfaces is close to that of a packed monolayer. (See above and ref 30.) Moreover, it has been shown[42] that increased hydrophobicity above that on TMCS-derivatized surfaces (contact angle ∼70°) is associated with a lower HMM-propelled actin velocity. Indeed, a similar effect (i.e., with a lower velocity) was observed on surfaces silanized using chlorosilanes with longer side chains.[31] In these cases, the contact angle was actually lower than 70° probably because of steric effects of the side chains that were believed to limit the degree of silanization. To summarize the above discussion, the chemically very simple trimethylchlorosilanized surfaces seem to be nearly ideal for use in bionanodevices. They are readily nanopatterned[9,43] and give very reproducible high-quality motility[21,31,32] close to room temperature, and as shown here, the preservation of actomyosin function during freeze−thaw cycles is excellent. In this connection, it is important to emphasize that the experiments were performed in the simplest possible way (i.e., by directly freezing the flow cells subsequent to initial observation and then thawing them at room temperature). There would thus be substantial potential for further optimization of the conditions (e.g., the addition of polyols and/or other cryoprotectants[2] and appropriate titrations of the freezing and thawing rates[38]). The exact process of ice formation (e.g., the freezing point[44]) seems to vary with the hydrophobicity of the surface (e.g., the freezing point on TMCS surfaces would be expected to be intermediate between those of hydrophilic and more strongly hydrophobic surfaces). This may be important to consider in future efforts to optimize the storage conditions. The excellent preservation without optimization and evidence that the freeze−thaw cycles rather than the storage itself is detrimental suggest that surface-immobilized actomyosin-based nanodevices can be readily stored at −20 °C for a longer time than observed here (i.e., considerably more than 1 month).

## CONCLUSIONS

We have demonstrated that the long-term storage of myosin-based nanodevices is possible at −20 °C (e.g., using an ordinary household freezer). This finding reaffirms the possibility of using such nanodevices at remote locations (e.g., in point-of-care diagnostics because no expensive or sophisticated laboratory equipment is required for storage). The method of storage may also be useful in high-throughput studies of actomyosin function (e.g., for drug-discovery applications[9] or other routine investigations). Moreover, the results may be possible to extrapolate to the storage of other complex protein nanomachines. Thus, it may be of interest to consider storage at −20 °C after immobilization of the proteins to biosensor surfaces or, alternatively, to nanoparticles or liposomes in solution.

## ASSOCIATED CONTENT

Ⓢ **Supporting Information.** Extended methods and four movies. This material is available free of charge via the Internet at http://pubs.acs.org.

## AUTHOR INFORMATION

**Corresponding Author**
*Corresponding author. E-mail: nuria.albettorres@lnu.se. Correspondence also to: alf.mansson@lnu.se.

## ■ REFERENCES

(1) Arakawa, T.; Prestrelski, S. J.; Kenney, W. C.; Carpenter, J. F. *Adv Drug Delivery Rev.* **2001**, *46*, 307–326.

(2) Chaniotakis, N. A. *Anal. Bioanal. Chem.* **2004**, *378*, 89–95.

(3) Singh, A. K.; Flounders, A. W.; Volponi, J. V.; Ashley, C. S.; Wally, K.; Schoeniger, J. S. *Biosens. Bioelectron.* **1999**, *14*, 703–713.

(4) Pardee, J. D.; Spudich, J. A. *Methods Cell Biol.* **1982**, *24*, 271–89.

(5) Kron, S. J.; Toyoshima, Y. Y.; Uyeda, T. Q.; Spudich, J. A. *Methods Enzymol.* **1991**, *196*, 399–416.

(6) Månsson, A.; Balaz, M.; Albet-Torres, N.; Rosengren, K. J. *Front Biosci.* **2008**, *13*, 5732–54.

(7) Korten, T.; Månsson, A.; Diez, S. *Curr. Opin. Biotechnol.* **2010**, *21*, 477–488.

(8) Agarwal, A.; Hess, H. *Prog. Polym. Sci.* **2010**, *35*, 252–277.

(9) Sundberg, M.; Bunk, R.; Albet-Torres, N.; Kvennefors, A.; Persson, F.; Montelius, L.; Nicholls, I. A.; Ghatnekar-Nilsson, S.; Omling, P.; Tågerud, S.; Månsson, A. *Langmuir* **2006**, *22*, 7286–95.

(10) van den Heuvel, M. G. L.; de Graaff, M. P.; Dekker, C. *Science* **2006**, *312*, 910–4.

(11) Ionov, L.; Stamm, M.; Diez, S. *Nano Lett.* **2005**, *5*, 1910–1914.

(12) Kim, T.; Cheng, L.-J.; Kao, M.-T.; Hasselbrink, E. F.; Guo, L.; Meyhöfer, E. *Lab Chip* **2009**, *9*, 1282–1285.

(13) Fischer, T.; Agarwal, A.; Hess, H. *Nat. Nanotechnol.* **2009**, *4*, 162–166.

(14) Månsson, A.; Sundberg, M.; Bunk, R.; Balaz, M.; Nicholls, I. A.; Omling, P.; Tegenfeldt, J. O.; Tagerud, S.; Montelius, L. *IEEE Trans. Adv. Packag.* **2005**, *28*, 547–555.

(15) Nitta, T.; Tanahashi, A.; Obara, Y.; Hirano, M.; Razumova, M.; Regnier, M.; Hess, H. *Nano Lett.* **2008**, *8*, 2305–2309.

(16) Vikhorev, P. G.; Vikhoreva, N. N.; Månsson, A. *Biophys. J.* **2008**, *95*, 5809–5819.

(17) Ramachandran, S.; Ernst, K. H.; Bachand, G. D.; Vogel, V.; Hess, H. *Small* **2006**, *2*, 330–334.

(18) Brunner, C.; Wahnes, C.; Vogel, V. *Lab Chip* **2007**, *7*, 1263–1271.

(19) Seetharam, R.; Wada, Y.; Ramachandran, S.; Hess, H.; Satir, P. *Lab Chip* **2006**, *6*, 1239.

(20) Uppalapati, M.; Huang, Y.-M.; Jackson, T. N.; Hancock, W. O. *Lab Chip* **2008**, *8*, 358.

(21) Sundberg, M.; Rosengren, J. P.; Bunk, R.; Lindahl, J.; Nicholls, I. A.; Tågerud, S.; Omling, P.; Montelius, L.; Månsson, A. *Anal. Biochem.* **2003**, *323*, 127–38.

(22) Grove, T. J.; Puckett, K. A.; Brunet, N. M.; Mihajlovic, G.; McFadden, L. A.; Xiong, Peng; von Molnar, S.; Moerland, T. S.; Chase, P. B. *IEEE Trans. Adv. Packag.* **2005**, *28*, 556–563.

(23) Månsson, A.; Tågerud, S. *Anal. Biochem.* **2003**, *314*, 281–93.

(24) Allison, S. D.; Randolph, T. W.; Manning, M. C.; Middleton, K.; Davis, A.; Carpenter, J. F. *Arch. Biochem. Biophys.* **1998**, *358*, 171–181.

(25) Chang, B. S.; Kendrick, B. S.; Carpenter, J. F. *J. Pharm. Sci.* **1996**, *85*, 1325–1330.

(26) Cao, E.; Chen, Y.; Cui, Z.; Foster, P. R. *Biotechnol. Bioeng.* **2003**, *82*, 684–690.

(27) Homsher, E.; Wang, F.; Sellers, J. R. *Am. J. Physiol.* **1992**, *262*, C714–23.

(28) Murakami, K.; Yasunaga, T.; Noguchi, T. Q. P.; Gomibuchi, Y.; Ngo, K. X.; Uyeda, T. Q. P.; Wakabayashi, T. *Cell* **2010**, *143*, 275–287.

(29) Geeves, M. A.; Holmes, K. C. *Adv. Protein Chem.* **2005**, *71*, 161–93.

(30) Persson, M.; Albet-Torres, N.; Ionov, L.; Sundberg, M.; Hook, F.; Diez, S.; Månsson, A.; Balaz, M. *Langmuir* **2010**, *26*, 9927–9936.

(31) Albet-Torres, N.; O'Mahony, J.; Charlton, C.; Balaz, M.; Lisboa, P.; Aastrup, T.; Månsson, A.; Nicholls, I. A. *Langmuir* **2007**, *23*, 11147–56.

(32) Sundberg, M.; Balaz, M.; Bunk, R.; Rosengren-Holmberg, J. P.; Montelius, L.; Nicholls, I. A.; Omling, P.; Tågerud, S.; Månsson, A. *Langmuir* **2006**, *22*, 7302–12.

(33) Uyeda, T. Q.; Kron, S. J.; Spudich, J. A. *J. Mol. Biol.* **1990**, *214*, 699–710.

(34) Howard, J.; Hudspeth, A. J.; Vale, R. D. *Nature* **1989**, *342*, 154–8.

(35) Chase, P. B.; Chen, Y.; Kulin, K. L.; Daniel, T. L. *Am. J. Physiol. Cell Physiol.* **2000**, *278*, C1088–C1098.

(36) Harada, Y.; Sakurada, K.; Aoki, T.; Thomas, D. D.; Yanagida, T. *J. Mol. Biol.* **1990**, *216*, 49–68.

(37) Prochniewicz, E.; Katayama, E.; Yanagida, T.; Thomas, D. D. *Biophys. J.* **1993**, *65*, 113–123.

(38) Bhatnagar, B. S.; Bogner, R. H.; Pikal, M. J. *Pharm. Dev. Technol.* **2007**, *12*, 505–523.

(39) Warrick, H. M.; Simmons, R. M.; Finer, J. T.; Uyeda, T. Q.; Chu, S.; Spudich, J. A. *Methods Cell Biol.* **1993**, *39*, 1–21.

(40) Jiang, S.; Nail, S. L. *Eur. J. Pharm. Biopharm.* **1998**, *45*, 249–257.

(41) Sigal, G. B.; Mrksich, M.; Whitesides, G. M. *J. Am. Chem. Soc.* **1998**, *120*, 3464–3473.

(42) Nicolau, D. V.; Solana, G.; Kekic, M.; Fulga, F.; Mahanivong, C.; Wright, J.; Ivanova, E. P.; dos Remedios, C. G. *Langmuir* **2007**, *23*, 10846–54.

(43) Bunk, R.; Sundberg, M.; Månsson, A.; Nicholls, I. A.; Omling, P.; Tågerud, S.; Montelius, L. *Nanotechnology* **2005**, *16*, 710–717.

(44) Yin, L.; Xia, Q.; Xue, J.; Yang, S.; Wang, Q.; Chen, Q. *Appl. Surf. Sci.* **2010**, *256*, 6764–6769.