Actin Nanotracks for Hybrid Nanodevices Based on Linear Protein Molecular Motors

G. S. Watson¹, C. Cahill¹, J. Blach¹, S. Myhra¹, Y. Alexeeva, E.P. Ivanova, D. V. Nicolau
BioNanoEngineering Lab, Swinburne University of Technology

Hawthorn, VIC 3122, Australia

¹School of Science, Griffith University,
Nathan, QLD 4111, Australia

ABSTRACT

Hybrid nano-devices based on linear protein molecular motors working on micro/nano-engineered surfaces that operate in a "cargo architecture", i.e. motor functionalized nano-objects running on nano-tracks, offer more opportunities than the inverse "sliding architecture" because it fully uses the information regarding directionality which is encoded in tracks, i.e. actin filaments or microtubules. However, this architecture requires the development of techniques for nanolithography with actin filaments (or microtubules) based on molecular self-assembly on engineered surfaces. The present contribution reports on the progress we have made regarding the building of actin nanostructures that would preserve the inherent information over extended micro-sized areas.

INTRODUCTION

Many of the biological functions of the cell, as diverse as cell movement and division, transport of vesicles and muscle function, are performed by ubiquitous protein linear molecular motors. These work as a pair in tandem, transforming chemical energy, through the hydrolysis of adenosin-triphosphate (ATP), in mechanical energy – movement. Despite many differences, most notably in molecular weight, direction of movement, etc. the linear motor proteins, either myosin, or kinesin or the less studied dynein, share a structural architectural similarity. First, the molecular motors *per se* are long proteins that end in a forked molecular structure with two arms, which actually perform the mechanical work. Second, and more important for the purposes of this contribution, the non-motor protein, either actin in the actin-myosin pair, or microtubules in the kinesin-tubulin pair, are filamentous constructions, presenting a helical structured surface with "stepping pads" for the respective motors, which "walk" along the filaments through a grip-and-push sequence of events. Some 'rule-of thumb' (approximate) values may be useful to put in context the forces involved in molecular motors. The weight of a cell's nucleus (in water) is 0.2 pN; the actin-gelsolin bonding force is 20 pN (gelsolin is a protein which binds exclusively at one end of actin); the dissociation of actin from myosin and the power stroke of myosin is 9 pN; the strength of the actin-actin bond is a 110 pN (actin filaments are formed via the polymerization of individual actin molecules); the biotin-streptavidin bond is 250 pN (almost similar to a weak true covalent bond); and a carbon-carbon bond is 30K pN.

Given the ubiquity and efficiency of molecular motors, the last decade or so witnessed an increasing number of efforts to build hybrid nano-devices using linear molecular motors. Early attempts to demonstrate the principles for future nanodevices, e.g. confining the motility in microstructures [1], [2], [3], [4], [5], [6], [7] and unidirectionality [8], [9], [10] have been followed by the demonstration or proposal of several primitive prototype devices that perform

different functions, e.g. moving cargoes [11], [12-unger], force measurement [13], imaging [14] and computation [15]. Essentially these proposed devices are off-springs of the motility assays, which can have either a “sliding assay” geometry [16] sliding geometry architecture, i.e. filaments/microtubules running on motor functionalized micro- or nanostructures, or a “bead assay” geometry [17], i.e. motor-functionalised cargoes running on filaments/microtubules.

Despite the clear *operational* advantages of the devices with bead geometry, many proposed primitive devices have a sliding geometry because of the *fabrication* advantages. However, in principle the sliding geometry brings the added complexity of ‘selecting’ microstructures to ensure unidirectionality of filaments/microtubules – if this is needed. Also, and perhaps more importantly, a major asset of is lost: the information regarding directionality of movement is encoded in the filaments/microtubules! Other problems aside, the logical way to build devices based on molecular motors is to do it Nature’s way, i.e. encode paths in filaments (or microtubules) and let the motors run on the nano-fabricated tracks. However, this –arguably more- logical approach raises several nano-fabrication problems, in particular the preservation of the information encoded in the filaments/microtubules. This is particularly difficult, but equally more rewarding if it can be achieved, for actin which is sued not only for directional transport but also for directional cytoskeleton building.

The present contribution reports on the progress we have made regarding the building of actin nanostructures that would preserve the inherent information over extended micro-sized areas.

RESULTS AND DISCUSSION

Actin: structure and properties

Actin is a highly asymmetrical molecule (figure 1) with two domains separated by a cleft which can host an ATP or ADP molecule and one divalent cation [18]. The monomeric form of actin (G-actin) self-assemble in filaments (F-actin), a process called “polymerization”, despite the fact that not new chemical bonds are formed. The transformation of G-actin to F-actin require the presence of salts and dephosphorylation of ATP into ADP and inorganic phosphate. The sites where the polymerization occurs are at the ‘ends’ of the actin molecule, denominated as the ‘pointed end’ and the ‘barbed end’, that is the self-assembly process progresses in a head-to-tail fashion [19].

Like almost any protein, but more critical, actin can irreversibly denaturate, in particular when bound nucleotides and cations are lost. ATP is then a ‘dynamic’ stabilizer whereas toxins (e.g. phalloidin, which essentially caps the growing end of the filament) are irreversible stabilizers. The sensitiveness of actin polymerization translates in the environment-controlled modulation of the properties of actin filaments, i.e. preparation, polymerization conditions and storage of actin monomers [20] or presence of actin related proteins, e.g. [21]. Table 1 reflects the variance of the mechanical properties actin filament *networks* as modulated by polymerization conditions [22], with stiffness $F/\Delta L$ (i.e. the force F needed to effect a length change, ΔL) being equal to $E/(aL)$ where E is the Young modulus and a is the cross-sectional area of a cylinder of the material on which the force F is applied. A single filament appears to have a stiffness of 65pN/nm and a Young modulus of $2 \cdot 10^9$ N/m² [24].

Figure 1. Electrostatic potential map of actin monomer (PDB code: 1HLU [24]).

Table 1. Mechanical properties of actin-related materials (adapted from [23])

Ref.	Method	Stiffness (pN/nm)	$\Delta L/L$	F (pN)	a (nm ²)	E (N/m ²)
[24]	Micro needle	65.3 ± 6.3	few nm/20 μ m	35-170	25	1.8×10^9
[25]	X-ray diffraction	-	$\approx 0.2\%$	250	50.3	2.5×10^9
[26]	Optical diffraction	45.7–67.6	<4nm/ μ m	<220	-	$\approx 2 \times 10^9$
[27]	X-ray diffraction	-	0.25 – 0.3 %	<250	-	$\approx 2 \times 10^9$

The (positive) conclusion is that if one can manipulate the properties of actin filaments via environmental conditions, one can use the environment, be that fluid- or surface-related, to manipulate the self-assembly of actin on surfaces.

Flow modulated actin nanolithography

The easiest alternative to obtain ordered structures with actin filaments is to use “designed” flow paths (figure 2). This was the simplest method for fabrication, which is naturally occurring, albeit parasitically, in classical flow cells. We could obtain either parallel or anti-parallel arrangements of filaments on surfaces. Although this method gives quite reproducible results in terms of filament alignment and it is easy to implement, the orientation of filaments is random. Therefore the method should be supplemented with another orientation-active method.

Figure 2. Actin filaments aligned by flow in grooves on flat surfaces (right) and in capillaries (left).

Moreover, actin filaments are fragile and the strong shear stresses could easily rupture them.

Application of electric fields

If electrodes are mounted at both ends of the flow cell, then in particular conditions the actin filaments are organized –most of the time– in a parallel arrangement (figure 3). This method or alignment has been studied before [10], but in the context of acceleration of the movement of actin filaments on myosin-functionalised channels. The orientation of the filaments is uncertain, but as actin filaments are supposed to have a dipole moment the unidirectionality could be assumed. Two problems arise with this method. Firstly, the electrodes have to be placed far away from the working cell, or electrolysis may start to occur. This experimental set-up imposes high voltages (up to 10V/cm) with associated experimental problems. A more delicate handling would require Pt patterned electrodes and possibly a combination of AC and DC electrical currents. Secondly, the application of electrical currents translates to an “ionic current” and subsequently a flow in the cell, resulting in an overlap of mechanical and electrical-induced alignment.

Figure 3. Actin filaments aligned parallel and parallel/perpendicular to an applied electric field (10kV/m).

Interestingly, in a number of instances, the orientation of the filaments appears to be perpendicular to the lines of the “ionic current”. Further work with micropatterned electrodes is underway.

Self-assembly of actin filaments around Au nanorods

Gold nanoparticles, in particular 10x40nm nanorods, have the similar dimensions with actin filaments (~8nm diameter). When mixed with gold nanorods, actin filaments form aggregates in the form of rectangular rafts on surfaces. Interestingly, and similarly with the experiments regarding the alignment in electric fields, the aggregates comprising actin filaments also align quasi-perpendicular to the flow in the cell (figure 4, right). Despite the high reproducibility of the method, the actin nano-structures are not very useful for hybrid nano-devices having a cargo architecture.

Figure 4. Rafts of aggregates of actin filaments/gold nanorods.

Patterning actin on ablated micro-channels

More classical microlithography can be used for the patterning of actin filaments in conjunction with one of the methods above. For instance, we could pattern actin filaments organized in micro-channels fabricated through the ablation of thin layers of polymers containing O-acryloyl acetophenone oxime (AAPO) which generate amino groups upon exposure [28].

Figure 5. F-actin (right) and myosin-functionalised microchannels fabricated in AAPO.

As actin is in normal buffer conditions negatively charged, actin filaments strongly attach on the bottom of microfabricated channels (figure 5).

Patterning actin via polymerization on surfaces or in micro-channels

All the methods reported so far used 'prefabricated' F-actin. However, in order to fully use the information-rich self-assembly one would need to somehow 'polymerize-actin-on-the-fly' and adsorb it on prefabricated structures. One attempt is to polymerize actin on microstructures using a flow cell. The best results have been obtained via the covalent immobilization of G-actin on Poly(styrene-co-maleic anhydride) followed by continuous flow of protein over 1.5 hours (figure 6). This procedure allows the filaments retain their ability to support the HMM-anti-HMM-covered-beads movement. Alignment along the linear channels fabricated on the PSMA polymeric surface and progressive formation of F-actin bundles assembled either from native filaments or from $\sim 2\text{-}\mu\text{m}$ -actin filaments with their barbed ends blocked by gelsolin and further condensed with Ba^{++} (according to a procedure described elsewhere [29]) was also achieved.

Figure 6. Binding of self-assembled F-actin (23 nM) to HMM on functionalized PSMA polymeric surfaces. Dark field observation shows 10 μm channels fabricated on PSMA polymeric surfaces (left). Fluorescent observation shows self-assembled F-actin filaments on the same field (middle). F-actin filaments aligned on the PSMA polymeric surfaces after 1.5 h in the continuous flow with the flow rate of 0.06 ml/min (right) with antiHMM-HMM beads binding on F-actin.

Actin nanostructures self-assembled on nano-organized surfaces

Perhaps the most interesting method of fabrication of actin nanostructures uses nano-structure surfaces (e.g. HOPG and mica) as 'templates' for self-assembly.

We used frozen G-actin stock that was thawed rapidly in DI water at 37°C. The polymerization was initiated in 5 mM potassium phosphate buffer at pH 7.5 with 100 mM KCl and 0.25 mM Mg ATP. The incubation took place at 37°C for 30 min. The incubated solution was spun at 45K rpm for 60 min at RT, whereupon the supernatant was decanted. The actin pellet was then rinsed/washed in a buffer solution of 10 mM imidazole at pH 6.8, 40 mM KCl, 1 mM MgCl_2 , and 0.5 mM DTT. Additional buffer was added for storage overnight at 4°C. The F-actin was then resuspended, resulting in a stock solution of 6 mg/mL. Two dilutions were carried out, resulting in 0.06 mg/mL (S1) and 6×10^{-4} mg/mL (S2). Drops of 5 – 25 μL from S1 or S2 were placed on substrate surfaces and allowed to dry at room temperature (ambient humidity ca. 60%).

Two substrates were employed during the present study. Highly oriented pyrolytic graphite (HOPG) will present an atomically flat hydrophobic surface with sp² bonding. Clean surfaces were prepared by cleavage immediately before exposure to solution. Mica will likewise present an atomically flat surface composed of a silicate tetrahedral network. That surface is hydrophilic and will be covered with an adsorbed thin aqueous layer. Hydroxylation will then take place, thus generating a negative surface charge. However, ion exchange with the divalent cation ($K^+ \rightleftharpoons Mg^{2+}$) in the aqueous phase is likely to produce a positive surface charge density.

The AFM analysis and tip-induced manipulation were carried out with a JEOL JSPM-4200 multi-technique/multi-mode instrument under air ambient conditions. Imaging was performed in the constant force contact mode. Beam-shaped probes with nominal force constants of 0.06 and 0.58 N/m were used.

Deposition of S1 actin on a hydrophobic HOPG substrate resulted in formation of paracrystalline rafts (figure 7), consisting of filamentary strands with longitudinal integrity being aligned at spacings of ca. 200 nm. The structures exhibited superficial resemblance to those reported in an earlier study [30]. However, closer inspection in combination with the outcome of tip-induced manipulation has revealed distinct differences.

Figure 7. Paracrystalline structure obtained from deposition of actin in solution onto HOPG. The high-resolution image (right) reveals that the height (ca. 10 nm) and periodicity (ca. 40 nm) were consistent with SEM results for F-actin.

The spacing between the parallel rows was a factor of 4-5 greater than those of the earlier study. That study was based on transferring a self-assembled bi-layer consisting of a lipid monolayer and an F-actin layer onto a DPPC coated mica substrate (or an aged carbon-coated grid for EM analysis). The repeat distance perpendicular to the longitudinal structure was ascribed to the nodal distance, ca. 36 nm, along the double-stranded F-actin chain. The present ripple spacing must therefore have a different origin. The spacing between rows (in figure 7) was found to be a function of interaction with the tip. The dependence is illustrated in figure 8 by the break in order at the edge of a second scan over a smaller field of view.

Manipulation appeared to result in successive 'herding' of alternate strands, due to the delicate interplay of tip-induced shear stress, with in-plane and out-of-plane interactions within the raft and with the substrate.

Adsorption onto HOPG has the merit of effectively turning off double-layer interaction between the actin and the substrate. However, while HOPG is hydrophobic, the actin will be hydrophilic

and will present a negative 'surface' charge. Thus ordering and spacings will be due to a subtle interplay between relatively weak and short-range out-of-plane van der Waals interaction with the substrate, longer-range in-plane repulsive electrostatic interaction, and attractive in-plane meniscus forces

Figure 8. The two contact mode images show: (left) the boundary between two successive scans over over-lapping fields of view, where the top half has been subjected to two raster scans, resulting in translation of every second row; (right) a third scan has been carried out over the full field of view, illustrating translation of every second row over the full field of view (note that the structure due to the original manipulation remained stable, top half of image).

The second nano-structured material that has been used was mica. Circular structures (figure 9) have been obtained from the exposure of a hydrophilic mica surface to actin in S2 solution. The features are due to selective deposition of actin at preferred sites of low interface energy. Those sites are most likely located at the air/fluid/solid intersection where an adhering air bubble is attached to the mica surface. The existence of such bubbles is thought to account for anomalies in double-layer interactions [31]. The features are stable against contact mode tip-manipulation, due to the double-layer force between a substrate with positive surface charge and a negative charge on the actin [32].

Figure 9. Circular actin nanostructures fabricated via the condensation of actin solutions on mica.

Higher resolution images of circular features on mica indicate that most of the features exhibited adsorption along a 'triple-point' line (figure 10, left). However, in some cases the circles were filled in (right), presumably due to availability of excess actin being forced into the circular confinement during evaporation. Interestingly, at higher concentrations, in the inner space of the circle F-actin self-organizes in parallel features similar to those obtained before (figure 7).

Figure 10. Higher resolution images of circular features on mica shown in pseudo-3D representation. The scales are in nm, and the heights of the features are consistent with that of a single layer of partially denatured actin. The contour lines are drawn through the center of the circle.

Actin nanostructures – perspectives and future work

While the many methods presented above can be improved to deliver finer, more reproducible and spatially more controllable actin filament tracks that are able to sustain the motility of motor-functionalized cargos, one essential characteristic is unlikely to be achieved in the near future: the automatic self-assembly *and* disassembly of actin patterns according to a program. This process of 'smart' fabrication and reuse of actin for cytoskeleton seems trivial for the simplest life forms, e.g. fungi solving mazes (figure 11). It appears that this natural method of fabrication & recycle does not use a bottom-up (and certainly not a top-down approach) but rather an 'inside-out' philosophy. While the progress towards the understanding of the flexible programming of the formation of actin-based cytoskeleton will certainly improve our chances of reproducing this smart and efficient nano-fabrication, in the short term we have to improve on a combination of bottom-up (e.g. use of 'pre-fabricated' nanostructured surfaces) and top-down (e.g. electric fields) methods for the fabrication of future hybrid devices based on molecular motors.

Figure 11. Fungus filaments solving a microfabricated maze.

CONCLUSION

The fabrication of actin nanostructures is a prerequisite for the development and operation of future hybrid nano-devices based on linear protein molecular motors working on micro/nano-engineered surfaces that operate in a “cargo architecture”. However, this requirement brings important challenges regarding appropriate techniques for the nanolithography *with* actin filaments (or microtubules) based on molecular self-assembly on engineered surfaces. Although the future will ask for programmed fabrication & recycle methods, in the short term we have to rely on the improvements coming from a combination of bottom-up (e.g. use of ‘pre-fabricated’ nanostructured surfaces) and top-down (e.g. electric fields) methods.

ACKNOWLEDGMENTS

Many colleagues contributed in various ways to this work, in particular the team at University of Sydney - Murat Kekic, Vlado Buljan and Cris dos Remedios and at Swinburne University – Jon Wright and Chittlada Mahanivong, and more recently Luisa Filippini and Kristi Hanson. The research has been supported by Defence Advanced Research Projects Agency (DARPA) and Australian Research Council (ARC) grants.

REFERENCES

1. Suzuki, H., Oiwa, K., Yamada, A., Sakakibara, H., H. Nakayama, Mashiko S. *Jpn. J. Appl. Phys.* 34:3937-3941 (1995).
2. Turner, D. C., C. Chang, K. Fang, S. L. Brandow, Murphy, D. B. *Biophys. J.* 69:2782–2789, (1995).
3. Suzuki, H., Yamada, A., Oiwa K., H. Nakayama and S. Mashiko. 1997. *Biophys. J.* 72:1997- (2001).
4. Nicolau, D.V., Suzuki, H., Mashiko, S., Taguchi, T., Yoshikawa, S. *Biophys. J.* 77 (2), 99044-99065, (1999).
5. Dennis, J. R., J. Howard, and V. Vogel. *Nanotechnology.* 10:232–236, (1999).
6. Mahanivong, C., Wright, J. P., Kekic, M., Pham, D. K., dos Remedios, C., Nicolau, D.V. *Biomedical Microdevices* 4(2): 111-116; (2002).
7. Bunk, R., Klinth, J., Rosengren, J., Nicholls, I., Tagerud, S., Omling, P., Mansson, A., Montelius, L. *Microelectronic Engineering* 67–68 899–904 (2003).

8. Hiratsuka, Y., Tada, T., Oiwa, K., Kanayama, T., Uyeda, T.Q. P. *Biophys. J.* 81: 1555–1561 (2001).
9. Clemmens, J., Hess, H., Howard, J., Vogel, V. *Langmuir*; 19(5); 1738–1744 (2003).
10. Riveline, D., Ott, A., Julicher, F., Winkelmann, D. A., Cardoso, O., Lacapere, J. J., Magnusdottir, S., Viovy, J. L., Gorre-Talini, L. & Prost, J. *Eur Biophys J.* 27, 403–8 (1998).
11. Hess, H., Clemmens, J., Qin, D., Howard, J. & Vogel, V. *Nano Letters* 1, 235–239, (2001).
12. Stracke, R., Bohm, K. J., Burgold, J., Schacht, H., Unger, E. *Nanotechnology*. 11:52–56, (2000).
13. Hess, H.; Howard, J.; Vogel, V. *Nano Lett.* 2(10); 1113–1115 (2002).
14. Hess, H.; Clemmens, J.; Howard, J.; Vogel, V. *Nano Lett.* 2(2); 113–116 (2002).
15. Nicolau, D. V., Jr.; Nicolau, D. V. In Biomedical Applications of Micro- and Nanoengineering. *SPIE Proc.* 4937, 219–225, (2002).
16. Yanagida, T., Nakase, M., Nishiyama, K., Oosawa, F. *Nature* 307, 58–60, (1984).
17. Spudich, J. A., Kron, S. J., Sheetz, M. P. *Nature*, 315, 584–586, (1985).
18. Kabsh, W., Mannherz, H.G., Such, D., Pai, E.F., Holmes, K.C. *Nature* 347, 37–44 (1990).
19. Pollard, T.D. *Annu. Rev. Biochem.* 55, 987–1035 (1986).
20. Xu, J., Schwarz, W. H., Kas, J. A., Stossel, T. P., Janmey, P. A., Pollard, T. D. *Biophys. J.* 74, 2731–2740 (1998).
21. Higgs, H. N. Pollard, T. D. *Annu. Rev. Biochem.* 70:649–76 (2001).
22. Janmey, P. A., Tang, J.X., Schmidt, C.F. <http://www.biophysics.org/btol/img/Janmey.P.pdf>
23. Chik, J. K., Lindberg, U., Schutt, C. E. *J Mol Biol* 263, 607 (1996). From Protein Data Bank: Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., Bourne, P.E. *Nucleic Acids Research*, 28,, 235–242 (2000).
24. Kojima, H., A. Ishijima, and T. Yanagida. *Proc Natl Acad Sci USA*. 91:12962–6 (1994).
25. Huxley, H. E., A. Stewart, H. Sosa, and T. Irving. *Biophys. J.* 67:2411–21 (1994).
26. Higuchi, H., T. Yanagida, and Y. E. Goldman. *Biophys. J.* 69:1000–10 (1995).
27. Wakabayashi, K., Y. Sugimoto, H. Tanaka, Y. Ueno, Y. Takezawa, Y. Amemiya. *Biophys. J.* 67, 2422–2435. (1994).
28. Wright, J.P., Pham, D.K., Mahanivong, C., Kekic, M., dos Remedios, C.G., Nicolau, D.V. *Biomedical Microdevices* 4(3): 205–211; (2002).
29. Angelini, T.E., Liang, H., Wriggers, W., Wong, G.C.L. *Proc. Natl. Acad. Sci. USA* 100, (2003).
30. Shi D. Somlyo A V, Somlyo A P, Shao Z, *J. Microsc.* 201: 377–382 (2001).
31. Considine RF, Hayes RA, Horn RG, *Langmuir*, 15: 1657–1659 (1999).
32. St-Onge D, Gicquaud C. *Cell. Biol.* 67: 297–300 (1989).