small
NANO MICRO

Artificial Bacterial Flagella for Remote-Controlled Targeted Single-Cell Drug Delivery

Rami Mhanna, Famin Qiu, Li Zhang, Yun Ding, Kaori Sugihara, Marcy Zenobi-Wong, and Bradley J. Nelson*

yellow for fabrication section of the report

In 1965 Bangham and colleagues described the diffusion of ions through spontaneously formed liquid crystal membranes later know as liposomes.[1] The ability of liposomes to entrap liquids encouraged research on their use as drug delivery vehicles.[2] The successful delivery of drugs using liposome-based systems requires a precondition that the liposomes circulate in the blood stream long enough without being destroyed or being cleared by the reticuloendothelial system (RES).[3] Targeting specific sites in the body is a more cumbersome task requiring prolonged circulation.[3b] For this purpose polyethylene glycol (PEG)-modified stealth liposomes were proposed and were shown to highly improve the half-life of liposomes.[4] Liposomes are among the first nanocarriers that have been functionalized with antibodies to target malignant cells and are currently available in several commercial products.[5] The precise delivery of drugs into single cells can only be achieved using micro-injection and techniques based on atomic force microscopy in vitro, while in vivo there has been no reports to our knowledge.[6] Recently, Wang and co-workers also demonstrated the feasibility of using magnetically driven nanowires for transport of drug carriers and targeted drug delivery.[7]

Bacterial flagella are capable of swimming in liquids driven by rotary motors.[8] Inspired by these natural systems, a variety of micro/nanorotors have been proposed.[9] The use of an external low magnetic field (≈2 mT) to control the swimming of helical microrobots was developed in our laboratory.[10] These devices, which we call artificial bacterial flagella (ABFs), are capable of performing precise 3D swimming in liquids similar to natural bacterial flagella (e.g., *E. coli*) which makes them suitable for several in vivo and in vitro applications.[11] ABFs can also be coated with a biocompatible layer such as titanium to reduce cytotoxicity.[12]

We hypothesized that ABFs can be functionalized with liposomes, thus allowing these micromachines to perform biological or biomedical tasks in a remotely controlled fashion. To test this hypothesis fluorescently labeled liposomes and calcein-loaded liposomes were adsorbed on the surface of ABFs. The adsorption of liposomes on the ABFs was confirmed by quartz crystal microbalance with dissipation monitoring (QCM-D) and fluorescence recovery after photobleaching (FRAP). The liposome-functionalized ABFs were then placed in contact with cells in vitro and the uptake of calcein (a model water soluble drug) by cells was monitored using fluorescence microscopy.

The fabrication of ABFs was followed as described earlier by Tottori et al.[12] with the exception that the thickness of the Ni layer was 25 nm and the thickness of the Ti layer was 15 nm for the devices used in the current study. In our previous work, we showed the ability of Ni/Ti helical microswimmers to perform controlled swimming and cargo transport in 3D liquids.[12] The thickness of Ni used ranged from 50 to 100 nm while the Ti thickness ranged from 0 to 5 nm. In the current study, the Ni thickness was reduced to 25 nm to improve the transparency of the microswimmers and consequently the quality of acquired confocal laser scanning microscope (CLSM) images. The Ti thickness on the other hand was increased to 15 nm to ensure complete coating of the Ni layer resulting in better cytocompatibility and adsorption of intact liposomes. ABFs used in the current work had three turns and were fabricated with horizontal arrays (**Figure 1**a). An individual ABF had a length of 16 μm and a diameter of 5 μm (Figure 1a). The filament cross section had an ellipsoidal shape with a thickness of approximately 1.19 μm along the short axis and 2.23 μm along the long axis (Supporting Information Figure S1). ABFs exhibit corkscrew swimming in liquids in response to a rotating magnetic field B at a frequency f. When a magnetic field of 3 mT was applied, ABFs wobbled about their longitudinal axis in response to a frequency below ≈1 Hz. Smooth corkscrew motion was achieved at higher frequencies below the step-out frequency after

Dr. R. Mhanna, F. Qiu, Y. Ding, Prof. B. J. Nelson
Institute of Robotics and Intelligent Systems
ETH Zurich
Zurich, CH-8092, Switzerland
E-mail: rami.mhanna@dep.uminho.pt
Prof. L. Zhang
Department of Mechanical and Automation Engineering
The Chinese University of Hong Kong
Hong Kong SAR, China
Dr. K. Sugihara
Laboratory of Biosensors and Bioelectronics
ETH Z
Zuric
Prof. M. Zenobi-Wong
Cartilage Engineering + Regeneration
ETH Zurich
Zurich

table of thickness of the Ti/Ni

green is for design section

DOI: 10.1002/smll.201303538

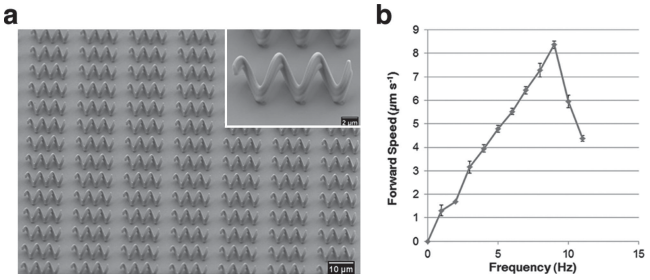

Figure 1. ABF structure and swimming properties. a) SEM image of a horizontal array of artificial bacterial flagella (ABFs), the inset shows a helical swimmer at a higher magnification. b) Forward swimming speed of ABFs as a function of input frequency. The error bars represent the standard deviation of mean values from three measurements at each frequency. Measurements were performed at room temperature in water by applying an electromagnetic field of 3 mT using three pairs of Helmholtz coils to induce the swimming of the ABFs.

which ABFs exhibited irregular swimming and a dip in their forward speed. Figure 1b shows the linear increase in the forward speed of ABFs as a function of the applied frequency below the step-out frequency.[13] The highest swimming speed achieved was 8.4 ± 0.16 μm s^{-1}. Steering of the ABFs was possible with micrometer precision by simply changing the rotating axis of the field, an example of which is shown in Figure 4a and Supplementary Movie S1.

Functionalized-ABFs (f-ABFs) for drug delivery applications were prepared by incubating the ABFs with liposomes (**Figure 2**). In the first step, the ABF array was UV treated to saturate the titanium surface with oxides and remove organic contaminants (Figure 2a).[14] The arrays were then sonicated in HEPES buffer to release the ABFs from the substrate resulting in a suspension of ABFs (Figure 2b and Supporting Information Figure S2).[15] Finally, the ABFs in suspension were mixed with DOPE/DOTAP (1:3) liposomes (200 nm diameter), which adsorb on the surface of the ABFs resulting in f-ABFs (Figure 2c). The DOPE lipids are known to be fusogenic while positively charged DOTAP lipids improve liposome uptake by cells.[16] This combination is also known to induce successful gene delivery and therefore we adopted the system with the aim of using it also for gene delivery in future studies.[17] The liposomes were loaded with calcein which is a fluorophore that does not readily penetrate the cell membranes and, therefore, active uptake of calcein-loaded vesicles is required for cells to show the calcein signal.

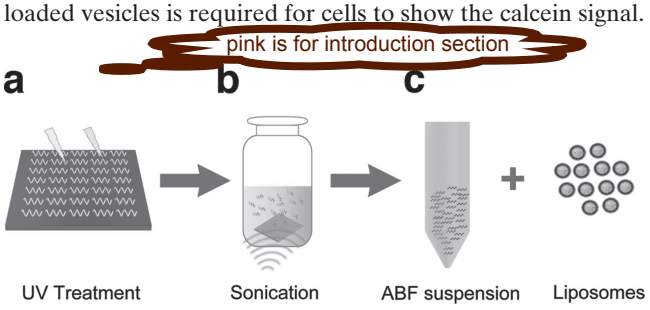

Figure 2. Schematic of the process of batch preparation of liposome functionalized ABFs. a) The process begins with UV treatment of the ABF array followed by b) sonication to detach the helical swimmers and finally c) incubation with a liposome suspension to allow adsorption of liposomes on the surface of ABFs.

Calcein is hydrophilic and can thus, be used as a model for a water soluble drug.[18]

In order to confirm that vesicles used in the current study adsorbed in an intact state on TiO_2 surfaces, we used QCM-D. **Figure 3**a shows the adsorption of calcein loaded DOPE/DOTAP (3:1) liposomes on TiO_2 coated QCM crystals. The adsorption of liposomes resulted in a final strong decrease in the frequency (Δf = –151.3 ± 19.9 Hz) and an increase in the dissipation (ΔD = 21.9 ± 1.7). The adsorption curve was roughly saturated after three hours; therefore, the incubation time of the ABFs with liposomes was set to three hours in all the experiments of this study. TiO_2 is known to support the adsorption of intact vesicles, as shown in previous literature.[19] The typical observed response of the QCM-D frequency and dissipation signals to the injection of liposomes is in accordance with previous literature.[19] Other liposome formulations such as DOPC can also be used and the formation of more than one layer of liposomes to maximize the drug loading capacity is also possible by alternating positively charged poly-l-lysine layers and negatively charged DOPS liposomes.[20] QCM-D analysis of the adsorption of the above and other liposome formulations are shown in Supplementary Figure S3.

To determine if the liposome-coating process was also applicable on the surface of TiO_2-coated ABFs, calcein loaded vesicles were adsorbed on the surface of ABFs and images of the f-ABFs were acquired with a CLSM. Because the curvature of the helical rods and their roughness might affect the adsorption behavior of liposomes on helical microswimmers, it is important to investigate if adsorption to planar substrates (QCM-D crystal) also applies to the helices. Figure 3b depicts a representative CLSM image of ABFs functionalized with calcein-loaded liposomes where a clear signal can be observed throughout the ABF. This is evidence of the formation of intact liposomes, as calcein would not be present in case of the formation of lipid bilayers/monolayers on the surface of ABFs. In order to further confirm that the adsorbed vesicles were intact and did not form lipid bilayers, FRAP was used. Figure 3c shows a representative image of ABFs functionalized with NBD-labeled vesicles before photobleaching, immediately after photobleaching the middle area of the ABFs, and one hour after photobleaching. In this experiment, the fluorescence is in the bilayer membrane but not inside the vesicle. When intact vesicles adsorb on a substrate, lipids are not mobile over micrometer distances, thus fluorescence in a bleached spot does not recover. However, supported lipid bilayers are mobile, and the photobleached area recovers its fluorescent signal within a short period.[21] The fluorescent signal of the photobleached spot on f-ABFs did not recover even after 1 h of photobleaching (Figure 3c); we can thus confirm that liposomes adsorbed to the ABF surfaces were intact.

Liposome-functionalized ABFs were able to swim with high precision and deliver the model drug calcein to single cells in vitro. In **Figure 4**a and Supplementary Video S1, a representative ABF functionalized with calcein-loaded vesicles was subjected to a rotating magnetic field at a frequency of 8 Hz using a manual magnetic motor. The resulting swimming speed of the f-ABF was 23 μm s^{-1}. The fact that f-ABFs

small 2014,
DOI: 10.1002/smll.201303538

NANO MICRO small

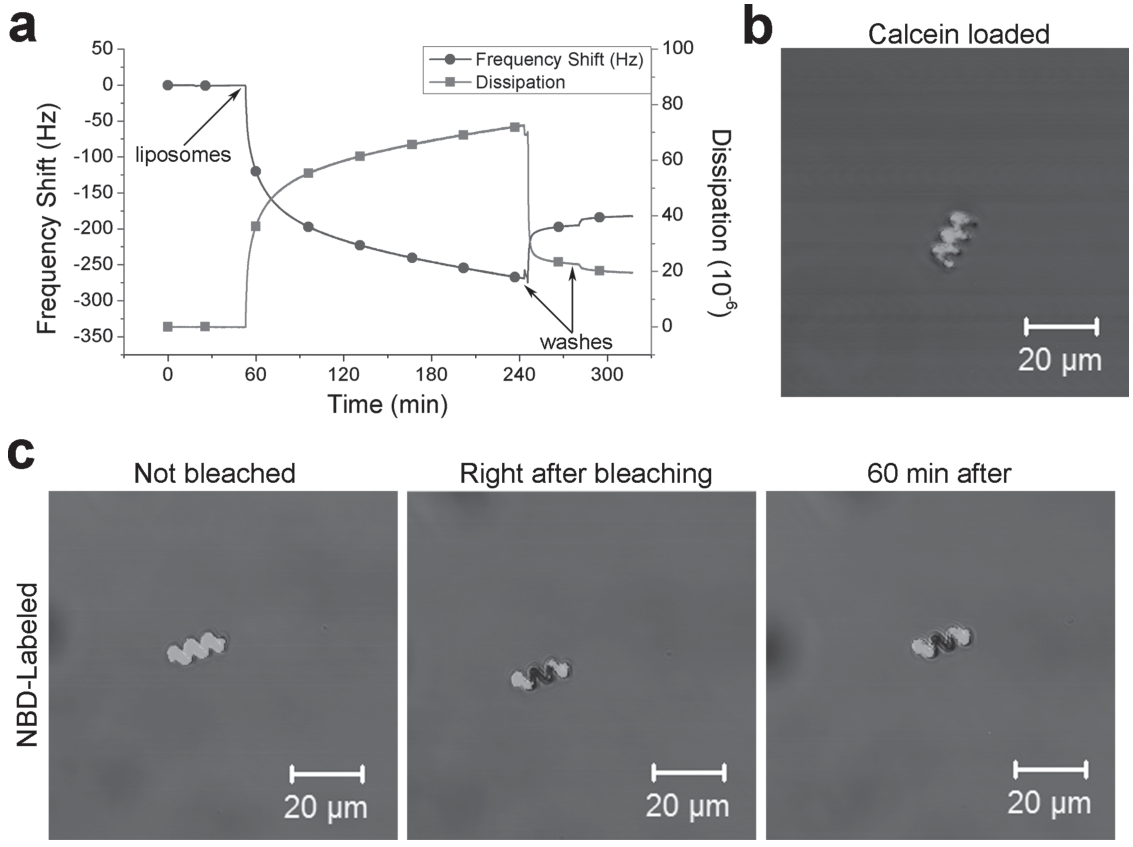

Figure 3. Liposomes adsorb in an intact state on the surface of ABFs. a) Normalized resonance frequency shift and dissipation shift of the 15 MHz detection frequency for the adsorption of DOPE/DOTAP (3:1) liposomes on titanium coated quartz crystal microbalance chips. b) A representative image of ABFs coated with liposomes loaded with 50 mM calcein. c) An ABF coated with NBD-Labeled liposomes before bleaching (left), after bleaching the center of the swimming microrobot (middle), and 60 min following the bleaching (right). Since no recovery was observed, it can be concluded that the liposomes are intact on the surface of the helical swimmers.

could swim without significant loss of the calcein fluorescent signal ($p = 0.2$, Supplementary Table S1) suggests the ability of these functionalized microswimmers to deliver their cargo to cells.

The delivery of calcein to single C2C12 cells in contact with f-ABF is shown in Figure 4b and Supporting Information Figure S4. It is noteworthy that even when two cells were in direct contact, the calcein was only delivered to the cell in contact with the ABF and not the other. Additionally, nearby cells showed no calcein signal (Figure 4b). When the ABF was removed from the surface of the cell using an external magnetic source, the calcein signal was still clearly present

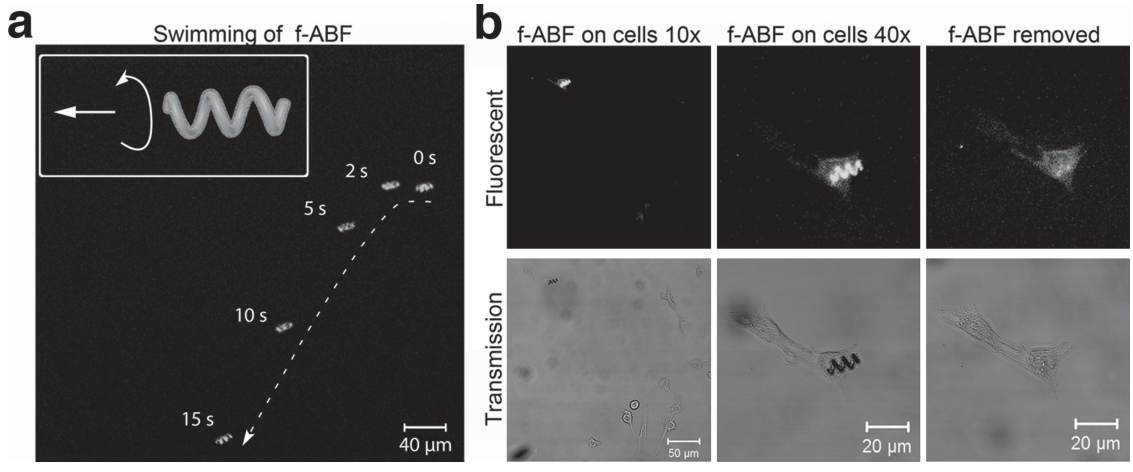

Figure 4. f-ABF swimming and calcein delivery to single cells. a) A representative time-lapse follow-up of the swimming of functionalized artificial bacterial flagella (f-ABF) coated with calcein-loaded liposomes. b) Representative calcein delivery from f-ABF to single cells at low magnification (left), high magnification of calcein delivery (middle) and calcein delivered to cells after removing the f-ABF (right).

DOI: 10.1002/smll.201303538

(Figure 4b). This data indicates that f-ABFs can perform precise swimming and deliver the loaded model drugs into single cells. The delivery of calcein might occur through fusion of the cationic vesicles with the membrane of the cultured cells or through endocytosis.[16b,17b,22] Further improvements of the system could be achieved by including targetting units in the liposomes such as antibodies and cell recognition sites.

In conclusion, we have demonstrated a method for batch preparation of liposome functionalized artificial bacterial flagella. These functionalized helical magnetic micromachines were able to deliver the hydrophilic model drug calcein to C2C12 mouse myoblasts in vitro. The data of the current study demonstrates the feasibility of using functionalized helical microswimmers that are steerable with an external magnetic field to perform single-cell-targeted drug delivery in vitro. The current data also points to the possibility of delivering other materials such as DNA or enzymes to single cells and the possible use of this system for in vivo applications. However, potential difficulties might be encountered for in vivo applications due to clearance by the RES as stated in the introduction. Although, ABFs provide a stabilization substrate for adsorbed liposomes, stealth liposomes or a PEG final coating as demonstrated in Supplementary Figure S3 may be necessary to overcome RES clearance.

purpel for literature review

Experimental Section

Materials: photoresist IP-L (from Nanoscribe GmbH, Germany), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP), 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine (DOPE), 1-oleoyl-2-[12-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino] lauroyl]-*sn*-glycero-3-phosphocholine (NBD-PC) were purchased from Avanti Polar Lipids. Phosphate buffer saline (PBS), fetal bovine serum (FBS), cell culture media [Dulbecco's modified eagle's medium (DMEM)-Glutamax], antibiotic-antimycotic, trypsin/EDTA were from Invitrogen AG, Basel, Switzerland. Calcein disodium salt, sodium chloride (NaCl), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), were purchased from Sigma Aldrich Chemie GmbH, Buchs, Switzerland.

Instrumentation: Liposomes were prepared using the extrusion approach where two glass syringes (1 mL each) were connected to a teflon/steel extruder equipped with two filters (200 nm pore size).[23] Calcein loaded vesicles were prepared by dissolving a DOPE/DOTAP (3:1) lipid film in calcein (50 mM in deionized water). Assessment of liposome adsorption on titanium-coated crystals was followed using QCM-D (Q-Sense E4, Gothenburg, Sweden). The ABFs were fabricated using 3D direct laser writing (DLW) and physical vapor evaporation.[24] ABF arrays were written in a photoresist, IP-L, and the unpolymerized photoresist was removed by incubating the arrays in a developer. Electron beam evaporation was then used to deposit an Ni/Ti bilayer on the surface of the ABFs. Electron beam evaporation was performed with a tilt angle of 15° to reduce shadowing effects. The detailed fabrication process can be found in other literature.[12] The length of a single ABF was 16 μm and the diameter was 5 μm. Imaging of cells and ABFs and FRAP were acquired using a confocal microscope (Carl Zeiss AG/LSM 510, equipped with a 40× 0.6 NA objective and a 488 nm argon laser). Time-lapse images of swimming liposome-coated ABFs were acquired using a Zeiss Axio Observer equipped with a Hamamatsu C9100–13 – Super sensitive fluorescence camera.

Batch preparation of Liposome-Functionalized ABFs: Unilamellar DOPE/DOTAP (3:1) liposomes were loaded with calcein (50 mM) or fluorescein labeled by adding NBD-PC [(2% (w/w) final percentage of the above lipid composition]. Liposomes were prepared at a final lipid concentration of 2.5 mg mL^{-1} and used at 0.5 mg mL^{-1} in all experiments. A chip containing 10000 ABFs was UV/ozone cleaned (30 minutes) to clean the surface and create free hydroxyl groups on the Ti surface. ABFs were then released by ultrasonication and incubated for 3 hours under gentle rotation with a liposome solution (0.5 mg mL^{-1}) to obtain functionalized-ABFs (f-ABFs). F-ABFs were washed in HEPES buffer 5 times by centrifuging the f-ABF containing solution (4000 rpm for 3 minutes each) to remove unbound liposomes.

Cell Culture: C_2C_{12} mouse myoblasts were purchased from American Type Culture Collection (LGC Standards, Molsheim, France) and seeded at 5000 cells/cm^2 on glass bottom 24-well-plates in DMEM supplemented with 1% fetal bovine serum and 1% antibiotic-antimycotic. Cultures were carried on in a humidified incubator (37 °C, 7% CO_2) and media was changed every 3 days. For calcein delivery experiments, cells were washed with HEPES buffer followed by 1 hour incubation with the ABF solution then cultured for 24–48 hours with new cell culture media.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

We thank the FIRST lab of ETH Zurich for technical support. The authors are also grateful to Dr. Bradley Kratochvil (ETH Zurich) for providing technical support on the experimental setup, Ruedi Borer (ETH Zurich) for the construction of the power amplifier for the Helmholtz coils setup, Kathrin Peyer and Erdem Siringil (ETH Zurich) for fruitful discussions. We also thank Prof. Janos Vörös (Laboratory of Biosensors and Bioelectronics, ETH Zurich, Switzerland) for his valuable scientific input. Funding for this research was partially provided by the European Research Council Advanced Grant "Microrobotics and Nanomedicine (BOTMED)" and the Swiss National Science Foundation (SNSF) Project No. 200021–130069.

[1] A. D. Bangham, M. M. Standish, J. C. Watkins, *J. Mol. Biol.* **1965**, *13*, 238.
[2] a) D. D. Lasic, *Trends Biotechnol.* **1998**, *16*, 307; b) G. Gregoriadis, *FEBS Lett.* **1973**, *36*, 292.
[3] a) G. Gregoriadis, C. P. Swain, E. J. Wills, A. S. Tavill, *Lancet* **1974**, *303*, 1313; b) G. Gregoriadis, *Trends Biotechnol.* **1995**, *13*, 527.
[4] a) A. L. Klibanov, K. Maruyama, V. P. Torchilin, L. Huang, *FEBS Lett.* **1990**, *268*, 235; b) M. L. Immordino, F. Dosio, L. Cattel, *Int. J. Nanomed.* **2006**, *1*, 297.

DOI: 10.1002/smll.201303538

[5] a) D. Peer, J. M. Karp, S. Hong, O. C. Farokhzad, R. Margalit, R. Langer, *Nat. Nanotechnol.* **2007**, *2*, 751; b) T. M. Allen, P. R. Cullis, *Science* **2004**, *303*, 1818.
[6] a) A. Graessmann, M. Graessmann, C. Mueller, in Method. Enzymol., Vol. *65* (Ed: K. M. Lawrence Grossman), Academic Press, New York, US **1980**, p. 816–825; b) M. Rechsteiner, L. Kuehl, *Cell* **1979**, *16*, 901; c) O. Guillaume-Gentil, E. Potthoff, D. Ossola, P. Dörig, T. Zambelli, J. A. Vorholt, *Small* **2013**, *9*, 1904; d) A. Meister, M. Gabi, P. Behr, P. Studer, J. Vörös, P. Niedermann, J. Bitterli, J. Polesel-Maris, M. Liley, H. Heinzelmann, T. Zambelli, *Nano Lett.* **2009**, *9*, 2501; e) M. G. Schrlau, E. M. Falls, B. L. Ziober, H. H. Bau, *Nanotechnology* **2008**, *19*, 015101.
[7] a) D. Kagan, R. Laocharoensuk, M. Zimmerman, C. Clawson, S. Balasubramanian, D. Kang, D. Bishop, S. Sattayasamitsathit, L. Zhang, J. Wang, *Small* **2010**, *6*, 2741; b) W. Gao, D. Kagan, O. S. Pak, C. Clawson, S. Campuzano, E. Chuluun-Erdene, E. Shipton, E. E. Fullerton, L. Zhang, E. Lauga, J. Wang, *Small* **2012**, *8*, 460.
[8] a) K. Kinbara, T. Aida, *Chem. Rev.* **2005**, *105*, 1377; b) H. C. Berg, R. A. Anderson, *Nature* **1973**, *245*, 380.
[9] a) R. Eelkema, M. M. Pollard, J. Vicario, N. Katsonis, B. S. Ramon, C. W. M. Bastiaansen, D. J. Broer, B. L. Feringa, *Nature* **2006**, *440*, 163; b) M. G. L. van den Heuvel, C. Dekker, *Science* **2007**, *317*, 333; c) G. A. Ozin, I. Manners, S. Fournier-Bidoz, A. Arsenault, *Adv. Mat.* **2005**, *17*, 3011; d) A. Ghosh, P. Fischer, *Nano Lett.* **2009**, *9*, 2243.
[10] L. Zhang, J. J. Abbott, L. Dong, B. E. Kratochvil, D. Bell, B. J. Nelson, *Appl. Phys. Lett.* **2009**, *94*, 064107.
[11] a) L. Zhang, K. E. Peyer, B. J. Nelson, *Lab Chip* **2010**, *10*, 2203; b) K. E. Peyer, L. Zhang, B. J. Nelson, *Appl. Phys. Lett.* **2011**, *99*, 174101; c) B. J. Nelson, I. K. Kaliakatsos, J. J. Abbott, *Annu. Rev. Biomed. Eng.* **2010**, *12*, 55.
[12] S. Tottori, L. Zhang, F. Qiu, K. K. Krawczyk, A. Franco-Obregón, B. J. Nelson, *Adv. Mater.* **2012**, *24*, 811.
[13] C. E. Sing, L. Schmid, M. F. Schneider, T. Franke, A. Alexander-Katz, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 535.
[14] a) J. R. Vig, *J. Vac. Sci. Technol A* **1985**, *3*, 1027; b) A.-S. Andersson, F. Bäckhed, A. von Euler, A. Richter-Dahlfors, D. Sutherland, B. Kasemo, *Biomaterials* **2003**, *24*, 3427.
[15] K. E. Peyer, S. Tottori, F. Qiu, L. Zhang, B. J. Nelson, *Chem. Eur. J.* **2013**, *19*, 28.
[16] a) N. Sakaguchi, C. Kojima, A. Harada, K. Koiwai, K. Kono, *Biomaterials* **2008**, *29*, 4029; b) I. Wrobel, D. Collins, *BBA Biomembr.* **1995**, *1235*, 296; c) Y. Ma, Z. Wang, W. Zhao, T. Lu, R. Wang, Q. Mei, T. Chen, *Int. J. Nanomed.* **2013**, *8*, 2351; d) D. Nicolosi, M. Scalia, V. M. Nicolosi, R. Pignatello, *Int. J. Antimicrob. Ag.* **2010**, *35*, 553; e) V. Budker, V. Gurevich, J. E. Hagstrom, F. Bortzov, J. A. Wolff, *Nat. Biotechnol.* **1996**, *14*, 760.
[17] a) L. Huang, S. Li, *Nat. Biotechnol.* **1997**, *15*, 620; b) N. Düzgüneş, S. Nir, *Adv. Drug Delivery Rev.* **1999**, *40*, 3.
[18] a) A. P. C. O. Bahia, E. G. Azevedo, L. A. M. Ferreira, F. Frézard, *Eur. J. Pharm. Sci.* **2010**, *39*, 90; b) J. Liu, X. Jiang, C. Ashley, C. J. Brinker, *J. Am. Chem. Soc.* **2009**, *131*, 7567.
[19] E. Reimhult, F. Höök, B. Kasemo, *Langmuir* **2002**, *19*, 1681.
[20] N. Graf, E. Thomasson, A. Tanno, J. Vörös, T. Zambelli, *J. Phys. Chem. B* **2011**, *115*, 12386.
[21] K. Sugihara, J. Vörös, T. Zambelli, *ACS Nano* **2010**, *4*, 5047.
[22] R. Leventis, J. R. Silvius, *BBA Biomembr.* **1990**, *1023*, 124.
[23] F. Olson, C. A. Hunt, F. C. Szoka, W. J. Vail, D. Papahadjopoulos, *BBA Biomembr.* **1979**, *557*, 9.
[24] C. N. LaFratta, J. T. Fourkas, T. Baldacchini, R. A. Farrer, *Angew. Chem. Int. Edi.* **2007**, *46*, 6238.

Received: November 14, 2013
Revised: January 20, 2014
Published online:

DOI: 10.1002/smll.201303538