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Sensitive, High Throughput Detection of Proteins in Individual, Surfactant-Stabilized Picoliter Droplets Using Nanoelectrospray Ionization Mass Spectrometry

Clive A. Smith,[#,†] Xin Li,[#,†] Todd H. Mize,[#,‡] Timothy D. Sharpe,[#,§] Edmund I. Graziani,[∥] Chris Abell,*[,#] and Wilhelm T. S. Huck*[,¶]

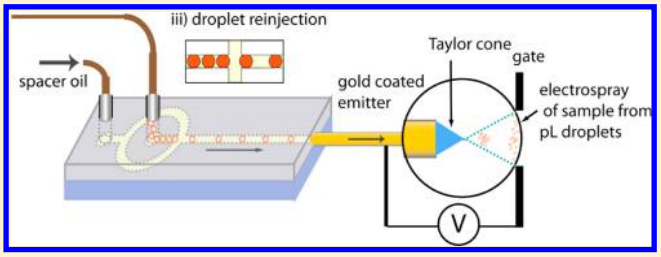
[#]Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, United Kingdom

[∥]Worldwide Medicinal Chemistry, Pfizer, Eastern Pt. Road, Groton, Connecticut 06340, United States

[¶]Radboud University Nijmegen, Institute for Molecules and Materials, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands

Supporting Information

ABSTRACT: Droplet-based fluidics is emerging as a powerful platform for single cell analysis, directed evolution of enzymes, and high throughput screening studies. Due to the small amounts of compound compartmentalized in each droplet, detection has been primarily by fluorescence. To extend the range of experiments that can be carried out in droplets, we have developed the use of electrospray ionization mass spectrometry (ESI-MS) to measure femtomole quantities of proteins in individual pico- to nanoliter droplets. Surfactant-stabilized droplets containing analyte were produced in a flow-focusing droplet generation microfluidic device using fluorocarbon oil as the continuous phase. The droplets were collected off-chip for storage and reinjected into microfluidic devices prior to spraying the emulsion into an ESI mass spectrometer. Crucially, high quality mass spectra of individual droplets were obtained from emulsions containing a mixture of droplets at >150 per minute, opening up new routes to high throughput screening studies.



Micrometer-sized aqueous droplets carried in a (fluorocarbon) oil phase in microfluidic devices and stabilized by a surfactant monolayer are emerging as a potentially powerful technology to study compartmentalized reactions at the picoliter to nanoliter scale.[1−4] In recent years, there has been tremendous progress in the development of microfluidics-based droplet platforms for the on-chip formation[5,6] and manipulation of monodisperse droplets.[7−12] A suite of fluorescence-based techniques can be used for high-throughput and highly sensitive analysis of droplet contents, enabling the picodroplets platform to be exploited in diverse applications including single cell-based assays,[13,14] and protein expression from single copies of DNA.[15] Despite the obvious potential of fluorescence-based techniques to investigate cellular or enzymatic activity in droplets, they are limited by the need for specific labeling of the natural substrate, sense-partner, antigen, or probe molecule. There are many potential applications, e.g., in proteomics and the analysis of cell-secretion products, where fluorescence is not the tool of choice, but where mass spectrometry (MS) offers unique analytical power. This consideration prompted us to develop a robust mass spectrometric interface to analyze proteins and peptides in droplets.

Initial attempts to analyze individual droplet contents by electrospray ionization mass spectrometry (ESI-MS) relied on removing the oil carrier phase via on-chip de-emulsification by extracting sample droplets from the oil phase into a continuous aqueous flow interfaced with ESI-MS.[16−19] Subsequently, there have been a number of advances in mass spectrometric analysis of plugs, generated in capillaries with or without series of T-junctions for the addition of further reagents and directly interfaced with a mass spectrometer,[20−22] or of droplets, generated in microfluidic devices with on-chip electrospray ionization emitter and containing HPLC effluent and proteolysis buffer.[23] However, no advances to date have reported the recording of mass spectra from individual surfactant-stabilized droplets. This is extremely important because the full potential of droplet technology in many applications can only be realized by the use of large numbers of stable droplets that can be manipulated and stored for extended time periods.[24] Thus, surfactant-stabilized droplets are required that can be injected into a device that interfaces with the mass spectrometer at the appropriate time. In this paper, we describe for the first time how femtomole quantities of proteins compartmentalized in individual surfactant-stabilized pico- to nanoliter droplets can be analyzed by spraying a remotely generated droplet emulsion directly into a mass spectrometer.

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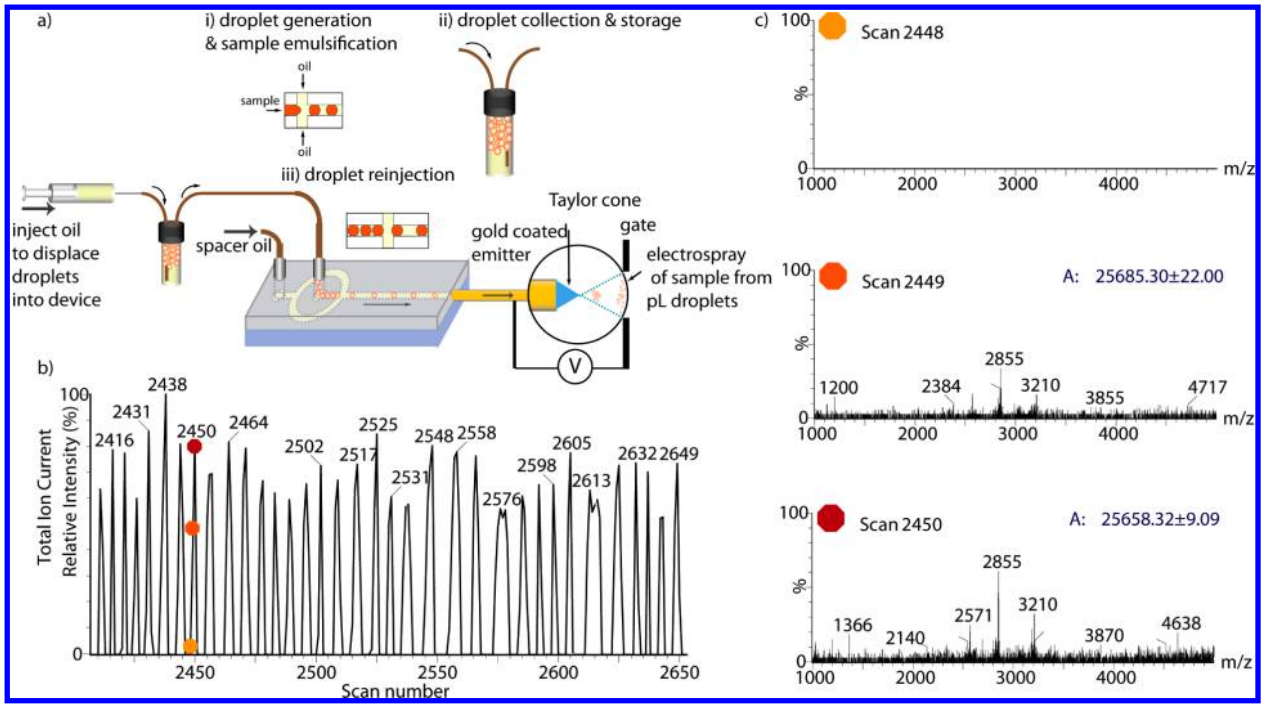

Figure 1. (a) Picoliter droplets containing α-chymotrypsinogen A (50 μM) were generated at 171 Hz in a flow-focusing droplet generation module. The droplets were collected off-chip, and the emulsion was stored overnight at 4 °C. For mass spectrometric analysis, droplets were reinjected into the emitter device. (b) 21 s section of TIC. The colored dots correspond to the individual scans of α-chymotrypsinogen A and are shown in (c).

RESULTS AND DISCUSSION

Choice of Oils and Surfactants for Direct Droplet Injection. The choice of the continuous phase and appropriate surfactant is important in microdroplet ESI-MS, not least because they exist in high concentration in relation to the analytes of interest. Fluorous oils and surfactants, with their characteristically strong electron withdrawing properties, should significantly reduce their ionization in positive ion mode. Initial scoping studies on bradykinin (5 μM) in Novec7500, FC-40, and FC-77 showed significant noise (Supporting Information, S4), which could be suppressed by the addition of PFOH (1*H*,1*H*,2*H*,2*H*-perfluorooctan-1-ol). Even when lowering the concentration of bradykinin to 100 nM (in perfluorooctane containing 30% [vol/vol] PFOH) and injecting 833 pL of (80 attomole of bradykinin) droplets, the ion current of the major charge state of bradykinin species $(BkH_2)^{2+}$ from a single scan was very strong and clearly showed individual droplets (see Supporting Information, S5).

Mass Spectrometry on Individual, Surfactant-Stabilized Droplets. Unfortunately, the preferred surfactant for stable droplets, a Krytox-Jeffamine triblock copolymer (see Figure 2: PEG-PFPE, in ref 13; commercially available as PicoSurf-2 (Sphere Fluidics Ltd.)) significantly reduced the noise from the oil but also strongly suppressed the bradykinin signal (Supporting Information, S1). Nevertheless, we were able to record high quality mass spectra from individual picoliter droplets by producing droplets of diameter ca. 107 μm (650 pL) containing a high concentration of α-chymotrypsinogen A (50 μM) at a frequency of 171 Hz in a flow-focusing droplet generation module using Novec 7500 containing 2% w/w PicoSurf-2 as the continuous phase. These droplets, each containing approximately 32.5 fmol of protein, were collected off-chip and the stable emulsion was stored overnight at 4 °C. Subsequently, droplets were reinjected at a flow rate of 50 nL/min into a microfluidic emitter device using Novec 7500 as the spacing oil at a flow rate of 167 nL/min (Figure 1a). Figure 1b shows a 21 s section of the total ion current (TIC) chromatogram of positive ion MS, with a droplet reinjection frequency of ca. 1.6 Hz, recorded at a scan rate of 11.1 Hz (0.08 s scan duration and 0.01 s interscan delay, V-mode time-of-flight, TOF) and using a capillary voltage of 2.54 kV. At these flow rates, the so-called nanoelectrospray ionization (nano-ESI)[25] regime, the direct injection of the biphasic flow into the mass spectrometer, leads to rises in the TIC each time individual droplets are emitted from the device. Significantly, the peaks for the droplets are well-separated as the TIC drops to a minimum during the injection of the oil phase, consistent with a significant reduction in the efficiency of ionization from sprayed oil. Each peak in the TIC chromatogram of Figure 1b consists of 3−5 scans of an individual droplet. The colored dots represent the single scan spectra presented in Figure 1c. These individual scans are remarkably free of interfering signals of the oil phase and clearly show the emergence of the signal for the protein α-chymotrypsinogen A (25 666 Da) as well as its rapid disappearance. Importantly, each of the scans containing the protein allows an accurate mass for the protein to be determined; the figures show the deconvoluted masses obtained from the single scan, which are all close to the expected mass of α-chymotrypsinogen A.

Injection of a Cocktail of Droplets. To demonstrate that mass spectrometry could be used to distinguish between different proteins in different droplets, 104−114 μm diameter (593−778 pL) droplets were produced from solutions of four different proteins, each droplet population containing 25 μM of protein (14.8−19.5 fmol of analyte in each droplet). These droplets were collected off-chip and stored overnight at 4 °C. Aliquots (8.0 μL) of each protein emulsion were then mixed to create a cocktail containing the four individual protein droplets (Figure 2a). For mass spectrometric analysis, the droplet

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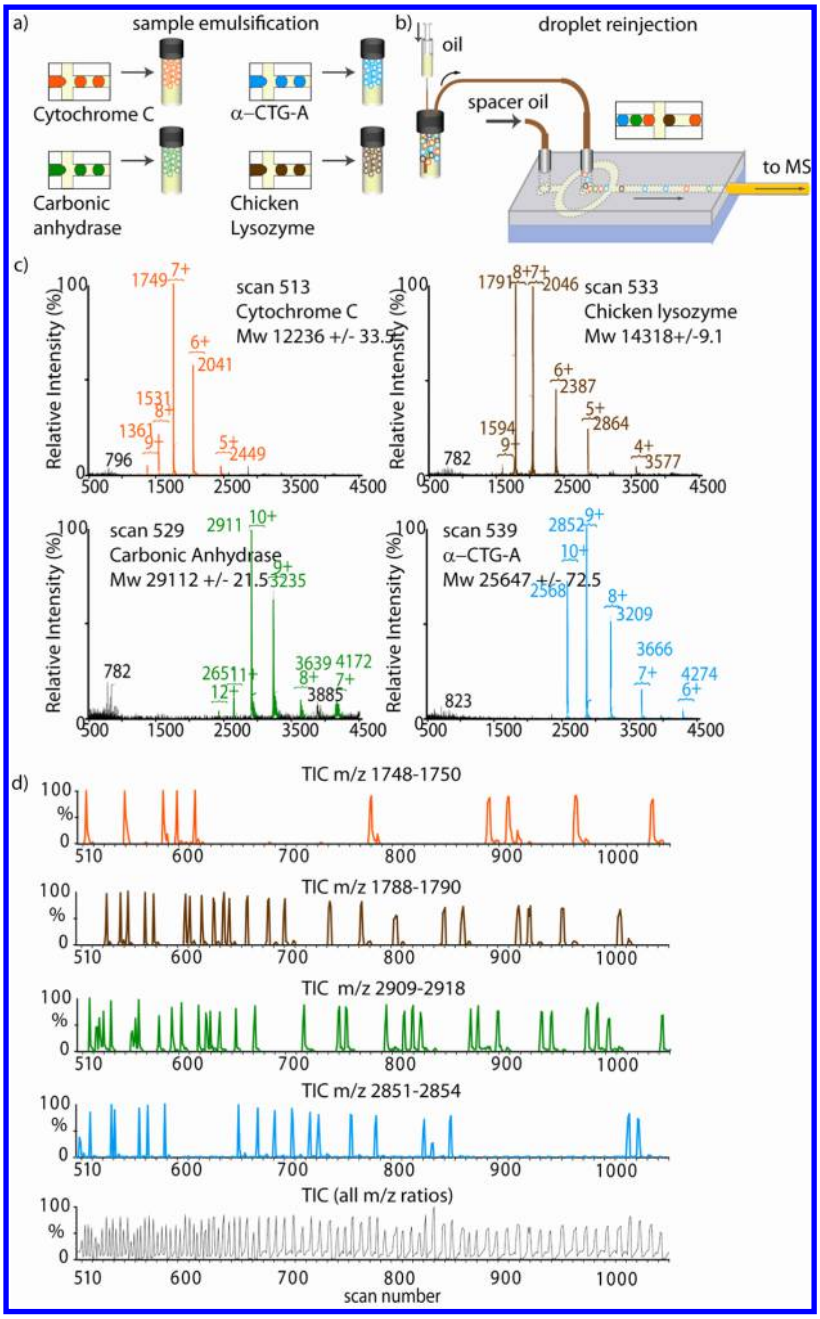

Figure 2. (a) Four populations of droplets containing either cytochrome C, α-chymotrypsinogen A, carbonic anhydrase, or chicken lysozyme (each 25 μM) were generated in a flow-focusing droplet generation module. The droplets were collected off-chip and stored at 4 °C overnight. Aliquots of each protein emulsion were then mixed to create a cocktail containing the four individual protein droplets. (b) The droplet cocktail was reinjected into the emitter device connected to the ESI-MS. (c) Individual scans of droplets containing cytochrome C (scan 513), carbonic anhydrase (scan 529), chicken lysozyme (scan 533), and α-chymotrypsinogen A (scan 539) respectively. (d) The TIC (551 scans, 33 s of data) and ion currents for 86 droplets recorded at *m*/*z* ranges 2851−2854, 2909−2918, 1788−1790, and 1748−1750 corresponding to the major charge states of α-chymotrypsinogen A, carbonic anhydrase, chicken lysozyme, and cytochrome C,, respectively.

cocktail was reinjected at a flow rate of 66.7 nL/min into the microfluidic emitter device using FC-3283 containing 2% w/w PicoSurf-2 surfactant (133.3 nL/min) as the spacing oil (Figure 2b). Analysis was carried out at a high scan rate of the MS (positive ion, V-mode for TOF, 0.05 s scan duration, 0.01 s interscan delay, scan rate of 16.7 Hz), enabling the four individual proteins in separate droplets, and in random order, to be observed and resolved at 2.6 Hz. Figure 2c shows the individual ion currents for the major charge states for each of the proteins; the quality of the single scan spectra allow each protein to be identified easily. It can be seen that these four ion currents collectively form the TIC in Figure 2d, showing remarkably stable periodic increases for 86 droplets over 33 s and over 551 scans. These data indicate that the signal strength could be used to estimate the amount of analyte in each droplet, although a quantitative analysis is beyond the scope of this paper. On the basis of the flow rate and the duration of the scan, we estimate that a mass spectrum is obtained from a volume of approximately 200 pL, containing a maximum of 5 femtomole of protein.

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Careful analysis of the mass spectra during injection of the oil in Figure 2d (Supporting Information, Figure S6) shows a range of peaks that can be attributed to surfactant molecules (see Supporting Information). In addition to surfactant ions, we also see residual traces of proteins, which indicate that some cause cross-contamination of the following droplet probably due to incomplete spraying of the sample before the next droplet arrives at the emitter, as we see no evidence of droplet fusion and proteins cannot diffuse through the oil phase in surfactant-stabilized emulsions.[26] The addition of surfactant-free spacing oil during the reinjection of droplets into the MS chip will improve the recording from single droplets.

Detection of Antibodies. Finally, we explored the limits of the technique with respect to the nature of the detected protein and the sensitivity of detection. As the molecular weight of proteins increases above 100 kDa, they become progressively more difficult to characterize by mass spectrometry. We therefore addressed this challenge by studying the antibody Trastuzumab (Herceptin) in droplets. Trastuzumab (hIgG1) exhibits a degree of microheterogeneity as a result of post-translational glycosylation, however, a raw molecular weight has recently been calculated at 148 057 Da.[27] This microheterogeneity of the glycoprotein sample should make the derivation of a molecular weight even more challenging from a single picodroplet. Trastuzumab-containing droplets (12.5 μM, 410 pL, 92 μm diameter, ca. 5.1 fmol antibody per droplet) were formed at a frequency of 134 Hz in a flow focusing droplet generation module using 2% w/w PicoSurf-2 surfactant in FC3283 and stored overnight at 4 °C. The droplets were reinjected into a microfluidic emitter chip (250 nL/min), with FC3283 (333 nL/min) as the spacing oil. Figure 3a shows the detection of individual droplets (MS configured in positive ion V-mode for TOF, with a sample cone voltage of 3.67 kV, 0.05 s scan duration, and 0.01 s interscan delay (scan rate 16.7 Hz)). Despite the large molecular mass of the analyte, single scan ESI MS spectra (Figure 3b, left) from individual droplets show five charge states, similar to the mass spectrum obtained from Trastuzumab sprayed under normal conditions (Figure 3c, left). Charge state deconvolution (using settings of minimal mass of 100 kDa and maximum of 200 kDa and a resolution of 0.125 Da/channel) from single scan spectrum obtained from a single droplet (scan 4561, containing around 5 femtomole of analyte) leads to a measured molecular weight for the antibody of 147.6 ± 0.016 kDa (Figure 3b, right), well within the expected mass range for the different glycosylated forms and very similar to the number obtained for Trastuzumab sprayed in a conventional manner into the ESI MS (147.7 ± 0.042 kDa, Figure 3c, right).

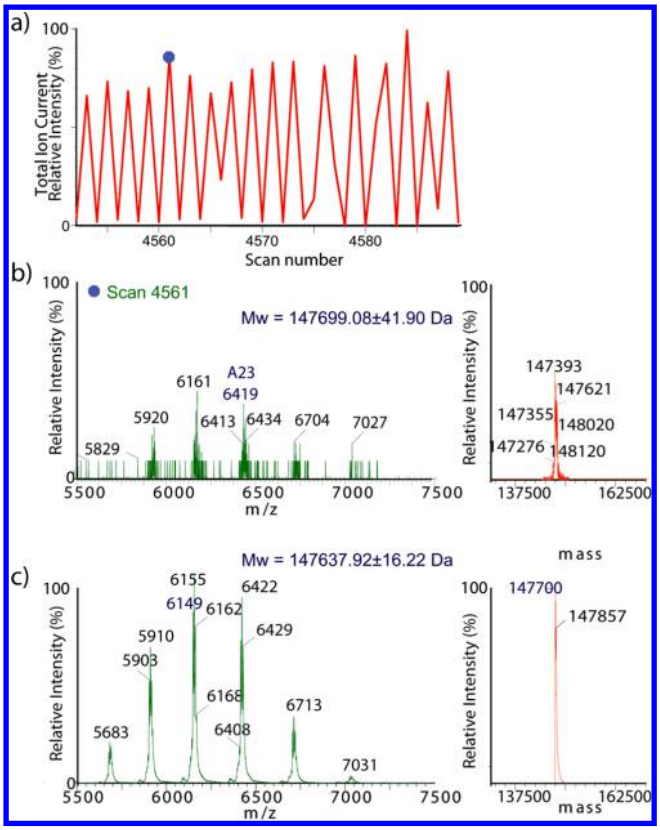

Figure 3. (a) Droplets (ca. 92 μm,408pL) containing Trastuzumab (12.5 μM) were spaced and injected into the mass spectrometer using a microfluidic emitter device. The total ion current peaked as individual droplets are emitted from the device. (b) Close-up of single scan 4561 (original range of 500−8000, no other protein peaks visible) and corresponding deconvoluted mass spectrum of Trastuzumab. (c) Conventionally sprayed ESI MS spectrum of Trastuzumab and corresponding deconvoluted mass spectrum.

CONCLUSION

We have demonstrated, for the first time, that it is possible to record high resolution mass spectra of individual surfactant-stabilized droplets containing subfemtomole amounts of proteins at scan rates of (10 Hz). The method is robust and can be applied to droplets as small as several hundred picoliter to several nanoliters, with a few femtomoles of analyte in droplets, and molecular weights of 1 to >100 kDa. The label-free detection of very small amounts of antibodies and other proteins in stabilized droplets will aid in the development in new assays, e.g., to detect the chemical modification of proteins. With further refinement of the technology, we believe that mass spectrometry on individual droplets offers a unique tool to not only directly study the secretion of molecules (cytokines, antibodies) from compartmentalized cells but also study their cytosolic constituents as well, following cell lysis.

ASSOCIATED CONTENT

Supporting Information

Full experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ca26@cam.ac.uk (C.A.); w.huck@science.ru.nl (W.T.S.H.).

Present Addresses

[†]Sphere Fluidics Limited, Wellington House, East Road, Cambridge CB1 1BH, UK.

[‡]Department of Chemistry, Physical and Theoretical Chemistry Laboratory, South Parks Road, Oxford OX1 3QZ, UK.

[§]Biozentrum University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) El Debs, B.; Utharala, R.; Balyasnikova, I. V.; Griffiths, A. D.; Merten, C. A. *Proc. Nat. Acad. Sci. U. S. A.* **2012**, *109*, 11570−11575.
(2) Guo, M. T.; Rotem, A.; Heyman, J. A.; Weitz, D. A. *Lab Chip* **2012**, *12*, 2146−2155.
(3) Tewhey, R.; Warner, J. B.; Nakano, M.; Libby, B.; Medkova, M.; David, P. H.; Kotsopoulos, S. K.; Samuels, M. L.; Hutchison, J. B.; Larson, J. W.; Topol, E. J.; Weiner, M. P.; Harismendy, O.; Olson, J.; Link, D. R.; Frazer, K. A. *Nat. Biotechnol.* **2009**, *27*, 1025−1031.
(4) Agresti, J. J.; Antipov, E.; Abate, A. R.; Ahn, K.; Rowat, A. C.; Baret, J.-C.; Marquez, J. M.; Klibanov, A. M.; Griffiths, A. D.; Weitz, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 4004−4009.
(5) Thorsen, T.; Roberts, R. W.; Arnold, F. H.; Quake, S. R. *Phys. Rev. Lett.* **2001**, *86*, 4163−4166.
(6) Anna, S. L.; Bontoux, N.; Stone, H. A. *Appl. Phys. Lett.* **2003**, *82*, 364−366.
(7) Theberge, A. B.; Courtois, F.; Schaerli, Y.; Fischlechner, M.; Abell, C.; Hollfelder, F.; Huck, W. T. S. *Angew. Chem., Int. Ed. Engl.* **2010**, *49*, 5846−5868.
(8) Ahn, K.; Kerbage, C.; Hunt, T. P.; Westervelt, R. M.; Link, D. R.; Weitz, D. A. *Appl. Phys. Lett.* **2006**, *88*, 024104.
(9) Link, D. R.; Grasland-Mongrain, E.; Duri, A.; Sarrazin, F.; Cheng, Z.; Cristobal, G.; Marquez, M.; Weitz, D. A. *Angew. Chem., Int. Ed.* **2006**, *45*, 2556−2560.
(10) Baret, J.-C.; Miller, O. J.; Taly, V.; Ryckelynck, M.; El-Harrak, A.; Frenz, L.; Rick, C.; Samuels, M. L.; Hutchison, J. B.; Agresti, J. J.; Link, D. L.; Weitz, D. A.; Griffiths, A. D. *Lab Chip* **2009**, *9*, 1850−1858.
(11) Ahn, K.; Agresti, J.; Chong, H.; Marquez, M.; Weitz, D. A. *Appl. Phys. Lett.* **2006**, *88*, 264105.
(12) Lorenz, R. M.; Edgar, J. S.; Jeffries, G. D. M.; Chiu, D. T. *Anal. Chem.* **2006**, *78*, 6433−6439.
(13) Clausell-Tormos, J.; Lieber, D.; Baret, J.-C.; El-Harrak, A.; Miller, O. J.; Frenz, L.; Blouwolff, J.; Humphry, K. J.; Köster, S.; Duan, H.; Holtze, C.; Weitz, D. A.; Griffiths, A. D.; Merten, C. A. *Chem. Biol.* **2008**, *15*, 427−437.
(14) Brouzes, E.; Medkova, M.; Savenelli, N.; Marran, D.; Twardowski, M.; Hutchinson, J. B.; Rothberg, J. M.; Link, D. R.; Perrimon, N.; Samuels, M. L. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 14195−14200.
(15) Courtois, F.; Olguin, L. F.; Whyte, G.; Bratton, D.; Huck, W. T. S.; Abell, C.; Hollfelder, F. *ChemBioChem* **2008**, *9*, 439−446.
(16) Fidalgo, L. M.; Whyte, G.; Bratton, D.; Kaminski, C. F.; Abell, C.; Huck, W. T. S. *Angew. Chem., Int. Ed.* **2008**, *47*, 2042−2045.
(17) Fidalgo, L. M.; Whyte, G.; Ruotolo, B. T.; Benesch, J. L. P.; Stengl, F.; Abell, C.; Robinson, C. V.; Huck, W. T. S. *Angew. Chem., Int. Ed.* **2009**, *48*, 3665−3668.
(18) Kelly, R. T.; Page, J. S.; Marginean, I.; Tang, K.; Smith, R. *Angew. Chem., Int. Ed.* **2009**, *48*, 6832−6835.
(19) Zhu, Y.; Fang, Q. *Anal. Chem.* **2010**, *82*, 8361−8366.
(20) Pei, J.; Qiang, L.; Lee, M. S.; Valaskovich, G. A.; Kennedy, R. T. *Anal. Chem.* **2009**, *81*, 6558−6561.
(21) Li, Q.; Pei, J.; Song, P.; Kennedy, R. T. *Anal. Chem.* **2010**, *82*, 5260−5267.
(22) Sun, S.; Slaney, T. R.; Kennedy, R. T. *Anal. Chem.* **2012**, *84*, 5794−5800.
(23) Ji, J.; Nie, L.; Qiao, L.; Li, Y.; Guo, L.; Liu, B.; Yang, P.; Girault, H. H. *Lab Chip* **2012**, *12*, 2625−2629.
(24) Baret, J.-C. *Lab Chip* **2012**, *12*, 422−433.
(25) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64−71.
(26) Zheng, B.; Roach, L. S.; Ismagilov, R. F. *J. Am. Chem. Soc.* **2003**, *125*, 11170−11171.
(27) Delobel, A.; Cantais, F.; Catrin, A.; Dereux, E.; Van Vyncht, G. *Glycosylation Engineering of Biopharmaceuticals, Methods in Molecular Biology*; Beck, A., Ed.; Springer: New York, 2013; Chapter 8.