Internalization Sensor

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A Programmable Sensor to Probe the Internalization of Proteins and Nanoparticles in Live Cells**

*Haiyin Liu and Angus P. R. Johnston**

The uptake of proteins, nanoparticles, and viruses into cells plays a critical role in drug delivery, immunity, and cell development.[1–3] Understanding the internalization of these materials allows us to design better drugs and restrict the proliferation of aberrant cells. However, current methods for studying internalization are limited by low throughput, low sensitivity, or are restricted to single cell populations. Herein we present a simple, high-throughput method for determining the cellular uptake of a range of materials that is compatible with conventional cell phenotyping and is independent of the cellular fate of the material. By using a nucleic acid sensor that specifically quenches the fluorescence of material on the surface of the cell but does not affect the fluorescence of internalized material (Figure 1), we were able to specifically examine the kinetics of nanoparticle and protein internalization in live cells. We believe this technique will broaden the application of internalization studies in complex biological systems.

To determine if material is inside or outside the cell, high-resolution microscopy can be used, however it is low throughput, and in the early stages of endocytosis it can be difficult to distinguish between material localized on the membrane, or internalized material that remains within 500 nm of the surface. While imaging flow cytometry and high-content screening can be used to improve sample throughput, this is at the expense of resolution.[4,5] Therefore, to study mechanisms of internalization in complex biological systems, there is a need for a simple, high-throughput technique to unambiguously determine if material has been internalized.

Flow cytometry allows high-throughput analysis of cells, but localization information must be obtained indirectly through changes in the fluorescent signal induced by internalization. A number of strategies have been developed to study internalization using flow cytometry, however they are either limited to studying a single fluorophore, eliminating the possibility of phenotyping,[6,7] or only applicable to a subset of cells and materials.[8] By taking advantage of advances in DNA nanotechnology,[9] we have developed a DNA molecular switch that signals when material has been internalized into a cell (specific hybridization internalization probe; SHIP; Figure 1). The SHIP sensor comprises of two 20-mer ssDNA sequences: 1) a probe with a 5′ fluorescent modification (Cy5) and a 3′ azide that enables the probe to be "clicked" onto the material of interest (fluorescent internalization probe; FIP_{Cy5}); and 2) a complementary sequence with a 3′ Black Hole Quencher 2 (BHQ2) that can hybridize to the FIP and is added to the assay after the material has been allowed to internalize (quencher probe; QP_C). The QP_C binds to the FIP on the surface of the cell, quenching its fluorescence, while the fluorescence of the internalized FIP is maintained because the QP_C cannot access internalized material. This step is performed at 4 °C, which prevents any of the QP_C from being taken up by the cells. The specificity of DNA hybridization leaves all other fluorophores unaffected by the quenching assay, which is a major advantage when phenotyping is required to identify cells of interest in a mixed sample. Furthermore, it enables the full spectrum of fluorescent probes to be used and allows for more than one molecule to be investigated in each assay.

To demonstrate the applicability of this sensor, we investigated the internalization of transferrin (Tf) into CEM-NKR cells and used anti-CD4 ($CD4_{PE}$) as both a plasma membrane and phenotyping stain. FIP_{Cy5} was attached to Tf using a strained cyclooctyne/succinimidyl ester linker (DIBO-SE).[11] The succinimidyl ester reacts with lysine residues on the protein, and the DIBO enables a copper-free click reaction[12] with the azide on FIP_{Cy5}. High-resolution microscopy confirmed that SHIP accurately distinguishes between surface-bound and internalized material. $Tf_{FIP\text{-}Cy5}$ (red) was incubated with CEM-NKR cells for 30 min at 4 °C to inhibit internalization (Figure 2a). The cross-section image confirms that the $Tf_{FIP\text{-}Cy5}$ fluorescence remains exclusively on the surface of the cell. Addition of QP_C efficiently quenches the fluorescent signal from $Tf_{FIP\text{-}Cy5}$ without affecting the fluorescence of the $CD4_{PE}$ (green) phenotyping stain (Figure 2b). To confirm the specificity of the QP_C, a mismatched quencher sequence (QP_M) was added to the cells, and the fluorescent signal from the $Tf_{FIP\text{-}Cy5}$ was unaffected (see Supporting Information, Figure S1). To demonstrate this assay can be applied to high throughput analysis, flow cytometry was used to quantify the fluorescent signal from the cells (Figure 2c). The signal from $Tf_{FIP\text{-}Cy5}$ dropped from intensity 5700 to 110 upon the addition of QP_C, whereas the addition of QP_M resulted in a slight increase in fluorescence (intensity 6100). This slight increase is assumed to be due to an excess of BHQ2 in solution, which lowers the background signal measured by the flow cytometer. If the excess BHQ2 is washed from the sample, the signal is equivalent to the non-quenched sample (Figure S2).

[*] H. Liu, Dr. A. P. R. Johnston
Nanomaterials for Biology Group, Department of Chemical and Biomolecular Engineering, The University of Melbourne
Parkville, Melbourne, 3010 (Australia)
E-mail: angusj@unimelb.edu.au

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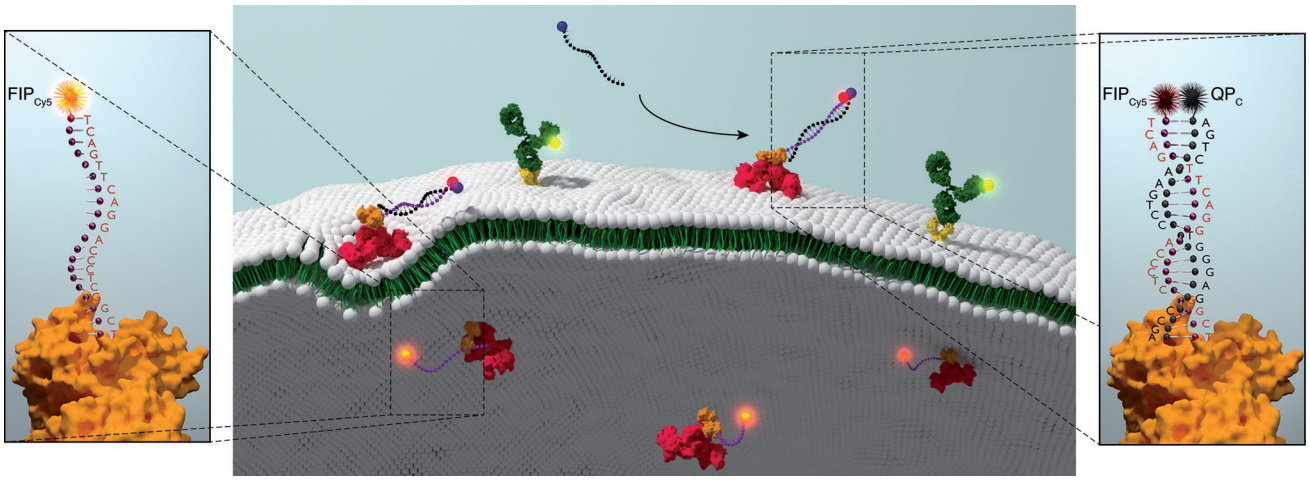

Figure 1. Specific hybridization internalization probe (SHIP). The material of interest (protein or nanoparticle) is labeled with a fluorescent DNA sequence, the fluorescent internalization probe, FIP (red). Once the labeled material has been incubated with the cells for the appropriate period of time, a quenching probe (QP_C) is added. The QP_C specifically quenches the fluorescence of the FIP on the surface, but does not affect the fluorescence of internalized FIP, or other surface markers (green).

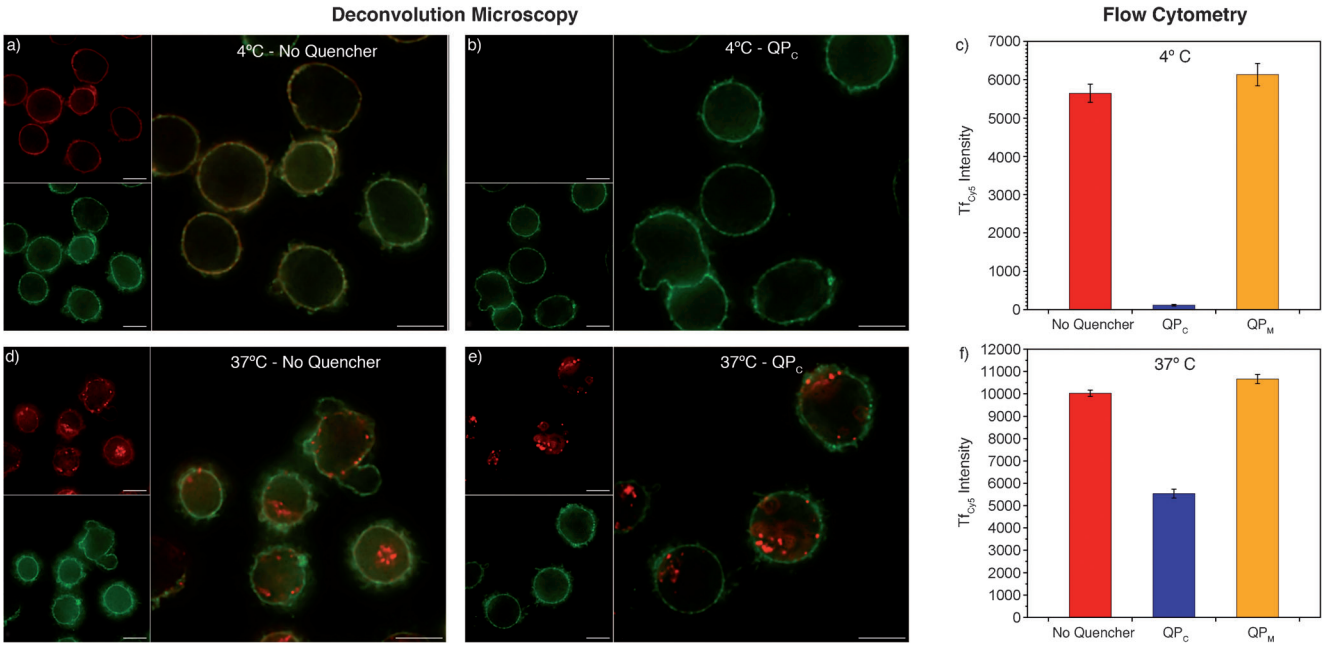

Figure 2. Surface-bound transferrin ($Tf_{FIP\text{-}Cy5}$) is specifically quenched by the DNA quenching probe (QP_C). a,b,d,e) Denoised[10] and deconvolved fluorescence microscopy images of CEM-NKR cells. Cells incubated with $Tf_{FIP\text{-}Cy5}$ (red) a) at 4 °C for 30 min, b) at 4 °C for 30 min, followed by the addition of 100 nM QP_C, d) at 37 °C for 10 min, e) at 37 °C for 10 min, followed by the addition of 100 nM QP_C. All samples were phenotyped with $CD4_{PE}$ (green). Scale bar 10 μm. c,f) Flow cytometry analysis of Tf_{Cy5} fluorescence intensity. Histograms comparing $Tf_{FIP\text{-}Cy5}$ incubated at c) 4 °C for 30 min, and f) 37 °C for 10 min. Data represents the mean intensity of 10000 cells ± s.d. for $n = 3$ samples.

At 37 °C Tf is rapidly internalized into the cells, as shown in Figure 2d, where $Tf_{FIP\text{-}Cy5}$ can clearly be observed both on the surface and inside the CEM-NKR cells. Addition of QP_C (Figure 2e) did not affect the fluorescence of the internalized $Tf_{FIP\text{-}Cy5}$, however, as expected the surface-bound $Tf_{FIP\text{-}Cy5}$ fluorescence was completely quenched. Figure 2e also highlights how the SHIP sensor makes it easier to unambiguously identify internalized Tf that remains close to the cell membrane. Again, flow cytometry was used to quantify the internalization process (Figure 2f). The fluorescent signal from $Tf_{FIP-Cy5}$ dropped from intensity 10000 to 5500 upon the addition of QP_C indicating that around 55% of the Tf associated with the cell was internalized within 10 min.

To demonstrate the advantages of the SHIP assay over other common internalization assays, we compared SHIP with the trypan blue (TB)[6] and acid wash (AW) methods,[7] and a pH sensor that increases fluorescence when internalized (Figure 3).[8] Similar to the SHIP assay, trypan blue can be used to rapidly quench surface fluorescence without affecting the signal from internalized material.[6] However, trypan blue

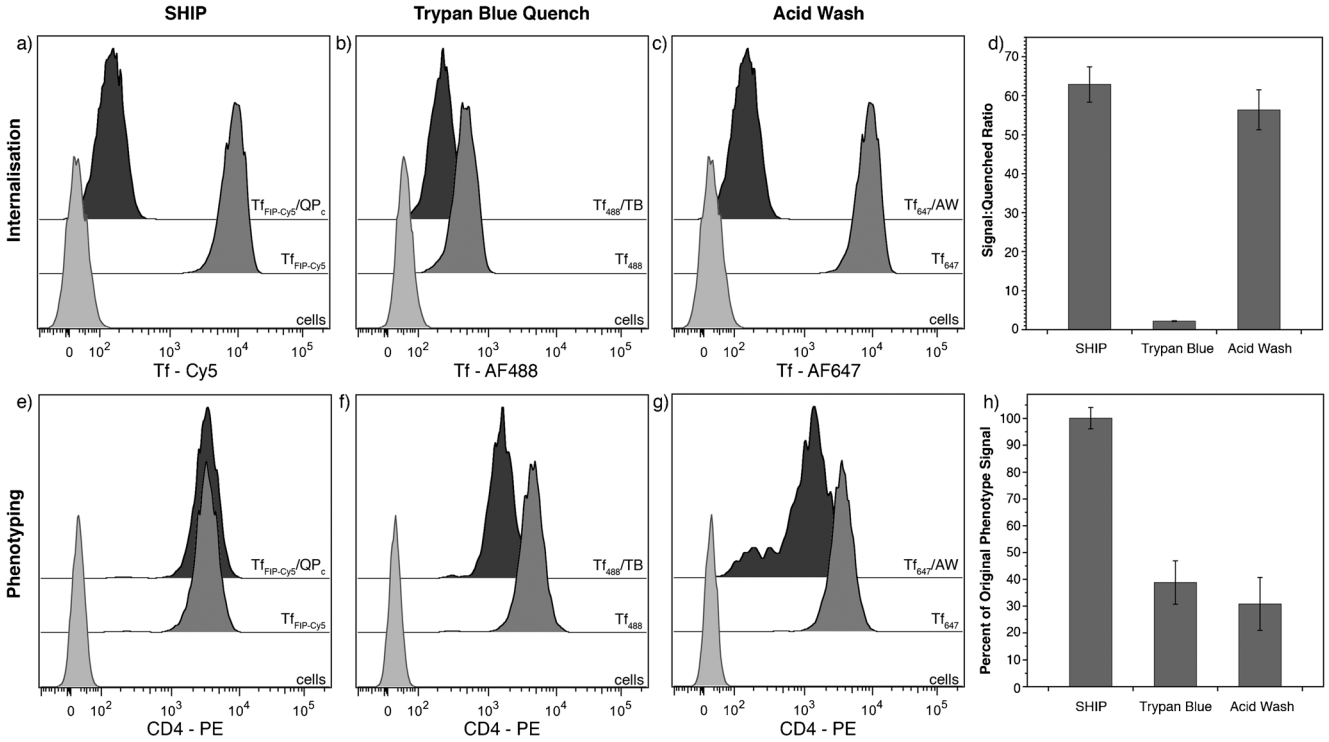

Figure 3. Comparison of SHIP with trypan blue and acid wash assays shows SHIP has a higher signal:quenched ratio and maintains the phenotyping signal. a–c) Flow cytometry histograms comparing the fluorescent signal from unlabeled CEM-NKR cells (light gray), CEM-NKR cells incubated with Tf (gray), and CEM-NKR cells incubated with Tf with surface fluorescence quenched (dark gray). d) Comparison of the signal:quenched ratio for the different internalization sensors. e–g) Flow cytometry histograms comparing the phenotyping signal ($CD4_{PE}$) from unlabeled CEM-NKR cells (light gray), CEM-NKR cells incubated with Tf (gray), and CEM-NKR cells incubated with Tf with surface fluorescence quenched (dark gray). h) Comparison of the phenotype signal before and after surface quenching for the different internalization sensors. Data represents the mean intensity of 10000 cells $\pm$ s.d. for $n = 3$ samples, except for the acid wash, where only 2500 cells were analyzed (due to the loss of cells during the washing process).

requires a high concentration (1 mM) and quenches fluorescence non-specifically, affecting any fluorophores used for phenotyping or marking other surface proteins. As shown in Figure 3 f, $CD4_{PE}$ lost 65% of its original signal in the presence of trypan blue, whereas in the SHIP assay, the addition of QP_C resulted in the loss of $<1\%$ of the signal (Figure 3 e). Furthermore, trypan blue exhibits significant autofluorescence which prevents multiple fluorophores being used in the assay (Figure S3), and limits its application to quenching in the 500–550 nm range. The autofluorescence of cells in this region means the signal-to-quenched ratio in the trypan blue assay is significantly lower than in assays that use dark red fluorophores like Cy5 or AF647 (Figure 3 d).

A more flexible alternative to trypan blue quenching is to use an acidic solution (such as 0.2 M acetic acid) to strip proteins and material from the surface of cells.[7] This assay has the advantage of being compatible with any fluorophore, however it is still limited to single-color investigations. When CEM-NKR cells were phenotyped with $CD4_{PE}$ after the acid wash, they lost more than 70% of their original $CD4_{PE}$ signal, compared to $<1\%$ in the SHIP assay. The additional processing steps in the acid wash protocol can also lead to the loss of a significant number of cells during the washing and resuspension steps. Furthermore, cell morphology is affected by the relatively harsh conditions of the acid wash, as observed in the FSC vs SSC flow cytometry plots (Figure S4).

To overcome the limitation of not being able to phenotype cells, pH sensitive probes, such as pHrodo, can be used.[8,13] This technique measures the increase in fluorescence of the sensor when it migrates from a neutral (extracellular) to an acidic (lysosomal) environment.[8] The assay relies on the internalized material localizing in an acidic environment, which is a limitation when studying cells with weakly or slowly acidifying endosomes (such as dendritic cells),[14] or with materials that are designed to undergo endosomal escape.[15–17] Additionally, while an increase in fluorescence is observed when pHrodo is localized in the lysosomal environment (pH 5) compared to the extracellular environment (pH 7.4), a fluorescent signal is still observed when pHrodo remains on the surface of the cell (Figure S5), which makes it difficult to distinguish between a large amount of material on the surface and small amount of internalized material. Development of the SHIP assay means that for the first time we are able to quantify internalization in a high-throughput fashion, while still being able to identify other surface markers and phenotype cells.

To investigate if the FIP sensor could interfere with the internalization of the material (which is a possibility with all fluorescent probes), $Tf_{FIP\text{-}Cy5}$ and Tf labeled with a conven-

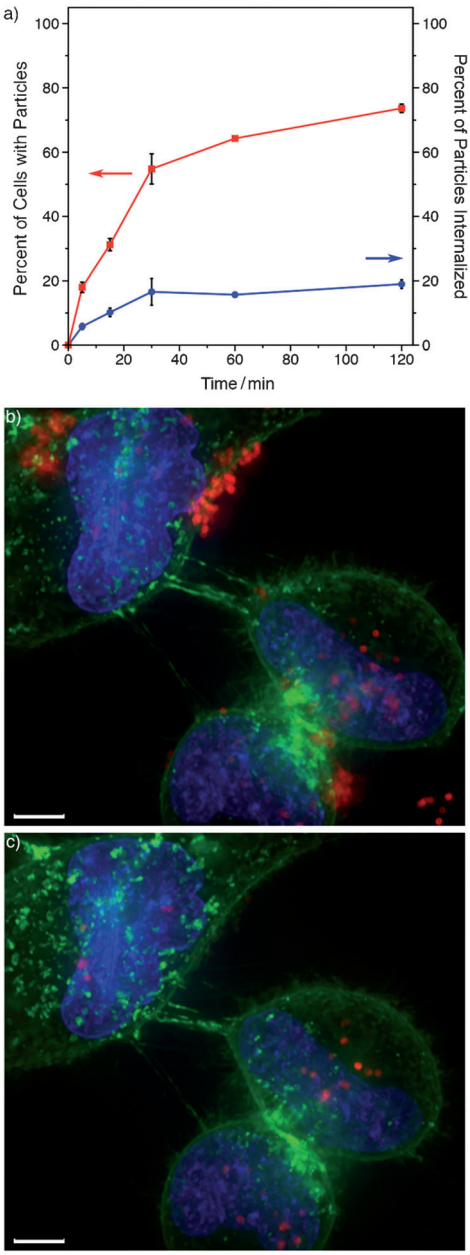

Figure 4. Kinetics of nanoparticle internalization quantified using SHIP. a) Flow cytometry analysis of the binding (■, left axis) and internalization (●, right axis) of $NP_{FIP\text{-}Cy5}$ in HeLa cells. Data represents the mean intensity of 10000 cells ± s.d. for $n = 2$ samples. b,c) Maximum intensity projection, fluorescence microscopy images of HeLa cells incubated with 500 nm particles for 2 h. b) No quencher, c) 100 nM QP_c. $NP_{FIP\text{-}Cy5}$ red, wheat germ agglutinin green, DAPI blue. Scale bar 5 μm.

tional succinimidyl ester fluorophore (AlexaFluor 488-Tf_{488}) were co-incubated at 37 °C with CEM-NKR cells (Figure S6). Both signals were completely co-localized, which demonstrates that in this case, both conventionally and FIP-labeled Tf are internalized and processed in a similar way. Although ssDNA may be susceptible to degradation during the internalization assay, we did not observe instability of the FIP sequences in these experiments. However if longer in vitro or in vivo experiments were required, a peptide nucleic acid (PNA) probe could be used in the place of DNA.[18]

The ability to quantify cellular internalization is important for more than just proteins. In particular, understanding the internalization of nanoengineered drug carriers[19] has the potential to greatly improve the therapeutic outcomes for a range of diseases.[16,20–24] To demonstrate SHIP is applicable to nanoparticles as well as proteins, we looked at the kinetics of nanoparticle uptake by CEM-NKR and HeLa cells. 500 nm diameter, alkyne-functionalized, poly(vinylpyrrolidone)-coated silica particles were functionalized with FIP_{Cy5} ($NP_{FIP\text{-}Cy5}$) and incubated with the cells for 2 h. Only 10% of the CEM-NKR cells had $NP_{FIP\text{-}Cy5}$ bound after 2 h, and no appreciable internalization was observed (Figure S7). In contrast, $NP_{FIP\text{-}Cy5}$ readily bound to HeLa cells, with more than 50% of the HeLa cells having particles bound after 30 min (Figure 4a). Internalization of $NP_{FIP\text{-}Cy5}$ occurred slowly, with only ca. 14% of the $NP_{FIP\text{-}Cy5}$ bound to HeLa cells being internalized after 2 h. This was confirmed with fluorescence microscopy (Figure 4b,c). These results demonstrate the SHIP sensor is useful for probing the internalization of materials ranging from proteins to nanoparticles.

In summary, we have demonstrated a simple, high-throughput method for probing the internalization of proteins and nanoparticles into cells. This method is compatible with a range of fluorophores and specifically quenches the fluorescent probe attached to the material of interest, enabling multi-color assays. No specialized equipment is required (other than a flow cytometer) and the straightforward assay can be routinely performed with minimal training. The FIP can be easily attached to a range of materials using click chemistry, and fluorescence of surface-bound material is quenched by simply adding the QP_C to the sample before analysis. SHIP delivers a high signal:noise ratio without the need for washing steps and is independent of intracellular localization of the material. We anticipate this new technique will provide a simple yet powerful tool to study the internalization of a range of materials, including proteins, viruses and nanoparticles and find application in the fields of drug delivery, cell biology, and immunology.

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[1] S. Conner, S. Schmid, *Nature* **2003**, *422*, 37–44.
[2] I. Canton, G. Battaglia, *Chem. Soc. Rev.* **2012**, *41*, 2718–2739.
[3] A. E. Smith, A. Helenius, *Science* **2004**, *304*, 237–242.
[4] A. P. R. Johnston, M. M. J. Kamphuis, G. K. Such, A. M. Scott, E. C. Nice, J. K. Heath, F. Caruso, *ACS Nano* **2012**, *6*, 6667–6674.
[5] J. Mintern, C. Percival, M. M. J. Kamphuis, W. J. Chin, F. Caruso, A. P. R. Johnston, *Adv. Healthcare Mater.* **2013**, DOI: 10.1002/anie.201301243.

[6] E. S. Van Amersfoort, J. A. G. Van Strijp, *Cytometry* **1994**, *17*, 294 – 301.
[7] B. Sönnichsen, S. De Renzis, E. Nielsen, J. Rietdorf, M. Zerial, *J. Cell Biol.* **2000**, *149*, 901 – 913.
[8] E. R. Simons, *Measurement of Phagocytosis and of the Phagosomal Environment in Polymorphonuclear Phagocytes by Flow Cytometry*, Wiley, Hoboken, **2010**.
[9] J. Bath, A. J. Turberfield, *Nat. Nanotechnol.* **2007**, *2*, 275 – 284.
[10] J. Boulanger, C. Kervrann, P. Bouthemy, *IEEE Trans. Pattern Anal.* **2007**, *29*, 1096 – 1102.
[11] X. Ning, J. Guo, M. A. Wolfert, G.-J. Boons, *Angew. Chem.* **2008**, *120*, 2285 – 2287; *Angew. Chem. Int. Ed.* **2008**, *47*, 2253 – 2255.
[12] J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 16793 – 16797.
[13] A. Muñoz Javier, O. Kreft, M. Semmling, S. Kempter, A. G. Skirtach, O. T. Bruns, P. del Pino, M. F. Bedard, J. Rädler, J. Käs, C. Plank, G. B. Sukhorukov, W. J. Parak, *Adv. Mater.* **2008**, *20*, 4281 – 4287.
[14] A. Savina, C. Jancic, S. Hugues, P. Guermonprez, P. Vargas, I. C. Moura, A.-M. Lennon-Duménil, M. C. Seabra, G. Raposo, S. Amigorena, *Cell* **2006**, *126*, 205 – 218.
[15] A. Varkouhi, M. Scholte, G. Storm, H. J. Haisma, *J. Controlled Release* **2011**, *151*, 220 – 228.
[16] A. P. R. Johnston, G. K. Such, S. L. Ng, F. Caruso, *Curr. Opin. Colloid Interface Sci.* **2011**, *16*, 171 – 181.
[17] M. Massignani, C. LoPresti, A. Blanazs, J. Madsen, S. P. Armes, A. L. Lewis, G. Battaglia, *Small* **2009**, *5*, 2424 – 2432.
[18] V. V. Demidov, V. N. Potaman, M. D. Frank-Kamenetskil, M. Egholm, O. Buchard, S. H. Sönnichsen, P. E. Nlelsen, *Biochem. Pharmacol.* **1994**, *48*, 1310 – 1313.
[19] C. Garnacho, S. M. Albelda, V. R. Muzykantov, S. Muro, *J. Controlled Release* **2008**, *130*, 226 – 233.
[20] B. G. De Geest, M. A. Willart, H. Hammad, B. N. Lambrecht, C. Pollard, P. Bogaert, M. De Filette, X. Saelens, C. Vervaet, J. P. Remon, et al., *ACS Nano* **2012**, *6*, 2136 – 2149.
[21] S. F. M. van Dongen, H.-P. M. de Hoog, R. J. R. W. Peters, M. Nallani, R. J. M. Nolte, J. C. M. van Hest, *Chem. Rev.* **2009**, *109*, 6212 – 6274.
[22] R. J. Passarella, D. E. Spratt, A. E. van der Ende, J. G. Phillips, H. Wu, V. Sathiyakumar, L. Zhou, D. E. Hallahan, E. Harth, R. Diaz, *Cancer Res.* **2010**, *70*, 4550 – 4559.
[23] J. A. Kim, C. Åberg, A. Salvati, K. A. Dawson, *Nat. Nanotechnol.* **2011**, *7*, 62 – 68.
[24] J. J. Moon, H. Suh, A. Bershteyn, M. T. Stephan, H. Liu, B. Huang, M. Sohail, S. Luo, S. H. Um, H. Khant, J. T. Goodwin, J. Ramos, W. Chiu, D. J. Irvine, *Nat. Mater.* **2011**, *10*, 243 – 251.

