Flow Cytometry of HEK 293T Cells Interacting with Polyelectrolyte Multilayer Capsules Containing Fluorescein-Labeled Poly(acrylic acid) as a pH Sensor

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Received December 19, 2006; Revised Manuscript Received March 9, 2007

Polyelectrolyte multilayer sensor capsules, 5 μm in diameter, which contained fluorescein-labeled poly(acrylic acid) (PAA_{AF}) as pH-sensitive reporter molecules, were fabricated and employed to explore their endocytotic uptake into HEK 293T cells by flow cytometry. The percentage of capsules residing in the endolysosomal compartment was estimated from the fluorescence intensity decrease caused by acidification. Capsules attached to the extracellular surface of the plasma membrane were identified by trypan blue quenching. The number of capsules in the cytoplasm was rather small, being below the detection limit of the method. The advantages of polyelectrolyte multilayer capsules are that the fluorophore is protected from interaction with cellular compartments and that the multilayer can be equipped with additional functions.

Introduction

Uptake and processing of capsules or particles by cells are important issues with regard to the transport of active agents into the cytoplasm.

A recently introduced delivery concept takes advantages of layer-by-layer (LbL)-coated particles as delivery devices.[1−4] The principle of the LbL coating is the alternating self-assembly of polycations and polyanions on flat or colloidal templates.[5,6] A large variety of synthetic (poly(allylamine hydrochloride) or poly(sodium 4-styrenesulfonate)) and biocompatible (protamine, dextran sulfate, chitosan, or nucleic acids) polyelectrolytes can be used for multilayer buildup.

The LbL coating with DNA or RNA as multilayer constituents and the subsequent interaction with cells open an interesting field of applications in cell biology. The LbL concept can be utilized in two ways: First, multilayers of biocompatible polymers and DNA can be employed as substrates for cell cultures to facilitate cell transfection with high rates due to the decomposition of the multilayer.[7−10] Second, colloids coated with biopolymers and DNA can be used as tools to explore the pathway of uptake and release of DNA or other bioactive species into the cytoplasm. The agents as well as sensor molecules can be either integrated within the polymer multilayer[1] or encapsulated in the interior.[11,12]

Recent work, employing plasmids encoding for the green fluorescent protein (GFP) or a red fluorescent protein (DsRed) as reporter molecules within the multilayer, has demonstrated that only a small fraction of particles was released from the endolysosomal compartment into the cytoplasm.[1] These reporter plasmids can only be expressed after endosomal uptake of the particles, subsequent release into the cytoplasm, and, finally, defoliation of the multilayer. One hypothesis to explain the low rate of expression is that, after uptake has been accomplished, most of the particles remain located in endolysosomes and undergo the lysosomal degradation process, which leads to the decomposition of the polymer shell and the inactivation of the plasmid. To verify this hypothesis it was necessary to develop a tool that would permit us to localize polyelectrolyte-coated particles or capsules in their intracellular environment under in vivo conditions and to quantify the number of them in the cytoplasm in relation to those in endolysosomes.

The aim of this study was thus to engineer particles or capsules with an integrated sensor function to track them along their path inside the cells. This reporter function is based on a pH-sensitive fluorescent probe that was integrated into the device to protect it from undesirable interaction with cellular components.

The intracellular pH can be used as a marker to localize the particles. The pH value within the cells varies, depending on the cell type, from about pH 4.5−5 within lysosomes to pH 7.3 in the cytoplasm and pH 8 within the mitochondria.[13−15] Particles within endolysosomes will experience an acidic environment, while capsules or particles in the cytoplasm are in a neutral medium.

There are numerous studies dealing with intracellular detection of pH using different sensors.[16−18] Optical sensors have been designed for detecting pH as well as for calcium, zinc, or glucose in different cell compartments[19] using a variety of fluorophores.[20,21] Carriers for the fluorophores range from fluorophore−polymer conjugates[22] to lipid-covered fluorophores (lipo-beads)[23,24] and dyes entrapped in a polymer matrix.[21,25,26] The major drawback of these systems concerning their application in uptake studies is that they are largely restricted to the function of a pH sensor. Other functions, for example, surface modifications as well as the integration of functional molecules for optimal release from the endolysosome, are quite difficult to add.

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10.1021/bm061200r CCC: $37.00
Published on Web 05/18/2007

LbL capsules or particles represent by their very nature multifunctional constructs, which may carry different functions engineered into the core itself or incorporated as layer constituents. This is advantageous considering further investigations with regard to biocompatibility. The surface of the capsules can be engineered to avoid decomposition in the endolysosomes or to promote release into the cytoplasm. The encapsulated fluorescent probe is protected from interactions with cell constituents such as cytoplasmatic proteins that may influence the fluorescence.[27] Reporter molecules other than the fluorescent pH sensor can be introduced into the capsule wall.

In the present study, LbL particles and capsules, containing amino fluorescein-labeled poly(acrylic acid) (PAA_{AF}) as the pH sensor, were fabricated and employed as a tracking device for studying their uptake and localization within cells by fluorescence-activated cell sorting (FACS) and confocal laser scanning microscopy (CLSM).

Experimental Section

Materials. Poly(acrylic acid) (M_w = 50 kDa, 25% w/w aqueous solution) was obtained from Polyscience (USA). Dimethylformamide (DMF) was obtained from Roth (Germany). Poly(allylamine hydrochloride) (PAH) (M_w = 70 kDa), poly(sodium 4-styrenesulfonate) (PSS) (M_w = 70 kDa), 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDAC), 2-morpholinoethanesulfonic acid monohydrate (MES), 5-aminofluorescein (AF), rhodamine isothiocyanate (RITC), and phosphate-buffered saline (PBS) were obtained form Sigma-Aldrich (Germany). Na_2CO_3 was obtained from Riedel-deHaën (Germany), KCl and $CaCl_2$ from Fluka (Germany), and NaCl from KMF Optichem (Germany).

Fluorescein-isothiocyanate-labeled cholera toxin subunit B, paraformaldehyde, sephadex G25 chromatography gel, and trypan blue (TB) were obtained from Sigma-Aldrich (Deisenhofen, Germany). Dulbecco's modified Eagle medium (DMEM) with L-glutamine was obtained from PAA Laboratories, and fetal bovine serum (FBS) was purchased from Biochrom AG.

Preparation. *Labeling of Polyelectrolytes.* PAA_{AF} was covalently labeled with AF with a labeling degree of 0.7% as described in ref 28. The conjugation of RITC to poly(allylamine hydrochloride) with a labeling degree of 0.5 was conducted according to ref 1.

Fabrication of $CaCO_3$ Particles Containing PAA_{AF}. $CaCO_3$ particles with a narrow size distribution around 5 ± 0.5 μm were prepared by recrystallization from saturated solutions.[29,30] Equal volumes of 0.33 M Na_2CO_3 containing 1 mg/mL PAA_{AF} and 0.33 M $CaCl_2$ solutions were rapidly mixed and intensively agitated with a magnetic stirrer at room temperature. After 30 s the agitation was stopped. The mixture was left without stirring for 15 min. During this time precipitation of $CaCO_3$ occurs, and spherical $CaCO_3$ particles are formed. The particles were thoroughly washed four times with water. About 95% of the added PAA_{AF} was taken up by $CaCO_3$ particles during their formation.

Preparation of LbL-Coated Capsules Containing PAA_{AF}. Capsules were prepared by sequential adsorption of positively charged PAH and negatively charged PSS on $CaCO_3$ particles containing PAA_{AF}. The adsorption of polyelectrolytes was conducted in 4 mg/mL solutions in 0.2 M NaCl. The pH of the PAH solution was adjusted to 6.5 by addition of NaOH. The adsorbing protocol started with PAH and finished with PSS. Eight layer pairs were adsorbed. Each adsorption step was carried out for 15 min with gentle agitation in a minishaker. Three washing and centrifugation steps followed to remove the nonadsorbed polyelectrolyte. After each cycle the $CaCO_3$ suspension was resuspended with ultrasound pulses to prevent aggregation. The $CaCO_3$ cores were removed in 0.1 M HCl followed by 10 washing steps in water. The coated $CaCO_3$ particles as well as the capsules were finally stored as an aqueous suspension (pH 6) at 4 °C.

pH Calibration of $CaCO_3$ Particle and Capsule Fluorescence. Approximately 3×10^5 particles or capsules were added to 600 μL of PBS with 0.5% FBS. The pH of PBS was adjusted between pH 2 and 8 with HCl and NaOH. The fluorescence as a function of pH was recorded in a FACSCalibur (Becton-Dickinson).

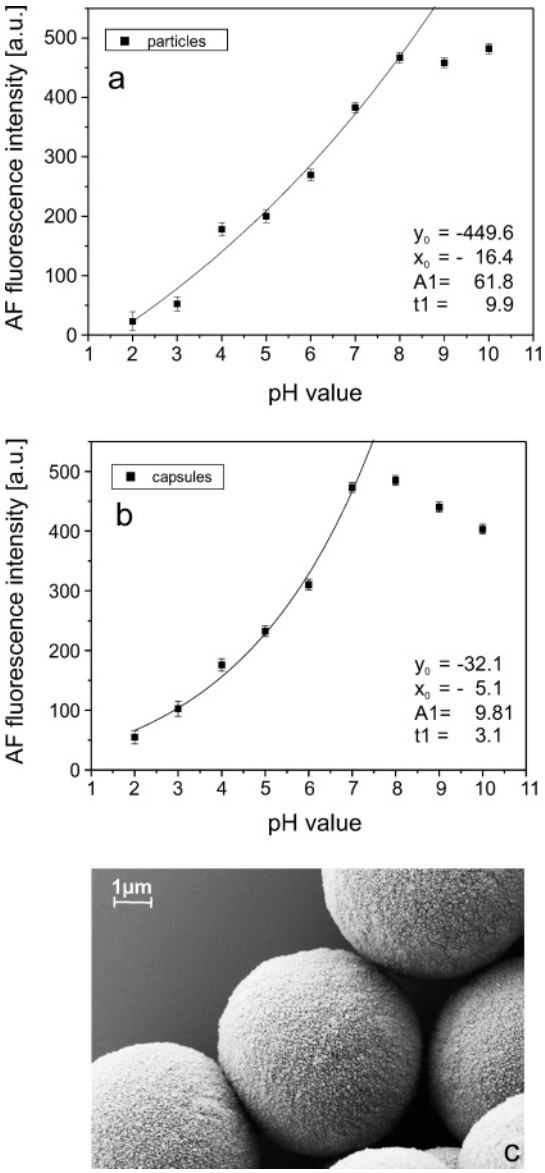
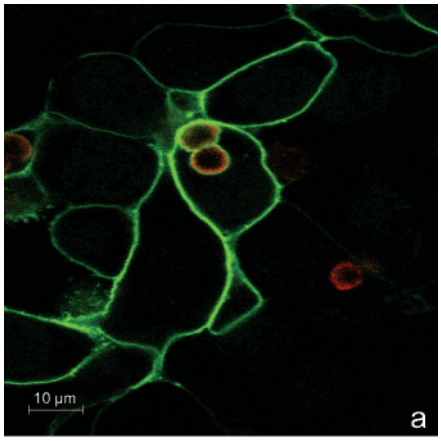


Figure 1. Fluorescence intensity of PAA_{AF} in (a) particles and (b) capsules as a function of the pH of the medium. The lines represent least-square fits to a single-exponential curve ($y = y_0 + A_1 \exp((x - x_0)/t_1)$) used later for converting fluorescence intensities into pH values. (c) Scanning electron micrograph of the calcium carbonate particles.

Particle Uptake. Adherent HEK 293T cells were used in accordance with previous studies.[1] They can be efficiently transfected and show a high phagocytotic activity. The cells were seeded in eight-chamber slides purchased from Nunc (size of one well, 0.7 cm × 0.7 cm; each well contained 2×10^4 cells). An incubation of 24 h in culture medium containing 5% FBS followed. Then 4×10^4 particles or capsules, respectively, were added to each well. Cell damage was not observed using this ratio. Particle−cell incubation was conducted for 48 h. This relatively low particle count was used to avoid multiple particle uptake, which is an essential requirement concerning the quantification of uptake by flow cytometry.

FITC−Cholera Toxin Labeling, NH_4Cl Treatment, and TB Quenching. Samples were fixed with 2% paraformaldehyde (PFA) for 20 min at room temperature and stored in PBS for 2 days. Then the cell membranes were labeled with FITC−cholera toxin (FITC−CTx) for 1 h at 37 °C according to ref 1.



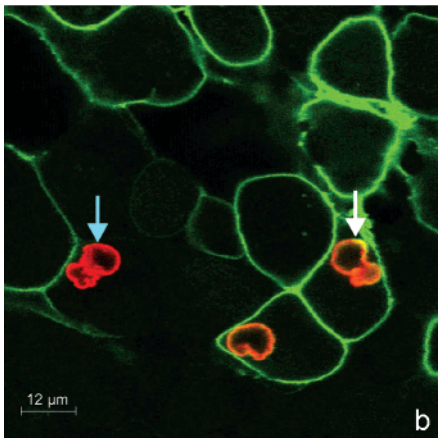


Figure 2. Confocal images of particles. (a) The endocytotic uptake of HEK 293T cells is demonstrated. The image shows red fluorescent particles, while the green fluorescence was emitted by FITC−CTx bound to membranes. It is possible to identify two particles that are surrounded by an endosomal membrane. Some particles, however, may have escaped into the cytoplasm, as demonstrated in part b, where the white arrow indicates a particle with an incomplete membrane coverage. The particle marked by the blue arrow was either not accessible for the FITC−CTx due to an impermeable plasma membrane or free in the cytoplasm.

The ammonium chloride treatment, inhibiting the acidification of the endolysosomes, was conducted as follows: Unless otherwise noted, 20×10^{-3} M NH_4Cl was added to 400 μL of culture medium per well 3 h before particle or capsule addition. After another 6 h of incubation the unstimulated endocytotic uptake is completed, and the NH_4Cl-containing medium was substituted by NH_4Cl-free one. Then, after further 42 h, the cells were washed with PBS, harvested with trypsin and culture medium, centrifuged, and resuspended in PBS with 0.5% FBS. Subsequently, culture cells as detailed below were investigated in either PBS or PBS containing NH_4Cl.

Quenching of the PAA_{AF} fluorescence with TB was induced by adding 10 μL of 0.4% TB solution to 400 μL of cell suspension followed by an incubation for 60 min.

Methods. *Flow Cytometry.* Fluorescence and scattering intensity distributions of particles and capsules were obtained by flow cytometry (FACSCalibur, Becton Dickinson, USA) with a laser excitation wavelength of 488 nm. Experiments were conducted in 137 mM NaCl, 2.8 mM KCl solution as the sheath fluid. Exactly 10 000 events were recorded. For the TB quenching experiments the sample was divided into two aliquots. One served as the control; the other was subjected to TB quenching. Data were analyzed by WinMDI2.8 software.

Confocal Laser Scanning Microscopy. Images were obtained by means of CLSM using a Leica TCS SP2. FITC−CTx and PAA_{AF} fluorescence were studied with the Ar/Kr laser, while the RITC fluorescence was excited with the He/Ne laser. Images were recorded

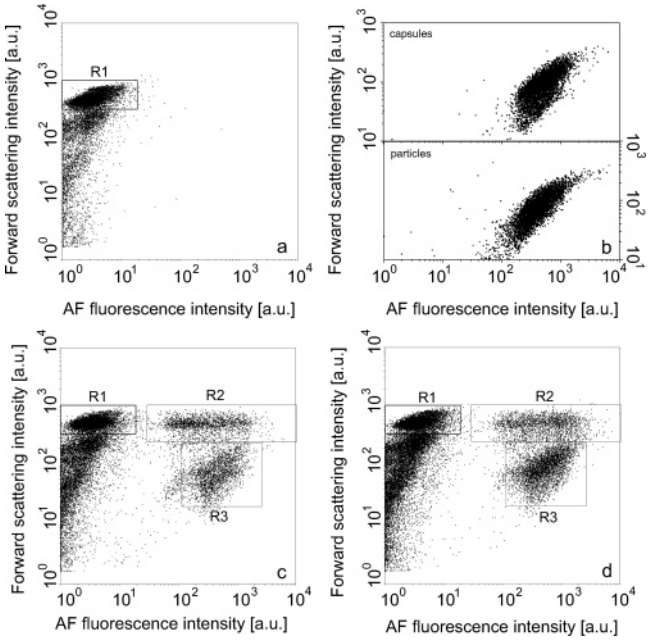


Figure 3. FACS dot plots obtained after incubation of (c) pH sensor particles and (d) pH sensor capsules with HEK 293T cells. The cell control and the particle/capsule control are shown in parts a and b. R1 indicates cells, R2 shows cells with capsule or particle association, and R3 denotes bulk particles or capsules.

in sequential mode between frames to separate green and red emission. The images were obtained with a HCX PL APO 63.0 × 1.32 oil objective with a lateral resolution of 1024 pixels.

Scanning Electron Microscopy. Scanning electron microscopy micrographs were conducted with a Gemini Leo 1550 instrument at an operation voltage of $U = 3.00$ kV. The sample was placed onto a glass slide, dried at room temperature, and sputtered with gold.

Results and Discussion

First, the pH-dependent PAA_{AF} fluorescence of particles prior to the removal of the core and, subsequently, of the capsules was studied for calibration purposes. The fluorescence intensities are presented in Figure 1a for particles and in Figure 1b for capsules. Both graphs show an increase of the fluorescence intensity in the range between pH 2 and 8. The intensity shift due to the changing of the pH value is reversible because the label is covalently bound to PAA and stable over a wide range of pH values. The fluorescence response of the capsules was almost identical to the fluorescence response of the LbL-coated $CaCO_3$ cores. This indicates that the fluorophore in the calcium carbonate core senses the pH of the bulk solution, which would be only possible if the cores are porous (Figure 1c) and PAA_{AF} is in direct contact with the aqueous environment.[31]

Before the capsule uptake was quantified by flow cytometry it was useful to visualize the uptake by means of confocal microscopy. Membrane staining allows localization of intracellular particles in qualitative terms. Plasma membrane compartments can be labeled by employing the specific binding of FITC−CTx to the ganglioside M1 (GM1).[32] Particles are identified by the red fluorescence of a RITC−PAH layer as a multilayer constituent. In this part of the study green fluorescent PAA_{AF} within the core was not present to avoid misinterpretation of the fluorescence of the core and the surrounding membrane.

As seen in Figure 2a, the plasma membrane as well as a membrane surrounding the red fluorescent particles appear in green fluorescence. In Figure 2b, particles that are not covered

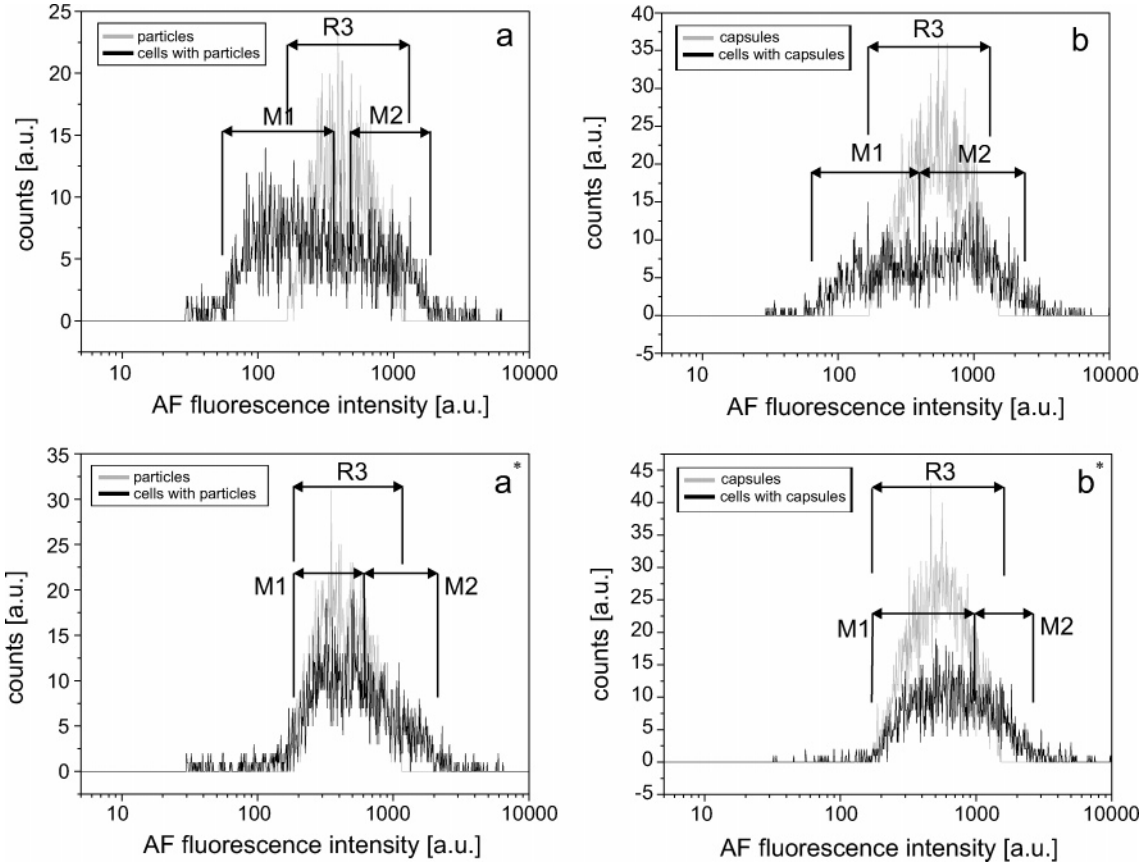


Figure 4. FACS histograms of the regions R2 = M1 + M2 and R3 of the dot plots for (a) cell-associated particles and (b) cell-associated capsules in PBS. Parts a* and b* represent the distributions of the same sample in NH_4Cl solution after 30 min of incubation.

by an additional fluorescent shell as well as a partially covered particle can be found. The green fluorescence around the particles indicates that the particles are inside a lipid membrane as a result of an endocytotic uptake process as described in ref 1. Particles without visible membranes have been either released into the cytoplasm or are surrounded by a nonstained membrane. This may occur if the plasma membrane was impermeable for FITC−CTx, which can happen if the PFA treatment was incomplete.

Next, sensor capsules showing the pH-dependent fluorescence were used to assess the pH of the environment of capsules and particles inside cells.

This would allow us to attribute particles and capsules to either the endolysosomal compartment or the cytoplasm. Figure 3 shows fluorescence FACS measurements obtained after incubation of HEK 293T cells with particles or capsules. The dot plot in Figure 3a represents control cells in the absence of particles or capsules. The region R1 denotes the population of HEK 293T cells. It is characterized by its large scattering with a small amount of self-fluorescence. The dot plots in Figure 3b represent the control particles as well as capsules; the regions R3 in Figures 3c and 3d denote free capsules and particles in the supernatant, respectively. The free capsule and particle region is characterized by its large fluorescence intensity and a forward scattering smaller than that observed for cells.

Region R2 combines the forward scattering intensity characteristic for cells together with the fluorescence intensity characteristic for capsules or particles. Hence, it represents either particles or capsules within cells or attached to the outside. To discriminate between these two possibilities requires further studies as described later. The intensity distribution in R2 is broad. The regions of bulk (R3) as well as the cell-associated (R2) particles and capsules in Figures 3c and 3d are similar concerning the intensity distribution and the event number. It can therefore be assumed that particles and capsules behave likewise with regard to their surface properties concerning interactions with cells.

About 18% of the cells are associated with particles or capsules. This follows from a comparison of the event numbers in R1 and R2. This result was expected because a relatively small amount of particles (two particles per cell) has been added to avoid the multiple uptake of particles, which would have interfered with the quantitative analysis of the FACS data. Since a considerable number of particles may have been sticking to the walls and cells were dividing during incubation, it is not surprising that only about 20% of the cells were found to be associated with particles. It is also worth noting that the presence of NH_4Cl during the endocytotic uptake did not influence the number of cells associated with particles or capsules. NH_4Cl is known to inhibit the acidification of the endolysosome[33] and, as shown later, NH_4Cl only changes the intralysosomal pH value[22,33,34] from acidic to neutral but did not interfere with endocytosis.

Figure 4 displays the histograms of the intensity distribution of cell-associated (a) particles and (b) capsules in region R2.

For the sake of comparison the respective distribution of free particles or capsules are overlaid in light gray. It is obvious that the distributions of cell-associated particles and capsules are broader and extend to lower intensities than those observed for particles or capsules themselves, respectively.

The monomodal fluorescence intensities of bulk particles and capsules are characterized by geometric mean (gmean) values of 430 ± 15 and 526 ± 30 a.u., respectively. The cell-associated intensity distributions in Figures 4a and 4b, however, appear

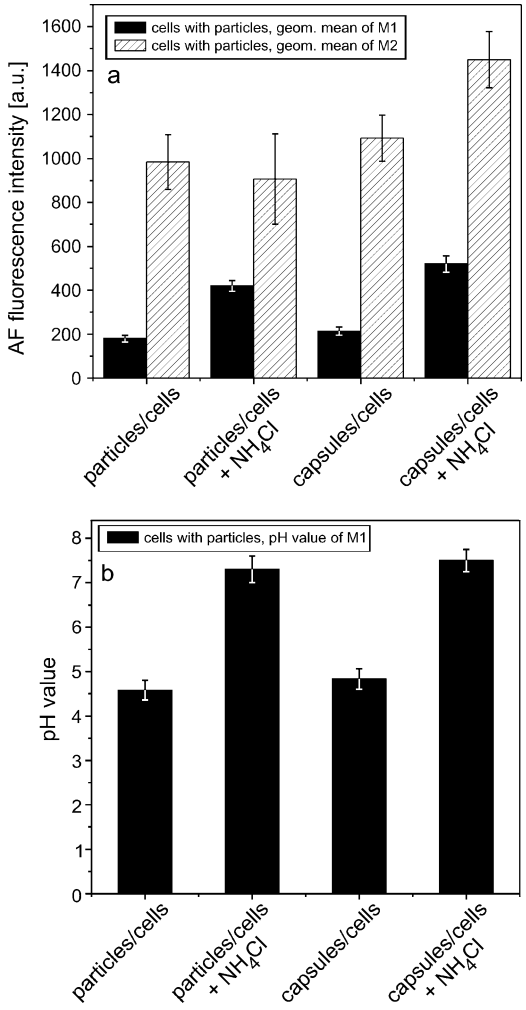


Figure 5. (a) Geometric mean values of the intensity distributions of R2: filled bars, low-intensity peak M1 (endolysosomal compartment); hatched bars, high-intensity peak M2 of particles and capsules associated with cells. (b) Calculated pH values for the low-intensity peak M1 based on the calibration curve given in Figure 1.

bimodal. They were divided accordingly into M1 and M2. The gmean value of the region M1 was about 180 ± 15 a.u. for particles and about 214 ± 18 a.u. for capsules. The gmean values for the region M2 were on the order of 1000−1100 a.u. Neither M1 nor M2 was a good match for the fluorescence distribution of free particles and free capsules. While the gmean value of M1 was about 2.5 times lower than that of R3, the gmean value of M2 exceeded that of R2 by a factor of 2. Our hypothesis, therefore, was that M1 represents particles or capsules in the endolysosomal compartment showing a diminished fluorescence in the acidic environment.

To prove this hypothesis, NH_4Cl was added to reverse the acidification of the endolysosomal compartment. Indeed, the distribution became significantly more narrow after incubation with NH_4Cl as shown in Figures 4a* and 4b*. An intensity shift of M1 toward larger values occurred upon addition of NH_4Cl. As a result of this shift, the bimodal shape of the distribution disappeared. The intensity in the presence of NH_4Cl increased to gmean values of 420 ± 25 a.u. (particles) and 520 ± 37 a.u. (capsules), respectively.

The fluorescence intensities in the histograms can be converted into pH values using the calibration curves given in Figure 1. The gmean values of fluorescence intensity as related to the calculated pH values are presented in Figure 5.

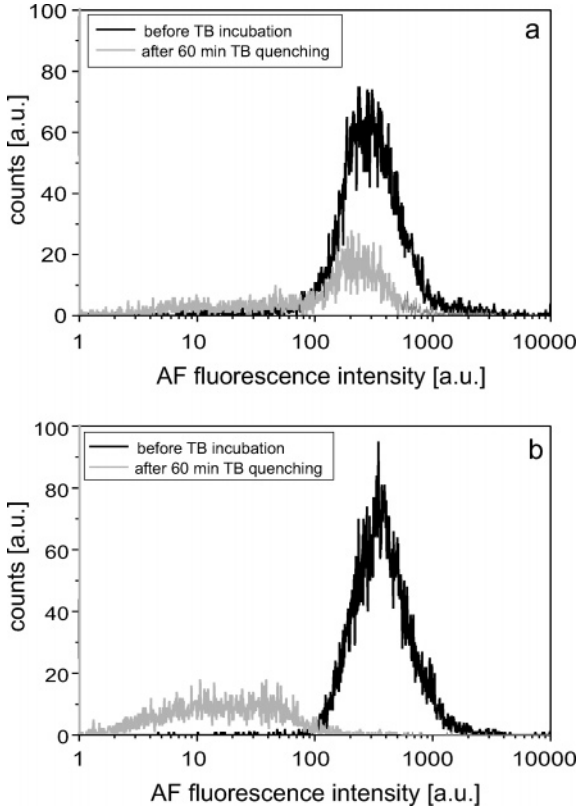


Figure 6. FACS histograms of (a) particle and (b) capsule PAA_{AF} fluorescence measured in PBS before and 60 min after TB incubation.

M1 provides a pH of 4.6 ± 0.2 for particles and a pH of 4.8 ± 0.2 for capsules (Figures 5b). These values are in good agreement with the pH values described for the interior of lysosomes.[23] In about 60% of cells with associated particles or capsules the events thus can be related to particles present in the endolysosomal compartment. This follows from an analysis of the event number in the histograms.

Further evidence for the endolysosomal compartment as the particle and capsule environment is provided by the PAA_{AF} fluorescence intensity shift induced by addition of NH_4Cl. This shift corresponded to pH values of 7.5 ± 0.25 and 7.3 ± 0.21 a.u., respectively, for the local environment of incorporated particles and capsules after addition of ammonium chloride. This is a good agreement with the expected neutralization of the endolysosome by NH_4Cl.

The intensity denoted as M2 could not be correlated with pH values, because the recorded fluorescence intensity values exceeded those observed for single particles or capsules. These high fluorescence intensities can thus only be attributed to multiple particles and capsules associated with one cell.

For the determination of the ratio of particles and capsules within the endolysosomes in relation to those in the cytoplasm, it is necessary to discriminate between an attachment to the cell from outside and the release into the cytoplasm. This can be accomplished by incubation with TB, a quencher for the PAA_{AF} fluorescence. The plasma membranes of living cells are impermeable to TB.[35] Therefore, only the fluorescence of those particles and capsules that are attached to the cell surface from the bulk will be quenched.

Prior to quenching experiments in cell suspensions, it was tested whether and to what degree the PAA_{AF} in particles or capsules were accessible to TB (Figure 6).

After an incubation of 60 min with TB, particles show a broad fluorescence intensity distribution with a pronounced peak in

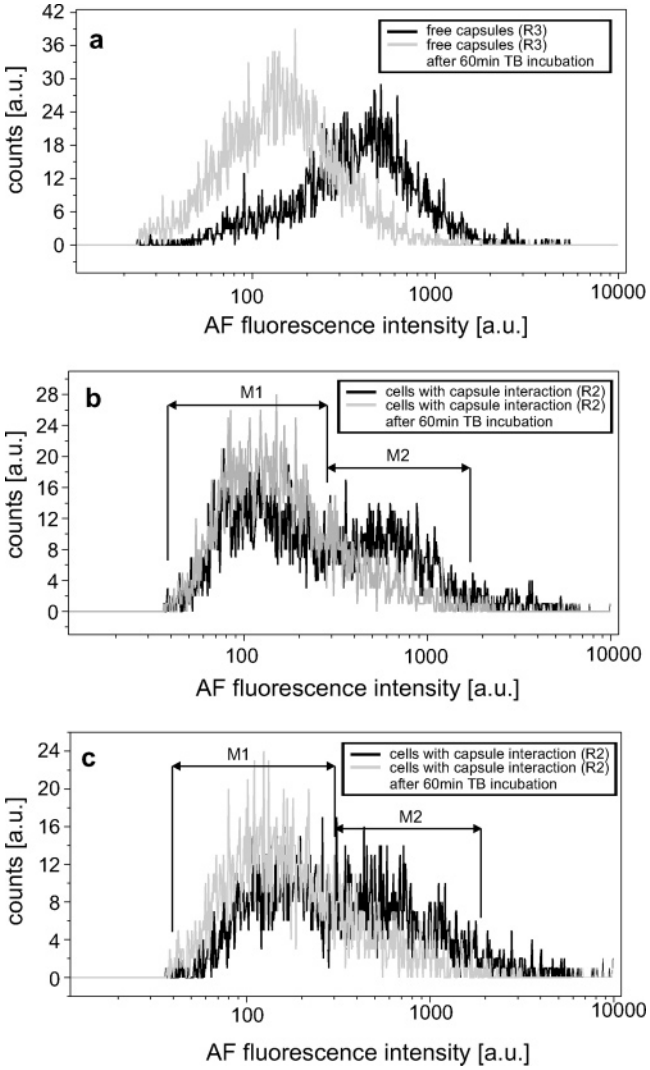


Figure 7. FACS histograms of region R2 of the dot plots using TB as a PAA_{AF} fluorescence quencher for capsules; measurements before (black) and after (gray) TB incubation for 60 min: (a) control capsules in supernatant, (b) cells with capsules in the absence of NH_4Cl during endocytosis, and (c) cells after a 2 day incubation in presence of NH_4Cl.

the region of control particles. In contrast, the fluorescence intensity of the capsule population decreased remarkably. This experiment demonstrated that there was a fraction of particles whose fluorescence cannot be quenched with TB. The fluorescence of capsules, however, was quenched by almost 2 orders of magnitude. Thus, only capsules are suitable for quantitative quenching experiments.

The quenching of capsules by TB in relation to the uptake is shown in Figure 7. Figure 7a provides as a control the decrease of the fluorescence intensity of bulk capsules in the presence of cells and, hence, culture medium after adding TB. Here, the TB-induced quenching is clearly pronounced but less than that in PBS alone (Figure 6b). We attribute this difference in behavior to the adsorption of proteins to capsules preventing partially the interaction of the latter with TB. The intensity distributions of cell-associated capsules are shown in Figures 7b and 7c. Regardless of whether NH_4Cl was present during the incubation (Figure 7c), the high-intensity peak M2 largely disappeared after TB incubation. The remaining intensity in this region can be best described as a tail of low frequency. The intensity of M1 was not shifted by TB. Thus, it can be assumed that a large fraction of the capsules in M2 was attached to the cell surface from outside and only a small fraction of capsules was located within the cytoplasm.

So, it can be finally concluded that the events in M1 represent capsules inside endolysosomes. Although the particle size is large, most of the particles were nevertheless located in the endolysosomes. Furthermore, as can be seen in Figure 2, the lysosomal membrane is rather closely attached to the particle surface, apparently leaving little free space inside the endosomes. But obviously the functionality of the endolysosomal membrane and the activity of the proton pumps are not affected by the close proximity of the capsule or particle surface as demonstrated by the low pH values.

As a result of the incubation of cells with NH_4Cl during the endocytotic process, the number of particles in the cytoplasm increased as described in ref 1. The data obtained in this study led to the conclusion that the number of capsules in the cytoplasmatic compartment, regardless of the ammonium-induced increase, is still small. It was below the detection limit of flow cytometry taking into account the background fluorescence from attached capsules and multiple capsules associated with one cell. This can be inferred from the similarity of the intensity distribution in Figures 7b and 7c.

Conclusion

It has been demonstrated that the fluorescence intensity of $PAA_{AF}/CaCO_3$ particles coated with a PAH/PSS multilayer as well as capsules fabricated by core dissolution in HCl responds to changes in the pH of the environment. Thus, particles and capsules can be used as fluorescence sensors for tracking particles inside living cells. Capsules are advantageous with respect to coated particles because the latter cannot be effectively quenched by TB.

Capsules with encapsulated PAA_{AF} can be useful as sensor systems with an encapsulated pH-sensitive fluorophore for locating the capsules within the cell compartments. Other reporter molecules can be integrated into the multilayer, as described in ref 1, thus forming a multifunctional sensor.

The developed LbL-based reporter device may allow us to study the uptake of LbL-coated colloids and capsules in more detail with the aim of optimizing them for delivery. The quantification of particles or capsules within different cell compartments offers a way to control and influence on the release from phagolysosome into the cytoplasm. It may be possible to develop means to provide the particles with additional surface modifications such as lipid layers with functional surfaces or special surface polymer layers to stabilize the multilayer and to facilitate endosomal escape.

Acknowledgment. We thank Michelle Prevot for assisting with SEM. D.H. acknowledges G. B. Sukhorukov for introducing him to the $CaCO_3$ core approach.

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BM061200R