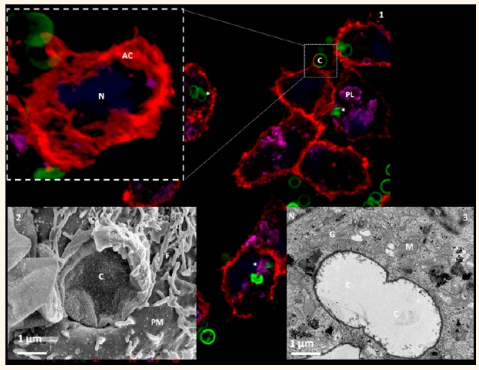
Multiple Internalization Pathways of Polyelectrolyte Multilayer Capsules into Mammalian Cells

Lena Kastl,[†,#] Daniel Sasse,[†,#] Verena Wulf,[†] Raimo Hartmann,[†] Josif Mircheski,[‡] Christiane Ranke,[†] Susana Carregal-Romero,[†,§] José Antonio Martínez-López,[‡] Rafael Fernández-Chacón,[‡] Wolfgang J. Parak,[†] Hans-Peter Elsasser,[⊥] and Pilar Rivera_Gil[†,∥,*]

[†]Fachbereich Physik, Philipps-Universität Marburg, Renthof 7, 35037 Marburg, Germany, [‡]Instituto de Biomedicina de Sevilla (IBiS) Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, Departamento de Fisiologia Medica y Biofísica, and CIBERNED, Avenida Manuel Siurot s/n, 41013, Sevilla, Spain, [§]Andalusian Center for Nanomedicine and Biotechnology (BIONAND), Andalusian Technological Park, C/Severo Ochoa, 35, 29590 Campanillas, Málaga, Spain, and [⊥]Institute of Cytology and Cytopathology, Philipps-Universität Marburg, Robert-Koch Straße 6, 35033 Marburg, Germany. [#]L. Kastl and D. Sasse contributed equally. [∥]Present address: Pilar Rivera_Gil, ICMAB-CSIC, UAB Campus, 08193 Bellaterra (Barcelona), Spain. E-mail: privera@icmab.es.

ABSTRACT Polyelectrolyte multilayer (PEM) capsules are carrier vehicles with great potential for biomedical applications. With the future aim of designing biocompatible, effective therapeutic delivery systems (*e.g.*, for cancer), the pathway of internalization (uptake and fate) of PEM capsules was investigated. In particular the following experiments were performed: (i) the study of capsule co-localization with established endocytic markers, (ii) switching-off endocytotic pathways with pharmaceutical/chemical inhibitors, and (iii) characterization and quantification of capsule uptake with confocal and electron microscopy. As result, capsules co-localized with lipid rafts and with phagolysosomes, but not with other endocytic vesicles. Chemical interference of endocytosis with chemical blockers indicated that PEM capsules enter the investigated cell lines through a mechanism slightly sensitive to electrostatic interactions, independent of clathrin and caveolae, and strongly dependent on cholesterol-rich domains and organelle acidification. Microscopic characterization of cells during capsule uptake showed the formation of phagocytic cups (vesicles) to engulf the capsules, an increased number of mitochondria, and a final localization in the perinuclear cytoplasma. Combining all these indicators we conclude that PEM capsule internalization in general occurs as a combination of different sequential mechanisms. Initially, an adsorptive mechanism due to strong electrostatic interactions governs the stabilization of the capsules at the cell surface. Membrane ruffling and filopodia extensions are responsible for capsule engulfing through the formation of a phagocytic cup. Co-localization with lipid raft domains activates the cell to initiate a lipid-raft-mediated macropinocytosis. Internalization vesicles are very acidic and co-localize only with phagolysosome markers, excluding caveolin-mediated pathways and indicating that upon phagocytosis the capsules are sorted to heterophagolysosomes.



KEYWORDS: polyelectrolyte multilayer capsules · mechanisms of internalization · cancer cells · pharmacological inhibitors · phagocytosis · macropinocytosis · clathrin-mediated uptake · lipid rafts · caveolin-mediated uptake · intracellular localization · lysosomes · endosomes · actin cytoskeleton · colloids · layer-by-layer assembly

Polyelectrolyte multilayer (PEM) capsules constitute a promising delivery system[1] for biomedical applications. The capsules are formed by sequential addition of oppositely charged polyelectrolytes onto a spherical template in a process called layer-by-layer assembly.[2−4] The whole system is stabilized predominantly by electrostatic interaction of the components. However, other types of stabilization processes have been used to create more sophisticated capsule systems.[5,6] After addition of a defined number of layers to the template, the template is chemically dissolved to obtain hollow capsules. The great advantage of these carrier systems is that PEM capsules can be modified with different functionalities.[7,8] For example, cargo molecules (*e.g.*, analyte-sensitive fluorophores, drugs, oligonucleotides, proteins) have been encapsulated within the cavity of PEM capsules.[9,10] Analyte-sensitive fluorophores in the cavity provide information about the environment in which the capsules are

* Address correspondence to pilar.riveragil@physik.uni-marburg.de.

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dispersed.[11,12] Biodegradable capsules promote the release of cargo from the cavity over time independently from an external trigger.[13−15] Other functional groups (*e.g.*, polyethylene glycol (PEG), colloidal nanoparticles, and proteins) can be integrated in the polyelectrolyte layer or attached to the surface of the PEM capsules. Surface modification of these capsules with PEG molecules increases the colloidal stability of the system due to the low fouling properties of these molecules, but their uptake by cells is reduced.[16,17] By embedding magnetic nanoparticles[18,19] or by linking molecules with molecular recognition properties (*e.g.*, DNA, antibodies, proteins),[20] PEM capsules can be either locally accumulated or targeted to specific cell populations,[21,22] respectively. Modifying the capsule wall with plasmonic nanoparticles[23−25] allows for controlled opening of single capsules and release of cargo inside the cells with light as an external trigger.[26,27] The materials selected to synthesize as well as to functionalize the capsules (*i.e.*, the polyelectrolytes for the shell and the type of core template) determine the physicochemical properties of the PEM capsules (*e.g.*, biodegradability, size, charge density, colloidal stability, molecular recognition, stimuli sensitivity, responsiveness).[28−31]

Despite the great biomedical potential of these carrier systems and the multiple biological potential applications already described in the literature, pathways for the internalization of PEM capsules and a correlation of their physicochemical properties and their intracellular fate still require further investigation. Reports in the literature indicate that incorporation of the capsules occurs spontaneously. For a more specific uptake, an improvement of the molecular recognition properties of the capsules would be required. Internalization has been demonstrated to be non-cell specific for a variety of cell lines.[32] Several groups have suggested macropinocytosis or lipid rafts as main entry pathways into eukaryotic cells, based on the use of one or two pharmacological inhibitors.[14,33,34] However, it is well known that no chemical inhibitor is absolutely specific for exclusively one pathway,[35] and thus additional studies are required. In general, the internalization of capsules by cells appears to be a highly energy-consuming, actin-mediated process, which depends on microcapsules' intrinsic factors, such as their physicochemical properties, rather than on the cell type. Although the exact mechanism of cellular uptake is still not fully unraveled, some important parameters that regulate this process have been recently elucidated. For instance, the overall charge of capsules has been demonstrated to play a role in capsule uptake. In analogy to smaller colloidal nanoparticles, charged capsules are ingested faster than uncharged ones,[16] and positively charged capsules are found to be engulfed more than negatively charged ones.[36] Anyhow, adsorption of cell medium proteins to the capsule surface tends to reduce differences in surface chemistry in the case of long incubation times. Softness also plays a role, as hydrogel-based capsules tend to accumulate in late endosomes,[10,37] in comparison to more rigid capsules, which end up in the phagolysosome.

In order to understand the uptake of PEM capsules by cells, it is helpful to understand the available body of literature on particle incorporation by cells in general. The plasma membrane of eukaryotic cells is a crucial interface that delimits the intracellular from the extracellular space. Since the 1970s, the plasma membrane is described according to the fluid mosaic model, which gives fluidity, dynamics, and asymmetry to this interface.[38] It is formed by a lipid bilayer made out of amphipathic lipids (phospholipids, glycolipids, and cholesterol) as well as of several kinds of associated proteins (integral and peripheral proteins, as well as glycoproteins). Cholesterol hinders solidification at low (room) temperature and prevents the membranes from becoming too fluid at high temperatures. By forming plasma membrane vesicles, cells can incorporate solids and liquids ranging from the nanometer up to the micrometer scale by numerous endocytic processes.[39,40] Roughly, endocytosis can be divided into phagocytosis (engulfment of large objects) and pinocytosis (macropinocytosis, lipid rafts, clathrin-dependent, caveolin-dependent, and clathrin- and caveolin-independent pathways).[41,42] Despite the common thought that only phagocytes are able to perform phagocytosis, most eukaryotic cells preserve this function.[43] The entry of solutes and liquids into the cell *via* endocytic pathways involves multiple stages. First, objects are taken up in plasma membrane invaginations toward the cytosol. Inward invaginations include lipid rafts, clathrin- or caveolin-mediated pathways, as well as clathrin- and lipid-rafts-independent pathways, whereas an outward engulfment of objects is more characteristic for phagocytosis and macropinocytosis. These invaginations are then pinched-off to form plasma-membrane-containing vesicles. Second, these vesicles mature into different specialized structures, *i.e.*, phagosomes, endosomes, caveosomes, and macropynosomes, depending on the endocytic machinery being activated, thus enabling sorting of the cargo. Finally, the cargo is delivered into intracellular compartments (*e.g.*, lysosomes, phagolysosomes), recycled back to the plasma membrane, or transported across the cells (*i.e.*, transcytosis). The morphological or biochemical distinction between the different endocytic pathways (clathrin- and caveolin-(in)dependent mechanisms, macropinocytosis, and phagocytosis) is quite difficult. During vesicle maturation there is a sequential fusion and fission of different endocytic vesicles and molecular markers where vesicular contents are bidirectionally exchanged. Thus, constituents from the plasma membrane, endosomes, and lysosomes appear in all of these endocytic vesicles.[43] Nevertheless, each

internalization mechanism exhibits several characteristic patterns, which are not entirely selective. As pointed out before, phagocytosis and macropinocytosis imply the formation of membrane protrusions to incorporate cargo, whereas all other pathways involve inward invaginations. Furthermore, phagocytosis can be distinguished from macropinocytosis by the type of exvagination formed. In the case of the macropinocytosis, the plasma membrane forms a kind of "arm" to surround the loose cargo (mostly liquids and small solutes). However, in the case of phagocytosis there is a formation of a sort of "cup" that tightly wraps around the cargo (mostly big solids). In both cases, the kinds of vesicles formed are on the micrometer scale, which fits the size of the capsules as investigated in this study. Other types of endocytic pathways (*i.e.*, clathrin- and caveolin-dependent and -independent mechanisms as well as lipid rafts) involve rather small membrane-bound vesicles (usually below 300 nm), and the scission from the plasma membrane is mostly dependent on the presence of dynamin.[40] Clathrin-dependent internalization processes are characterized by the presence of several adaptor proteins and by a clathrin coating. These routes are mostly associated with receptor-mediated endocytosis.[41] Lipid rafts are stationary vesicles on the plasma membrane. Like caveolin vesicles, they are cellular domains rich in cholesterol and sphingolipids, which lead to regions of enhanced packing and reduced fluidity. The presence of caveolae in the vesicles distinguishes lipid rafts from caveolin-dependent uptake processes. Cholesterol appears to be a key player in the formation of lipid rafts. The pH also plays a role in distinguishing the different internalizing vesicles. Most of the sorting vesicles reduce their pH upon maturation, thus reaching the lowest value when meeting the lysosomes or phagolysosomes, with the exception of the caveosomes, which are characterized by a neutral/slightly alkaline pH.[44]

In this study, the internalization mechanism of PEM capsules by mammalian cells was elucidated. The contribution of this article is the systematic study of the role of different endocytic processes on the internalization of PEM capsules by cells.

RESULTS

Microscopic Characterization of Capsule Uptake. The internalization of PEM capsules of different sizes by living cells is a well-established process, independently shown by several groups.[32,45] Despite their relatively "large" size (normally a few μm) (Figure SI-1), PEM capsules are easily incorporated not only by specialized phagocytic cells (*e.g.*, macrophages, dendritic cells) but also by a great variety of different cells (*e.g.*, embryonic fibroblasts, neuro- and glioblastoma cells, epithelial cancer cells) including primary cells in culture (Figures 1, 2, and SI-3) (*e.g.*, monocyte-derived dendritic cells, bone marrow mouse cells, and astrocytes)[14,46] (Figure SI-3.2.A–H). However, primary hippocampal neurons in culture took up capsules very inefficiently (Figure SI-3.1.D–F). In our study PEM capsules composed of PSS/PAH (polystyrene sulfonate/poly(allylamine hydrochloride)) as a prototype of synthetic nondegradable capsules and of pARG/DEXS (poly-L-arginine/dextrane sulfate) as a prototype of biodegradable capsules were used. Experiments were mainly performed on the example of the breast cancer cell line MDA-MB-231, but for comparison of the results obtained, A549 lung cancer cells were also used (Figure SI-7). Electron microscopy images clearly show that the uptake of the capsules by the cells does not interfere with their viability. Infoldings of the inner membrane of the mitochondria were perfectly defined and did not collapse. The number and the size of some mitochondria were, however, increased. This would be consistent with the assumptions that (i) the uptake is an energy-consuming process and that (ii) lipid recovery to the plasma membrane is being performed. Furthermore, the nuclear membranes were also found to be intact and the chromatin to be perfectly distributed over the nucleus. All these signs indicate that cell viability was not impaired by uptake of the capsules. The idea that the internalization of PEM capsules is an active process was confirmed upon the sensitivity of uptake to a decrease in the incubation temperature (Figure 3.A).

A closer look at the interaction of PEM capsules with MDA-MB-231 breast cancer cells shows that the filopodia of the cells surround the capsules to stabilize them at the cell membrane (Figure 1.A). Extension of the plasma membrane (membrane ruffling and redistribution of the actin cytoskeleton) to engulf the capsules occurs (*cf.* Figure 1), rather than the formation of invaginations, which would have to be pinched off. This suggests the exclusion of pathways like clathrin- and caveolin/lipid rafts-(in)dependent mechanisms. The formation of a phagocytic cup can be clearly seen in Figure 1.B and 1.C, thus pointing out phagocytosis rather than macropinocytosis as the internalization pathway (for more images concerning the internalization of PEM capsules by different kinds of cells *via* CLSM, TEM, and SEM see Figure SI-3).

Intracellular Localization of PEM Capsules. The study of intracellular trafficking was performed by observing the co-localization of PEM capsules with endocytic markers (see Table 2). There are two common ways to perform co-localization. One method uses the labeling of constituents (*e.g.*, lysosomal-associated membrane protein 1 (LAMP1), caveolin, early endosome antigen 1 (EEA1), clathrin) of specific organelles involved in internalization pathways (*i.e.*, phagolysosomes, caveolae vesicles, early endosomes, clathrin pits, respectively) with fluorescent molecules. In the other method, the so-called "pulse-chase" analysis,[47,48] the cells are exposed to markers with known uptake pathways (*e.g.*, cholera toxin B1 (CTXB) as a marker for lipid rafts). In both cases the location of the fluorescent PEM capsules within these organelles can be confirmed by the

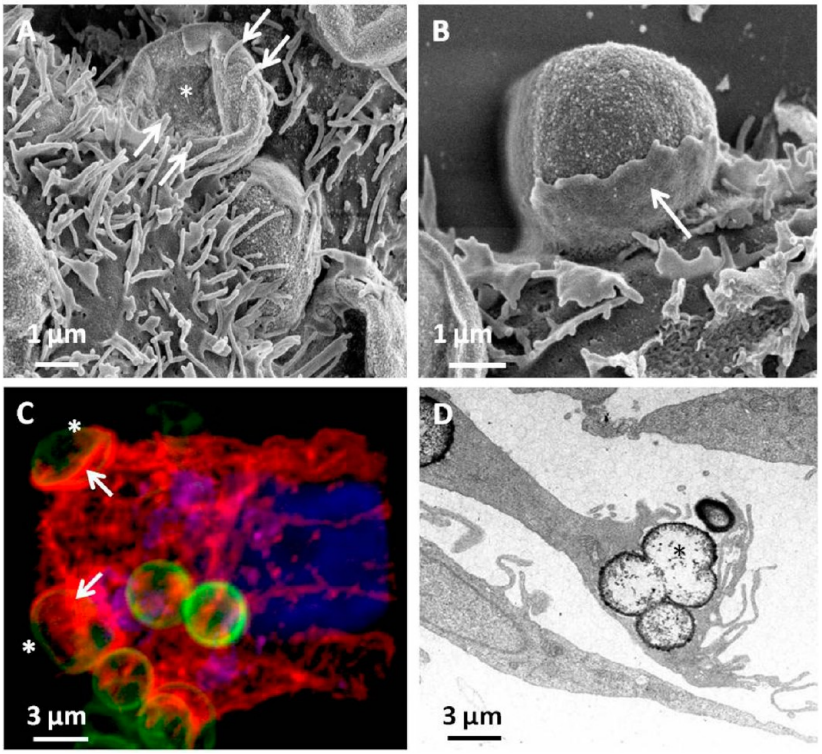

Figure 1. Microscopic characterization of capsule−cell interactions. Cultured MDA-MB-231 breast cancer cells were incubated with fluorescently labeled PSS/PAH capsules whose walls were modified with electron-dense gold nanoparticles (highlighted with a star). (A, B) Scanning electrom microscopy (SEM) images of the cells' plasma membranes. (A) The formation of filopodia (highlighted with arrows) to stabilize the capsules as well as (B) the formation of a phagocytic cup (highlighted with arrows) to engulf the PEM capsules can be clearly seen in these pictures. (C) Confocal laser scanning microscope (CLSM) images showing the reorganization of actin cytoskeleton (red) to internalized PEM capsules (green, highlighted with a star) in the same kind of phagocytic cup. The cell nucleus is shown in blue. (D) Transmission electron microscope (TEM) image of a cell elongating its body to engulf several capsules.[13]

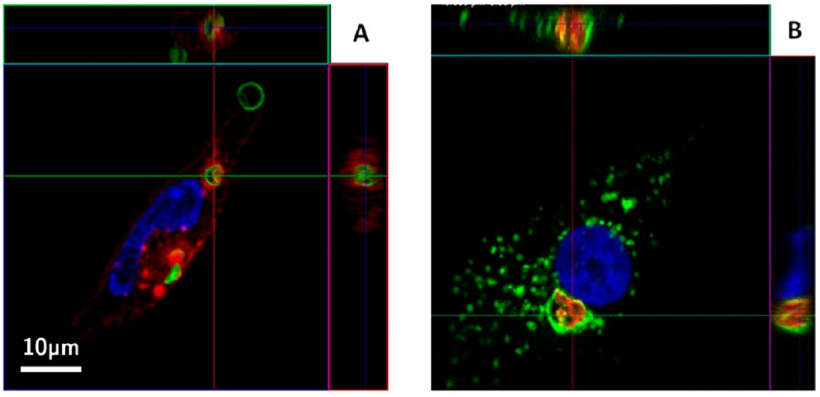

Figure 2. Co-localization of fluorescently labeled PSS/PAH capsules (green in A or red in B) with a marker for lipid rafts (cholera toxin B1 (red) in A) and with LAMP1 (green), a protein highly expressed in phagolysosomes' membrane with CLSM (B). The MDA-MB-231 cell's nucleus is shown in blue. An orthogonal view from different planes (*x*/*y*, *x*/*z*, or *y*/*z*) of the confocal microscope image shows the co-localization of the capsules with the different cellular structures.

co-localization between the fluorescent marker and the capsule. We observed that after an initial engulfment of capsules in types of phagocytic cups (Figure 1), there is a co-localization of the capsules with lipid rafts (marked with CTXB) (Figure 2.A). Lipid rafts are functional membrane microdomains enriched in cholesterol, specific proteins, and glycosphingolipids.[49] The observed co-localization of PEM capsules with structures containing LAMP1, which is a marker for phagolysosomes at a very early and later stage of phagocytosis[48,50] (Figure 2.B), demonstrates that phagosomes are the final localization of the capsules. Few signs of co-localization with other endocytic vesicles such as endoplasmic reticulum (ER), caveolin, endosomes,[10] and clathrin were observed (Figure SI-4.1).

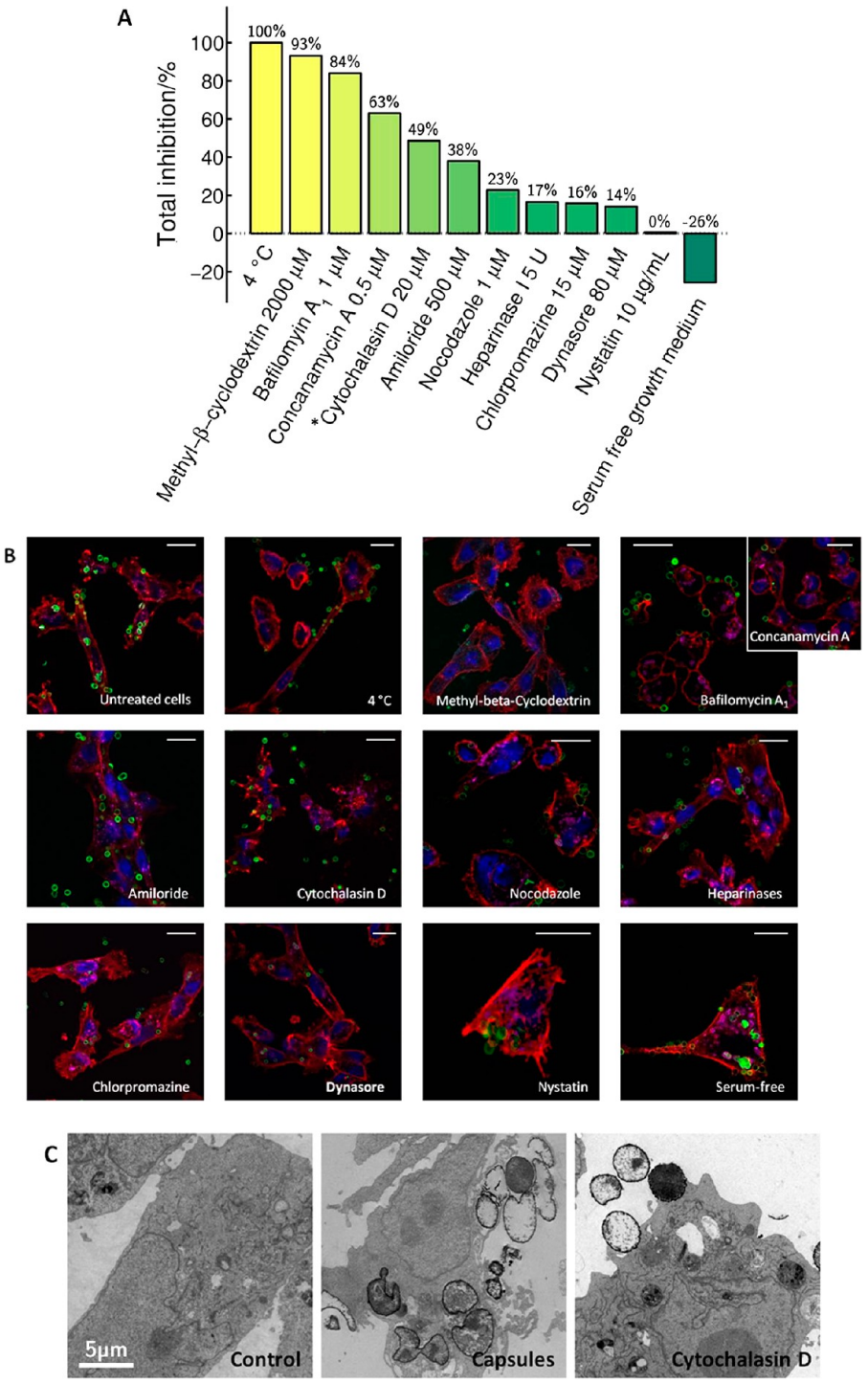

Figure 3. Pharmacological/chemical inhibition of all known internalization pathways. (A) Quantification of capsule uptake inhibition as described in the Methods section. *See comments in Table 1. (B) CLSM images of MDA-MB-231 breast cancer cells in the presence or absence (control cells) of the different inhibitors. The cytoskeleton (red), phagolysosomes (pink), and the nucleus (blue) of the cells were stained to assess internalization of FITC-modified PSS/PAH capsules (green). The length of the scale bar is 20 μm. (C) Positive effect of cytochalasin D on the inhibition of gold nanoparticle-modified PSS/PAH capsule internalization assessed in more detail. TEM images of MDA-MB-231 breast cancer cells in the absence of capsules and inhibitor (control), absence of inhibitor but incubated with capsules (capsules), and treated with cytochalasin D previous to capsule incubation (cytochalasin D).

The results obtained by the co-localization of capsules with different endocytic markers (Figure 2 and Figure SI-4.1 and SI-4.2) together with the results obtained by the microscopic analysis of internalization (Figure 1) suggest an exclusion of pathways such as clathrin- or caveolin-mediated endocytosis. On the contrary, they indicate that other processes, lipid rafts, macropinocytosis, or phagocytosis, are involved in the uptake of PEM capsules.

TABLE 1. Summary of the Results (in descending order) Obtained by Quantification of PSS/PAH Capsule Internalization by MDA-MB-231 Breast Cancer Cells upon Interfering with All Known Pathways

pharmacological/chemical inhibitor or alteration of the cellular environment	concentration	associated pathway	quantification of inhibition	references
cell incubation at 4 °C		all	100%	
methyl-beta-cyclodextrin	2000 μM	lipid rafts and lipid-raft-mediated macropinocytosis	93%	35, 59
bafilomycin A_1	1 μM	phagocytosis	84%	64, 65, 67, 68
concanamycin A	0.5 μM	phagocytosis	63%	64, 65, 67, 68
cytochalasin D	20 μM	phagocytosis	49%[a]	43
amiloride	500 μM	macropinocytosis	38%	35, 52, 59
nocodazole	1 μM	endocytosis	23%	69−72, 43, 53
heparinases	5 U	adsortive mechanisms	17%	73
chlorpromazine	15 μM	clathrin-mediated endocytosis	16%	35, 52
dynasore	80 μM	clathrin- and lipid rafts/caveolae-mediated endocytosis	14%	74
potassium depletion		clathrin-mediated endocytosis	→0%	35
nystatin	10 μg/mL	lipid rafts/caveolae	1%	35
cell incubation in serum-free medium		determined by the adsorbed proteins	−26%	

[a] CLSM delivered erroneous results regarding the efficiency of inhibition, due to lack of optical resolution. TEM characterization showed a near-complete loss on internalization upon disruption of the actin cytoskeleton with cytochalasin D (Figures 3.C, 4, and SI-8). However we were unable to do quantification due to the limitation of this technique to process a significant amount of images over time.

Pharmacological/Chemical Inhibition of Endocytic Pathways and Quantification of PEM Capsule Uptake. To date, the most efficient, reliable, and affordable tool for testing endocytosis is chemical inhibition of internalization.[35] Therefore, the endocytic pathways used by PEM capsules were characterized on the basis of their differential sensitivity to pharmacological/chemical inhibitors (Figure 3 and Figure SI-6). However, nonspecific effects of such inhibitors have been reported and thus need to be considered upon data analysis.[35] MDA-MB-231 breast cancer cells were treated with different inhibitors of known internalization pathways (see Table 1 and SI §2 for a precise description of all inhibitors used), and the number of internalized capsules per cell was calculated (Figure 3). Concentrations were chosen in a way that the inhibitors are active, but still not harmful to cells, as determined *via* viability assays (Figure SI-5). From the statistical analysis of capsule uptake (cumulative distribution functions, CDFs), which is available in the Supporting Information, we derived a mean blocking efficiency of each inhibitor. The results of inibition of capsule uptake for PSS/PAH-based capsules and MDA-MB-231 breast cancer cells are summarized in Table 1. Similar results were obtained for pARG/DEXS-based capsules and MDA-MB-231 breast cancer cells and PSS/PAH-based capsules and A549 human lung cancer cells (*cf.* the Supporting Information).

(i) Cell surface heparan sulfate proteoglycan-mediated internalization is ruled by electrostatic interaction between the negatively charged glycocalix and the charged particles.[51] Atomic force microscopy measurements of adhesive forces between capsules and cell membranes showed that the uptake of PEM capsules correlates with the adhesion of capsules to the outer cell membrane.[36] Indeed, treatment with heparinases that hydrolyze proteoglycans[52] showed a 17% decrease in the uptake of PEM capsules (Figures 3.A, 3.B, and SI-6.1). Thus, electrostatic interactions are indeed responsible for an effective cell−capsule interaction by improving capsule attachment to the negatively charged cell surface.

(ii) Cytoskeleton assembly: The intracellular localization and motility of endocytic organelles is linked to microtubules and to actin filaments, which provide molecular motors to sort, transport, and mature the different endocytic/phagocytic vesicles. Mostly all uptake processes, except macropinocytosis and phagocytosis, appear to be enhanced by, but are not strictly dependent on, a functional cytoskeleton. Intact microtubules are required for vesicle maturation.[43,53] Actin and microtubule polymerization around the nascent vesicles (*i.e.*, macropinosomes and phagosomes) is responsible for the formation of large F-actin-coated vesicles. This constitutes a feature of all types of phagocytosis[54] and macropinocytosis. Use of F-actin and microtubule depolymerizing drugs such as cytochalasins and nocodazole, respectively, completely blocks these processes.[55] Nevertheless, F-actin has been lately described to be involved in the plasma membrane rearrangement during processes such as ruffling, migration, and phagocytosis.[43] Furthermore, actin polymerization around the emerging phagosome is a feature of all types of phagocytosis.[54]

Incubation of MDA-MB-231 breast cancer cells with cytochalasin D clearly disturbed the internalization of PEM capsules. Unexpectedly, a reduction of "only" 49% of capsule uptake could be stated by CLSM (Figures 3.A and 3.B, and SI-6.2). However, a closer look at the cell membrane with TEM confirmed a complete abolishment of capsule uptake when actin polymerization was blocked (Figures 3.C, 4, and SI-8). It seems that although attachment to the plasma membrane and an attempt at internalization were visible with the CLSM, the actin

cytoskeleton was not able to reorganize properly and invagination of the vesicles into the cytosolic side of the cells was not completed (Figures 3.C, 4, and SI-8). On the other hand, the cellular uptake of capsules was slightly sensitive (23% reduction of uptake) to microtubule polymerization, as demonstrated upon incubation with nocodazole (Figures 3.A, 3.B, and SI-6.2). The higher sensitivity to cytochalasin compared to nocodazole and the fact that to internalize these kind of capsules, the endocytic vesicles have to be around 2−4 μm in diameter (which is considered large) confirmed the assumption that the phagosome size governs the type of motor system used.[56] Whereas small endocytic vesicles (<1 μm) are transported along the microtubules, bigger endocytic vesicles (*e.g.*, phagosomes) travel along the actin cytoskeleton. These results highlight the utilization of the actin microfilaments to propel the capsule-containing endocytic vesicle and give additional evidence for phagocytosis as a final uptake modus.

(iii) Clathrin- and caveolin/lipid-raft-mediated endocytosis: Certain cellular internalization proteins involved in the clathrin- and caveolin-mediated endocytosis, such as clathrin or EEA1 and caveolin, respectively, did not colocalize with the PEM capsules (Figure SI-4.1). However, capsules did co-localize with lipid rafts and phagocytosis markers such as CTXB and LAMP1 (Figures 2 and SI-4). CTXB binding does not necessarily correlate with caveolae vesicles,[49] thus establishing a difference between caveolin-dependent pathways and lipid rafts. These results suggested an exclusion of clathrin- and caveolin-mediated endocytosis. However, they do not exclude lipid rafts, macropinocytosis, or phagocytosis as uptake pathways. Indeed, PEM capsules were not internalized through clathrin-mediated endocytosis, as chlorpromazine (Figures 3.A and SI-6.4) or potassium depletion (Figure 4), which are specific blockers of this pathway,[35] did not influence their uptake. The caveolin-mediated pathway can be excluded because the pH of the endocytic vesicles was not alkaline[44] (Figure 5). The pH of these vesicles during capsule uptake was measured by tracking pH-sensitive capsules in confocal images.[57] The results indicated that the capsules were in an acidic environment, thus being in accordance with the co-localization studies (Figure SI-4) showing no signs of localization of the capsules with caveolin. Furthermore, treatment of breast cancer cells with dynasore (Figures 3.A, 3.B, and SI-6.4), which inhibits the scission of dynamin-dependent vesicles from the plasma membrane[58] and the induction of clathrin- and lipid raft-dependent and -independent endocytosis had no effect on the uptake of PEM capsules. Taking these results together, it can be concluded that neither clathrin- nor caveolin-mediated endocytosis is participating in the internalization of PEM capsules. However, the results do not provide a profound statement about the involvement of lipid rafts and do not exclude macropinocytosis or phagocytosis.

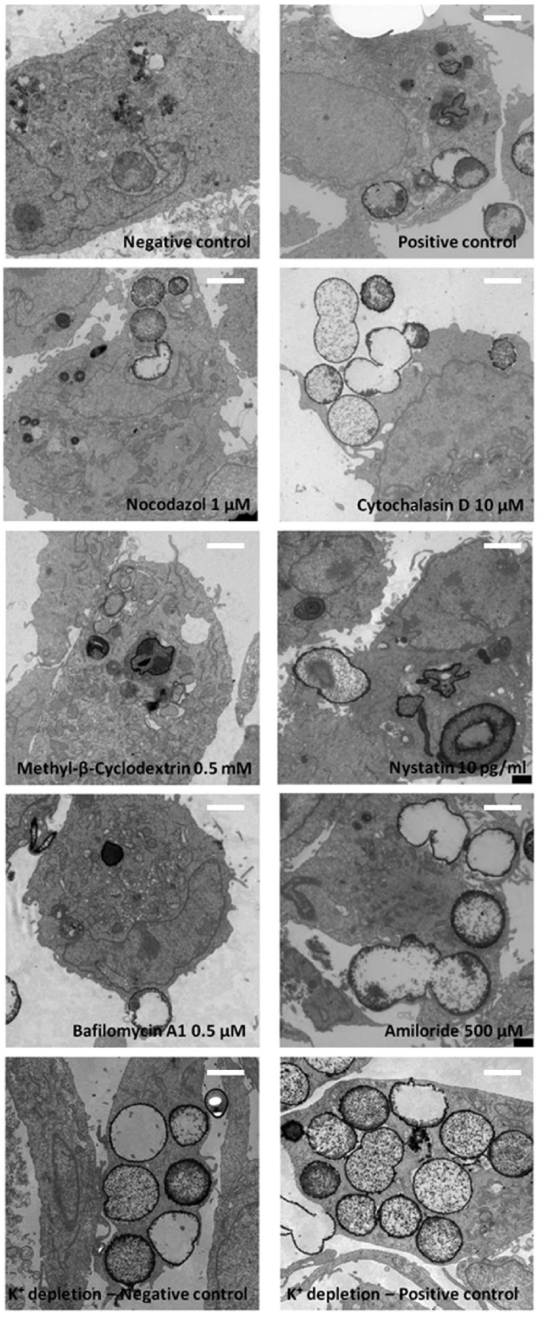

Figure 4. TEM characterization of the internalization of gold nanoparticle-modified PSS/PAH capsules by MDA-MB-231 breast cancer cells in the presence and absence (control) of the different pharmacological/chemical inhibitors of different cellular processes that have been associated with specific pathways. Negative and positive controls are cells treated without and with capsules, respectively. The length of the scale bar is 3 μm.

Lipid-raft-mediated endocytosis (which may be dependent or independent of caveolae) as well as macropinocytosis, which has been described as a sort of lipid raft-dependent and receptor-independent form of endocytosis,[59] are sensitive to drugs that alter the structure of cholesterol-rich plasma membrane domains.[60] These drugs include methyl-beta-cyclodextrin (MβCD) and nystatin, which respectively deplete and sequestrate

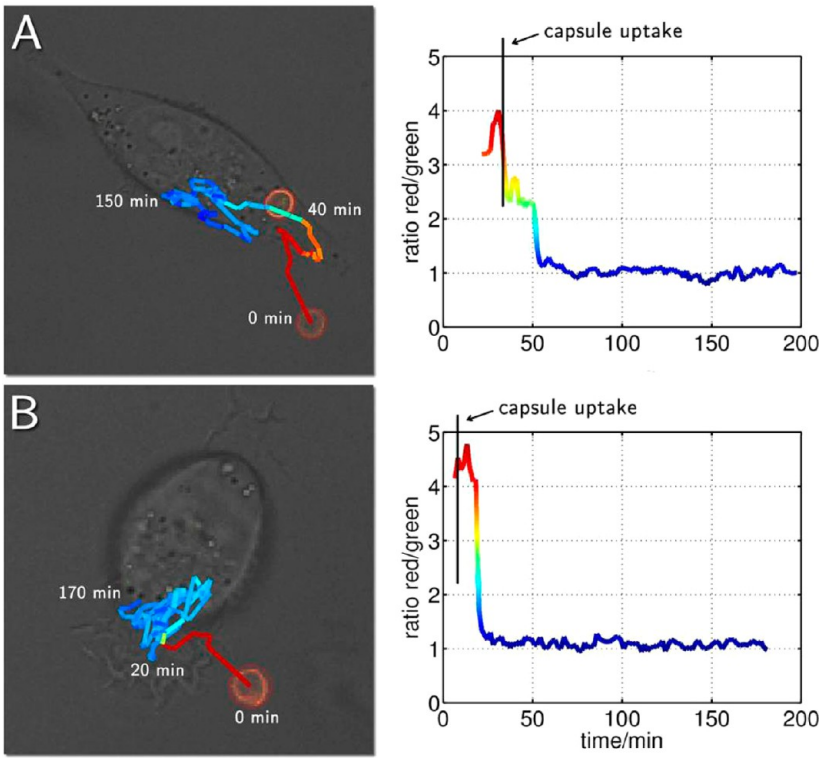

Figure 5. The acidification of endocytic vesicles of MDA-MB-231 breast cancer cells was measured by tracking pH-sensitive, SNARF-filled PSS/PAH capsules in confocal images. The intensity ratio of the two emissions of each capsule can be used to estimate the local pH around the capsule (depicted in red ≈ pH 7 to blue ≈ pH 5 in the graphs).

cholesterol from the plasma membrane of the cells. Surprisingly, only cholesterol depletion by treatment with MβCD induces an almost complete (93%) loss of capsule internalization (Figures 3.A, 3.B, 4, and SI-6.3), whereas cholesterol sequestration evoked by nystatin had no effect on the internalization of the capsules (Figures 3.A, 3.B, 4, and SI-6.3). These results demonstrate a crucial role of cholesterol in the packing of the plasma membrane to engulf the capsules. However, they do not distinguish between lipid rafts and the related macropinocytosis. In summary we have observed that PEM capsules co-localize with lipid rafts (marked with CTXB), and the uptake is strongly sensitive to MβCD, which sequestrates cholesterol from the plasma membrane and has a significant effect on the cytoskeleton, especially on the dispersion of a cortical F-actin.[61] However, it is not sensitive to dynasore or potassium depletion. Furthermore, the size of the lipid rafts is normally significantly smaller than the size of the capsule. Gathering up the data concerning the role of lipid rafts and macropinocytosis in the uptake of capsules, it can be concluded that lipid-raft-mediated macropinocytosis, but not lipid rafts, plays an important role in the uptake of PEM capsules at the level of capsule invagination to the cytosolic side.

(iv) Phagocytosis *vs* macropinocytosis: These forms of cellular entry lack distinctive pharmacological tools to be distinguished. As already introduced, they can be differentiated from other forms of endocytosis due to the formation of large, outward vesicles (phagosomes and macropinosomes). The formed vacuoles are plasma membrane derivates and lack specific proteins/lipids or coatings. These vesicles undergo a series of fusion events with other intracellular vesicles, thus changing their composition upon maturation. Due to the lack of pharmacological targets, it is very difficult to selectively distinguish one from another. Alteration of the intracellular/intravacuolar acidification with selective drugs is the best choice to study macropinocytosis and phagocytosis because these drugs have the fewest collateral effects on other pathways.[35] Amiloride inhibits the sodium−proton (Na^+/H^+) exchange and causes strong cytosolic acidification. As a result, amiloride is commonly used to inhibit (macro)pinocytosis.[52,62] However, its specificity is limited, as amiloride derivatives have been reported to also induce actin reorganization.[35] Bafilomycin A_1 is a strong inhibitor of the vacuolar type H^+-ATPase (V-ATPase), which is responsible for the acidity of lysosomes, the biogenesis of phagolysosomes and for the degradation of endocytosed material.[63] During phagosome biogenesis a proper lysosomal acidification is required, thus making phagocytosis sensitive to this antibiotic.[55,64,65]

As seen in Figures 3.A, 3.B, and SI-6.5, treatment of breast cancer cells with bafilomycin A_1 exhibited a significantly strong reduction (84%) of PEM capsule uptake, whereas incubation with amiloride moderately (38%) decreased their uptake. Treatment with concanamycin A, an analogue of bafilomycin A_1, showed the

same effect on diminishing the capsule uptake. These results were confirmed qualitatively with TEM (Figures 4 and SI-8).

The reduction of internalization mediated by amiloride together with the results obtained previously (lipid raft co-localization and MβCD sensitivity) confirms the role of lipid-raft-mediated macropinocytosis as stated before. However, the almost complete reduction of capsule uptake obtained by disrupting the acidification and biogenesis of the phagolysosome after bafilomycin A_1 treatment strongly involves phagocytosis at the internalization stage of capsule sorting. This is also supported by the co-localization of capsules with LAMP1, the strong sensitivity of uptake to cytochalasin D, and the formation of large phagocytic cups observed before.

(v) Disruption of plasma membrane fluidity: When MDA-MB-231 breast cancer cells were cooled from 37 °C to 4 °C, there was a complete inhibition (100%) of the internalization of the capsules (Figures 3.A, 3.B, and SI-6.6). At low temperatures the plasma membrane loses fluidity and increases rigidity; that is, the rapid lateral and slow axial movement of proteins and lipids is impaired. These movements are essential, for example, for receptor-mediated endocytosis. However, an increase in the plasma membrane rigidity also has a negative effect on the formation of protrusions that engulf the capsules. This kind of treatment served additionally as a control for achieving a complete uptake inhibition.

(vi) Inhibition of the opsonization of the capsules by pretreating the MDA-MB-231 breast cancer cells without nutrients (preincubation in serum-free medium) previous to and during the cell treatment with capsules significantly increased the internalization of the capsules compared to control cells (Figures 3.A, 3.B, and SI-6.7). These results give evidence that the mechanism of internalization of the capsules might not be determined by the serum proteins (mostly receptor-mediated[66]) that could adhere to the surface of bare capsules. These results help to relate the internalization patterns of the capsules with their physicochemical properties. Thus, providing the capsules with a surface chemistry that blocks protein coating might help to predict the behavior of these capsules. Additionally, by avoiding capsule−serum protein interaction, the charge density of the capsules is maintained and the electrostatic interaction with the negatively charged glycocalix of the cells is preserved. Thus, the important role of an adsorptive mechanism of internalization to stabilize the capsules at the cell membrane previous to internalization is confirmed.

DISCUSSION

The results presented provide a more detailed analysis compared to other publications that aim only at a single pathway.[10,14,33,66] Previous reports focused on collateral studies concerning either the co-localization of PEM capsules with one or a few markers of endocytic vesicles, or inhibited only one pathway. In the frame of this work, several markers and inhibitors of mostly all uptake pathways were studied *via* optical and electron microscopy. The internalization of PEM capsules was quantified with several approaches.

Within the frame of this systematic study it can be concluded that different internalization modes are activated to incorporate PSS/PAH-based PEM capsules by MDA-MB-231 breast cancer cells. Our results show (i) an adsorptive mechanism responsible for the primary cell−capsule contact due to electrostatic interactions between the capsules and the negatively charged glycocalix; (ii) the formation of filopodia and a strong actin reorganization to form large protrusions (in the form of a cup) to engulf the capsules similar to phagocytic processes; (iii) a co-localization with lipid rafts and phagolysosomes, but not with caveolin, clathrin, or ER; (iv) dynamin-independent internalization; (v) the absence of inhibition by nystatin, chlorpromazin, and potassium depletion; and (vi) the inhibition of uptake by cytochalasin D, MβCD, bafilomycin A_1 and its analogue, concanamycin A, and to a lesser extent amiloride and nocodazole.

On one hand, we have presented evidence, in accordance with other publications,[14] that discards clathrin-mediated endocytosis and assesses the involvement of lipid rafts in the uptake of PEM capsules. However, our results indicate that the involvement of lipid rafts contributes to a more complex pathway rather than becoming solely responsible for it. Due to the large size of the capsules and the small size of clathrin or caveolin/lipid raft vesicles,[40−42] it was expected that these processes were not involved in the direct uptake of capsules. We could easily exclude associated internalization routes. Nevertheless, since previous works showed that 300 nm to 1 μm, soft, hydrogel, silica capsules were internalized *via* clathrin-mediated endocytosis,[37] we wanted to test if this mechanism was involved in the uptake of bigger, more rigid capsules.

According to the results, there was a lack of co-localization of the capsules with characteristic markers for clathrin-mediated endocytosis (EEA1, clathrin), and the uptake was insensitive to all pharmacological/chemical inhibitors used for this pathway (chlorpromazin, dynasore, and potassium depletion).

Interestingly, regarding lipid rafts, our results excluded caveolin-mediated endocytosis but not caveolin-independent lipid rafts. First, there was not a co-localization of the capsules with the caveosomes (stained by caveolin). Second, caveosomes are transported within the cell along microtubules, and inhibition of microtubule formation with nocodazole had little effect on the internalization of the capsules. Finally, caveosomes have a different pH (neutral) and a different content compared to endosomes (acid) and do not end up fusing with lysosomes.[75] In contrast,

the vesicles involved in the internalization of the capsules are clearly acidic[11,57] and possess lysosomal markers (LAMP1).[48] As seen in Figure 5, the pH of the internalized vesicles is acidic from almost the beginning of the uptake process. Lipid rafts are commonly stabilized with caveolin. If caveolin-mediated pathways were involved, the internalizing vesicles should show, at least initially, a more neutral pH proper for the caveosomes. The steady decline in the luminal pH of the vesicles transporting the capsules is associated with phagosome maturation, and therefore this result represents a sign of phagocytosis.[50] Additionally, it is well known that the surface area of phagosomes can be increased by replenishing the plasma membrane with endosomal, ER, and post-Golgi membranes.[76−78] In Figure 2.B, a large phagolysosome (stained by LAMP1) containing approximately four capsules is clearly visible, thus confirming the ability of these cells to increase the surface area of phagolysosomes to engulf large objects. Proteomics and biochemical analysis have demonstrated that several proteins from the ER (*e.g.*, calnexin) are present in the membrane of phagolysosomes or phagosomes during their biogenesis.[65,79,80] Furthermore, Desjardins *et al*. proposed an ER-mediated phagocytosis where ER is recruited to the plasma membrane to supply membrane for the formation of the phagocytic cup.[65] This would explain the drastic increase in the size of phagolysosomes upon capsule uptake observed in Figure 2.B, where a vesicle containing several capsules can be clearly seen. Although our results showed few signs of co-localization of the ER (labeled by calnexin) with the capsules after 4 h (Figure SI-4.1.F), we observed a co-localization with the plasma membrane (stained with wheat germ agglutinin) (Figure SI-4.1.F). This gives an indication of a possible recruitment of the ER to the plasma membrane. The lack of co-localization with the ER can be explained either because the ER is not involved in this kind of internalization pathway (ER-independent phagocytosis)[48] or because of the (late) time frame selected for microscopic visualization (*i.e.*, 4 h). Calnexin is a protein present in early forming phagosomes at early stages of phagocytosis, such as the phagocytic cup, and suffers fast (after 1 h) degradation upon phagosome maturation.[65] During the biogenesis of phagocytic structures, the decrease in calnexin correlates with LAMP1 accumulation,[65] which indeed confirmed our results.

Concerning the caveolin-independent lipid rafts, macropinocytosis-mediated lipid rafts play an important role at an early stage of the capsule internalization process after the formation of the phagocytic cup, *i.e.*, at the level of invagination of endocytic vesicles containing the capsules to the cytosolic side of the cell. This is supported by the co-localization of the capsules with lipid rafts (CTXB) and the sensitivity of uptake to amiloride (a Na^+/H^+ pump blocker related to macropinocytosis[59]) but not to dynasore (an inhibitor influencing the lipid rafts), but also by the size of lipid rafts compared to the capsules and to the initial formation of a phagocytic cup, which strongly depends on the actin cytoskeleton. Furthermore, the uptake of the capsules after 4 h was completely inhibited by MβCD, which removes cholesterol from cultured cells, thus inhibiting membrane packing at a first stage but also uptake-associated processes such as macropinocytosis and lipid rafts.

Cellular incubation with bafilomycin A_1 and its analogue concanamycin A disturbed the capsule uptake in a concentration-dependent manner. Both are specific inhibitors of the vacuolar-type H^+-ATPase, which prevents reacidification of the lysosome,[11] which is required for the maturation of the phagosomes. Treatment with amiloride (which also affects acidification) had a less significant effect on the uptake of PEM capsules compared to MβCD or bafilomycin A_1.

These results, together with the co-localization studies that demonstrate a primary localization in plasma membrane invaginations (enriched in cholesterol and glycosphingolipids) and a final localization in phagolysosomes located in the perinuclear region, suggest a multistep mechanism of PEM capsule internalization. This mechanism combines lipid-raft-mediated macropinocytosis and phagocytosis. Such a synergism has already been described for the entry of the bacterium *Mycobacterium tuberculosis* and the HIV virus.[81] Furthermore, a possible role of lipid rafts in the phagolysosome maturation[80] cannot be excluded, as co-localization of CTXB with LAMP1 was observed in the presence of the capsules (Figure SI-4.2).

Obviously, the generality of results raises the question of whether the results are limited to one type of capsules (PSS/PAH-based) and one type of cell line (MDA-MB-231) as used here. The polyelectrolytes PSS/PAH have been chosen as a prototype, as these materials are synthetic and thus nondegradable, and most capsule-based studies in the literature are related to this system. There is no special feature based on which MDA-MB-231 have been chosen, besides the fact that several of our previous studies were based on these cells. We repeated some of the inhibition experiments for two other configurations. In the first set we used the same cell type (*i.e.*, MDA-MB-231) but incubated them with capsules produced with different polyelectrolytes (pARG/DEXS) and, thus, different surface chemistries. The pARG/DEXS system is also known to be a prototype of biodegradable capsules. In the second set of experiments, we took a different cell line (A549 human lung cancer cells) and incubated them with the same capsules (*i.e.*, PSS/PAH). As can be seen in the data presented in the Supporting Information (§7), the results are highly similar, which suggests that the results obtained in this work are not limited to the special capsule−cell configuration but rather are valid in a broader context. It has to

be pointed out that for the work presented here no functionalization of the capsule surface with active (*e.g.*, targeting) molecules such as antibodies, PEG, and peptides was performed, and thus capsule uptake has to be considered nonspecific. Therefore, we cannot exclude that the internalization pathway might be altered in the case where an active modification of the surface of the capsules is performed.

Summarizing, PEM capsule entry begins with the formation of a phagocytic cup, followed by a lipid-raft-mediated macropinocytosis and a final activation of the phagocytic machinery to sort PEM capsules in the heterophagolysosomes.

CONCLUSIONS

The aim of this research article was to elucidate and to fully describe the role of all endocytic pathways in the uptake patterns of PEM capsules. The comprehensiveness of this work is based on using different methods, *i.e.*, morphological characterization and pharmacological/chemical inhibition of capsule internalization and capsule−organelle co-localization. Data suggest that multiple internalization pathways are involved at different stages of PEM capsule uptake. First of all, adsorptive mechanisms due to the electrostatic interactions induce a nonspecific, initial interaction of the capsules with the cells. Second, strong actin reorganization and filopodia formation occur to form a phagocytic cup (proper for phagocytosis[65]) to stabilize the capsules at the plasma membrane. At the early stage of engulfment, the capsules co-localize with the lipid rafts and require cholesterol-rich lipid domains, which are responsible for a proper cytosolic invagination. Due to the capsule size being larger than lipid rafts, the independence on an active dynamin, and the acidity of the internalizing vesicles (which exclude caveolin-mediated endocytosis), lipid-raft-mediated macropinocytosis plays a decisive role at this early stage of internalization. Clathrin-mediated endocytosis can be excluded due to the insensitivity of capsule uptake to chlorpromazine as well as to K^+-depletion and to dynasore. The absence of co-localization with calnexin, a marker for early, new-forming phagosomes, suggests that phagocytosis is not involved at this early stage of endocytosis. Upon cytosolic invagination, the PEM capsules are transported to the perinuclear cytoplasma of the cells within acidic vesicles. The transmembrane signaling cascade that might be activated remains unclear. However, a local membrane-induced polymerization of actin on the cell surface, as well as the requirement of vesicle acidification and the strong co-localization with LAMP1 is typical at all stages of phagolysosome maturation.[79,82] Consequently, in the last step of capsule uptake the phagocytic machinery is activated to transport PEM capsules until they reach their final location, the heterophagolysosomes.

METHODS

For more details regarding the experimental section and additional results, the reader is referred to the Supporting Information.

Synthesis of Fluorescent Gold Nanoparticle-Functionalized PEM capsules. The synthesis (SI §1) of poly(sodium 4-styrenesulfonate) and poly(allylamine hydrochloride) (PSS/PAH) and of poly-L-arginine and dextran sulfate (pARG/DEXS)[13] capsules functionalized with gold nanoparticles and fluorescein isothiocyanate (FITC) was carried out following our previous protocols.[83] Gold nanoparticles and FITC were used for visualization with the electron microscope and with the optical microscope, respectively.

Cell Culture and Treatment. MDA-MB-231 human breast adenocarcinoma cells and A549 human lung cancer cells (see SI §2) were seeded and grown overnight in growth medium (DMEM supplemented with 10% fetal bovine serum, insulin, and 1% penicillin/streptomycin). PEM capsules were added at a concentration of 20 capsules per cell and incubated for 4 h, at 37 °C and 5% CO_2. In the case of incubation with the different pharmacological/chemical inhibitors (see SI §2), the inhibitors were diluted in medium at concentrations (given in Table 1) that were determined to be nontoxic to the cells by cytotoxicity assays (see §5 and Figure SI-5 of the Supporting Information). Control cells were treated with medium only, instead of inhibitor solution. The inhibitors were added 1 h previous to capsule incubation and were maintained for 4 h.

In the case of potassium depletion, the cells were first incubated with a hypotonic medium (EMEM/H_2O, 1:1) for 5 min at 37 °C, then with isotonic medium A (100 mM NaCl in 50 mM HEPES pH 7.4) for 10 min at 37 °C, and finally with isotonic medium B (100 mM NaCl, 1 mM $CaCl_2$, 2.5% w/v BSA in 50 mM HEPES pH 7.4) for 30 min at 37 °C. Control cells were incubated with both isotonic media (A and B), supplemented with 10 mM KCl in order to avoid K^+-ion exchange.

Immunofluorescence. MDA-MB-231 cells were fixed and permeabilized previous to incubation with the primary and the secondary antibodies (see Table 1 in the SI, §2) and to the staining of the cytoskeleton with phalloidin/tetramethylrhodamine and of the cells' nuclei with DAPI (4′,6-diamidino-2-phenylindole). If required, staining of the cell membrane with wheat germ agglutinin/tetramethylrhodamine was performed just before permeabilization. Finally, the cells were mounted on slides using Flouromount-G.

Confocal Laser Scanning Microscopy. To visualize the capsules and different stained cellular structures, a CLSM 510 Meta (Zeiss) microscope was used. It was equipped with a laser diode emitting at 405 nm, an argon laser with a line at 488 nm, and a helium−neon laser for excitation at 543 and 633 nm, respectively. Images were taken with a Plan-Apochromat 63×/1.40 oil DIC M27 objective, and the pinhole was set to 0.86−1.32 airy units.

Tracking the pH of the Medium Surrounding Capsules to Establish the Acidification of Endocytic Vesicles Involved in the Uptake of PEM Capsules. The acidification of endocytic vesicles of MDA-MB-231 cells was measured by tracking pH-sensitive, SNARF-filled PEM capsules in confocal images. The emission of SNARF (excitation: 488 and 543 nm) was recorded at 550−615 and 615−700 nm. The ratio of both emission intensities was plotted against the time.

Ultrastructural Analysis *via* TEM. For transmission electron microscopy, MDA-MB-231 cells were treated according to Ito and Karnovsky.[84] Postfixation was performed in 1% osmium tetroxide for 1 h at room temperature followed by an overnight incubation with 0.3% uranyl acetate dissolved in 50 mM maleate buffer (pH 5.0). Samples were embedded in Epon according to standard procedures. Thin sections were contrasted with lead citrate and examined with a Zeiss EM 109S electron microscope equipped with a TRS wide-angle dual-speed CCD camera.

TABLE 2. Illustration of the Different Cellular Structures Used to Study Their Co-localization with the PSS/PAH Capsules[a]

cellular structure	target/*dye*	co-localization	endocytic pathway
endosomes	EEA1	no	clathrin- and caveolin-(in)dependent endocytosis
clathrin-coated vesicles	clathrin	no	clathrin-mediated endocytosis
caveolin-coated lipid rafts	caveolin 1	no	caveolin-mediated lipid rafts
lipid rafts	CTXB	yes	lipid rafts
endoplasmic reticulum (ER)	calnexin	no	ER-mediated phagocytosis
phagolysosomes	LAMP1[b]	yes	phagocytosis
cell membrane	*WGA*	*n/a*	*n/a*
actin cytoskeleton	*phalloidin*	*n/a*	*n/a*
nucleus	*DAPI*	*n/a*	*n/a*

[a] Their associated pathways of entry and the degree of co-localization with the capsules observed *via* CLSM after 4 h incubation with MDA-MB-231 cells are also presented. The staining of structures (italicized text), such as the cell membrane or the actin cytoskeleton and the nucleus, were done to help quantify the number of internalized capsules and to facilitate imaging, respectively. AF: Alexa fluor. EEA1: early endosome antigen 1. CTXB: cholera toxin B1. LAMP1: lysosomal-associated membrane protein 1. WGA: wheat germ agglutinin. [b] The hybridoma antibody LAMP1 developed by J. Thomas August and James E. K. Hildreth was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242, USA.

Quantification of the Inhibition of Capsule Uptake. The cellular uptake of capsules was quantified by manual CLSM image analysis. All images were acquired and evaluated as *z*-stacks. Capsules surrounded by lysosomal-associated membrane protein 1 were always classified as intracellular. Capsules clearly surrounded by actin were classified as intracellular, but were distinguished from capsules that were only located between densely grown cells. Also, visible deformation of capsules, which can be frequently observed after capsule uptake (once they are already located in the phagolysosome), was considered as an indication of intracellular localization. Dividing and not fully depicted cells were not taken into account. Please note that we purposely decided against flow cytometry, although this would have allowed for a higher number of investigated cells. With flow cytometry it is not straightforward to distinguish between capsules just adherent to the outer cell membrane and actually internalized capsules,[85] whereas this was easily possible with CLSM with the above-described criteria.

Following the scheme for CLSM detection of capsules, the number of intracellular capsules was individually determined for each cell. For every inhibitor concentration, two or three independent experiments with at least 100 cells analyzed per experiment were conducted. To check for classification bias, parts of the image analysis were performed as single blinded analyses.

Instead of plotting the number of internalized capsules per cell in the format of a histogram, data are presented as cumulative distribution functions (see SI §6). The CDF $0 \leq p(N_{in}) \leq 100\%$ represents the probability of a cell having 0 to N_{in} capsules internalized. As an example, $p(2) = 0.87$ signifies that 87% of the analyzed cells do not have more than two capsules internalized (*i.e.*, either 0, 1, or 2 capsules per cell). CDF plots for at least three independent experiments were arithmetically averaged directly without weighting and merged into one diagram. The standard deviation was calculated from the deviation of the independent CDFs of the three different experiments per each data set.

For the sake of clarity and for a better comparison of the results obtained for all inhibitors, the total efficiency of inhibition was presented. To address the total efficiency E_T of inhibition with one single value, the area A_I lined by the CDF $I(N)$ for inhibited cells and the line $p = 1$ was determined and divided by the area A_C enclosed by the graph of the control $C(N)$ and $p = 1$. Finally, the effectiveness of one inhibitor was calculated as $E_T = 1 - A_I/A_C$.

Conflict of Interest: The authors declare no competing financial interest.

Supporting Information Available: A detailed description of the methods followed in this work and additional results are available free of charge *via* the Internet at http://pubs.acs.org.

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