

Optical ratiometric silica pH sensors unveil active transport and subcellular particle localization in protoplasts

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ABSTRACT: Understanding the interaction mechanisms of engineered nanomaterials (ENMs) with plant membranes is crucial for their effective use in various applications. While passive transport of smaller ENMs is well-documented, the mechanisms underlying active transport of larger ENMs remain poorly understood. This study systematically investigates the active transport and subcellular distribution of ENMs (100–1000 nm) within protoplasts using optical ratiometric silica pH sensors for localization. Highly monodispersed ratiometric pH sensors, based on silica particles modified with fluorescein-5-isothiocyanate (FITC) and cyanine3 NHS ester (CY3) dyes, were employed to elucidate internalization mechanisms. Protoplasts from *Nicotiana tabacum* L. leaves successfully internalized the sensors. 3D segmentation of protoplasts revealed distinct pH gradients, indicating vacuole accumulation. Colocalization analysis and cellular compartments staining further confirmed sensor distribution. High-throughput imaging flow cytometry showed efficient internalization rates, which decreased after cell wall regeneration. Notably, inhibition experiments with the salicylic acid (SA) and Tyrphostin A23 (TyrA23) inhibitors confirmed clathrin-mediated endocytosis in particle uptake. This study establishes rational design principles for controlling active ENM uptake and subcellular localization via optical pH sensing in protoplasts. The findings enhance our understanding of plant cell trafficking mechanisms and hold promise for targeted delivery and applications in plant biology research.

KEYWORDS: optical ratiometric pH-sensors, intracellular pH tracking, protoplast, endocytic pathway, computational analysis

1 Introduction

Engineered nanomaterials (ENMs) are a class of materials in the nanoscale that exhibit exclusive properties compared to bulk materials [1]. ENMs can be manipulated in terms of dimensions,

shapes, sizes, compositions, porosity, phases, and uniformity to bring new solutions in the healthcare, agriculture, energetic, environmental and food field among all [1]. They have proved to successfully penetrate both mammalian and plant biological membranes. Therefore, ENMs have been massively employed as biomolecular cargo nanocarriers in mammalian and lately into plant cells thanks to their advantageous features. Indeed, ENMs are characterized by high performance, low cytotoxicity, efficient cargo shielding from enzymatic degradation within biological environments [2, 3], high tunability that allows them to be targeted to specific organelles or tissues [4] and compatibility to a wide range of plant species [5, 6].

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ENMs are internalized into mammalian cells across a cell membrane mainly via active transport (i.e. endocytosis, phagocytosis or pinocytosis) [7]. However, ENMs uptake into plants is challenging due to the presence of the plant cell wall, a dynamic bio-physical barrier that causes a significantly lower endocytic rate than that observed in animal cells [8, 9].

A study conducted on protoplasts isolated from *Arabidopsis thaliana* L. leaves reports the mechanisms that regulate the passive transport of ENMs with different shapes, charge and dimension in the range of the natural membranes pore size (5–20 nm) [10], elucidating that the uptake and the subcellular distribution of ENMs in protoplasts is not only dictated by the particle size but also by the magnitude, but not the sign, of the zeta potential.

On the other hand, little is known about the mechanisms that regulate the active transport of bigger ENMs (≥ 100 nm) into protoplasts where the internalization occurs through endocytosis in which the ENMs are surrounded by the plasma membrane phospholipid bilayer portions in the form of a cavity-like structure, before being endocytosed [11].

In this work, we have used protoplasts prepared from *Nicotiana tabacum* L. leaves to investigate the internalization of ENMs in the range 100–1000 nm since it provides a model system for gene transformation studies [12, 13] and for the scaling up from the laboratory to the field. On top of that, tobacco is tightly related to major economic crops such as eggplant, pepper, potato and tomato, and itself has relevant commercial applications [14].

We selected silica-based ENMs since the earth's crust is mainly composed of silicates. Indeed, some higher plants produce the so-called “silica bodies”, which are opal phytoliths, when soluble silica present in the soil is taken up by the roots and translocated to different organs of the plant through the vascular system [15]. Silicon does not represent an essential element in plant growth and development, however, numerous studies demonstrate that silicon improves plant physiology [16] and ameliorates plants vigor [17]. Moreover, experimental evidences attest that silicon plays an important role when plants experiment exogenous abiotic [18] and biotic stress conditions [19]. Furthermore, SiO_2 storage plays a role as physical and mechanical protection in plants [20] and it also enhances plants growth, total protein content and photosynthesis without altering the activity of antioxidant enzymes [21]. Silica-based ENMs can be produced in different shapes, sizes and wettability thanks to their simple, customizable and low-cost synthesis. We exploited the Stöber process for the rational synthesis of different silica particles in the dimensional range of interest of 100–1000 nm since it represents a convenient, one-step process that allows to produce microscopic particles of colloidal silica with diameters ranging from 50 to 10,000 nm with a fairly uniform size distribution simply tuning the conditions of the reaction such as the concentration and type of reactants, catalysts and electrolytes employed [22]. Then, we functionalized the plain silica particles with fluorophores that allowed us to both tune their superficial charge for facilitating their internalization, and to optically monitor pH changes by means of confocal imaging. This approach provides a non-invasive method for measuring the *in vivo* pH of specific subcellular compartments [23]. It is known that in *Arabidopsis* protoplasts, the matrices of subcellular organelles in plants have different pH values, starting from the pH value of the cytoplasm (~ 7.3) to the lowest value measured in the vacuole (~ 5.2), with decreasing intermediate values for the organelles of the secretion pathway (endoplasmatic reticulum (ER) ~ 7.1 , Golgi ~ 6.8 and trans Golgi network (TGN) ~ 6.3) [24]. pH homeostasis in the different

organelles is essential for their morphology and function. Moreover, extra- and intra-cellular pH regulation are essential for cell viability, thus, the detection of impaired pH from the expected physiological values could allow to diagnose plant genome anomalies [25] or the effect of abiotic [26] or biotic [27] stresses. Thanks to these experimental approaches, we demonstrated that ENMs in the range 350–650 nm can be internalized via active uptake into viable protoplasts, we clarified their subsequent subcellular distribution exploiting the optical ratiometric pH tracking. Furthermore, we confirmed the distribution of the silica-based pH sensors into the *Nicotiana tabacum* L. protoplasts by performing experiments of co-localization through the staining of different subcellular compartments of the plant cell. Lastly, through the use of specific inhibitors coupled with flow cytometry measurements, we demonstrated that clathrin-dependent mechanisms are involved in the uptake of ENMs into protoplasts (Fig. S21 in the Electronic Supplementary Material (ESM)). Our work represents one of the first study that highlights the mechanism of active ENMs transport into protoplasts according to the material nature, dimension and superficial charge, which differs from ENMs uptake mechanisms stated for mammalian cells.

2 Results and discussion

2.1 Syntheses of the pH sensing silica particles

In order to assemble ratiometric optical pH sensing particles, we employed a simple bottom-up one pot batch-process. We started from synthesizing monodisperse non-porous solid silica seed particles with selected diameters in the micrometric range by using the hydrolysis of tetraethyl orthosilicate (TEOS) for the sol-gel transition, subsequently we functionalized them as ormosil (organically modified silica), with the thiourea obtained from the reaction between (3-Aminopropyl)triethoxysilane (APTES) and fluorescein-5-isothiocyanate (FITC) and the amide obtained from the reaction between APTES and cyanine3 NHS Ester (succinimidyl ester) (CY3) as shown in Fig. 1.

The production of monodisperse non-porous solid silica seed particles were optimized adopting the response surface methodology (RSM) and were designed considering previously proposed nucleation and growth mechanism of silica particles [28]. In particular, they were carried out in a fixed ethanoic solution, under low concentration of TEOS, to generate a small number of nucleation points in the early stage of the reaction and high ionic strength in the reactant solution to increase the final particle size since it promoted particle coagulation in early stage of the reaction [29, 30]. KCl was selected as electrolyte, as previews batch-process experiments indicated that different cationic species made crucial effects on the size distributions, in particular, the order of the silica particles size formed in the presence of electrolytes was $\text{LiCl} < \text{NaCl} < \text{KCl}$. Moreover, KCl was solubilized in a small, fixed volume of water since experimental evidences assessed that the average size for the KCl addition remarkably increased with the decrease in the water concentration. This might be associated to a decrease in hydration number of K^+ ions with the decrease in the water concentration, which destabilized the K^+ ions in the solution and promoted their adsorption onto the silica particles [31]. Finally, the reaction was catalyzed via basic hydrolysis adopting different concentrations of NH_4OH without the addition of a porogen [32] to keep the silica particles non porous. The selected protocols allowed to produce monodispersed silica seeds of 0.3, 0.6, 0.9 μm of

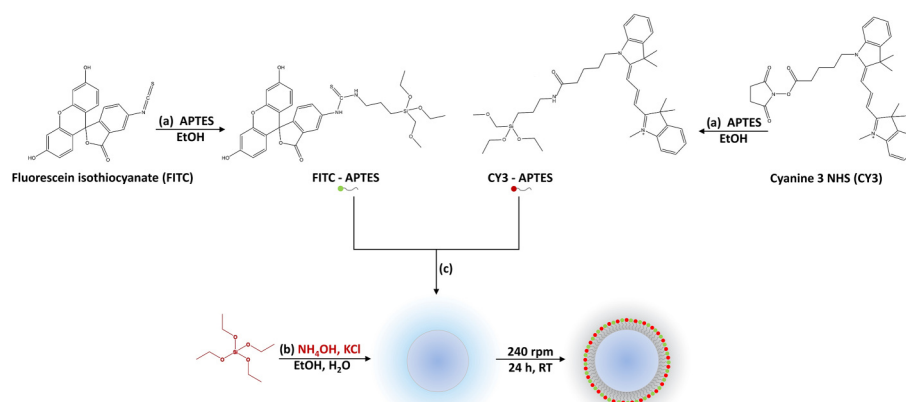


Figure 1 Rational synthesis of FITC-CY3 pH sensing silica particles: (a) FITC sensing dye and CY3 reference dye coupling to APTES linker; (b) silica core particles formation in basic environment and in presence of KCl salt starting from TEOS as monomer; (c) silanization of the silica core particles with FITC-APTES thiourea and CY3-APTES amide.

diameter, respectively, that were further silanized, adopting the same protocol, to assemble ratiometric optical pH sensing particles.

The chosen synthetic route offers several advantages, since it is carried out under mild conditions, requires few purification steps and can be easily scaled up, resulting in affordable and straightforward.

2.2 Morphological characterization of the pH sensing silica particles

Diameter, superficial charge and number per volume of FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm pH sensing particles were measured by scanning electron microscopy (SEM), dynamic light scattering (DLS) and flow cytometry. As reported in Fig. 2(a), the average dried diameters of FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm were 364.23 ± 27.49 , 662.48 ± 82.15 and 960.22 ± 79.98 nm, while their hydrodynamic diameters and polydispersity index (PDI) were 583 ± 16 (PDI 0.14), 1151 ± 55 (PDI 0.19), and 1857 ± 169 (PDI 0.08), respectively. The hydrodynamic diameter evaluated via DLS appeared approximately double of the size than those evaluated in dry condition via SEM analysis for each type of pH sensing particle. Nevertheless, such difference has to be attributed to the dynamic coordination of water molecules around the assembled probes that are colloidal silicas in suspension [33–35].

The apparent zeta potential of FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm pH sensing particles ascertained by DLS were in order -24.7 ± 0.73 , -20.7 ± 0.25 and -69.4 ± 0.87 mV. Superficial charge measurements revealed satisfactory silica functionalization and FITC/CY3 cargo for all probes that were characterized by high negative charge compared to that of plain SiO_2 and a switch to positive zeta potential values after the poly(allylamine hydrochloride) (PAH) layer deposition (Fig. S19 in the ESM). Moreover, the absolute value of the charge resulted > 20 , thus, it might also be beneficial in facilitating probes penetration and subcellular distribution into protoplasts derived from *Nicotiana tabacum* L. leaves since not only the size but also the magnitude of the charge dictate their internalization [10].

Water contact angles (WCA) measurements revealed all particles are hydrophilic with $\theta \leq 90^\circ$. Plain SiO_2 resulted more hydrophilic than FITC-CY3 sensors before and after PAH treatment except for FITC-CY3 0.9 μm before and after PAH treatment (Fig. S19 in the ESM).

The concentration of FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and

FITC-CY3 0.9 μm pH sensing particles ascertained via flow cytometry analysis was inversely related to the sensors' dimension and resulted 7935 ± 557 particles/ μL (396,750 particles/mg), 6097 ± 1256 particles/ μL (304,850 particles/mg) and 4902 ± 1114 particles/ μL (245,100 particles/mg), respectively.

The morphology was assessed via SEM analyses and FITC-CY3 0.3 μm , FITC-CY3 0.6 μm , and FITC-CY3 0.9 μm pH sensing particles micrographs at different magnifications are reported in Figs. 2(b) and 2(e), 2(c) and 2(f), and 2(d) and 2(g), respectively. Examples of SEM images segmentation acquired at 30,000 $\times$, 50,000 $\times$, 100,000 $\times$ magnifications and the relative descriptive statistics are reported in Fig. S1 and Table S1 in the ESM. Notably, SEM analyses, together with the average particle diameters, revealed morphological similarities among the different functionalized silica particles, since all probes were assembled adopting the same synthetic approach. For instance, all silica sensors (Figs. 2(b)–2(g)) exhibited a regular spherical shape and slight corrugations on the surface, due to random silica porosity [32] and uniform diameter distribution. The good monodispersity of FITC-CY3 0.3 μm , FITC-CY3 0.6 μm , FITC-CY3 0.9 μm sensors is also shown in Fig. 2(h) that reports the box-plot that compares their SEM diameter distribution, in Fig. 2(i) that displays SEM diameter distribution histograms and their representative fitted Gaussian curve, and in Fig. 2(j) that reports DLS hydrodynamic diameter distribution histograms.

2.3 Optical properties of the pH sensing silica particles

Spectrofluorimetric analyses were performed to calibrate FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm sensors and to assess their performances in terms of accuracy and robustness over time. The three biosensors were calibrated adopting K3 medium with known pH in the physiological range (4.5–7.5) with a pH resolution of 0.5 (Figs. S2(a)–S2(c) in the ESM). All sensing particles showed simultaneous response to pH variations attesting that different sizes of the particles did not affect the diffusion of H^+ [36]. The pK_a constants of the conjugated sensing dye to the different silica cores were calculated from the calibration curves adopting a sigmoidal fit [37]. They didn't differ much from the pK_a 6.5 of the dye in the free form [38] and approached its value as the silica core size increased. In particular, FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm sensors showed an equivalence point at $\text{pH } 5.18 \pm 0.10$, $\text{pH } 5.65 \pm 0.06$, $\text{pH } 6.21 \pm 0.37$, respectively.

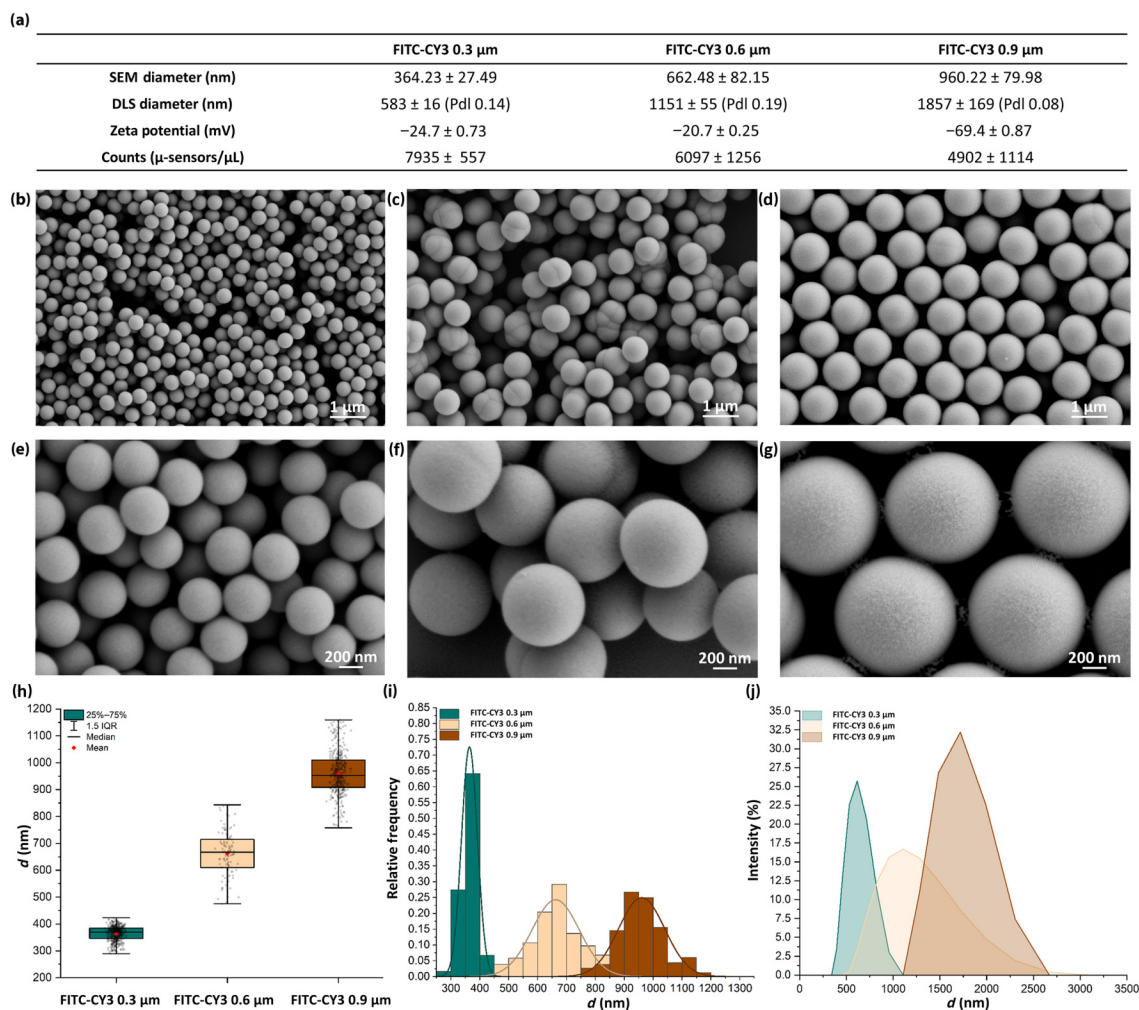


Figure 2 SEM micrographs and diameter distributions related to FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm pH sensing particles. (a) SEM, DLS sizes and zeta potential values of FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm pH sensing particles (refractive index in water: 1.458; absorption: 0.010; 25 $^{\circ}\text{C}$) and fluorescence-activated cell sorting (FACS) number of particles per volume (sensors concentration 0.02 mg/mL; 10 μL volume; phosphate buffered saline (PBS) 1 $\times$, fetal bovine serum (FBS) 5% v/v, Triton 0.5% (w/v) solution). Values were expressed as mean $\pm$ standard deviation (SD) of three independent experiments ($n = 3$). SEM micrographs of (b) FITC-CY3 0.3 μm with (e) enlarged view, (c) FITC-CY3 0.6 μm with (f) enlarged view, and (d) FITC-CY3 0.9 μm with (g) enlarged view pH sensing silica particles. Scale bars: 1 μm , enlarged view: 200 nm. (h) Box-plot comparing FITC-CY3 0.3 μm , FITC-CY3 0.6 μm , FITC-CY3 0.9 μm diameter distribution arose from SEM analysis. (i) Diameter distribution histograms arose from SEM analysis of FITC-CY3 0.3 μm , FITC-CY3 0.6 μm , FITC-CY3 0.9 μm . The superimposed black lines represent the Gaussian best fits. (j) Hydrodynamic diameters distribution histograms evaluated via DLS analysis of FITC-CY3 0.3 μm , FITC-CY3 0.6 μm , FITC-CY3 0.9 μm .

Indeed, the calibration curves of all sensors demonstrated that FITC-conjugated followed the photophysical properties of the free FITC pH indicator in solution and proved that dye conjugation did not impair its sensitivity towards protons. In fact, the pK_a value calculated from the fitted calibration curve. The small difference in pK_a could be associated with the presence of charged functional groups on the surface of silica particles that can interact with the FITC molecules in the proximity by hydrogen bonding, causing the shift of pK_a [29, 30]. Moreover, the different curvature of the particles, defined as the inverse of the radius squared, could play a role in facilitating enhanced Förster resonance energy transfer (FRET) between FITC-CY3 dyes and causing a pK_a shift [39, 40].

Figure 3(a) reports the representative overlay G/R micrographs of FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm sensors imaged at pH 4.5, 5.5, 6.5 and 7.5 (full panels Figs. S3–S5 in the ESM). FITC is characterized by a high quantum yield of $\phi = 0.93$ [41, 42] that allows to precisely appreciate pH variations thanks to its marked color change. The confocal laser scanning microscope

(CLSM) micrographs show that the overall emission of the sensing particles depends on the local pH. In particular, the emission results predominantly in the orange at acidic pH values, while it shifts in the green region of the visible spectrum at basic pH values. Figure 3(b) reports the plots of the fluorescence intensity ratio ($I_{525\text{ nm}}/I_{570\text{ nm}}$) as a function of pH derived from the measurement of the probe's fluorescence intensity at different pH values. Notably, CLSM calibration data were in accordance with the spectrofluorimetric bulk analysis (Figs. S2(a)–S2(c) in the ESM) and confirmed an inverse relationship between FITC fluorescence and protons concentration [43, 44].

2.4 pH sensing silica particles rapidly respond to pH variations in protoplasts

Tobacco protoplasts incubated in dark conditions and static mode, at 26 $^{\circ}\text{C}$ for 18 h in K3 medium along with known concentration of FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm

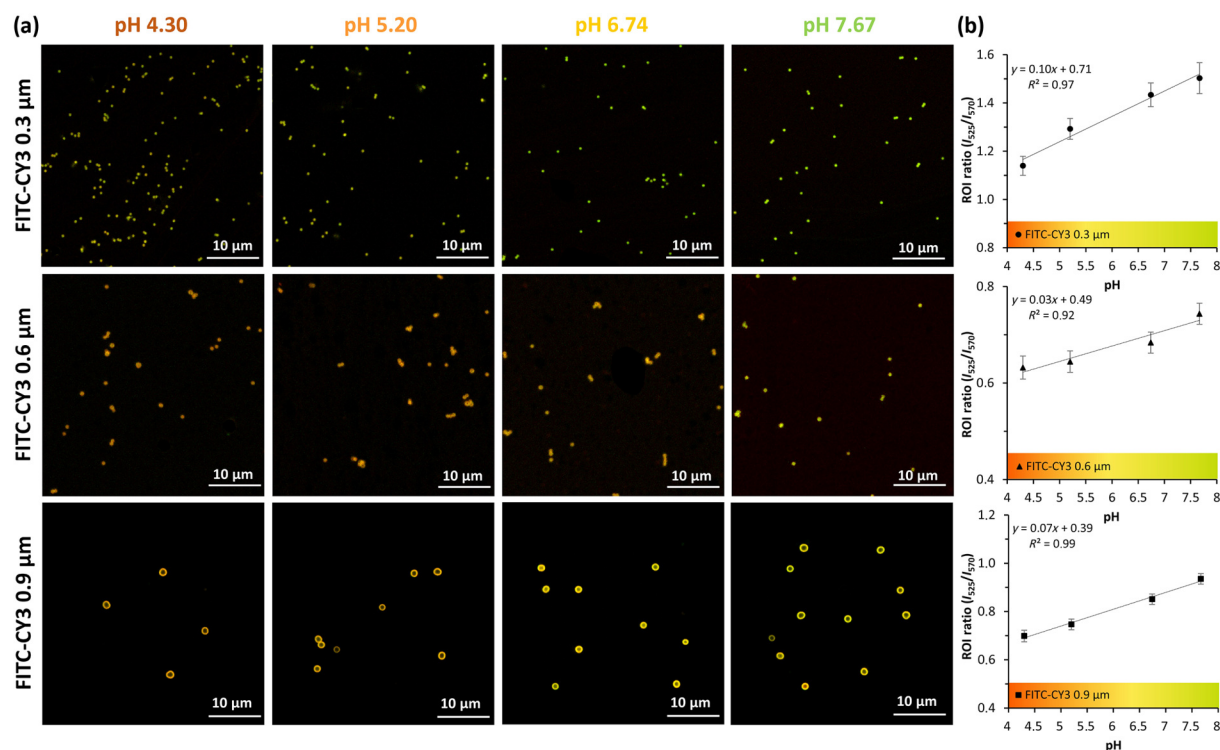


Figure 3 CLSM calibration of FITC-CY3 0.3 μm, FITC-CY3 0.6 μm and FITC-CY3 0.9 μm pH sensing silica particles: (a) representative CLSM micrographs showing the pH dependence of the fluorescence of the sensors in K3 pH-adjusted media. G/R overlays of the fluorescence channels at pH 4.30, 5.20, 6.74, 7.67 are reported. Green channel ($\lambda_{EM} = 488\text{--}560\text{ nm}$, $\lambda_{EX} = 488\text{ nm}$)/Red channel ($\lambda_{EM} = 560\text{--}630\text{ nm}$, $\lambda_{EX} = 555\text{ nm}$). Obj 40×/1.4× Oil, zoom 5, pinhole 1 AU. Scale bars: 10 μm. (b) Ratiometric calibration curves of FITC-CY3 0.3, 0.6 and 0.9 μm on fluorescence intensity ratio of green and red channels derived from the relative CSLM micrographs. Values expressed as mean $\pm$ standard error (SE) of nine independent experiments ($n = 9$).

sensors were further investigated via 4D (X, Y, Z, t) CLSM live imaging to record particles uptake and quantify pH variations. Notably, FITC-CY3 0.3 μm and FITC-CY3 0.6 μm sensors were successfully internalized by protoplasts, while the FITC-CY3 0.9 μm sensors were not, as depicted in Figs. 4(a), 4(d), and 4(g), respectively (full panels Fig. S6 in the ESM). Several protoplasts per condition were segmented and the mean value of pH read-outs inside and outside the cell membrane were calculated. In particular, FITC-CY3 0.3 μm and FITC-CY3 0.6 μm pH sensing particles measured internal pH values of 4.48 ± 0.78 and 4.74 ± 0.75 and external pH values of 6.02 ± 0.82 and 6.00 ± 0.34 , respectively, while FITC-CY3 0.9 μm sensors measured an external pH of 5.26 ± 0.15 . All external pH values measurements obtained from different probes were mildly acidic, with an average value close to that of K3 culture media. In contrast, pH measured inside protoplasts by FITC-CY3 0.3 μm and FITC-CY3 0.6 μm sensors resulted significantly more acidic, around 4.5. This suggests that intracellularly, the sensors localized and accumulated inside the vacuole, which is characterized by more acidic pH values [45–48]. The internalized probes move rapidly within the vacuole, as shown in (Video S1 in the ESM), following the complex ion fluxes. Some protoplasts exhibited a notable accumulation of sensors inside the vacuoles. These sensors appeared to cluster in vesicles, as indicated by the bright field confocal acquisition, forming the so-called “silica bodies” (Video S2 in the ESM) [15, 48]. The location and pH values of FITC-CY3 0.3 μm, FITC-CY3 0.6 μm and FITC-CY3 0.9 μm sensors are further corroborated by the pH colormaps of their Z-projection (Figs. 4(b), 4(e), and 4(h)) and the 3D scatter plot (Figs. 4(c), 4(f), and 4(i)) obtained from image segmentation.

2.5 pH sensing silica particles are internalized by protoplasts following the endocytic pathway

To investigate the mechanisms underlying the uptake of FITC-CY3 0.3 μm and FITC-CY3 0.6 μm sensors in tobacco protoplasts, the amphiphilic styryl dye FM4-64, N-(3-(triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide) was employed to monitor organelle dynamics within the endocytic pathway. Immediately after application, FM4-64 stains the plasma membrane and, over time, labels early endosomes, tracking the endocytic pathway towards the vacuole [49, 50]. In our experimental conditions, FM4-64 clearly stained the plasma membrane of protoplasts incubated with FITC-CY3 0.3 μm or FITC-CY3 0.6 μm sensors (Figs. 5(a) and 5(c)), evidencing the presence of sensors within the plasma membrane, consequently, inside the protoplast. In full panels of Figs. S7 and S9 in the ESM, a weak signal in the FITC channel can be observed, indicating the presence of the sensors colocalizing with small endocytic compartments labelled by FM4-64. These observations suggest the involvement of early endosomes in the pathway that transports the sensors to the vacuole. Clear evidence that the pH sensing silica particles were ultimately localized in the vacuole was obtained transforming the protoplasts with the vacuolar marker GFP-CHI [51], which is targeted to the vacuole via the specific vacuolar sorting determinant of tobacco chitinase A (CHI). Sixteen hours after transformation and incubation with FITC-CY3 0.6 μm and FITC-CY3 0.3 μm sensors, GFP-CHI labelled the vacuole of the protoplasts, clearly showing the sensors, visible in red due to their response to pH variations in the vacuole (Figs. 5(b) and 5(d)). GFP-CHI reached the vacuole after being synthesized and exported from

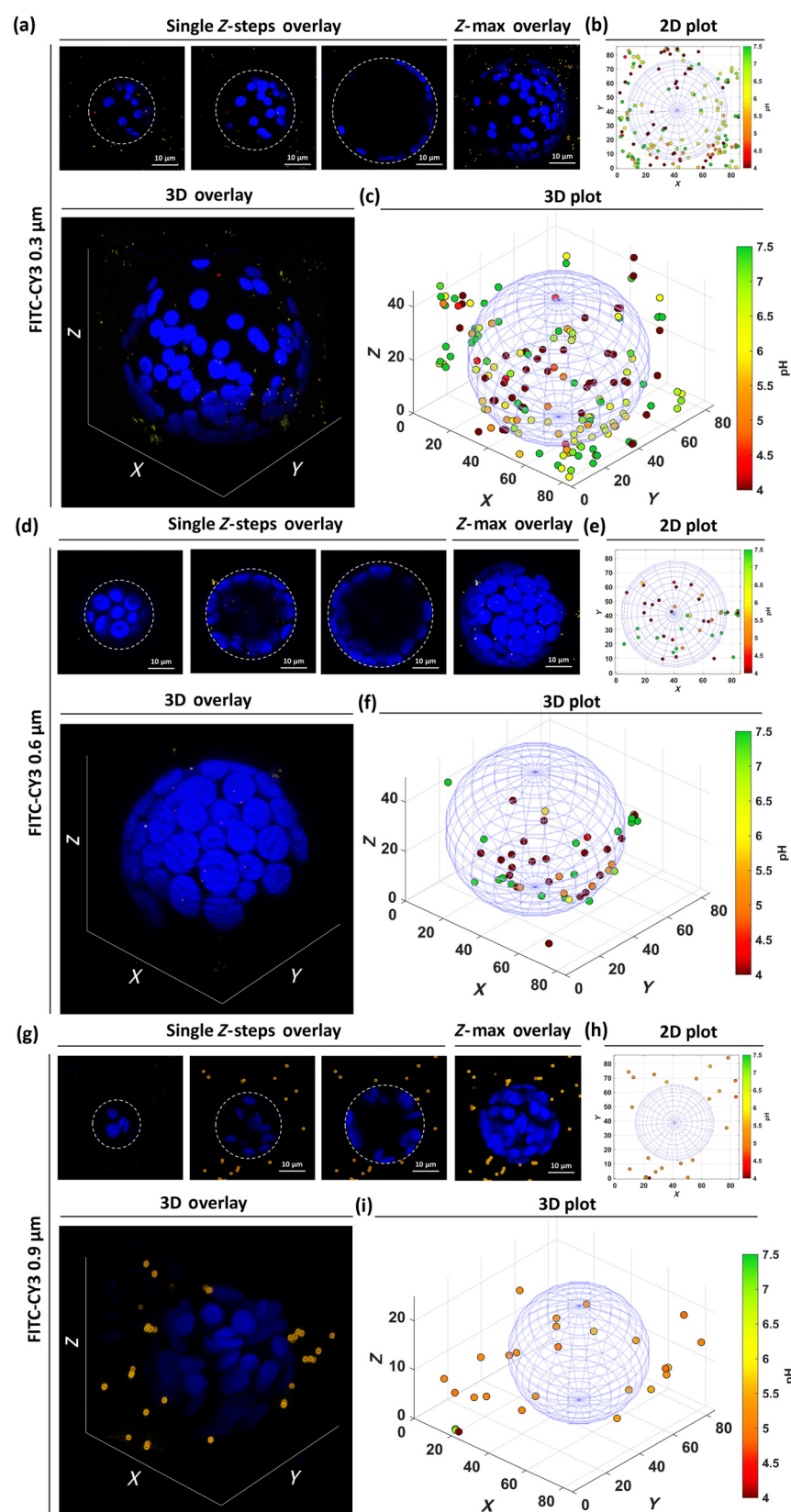


Figure 4 Representative CLSM micrographs, maximum Z-projection and 3D reconstruction showing (a) FITC-CY3 0.3 μm , (d) FITC-CY3 0.6 μm , and (g) FITC-CY3 0.9 μm pH sensing silica particles with a single protoplast obtained from *Nicotiana tabacum* L. leaves in K3 medium after overnight incubation. (b) FITC-CY3 0.3 μm Z-projection map and (c) 3D scatter plot of the experiment shown in (a) with relative pH colormaps. (e) FITC-CY3 0.6 μm Z-projection map and (f) 3D scatter plot of the experiment shown in (b) with relative pH colormaps. (h) FITC-CY3 0.9 μm Z-projection map and (i) 3D scatter plot of the experiment shown in (c) with relative pH colormaps. Obj 40 $\times$ /1.4 $\times$ OIL, zoom 2.5, pinhole 1 AU. Scale bars: 10 μm (resolution 1024 $\times$ 1024; field of view in 85.02 μm $\times$ 85.02 μm ; step size 2.5 μm), FITC (green channel λ_{EM} = 488–560 nm, λ_{EX} = 488 nm), CY3 (red channel λ_{EM} = 560–630 nm, λ_{EX} = 543 nm) and Chlorophyll (blue channel λ_{EM} = 645–735 nm, λ_{EX} = 488 nm).

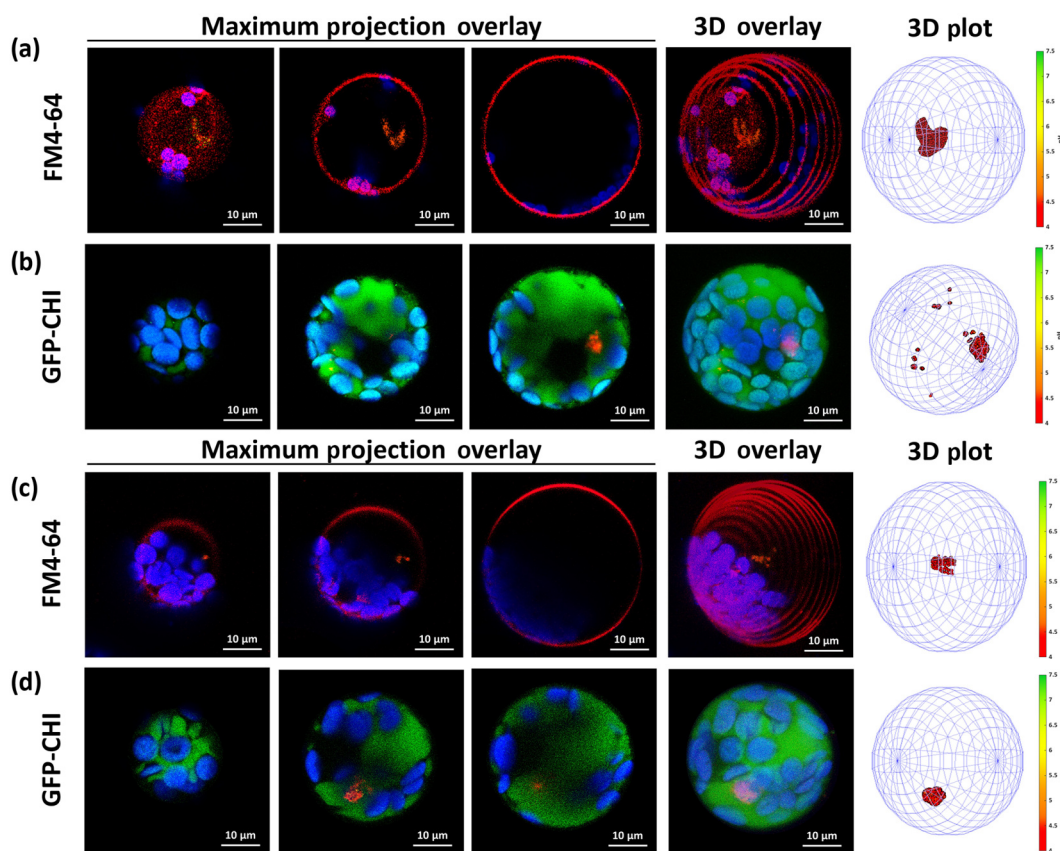


Figure 5 Representative CLSM micrographs showing the co-localization of the pH sensing silica particles into protoplasts obtained from *Nicotiana tabacum* L. leaves. Z-projection of the overlay, 3D reconstruction and 3D scatter plot of protoplast with FM4-64 stained plasma membrane and early endosomes in presence of (a) FITC-CY3 0.3 μm and (c) FITC-CY3 0.6 μm . Z-projection of the overlay, 3D reconstruction and 3D scatter plot of transformed GFP-CHI protoplasts in presence of (b) FITC-CY3 0.3 μm and (d) FITC-CY3 0.6 μm . FITC and GFP-CHI (green channel, $\lambda_{\text{EM}} = 488\text{--}560\text{ nm}$, $\lambda_{\text{EX}} = 488\text{ nm}$), CY3 and FM-64 (red channel, $\lambda_{\text{EM}} = 560\text{--}630\text{ nm}$, $\lambda_{\text{EX}} = 543\text{ nm}$) and Chlorophyll (blue channel $\lambda_{\text{EM}} = 645\text{--}735\text{ nm}$, $\lambda_{\text{EX}} = 488\text{ nm}$). Obj 40 $\times$ 1.4 $\times$ OIL, zoom 2.5, pinhole 1 AU. Scale bars: 10 μm (resolution 1024 $\times$ 1024; field of view in 85.02 $\mu\text{m} \times 85.02\text{ }\mu\text{m}$; step size 2.5 μm).

the ER, which showed green fluorescence in the initial hours following transformation (Figs. S8 and S10 in the ESM).

2.6 pH sensing silica particles cytotoxicity, uptake efficiency and mechanism of internalization in protoplasts

To determine the toxicity of FITC-CY3 sensors with three different sizes (0.3, 0.6, and 0.9 μm) on tobacco protoplasts, we used increasing concentrations of sensors (1, 2, and 3 mg/mL) dissolved in ethanol (25, 50, 75 $\mu\text{L/mL}$) as reported in Fig. 6(a). Microscopy cell counts revealed that no statistically significant differences in viability were observed compared to the control at concentrations of 1 and 2 mg/mL (25 and 50 $\mu\text{L/mL}$ of ethanol). On the contrary, at the highest concentration of 3 mg/mL (75 $\mu\text{L/mL}$ of ethanol), the viability of protoplasts was strongly reduced. Therefore, the particles concentration of 2 mg/mL (50 $\mu\text{L/mL}$ of ethanol) was used for subsequent experiments. In order to estimate the uptake efficiency of protoplasts, a quantitative flow cytometry analysis was performed. Protoplasts were incubated with FITC-CY3 0.3 μm or FITC-CY3 0.6 μm sensors for 16 h and their internalization was measured in all experimental conditions. Representative dot plots and histograms (Figs. 6(b), 6(c) and 6(d)) show the percentages of cyanine-positive protoplasts, indicating successful uptake of the silica particles with different sizes. In particular, 0.3 μm sensors were internalized more efficiently than larger 0.6 μm sensors after 16 h of incubation with freshly prepared protoplasts (Figs. 6(b) and 6(d)).

The same experiment was conducted on the positively charged (FITC-CY3)₂PAH 0.3 μm , (FITC-CY3)₂PAH 0.6 μm and (FITC-CY3)₂PAH 0.9 μm sensors to evaluate whether the superficial charge could influence sensors uptake. The results revealed no uptake of the positively charged FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm into protoplasts after 16 h of sensors incubation as shown in Fig. S20 in the ESM. Then, to study the endocytosis mechanisms used by nanoparticles to enter protoplasts, we used two inhibitors of the clathrin-mediated endocytosis pathway such as salicylic acid (SA) and Tyrphostin A23 (TyrA23). It has been reported that in plant cells SA acts as an endogenous signal that hinders clathrin-mediated endocytosis [52, 53], similarly, TyrA23, a tyrosine analog that blocks specifically the internalization of clathrin-coated vesicles from plasma membrane in both animal [54] and plant cells [55, 56]. The freshly obtained protoplasts were treated for 16 h with either SA or TyrA23.

Interestingly, the uptake efficiency was drastically inhibited under all conditions after the different treatments, indicating that the mechanism of internalization into protoplasts is clathrin-mediated. Moreover, analysis of 16 h uptake efficiency of nanoparticles added after 16 h of cell wall regeneration (Figs. 6(c) and 6(d)) showed very low internalization rates for both silica particle sizes, which decreased even further following SA or TyrA23 treatments. To further validate the mechanism of clathrin-mediated endocytosis, the effect of two drugs that are not involved in clathrin-

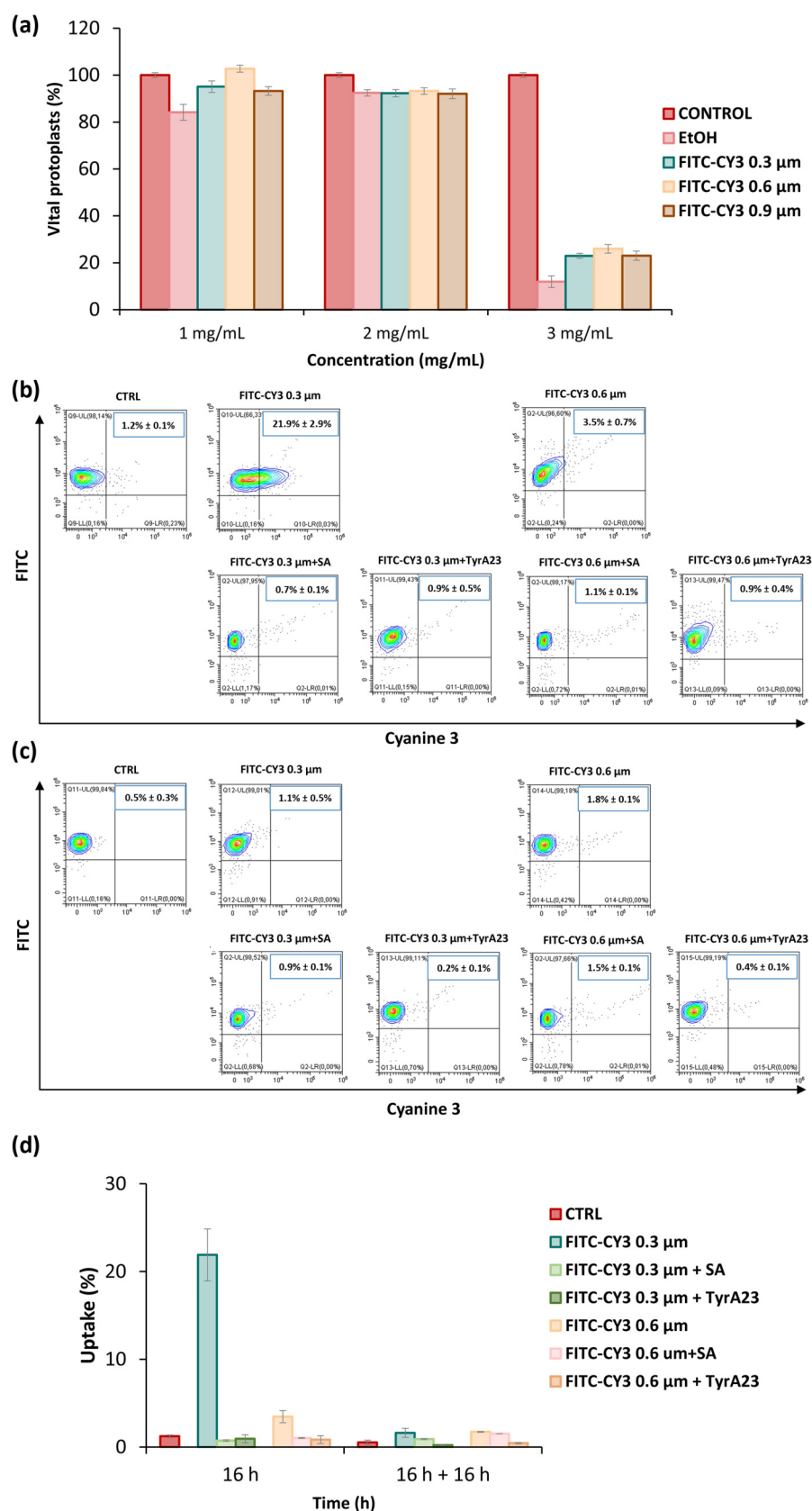


Figure 6 Uptake efficiency and cytotoxicity of FITC-CY3 0.3 μm and FITC-CY3 0.6 μm pH sensing silica particles into protoplasts: (a) FITC-CY3 0.3 μm and FITC-CY3 0.6 μm cytotoxicity; (b) and (c) representative dot plots analyzed by flow cytometry showing the uptake of FITC-CY3 0.3 μm and FITC-CY3 0.6 μm into protoplasts in the presence or absence of SA or TyrA23. Protoplasts were incubated immediately with sensors (b) for 16 h (16 h) or (c) for an additional 16 h from their preparation (16 h + 16 h). (d) Graph showing the percentage of FITC-CY3 sensors uptake extrapolated from (b) and (c). Values were expressed as mean $\pm$ SE of three independent experiments ($n = 3$).

mediated endocytosis was tested on nanosensor uptake efficiency. Typhostin A51 (TyrA51), the negative control used with the TyrA23, has no inhibitory effects on AP2-mediated recognition of endocytic signals [54, 55]. Furthermore, Endosidin 5 (ES5) was used, it blocks proteins recycling from plasma membrane, but has no inhibitory effect on clathrin mediated endocytosis [52, 57]. Both inhibitors did not block the nanosensors uptake (Fig. S18 in the ESM).

These results demonstrate that the uptake efficiency of protoplasts is strongly influenced by the size of the particles and the thin layer of newly synthesized cell wall. It has been reported that nanoparticles with small diameter (ranged 5–100 nm) can passively cross the plasma membrane via fluid-phase endocytosis [58, 59].

The nanoparticles with higher diameter are unable to pass passively through the plasma membrane, their crossing of the plasma membrane requires an active endocytosis process.

In this study, we demonstrated that high diameter (0.3 and 0.6 μm) nanosensors entered the cells via clathrin-mediated endocytosis, but we also found that the uptake efficiency was inversely related to nanoparticle size. This trend could be explained by the ability of nanoparticles to be incorporated into endocytic vesicles, presumably 0.3 μm particles are more easily packed into clathrin-coated vesicles budding from the plasma membrane respect to 0.6 μm nanoparticles.

Furthermore, the significant inhibition of nanosensor uptake, even of the smallest ones, in the presence of clathrin-mediated endocytosis inhibitors is the definitive proof that 0.3 and 0.6 μm nanosensors are internalized via the clathrin-mediated pathway.

This work highlights that the size, surface charge and curvature angle of nanosensors are fundamental for the uptake and intracellular fate of nanoparticles in viable plant protoplasts.

3 Conclusions

The interaction mechanisms of larger ENMs with plant biological membranes remain largely unexplored. Existing models have clarified the uptake and subcellular localization of smaller ENMs via passive transport, but comprehensive studies on active transportation of nanostructured materials are lacking. This study systematically investigated the mechanisms and subcellular distribution of ENMs, ranging from 100 to 1000 nm, within protoplasts endomembrane and newly synthesized cell walls using pH sensing for localization. Silica particles were selected as the ENM model due to their natural abundance in soil and their potential in improving plant physiology. We rationally designed ratiometric pH sensors based on silica particles modified with FITC and CY3 as sensing and reference dyes, respectively. We selected the synthetic protocols that produced highly monodispersed sensing particles with diameters of 364.23 ± 27.49 nm (FITC-CY3 0.3 μm), 662.48 ± 82.15 nm (FITC-CY3 0.6 μm) and 960.22 ± 79.98 nm (FITC-CY3 0.9 μm), characterized through SEM, DLS, and high-throughput imaging flow cytometry. Performance was assessed via spectrofluorimetric and CLSM analysis. When incubated with protoplasts from *Nicotiana tabacum* L. leaves, FITC-CY3 sensors of 364.23 ± 27.49 nm and 662.48 ± 82.15 nm were successfully internalized. Protoplasts 3D segmentation revealed mildly acidic extracellular pH and strongly acidic intracellular pH (4.5), indicating vacuole accumulation. This vacuole localization of nanosensors was confirmed and further validated by colocalization analysis by staining the early endocytic pathway, with FM4-64, leading to the vacuole, via GFP-CHI protoplasts transformation.

High-throughput imaging flow cytometry evaluated internalization efficiency and mechanisms before and after new cell wall regeneration. After 16 h, we observed high internalization rates ($21.9\% \pm 2.9\%$ for 0.3 μm and $3.5\% \pm 0.7\%$ for 0.6 μm), which significantly decreased after 32 h of cell wall regeneration. The presence of the SA and TyrA23 inhibitor, which blocks clathrin-mediated endocytosis, drastically reduced uptake, confirming clathrin-mediated endocytosis as the internalization mechanism. This study is the first to establish rational design principles for controlling active ENM uptake and subcellular localization via optical ratiometric pH sensing in protoplasts. Our model is expected to be widely useful in plant cell trafficking studies, targeted delivery into plants cells, and enhancing the fundamental understanding of plant biology.

4 Experimental

4.1 Chemicals

FITC, K3 buffer, PBS buffer, ammonium hydroxide 28% (NH_4OH), Triton X-100, Lebovitz (L15 medium), FBS, TyrA23 were purchased from Sigma Aldrich, Cyanine3 NHS Ester (succinimidyl ester) (CY3) was from Lumiprobe, tetraethyl orthosilicate (TEOS) was from Aldrich Chemistry, (3-aminopropyl)triethoxysilane (APTES) was from Aldrich chemistry, Cellulase “Onozuka R-10” and Macerozyme R-10 were from Duchefa Biochemie, (N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide) (FM[™]-4-64 Dye) was from Thermo Fisher, anhydrous ethanol was from VWR, ethanol (EtOH) was from Honeywell, potassium chloride (KCl) was from Sigma life science. TyrA23, SA, TyrA51 were purchased from Sigma Aldrich, ES5 was purchased from ChemBridge Corporation.

4.2 Instruments

FITC-CY3 pH sensing silica particles synthesis was carried out at controlled flow rate employing a syringe pump (RWD 462 Double Syringe Pump). pH buffers and pH switches were monitored adopting an XS benchtop pH meter pH 50 equipped with an XS Sensor Standard T DHS BNC pH probe. Chemical structures, substructures and reactions of FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm sensors were drawn adopting ChemDraw while the protoplast sketch was created with Biorender.com. Fluorescence analyses were adopted to build calibration curves of emission and were performed employing a CLARIOstar[®] Plus plate reader (CLARIOstar, BMG LABTECH Inc., USA) while data were acquired and analyzed using MARS Data Analysis software provided by the manufacturer. DLS were conducted to assess FITC-CY3 pH sensing particles hydrodynamic dimensions, monodispersity and surface charge were performed with a Zetasizer Nano ZS purchased from MALVERN and data analyzed Zetasizer 7.12 software provided by the manufacturer. The hydrophilicity of FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm sensors was assessed by measuring their static WCA adopting a CAM 200 (KSV Instruments Ltd., Finland) instrument. Sensors surface morphology was investigated by scanning electron microscopy (Sigma 300VP, Zeiss, Germany). Prior SEM acquisitions FITC-CY3 pH sensing particles were sonicated (DU-32 Digital ultrasonic cleaner) and sputtered with gold in order to become conductive (Safematic CCU-010 LV Vacuum Coating). FITC-CY3 pH sensing particles and protoplasts were isolated after

washing adopting a centrifuge (SL 40R Centrifuge, Thermo Scientific). FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm number of particles per volume, rate and the mechanism of internalization into protoplasts derived from *Nicotiana tabacum* L. leaves was determined employing a flow cytometer CytoFLEX S Beckman Coulter. A Zeiss LSM 710 microscope was adopted to calibrate FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm sensors, to perform tests of sensors internalization and tests of sensors co-localization into protoplasts derived from *Nicotiana tabacum* L. leaves. CLSM images were further analyzed by ImageJ software.

4.3 Optical sensors synthesis

Ratiometric optical pH sensing particles were synthesized in a one-pot synthesis in the range 0.1–1.0 μm with low polydispersity adopting modified Stöber protocols and a non-optimized response surface methodology (RSM) approach. In particular, non-porous solid silica seed particles with selected diameters in the range 100–1000 nm were synthesized via basic hydrolysis adding in the batch reactor to an ethanolic solution of water (12 mL EtOH, 1.12 mL H_2O) the TEOS monomer (150, 150, 100 μL), the NH_4OH catalyst (1270, 1270, 760 μL) and the KCl electrolyte (1.1, 4.6, 2.8 mg) to produce monodispersed silica seeds of 0.3, 0.6, and 0.9 μm of diameter, respectively. The reaction was carried on at room temperature (room T), for 30 min under controlled magnetic stirring of 240 rpm. The silica seeds were further silanized to assemble optical ratiometric sensors adopting APTES as linker [60, 61], while FITC and CY3 as proton and reference probes, respectively. Firstly, FITC-APTES and CY3-APTES conjugated were synthesized in tandem for 2 h solubilizing 0.5 mg of FITC/CY3 in 2 mL anhydrous EtOH each adding 0.37 μL of APTES, under controlled stirring (500 rpm) at room T. Crude products were united upon completion of their reactions. Then, they were filled into a syringe and injected into the silica seed batch reactor with a controlled flow rate of 0.05 mL/h. The reaction mixture was kept under stirring at 240 rpm at room T for 24 h. The final product was centrifuged (2500 rpm, 5 min, room T), and the supernatant was discarded. The pellet was further washed three times in 50 mL of ethanol and then three times in 50 mL of deionized (D.I.) water (2500 rpm, 5 min, room T). Lastly, the clean sensors were resuspended in ethanol to obtain a 40 mg/mL stock solution and were stored in the dark at room T. FITC-CY3 0.3 μm , 0.6 μm and 0.9 μm were coated by a layer of polyelectrolyte to make their superficial charge positive to further investigate whether their superficial charge could influence either particles hydrophilicity or uptake. For instance, 500 μL of each ethanoic stock solution (40 mg/mL) was centrifuged, then the recovered pellet was dissolved in 1 mL of PAH ($M_w \sim 56,000$) (2 mg/mL, 0.5 M NaCl, pH = 6.5) for 15 min under vigorous stirring (800 rpm). After the treatment, the particles were centrifuged and their pellet was dissolved again in ethanol (40 mg/mL) prior further analysis.

4.4 DLS characterization

Hydrodynamic diameter, monodispersity and surface charge of the pH sensors dispersed in D.I. water were measured by DLS (refractive index in water 1.458, absorption 0.010, 25 $^\circ\text{C}$, 3 min equilibrium time) [62].

4.5 WCA measurements

The hydrophilicity of FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm sensors was assessed by measuring their static

WCA. First, a thin and homogeneous layer of sensors stock solution ($\sim 100 \mu\text{L}$) on the surface of a cover glass (24 $\times$ 24 mm, 0.13–0.17 mm thickness). The samples were left drying overnight at room temperature. For the experiment set up, the liquid (heavy phase) was represented by water ($d = 0.9986 \text{ g/cm}^3$) and the liquid (light phase) was air ($d = 0.0013 \text{ g/cm}^3$). At least three independent measurements per each sample were performed by loading on the tip of the needle a drop of 5 μL of volume that was stroke on the surface of the sample.

4.6 SEM characterization

The morphology of FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm sensors was characterized by means of SEM (Sigma 300VP, Zeiss, Germany) with accelerating voltage of 5 kV, secondary electron detector (SE2), and 30,000 $\times$, 50,000 $\times$, 100,000 $\times$ magnifications. For sample preparation, 1 μL of sensors from stock solutions in ethanol were deposited onto a silicon wafer, air-dried at room T overnight and sputter coated with a 10 nm thick gold layer (Safematic CCU-010 LV Vacuum Coating).

4.6.1 SEM images processing and analysis

The image segmentation was performed following the protocol shown in Fig. S11 in the ESM, using python (v. 3.9.16), Spyder IDE (v. 5.4.3) and the scikit-image libraries (v. 0.23.2). Briefly, the image was cropped to eliminate the information bar (Fig. S11(a) in the ESM), then a global thresholding was applied to include all the particles. It should be noticed that the threshold was set case-by-case, in fact it strongly depends on the image contrast (Fig. S11(b) in the ESM). The particle's border was extracted by the active contour model (Fig. S11(c) in the ESM). The contours were subtracted to the thresholded image by a Boolean operation (Fig. S11(d) in the ESM). The single particles were then segmented using watershed (Fig. S11(e) in the ESM). A threshold was used on the circularity to remove particles that were incorrectly bounded together during the segmentation (Fig. S11(f) in the ESM). The particles equivalent diameter was calculated following Eq. (1).

$$d = 2\sqrt{\frac{A}{\pi}} \quad (1)$$

The statistic was computed calculating the mean, the SD, the median, the interquartile range (IQR), the first quartile (Q1), the third quartile (Q3). Outliers outside the region comprised between the $Q1 - 1.5\text{IQR}$ and $Q3 + 1.5\text{IQR}$ were interactively excluded, until no point was out of the range, this allows to clean the data from particles outside the main plane and other segmentation artifacts.

4.7 Fluorimetric calibration

FITC-CY3 0.3 μm , FITC-CY3 0.6 μm and FITC-CY3 0.9 μm sensors were calibrated in K3 medium in their full pH range of sensibility with a pH resolution of 0.5 (4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5) (room T). Calibration procedure was the same for every system: Firstly the pH of K3 medium was adjusted at the selected values adding NaOH (1 M) or HCl (1 M) to its solution under magnetic stirring while continuously monitoring its value adopting a pH meter, then sensors stock solutions (40 mg/mL) (EtOH, room T, dark) were properly dispersed by vortexing, then 1 μL of the stock solution was added to 300 μL of K3 with adjusted pH and analyzed at the plate reader. FITC-CY3 sensors were excited at: λ_{EX} FITC = $472 \pm 16 \text{ nm}$, and λ_{EX} CY3 = $550 \pm 8 \text{ nm}$, signals were

collected between 500 ± 10 nm, 700 ± 10 nm, 570 ± 10 nm, and 700 ± 10 nm, respectively. Ratios were calculated as $\lambda_{EM\ MAX\ FITC} (525\ nm)/\lambda_{EM\ MAX\ CY3} (570\ nm)$. Spectra were processed adopting a MARS Data Analysis software.

4.8 Protoplasts preparation, sensors incubation and internalization analyses

4.8.1 Protoplasts preparation

Nicotiana tabacum L. cv SR1 plants were cultivated as described by Neumann et al. (2002) [54]. Tobacco leaf protoplasts were prepared as described by Leucci et al. (2007) [63]. Briefly, leaves of 6-week-old tobacco seedlings were harvested and washed first with NaClO and then in D.I. H₂O. Next, the leaves were digested overnight at 26 °C with 10 mL of K3 medium containing sucrose 0.2%, cellulase Onozuka R-10 1.2% and macerozyme R-10 0.4%. After incubation, protoplasts were separated from leaves debris still present in the digestion solution via filtration with the help of a metallic sieve (150 mesh). After purification, the protoplasts were washed 3 times with 1 mL of W5 solution (600 rpm, 5 min, 22 °C) and were suspended in 1 mL of K3 medium. The pH sensing silica particles were added in freshly prepared protoplasts or in those incubated in K3 medium for 16 h and kept for 16 h, in the dark and static mode to perform the programmed experiments.

4.8.2 Protoplast transformation

Protoplasts transformation followed the protocol described by Leucci et al. (2007) [60]. Briefly, 10 µg of GFP-CHI plasmid was used for the transformation of around 600,000 protoplasts. After 2 h, the protoplasts were rinsed to remove the PEG 40% suspended in 1 mL of K3 medium and immediately incubated with the pH sensing silica particles for performing co-localization experiments.

4.8.3 pH sensing silica particles uptake

pH sensing experiments were performed adding 50 µL of the fluorescent optical sensors (EtOH, 40 mg/mL) spiked in 1 mL of K3 medium containing: (i) freshly prepared (time 0 h) protoplasts, (ii) before incubated for 16 h in K3 medium (time 16 h) protoplasts, (iii) freshly transformed protoplasts. The fluorescent optical incubation was performed for 16 h at 26 °C in the dark, in a static mode in all experimental conditions.

4.8.4 Protoplast endocytic pathway staining

The protoplast endocytic pathway was stained using FM4-64 dye (molecular probe) as described in De Caroli et al., in 2011 [56]. Briefly, isolated protoplasts suspended in K3 medium were incubated with the pH sensing silica particles overnight (16 h, in the dark, static mode), then, 1 µL of FM4-64 (100 µM) was added to 100 µL of protoplasts (30 min, room T). Finally, the solution was washed adding 100 µL of W5 and centrifuged (30 s, 600 rpm, room T). Washed recovered protoplasts were resuspended in K3 medium and, immediately, observed to visualize the plasma membrane. Early endosomes were visible 20 min after FM4-64 staining.

4.8.5 SA, TyrA23, TyrA51 and ES5 treatments

Protoplasts were incubated in K3 medium with SA, or TyrA23, or TyrA51, or ES5 following the protocol described by De Caroli et al., in 2011 and 2020 [52, 56]. 100 µM of SA, or 350 µM of TyrA23, or 350 µM of TyrA51, or 50 µM of ES5 was added to the protoplasts at time 0 or after 16 h from their preparation (time 16 h) together

with 50 µL of the fluorescent optical sensors (EtOH, 40 mg/mL) as described above.

4.9 Sensors' cytotoxicity

For vitality test, protoplasts were incubated in K3 medium with increasing concentrations (1, 2, and 3 mg/mL) of FITC-CY3 sensors dissolved in ethanol (25, 50, and 75 µL/mL). The toxicity of sensors with three different sizes (0.3, 0.6, and 0.9 µm) were tested and protoplasts without particles or protoplasts treated with only ethanol were used as control. Dead and alive protoplasts count was performed acquiring confocal images for each condition, employing Obj 10×/0.25, acquisition speed of 400 Hz, 1 as zoom and 1 as pinhole (tile scan 10 × 1; field of view in 848.53 µm × 848.53 µm). Images were then analyzed via ImageJ adopting the plugin analyze cell counter.

4.10 Flow cytometry analysis

The number of FITC-CY3 sensors per volume was measured by flow cytometry. Sensors were prepared at the concentration of 0.02 mg/mL in PBS 1× containing 5% FBS and 0.5% Triton X-100 in order to avoid their aggregation. This aliquot was analysed on a flow cytometer (CytoFLEX S, Beckman Coulter) with high resolution for nano- and microparticles measurement reached by using the side scatter of the 405 nm laser. The number of single events contained in 10 µL of solution was acquired and analysed using CytExpert software. Uptake efficiency of FITC-CY3 sensors was measured into protoplasts derived from *Nicotiana tabacum* L. leaves by flow cytometry. Protoplasts were incubated with 2 mg/mL of sensors dissolved in K3 medium for 16 h and after a further 16 h, prior and after the initial of new cell wall regeneration, respectively. The same experiments were repeated treating the protoplasts with inhibitor SA or TyrA23 at time 0 and at time 16 h. The samples were washed with W5 solution adopting the same procedure employed for protoplasts isolation. Protoplasts were then resuspended in 1 mL of MM solution (0.4 M mannitol, 0.004 M 2-(N-morpholino)ethanesulfonic acid hydrate (MES), pH 5.7) prior flow cytometry analysis. Approximately 20,000 events were acquired for each sample and analyzed by CytExpert software. Dead cells, debris, and doublets were excluded based upon forward scatter and side scatter measurements. The gating strategy was designed to account for the autofluorescence emitted by protoplasts in the green spectrum, primarily due to chlorophyll. For this reason, Cy3 was selected as the primary indicator for particle uptake because it offers several advantages over FITC. Specifically, FITC signals in the green spectrum overlap with the protoplast autofluorescence, making it less reliable for discerning internalized particles. Unlike FITC, Cy3 is insensitive to pH fluctuations. This ensures a stable and consistent signal, even in acidic environments where FITC fluorescence can quench. Given these considerations, we determined that Cy3 provides a more accurate and stable indication of internalized particles. The Cy3- and FITC+ group likely represents autofluorescence from the whole protoplast population rather than actual uptake of labeled particles.

4.11 CLSM characterization

FITC-CY3 0.3 µm, FITC-CY3 0.6 µm and FITC-CY3 0.9 µm sensors were characterized by confocal microscopy and were calibrated in K3 medium in their range of sensibility with a pH resolution of 1.0 (4.5, 5.5, 6.5, and 7.5). Confocal acquisitions were performed employing Obj 40×/1.40× OIL, acquisition speed of

400 Hz, 2.5 and 5 as zoom and 1 as pinhole. Emission signals were acquired simultaneously for each system. In particular: FITC-CY3 pH sensing particles and protoplasts were characterized exciting FITC and chlorophyll with laser $\lambda = 488$ nm at 1% collecting their emission signal in the range 488–560 nm and 645–735 nm, respectively, CY3 was excited with laser $\lambda = 543$ nm at 1% collecting its emission signal in the range 560–630 nm. FM4-64 dye (molecular probes) was excited adopting the laser $\lambda = 543$ nm at 1% while collecting its emission signal in the range 645–735 nm. GFP-CHI signal was excited adopting the laser $\lambda = 488$ nm at 1% while collecting its emission signal in the range 488–560 nm.

Experiments of pH sensing were performed adding approximately 800 μL of K3 medium containing protoplasts incubated overnight along with the pH sensing particles into a μ -Slide 4 Well Chamber Slide (IBIDI) previously treated with 0.3 mg/mL of poly-L-lysine (37 °C, 30 min). At least 6 protoplasts per condition were segmented to establish if the sensors were located inside or outside of the protoplast and to measure the relative local pH, representative segmentations are reported in Figs. S14–S16 in the ESM. Experiments of co-localization were performed embedding the transformed protoplasts incubated overnight along with the pH sensing particles into agarose (final agarose concentration of 0.3% w/v) and then depositing some drops between 2 coverslips.

4.11.1 CLSM images processing and analysis

CLSM images in 3D (X, Y, Z) were automatically analyzed in GNU Octave (version 7.2.0), with a custom algorithm modified starting from [43, 44]. Inputs of the algorithm were the fluorescence indicator (FITC) and reference (CY3) signals of the 0.3, 0.6 or 0.9 μm pH sensing particles. Briefly, the reference channel was pre-processed Z-by-Z, converted to grayscale, binarized and reconstructed in 3D by direct connectivity (Fig. S17(1a) in the ESM). The resulting 3D binary matrix was used as a mask to extract positions and mean fluorescence intensity ratios ($I_{\text{FITC}}/I_{\text{CY3}}$) of the sensors belonging to the original FITC and CY3 signal images (Fig. S17(2a) in the ESM). pH values were extracted by passing $I_{\text{FITC}}/I_{\text{CY3}}$ data (Fig. S17(3a) in the ESM) in a previously obtained calibration curve (Fig. 3). Contemporarily, the bright field channel images were processed using ImageJ software [64] to extract the center C_p (X, Y, Z) of the protoplast and its ray R_p (Fig. S17(1b) in the ESM). Then, the algorithm computed the Euclidean distance d_{ps} between C_p and each single sensor, establishing if the sensors were located inside ($d_{ps} \leq R_p$) or outside ($d_{ps} > R_p$) of the protoplast (Fig. S17(c) in the ESM).

4.12 Statistical analysis

At least of one triplicate sample was consisted for each type of analysis quality control; analysis results were expressed as mean $\pm$ standard deviation/radq(n). EXCEL software was employed for the statistical analysis. Analysis of variance (ANOVA) with single factor and least significant differences (LSD) at the 5% significant level were adopted to identify statistically significant differences.

Electronic Supplementary Material: Supplementary material (SEM images of various ratiometric pH sensors, segmentation analyses, calibration data, CLSM micrographs, CLSM time-series of pH-sensing particles incubated with protoplasts, FACS analyses of uptake efficiency, zeta potential analyses, flow cytometry analyses of uptake efficiency, and a schematic of the optical pH sensors'

functioning mechanism) is available in the online version of this article at <https://doi.org/10.26599/NR.2025.94907201>.

Data availability

All data needed to support the conclusions in the paper are presented in the manuscript and the Electronic Supplementary Material. Additional data related to this paper may be requested from the corresponding author upon request.

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Declaration of competing interest

All the contributing authors report no conflict of interests in this work.

Author contribution statement

H. I.: Conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft, writing – review & editing, visualization, supervision. L. M. C.: Methodology, validation, investigation, data curation, writing – review & editing. A. B.: Methodology, software, validation, formal analysis, investigation, data curation, writing – original draft visualization. V. O.: Methodology, software, validation, formal analysis, investigation, data curation, writing – original draft, writing – review & editing. S. F.: Validation, formal analysis, investigation, writing – original draft, writing – review & editing. G. G.: Funding acquisition. L. L. d. M.: Conceptualization, writing – review & editing, supervision, project administration, funding acquisition. M. D. C.: Conceptualization, methodology, investigation, data curation, writing – review & editing, supervision, project administration, funding acquisition. G. P.: Conceptualization, writing – review & editing, supervision, project administration, funding acquisition.

Informed consent

Not applicable.

Ethics statement

Not applicable.

Use of AI statement

None.

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