Conjugation of oligo-His peptides to magnetic γ-Fe_2O_3@SiO_2 core-shell nanoparticles promotes their access to the cytosol

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ABSTRACT:

The endosomal entrapment of functional nanoparticles is a severe limitation to their use for biomedical applications. In the case of magnetic nanoparticles (MNPs), this entrapment leads to poor heating efficiency for magnetic hyperthermia and suppresses the possibility to manipulate them in the cytosol. Current strategies to limit their entrapment are based on their functionalization with cell-penetrating peptides in order to promote their translocation directly across the cell membrane or their endosomal escape. However, these strategies suffer from potential release of free peptides in cell and to the best of our knowledge there is currently a lack of effective methods for the cytosolic delivery of MNPs after incubation with cells.

Herein, we report the conjugation of fluorescently labelled cationic peptides to γ-Fe_2O_3@SiO_2 core-shell nanoparticles by click chemistry to improve MNP access to the cytosol. We compare the effect of Arg_9 and His_4 peptides. On one hand, Arg_9 is a classical cell-penetrating peptide, able to enter cells by direct translocation and on the other hand, it has been demonstrated that sequences rich in histidine residues promote endosomal escape, most probably by the proton sponge effect. The methodology developed allows to have a high co-localization of the peptides and core-shell nanoparticles in cells and to attest that the grafting onto nanoparticles of peptides rich in histidine promotes NP access to the cytosol. The endosomal escape was confirmed by a calcein leakage assay and by ultrastructural analysis in transmission electron microscopy. No toxicity of the nanoparticles functionalized with peptides was found. We show that our conjugation strategy is compatible with the addition of multiple substrates and can thus be used for the delivery of cytoplasm-targeted therapeutics.

1. INTRODUCTION

Magnetic nanoparticles (MNPs) have emerged as an important class of functional nanomaterials in the biomedical field for various applications such as magnetic hyperthermia to kill cancer cells, magnetic resonance imaging, drug delivery, or cellular engineering.[1,2] For some of these applications, MNP entry inside cells is required. However, as most other types of nanoparticles, MNPs enter cells by endocytosis and often remain trapped inside endosomes. Confinement of MNPs in these small intracellular vesicles, restricts the ease of magnetic manipulation[3,4] and also prevents MNPs from interacting with targets present in the cytosol or other organelles. Moreover the aggregation of the MNPs inside the endosomes has been shown to negatively impact their heating efficiency in magnetic hyperthermia due to dipolar interactions between the MNPs, and thus limits the development of magnetic hyperthermia-based treatment of cancer.[3] In cellular engineering, MNPs have recently been used to remotely control cellular functions.[5–7] This often-called "magnetogenetics" approach is mostly used for the control of extracellular proteins or ion channels due to the difficulties to avoid endosomal confinement of MNPs after their cellular uptake. An easy access to intracellular proteins capable of triggering a cascade of signaling event would greatly expand the potential of magnetic cellular engineering. For example, the GTPases Ras, Rac or Cdc42 are intracellular proteins which are involved in signaling pathways in neurons, and are targets of choice for magnetic cellular manipulation to induce neuronal growth. Their activation with functionalized MNPs has been achieved, but with particles that were directly micro-injected in cells.[5,6,8] Unfortunately, micro-injection can only be done on a small number of cells, making the process difficult to parallelize. Finding a way to overcome the endosomal entrapment of MNPs and to enable their diffusion into the cytosol would be a key step for the development of applications such as magnetic hyperthermia for cancer treatment or as the remote control of cellular processes.

In recent years, cell penetrating peptides (CPPs), often relatively short purely cationic or amphipathic peptides, have shown promises in mediating the transport of a wide range of modalities such as peptides, proteins and oligonucleotides.[9,10] CPP cellular uptake mostly occurs by endocytosis. However, some CPPs can also enter cells by direct translocation, a process which involves a transient perturbation of the lipid bilayer of the plasma membrane and allows the CPP and its cargo to directly reach the cytosol.[11–15] The conjugation of CPPs onto the surface of nanoparticles has been presented in previous work by different research groups.[16] For example, the TAT peptide has been grafted on CdSe-ZnS quantum dots[17] and poly-arginine CPPs (or lipidated derivatives) on superparamagnetic iron oxide nanoparticles (SPION) and gold nanoparticles.[18,19] In all cases the CPPs were found to improve nanoparticle (NP) cellular uptake although the endocytosis pathways remained predominant and NPs were not visualized in the cytosol. Interestingly, efficient siRNA nanocarriers have been engineered combining NPs and CPPs, where the CPP was used both to easily load the siRNAs *via* electrostatic interactions and to improve siRNA cytosolic delivery. In particular, SPIONs derivatized with both poly-arginine and gH625 CPPs[20] and chitosan NPs functionalized with the poly(histidine-arginine)$_6$ peptide[21] were shown to promote siRNA endosomal escape. While the cellular distribution of the chitosan nanocarrier was not analyzed,[21] Ben Djemaa *et al*., mentioned in their study that the SPION nanocarriers themselves remained trapped inside endosomes/lysosomes.[20]

Our scope in this study was to improve access to the cytosol of the MNPs themselves, which is essential for innovative cellular engineering applications. We have investigated for this the impact of MNP functionalization with two basic peptides, a poly-Arg peptide (Arg_9) and a short His-rich

peptide (His_4-Trp), on their internalization process. Arg_9 is a classical CPP, which has been shown to enter cells by both endocytosis and direct translocation.[22–24] Its capacity to transiently destabilize the plasma membrane or endosomal membranes to reach the cytosol is however strongly dependent on different parameters including its concentration and the nature and size of the transported cargo.[13,16,22–24] On the other hand, oligo-His sequences have been shown to promote endosomal escape, probably by the so-called "proton sponge effect". It has indeed been proposed that protonation of the imidazole group of His residues which occurs in the acidic lumen of late endosomes can induce proton and chloride ion accumulation, osmotic swelling of endosomes and burst.[25–27]

The MNPs used in this study are core-shell γ-Fe_2O_3@SiO_2 nanoparticles. Those were chosen for several reasons. They can be synthesized with sizes suitable for biomedical applications, and by functionalizing their surface they have a good colloidal stability, even in complex medium such as the cell cytosol. They can also easily be rendered fluorescent by the addition of a fluorophore in the silica shell, which enables their visualization through fluorescence and confocal microscopy. Finally, the silica chemistry being well described, different types of functionalization are possible.

A covalent grafting of the peptides on core-shell nanoparticles was favored in this study because, unlike non-covalent bonds (electrostatic interactions, adsorption, hydrogen bonds), it allows to follow unambiguously the nanoparticle-peptide conjugates. The covalent bond also prevents uncontrolled peptide release in biological media.

Among the different chemoselective ligation methodologies, the copper-free click chemistry based on the reaction between a constrained alkyne, for example the dibenzocyclooctyne (DBCO) group, and an azide function is widely studied for its ease of implementation, its high yield and its non-cytotoxicity.[28] In this work the γ-Fe_2O_3@SiO_2 nanoparticles were easily functionalized with a DBCO group, while the peptides were designed to carry an azido function. We report here an optimized protocol that allowed the efficient covalent grafting of the peptides on the core-shell nanoparticles and ensured the complete removal from their surface of the adsorbed unreacted species. We also showed the possibility to graft one or two substrates at the surface of the MNPs. After incubation with cells, the polyhistidine-functionalized nanoparticles showed partially diffuse cytosolic fluorescence by confocal microscopy, suggestive of an enhanced endosomal escape, in contrast to the non-functionalized and the polyarginine-functionalized MNPs which exhibited exclusively a punctuate fluorescent signal. The capacity of the polyhistidine-functionalized nanoparticles to perturb the membrane of endosomes was further confirmed by a calcein leakage assay. Importantly, the rupture of the membrane of some of the endosomes could be visualized and the presence of MNPs free in the cytosol could be confirmed by ultrastructural analysis in transmission electron microscopy.

2. EXPERIMENTAL SECTION

2.1 Materials

Standard Fmoc amino acids, were purchased from Iris Biotech (Germany). Rink Amide AM resin, *N,N'*-Diisopropylcarbodiimide (DIC), 1-Hydroxybenzotriazole hydrate (HOBt), *N,N,N',N'*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), *N,N*-Diisopropylethylamine (DIEA), ethylenediaminetetraacetic acid (EDTA) $Pd(PPh_3)_4$, $PhSiH_3$, Fmoc-ε-Ahx-OH and N_3-CH_2-COOH, Tetraethylorthosilicate (TEOS), citric acid, 3-aminopropyltriethoxysilane (APTS), rhodamine B isothiocynate, Dimethylsulfoxyde (DMSO), dibenzocyclooctyne-PEG_4-N-hydroxysuccinimidyl ester, tri-sodium citrate dihydrate, 3-morpholinopropane-1-sulfonic acid (MOPS) buffer, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES), Hanks balanced salt solution (HBSS), fetal bovine serum (FBS), Hoechst 33342 dye, calcein, trypan blue 0.4%, glutaraldehyde, fluorescamine and Epon resin were purchased from Sigma-Aldrich (France). Dimethylformamide (DMF) (Peptide synthesis grade), acetonitrile (HPLC grade), trifluoroacetic acid (TFA) (Optical spectroscopy grade), dichloromethane (Analysis grade), piperidine (Peptide synthesis grade) and citric acid were obtained from Carlo Erba (France). Ferrous chloride, ferric chloride, ferric nitrate, acetone, diethyl ether, ethanol (96%), nitric acid (68%), hydrochloric acid 37% and ammonia (20%) were provided by VWR (France). 2-(methoxy(polyethyleneoxy)propyl)trimethoxysilane (PEOS) was obtained from ABCR (Germany). Dulbecco's modified Eagle's-F12 medium (DMEM-F12), penicillin, streptomycin, amphotericin B and trypsin were purchased from Gibco (France). Lactacte dehydrogenase (LDH) was provided by Invitrogen (France). Wild type Chinese Hamster Ovary (CHO-K1) cells (reference CCL-61) were obtained from ATCC (USA).

2.2 Peptide synthesis

2.2.1 Fluorescent azidopeptide derivatives CF-R9 and CF-H4

The fluorescent peptides **CF-R9** (CF-Ahx-Lys(CO-CH_2-N_3)-$(Arg)_9$-NH_2, CF: 5(6)-carboxyfluorescein) and **CF-H4** (CF-Ahx-Lys(CO-CH_2-N_3)-$(His)_4$-Trp-NH_2) were obtained by Fmoc solid-phase peptide synthesis (SPPS). The Arg_9 sequence was assembled on Rink amide Protide LL (CEM Corporation) by automated microwave-assisted SPPS using a Liberty Blue synthesizer (CEM Corporation) at the "Plateforme d'ingénierie des protéines" (IBPS, Sorbonne Université). It was then elongated manually to give **CF-R9**. The **CF-H4** peptide was synthesized manually in a polypropylene vessel on Rink Amide AM resin (0.35 mmol/g resin, 0.1 mmol scale). For the manual elongations of both peptides, Fmoc protected amino acids and Ahx linker (5 equiv.) were activated for 3 min with HBTU (4.5 equiv.) and DIEA (10 equiv.) in DMF before addition to the peptide-resin. The coupling reactions were then allowed to proceed for 30 min at room temperature (RT). Fmoc deprotections were performed using a 20% v/v solution of piperidine in DMF (3 + 7 min). 5(6)-carboxyfluorescein (5 equiv.) was coupled on the *N*-terminus of the peptide using DIC (5 equiv.) and HOBt (10 equiv.) in DMF (15 min activation, 15 h coupling at RT). The resin was then washed several times with a 20% v/v solution of piperidine in DMF. Selective Alloc removal from the Lys side chain was then performed by treatment of the peptide-resin with a solution of $Pd(PPh_3)_4$ (0.25 equiv.), $PhSiH_3$ (25 equiv.) in dichloromethane for 90 min at RT under argon, then the resin was washed using a 20% v/v solution of piperidine in DMF. This was followed by coupling of N_3-CH_2-COOH (5 equiv.) on the Lys side chain using DIC (5 equiv.) and

HOBt (10 equiv.) in DMF (15 min activation, 15 h coupling at RT). Final peptide deprotection and cleavage from the resin was achieved by treatment with a solution containing 95% TFA, 2.5% H_2O and 2.5% triisopropylsilane for 4 h at RT. The peptides were precipitated with cold diethyl ether and incubated for at least 30 min at -20 °C, and pelleted by centrifugation. Peptides were purified by reverse phase HPLC (RP-C18 column, 5 µm, 250 x 16 mm, Macherey Nagel) using a flow rate of 10 mL/min and a linear gradient over 30 min of 10-40% of solvent B in A for **CF-R9** and 1-30% B in A for **CF-H4** (A: 0.1% TFA in H_2O, B: 0.1% TFA in CH_3CN). Peptides were monitored at 220 nm. The purity of the fractions was assessed by HPLC (RP-C18 column, 5 µm, 100 x 4.6 mm, Higgins Analytical) at a flow rate of 1 mL/min with a linear gradient of solvent B in A over 10 min (Figure S1). Peptides were monitored at 220 nm. Fractions containing the pure desired peptide were combined and lyophilized.

The peptides were characterized by Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) mass spectrometry (Applied Biosystems 4700 or AB Sciex Voyager DE-PRO spectrometer) in positive ion mode, reflector, using CHCA (α-cyano-4-hydroxycinnamic acid) at 10 mg/mL in H_2O/CH_3CN/TFA (1/1/0.01) as matrix. **CF-R9**, expected m/z $[M+H]^+$: 2106.19, observed m/z $[M+H]^+$: 2106.41. **CF-H4**, expected m/z $[M+H]^+$: 1435.59, observed m/z $[M+H]^+$: 1435.92. In both cases, an additional peak was observed (Δ m/z: -28), suggesting a fragmentation of the azido group *via* expulsion of N_2 during MALDI-TOF analysis.[29]

2.2.2 Azidopeptide derivatives R9 and H4**

Peptides **R9*** (N_3-CH_2-CO-$(Arg)_9$-NH_2) and **H4*** (N_3-CH_2-CO-$(His)_4$-Trp-NH_2) were synthesized as described above by Fmoc solid-phase chemistry. N_3-CH_2-COOH was in this case introduced on the peptide *N*-terminus. Peptides were purified by reverse phase HPLC (RP-C18 column, 5 µm, 250 x 16 mm, Macherey Nagel) using a flow rate of 10 mL/min and a linear gradient over 30 min of 0-40% solvent B in A for **R9*** and 1-30% of solvent B in A for **H4***. The purity of the fractions was checked by analytical HPLC (Figure S1). The peptides were characterized by MALDI-TOF MS as described above (Figure S2). **R9***, m/z $[M+H]^+$: 1505.95 (expected), 1505.79 (observed). **H4***, m/z $[M+H]^+$:835.36 (expected), 835.28 (observed). In both cases, an additional peak was observed (Δ m/z: -28), suggesting a fragmentation of the azido group *via* expulsion of N_2 during MALDI-TOF analysis.[29]

2.3 Nanoparticles synthesis

2.3.1 Maghemite nanoparticles γ-Fe_2O_3

The sorted maghemite nanoparticles were obtained by alkaline co-precipitation.[30] A solution of ammonia 22.5% in H_2O (1 L) was added to an acidic iron (II) and iron (III) ions solution (180 g of $FeCl_2$, 100 mL of HCl 37%, 500 mL of deionized (DI) H_2O, 715 mL of $FeCl_3$ 27%) and left at room temperature under stirring for 30 min. After rinsing with DI H_2O, the obtained Fe_3O_4 nanoparticles were redispersed in 360 mL of nitric acid (52%), the solution was agitated for 10 min, and the particles were magnetically separated from the solution. A solution of iron (III) nitrate (323 g) in DI H_2O (800 mL) was then added, and the combined mixture was boiled (150 °C) for 30 min to give γ-Fe_2O_3 nanoparticles.

The resulting nanoparticles were acidified with nitric acid (52%, 360 mL) and washed with acetone (3x, 1 L) and diethyl ether (2x, 0.5 L) before they were redispersed in DI H_2O (1 L), resulting in γ-Fe_2O_3 nanoparticles which are polydisperse in size. To decrease the polydispersity,

a size sorting process was performed.[31] The addition of nitric acid (52.5%, 30 mL) increased the ionic strength, leading to the flocculation of the larger, thus less stable, nanoparticles. These precipitated nanoparticles were separated from the rest of the magnetic fluid, washed with acetone (0.5 L) and diethyl ether (0.3 L), and finally redispersed in DI H_2O. To ensure their stability and dispersion at neutral pH, the nanoparticles were citrated by heating the dispersion (150 °C) with sodium citrate (4.5 g, 0.125 M) for 30 min. After washing with acetone (0.5 L) and diethyl ether (0.3 L), the resulting nanoparticles were dispersed in DI H_2O to give a final iron concentration of 1.07 M.

2.3.2 γ-Fe_2O_3@SiO_2 Core-Shell Nanoparticles CS

Tetraethyl orthosilicate (112 µL, 0.50 mmol TEOS), 30% ammonia solution (250 µL, 3.9 mmol), and aminopropyltriethoxysilane functionalized rhodamine B (18.6 µL, 0.076 µmol APTS) were added to a solution of γ-Fe_2O_3 nanoparticles (125 µL, [Fe] = 0.13 mmol) in 1:2 DI H_2O/ethanol (15 mL). The mixture was agitated for 2 h at RT. The silica shell was functionalized by the addition of TEOS (39.1 µL, 0.18 mmol), trimethoxysilane 3-[methoxy(polyethyleneoxy) propyl] (PEOS) (40.7 µL, 0.075 mmol) and APTS (19.7 µL, 0.084 mmol). The mixture was stirred for 15 h at RT. The resulting nanoparticles were then rinsed with diethyl ether/ethanol 15:1 (3x, 30 mL) and redispersed in 5 mL of a 3-morpholinopropane-1-sulfonic acid (MOPS) buffer (0.1 M, pH = 7.4). The obtained core-shell nanoparticles were characterized by TEM. The iron concentration of **CS** was 24 mM. The suspension was stable at 4 °C for six months.

2.3.3 Dibenzocyclooctyne-Functionalized Core-Shell Nanoparticles DBCO-CS

DBCO(a)-CS and **DBCO(b)-CS** were synthesized by adding 3.8 µL and 1.9 µL dibenzocyclooctyne-PEG_4-*N*-hydroxysuccinimidyl ester (10 mM in dimethylsulfoxyde (DMSO)) respectively to a solution of core-shell nanoparticles **CS** (100 µL, [Fe] = 24 mM) dispersion in MOPS (0.1 M, pH 7.4). The mixtures were incubated for 1 h at RT. The DBCO-functionalized nanoparticles were then applied onto Sephadex G-25 steric exclusion column (PD10 columns, GE Healthcare) and washed with HEPES buffer (0.2 M, pH = 7.4). The iron concentrations of **DBCO(a)-CS** and **DBCO(b)-CS** were 18 mM.

2.3.4 Carboxyfluorescein Peptide-Functionalized Core-Shell Nanoparticles CF-R9-CS

A solution of **CF-R9** peptide in H_2O (19.0 µL, 1.00 mM) was incubated with a suspension of **DBCO(a)-CS** (100 µL, [Fe] = 18 mM) at a molar ratio 2:1 (DBCO:N_3-peptide) at RT for 15 h. **CF-R9-CS** nanoparticles were purified using Nanosep with a cut-off of 10 kDa eluting with EDTA 2 mM in HEPES buffer (0.2 M, pH 7.4). The iron concentration of **CF-R9-CS** was 13 mM. The suspensions were stable for one month.

2.3.5 Carboxyfluorescein Peptide-Functionalized Core-Shell Nanoparticles CF-H4-CS

A solution of **CF-H4** peptide in H_2O (9.5 µL, 1.00 mM) was incubated with a suspension of **DBCO(b)-CS** (100 µL, [Fe] = 18 mM) at a molar ratio 2:1 (DBCO:N_3-peptide) at RT for 15 h. **CF-H4-CS** nanoparticles were purified using Nanosep with a cut-off of 10 kDa eluting with EDTA 2 mM in HEPES buffer (0.2 M, pH 7.4). The iron concentration of **CF-H4-CS** was 13 mM. The suspensions were stable for two weeks.

2.3.6 Bi-Functionalized Nanoparticles cy5/CF-R9-CS

A solution of peptide **CF-R9** (9.5 µL, 1.00 mM) in H_2O was incubated with a suspension of **DBCO(a)-CS** (100 µL, [Fe] = 18 mM) at RT for 5 h, then a solution of cy5-N_3 (9.5 µL, 1.00 mM) in H_2O was added to the suspension at RT and reacted for 15 h. **cy5/CF-R9-CS** was purified using Nanosep with a cut-off of 10 kDa eluting with EDTA 2 mM in HEPES buffer (0.2 M, pH 7.4). The iron concentration of **cy5/CF-R9-CS** was 11 mM.

2.4 Nanoparticles characterization techniques

2.4.1 Atomic Absorption Spectroscopy (AAS)

The total iron concentration was determined by atomic absorption spectrophotometry at 248 nm (PerkinElmer Pinaacle 500). Before AAS measurements, nanoparticles were mineralized using concentrated hydrochloric acid (37%) and then diluted with nitric acid (2%).

2.4.2 Fluorescence spectroscopy

The presence of rhodamine was investigated by fluorescence spectroscopy. The measurements were performed on a fluorescence spectrophotometer (Cary Eclipse). Fluorescence emission spectra were recorded in the range from 550 to 650 nm, using the excitation wavelength at 540 nm (Figure S5).

2.4.3 Transmission Electron Microscopy (TEM)

The morphology and size of nanoparticles were characterized using a JEOL-1011 transmission electron microscope. A droplet of diluted nanoparticle suspension in H_2O was deposited on a carbon-coated copper grid, and the excess was drained using a filter paper. Size analysis was achieved in TEM images using ImageJ software.[32]

2.4.4 Magnetic measurement

A suspension of γ-Fe_2O_3 nanoparticles was introduced in a sample capsule for vibrating sample magnetometry (VSM) analysis (Quantum Design, Versalab). Field-dependent magnetization curves were measured at 300 K as a function of the external field, in the range of -7.10^5 to $+7.10^5$ A/m to obtain saturation magnetization. The magnetic moment recorded (in A/m) can be converted to give the magnetization at saturation of the material expressed in emu/g of γ-Fe_2O_3.

2.4.5 Size and zeta potential analysis

The hydrodynamic diameters and zeta potential of the samples were determined in H_2O (viscosity of 0.8872 cP, refractive index (RI) of 1.330) at 25 °C. Dynamic Light Scattering (Nano ZS, Malvern, United Kingdom) was set up with detection angle at 173° and automatic optimization of conditions.

2.4.6 Fluorescamine assay

APTS standard solutions, 0 µM, 2 µM, 8 µM, 14 µM, 20 µM and 26 µM were prepared in a solution of fluorescamine (0.2 M in EtOH). 20 µL of the nanoparticle suspensions were added in 5 mL of a fluorescamine solution (0.2 M in EtOH). The solutions were kept in the dark for 2 h. Fluorescence emissions were recorded at 480 nm, using an excitation wavelength of 390 nm. Based on the APTS calibration curve, the concentration of amine in each sample was determined.

2.5 Cell culture

Wild type Chinese Hamster Ovary (CHO-K1) cells were cultured in DMEM-F12 culture medium supplemented with 10% fetal bovine serum (heat inactivated FBS), penicillin (100,000 IU/L), streptomycin (100,000 IU/L), and amphotericin B (1 mg/L) in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.6 Live cell imaging of internalized nanoparticles

CHO-K1 cells were seeded in glass-bottom dishes treated beforehand with FBS for 1 h (10 000 cells/well in 200 µL DMEM-F12 containing 10% FBS, µ-Slide 8 Well ibiTreat). Cells were incubated at 37 °C in a 5% CO_2 atmosphere for 48 h before the medium was removed, and new medium containing the specified nanoparticles ([Fe] = 560 µM in 150 µL DMEM-F12) was added. The cells were incubated for a further 2 h or 6 h at 37 °C (for the 6 h incubation experiment, the medium was replaced after 4 h with fresh DMEM-F12 containing no nanoparticles). Cells were then washed twice with culture medium (200 µL) and three times with HBSS (200 µL) before Hoechst 33342 dye (4 µg per well) was added. After incubation for a further 15 min, cells were washed with culture medium (200 µL) before adding HBSS (200 µL). The cells were then immediately imaged on a Leica SP5 confocal microscope using a 63X oil immersion objective lens. When needed, trypan blue (100 µL) was added to the cells before imaging. Final images were generated as a maximum intensity projection of seven Z-stacked images (final image z = 0.9 µm) using FIJI ImageJ.[33] Pearson's correlation coefficient (PCC) values were obtained from coloc2 using FIJI ImageJ. Three representative cell images (> 30 cells) were used to calculate the PCC values, and the results are presented as mean ± standard deviation. The absolute PCC values of 1–0.7 indicate a relatively strong correlation, 0.69–0.36 indicate a moderate correlation.

2.7 Calcein leakage experiments

For monitoring calcein leakage, CHO-K1 cells were seeded in glass-bottom dishes treated beforehand with FBS for 1 h (10 000 cells/well in 200 µL DMEM-F12 containing 10% FBS, µ-Slide 8 Well ibiTreat). Cells were incubated at 37 °C in a 5% CO_2 atmosphere for 48 h, before the medium was removed. The cells were then incubated for 4 h with a new DMEM-F12 medium containing a mixture of the specified nanoparticles ([Fe] = 560 µM in 150 µL) and calcein (160 µM). The medium was then replaced with fresh DMEM-F12 containing no nanoparticles and the cells were incubated for a further 2 h. Treatment with calcein (160 µM in 150 µL) alone was also performed as a control. Cells were then processed and imaged as described above. Mean fluorescence intensity (MFI) was measured using FIJI ImageJ software. Three representative cell images with 10 nucleus/image were used to obtain the MFI for each condition. Data were expressed as mean ± standard deviation. Data were analyzed using one-way analysis of variance

(ANOVA). ****P-value < 0.0001 was considered statistically significant. Statistical analysis was performed using the Prism software package (PRISM 8.0; GraphPad Software, USA).

2.8 LDH Cytotoxicity assay

Cytotoxicity was measured after 6 h or 24 h cell incubation with the specified nanoparticles. CHO-K1 cells were seeded into 96-well flat bottom plates (15 000 cells/well for the 6 h cytotoxicity experiments, 5 000 cells/well for the 24 h cytotoxicity experiments). Cells were incubated at 37 °C in a 5% CO_2 atmosphere for 24 h before the medium was removed, and new medium (DMEM-F12) containing the specified nanoparticles at different iron concentration (0.14; 0.28; 0.56 and 1.12 mM in 60 µL) was added. For the 24 h cytotoxicity experiments, 50 µL of DMEM-F12 containing 5% FBS was added after 6 h cell incubation with the nanoparticles, and cells were incubated for further 18 h. At the end of the incubation times, LDH (Lactate dehydrogenase) release into cell media was analyzed using the CyQUANT™ LDH Cytotoxicity Assay Kit (Invitrogen) according to the manufacturer's protocol. In brief, each sample medium (50 µL) was transferred to a new 96-well flat bottom plate and the reaction mixture (50 µL) was added. After 30 min incubation, the stop solution (50 µL) was added and the absorbance was read at 490 nm and 680 nm. Controls (spontaneous LDH and maximum LDH activity) and percentage cytotoxicity calculations were conducted according to the manufacturer's protocol. Results were converted and represented as cell viability. Experiments were conducted in triplicate.

2.9 Transmission electron microscopy (TEM)

CHO-K1 cells were seeded in 12-well cell culture plates containing a 15 mm coverslip treated beforehand with FBS for 1 h (30 000 cells/well in 500 µL). Cells were incubated at 37 °C in a 5% CO_2 atmosphere for 48 h before the medium was removed, and new medium (DMEM-F12) containing the specified nanoparticles ([Fe] = 560 µM in 500 µL) was added. The cells were incubated for a further 6 h (the culture medium was replaced with fresh medium containing no nanoparticles after 4 h), then washed twice with culture medium (1 mL) and three times with HBSS (1 mL). The cells were fixed with glutaraldehyde (2%) in cacodylate buffer (0.1 M, pH 7.4) at RT for 2 h. The fixed cells were washed five times with cacodylate buffer (0.1 M, pH 7.4). Samples were then postfixed with 1% osmium tetroxide containing 1.5% potassium cyanoferrate on ice for 1 h, gradually dehydrated in ethanol (50% to 100%) and embedded in Agar 100 Epoxy resin. Thin sections (80 nm) were collected onto 200 mesh copper grids, and counterstained with uranyl acetate and lead citrate before examination in either conventional Transmission Electron Microscopy or Scanning Transmission Electron Microscopy (STEM-in-SEM). TEM was realized with a JEM-2100 (JEOL) operating at 80 kV with a LaB6 filament, and images were recorded with a side-mounted 2k x 2k Veleta CCD driven by iTEM software (Olympus). For STEM-in-SEM, counterstained grids were first coated with 2 nm carbon using ACE600 apparatus (Leica microsystems), before being imaged in STEM imaging mode within a Field-Emission Scanning Electron Microscope (FE-SEM) GeminiSEM 500 (Carl Zeiss Microscopy) operated at 20 kV, in bright field imaging mode with a 20 µm aperture (IBPS EM Facility, Sorbonne University, Paris, France).

2.10 Automatic acquisition in STEM-in-STEM and ultrastructural analysis

Previously observed TEM grids were automatically imaged in the STEM imaging mode of the GeminiSEM 500 SEM (20 kV, 20 µm aperture and high current mode) driven by Atlas 5 software (Carl Zeiss Microscopy). An automated mosaic process of acquisition (stitching of 25 adjacent ROI of 24.6 x 24.6 mm FOV) generated a large high-resolution map (FOV 100 x 100 mm, 1,7 Gpix, 3 nm / pixel). All images were acquired by mixing electrons collected simultaneously in Bright Field and in High Angular Annular Dark Field modes.

Two of these maps were examined for **CS** and for **CF-H4-CS**, representing more than 40 cells per condition. The nanoparticles were divided in 5 classes, depending on their location: endosome, multi-vesicular body (MVB), autophagosome, rupture of endosomal membrane and cytosol. Two manual counting were realized using FIJI ImageJ software[33] and Cell Counter plugin (Total of events: 820 for **CS** and 834 for **CF-H4-CS**). Data were analyzed using the χ^2 test of independence in Excel, according to the formula in the equation below (A_{ij}: observed values and E_{ij} theoretical values):

$$\chi^2 = \sum\sum \frac{((A_{ij}) - E_{ij})^2}{E_{ij}}$$

3. RESULTS AND DISCUSSION

3.1 Strategy for the preparation of core-shell nanoparticles functionalized with peptides

Our strategy was to use the strain promoted azide-alkyne cycloaddition (SPAAC) for the conjugation of the peptides to the surface of γ-Fe_2O_3@SiO_2 core-shell nanoparticles (Figure 1). This required the installation of the two mutually reactive functional groups involved in the biorthogonal ligation (azide and strained alkyne) onto the peptide and nanoparticle precursors. The peptides and nanoparticles were also labelled with different fluorophores (fluorescein and rhodamine respectively) to help monitoring the ligation reaction and to study the nanoparticle distribution inside cells by confocal microscopy. We chose to introduce the strained alkyne (DBCO) onto the nanoparticle and the azido group onto the peptide precursors. This avoided overdecoration of the peptides with hydrophobic groups and their attendant poor solubility and poor handling properties.

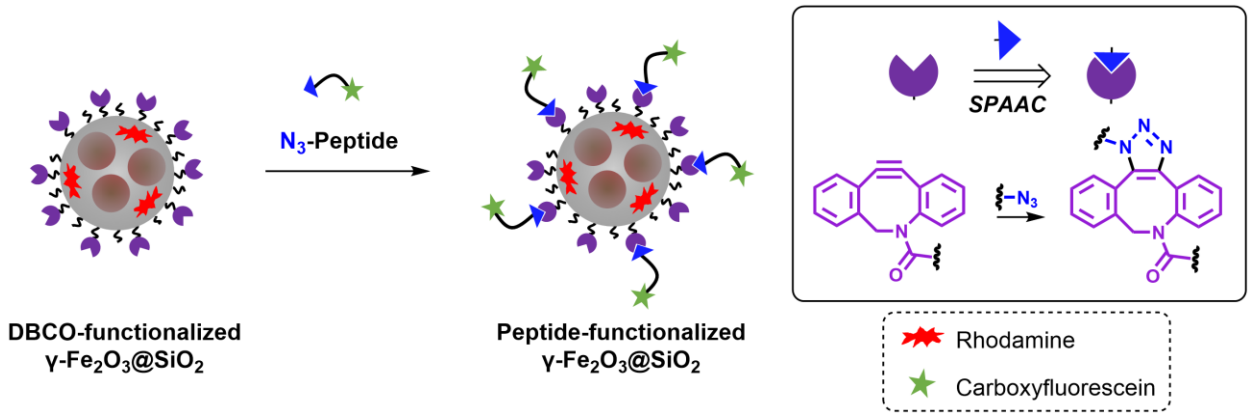

Figure 1. Click reaction between DBCO-functionalized core-shell nanoparticles and azido-functionalized peptides.

3.2 Synthesis and characterization of the azidopeptides

We started with the synthesis of the carboxyfluorescein (CF) labelled azidopeptides **CF-R9** (CF-Ahx-Lys(CO-CH_2-N_3)-$(Arg)_9$-NH_2) and **CF-H4** (CF-Ahx-Lys(CO-CH_2-N_3)-$(His)_4$-Trp-NH_2) (Figure 2a). The peptide segments Arg_9 and His_4-Trp (intermediates **1** and **2**) were first assembled using standard Fmoc solid phase peptide chemistry. Subsequently, a Lys residue orthogonally protected by an Alloc group was coupled to allow the later addition of an azido derivative on the Lys side chain. An aminohexanoic acid (Ahx) spacer was added prior to peptide *N*-terminal functionalization with 5(6)-carboxyfluorescein to afford intermediates **3** and **4**. Selective Alloc removal from the lysine side chain was achieved by palladium catalyzed reductive deprotection to reveal the free amine, which was subsequently reacted with azidoacetic acid in the presence of DIC/HOBt. Both peptides were finally fully deprotected and cleaved from the resin using standard TFA treatment, purified by reverse phase HPLC and characterized by MALDI-TOF mass spectrometry. The desired $[M+H]^+$ ion was observed for both **CF-R9** and **CF-H4** peptides (Figures 2b and S1). We also noted ion peaks (labelled by '◆', Figure 2b) corresponding to a loss of 28 compared to the expected m/z values, most probably due to the fragmentation during MALDI-TOF MS analysis of the azido functionality resulting in the expulsion of N_2, as previously reported.[29]

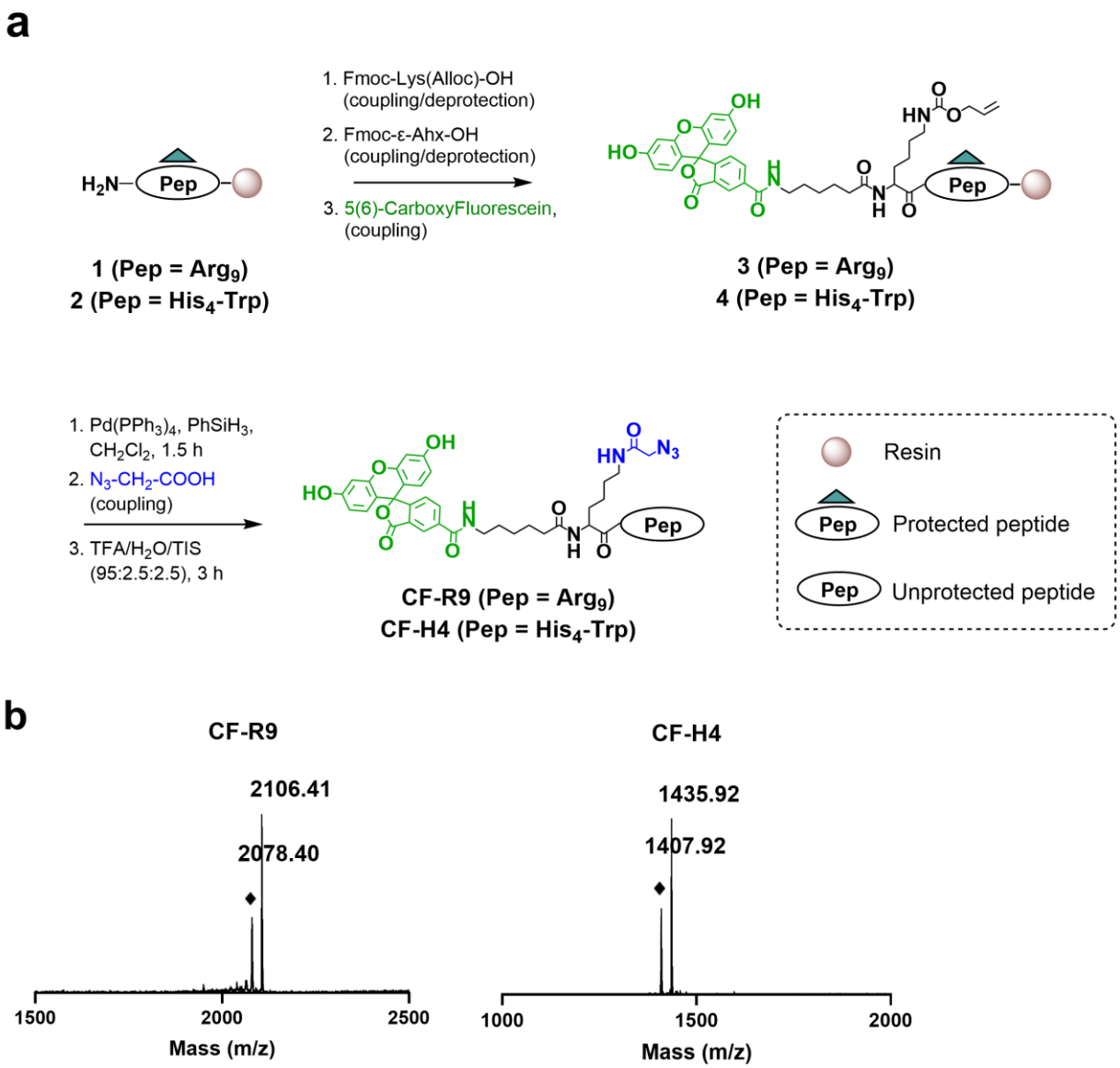

Figure 2. a) Synthesis of the azidopeptides **CF-R9** and **CF-H4**. Note that the peptides have a carboxamide function at their *C*-terminus. Pep = peptide, CF = 5(6)-carboxyfluorescein. **b)** MALDI-TOF mass spectra of **CF-R9** and **CF-H4**. Peaks labelled by ◆ correspond to a loss of 28 compared to the expected m/z values, presumably due to the expulsion of N_2 during MALDI-TOF MS analysis.

3.3 Synthesis and characterization of γ-Fe_2O_3@SiO_2 core-shell nanoparticles

Next, we moved onto the synthesis of rhodamine-labelled DBCO-functionalized nanoparticles **DBCO-CS** by adapting a previously reported procedure.[5] The approach uses amino groups grafted onto the surface of core-shell nanoparticles, that can readily undergo coupling with a *N*-hydroxysuccimidyl (NHS) activated DBCO (Figure 3a). The synthesis started with the preparation of magnetic nanoparticles (maghemite γ-Fe_2O_3) by alkaline co-precipitation of iron salts.[30] After

size sorting, the average diameter of the magnetic cores was determined by TEM images analysis (d_{TEM} = 11.1 nm, σ = 0.17 nm, Figure S3) and their saturation magnetization (M_S) was measured with a Vibrating Sample Magnetometer (MS = 73.1 emu/g, Figure S4). The γ-Fe_2O_3 nanoparticle cores were then co-encapsulated with rhodamine in a thick silica shell by simultaneous treatment with tetraethoxysilane (TEOS) and APTS-rhodamine (Figure S5). Subsequent functionalization at the shell surface with short PEG chains and amino groups yielded γ-Fe_2O_3@SiO_2 core-shell nanoparticles **CS**. The average size of **CS** was measured by TEM images analysis (38.7 nm, σ = 0.18 nm, average over 210 particles, Figure 3b, Table 1), that also confirmed the core-shell structure of the particles. The zeta potential of the **CS** nanoparticles was measured to be +22 mV (Table 1). The density of amino groups at the core-shell surface was determined by a fluorescamine assay (d= 2.0/nm², 3.4 mmol/L). In the final step, a NHS activated strained alkyne was coupled onto **CS** through the primary amine to produce **DBCO-CS**. A short PEG_4 linker was inserted to move the DBCO groups away from the core-shell surface and thus increase their reactivity. The reaction conditions were optimized in terms of NH_2:NHS-PEG_4-DBCO molar ratio. The optimal molar ratio of NH_2:NHS-PEG_4-DBCO was found to be 10:1 for attaching **CF-R9** peptide and 20:1 for attaching **CF-H4** peptide, at which no aggregation of the nanoparticles was observed after conjugation to the peptides.

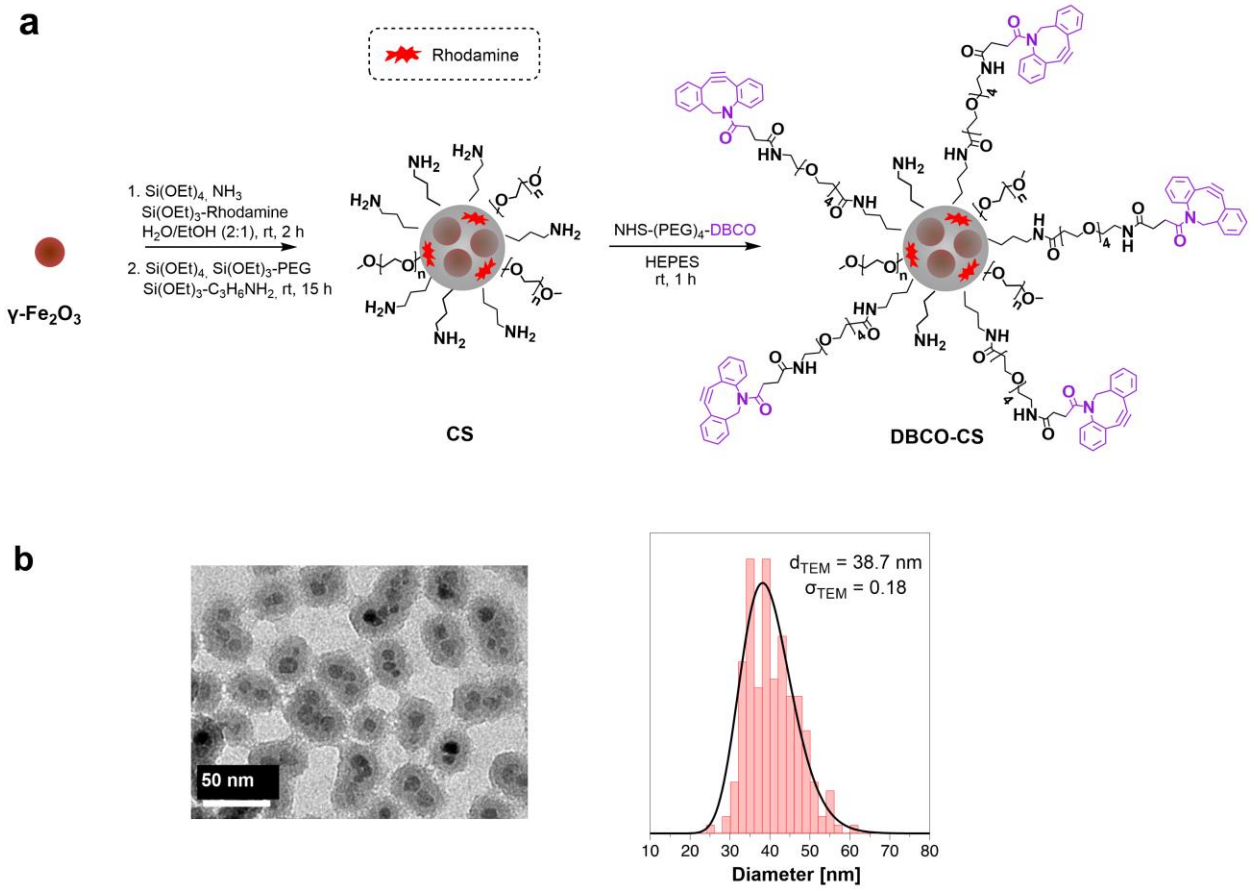

Figure 3. a) Preparation of DBCO-functionalized γ-Fe_2O_3@SiO_2 core-shell nanoparticles **DBCO-CS**. DBCO = dibenzocyclootyne. **b)** TEM image of **CS** and their size distribution. d_{TEM} = average diameter of core-shell nanoparticles determined by TEM analysis.

Sample	Hydrodynamic diameter (nm)[a]	PDI	Zeta Potential (mV)[a]
γ-Fe_2O_3	17.1	0.07	- 46.2
CS	43.2	0.14	+ 21.8
DBCO-CS	47.4	0.18	+ 30.7
CF-R9-CS	57.6	0.14	+ 22.9
CF-H4-CS	52.1	0.14	+ 21.1

[a] Mean (n=6)

Table 1. Summary of hydrodynamic diameter (analysis in number), polydispersity index (PDI), and zeta potential of the different nanoparticles, determined by DLS. The hydrodynamic size distributions of the core-shell nanoparticles are shown in Figure S6.

3.4 Functionalization of core-shell nanoparticles with cationic peptides by SPAAC click chemistry

With **CF-R9, CF-H4** peptides and **DBCO-CS** nanoparticles in hand, we applied the copper-free click reaction to generate the desired peptide-nanoparticle hybrids **CF-R9-CS** and **CF-H4-CS** by formation of a stable 1,2,3-triazole linkage (Figures 1 and 4a). For this, alkyne containing nanoparticles were treated with azidopeptides at a molar ratio of 2:1 (DBCO:N_3-Peptide) in HEPES buffer (pH 7.4). The first attempts to purify the resulting peptide-nanoparticle conjugates were performed by steric exclusion chromatography but removal of non-covalently bound peptide was unsuccessful. This was highlighted by imaging experiments of cells that had been incubated with the resulting sample of **CF-R9-CS**, which showed a weak colocalization between the rhodamine and carboxyfluorescein labels (See supplementary information Figure S7). The purification process was therefore changed to ultrafiltration (Nanosep, 10 kD) and applied to both **CF-R9-CS** and **CF-H4-CS**. Chinese hamster ovary (CHO-K1) cells were incubated with the new nanoparticle samples for 2 h. Confocal microscopy images showed this time an excellent colocalization of rhodamine and carboxyfluorescein, as confirmed by the calculated Pearson's colocalization coefficients[34] (PCC) (PCC = 0.94 ± 0.02 for **CF-R9-CS** and PCC = 0.91 ± 0.03 for **CF-H4-CS**, Figures 4 and S8). This indicated the successful covalent functionalization of the core-shell nanoparticles with the peptides and the complete removal of the unreacted peptide species. The conjugation of **CF-R9** or **CF-H4** peptides slightly increased the hydrodynamic diameter of the nanoparticles (**CF-R9-CS**= 58 nm, **CF-H4-CS**= 52 nm, Figure S6), without significantly affecting their zeta potential (Table 1). The polydispersity index remained below 0.2, indicating a narrow size distribution (Table 1).

This set of experiments confirmed that a covalent strategy is more suited when functionalizing γ-Fe_2O_3@SiO_2 CS with peptides (and in particular with CPPs which can strongly interact with anionic components of the cell membrane), to avoid peptide premature release in cellular context. They also showed that the final step of nanoparticle purification to remove unreacted (non-covalently linked) peptides is then particularly critical. Labeling the core-shell nanoparticles and the peptides with different fluorescent probes and analyzing their colocalization in cells was found to be very useful to optimize the protocol of peptide grafting and to check the efficiency of the purification of the final compounds.

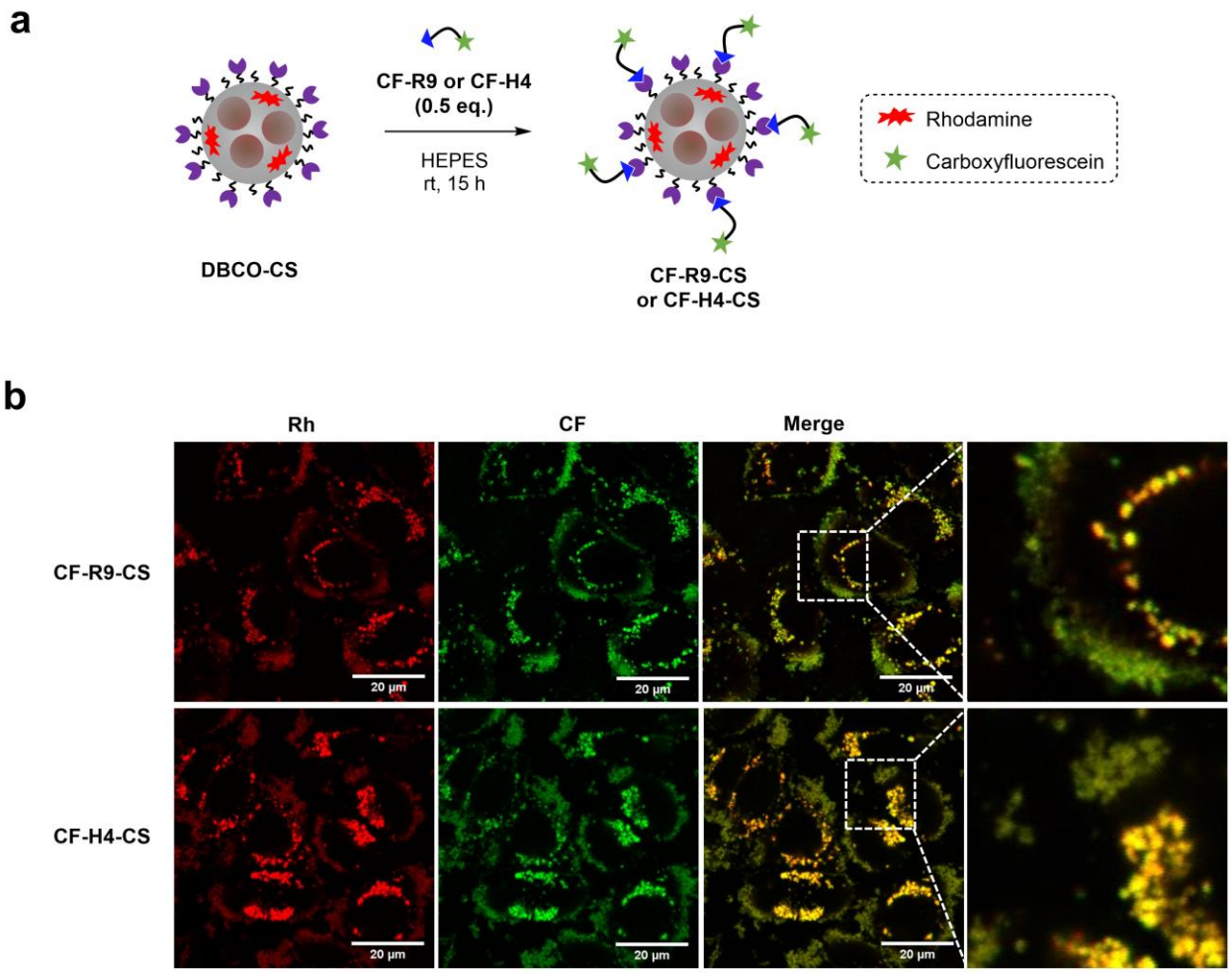

Figure 4. **a)** Click reaction between DBCO-functionalized core-shell nanoparticles **DBCO-CS** and azidopeptide **CF-R9** or **CF-H4**. **b)** Live cell confocal fluorescence microscopy images of CHO-K1 cells incubated for 2 h with **CF-R9-CS** or **CF-H4-CS** ([Fe] = 560 µM) in DMEM-F12 culture medium. Cells were washed twice with culture medium, thrice with HBSS before imaging. Rhodamine (Rh) is shown in red and carboxyfluorescein (CF) in green. Scale bars: 20 µm.

3.5 Simultaneous incorporation of mixed azido-substrates for the generation of bi-functionalized nanoparticles

Next, we explored the versatility of our functionalization methodology by incorporating two different substrates (**CF-R9** and cyanine5) onto the nanoparticle surface. For this, **DBCO-CS** was first conjugated with **CF-R9** by using a higher DBCO:N_3-peptide ratio (4:1), the remaining DBCO sites were then reacted with cyanine5-azide (molar ratio of 4:1 (DBCO:Cy5-N_3) (Figure 5a). The successful addition of the two azido-substrates was confirmed by visualization of all dyes by confocal fluorescence microscopy after CHO-K1 cells incubation with the nanoparticles (Figure 5b). Good Pearson's correlation coefficient (PCC) values were measured between rhodamine/carboxyfluorescein ($PCC_{Rh/CF}$ = 0.76) and rhodamine/cyanine5 ($PCC_{Rh/Cy5}$ = 0.87), respectively, showing the formation of the desired bi-functionalized nanoparticle conjugate

Cy5/CF-R9-CS (Figure 5b). This proof of concept work suggests that our conjugation strategy is compatible with the addition of multiple substrates, and can be used for exploring the synergistic effect of attaching mixed functionalities/modalities to core-shell nanoparticles.

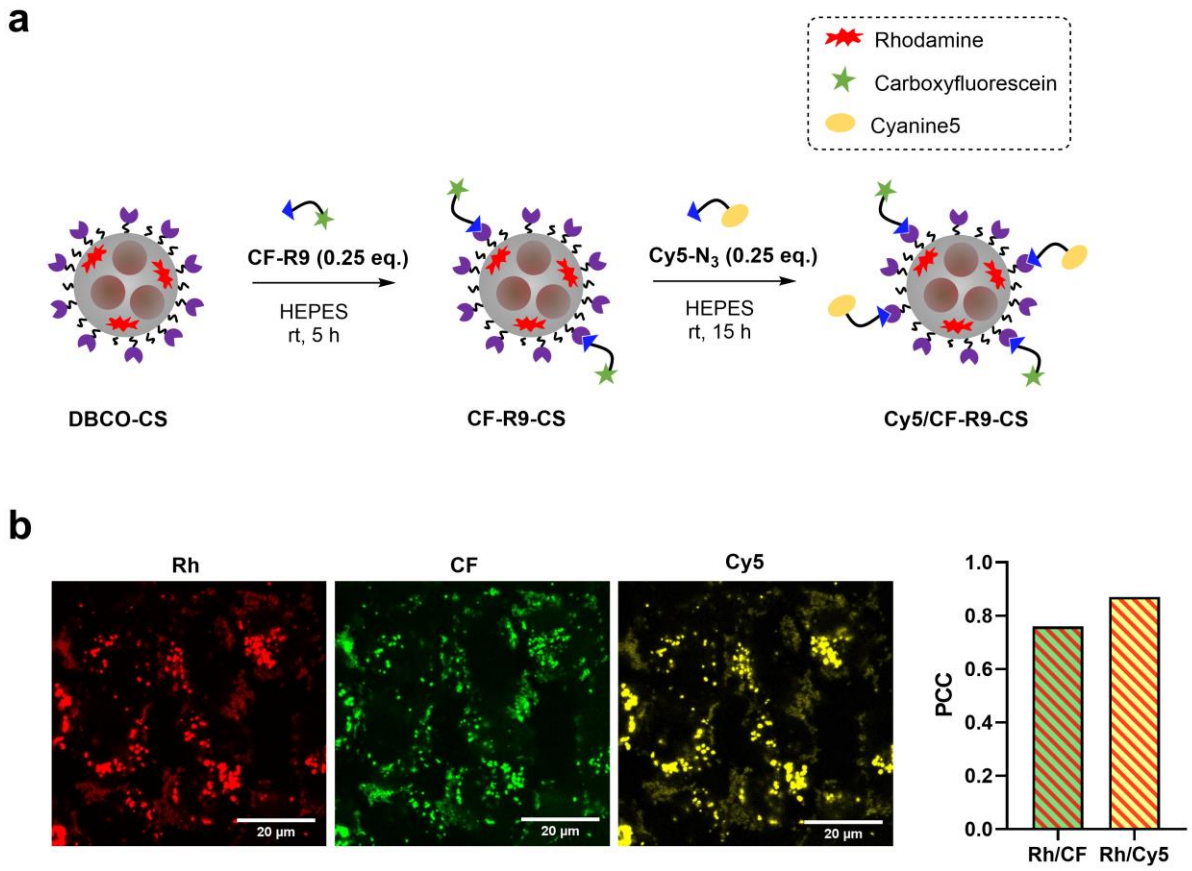

Figure 5. a) Bi-functionalization of **DBCO-CS** with **CF-R9** and Cy5-N_3 by click chemistry. **b)** Confocal fluorescence microscopy images of CHO-K1 cells incubated for 2 h with **Cy5/CF-R9-CS** ([Fe] = 560 µM) (live cell imaging) and measured PCC values. Rhodamine (Rh) is shown in red, carboxyfluorescein (CF) in green and cyanine5 (Cy5) in yellow. Scale bars: 20 µm.

3.6 Assessment of the intracellular distribution of the peptide-functionalized nanoparticles

3.6.1 Visualization of the fluorescently labelled conjugates by live cell confocal microscopy

To investigate if the conjugation of the cationic peptides can promote the nanoparticle access to the cytosol, we analyzed the intracellular distribution of the functionalized core-shell nanoparticles **CF-R9-CS** and **CF-H4-CS** by live cell confocal fluorescence imaging. In a first experiment, cells were imaged directly after they were incubated with the nanoparticles for 2 h and washed with culture medium. This experiment showed for both **CF-R9-CS** and **CF-H4-CS** appreciable levels of punctuate fluorescence surrounding the nucleus, as well as some diffuse fluorescence in the

cells (See supplementary information Figure S9a). In parallel, we also imaged cells that were treated with trypan blue after incubation with the nanoparticles in order to quench the fluorescence of membrane-bound species,[35] as the binding of nanoparticle conjugates to the cell membrane can result in misleading representations of their intracellular distribution. Trypan blue acts as a quenching agent for the green fluorescence of the carboxyfluorescein-labelled nanoparticle conjugates and because of its non-permeant nature, it only quenches extracellular conjugates. However, the use of trypan blue prevents the visualization of the rhodamine fluorescence, due to overlapping of their fluorescence spectra. Upon direct comparison of images from trypan blue treated and untreated cells, we found similar levels of punctuate fluorescence indicative of endosomal localization. In contrast, the diffuse fluorescence was here no longer detectable for the trypan blue treated cells (See supplementary information Figure S9b). We concluded that the addition of trypan blue prior to imaging was a key point in obtaining an accurate representation of nanoparticle localization in cells and that after 2 h incubation, all internalized species were present in endosomes. This showed that both types of nanoparticles enter cells exclusively by endocytosis, the cationic peptides **CF-R9** and **CF-H4** were therefore not able to promote their direct translocation. We reasoned that a longer incubation time could enhance the levels of endosomal escape facilitated by the peptides. Pleasingly, after increasing the incubation period from 2 to 6 h, we were able to observe significant levels of diffuse intracellular fluorescence for the histidine functionalized nanoparticle **CF-H4-CS**, suggestive of endosomal escape (Figure 6). In contrast, no change in the cellular localization was seen for the R9-linked conjugate.

As control experiments, unconjugated **CF-R9** and **CF-H4** peptides were incubated with cells and their intracellular distribution was analyzed (Figure S10). **CF-R9** gave strong punctuate and diffuse fluorescent signals, as expected from literature data.[22,24,36] In contrast, only a very weak punctuate fluorescent signal was observed for **CF-H4**, even after 6 hours incubation with cells, showing that the peptide is only poorly internalized by endocytosis, and is not able to escape from endosomes. Taken together, our experiments suggest that a high concentration of His residues in endosomes is required to induce endosomal escape, this can be reached when the **CF-H4** peptide is conjugated to the core-shell nanoparticles.

In parallel to these experiments, we conducted a lactate dehydrogenase (LDH) release assay after 6 and 24 h of incubation. This assay measures the cytotoxicity by quantifying the release of the cytosolic enzyme LDH into the extracellular environment upon damages to the plasma membrane. The LDH assay showed that the nanoparticle conjugates did not induce any general cytotoxicity in CHO-K1 cells after 6 or 24 h of incubation (See supplementary information Figure S11).

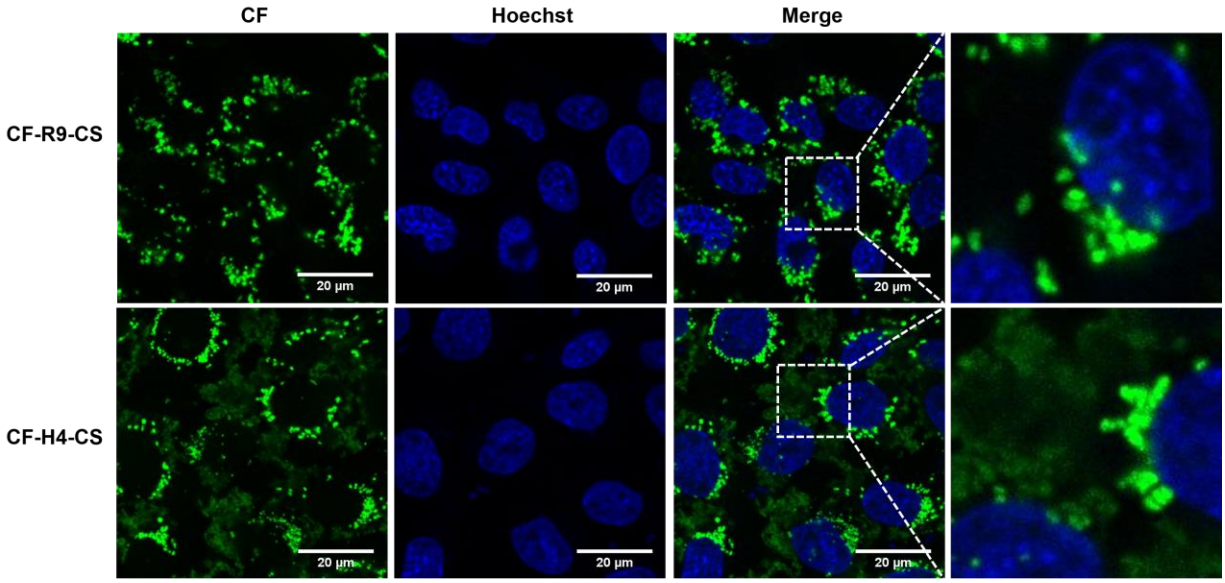

Figure 6. Confocal fluorescence microscopy images of CHO-K1 cells incubated for 6 h with **CF-R9-CS** or **CF-H4-CS** ([Fe] = 560 µM) and then treated with trypan blue (live cell imaging). Carboxyfluorescein (CF) is shown in green and Hoechst dye in blue. Scale bars: 20 µm.

3.6.2 Visualization of intracellular calcein distribution by live cell confocal microscopy

We further investigated the capabilities of **CF-H4-CS** at inducing endosomal escape by tracking the intracellular distribution of co-incubated calcein. Such a method has previously been used to evaluate the ability of nanomaterials in disrupting the stability of endocytic vesicles.[37,38] When incubated alone with cells, the green fluorescent calcein dye is internalized *via* the endocytic pathway and largely found trapped in endosomes. The release of calcein from the endosomes can be used as an indirect measure of vesicle disruption caused by an external stimulus.[39] To avoid competition when visualizing the green fluorescence of calcein, we synthesized peptides **R9*** and **H4***, which are non-fluorescent versions of our cationic azidopeptides (Figure 7a). Both were conjugated to the core-shell nanoparticles. The resulting hybrids **R9*-CS** and **H4*-CS** were then co-incubated with calcein for 6 h in CHO-K1 cells. Analysis of the calcein distribution by live cell confocal microscopy showed that only cells treated with **H4*-CS** displayed significant diffuse fluorescence throughout the cell (suggestive of calcein presence in the cytosol and nucleus), whereas treatment with **R9*-CS** or **CS** alone resulted in only punctuate cytoplasmic fluorescence (Figure 7b). We confirmed this observation by quantifying the mean fluorescence intensity observed in the nucleus upon incubation with the different nanoparticle conjugates, our results show a clear improvement for cells treated with histidine-bound nanoparticles (Figure 7c, control experiments were carried out with treatment of calcein alone). Our data strongly suggest a simultaneous internalization *via* endocytosis of calcein and nanoparticles (as confirmed by co-localization of calcein/rhodamine by live cell confocal fluorescence microscopy, see supplementary information Figure S12), which was followed by endosomal escape promoted by the tetra-histidine motif resulting in calcein redistribution into the cytosol and nucleus.

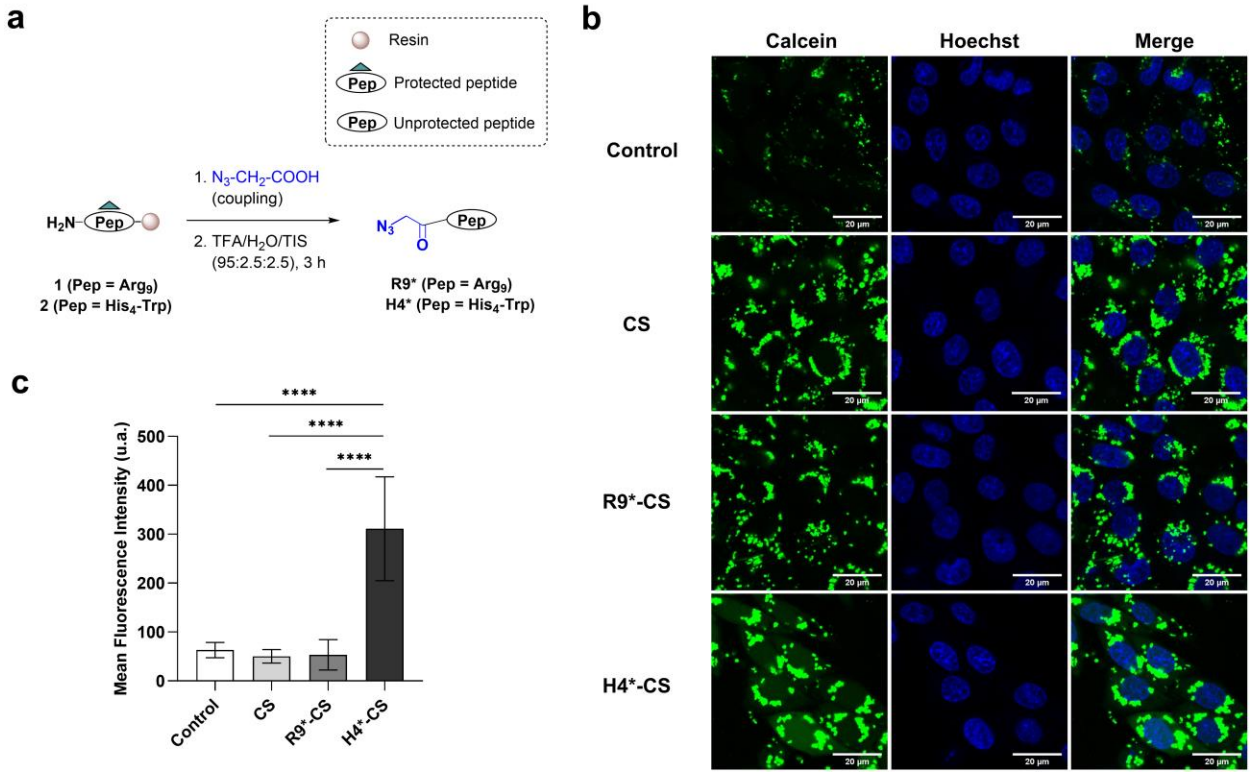

Figure 7. **a)** Synthesis of the azidopeptides **R9*** (N_3-CH_2-CO-$(Arg)_9$-NH_2) and **H4*** (N_3-CH_2-CO-$(His)_4$-Trp-NH_2). Pep = peptide. **b)** Confocal fluorescence microscopy images of CHO-K1 cells co-incubated for 6 h with calcein (160 µM) and **CS**, **R9*-CS** or **H4*-CS** ([Fe] = 560 µM) and then treated with trypan blue (live cell imaging). A control experiment was performed in the same conditions but with calcein alone (160 µM). Calcein is shown in green and Hoechst dye in blue. Scale bars: 20 µm. **c)** Corresponding mean fluorescence intensity of calcein found in the nucleus. Data are represented as mean ± SD (n = 30). ****: $p < 0.0001$.

3.6.3 Direct visualization of the intracellular localization of the core-shell nanoparticles using transmission electron microscopy

Finally, we performed transmission electron microscopy (TEM) and scanning transmission electron microscopy (STEM-in-SEM) on thin sections of fixed cells incubated with the different nanoparticles. This technique gives much higher resolution images compared to confocal microscopy. It allowed to directly identify cellular organelles and to define more precisely NP localization in cells. TEM and STEM images show that the majority of internalized nanoparticles are localized into enlarged endosomes (Figure 8), as described already by Di Corato *et al.* for different nanoparticles.[3] Interestingly, events of rupture of the endosomal membranes with nanoparticles escaping from the damaged endosomes could also be seen in TEM images. More specifically, we could observe nanoparticles being released from zones of endosomes with no visible membrane surrounding (white arrows in Figure 8). Importantly, and in accordance with the data obtained by confocal microscopy which showed that the poly-His peptide when grafted on the core-shell nanoparticles is able to promote endosomal escape, we observed more evidence of

these events of rupture of the endosomal membranes for cells incubated with the histidine functionalized nanoparticles **CF-H4-CS**, as compared to **CF-R9-CS** and **CS** (Figure 8).

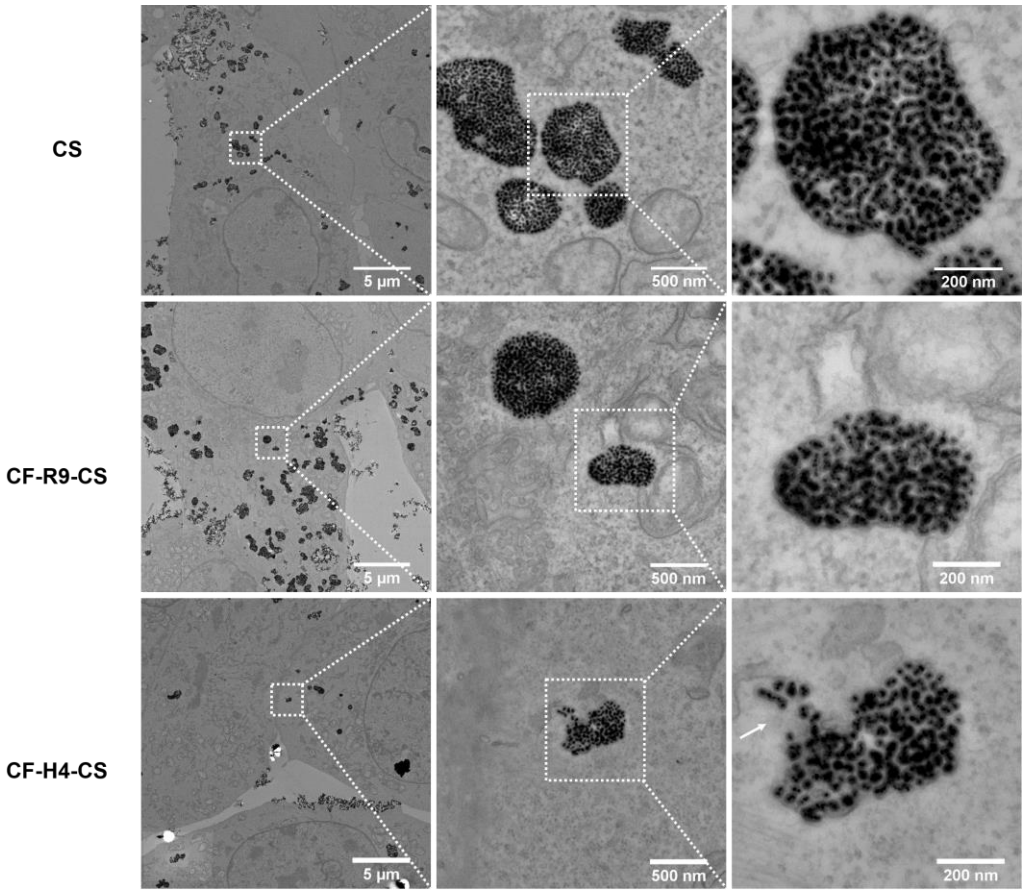

Figure 8. STEM-in-SEM images of CHO-K1 cells incubated with **CS**, **CF-R9-CS** or **CF-H4-CS** ([Fe] = 560 µM) for 6 h. The zoom images show **CS** and **CF-R9-CS** nanoparticles encapsulated in intact endosomes and **CF-H4-CS** nanoparticles escaping from damaged endosomes (in this later case the endosomal membrane is discontinuous, the white arrow points the membrane rupture zone).

To strengthen these preliminary observations, for cells incubated with **CS** or with **CF-H4-CS**, we automatized sample acquisitions using STEM-in-SEM to extensively classify and quantify events associated to the nanoparticles, according to the nanoparticle intracellular localizations (Figure 9). Importantly, in all cellular electron microscopy experiments, we never observed any evidence of mislocalization of NPs caused by sample preparation (*e.g.* NPs on a different focal plan or holes in the sections, both made by a displacement of NPs during ultramicrotomy). In addition, contrast of membranes was enhanced if necessary, to avoid any misinterpretation due to a punctually lack of contrast. We could distinguish five different situations in the STEM images: NPs were present in intact early endosomes, in intact late endosomes/multi-vesicular bodies or in intact autophagosomes, NPs were escaping from endosomal vesicles presenting a rupture of their membrane and finally, NPs were present in the cytosol (Figure 9). More than 800 events were classified into these 5 families for cells incubated with **CS** or with **CF-H4-CS (**Table **S1**).

The analysis of the different internalization events recorded showed a significant difference between the intracellular distributions of **CS** and **CF-H4-CS** (χ^2 test of independence, $p < 3.10^{-13}$). Importantly, we revealed a significant 1.7-fold increase in endosomal membrane rupture for cells incubated with **CF-H4-CS** as compared to **CS** (Figure 9), confirming a higher number of events corresponding to the endosomal escape of NP functionalized with the poly-histidine peptide to reach the cytosol.

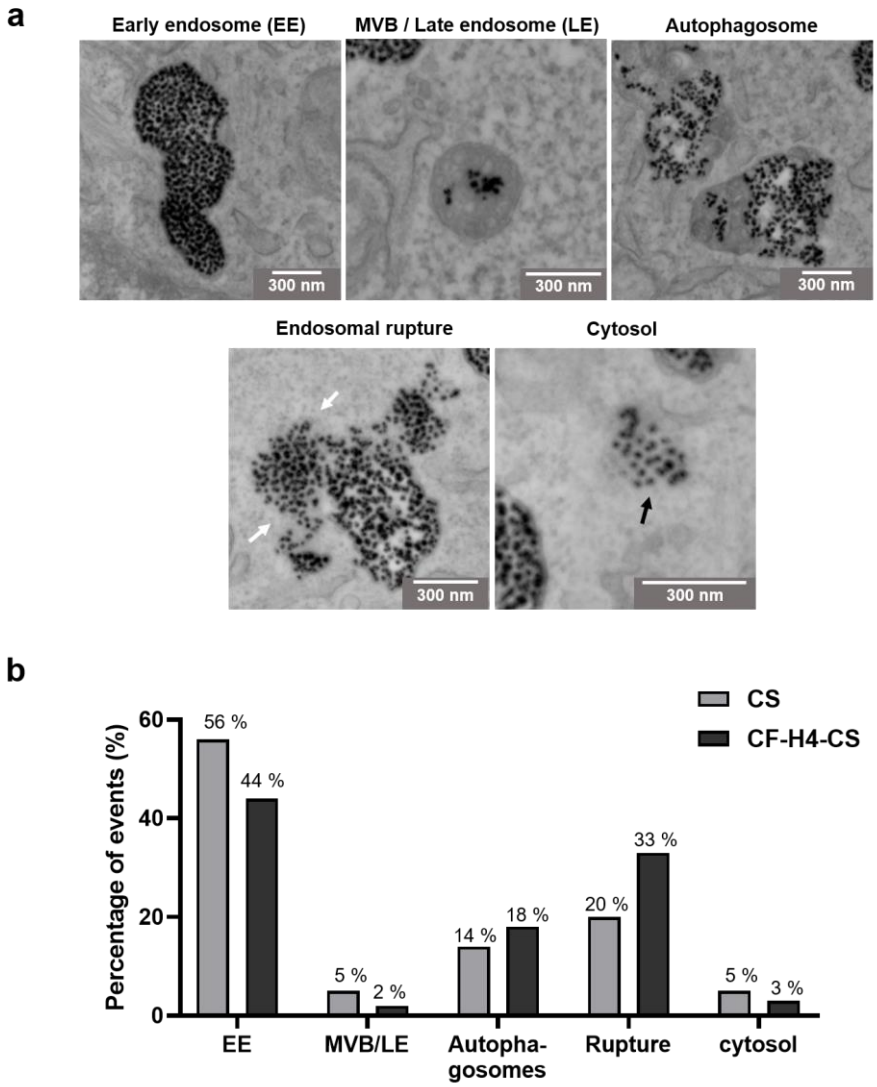

Figure 9. STEM analysis of ultrathin sections of cells incubated with **CS** or **CF-H4-CS** ([Fe] = 560 µM) for 6 h. **a)** Representative examples of STEM images showing: i) NPs in intact early endosomes (EE), ii) NPs in intact multi-vesicular bodies (MVB)/late endosomes (LE) (both characterized by the presence of intraluminal vesicles), iii) NPs in intact autophagosomes (characterized by a double membrane), iv) NPs escaping from endosomes showing a rupture of their membrane (white arrows) and v) NPs free in the cytosol (black arrow). Scale bars: 300 nm. **b)** Intracellular distribution of the nanoparticles (more than 800 events were analyzed per conditions). A χ^2 test of independence was performed showing a high significance difference between the distributions of **CS1** and **CF-H4-CS1**: $\chi^2 = 64.44$, df 4, $p < 3.10^{-13}$.

CONCLUSIONS

We have reported in this study a robust procedure to functionalize Fe_2O_3@SiO_2 core-shell nanoparticles with cationic peptides, using click chemistry. Both starting materials used in the click reaction, core-shell nanoparticle and peptide, were labeled with fluorescent probes. Their successful covalent conjugation could thereby be confirmed by fluorescence microscopy after incubation of the resulting functionalized nanoparticles with cells. Using this procedure, we could prepare well-defined and stable nanoparticles conjugated to a poly-arginine (**CF-R9**) or a poly-histidine (**CF-H4**) peptide.

By combining complementary techniques (live cells fluorescence confocal microscopy, calcein endosomal release experiments and ultrastructural analysis in TEM), we could analyze the process of cellular uptake of the nanoparticles and define their intracellular distribution. Unconjugated core-shell nanoparticles **CS** and nanoparticles conjugated with both types of cationic peptides (**CF-R9-CS** and **CF-H4-CS)** enter cells only by endocytosis, as shown by cell images after 2 hours incubation. Fluorescence observed in cells incubated with the nanoparticles for longer (6 hours), showed again endosomal localization for **CS** and **CF-R9-CS** but, gratifyingly, cytosolic localization was then also observed for **CF-H4-CS**. Calcein release in the nucleus proved the damage of endosomal membranes in the presence of the nanoparticles functionalized with the poly-histidine peptides. Importantly, ultrastructural images (*i.e.* in TEM and in STEM) showing the exact localization of the nanoparticles, confirmed the capacity of the **CF-H4-CS** nanoparticles to escape from endosomes. The His-rich peptide was therefore able to trigger a disruption of the endosomal membrane, most probably by the proton sponge effect. Efficient endocytosis, leading to a high concentration of His residues in endosomes seems to be required for this process to occur. This was achieved by covalent conjugation of the His-rich peptide to the core-shell nanoparticles. Cellular viability assaying demonstrated that the process of endosomal escape of **CF-H4-CS** did not induce toxicity.

Endosomal escape is a bottleneck in therapeutic delivery. The next step of the approach developed in this study will be to show whether the endosomal escape provides a significant therapeutic gain by grafting a cytosol-targeted therapeutic molecule to the surface of the core-shell nanoparticles.

The ability to modify the core-shell nanoparticles with multiple substrates, while retaining their endosomal escape properties, as we have shown, is crucial for their future development. One can imagine to attach a ligand targeting intracellular protein to the nanoparticles and to manipulate them in the cytosol in order to trigger a signalization pathway. This signalization pathway could also be activated by the heat generated by the magnetic nanoparticles under an alternative magnetic field. The simplicity and versatility of the click-chemistry and the non-toxicity of the conjugates **CF-H4-CS** coupled with their capacity to facilitate endosomal escape make them promising tools for magnetic cellular engineering.

ASSOCIATED CONTENT

Supporting Information

Figures S1, S2. Peptide characterization: Analytical HPLC chromatograms and MALDI-TOF mass spectra. **Figures S3 – S6. Magnetic nanoparticles characterization:** TEM image, magnetization curve, fluorescence emission spectrum and size distribution. Figures **S7, S8. Assessment of peptide-nanoparticle conjugation:** Fluorescence microscopy images on fixed cells and Pearson's correlation coefficient (PCC) values. Figures **S9 - S12, Table S1. Intracellular distribution and cytotoxicity studies:** Live cell confocal fluorescence microscopy images and LDH release assay.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interests.

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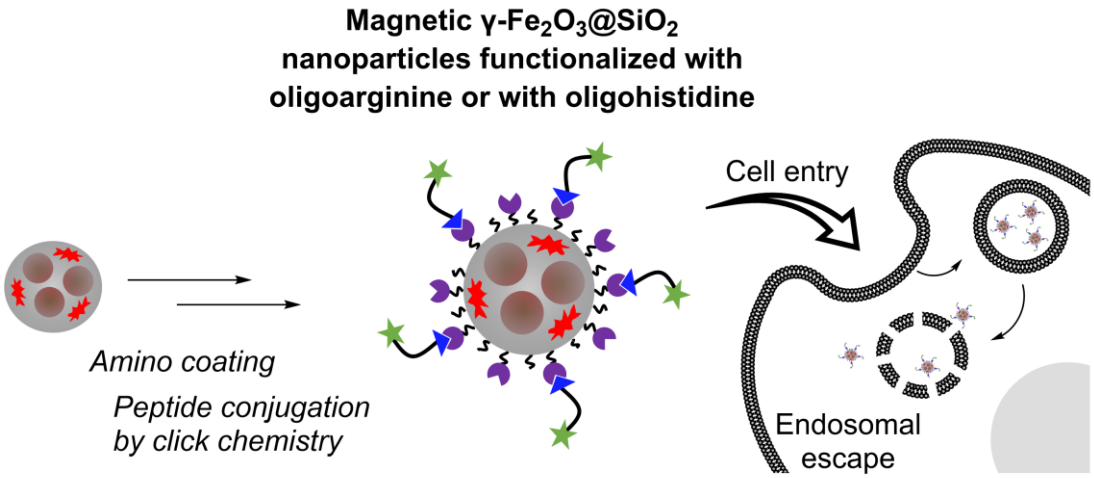
TOC graphic


Magnetic γ-Fe2O3@SiO2
nanoparticles functionalized with
oligoarginine or with oligohistidine
Amino coating
Peptide conjugation
by click chemistry
Cell entry
Endosomal
escape