Metal–Phenolic Coatings as a Platform to Trigger Endosomal Escape of Nanoparticles

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Abstract

The intracellular delivery of functional nanoparticles (NPs) and the release of therapeutic payloads at a target site are central issues for biomedical applications. However, the endosomal entrapment of NPs typically results in the degradation of active cargo, leading to poor therapeutic outcomes. Current advances to promote the endosomal escape of NPs largely involve the use of polycationic polymers and cell-penetrating peptides (CPPs), which both often suffer from potential toxicity and convoluted synthesis/conjugation processes. Herein, we report the use of metal–phenolic networks (MPNs) as versatile and nontoxic coatings to facilitate the escape of NPs from endo/lysosomal compartments. The MPNs, which were engineered from the polyphenol tannic acid and Fe^{III} or Al^{III} , enabled the endosomal escape of both inorganic (mesoporous silica) and organic (polystyrene and melamine resin) NPs owing to the “proton-sponge effect” arising from the buffering capacity of MPNs. Post-functionalization of the MPN-coated NPs with low-fouling polymers did not impair the endosomal escape, indicating the modular and generalizable nature of this approach. We envisage that the ease of fabrication, versatility, low cytotoxicity, and promising endosomal escape performance displayed by the MPN coatings offer opportunities for such coatings to be used for the efficient delivery of cytoplasm-targeted therapeutics using NPs.

Keywords: intracellular trafficking, polyphenols, escape mechanism, bio–nano interactions, surface modification.

Recent advances in colloidal synthesis have enabled the generation of various nanoparticles (NPs) composed of organic, inorganic, or hybrid materials.¹⁻⁴ Owing to their advantages, including tailorable composition, structure, surface chemistry, and functionality, NPs exhibit significant potential for biomedical applications such as imaging,⁵⁻⁷ drug delivery,^{8,9} and therapeutic regimens.^{10,11} However, NPs with diameters from ~50 nm to >500 nm are generally internalized *via* endocytosis and encapsulated in early endosomes (pH ~6.3).^{12,13} Subsequently, some of the NPs are transported to the plasma membrane by fusing with recycling endosomes, while the other NPs remain in early endosomes that are continuously acidified to late endosomes (pH ~5.5). The late endosomes can then either fuse with the plasma membrane to export their contents in the form of exosomes or fuse with lysosomes (pH ~4.7), whose contents become exposed to a variety of degradation enzymes.^{12,14} In the latter case, active biomacromolecules (*e.g.*, proteins, DNA and RNA) transported by the NPs can be readily degraded, which severely reduces the bioavailability of the therapeutics.¹⁵ Endosomal escape has therefore been regarded as a critical “bottleneck” for on-demand intracellular delivery.¹⁶

A number of materials have been developed to overcome sequestration into the lysosome including NPs functionalized with small drug molecules,¹⁷ cationic or pH-responsive polymers,¹⁸ cell-penetrating peptides (CPPs),¹⁸ and liquid-metal transformers.¹⁹ Among these materials, polycationic polymers, such as polyethyleneimine (PEI),²⁰ and CPPs derived from bacterial or viral proteins, such as HIV-derived transactivator of transcription peptides, are the most commonly used materials.²¹ PEI is widely considered as the gold standard to promote endosomal escape owing to its high density of amine groups that can be protonated and can trigger a large inflow of protons, chloride ions, and water into the endo/lysosomes, causing osmotic swelling and membrane rupture known as the “proton-sponge effect”.^{18,21} However, this intrinsic high charge density can also significantly reduce cell viability;²² thus, polycationic materials need to be appropriately engineered to balance the trade-off between escape

efficiency and toxicity.²² In contrast, CPPs generally exhibit low endosomal escape efficiency despite improving cellular uptake significantly.^{21,23} In addition, the release of most CPPs studied to date from endosomes necessitates auxiliary charged polymers, which can lead to increased toxicity.²⁴ Moreover, a large number of CPPs lack cell, tissue, and/or organ specificity, and their cellular uptake and escape mechanisms are still under investigation.^{18,24} Furthermore, it is notable that the conjugation and functionalization of NPs with these endosomal escape polymers/peptides often involves specific sophisticated and time-consuming reaction steps. Therefore, there is still a need for a simple, nontoxic, and general approach for enhancing endosomal escape of NPs.

In 2013, we introduced metal–phenolic networks (MPNs) as a versatile and reproducible method for depositing ~10 nm coating on a range of substrates including planar supports and NPs exhibiting different size, shape, surface charge, and composition.²⁵ The MPNs are obtained *via* rapid (<30 s) assembly through the coordination between phenolic ligands and metal ions.^{25,26} Phenolic ligands can be either naturally occurring or synthetic poly(ethylene glycol)-based polyphenols, and a variety of metals can be used to tailor the functional properties of the MPN-based materials, without significantly changing some materials properties, such as thickness.^{27,28} Of particular relevance to the present study, MPNs display pH-responsive multivalent coordination, which could potentially endow MPNs with pH-buffering capacity. For example, at pH > 7 (*e.g.*, intravenous and cytosolic environments), MPNs are primarily present as the tris-complex state.²⁵ In contrast, the endo/lysosomal vesicles are slightly acidic, which should give rise to predominately the bis-complexation state owing to the protonation of the phenolic molecules.

Herein, we demonstrate that MPN-coated NPs (NP@MPN) readily escape endosomes owing to the “proton-sponge effect” (**Scheme 1**). MPNs assembled from tannic acid (TA) and metal ions, including Fe^{III} and Al^{III}, facilitated the endosomal escape of NPs, both inorganic and

organic NPs. The mechanism of MPN-triggered NP endosomal escape was explored in terms of both the biological and material aspects. The results show that the transition of the dominant TA-Fe^{III} complexation state from tris to bis enabled disruption of the endo/lysosomal membranes by inducing the “proton-sponge effect”. As the MPN-coating strategy is reproducible and substrate-independent,²⁵ it is expected to be widely applicable to a range of nanoparticle systems.

Scheme 1. MPNs Facilitate Endosomal Escape *via* the “Proton-Sponge Effect”

(A) Pristine NPs struggle to escape from endo/lysosomes. (B) MPN-coated NPs (NP@MPN) escape from endo/lysosomes. However, treating cells with bafilomycin A₁ to inhibit proton influx to the endo/lysosomal compartment prevents NP@MPN escape, proving that MPNs trigger escape through the “proton-sponge effect”. (C) Post-functionalizing NP@MPNs with a poly(N-vinylpyrrolidone) (PVPON) layer does not impact the ability to escape the endosome, proving that escape is not mediated by membrane disruption.

Results and Discussion

NP@MPN Formation. MPNs were deposited on polystyrene NPs (PS·NPs), melamine formaldehyde NPs (MF·NPs), and mesoporous silica NPs (MS·NPs) by the assembly of TA/Fe^{III} to form PS·NPs@MPN, MF·NPs@MPN, and MS·NPs@MPN using our previously reported protocol that yields coatings with 10 nm thickness.²⁵ Transmission electron microscopy (TEM) was used to characterize the size and morphology of the NPs before (**Figure 1A–C**) and after (**Figure 1D–F**) MPN coating. The dark blue color of the NP suspensions (**Figure 1H**) following coating confirmed deposition of the MPNs. This was further confirmed by the presence of iron detected from energy-dispersive X-ray spectroscopy (EDX) mapping of the coated NPs (**Figure 1D–F, Figure S1**) and the existence of freestanding films (capsules) after template removal (**Figure S2A**). The appearance of the characteristic ligand-to-metal charge transfer band at ~570 nm also indicated the formation of TA/Fe^{III} complexes (**Figure S2B**).²⁵ Inductively coupled plasma–optical emission spectroscopy (ICP-OES) was used to quantify the number of iron atoms per NP. For example, there are approximately 1.4 million iron atoms per PS·NPs@TA/Fe^{III} (**Table S1**). In addition, TA/Fe^{III} coating of the MS·NPs resulted in a zeta (ζ)-potential shift from -10 ± 10 to -30 ± 9 mV owing to the binding of TA onto the MS·NPs, in agreement with our previous work.²⁵ Similarly, ζ-potentials of PS·NPs and MF·NPs shifted from -25 ± 10 to -46 ± 12 mV and from 48 ± 8 to -31 ± 10 mV, respectively, (**Figure 1G**) after MPN deposition. The TEM images along with the intensity size distribution and polydispersity index (PDI) data (**Table S2**) show that the NPs remain well dispersed after coating with TA/Fe^{III} (insets of **Figure 1D–F**).

Figure 1. TEM images of MS·NPs, PS·NPs, and MF·NPs (A–C) before and (D–F) after TA/Fe^{III} coating. (D–F) EDX mapping showing representative elements from NPs and iron present in the MPN films. (G) ζ -potentials of MS·NPs, PS·NPs, and MF·NPs before (red) and after (blue) MPN coating. (H) Photographs of uncoated (left frames) and TA/Fe^{III}-coated NPs (right frames) suspensions in water. (I) Intensity size distribution profiles of NPs@TA/Fe^{III}, where NPs denote MS·NPs, PS·NPs, or MF·NPs.

Internalization and Cytotoxicity of NPs and NP@MPNs. Pristine NPs and NP@TA/Fe^{III} (MS·NPs@TA/Fe^{III}, PS·NPs@TA/Fe^{III}, and MF·NPs@TA/Fe^{III}) were incubated with MDA-

MB-231, a common human adenocarcinoma cell, for 4 h at 4 °C and 37 °C at a particle-to-cell ratio of 2000:1. After incubation, cells were analyzed by imaging flow cytometry, and cell internalization was quantified by the internalization factor (IF). The latter factor is based on the spatial relationship between bright-field images of cells and fluorescent NPs where a positive IF indicates internalized particles and a negative IF denotes NPs that remain attached to the cell membrane.²⁹ Upon incubation at 4 °C, cells barely engulfed the NPs owing to the inhibition of endocytosis (**Figure S3**), whereas upon incubation at 37 °C, nearly all cells (~100%) displayed positive IF values for both pristine NPs and NP@TA/Fe^{III}, suggesting energy-dependent internalization pathways (**Figure S4**). The concentration-dependent cytotoxicity of pristine NPs and NP@TA/Fe^{III} in MDA-MB-231, Raw 2647 cells, and HeLa cells over 48 h was subsequently examined. As shown in **Figure S5**, the coated NPs displayed negligible cytotoxicity to both cancerous and noncancerous cell lines, which is similar to our previous report,²⁵ thus demonstrating that the materials examined herein are suitable for use in bio–nano systems.

Endo/Lysosomal Escape of NP@MPNs. To investigate the endosomal escape capabilities of NP@MPNs, colocalization analysis of both pristine MS·NPs and MS·NPs@TA/Fe^{III} with endo/lysosomes in MDA-MB-231 cells was conducted. MS·NPs were labeled with fluorescein isothiocyanate (FITC) (the resulting NPs can be seen as green spots in **Figure 2A**), whereas endo/lysosomes were stained with LysoTracker Red. After incubation with the cells for 4 h, most pristine MS·NPs remained inside the endo/lysosomes, as indicated by the yellow dots (overlap of red and green dots) in the merged confocal laser scanning microscopy (CLSM) images. In contrast, colocalization was significantly reduced when analyzing the MS·NPs@TA/Fe^{III}, suggesting that the MPN (TA/Fe^{III}) coating enabled successful endosomal escape of the NPs (**Figure 2A**). Additionally, color scatter plots and corresponding Pearson's correlation coefficient (PCC) values were used to provide a statistical correlation between the

red and green fluorescence signals in the images. Because of the strong positive correlation and high degree of colocalization for pristine MS·NPs with endo/lysosomes, almost every green fluorescent point resided on a red fluorescent point (**Figure 2B**, left frame), and the PCC value was therefore approximately 1 (**Figure 2C**).³⁰ In contrast, MS·NPs@TA/Fe^{III} showed distinct green and red signals (**Figure 2B**, right frame) and the PCC value was reduced to 0.5, indicating a low degree of NP colocalization with endo/lysosomal compartments (**Figure 2C**). The endosomal escape ability of MS·NPs@MPNs was higher than that of MS·NPs coated with PEI (MS·NPs@PEI) with a PEI molecular weight of 800 or 1300 Da but comparable to that of MS·NPs@PEI with a PEI molecular weight of 2000 Da (**Figure S6** and **Table S3**). Next, the kinetics of cell association and colocalization/escape were conducted, and 4 h was determined to be an optimal time point for both association and escape. Thus, all subsequent experiments were conducted at an incubation time of 4 h (**Figure S7**). The presence of MPNs as a coating also enabled the endosomal escape of organic NPs such as PS·NPs and MF·NPs (**Figures 3** and **S8**). In addition to enabling escape for three different types of NPs in MDA-MB-231 cells, MPN coatings also triggered endosomal escape in both Raw cells and HeLa cells (**Figures S9** and **S10**; **Tables S4** and **S5**), highlighting the general applicability of this approach. Moreover, this endosomal escape property was maintained if the coordinating metal was changed from Fe^{III} to Al^{III}, thus allowing further tuning of the degradability of the MPNs (**Figure S11**).^{28,31}

Figure 2. (A) Representative CLSM images of MDA-MB-231 cells incubated with pristine MS-NPs (top row), and MS-NPs@TA/Fe^{III} (bottom row) for 4 h at a particle-to-cell ratio of 2000:1. NPs (green) were fluorescently labeled *in situ* during synthesis. Endo/lysosomes (red) were stained with LysoTrakcer Red. Nuclei (blue) were stained with Hoechst 33342. The dashed lines in the merged images indicate the cellular boundaries based on the corresponding bright-field images. Scale bars are 20 μm. (B) Color scatter plots of pristine MS-NPs and MS-NPs@TA/Fe^{III} vs endo/lysosomes. (C) Corresponding Pearson's correlation coefficient (PCC) values of pristine MS-NPs and MS-NPs @TA/Fe^{III} vs endo/lysosomes (mean ± standard deviation, $n > 50$). **** $P < 0.0001$ with 95% confidence level from unpaired t -test.

Figure 3. (A) Color scatter plots of pristine PS-NPs and PS-NPs @TA/Fe^{III} vs endo/lysosomes. (B) PCC plots of pristine PS-NPs and PS-NPs@TA/Fe^{III} vs endo/lysosomes. (C) Color scatter plots of pristine MF-NPs and MF-NPs@TA/Fe^{III} vs endo/lysosomes. (D) PCC plots of pristine MF-NPs and MF-NPs@TA/Fe^{III} vs endo/lysosomes (mean $\pm$ standard deviation, $n > 50$). *** $P < 0.001$ and ** $P < 0.01$ with 95% confidence level from unpaired t -test.

Endo/Lysosomal Membrane Destabilization Induced by NP@MPN. To further study the membrane stability of endo/lysosomal compartments in cells treated with NP@MPNs, a calcein leakage assay was used.²¹ Calcein is a membrane-impermeable dye that is readily internalized by most cell types *via* micropinocytosis and has been used previously to assess the endosomal escape capabilities of various nanoparticles.²¹ Calcein displays punctuated fluorescence patterns when entrapped in endo/lysosomes, as it cannot escape (**Figure 4A–C**); however, it fluoresces brightly and uniformly when the endosomal membrane is disrupted and it escapes.³² Cells incubated with calcein and pristine NPs (**Figure 4D–F**) showed punctuated fluorescence similar to that observed for cells incubated solely with calcein. In contrast,

incubation with calcein and NP@TA/Fe^{III} resulted in diffuse fluorescence throughout the cell, similar to the result obtained with NP@PEI ($M_w = 2000$ Da), suggesting membrane destabilization of the endo/lysosomal compartments and subsequent calcein leakage into the cytosol (**Figure 4G–I, Figure S12**). The same effect was obtained regardless of the NP type used, indicating the suitability of MPNs as a generally applicable coating strategy to modulate the endo/lysosomal membrane destabilization properties of NPs. Combined with the colocalization studies, the calcein leakage assays confirm that MPNs can destabilize endo/lysosomal membranes and promote the endosomal escape of various NP systems.

Figure 4. CLSM images of MDA-MB-231 cells incubated with (A–C) calcein, (D–F) calcein and pristine NPs, and (G–I) calcein and NP@TA/Fe^{III} for 4 h at a particle-to-cell ratio of 2000:1. NPs were not fluorescently labeled to avoid interference with the calcein signal. Scale bars are 20 μ m.

Mechanistic Insights into MPN-Induced NP Endosomal Escape. We first investigated the biological aspects of the NP@MPN endosomal escape mechanism. We hypothesize that the escape mechanism is related to the “proton-sponge effect” owing to the pH-responsive nature of MPNs and their potential ability to buffer the pH and thereby trigger osmotic rupture and the cytosolic release of the NP@MPNs. Therefore, an inhibitor, bafilomycin A₁, was used to block the membrane-bound, vacuolar-type, proton-translocating ATPases (V-ATPases) and hence terminate the endo/lysosomal acidification process (**Figure 5A**).^{33–35} Calcein was used to track the influence of bafilomycin A₁ on the stability of the endo/lysosomal compartments, and MS·NPs and PS·NPs were used as representative inorganic and organic NPs, respectively. As shown in **Figure 5B**, diffuse fluorescence was observed when the cells were incubated with calcein and NP@TA/Fe^{III}. Nonetheless, the fluorescence weakened and became punctuated when the cells were further treated with bafilomycin A₁, suggesting V-ATPase-driven H⁺ influx was essential to enable NP endosomal escape, likely through osmotic swelling.²⁰ However, as the potential H⁺ buffering capacity of MPNs has not been previously investigated, a further investigation of the physicochemical properties of the MPN coatings was necessary.

Figure 5. (A) Schematic diagram of bafilomycin A₁ inhibiting V-ATPase and termination of the inflow of H⁺ and Cl⁻. (B) CLSM images of MDA-MB-231 cells incubated with calcein, calcein and MS·NPs @TA/Fe^{III}, or calcein and PS·NPs @TA/Fe^{III} in the absence (top row) and presence (bottom row) of inhibitor bafilomycin A₁ for 4 h at a particle-to-cell ratio of 2000:1. PS·NPs and MS·NPs were not fluorescently labeled to avoid interference with the calcein signal. Scale bars are 20 μ m.

We next investigated the material aspects of MPNs that contribute to endosomal escape. The buffering capacity of TA/Fe^{III} complexes was confirmed by conducting titration experiments

in particle suspensions and in solution. Pristine PS·NPs and PS·NPs@TA/Fe^{III} were dispersed in Milli-Q water followed by pH adjustment to ~7 to mimic physiological pH conditions. As shown in **Figure 6A**, the pH of the pristine NPs significantly decreased to less than 5 after adding 1 μ mol HCl, whereas roughly twice as much HCl was required in the presence of NP@TA/Fe^{III} to reach pH 5. In addition, the titration curve of NP@TA/Fe^{III} declined more gradually than that of pristine NPs. These results indicate that NP@TA/Fe^{III} exhibits better buffering capacity within the estimated endosomal “pH change window” (from pH ~7.2 to ~4.7) than the pristine NPs. Similar curves were obtained for the titration of TA/Fe^{III} complexes in solution (**Figure S13A**). That is, the NP@TA/Fe^{III} studied herein can buffer the endosomal acidification and trigger the “proton-sponge effect”.

Polyphenols, including TA, have been reported to complex with various biomolecules (*e.g.*, proteins).³⁶ Therefore, it is possible that the TA molecules alone or those resulting from disassembly of the TA/Fe^{III} complex could disrupt the stability of the endo/lysosomes. To investigate this, a control experiment was conducted using NPs coated with a monolayer of TA.²⁵ Briefly, FITC-labeled MS·NPs, PS·NPs, or MF·NPs were incubated with an excess amount of TA for 12 h to allow sufficient TA deposition followed by washing with Milli-Q water. Both ζ -potential measurements and Fourier-transform infrared (FTIR) spectroscopy analysis were performed to confirm the deposition of TA on the surface of the NPs (MS·NPs, PS·NPs, and MF·NPs). Following TA adsorption on the different NPs, ζ -potential of the NPs shifted to within a range of –20 to –30 mV (**Figure 6B**). Relative to the FTIR spectra of the pristine NPs, those of the TA-coated NPs (NP@TA) (air-dried) were shifted and featured TA-characteristic bands (O–H stretching, C–O stretching, C=O stretching vibration), further confirming the presence of TA (**Figure S13B**).^{28,37} Colocalization results of NP@TA with endo/lysosomes (**Figures 6C and S14, Table S6**) together with the calcein assays (**Figure S15**) illustrated that TA alone did not induce endosomal escape. Moreover, from the titration

curves shown in **Figure S13A**, TA has a weaker buffering capacity than TA/Fe^{III} complex in solution, which further confirms that TA alone cannot buffer enough proton inflow to trigger endosomal escape.

Figure 6. (A) Titration curves of NP and NP@TA/Fe^{III} (PS-NPs were used as a representative NP) in suspension as a function of HCl addition. (B) ζ -potentials of pristine NPs and NP@TA. (C) Merged CLSM images of MDA-MB-231 cells incubated with MS-NPs@TA, PS-NPs@TA, or MF-NPs@TA for 4 h at a particle-to-cell ratio of 2000:1. NPs (green) were fluorescently labeled with FITC. Endo/lysosomes (red) were stained with LysoTrakcer Red. Nuclei (blue) were stained with Hoechst 33342. The dashed lines indicate the cell boundary based on corresponding bright-field images. Scale bars are 20 μ m.

To investigate whether TA/Fe^{III} could also trigger escape through membrane interactions, a nonionic polymer (poly(*N*-vinylpyrrolidone), PVPON) layer was deposited onto the

NP@MPN to modify the surface chemistry and block any nonspecific physical interactions of TA without hindering the flow of protons. PVPON is a suitable polymer candidate as it can associate with TA through hydrogen bonding and has been shown to reduce the fouling and protein adsorption of NPs.^{38–40} The presence of PVPON on the NPs was confirmed by changes in ζ -potential values and FTIR spectra. Compared with the ζ -potential of pristine NPs and NP@MPNs, that of the PVPON-coated NP@MPNs (NP@MPN-PVPON) was less negative, owing to the inherent neutrality of PVPON (**Figure 7B**). Moreover, the vibration at 1661 cm^{-1} , which is indicative of C=O stretching, in the FTIR spectra of MS·NPs@MPN-PVPON and PS·NPs@MPN-PVPON demonstrated the presence of PVPON (**Figure 7C, D**).⁴¹ The NP@MPN-PVPON was incubated with cells to study their colocalization with acidic vesicles and potential to escape (**Figure 7A**). Neither PVPON-coated NPs (NP@PVPON) nor PVPON-TA-coated NPs (NP@PVPON-TA) escaped from endo/lysosomal compartments (**Figure 7E, Figure S16, Table S7**). However, when NP@MPNs are coated with a layer of PVPON (NP@MPN-PVPON), they could still escape (**Figure 7E, Table S7**), indicating that MPNs assist endosomal escape through the “proton-sponge effect” rather than through membrane interactions. These results also reveal the versatility of MPNs, in that the benefit of endosomal escape can be retained even after subsequent surface modification.

Figure 7. (A) Schematic diagram of NP@MPN-PVPON escaping from the endo/lysosomal compartment. (B) ζ -potentials of pristine NPs (uncoated), and PVPON-coated NPs (“@PVPON”), MPN-coated NPs (“@MPN”), and MPN-PVPON-coated NPs (“@MPN-PVPON”). (C) FTIR spectra of PVPON, MS-NPs, MS-NPs@PVPON, and MS-NPs@MPN-PVPON. (D) FTIR spectra of PVPON, PS-NPs, PS-NPs@PVPON, and PS-NPs@MPN-PVPON. (E) Merged CLSM images of MDA-MB-231 cells incubated with NP@PVPON and

NP@MPN-PVPON (NP refers to MS·NPs or PS·NPs) for 4 h at a particle-to-cell ratio of 2000:1. MPN refers to TA/Fe^{III}. Scale bars are 20 μ m.

Conclusions

We have introduced the use of MPN coatings as a facile, universal, and low cytotoxic approach to enhance the endosomal escape of both inorganic and organic NPs. Pristine and TA-coated NPs did not escape endo/lysosomal compartments following internalization by cells, demonstrating that the combination of TA with a metal was essential for escape. In contrast, the presence of MPN coatings on NPs enabled them to escape through the “proton-sponge effect”. From a biological perspective, in the presence of a v-type ATPase inhibitor, TA/Fe^{III}-coated NPs were no longer able to escape. This result suggests that MPNs buffer the influx of protons to cause osmotic rupture of the membrane. From a material perspective, TA/Fe^{III}-coated NPs had better buffering capacity than pristine NPs within the “endosomal pH change window”. In addition, a shielding layer of PVPON did not hamper the endosomal escape abilities of MPNs. The ability to modify MPNs, while retaining their endosomal escape properties, is crucial for the future development of MPN-based nanoparticle systems and is a fundamental step toward *in vitro* and *in vivo* application. Endosomal escape is a bottle-neck in therapeutic delivery, and the present investigation on the inherent escape properties of MPNs helps advance our understanding of MPNs for various biomedical applications.^{28,31,42} The simplicity, versatility, and negligible cytotoxicity of MPNs, coupled with their capacity to facilitate endosomal escape make MPN coatings promising for engineering the cytosolic delivery of functional NPs.

Experimental Section

Materials. FITC, (3-aminopropyl)triethoxysilane (APTES), cetyltrimethylammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), triethanolamine (TEA), sodium salicylate (NaSI), iron(III) chloride hexahydrate (FeCl₃·6H₂O), aluminum chloride hexahydrate

(AlCl₃·6H₂O), TA, 3-(*N*-morpholino)propane sulfonic acid (MOPS), Dulbecco's phosphate-buffered saline (DPBS), phenazine methosulfate (PMS), and PVPON (10 kDa) were purchased from Sigma-Aldrich (USA). 2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxyanilide inner salt (XTT), caclein (high purity), Dulbecco's modified Eagle's medium (DMEM), LysoTacker Red DND-99, trihydrochloride (Hoechst 33342, 10 mg/mL), and Alexa Fluor 594-wheat germ agglutinin conjugate (AF594-WGA) were obtained from Life Technologies (USA). Bafilomycin A₁ was purchased from InvivoGen (USA). PS·NPs and MF·NPs (with and without fluorescence labeling) were purchased from MicroParticles (GmbH). All chemicals were used as received without further purification. The pH values of the solutions were measured with a Mettler-Toledo MP220 pH meter. Milli-Q water with a resistivity greater than 18.2 MΩ cm was obtained from a three-stage Millipore Milli-Q plus 185 purification system (Millipore Corporation, USA). All aqueous solutions were filtered with 220 nm diameter membranes before use.

Minimum Information Reporting in Bio–Nano Experimental Literature (MIRIBEL). The studies conducted herein, including material characterization, biological characterization, and experimental details, conform to the MIRIBEL reporting standard for bio–nano research,⁴³ and we include a companion checklist of these components in the Supporting Information.

Synthesis of Fluorescently Labeled Mesoporous Silica Nanoparticles. Fluorescently labeled dendritic mesoporous silica nanoparticles were synthesized from the modified one-pot synthesis reported by Yang *et al.*⁴⁴ Briefly, 2.7 mg of FITC was dissolved in 400 μL of APTES and incubated for 3 h. TEA (68 mg) was dissolved in 500 μL of Milli-Q water and transferred to a flask containing 25 mL of Milli-Q water in an oil bath at 80 °C with magnetic stirring for 20 min. Then, 380 mg of CTAB and 168 mg of NaSI were added to the flask, and the mixture was stirred for 30 min. FITC-APTES solution was subsequently added to the flask and stirred for 5 min. Formation of nanoparticles was then initiated by introducing 4 mL of TEOS to the

above mixture, and the final product was obtained after reaction for 2 h with moderate stirring (~350 rpm). The product was purified by high-speed centrifugation (10000 g, 10 min) and washing thrice with ethanol. Residual surfactant was removed by extracting the collected product with HCl/methanol solution at 60 °C in an oil bath with gentle stirring for 6 h. Subsequently, the sample was washed three times with ethanol (10000 g, 10 min) and the final product was obtained after drying the sample in a furnace overnight at 80 °C.

TA/Fe^{III} Coating on Nanoparticle Templates. The assembly of TA/Fe^{III} coating on the nanoparticle template was based on our previous report.²⁵ First, 200 µL of MS·NPs (2 mg/mL) or 50 µL of PS·NPs was washed twice with Milli-Q water by centrifugation (10000 g, 10 min) and subsequently dispersed in 440 µL of Milli-Q water. Then, 3 µL of TA solution (24 mM) and 3 µL of FeCl₃·6H₂O (37 mM) were successively added to the particle suspension at room temperature (25 °C) followed by brief vortex and sonication after each addition. The pH was then raised by adding 500 µL of MOPS (20 mM, pH 7.4), and the suspension was thoroughly vortexed for 5 min to allow sufficient film formation and adherence. The obtained TA/Fe^{III}-coated NPs (NP@TA/Fe^{III}) were washed three times with Milli-Q water to remove excess material by centrifugation (10000 g, 10 min). During each washing step, the supernatant was removed, and the pellet was vortexed and sonicated for 30 s to avoid particle aggregation. After the final Milli-Q wash, the pellet was resuspended in 500 µL of Milli-Q water for future experiments. TA/Fe^{III} coating on MF·NPs was obtained using the same protocol except that centrifugation was conducted at 3000 g and for 3 min.

TA/Al^{III} Coating on Nanoparticle Templates. The preparation of TA/Al^{III}-coated NPs (NP@TA/Al^{III}) was modified from our previous report.²⁸ The coating steps were the same as the protocol provided in the previous section except that MOPS (100 mM, pH 8) and AlCl₃·6H₂O (24 mM) were used instead.

Material Characterization. The structure and elemental profiles of the nanoparticles were analyzed by TEM and EDX on a FEI Tecnai TF20 instrument at an operation voltage of 200 kV. To ensure sufficient signal for the element mapping analysis, the acquisition time was set to more than 50 min. For TEM characterization, nanoparticle suspensions were air-dried on formvar-coated gold grids. The ζ -potential, intensity size distribution, and PDI of the nanoparticles were measured in Milli-Q water on a Zetasizer Nano-ZS instrument (Malvern Instrument, UK). FTIR spectra of the freeze-dried nanoparticles were recorded on a Tensor II FTIR spectrophotometer (Bruker). UV–Vis absorption measurement was carried out on a Varian Cary 4000 UV–Vis spectrophotometer.

Quantification of Iron per Nanoparticle. ICP-OES and flow cytometry (Apogee A50-Micro flow cytometer) were used to determine the content of iron in the NPs. First, an iron ICP standard solution was diluted to 0.1, 0.5, 1, 5, and 10 ppm with 5% Suprapur nitric acid (HNO_3) to construct calibration curves. Four batches of TA/ Fe^{III} -coated MS·NPs, PS·NPs, and MF·NPs were prepared following the protocol described above. Nanoparticle concentration was determined by flow cytometry. To determine the content of iron, 1 mL 65% HNO_3 was added to 2 mL of NPs@TA/ Fe^{III} suspension and incubated for 1 h to disassemble the MPNs. Then, the NPs were spin down (10000 g, 10 min) and 2 mL of the supernatant was collected and diluted with Milli-Q water to 5% HNO_3 for ICP analysis.

Cell Culture. Human breast adenocarcinoma cell line, MDA-MB-231, was purchased from the American Type Culture Collection (USA) and used in all cell experiments. MDA-MB-231 cells with passage number from 20 to 47 were cultured in complete DMEM supplied with 10% fetal bovine serum (FBS) at 37 °C, 5% CO_2 , and 95% humidity.

Cell Internalization Assay. MDA-MB-231 cells were seeded in a 6-well plate (Costar3516, Corning, USA) at a density of 200000 cells per well in 2 mL of DMEM supplied with 10% FBS for 18 h. After incubation, the culture media was aspirated and replaced with 1 mL of

fresh media to reduce the sedimentation distance for NPs. Then, pristine and TA/Fe^{III}-coated fluorescent MS·NPs, PS·NPs, or MF·NPs were incubated with the cells for 4 h at a particle-to-cell ratio of 2000:1. Following incubation, the cells were gently washed twice with DPBS, detached from culture flask with trypsin, and collected by centrifugation (400 g, 5 min). The cells were then fixed with 200 μ L of 4% paraformaldehyde for 20 min at room temperature, and the membrane was stained with AF594-WGA (5 μ g/mL) for 5 min. Finally, the cells were further washed twice with DPBS and resuspended in 100 μ L of DPBS.

Cell internalization assay was conducted by imaging flow cytometry (AMNIS ImageStream[®]X MarkII, Amnis Corporation, USA) and 5000 cells were analyzed for each sample. The built-in internalization wizard of Amnis ImageStream IDEAS software was used to evaluate the internalization on focused single cells statistically.

Colocalization by Confocal Laser Scanning Microscopy. MDA-MB-231 cells were seeded in the 8-well Lab-Tek-Chamber slide at a density of 30000 cells per well in 400 μ L of DMEM supplied with 10% FBS for 18 h. After incubation, the culture media was removed and replaced with 200 μ L of fresh media to reduce the sedimentation distance for NPs. Fluorescently labeled MS·NPs, PS·NPs, or MF·NPs with and without MPN (TA/Fe^{III}) coating were subsequently introduced and incubated with cells for 4 h. To investigate the endosomal escape of the NPs, the samples were washed gently twice with DPBS to remove excessive NPs, and LysoTracker Red was added to the culture media to obtain a final concentration of 100 nM and incubated for 1 h following the supplier's protocol for endo/lysosomes staining. Cells were gently washed three times with DPBS and incubated with Hoechst 33342 (1 μ g/mL) for 10 min to stain the nucleus. Finally, the cells were live-imaged using a Nikon A1R confocal microscope with a 40 $\times$ water immersion objective. PCC and color scatter plots were obtained from WCIF ImageJ software. The experiments were repeated in triplicates, and five representative cell images (>50

cells) were used to calculate the PCC values and results are presented as mean $\pm$ standard deviation.

Calcein Leakage Assay to Monitor Endo/lysosomal Membrane Stability. MDA-MB-231 cells were seeded in 8-well Lab-Tek-Chamber (Costar, Corning, USA) at a density of 30000 cells per well in 400 μ L of DMEM supplied with 10% FBS for 18 h. Then, the culture media was aspirated and replaced with 180 μ L of fresh media to reduce the sedimentation distance for NPs. Calcein (20 μ L, 1.5 mg/mL in DPBS) was added to each well to obtain a final concentration of 150 μ g/mL in the presence or absence of NPs (pristine, TA-coated, and TA/Fe^{III}-coated MS·NPs, PS·NPs, or MF·NPs) and incubated with cells for 4 h. Non-fluorescent NPs (same size and shape) were used in this assay to avoid interference with the calcein signal. After incubation, four DPBS washing steps were applied to remove extracellular calcein and NPs. Finally, cells were imaged and captured by CLSM.

Mechanism Study with Bafilomycin A₁. MDA-MB-231 cells were seeded in 8-well Lab-Tek-Chamber (Costar, Corning, USA) at a density of 30000 cells per well in 400 μ L of DMEM supplied with 10%FBS for 18 h. Then, the culture media was replaced with 180 μ L of fresh media or media containing bafilomycin A₁. Calcein (20 μ L, 1.5 mg/mL) was added to each well to obtain a final concentration of 150 μ g/mL for calcein and 100 nM for bafilomycin A₁. TA/Fe^{III}-coated and nonfluorescently labeled MS·NPs and PS·NPs as representatives of inorganic and organic NPs were incubated with the treated cells for 4 h at a particle-to-cell ratio of 2000:1. After incubation, the samples were gently washed four times with DPBS to remove excess materials. Finally, cells were imaged and captured by CLSM.

Titration Experiments for Solutions of Nanoparticles and Complexes. For titration in nanoparticle suspensions, 10 batches of PS·NPs@TA/Fe^{III} were prepared by the protocol discussed in *TA/Fe^{III} Coating on Nanoparticle Templates*, but finally combined and dispersed in 5 mL Milli-Q water. The same number of pristine PS·NPs were washed twice with Milli-Q

and finally dispersed in 5 mL Milli-Q water as a control. The pH was subsequently adjusted to ~7 to mimic physiological pH. Then, titration was conducted by adding 0.1 M hydrochloric acid (HCl) dropwise to the nanoparticle suspensions followed by stirring, and the corresponding pH was measured using a Mettler-Toledo MP220 pH meter.

For titration in TA/Fe^{III} solution, 30 μ L of TA solution (24 mM) and 30 μ L of FeCl₃·6H₂O (37 mM) were successively added to 5mL of Milli-Q followed by stirring. A further 5mL of Milli-Q water was prepared as a control (blank), and the titration steps as described above were followed.

Mechanism Study with PVPON Shielding. TA/Fe^{III}-coated and fluorescently labeled MS·NPs and PS·NPs were synthesized as per the protocol used for TA/Fe^{III} coating on NP templates, as described previously.

The PVPON-coated NPs were prepared as follows. First a stock solution of PVPON (10 kDa) dissolved in Milli-Q water (20 mg/mL) was prepared. Then, 50 μ L of MS·NPs or PS·NPs was incubated with 50 μ L of PVPON (20 mg/mL) overnight. To prepare the PVPON-TA-coated NPs, 50 μ L of TA (24 mM) and 50 μ L of PVPON (20 mg/mL) were successively added to 50 μ L of MS·NPs or PS·NPs suspension with vigorous stirring. For MPN (TA/Fe^{III})-PVPON-coated NPs, 50 μ L of PVPON was incubated with 50 μ L of MPN-coated NPs. All samples were incubated overnight with stirring followed by washing thrice with Milli-Q to remove the excess material.

MDA-MB-231 cell seeding and staining, nanoparticle feeding, and investigation of endosomal escape were conducted as per the above protocol discussed in *Colocalization by Confocal Laser Scanning Microscopy*.

Cell Viability by XTT Assay. MDA-MB-231 cells were seeded in a 96-well plate (Costar, Corning, USA) at a density of 8000 cells per well in 100 μ L of DMEM supplied with 10% FBS for 18 h. Then, the culture media was removed and replaced with fresh media (for cell control)

and fresh media containing uncoated and TA/Fe^{III}-coated MS·NPs, PS·NPs, or MF·NPs at a particle-to-cell ratio from 100:1 to 5000:1 for 48 h. After the treatment, the media was replaced with fresh media containing activated XTT (9 mL of 0.2 mg/mL XTT in complete DMEM could be activated by adding 22.5 μ L 0.6 mg/mL PMS in DPBS), and cells were further incubated for 3 h. Finally, cells were screened on an Infinite M200 microplate reader (Tecan, Switzerland); absorbance readings were measured at 475 nm, and a reference wavelength of 675 nm was used. Cell viability was expressed as a percentage by normalizing absorbance to untreated cells. All experiments were performed quadruplicates and data are presented as mean $\pm$ standard deviation.

ASSOCIATED CONTENT

Supporting Information

EDX mapping images, TEM image of TA/Fe^{III} capsules, cell internalization data at 4 and 37 °C, cytotoxicity data, CLSM images, ζ -potential, colocalization and color scatter plot of TA/Al^{III}-coated NPs, FTIR spectra, Pearson's correlation coefficients, PDI, intensity size distribution, and MIRIBEL checklist for reporting research in bio–nano science. This material is available free of charge *via* the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

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