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ORIGINAL RESEARCH

An AIEgen-Based Carrier Enables Efficient Cytosolic Delivery of Bioactive Proteins

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Background: Cytosolic protein delivery enables the direct transport of exogenous proteins into the cytoplasm, offering a powerful approach for precise regulation of intracellular activities. This strategy facilitates the investigation of complex physiological processes in cellular and molecular biology and supports the development of protein-based biotechnologies and precision therapeutics, such as targeted protein supplementation for cellular repair or modulation of aberrant cellular functions. However, current delivery methods face some challenges, including cytotoxicity, immune activation, and the difficulty of maintaining protein stability and activity during transport.

Methods: Here, we report a novel AIEgen (Aggregation-induced emission luminogen) -based carrier, MTPABP-Guided-Intracellular-Carrier (MAGIC), that achieves high efficiency in delivering native proteins into the cytosol. MAGIC is capable of binding both negatively and positively charged proteins and mediates their intracellular transport via clathrin-mediated uptake with endosomal escape.

Results: Notably, the β-Gal and HRP proteins delivered by MAGIC maintain their biological activity after delivery, and the cytotoxicity of MAGIC is approximately 40% lower than that of Lipo8000 and TranEX. MAGIC efficiently delivered trypsin, RNase A, and saporin into the cytosol of mammalian cell lines while preserving their enzymatic or functional activity.

Conclusion: This rationally designed AIEgen-based platform provides a versatile and efficient solution for cytosolic protein delivery. The MAGIC delivery system holds significant promise for advancing the study of cellular physiology and enabling precise therapeutic interventions.

Keywords: AIEgen carrier, cytosolic delivery, protein transport, therapeutic proteins

Introduction

Proteins play dynamic and diverse roles in a wide range of biochemical reactions and physiological processes, including metabolism, signaling, energy production, gene regulation, and immune responses.[1,2] In medicine, proteins are of central importance, as many diseases result from the functional alteration, deficiency, or misregulation of intracellular proteins. Such disruptions may be caused by genetic mutations, structural abnormalities, or concentration imbalances, for example, the aggregation of β-amyloid into amyloid plaques, which interferes with neuronal signal transmission.[1,3] Traditionally, protein therapeutics have relied on the indirect delivery of genes encoding therapeutic proteins, typically through transfection of DNA or mRNA.[4] However, these approaches face several limitations, including low transfection efficiency, non-specific protein expression, potential immune responses, delivery system instability, and abnormal post-translational modifications. With the growing clinical application of protein therapeutics, the challenges associated with their efficient intracellular delivery have become increasingly prominent. However, despite decades of research, there remains no simple, safe, and broadly effective way to deliver intact functional proteins directly into the cytosol of living cells without loss of activity or toxicity. Consequently, there is a pressing need for a delivery strategy that not only ensures efficient cytosolic delivery but also preserves protein functionality and minimizes off-target effects.

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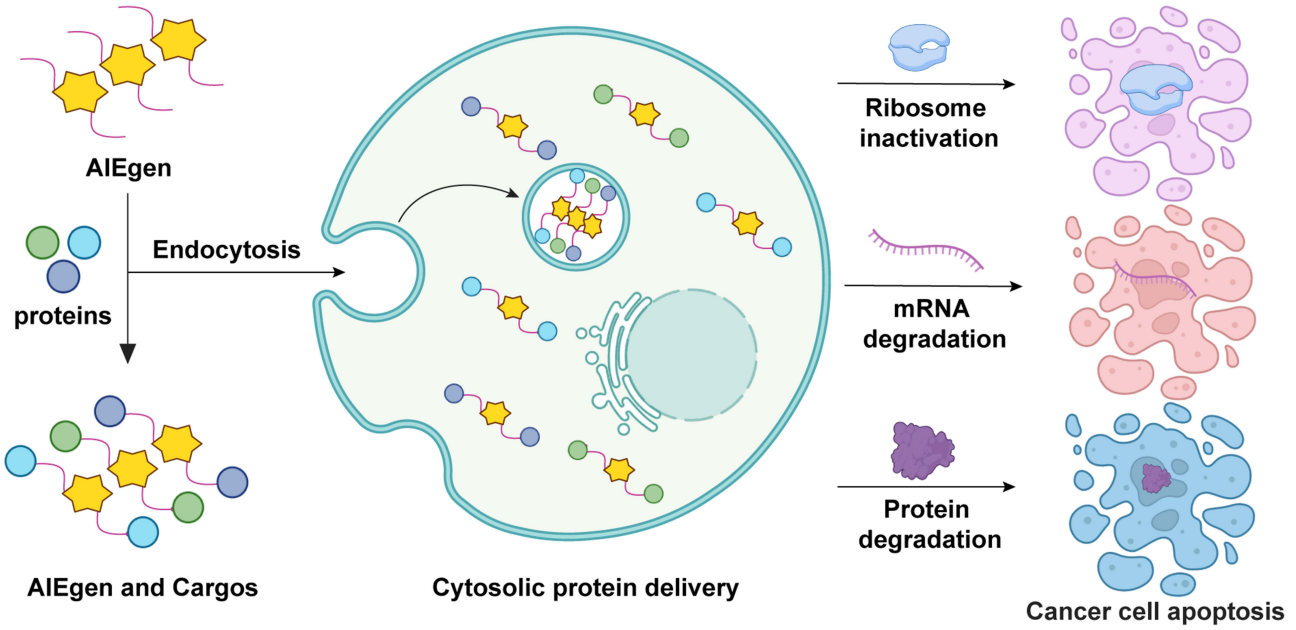
Graphical Abstract



Intracellular protein delivery is crucial for both biomedical research and protein-based therapeutics. Proteins possess high specificity, low toxicity, and a range of biological functions that cannot be fully replicated by small-molecule compounds.[2] Direct cytosolic delivery of proteins offers a promising strategy for the targeted and transient modulation of cellular functions and for the treatment of various diseases. This approach avoids many of the drawbacks associated with gene-based delivery, such as permanent or random genetic alterations, prolonged off-target effects, cellular stress responses, and potential carcinogenicity.[3,5–8] While the complex structures of proteins contribute to their enhanced potency and selectivity relative to small molecules, these same structural features often result in poor stability. As the clinical use of protein therapeutics has expanded, so too have the challenges associated with their effective intracellular delivery. Common strategies such as CPPs, lipid/polymer carriers, and pH-responsive endosomal-escape systems typically achieve limited cytosolic delivery efficiencies and often cause membrane disruption or cytotoxicity at micromolar levels.[9–12] These limitations underscore the need for safer and more efficient carriers for functional protein delivery. In this work, we address this unmet need by developing a rationally designed AIEgen (Aggregation-induced emission luminogen) -based carrier, termed MTPABP-Guided-Intracellular-Carrier (MAGIC), that can efficiently transport diverse proteins into the cytosol through clathrin-mediated uptake with endosomal escape while preserving their biological function and minimizing cytotoxicity.

Currently, several strategies have been developed for intracellular protein delivery, each with distinct advantages and limitations. Physical methods such as microporation and electroporation can directly introduce proteins into the cytoplasm, thereby avoiding endosomal entrapment. However, these techniques are typically low-throughput, invasive, require specialized equipment, and may permit unintended molecules to enter cells, potentially leading to side effects.[13–15] Carrier-mediated delivery systems enhance protein loading capacity and protect cargo from enzymatic degradation, thereby improving in vivo stability and offering tunable targeting properties. Nonetheless, these approaches often result in the formation of large nanoparticles with poor tissue penetration, limited ability to traverse biological barriers (eg, the blood-brain barrier), potential toxicity from carrier materials or degradation byproducts, and compositional heterogeneity particularly in polymer-based formulations.[16,17] Cell-penetrating peptide (CPP)-based delivery methods offer operational simplicity for in vitro applications. However, they generally suffer from low cytoplasmic delivery efficiency due to endosomal entrapment, and may compromise protein function via electrostatic interactions or elicit immune

responses.[18,19] Exosome-mediated delivery provides improved biocompatibility and reduced immunogenicity by leveraging the natural signaling roles of exosomes. Despite these advantages, challenges remain, including inefficient exosome production, low protein-loading capacity, and frequent endosomal entrapment of unmodified exosomes.[20–22] In summary, although many current approaches offer potential for direct cytoplasmic delivery, they are still hindered by limitations such as poor tissue penetration, material heterogeneity, endosomal sequestration, and immune activation. Therefore, innovative carriers that overcome these barriers are critically needed.

AIEgen is a class of fluorogenic molecules that exhibit enhanced emission upon aggregation and have recently gained attention as multifunctional platforms in bioimaging.[23–25] In this study, we propose an AIEgen-based carrier, MTPABP-Guided-Intracellular-Carrier (MAGIC), as a promising platform for binding a broad range of proteins and enabling their efficient cytosolic delivery. MAGIC efficiently delivers both positively (RNase A, trypsin, saporin) and negatively charged proteins (β-Gal, EGFP), demonstrating broad cargo compatibility across a wide charge range.[6,26,27] The carrier's scalable aqueous preparation and low cytotoxicity highlight its strong safety and translational potential for intracellular protein delivery. MAGIC enters cells via clathrin-mediated uptake with endosomal escape and achieves efficient cytosolic release of intact proteins through enhanced endosomal escape facilitated by the AIEgen core structure. To our knowledge, this represents one of the first demonstrations of an AIEgen-based carrier enabling efficient, functional cytosolic delivery of both native and cytotoxic proteins. These findings highlight the potential of MAGIC as a versatile and effective tool for intracellular protein delivery in various biomedical applications.

Materials and Methods

Materials

β-Galactosidase (β-Gal, 430 kDa, pI 5.0) was obtained from J&K Scientific (J&K Scientific Ltd., Shanghai, China). Ribonuclease A (RNase A, 13.7 kDa, pI 8.6) and trypsin (24.0 kDa, pI 10.5) were purchased from MCE (MedChemExpress Co., Ltd., Shanghai, China). Saporin (32.8 kDa, pI 9.3) was sourced from Merck (Merck & Co., Inc., Darmstadt, Germany). Amplex Red was obtained from Macklin Biochemical (Macklin Biochemical Co., Ltd., Shanghai, China). 3,3′,5,5′-Tetramethylbenzidine (TMB) and horseradish peroxidase (HRP, 40.0 kDa, pI 7.2) were purchased from Yuanye Biotechnology (Yuanye Biotechnology Co., Ltd., Shanghai, China). TransEx was generously provided by Cenji Biotech (Cenji Biotechnology Co., Ltd., Shanghai, China). Lipo8000™ Transfection Reagent was purchased from Beyotime (Beyotime Biotech Inc., Jiangsu, China).

Cell Culture

HeLa cells (human cervical carcinoma, ATCC, CCL-2), HEK 293T cells (human embryonic kidney, ATCC, CRL-3216), and HT22 cells (mouse hippocampal neurons, Pricella, CL-0697) were cultured under standard condition.[28] HeLa cells were maintained in Minimum Essential Medium (MEM; Pricella), while HEK 293T and HT22 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco). All media were supplemented with 10% fetal bovine serum (FBS; Gibco), 100 μg/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

MTPABP/EGFP Protein Cytosolic Delivery

HeLa, HEK 293T, and HT22 cells were seeded in 35 mm glass-bottomed dishes (Cellvis) at >70% confluency in MEM or DMEM supplemented with 10% FBS and cultured overnight. EGFP protein was mixed with MTPABP at designated weight ratios and diluted with 1 mL deionized water. The mixture was incubated overnight at 4°C to allow complex formation. The resulting solutions were ultrafiltered using 30 kDa molecular weight cutoff ultrafiltration tubes to remove unbound MTPABP and free protein. The retentates were diluted with serum-free DMEM to the desired working concentration.

Before treatment, cells were washed twice with PBS to remove residual serum. The protein-MTPABP complexes were then added to the culture dishes and incubated for 6 h at 37 °C under 5% CO_2. After incubation, the media were removed, and cells were washed three times with PBS. Fluorescence imaging was performed using a laser scanning confocal microscope (Zeiss LSM 980 with Airyscan, Jena, Germany).

MTPABP/β-Gal Cytosolic Delivery

HeLa cells were seeded at >70% confluency in 35 mm glass-bottomed dishes (Cellvis) using MEM supplemented with 10% FBS and incubated overnight. β-Galactosidase (β-Gal, 5 μg) was complexed with MTPABP (8 μg) at a 1:1.6 weight ratio, diluted in 100 mL of serum-free MEM, and incubated overnight at room temperature. The mixture was then further diluted with 900 mL of serum-free MEM. Prior to treatment, cells were washed twice with PBS, and the prepared complexes were added to the dishes. After 6 h of incubation at 37°C under 5% CO_2, the media were removed, and cells were washed three times with PBS. Commercial transfection reagents, Lipo8000 and TransEx, were used as positive controls following the manufacturers' protocols. Naked β-Gal, without complexation, served as a negative control. Experiments were performed by three independent replicates.

β-Gal Staining and Activity Assay

The cytosolic delivery and enzymatic activity of β-galactosidase (β-Gal) were assessed using both in situ staining and quantitative analysis. HeLa cells were treated with MTPABP/β-Gal (1.6:1 w/w) complexes for 6 h as described above, then washed twice with PBS and fixed for 10 min at room temperature. After three additional PBS washes, cells were incubated with X-Gal working solution (5%) for 4 h at 37°C in a CO_2-free environment and imaged using an optical microscope (SOPTOP ICX41, China). For quantitative analysis, treated cells were lysed and 50 μL of lysate was mixed with 50 μL of O-nitrophenyl-β-D-galactopyranoside (ONPG) substrate solution. After 30 min incubation at 37°C, 150 μL of stop solution was added. Absorbance was measured at 420 nm using a microplate reader (BioTek, USA). Native β-Gal at equivalent concentration served as a 100% activity reference. Experiments were performed by three independent replicates.

MTPABP/HRP Cytosolic Delivery and Activity Staining

Cytosolic delivery of HRP was evaluated using intracellular enzymatic activity assays. Delivered HRP catalyzes the oxidation of the non-fluorescent substrate Amplex Red to fluorescent resorufin in the presence of hydrogen peroxide. Briefly, HeLa cells were treated with MTPABP/HRP (8 μg, 6 μg; 1.3:1 w/w) complexes for 6 h as described above, then washed three times with PBS. Cells were incubated in serum-free MEM containing Amplex Red (50 μM) and hydrogen peroxide (500 μM) for 30 min at 37°C under 5% CO_2. After incubation, cells were washed and imaged using laser scanning confocal microscopy (LSCM). Experiments were performed by three independent replicates.

In a complementary colorimetric assay, tetramethylbenzidine (TMB) was used as a chromogenic substrate. HeLa cells treated with MTPABP/HRP (8 μg, 6 μg; 1.3:1 w/w) complexes were washed five times with PBS. TMB solution (1 mL, 50 μg/mL) in acetate buffer (pH 5.0) containing 10 mM hydrogen peroxide was added to each dish. After 30 min incubation at room temperature, color development was recorded using an optical microscope (SOPTOP ICX41, China). Native HRP without complexation was used as a negative control for cytosolic delivery. Experiments were performed by three independent replicates.

Cytosolic Delivery of Cytotoxic Proteins Saporin, RNase A, and Trypsin

To evaluate the cytosolic delivery of toxic proteins, HeLa cells were seeded in 96-well plates and cultured overnight. MTPABP (8 μg) complexes were prepared with saporin (5 μg/mL), RNase A (50 μg/mL), and trypsin (20 μg/mL), diluted in 100 μL serum-free MEM, and incubated overnight at room temperature. The complexes were then further diluted with an additional 100 μL of serum-free MEM. Following removal of the culture media, cells were washed with PBS and treated with 200 μL of the protein complexes per well. After 6 h of incubation at 37°C under 5% CO_2, the media were replaced with fresh MEM containing 10% FBS, and cells were incubated for an additional 24 h. Cytotoxicity was assessed using a CCK-8 assay. Each sample was tested in quintuplicate. Naked saporin, RNase A, trypsin, and MTPABP alone at equivalent concentrations were used as controls. Experiments were performed by three independent replicates.

Endocytosis Inhibition of Cellular Delivery

To investigate the intracellular delivery route, HeLa cells were seeded on 35 mm glass-bottom dishes (Cellvis) at >70% confluence in MEM supplemented with 10% FBS and cultured overnight. To assess the cellular uptake pathway, cells were

incubated with 5 μM MTPABP for 1 h at 37°C with 5% CO_2, in the presence or absence of 10 μM chlorpromazine. MTPABP/HRP complexes and HRP alone were prepared under similar conditions with or without 10 μM chlorpromazine, 10 μM dynasore, 200 μM genistein and 10 mM methyl-β-cyclodextrin (MβCD) following the procedures described above. Cytosolic enzyme activity of native HRP without MTPABP complexation was measured as a control. Cells were incubated in serum-free MEM containing Amplex Red (50 μM) and hydrogen peroxide (500 μM) for 30 min at 37°C under 5% CO_2. In addition, complexes were incubated with cells for 2h, 4h, 6 h and 8h and co-stained with LysoTracker Green (50 nM) for 30 min at 37°C with 5% CO_2, to capture early cellular uptake and endosomal escape events in the absence of endocytic inhibitors. After treatment, cells were washed three times with PBS and imaged using a laser scanning confocal microscope (Nikon Eclipse Ti2-E, Japan). Experiments were performed by three independent replicates.

Cell Viability Assay

Cell viability after incubation with MTPABP/protein complexes was assessed using the standard Cell Counting Kit-8 (CCK-8) assay. HeLa cells were seeded in 96-well plates at a density of 5000 cells per well and cultured overnight. The culture medium was then replaced with 200 μL of serum-free MEM containing MTPABP/protein complexes or native proteins at concentrations optimized for protein delivery. Cells were incubated for 6 h, after which the medium was replaced with 100 μL of fresh MEM supplemented with 10% FBS. Following an additional 24 h incubation, cell viability was measured using the CCK-8 assay to confirm the sustained functionality of delivered proteins and the absence of long-term cytotoxic effects. Each condition was tested in five replicates and the experiments were performed by three independent replicates.

MTPABP/Protein Complex Characterization

MTPABP was mixed with model proteins and incubated overnight at room temperature. The resulting mixtures were then diluted with deionized water prior to characterization. The size and zeta potential of the formed complexes were measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS 90 (Malvern, UK) at 25°C.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 9.0.[29] Data for fluorescence intensity, enzyme activity, and cell viability are presented as means ± standard deviation (SD). Statistical significance was assessed using Welch's corrected two-tailed t-tests. P-values are denoted as follows: **** for $p \leq 0.0001$, *** for $p \leq 0.001$, ** for $p \leq 0.01$, * for $p \leq 0.05$, and ns (not significant) for $p > 0.05$.

Results

Efficient Cytosolic Delivery of EGFP via AIEgen-Based Carrier MAGIC

We employed an aggregation-induced emission luminogen (AIEgen)-based carrier system for intracellular protein delivery. The carrier molecule, MTPABP, was designed using 4,4′-dimethoxytriphenylamine (as the electron-donating AIE unit) conjugated to 2,1,3-benzothiadiazole (as the electron-accepting unit), forming a donor–acceptor structure that supports long-wavelength fluorescence emission.[30] The photophysical results indicate that that MTPABP exhibited strong absorption at 460 nm and an emission wavelength peak at 660 nm (Figure S1A–B). MTPABP-Guided-Intracellular-Carrier (MAGIC) readily forms electrostatic complexes with protein cargos via charge-based interactions, facilitating efficient intracellular transport (Figure 1A). As a proof-of-concept, we selected Enhanced Green Fluorescent Protein (EGFP; 27.3 kDa, pI 5.8) as a model cargo. Following simple incubation with MTPABP, the resulting complexes were introduced to HeLa cells and imaged after 6 h. Striking intracellular fluorescence was observed, confirming efficient cytosolic delivery. Notably, this enhanced delivery performance was also evident in HEK 293T and HT22 cells, demonstrating the broad applicability and effectiveness of MAGIC across multiple cell types (Figure 1B, Figure S2A–B).

To further optimize delivery conditions, we systematically evaluated the impact of MAGIC concentration on intracellular protein uptake. HeLa cells were treated with MTPABP/EGFP complexes prepared at varying molar ratios, and the resulting delivery efficiencies were quantified. Interestingly, the intracellular fluorescence intensity exhibited

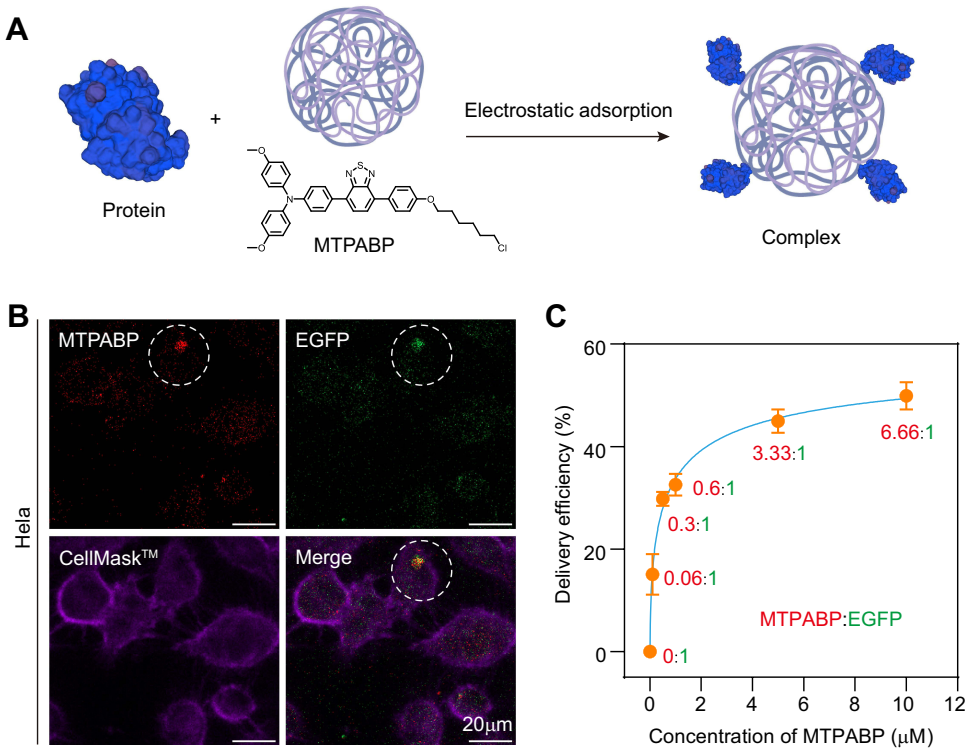


Figure 1 Efficient cytosolic protein delivery mediated by MTPABP-Guided-Intracellular-Carrier (MAGIC). (**A**) Schematic illustration of MTPABP forming complexes with proteins for intracellular delivery. Scale bar represents 20 μm. (**B**) Confocal microscopy images showing the delivery of EGFP protein into HeLa cells by MTPABP. White circles indicate regions of co-localization between MTPABP and EGFP. (**C**) Quantitative analysis of delivery efficiency at varying MTPABP concentrations and EGFP incubation ratios in HeLa cells.

a nonlinear increase as the concentration of MTPABP increased, suggesting a dose-dependent enhancement in cellular uptake and complex formation (Figure 1C). This finding highlights the tunable nature of the MAGIC system, enabling precise control over protein delivery efficiency through modulation of carrier concentration.

MAGIC Enables Functional Cytosolic Delivery of Bioactive β-Galactosidase with Low Cytotoxicity

Beyond achieving high delivery efficiency, it is crucial that intracellularly delivered proteins maintain their bioactivity while minimizing cytotoxic effects. To evaluate this, we selected β-galactosidase (β-Gal; 430 kDa, pI 5.0) as a representative bioactive enzyme[31] to assess whether MAGIC mediated cytosolic delivery preserves enzymatic functionality (Figure 2A). HeLa cells treated with MTPABP/β-Gal complexes were subsequently incubated with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), a chromogenic substrate that produces an insoluble blue product upon hydrolysis by β-Gal. Remarkably, cells receiving MTPABP/β-Gal treatment exhibited strong and widespread blue staining, clearly demonstrating efficient intracellular delivery and retention of enzymatic activity. This staining was significantly more intense than that observed in cells treated with control delivery materials, highlighting the superior performance of MAGIC in functional protein delivery (Figure 2B).

We assessed the biocompatibility of MAGIC by measuring cytotoxicity with a CCK-8 assay.[32] MTPABP showed significantly lower cytotoxicity than commercial transduction reagents (Figure 2C), demonstrating its strong potential for intracellular applications. Using a β-Gal activity assay kit, we quantified enzymatic activity and found that over 60% of β-Gal function remained after cytosolic delivery via MTPABP (Figure 2D), confirming effective preservation of protein activity. Dynamic light scattering (DLS) and zeta potential analyses revealed that MTPABP forms stable, uniform protein-loaded nanoparticles sized between 100 and 300 nm (Figure 2E), outperforming commercial carriers such as

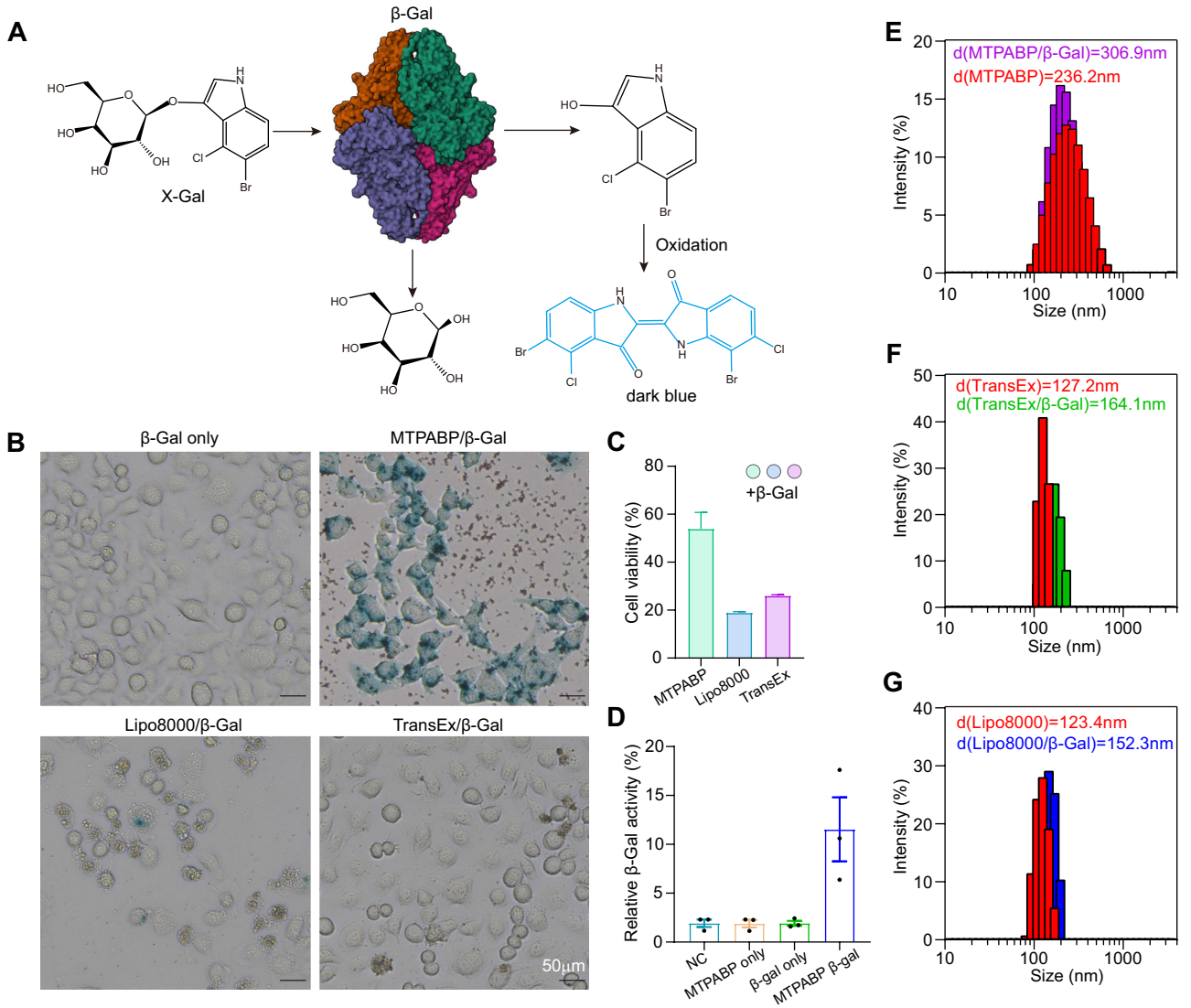


Figure 2 Cytosolic delivery of β-Gal by MAGIC. (**A**) Schematic of β-Gal catalyzing the hydrolysis of the colorless substrate X-Gal into a blue-colored product. (**B**) X-Gal staining of HeLa cells treated with MTPABP/β-Gal complexes for 4 h, showing enzymatic activity. Scale bar represents 50 μm. (**C**) Cell viability of HeLa cells after delivery of β-Gal using MTPABP, Lipo8000, and TransEx, assessed by CCK-8 assay. (**D**) Quantitative measurement of intracellular β-Gal enzymatic activity following delivery. The black dots represent the original data points of the sample. Protein dose was 8 μg per well; MAGIC concentration was 5 μM per well. (**E–G**) Dynamic light scattering (DLS) analysis showing size distributions of MTPABP and MTPABP/β-Gal complexes (E), TransEx and TransEx/β-Gal complexes (F), and Lipo8000 and Lipo8000/β-Gal complexes (G).

TransEx and Lipo8000 in nanoparticle stability and consistency (Figure 2F, G, and Figure S3A–C). These findings confirm that MAGIC efficiently delivers functional bioactive proteins intracellularly while minimizing cytotoxicity.

MAGIC Delivers Native Enzymes While Preserving Functionality and Reducing Cytotoxicity

To evaluate the generality of the AIEgen-based carrier system for intracellular protein delivery, we tested horseradish peroxidase (HRP; 40.0 kDa, pI 7.2), a native enzyme with diagnostic and therapeutic relevance.[33,34] HRP catalyzes the oxidation of nonfluorescent Amplex Red into a red fluorescent, membrane-impermeable product in the presence of hydrogen peroxide (Figure 3A). When complexed with MTPABP, HRP retained enzymatic activity, as indicated by red fluorescence in treated cells outperforming commercial delivery reagents such as TransEx and Lipo8000 (Figure 3B).

To assess biocompatibility, we performed CCK-8 assay to evaluate cytotoxicity. The results showed that MTPABP exhibited lower cytotoxicity than both control materials, suggesting improved cell compatibility (Figure 3C). In

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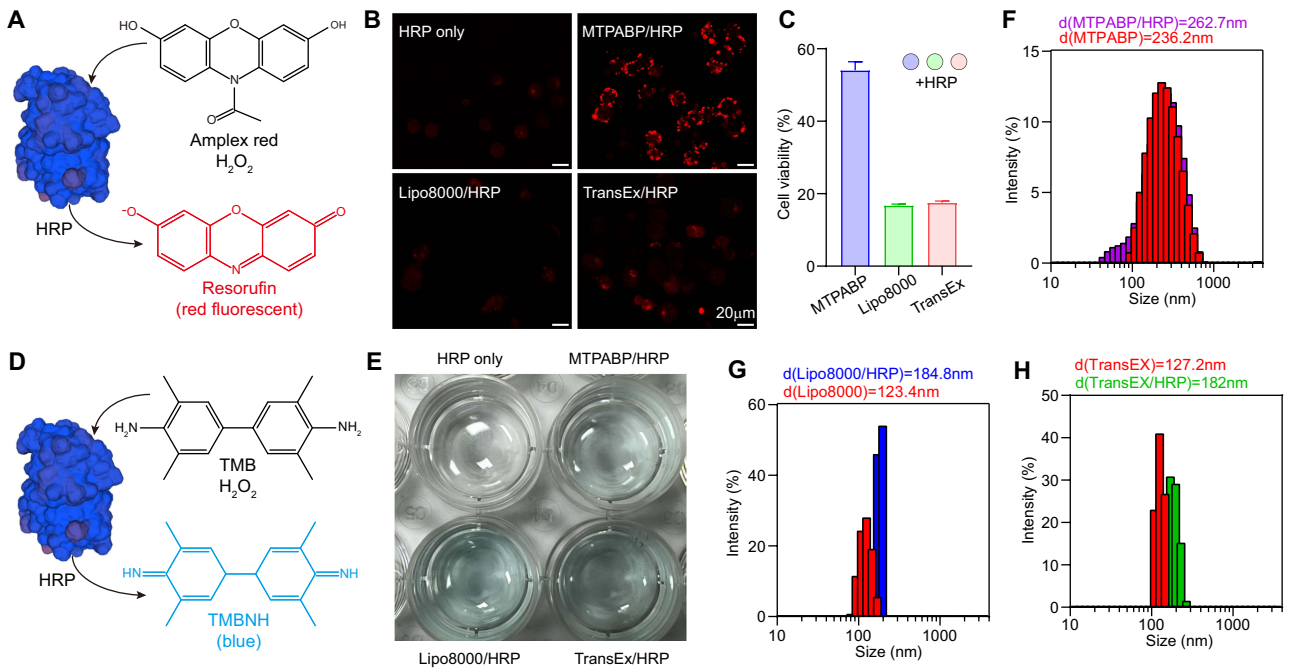

Figure 3 Cytosolic delivery of HRP by MAGIC. (**A**) Schematic showing HRP-catalyzed conversion of the colorless substrate Amplex Red into the red fluorescent product resorufin in the presence of hydrogen peroxide. (**B**) Confocal microscopy images of HeLa cells treated with MTPABP/HRP complexes and Amplex Red. Commercial reagents Lipo8000 and TransEx were included as positive controls. Scale bar represents 20 μm. (**C**) Cell viability of HeLa cells after delivery of HRP using MTPABP, Lipo8000, and TransEx, assessed by CCK-8 assay. (**D**) HRP-catalyzed conversion of the colorless substrate TMB into a blue-colored product in the presence of hydrogen peroxide. (**E**) Analysis of intracellular HRP enzymatic activity by TMB assay. Protein dose was 6 μg per well; MTPABP concentration was 5 μM per well. (**F–H**) Dynamic light scattering (DLS) analysis showing size distributions of MTPABP and MTPABP/HRP complexes (F), Lipo8000 and Lipo8000/HRP complexes (G), and TransEx and TransEx/HRP complexes (H).

a complementary assay, HRP catalyzed the conversion of tetramethylbenzidine (TMB), a colorless substrate, into a blue chromogenic product in the presence of hydrogen peroxide (Figure 3D). Cells treated with MTPABP/HRP showed the highest HRP activity among all delivery systems tested, as determined by TMB-based colorimetric analysis (Figure 3E). Dynamic light scattering (DLS) and zeta potential measurements confirmed that MTPABP forms stable, uniform HRP-loaded nanoparticles with diameters ranging from 100 to 300 nm (Figure 3F, Figure S4A). In contrast, commercial reagents such as Lipo8000 and TransEx formed less uniform complexes, as shown in Figure 3G–H and Figure S4B–C. These results highlight MAGIC's strong potential for safe, efficient, and functional delivery of native enzymes.

MAGIC Enables Efficient Cytosolic Delivery of Cytotoxic Proteins

To further demonstrate the versatility of MAGIC as a protein delivery platform, we evaluated its ability to deliver cytotoxic proteins, including trypsin (24.0 kDa, pI 10.5), ribonuclease A (RNase A; 13.7 kDa, pI 8.6), and saporin (32.8 kDa, pI 9.3). These proteins exert their cytotoxic effects through distinct intracellular mechanisms,[6,35–37] making them ideal candidates for testing delivery efficacy and functional release.

We first examined Trypsin, a protease that degrades intracellular proteins upon internalization (Figure 4A). We incubated MTPABP with Trypsin to form a delivery complex, and then applied the complex to HeLa cells. We used CCK-8 assay to assess cytotoxicity and found that cells treated with MTPABP/trypsin exhibited significantly reduced viability, indicating successful cytosolic delivery of active trypsin (Figure 4B). We performed dynamic light scattering (DLS) and zeta potential analyses, and the results confirmed the formation of stable MTPABP/trypsin nanoparticles with an average size of ~300 nm (Figure 4C and Figure S5A).

Next, we tested RNase A, a positively charged enzyme that catalyzes the degradation of RNA (Figure 4D). Following treatment of HeLa cells with MTPABP/RNase A complexes, reduced cell viability indicated successful intracellular delivery and retained enzymatic activity of RNase A (Figure 4E). DLS and zeta results showed stable nanoparticle formation with an average size of ~350 nm (Figure 4F and Figure S5B).

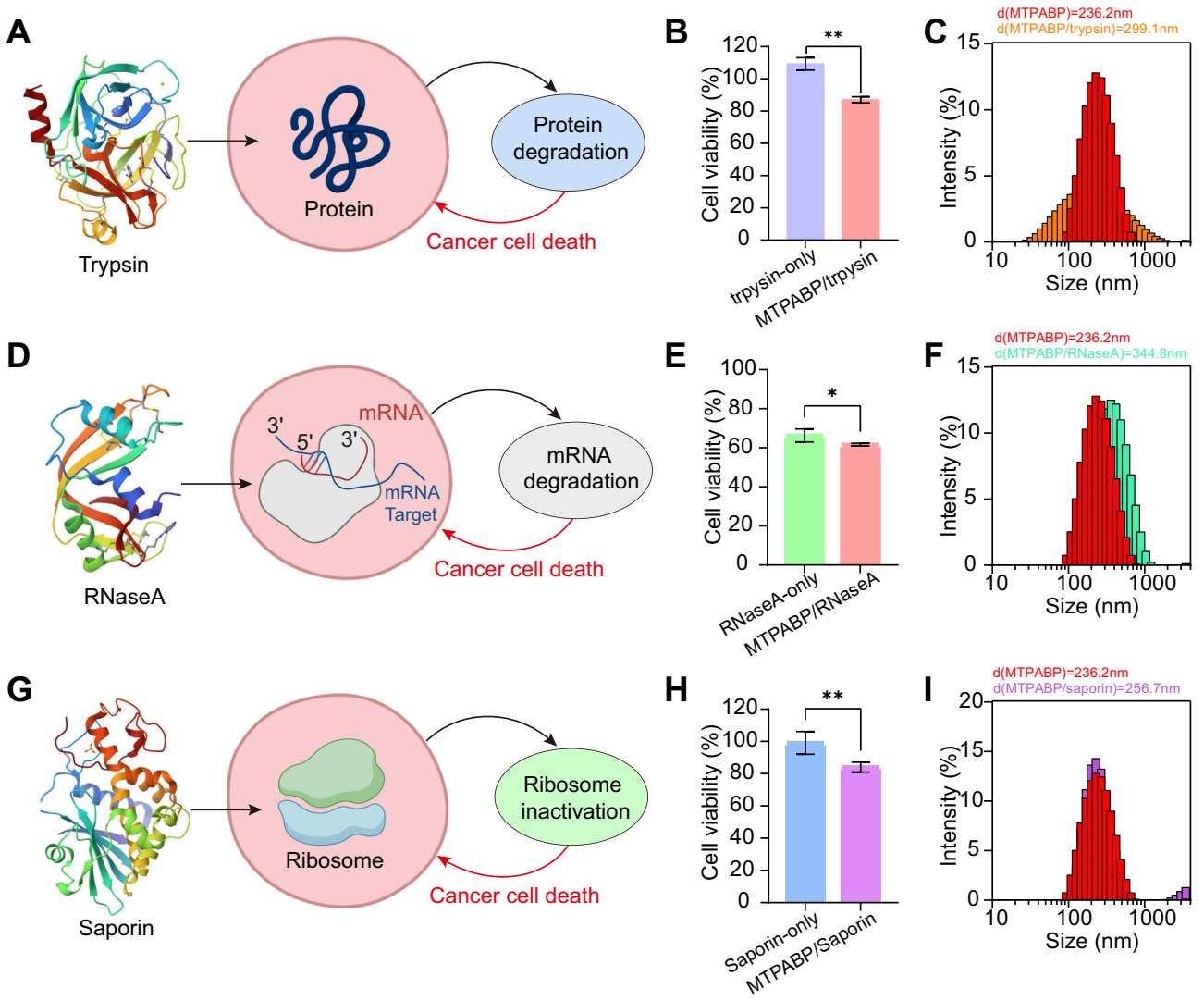

Figure 4 Cytosolic delivery of toxic proteins by MAGIC. (**A**) Schematic illustrating cytosolic delivery of trypsin into cancer cells, leading to intracellular protein hydrolysis and cell death. (**B**) Viability of HeLa cells treated with MTPABP/trypsin complexes, assessed by MTT assay. Trypsin concentration was 20 μg/mL. Free trypsin served as a control. (**C**) Dynamic light scattering (DLS) analysis of MTPABP and MTPABP/trypsin complexes. (**D**) Schematic illustrating cytosolic delivery of RNase A into cancer cells, causing RNA degradation and cell death. (**E**) Viability of HeLa cells treated with MTPABP/RNase A complexes, determined by MTT assay. RNase A concentration was 50 μg/mL. Free RNase A served as a control. (**F**) DLS analysis of MTPABP and MTPABP/RNase A complexes. (**G**) Schematic illustrating cytosolic delivery of saporin into cancer cells, resulting in ribosome inactivation and cell death. (**H**) Viability of HeLa cells treated with MTPABP/saporin complexes, evaluated by MTT assay. Saporin concentration was 5 μg/mL. Free saporin served as a control. (**I**) DLS analysis of MTPABP and MTPABP/saporin complexes. Note: The concentration of MTPABP used in all cytosolic delivery experiments with saporin, RNase A, and trypsin was 5 μM per well. (* indicates compared to the RNase A-only, saporin-only and trypsin-only. *p<0.05, **p<0.01).

Lastly, we examined saporin, a ribosome-inactivating protein that blocks protein synthesis by depurinating a specific nucleotide on the 28S rRNA subunit (Figure 4G). While saporin alone exhibited minimal cytotoxicity due to limited membrane permeability, its cytotoxicity increased significantly when delivered with MTPABP, indicating efficient cytosolic release and functional activity (Figure 4H). Notably, MTPABP alone did not contribute to this toxicity. DLS and zeta analysis revealed that MTPABP formed ~250 nm nanoparticles with saporin (Figure 4I and Figure S5C).

Together, these results demonstrate that MAGIC is a potent and versatile delivery platform capable of efficiently transporting diverse cytotoxic proteins into the cytosol while maintaining their functional activity.

Clathrin-Dependent Endocytosis Underlies Efficient Cytosolic Delivery of MAGIC

To investigate the cellular uptake mechanism of MTPABP/protein complexes, particularly whether they utilize endocytosis followed by efficient endosomal escape, we employed with a panel of pharmacological inhibitors targeting distinct

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endocytic pathways, delivered the MTPABP/HRP complex intracellular, and determined the HRP enzyme activity using Amplex Red. Cellular uptake was inhibited using: 10 μM chlorpromazine to block clathrin-mediated endocytosis;[38–41] 10 μM dynasore to inhibit dynamin-dependent endocytosis; 200 μM genistein to suppress caveolae-mediated endocytosis; and 10 mM methyl-β-cyclodextrin (MβCD), a cholesterol-depleting agent that disrupts caveolae/lipid raft-mediated endocytosis.[42,43] Following inhibitor treatments, the relative cellular fluorescence intensity decreased by approximately 61%, 35%, 40%, and 21%, respectively (Figure 5A–J, and Figure S6A–D). Conversely, confocal imaging of cells treated with MTPABP/HRP displayed strong red fluorescence in the absence of chlorpromazine, dynasore and genistein indicative of successful HRP delivery and enzymatic activity in the cytosol (Figure 5A–J, and Figure S6A–D).

Furthermore, the intracellular trafficking and endosomal escape of MTPABP/HRP complexes in HeLa cells were explored using confocal laser scanning microscope (CLSM) (Figure S7). MTPABP/HRP complexes were cultured with Hela cells for 2, 4, 6, 8 h, respectively. With the incubation time increasing, the amount of intracellular complexes increased. In the early stage of cell uptake, the localization of complexes and endo/lysosomes was overlapped. However, after 8 h, the complexes were separated from the endo/lysosomes, indicating that MTPABP/HRP complexes display excellent endosomal escape ability. Together, these findings affirm that MAGIC facilitates protein internalization through clathrin-mediated uptake with endosomal escape and promotes efficient endosomal escape while preserving protein bioactivity.

In summary, MAGIC emerges as a powerful and adaptable AIEgen-based carrier capable of efficiently delivering a wide variety of functional proteins, including enzymes and cytotoxins into the cytosol, with minimal toxicity and strong preservation of bioactivity.

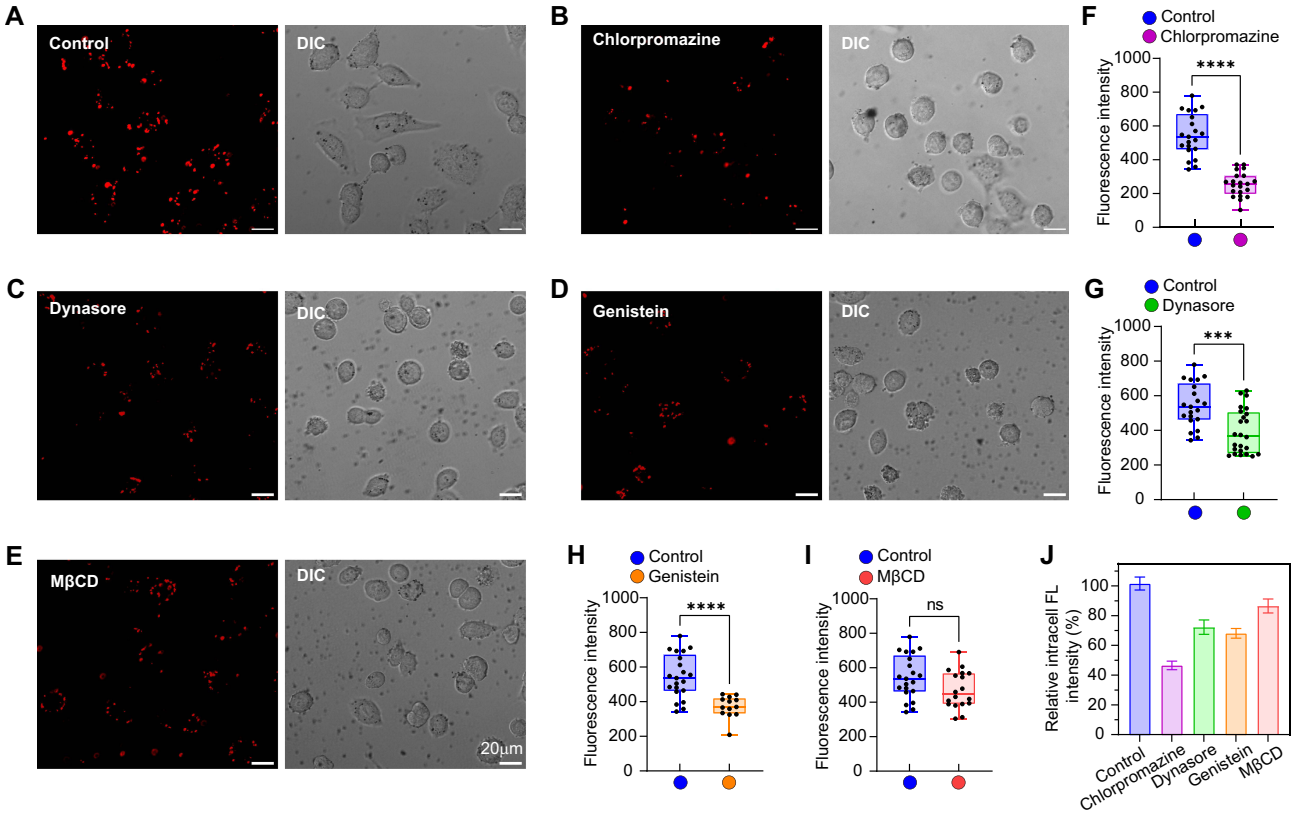

Figure 5 Cytosolic delivery route of MAGIC. (**A–E**) Live cell imaging of Hela cells after treatments with different pharmacological blocking reagents: (**A**) control, (**B**) chlorpromazine, (**C**) dynasore, (**D**) genistein, and (**E**) MβCD. Scale bar represents 20 μm. (**F–I**) Quantification of Amplex Red fluorescence intensity in HeLa cells treated with MTPABP/HRP complexes: (**F**) with or without chlorpromazine, (**G**) with or without dynasore, (**H**) with or without genistein, (**I**) with or without MβCD. (* indicates compared to the control group. ***p<0.001, ****p<0.0001 and ns (not significant) for p > 0.05) (**J**) Summary of relative fluorescence intensity of Amplex Red in cells treated with different uptake inhibitors.

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Discussion

In this study, we introduced MTPABP-Guided-Intracellular-Carrier (MAGIC), a novel AIEgen-based protein delivery platform that enables efficient, cytosolic delivery of a wide range of functional proteins. Our results demonstrate that MAGIC forms stable, nanoscale complexes with diverse proteins via electrostatic interactions and efficiently transports them into the cytosol of various mammalian cell lines. MAGIC not only achieves high delivery efficiency, but also preserves the enzymatic activity of bioactive proteins following intracellular delivery. Delivery was systematically tested within a range of MTPABP concentrations and across three representative cell lines (HeLa, HEK 293T, and HT22), thereby establishing the operational boundaries under which reproducible cytosolic delivery was achieved. Compared to commercial transduction reagents, MAGIC exhibited superior delivery performance with significantly lower cytotoxicity and improved nanoparticle stability.

MAGIC successfully delivered cytotoxic proteins into cells, leading to marked reductions in cell viability, underscoring its therapeutic potential. Mechanistic studies revealed that MAGIC enters cells via clathrin-mediated endocytosis and enables effective endosomal escape, facilitating the release of intact and functional protein cargos into the cytoplasm. Similar pathways have been reported in recent DNA delivery and lysosome-responsive systems. For instance, Zn(II)-Schiff base complexes were shown to undergo lysosome-associated uptake and pH-triggered release behaviors that facilitate cytosolic transport of payloads,[42] and low-generation dendrimer nanogels achieved improved cellular internalization and lysosomal escape through transient high-charge states that enhance endocytic trafficking efficiency.[43] Together, these findings position MAGIC as a powerful and versatile platform for intracellular protein delivery, with promising applications in biomedical research and therapeutic development.

Comparison with Existing Delivery Platforms

Cellular functions are predominantly carried out by proteins and their interactions. Direct cytosolic protein delivery represents a promising therapeutic strategy, particularly given the high specificity and ability of protein drugs to target otherwise undruggable molecules. Numerous approaches have been developed to facilitate cytosolic protein delivery. Cell-penetrating peptides (CPPs) can transport biomolecules across cell membranes but often suffer from endosomal entrapment and potential protein damage. In contrast, MAGIC achieves efficient endosomal escape and preserves protein integrity through simple non-covalent interactions, avoiding complex cargo modification.[44,45] Cell-derived vesicles and virus-like particles exploit natural mechanisms for entry and offer improved biocompatibility but may trigger immune responses or raise safety concerns.[3,46,47] Lipid-based nanoparticles (LNPs) are useful for in vivo delivery but tend to cause immune responses, exhibit poor stability, and are optimized mainly for nucleic acids. MAGIC, however, offers higher stability and biocompatibility, minimal immunogenicity, and broad compatibility with diverse proteins.[48,49] Exosome-based systems, though naturally biocompatible, face challenges in loading efficiency and targeting control.[50,51] Conversely, MAGIC is easy to synthesize, highly reproducible, and tunable for targeted delivery, while its intrinsic AIEgen fluorescence enables simultaneous delivery and real-time tracking. Nevertheless, many of these methods suffer from issues such as low stability, limited cargo versatility, or high toxicity. Compared with other emerging protein delivery strategies, MAGIC shows clear advantages in efficiency, versatility, and controllability.

In this study, we address these limitations by introducing MAGIC, a biocompatible, easy-to-synthesize AIEgen carrier composed of 4,4′-dimethoxytriphenylamine and 2,1,3-benzothiadiazole. Unlike traditional delivery systems that rely heavily on protein modifications or carrier proteins, MAGIC facilitates uptake via clathrin-mediated endocytosis and enables efficient endosomal escape. Its broad applicability to various proteins and cell types, along with real-time traceability and low cytotoxicity, suggests a significant advancement in intracellular delivery technology.

Biocompatibility and Clinical Translation Potential

An essential requirement for any intracellular delivery platform is high biocompatibility, particularly for applications involving repeated or systemic administration. Our results demonstrate that MAGIC consistently exhibits low cytotoxicity across multiple cell lines and protein cargos, including EGFP, β-Gal, HRP, trypsin, RNase A, and saporin. This broad safety profile is critical for minimizing off-target effects and ensuring cellular viability during therapeutic interventions or

long-term experimental studies. Notably, MAGIC outperforms widely used commercial reagents such as Lipo8000 and TransEx, both in terms of delivery efficiency and cell compatibility.

The simple synthesis of MTPABP from biocompatible building blocks (4,4′-dimethoxytriphenylamine and 2,1,3-benzothiadiazole) further supports its potential for clinical translation. Its ease of manufacture, scalability, and structural tunability enable adaptation for specific clinical needs, such as targeting disease-relevant cell types or optimizing pharmacokinetics. From a regulatory perspective, MAGIC's non-viral and non-genetic nature offers safety advantages over viral vectors and gene-editing platforms, reducing the risk of genomic integration, mutagenesis, or immunogenicity. Moreover, its ability to deliver cytotoxic proteins, such as saporin and RNase A without requiring genetic encoding enables transient, controllable therapeutic effects, which is particularly desirable in oncology and immunotherapy contexts. Given its compatibility with enzymatic cargos, MAGIC also holds promise for enzyme replacement therapies in inherited metabolic disorders, and for delivering genome-editing tools in ex vivo gene therapy protocols.

Collectively, these features make MAGIC a strong candidate for translational development in precision medicine, with applications spanning from fundamental cell biology to next-generation protein therapeutics.

Limitations and Future Directions

Despite the promising capabilities of MAGIC in intracellular protein delivery, several limitations must be addressed before its widespread adoption in clinical and research settings. First, while our results demonstrate efficient delivery across various cell lines, we have not yet assessed MAGIC's performance in more complex biological systems such as 3D organoids, or in vivo models. These systems often exhibit greater physiological relevance and present additional barriers, such as extracellular matrices or immune clearance mechanisms, which may affect delivery efficiency and safety. In addition, the delivery performance of MAGIC may vary depending on the physicochemical attributes of the cargo, such as protein size or net charge and environmental conditions, including serum concentration and cell type.

Second, although MAGIC enables clathrin-mediated uptake and promotes endosomal escape, the precise mechanism of escape remains unclear. Further studies using high-resolution imaging and molecular probes are warranted to elucidate the dynamics of endosomal trafficking, escape timing, and possible off-target effects. Understanding these processes will inform design improvements for even more efficient and selective delivery.

Third, while MAGIC exhibits low cytotoxicity in vitro, long-term biocompatibility and potential immunogenicity must be thoroughly evaluated. Comprehensive pharmacokinetic and biodistribution studies in animal models will be crucial for assessing systemic stability, clearance routes, and accumulation in non-target tissues key factors for eventual clinical translation.

Moreover, although our platform shows compatibility with a range of proteins, the physicochemical properties of the cargo (eg, size, charge, stability) may influence delivery efficacy.[52] Expanding the scope of test cargos, including large multi-subunit complexes or membrane-associated proteins, will help define the platform's versatility and limitations.

In future work, structural tuning of the MAGIC molecule could enhance tissue penetration, pH responsiveness, or target specificity. Conjugating ligands or antibodies to MAGIC for receptor-mediated targeting could further improve selective delivery to disease-relevant cell populations, such as tumor cells or neurons.

Conclusion

MAGIC provides a simple, effective, and broadly applicable strategy for intracellular protein delivery, enabling cytosolic access of functional proteins with high efficiency and minimal toxicity. MAGIC is capable of binding both negatively and positively charged proteins and mediates their intracellular transport via clathrin-mediated uptake with endosomal escape, but the exact molecular mechanism behind this process remains to be clarified. This AIEgen-based platform holds strong potential for advancing both fundamental research and translational applications in protein-based therapy. The current findings have been verified in a cultured cell model, so the conclusion is limited to the in vitro environment. The extrapolation of the system in vivo requires further experimental verification. In future work, we will focus on optimizing the targeted delivery of MAGIC in animal models and evaluating pharmacokinetics, safety and therapeutic effects.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author (He Liu) upon reasonable request.

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Disclosure

The authors report no conflicts of interest in this work.

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