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Nanoparticle-Based Artificial Mitochondrial DNA Transcription Regulator: *MitoScript*

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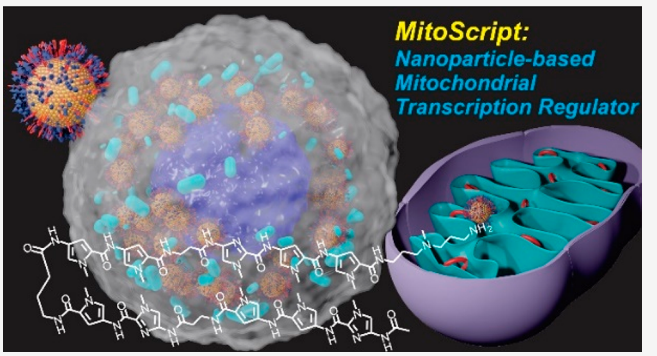
ABSTRACT: The growing knowledge of the links between aberrant mitochondrial gene transcription and human diseases necessitates both an effective and dynamic approach to control mitochondrial DNA (mtDNA) transcription. To address this challenge, we developed a nanoparticle-based synthetic mitochondrial transcription regulator (*MitoScript*). *MitoScript* provides great colloidal stability, excellent biocompatibility, efficient cell uptake, and selective mitochondria targeting and can be monitored in live cells using near-infrared fluorescence. Notably, *MitoScript* controlled mtDNA transcription in a human cell line in an effective and selective manner. *MitoScript* targeting the light strand promoter region of mtDNA resulted in the downregulation of ND6 gene silencing, which eventually affected cell redox status, with considerably increased reactive oxygen species (ROS) generation. In summary, we developed *MitoScript* for the efficient, nonviral modification of mitochondrial DNA transcription. Our platform technology can potentially contribute to understanding the fundamental mechanisms of mitochondrial disorders and developing effective treatments for mitochondrial diseases.



KEYWORDS: *Mitochondria DNA (mtDNA) manipulations, Artificial transcription factors, Mitochondria-targeted delivery, Nanoclusters, Nanomedicine*

Abnormal mitochondrial DNA (mtDNA) transcription has been associated with a multitude of human diseases.[1−3] Effective and innovative methods of site-specifically regulated mitochondrial DNA (mtDNA) transcription are thus urgently required for both investigating and treating these disorders.[4,5] For this purpose, one of the most difficult obstacles to overcome is the delivery of DNA binding motifs, such as DNAs, RNAs, and small molecules, into the targeted mitochondria efficiently and selectively.[6,7] Multiple copies of mtDNA exist in mammalian cells in the heteroplasmic form, comprising both wild-type alleles and mutations.[2] The current approaches to mtDNA transcription manipulation have primarily relied on exogenous delivery of transcription factors [e.g., mitochondrial transcription factor A (TFAM)] via DNA-based editing nucleases such as transcription activator-like effector nucleases (TALENs), CRISPR−Cas9 systems, and DddA-derived cytosine base editors (DdCBEs).[5,8−15] These methods can potentially bind to mutant mtDNA and trigger transcriptional repression or base editing to reduce the pathogenic burden while leaving wild-type alleles alone.[10] However, to translate these advanced tools into therapeutics or models for mitochondria-associated diseases, significant improvements in their delivery into the cytoplasm and mitochondria would be critically required. This is because most mitochondrial transcription factors and base editing tools encounter significant barriers during intracellular delivery or circulation in the blood. In addition, due to their enormous size, their mobility is hampered when passing through cellular membranes and mitochondrial membranes, which poses an additional obstacle to achieving maximum efficiency in mitochondrial transcription regulation.[8,9] Small-molecule-based approaches to mimic the function of mitochondrial transcription factors (TFs) have recently been demonstrated, albeit with limited effectiveness due to their poor solubility and delivery efficiency into targeted mitochondria.[9,16]

In response to the challenges outlined above, nanoparticle (NP)-mediated drug delivery systems (DDSs) have emerged as promising options for developing novel therapeutic interventions for mitochondria-associated diseases such as cancer and neurological diseases.[17−22] When binding to mitochondria-associated biomolecules, NP-mediated DDSs

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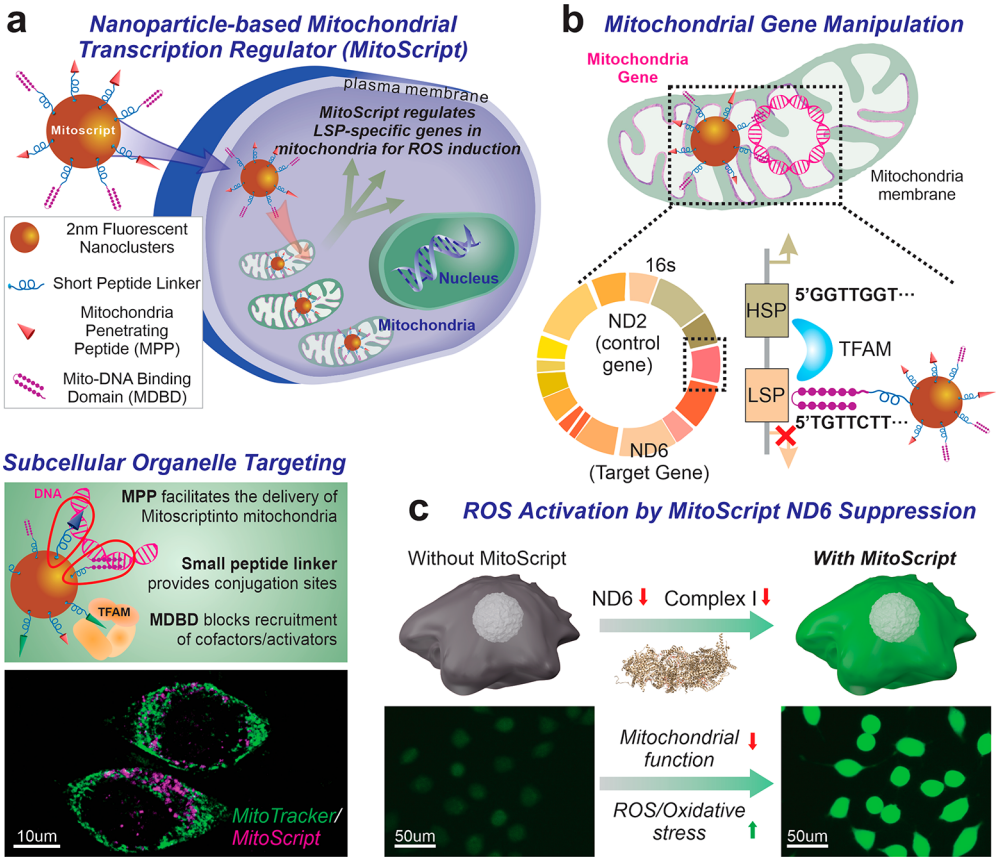

Figure 1. Schematic diagram illustrating *MitoScript*-based regulation of mitochondria gene transcription. (a) Overall design of *MitoScript*. To overcome the challenges associated with the regulation of mitochondrial gene expression and redox manipulation, a biomimetic *MitoScript*-based approach that facilitates overcoming the cell membrane and mitochondrial barriers is developed. The *MitoScript* is assembled from a glutathione peptide-capped gold nanocluster core, mitochondrial penetrating peptide, and a mito-DNA binding pyrrole imidazole polyamide (PIP) ligand that effectively translocates a DNA binding motif into the mitochondria instead of the nucleus. Inset image (200 × 200 μm) in the lower panel is cellular imaging of HeLa cells from the fluorescence of *MitoScript*. (b) Delivery of *MitoScript* into mitochondria for targeting the ND6 gene in mitochondria DNA. The schematic diagram shows the human mitochondrial genome, and the box indicates the location of the ND6 gene in the light strand. (c) Illustrations of ND6 mtDNA suppression-induced ROS activation. Specifically, *MitoScript*-based suppression of the ND6 gene would lead to the defects in Complex I in the mitochondria membrane which further leads to a reduction of the mitochondria function and increases ROS inside the cell. Inset images (200 × 200 μm) are dichlorodihydrofluorescein diacetate-based staining of intracellular ROS. HeLa cells were treated by *MitoScript* followed by imaging with 2′-7′-dichlorodihydrofluorescein diacetate-based detection of ROS.

can increase drug solubility, cellular targeting specificity, and multivalency effects.[23−27] Several NP-based DDSs have demonstrated the capacity to significantly impact mitochondrial activity by altering the cellular redox environment, increasing the temperature of the cytoplasm and delivering medicines that target mitochondrial pathways.[28−31] Nevertheless, their potential for direct, effective, and target-specific modulation of mitochondrial gene expression remains largely untapped, necessitating additional inventive research in this area.[32] The mitochondrial genome of mammals encodes 13 proteins.[3] Because each of them has a distinct function in mitochondria-mediated disease modeling and therapy, the ability to selectively regulate each mitochondrial gene transcription may promote a more precise understanding and improved treatment of mitochondria-associated diseases. Therefore, there is a clear need to develop an effective, systematic, and selective method to modulate mitochondrial gene expression levels by mimicking the functions and structures of mitochondrial TFs.

Herein, we demonstrated the development of a bioinspired nanoparticle-based artificial mitochondrial transcription factor (*MitoScript*, Figure 1). Our developed *MitoScript* platform is constructed from three components: (i) an ultrasmall nanocluster as a fluorescent core that allows for the conjugation of multiple biomolecular ligands,[33−35] (ii) synthetic PIP oligomers as mtDNA binding domains for site-specific transcription regulation, and (iii) mitochondria-penetrating peptides (MPPs) as mitochondrial localization domains (Figure 1a). Nanoparticles/nanoclusters are linked to the DNA binding and mitochondrial localization domains through hydrophilic glutathione (GSH) peptides, with two carboxylic groups for carbodiimide coupling with amine-terminated PIPs and MPPs. *MitoScript*'s unique properties allow it to be soluble in water and a range of buffers, and it can be delivered to targeted cellular suborganelles (e.g., mitochondria) in a biocompatible or nonviral way. Additionally, because of the zwitterionic nature of its surface ligands (anionic GSH linkers, cationic MPPs, and PIPs), *MitoScript* can be effectively uptaken by cells, which further leads to the targeting of the mitochondria.[36−38] In parallel, the endogenous fluorescence generated by quantum confinement at the nanoscale sizes of gold nanoclusters can be applied to visualize cellular uptake and mitochondria targeting.[39−42] Most crucially, *MitoScript* was effectively as well as selectively delivered to mitochondria

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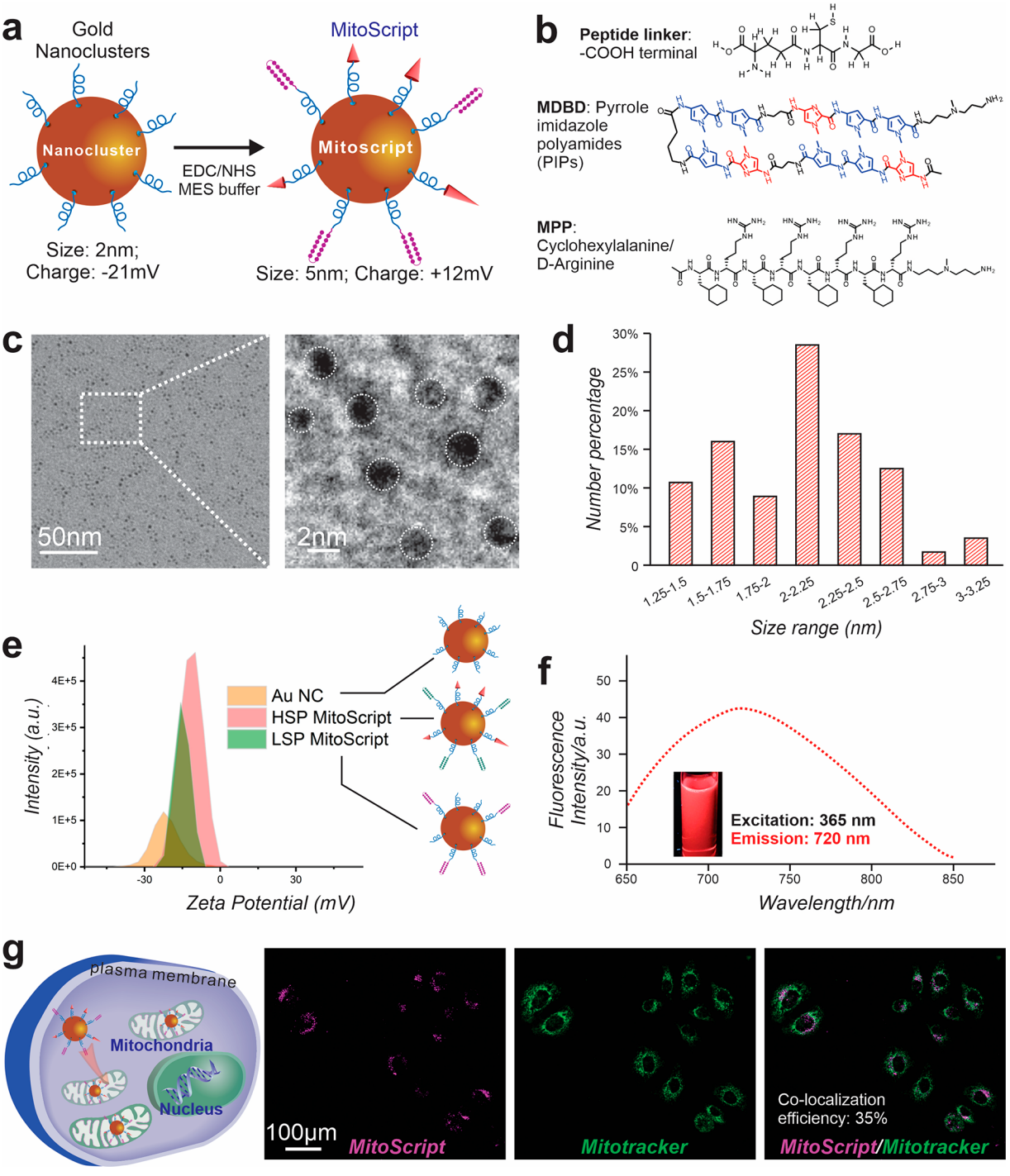

Figure 2. Synthesis of *MitoScript* for mitochondrial targeting. (a) A schematic diagram illustrating the conjugation of different domains onto a glutathione (GSH)-functionalized Au nanocluster to form the *MitoScript*. EDC/NHS coupling between the carboxylic group in the GSH and the primary amine group in the PIP and MPP was used for the conjugation. (b) Chemical structures of different domains used in the synthesis of *MitoScript*. (c, d) Transmission electron microscope (TEM, c) and quantification of TEM sizes of nanoclusters. (e) Zeta potential measurement confirming the conjugation of cationic/neutrally charged MPP and PIP ligands to the anionic nanoclusters which leads to a decrease of negative charge on the nanocluster surface. (f) Fluorescence microscope of the constructed *MitoScript*. The inset image shows the fluorescence from an aqueous nanocluster in solution under exposure of UV (λ = 325 nm). (g) Confocal microscope characterization of *MitoScript*-based selective targeting of mitochondria. A high colocalization coefficiency (calculated based on Pearson score) and minimal nanocluster fluorescence in the nucleus region indicate the efficient mitochondrial transportation of *MitoScript*.

and regulated mtDNA transcription. The NADH-ubiquinone oxidoreductase chain six protein (ND6) is a crucial subunit of NADH dehydrogenase in the electron transport chain and is transcribed by the light chain of circular mtDNA (Figure 1b).[43,44] When the mtDNA binding domain was specifically engineered for binding to the minor groove of the light chain TFAM binding sites, we selectively suppressed ND6 genes in HeLa cells. *MitoScript*-based manipulation of mtDNA transcription further altered the redox status of the cells, with significantly increased production of reactive oxygen species

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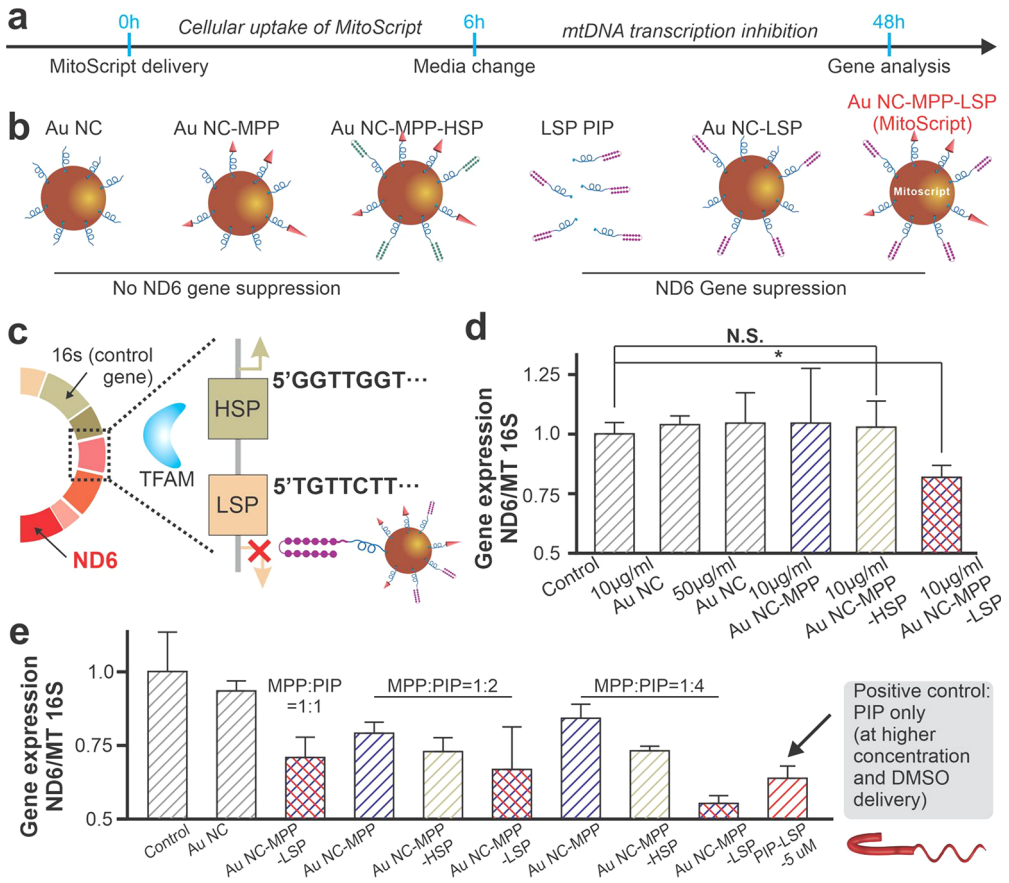

Figure 3. *MitoScript*-based mitochondrial gene regulation. (a) Schematic diagram showing the working principle of *MitoScript* for mitochondrial gene regulation. *MitoScript* is typically administered to cells in OptiMEM for 6 h followed by media change into growth media 48 h after cells are harvested for mitochondrial gene analysis. (b) Experimental design (timeline and control groups) of *in vitro* validation of the *MitoScript*-based regulation of mtDNA transcription. (c) A schematic diagram showing the *in vitro* model for studying gene transcription in mitochondria based on the ND6 mRNA level and using the 16S gene as a control gene. TFAM: Mitochondrial transcription factor A. HSP: Heavy strand promoter. LSP: Light strand promoter. (d) Selective suppression of ND6 by LSP-targeting *MitoScript*. In contrast, the nanocluster alone or nanoclusters conjugated with MPP only do not show any regulatory effects on mitochondrial gene expression. $N = 3$ biological replicates. *$P < 0.05$ by a one-way ANOVA test with Tuckey posthoc analysis. (e) Optimization of the *MitoScript* platform by modulating the conjugation ratio between MPP and PIP from 1:1, 1:2, to 1:4 and treating cells with different concentrations of *MitoScript* of ND6 by an LSP-targeting *MitoScript*. PIP molecule alone at a high concentration also induced suppression of ND6 gene expression, but their delivery requires DMSO which is toxic at high concentrations. $N = 3$ biological replicates.

(ROS) associated with suppression of the ND6 gene (Figure 1c). Thus, we showcased the development of a modular nanoparticle-based *MitoScript* platform for selective manipulation of mtDNA transcription, which provides a unique means of understanding and potentially treating mitochondria-associated diseases.

Natural mitochondrial transcription factors, such as **TFAM**, regulate gene regulation through their multidomain structures, typically including a mitochondria penetration domain, a DNA-binding domain, an activation/suppression domain, and a peptide backbone that bridges different domains.[45−47] *MitoScript* partially recapitulates the multidomain structures of natural mitochondria transcription factors (TFs), by grafting multiple mitochondria penetration domains (MPP peptide) and DNA binding domains (PIP oligomers) onto a single nanocluster (Figure 1a and Figures S1 and S2). *MitoScript* has sizes (2−3 nm) smaller than natural transcription factors, but it adds additional unique functions, such as an innate near-infrared (NIR) fluorescence for mitochondria tracking. More interestingly, a multivalency effect resulting from the higher densities of surface conjugation sites can be used to assemble MPP and DNA binding domains, as demonstrated in this study.[38,48] To prove this, fluorescent ultrasmall gold nanoclusters were first synthesized from a facile redox reaction between gold(III) chloride and a glutathione (GSH) peptide (Figure 2a). In this reaction, the thiol group in GSH serves as both a reducing agent and capping agent for restricted crystal growth, yielding 2−3 nm gold nanoclusters with 100−200 carboxylic groups on the surfaces of each particle.[48] Using liquid chromatography mass spectroscopy (LCMS), we calculated around 32% coverage in terms of ligand conjugation on *MitoScript* (Figures S3−S6), which is consistent with the literature.[48] The ligands were also found to be stable for at least 24 h after cellular uptake, thereby validating our conjugation strategy (Figure S7). In parallel, amine-functionalized hairpin polyamide was synthesized with amino acid sequences of ImPyPyβImPy-γ-PyPyβImPyPy-βDp-NH_2 using solid-phase synthesis (Figures 2b and S1). In hairpin polyamides, *N*-methylimidazole (Im) and *N*-methylpyrrole (Py) amino acids are known to selectively bind complementary G−C and A−T motifs on both nuclear and mitochondria DNA.[49−55] Specifically, the amino acid sequence is designed to

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bind to the light strand promoter (LSP, Figure S1), which can alter gene expression on the light strand of mitochondria DNA.

Similarly, MPP with an optimal ratio of hydrophobic cyclohexylalanine (Cha) and positive D-arginine (Arg) that allows for robust targeting of the mitochondrial membrane was also synthesized with a residual amine group.[5,52] By conjugating an equal amount of amine-functionalized PIP and MPP to carboxylic groups on gold nanoclusters, we assembled them into *MitoScript*. Considering its ultrasmall size, innate NIR fluoresce, multivalent surfaces, excellent cellular uptake, low batch-to-batch variation, and high biocompatibility (Figures 2c–f, S3, and S8–S10) compared to the nanocluster or PIP molecule alone, *MitoScript* could be suitable for mitochondria-based applications. To realize mitochondrial transcription regulation, efficient transportation across cellular membranes must be achieved. We studied the efficient delivery of *MitoScript* to HeLa cells using a fluorescence microscope using the intrinsic fluorescent properties of the nanoclusters at the core of *MitoScript* (Figure 2f). By avoiding using organic solvents or viral transfection vectors, we could enhance biocompatibility and potentially reduce immunogenicity. Notably, *MitoScript* was found to be stable in most physiological buffers, including phosphate-buffered saline (PBS) and cell growth media, which could be attributed to the dense hydrophilic GSH linker on its surfaces (Figure 2e). This contrasts with our previously reported PIP molecules, which also inhibit mitochondria gene expression but are poorly soluble in water or any physiologically relevant buffers.[49,56]

Overall, even at a relatively low concentration (10 μg/mL or 100 nM), *MitoScript* was rapidly (within 24 h) uptaken by nearly all cells, with an over 96% delivery efficiency (Figure S10). Also, because of its high biocompatibility and avoidance of using organic solvents during the delivery, *MitoScript* at both low (10 μg/mL) and high concentrations (100 μg/mL) was found to be nontoxic (Figures S8 and S9), not only to the standard HeLa cell line but also to more delicate human-induced pluripotent stem-cell-derived neural stem cells as well as human monocytes. The high efficiency of cellular uptake and increased biocompatibility of *MitoScript* represent clear advantages over conventional DNA binding motifs, including free PIP molecules.

Furthermore, *MitoScript* particles that were efficiently uptaken by cells effectively targeted the mitochondria, as demonstrated by confocal microscope-based live-cell imaging (Figures 2g and S11). Specifically, mitochondria in HeLa cells were stained with tetramethylrhodamine isocyanate (TRITC) labeled MitoTracker, and to make *MitoScript* visible using confocal microscopy, nanoclusters were conjugated with *N*-hydroxyl succinimide (NHS) conjugated cyanine 5 (Cy5) dye using the residual amine groups in the GSH linker (Figure S11). A high colocalization (approximately 35% from the Pearson score) of *MitoScript* fluorescence with TRITC signals in the live cell imaging experiment suggests a selective delivery of *MitoScript* into the mitochondria versus the cytosol (note that DAPI nuclei staining was not used as the fluorescence overlaps with *MitoScript*). Although this is not as target-specific as the PIP molecule alone, further optimization could be achieved by enhancing the MPP to PIP ratio. Likewise, as the delivery of DNA binding motifs into the nucleus has also been widely used to manipulate nuclear gene transcription, it is crucial to ensure a minimal presence of *MitoScript* in the nucleus.[9]

We proved this by observing minimal *MitoScript* fluorescence signals in nuclear regions in the confocal microscope images (Figure 2g). Such efficient and selective delivery of *MitoScript* into the mitochondria are prerequisites of mitochondrial gene manipulation and could be attributed to MPPs on *MitoScript*. Although DNA binding motifs have also been conjugated with MPPs for selective delivery into mitochondria, we achieved a high delivery efficiency at nanomolar concentrations without any organic solvents or viral vectors.[9] This result is consistent with previous literature in spherical nucleic acids, where oligonucleotides conjugated to nanoparticles result in better transfection efficiency than free oligonucleotides, because of not only the alteration of the cellular uptake mechanism but also reduced enzymatic degradation of DNA binding motifs and multivalent binding of target DNAs on nanoparticles.[57]

Next, we investigated the regulation of gene transcription in mitochondria by *MitoScript* (Figure 3). *MitoScript* is typically assembled from multiple DNA binding domains (PIP molecules), MPP domains, and a small-sized nanocluster as their cores. We did not include *MitoScript* with an LSP-targeting PIP ligand only as it is well established that MPP is essential for biomolecular delivery into mitochondria. We hypothesize that (i) both DNA binding domains and MPPs are essential for the selective manipulation of mitochondria gene transcription; (ii) multiple domains assembled on nanoclusters would have multivalency effects on gene expression; and (iii) the small size of nanoclusters is crucial for efficient mitochondria gene manipulation (Figure 3a,b).

To prove this, we isolated and specifically analyzed mitochondrial genes 48 h after delivery of *MitoScript* that targets the light strand promoter (LSP) region using 16S (mitochondrially encoded 16S RNA) as a baseline gene (Figure 3c). As controls, we synthesized and tested nanoclusters conjugated with LSP-targeting PIPs only, with MPPs only, or with both domains, but the PIP was engineered to target the HSP region in the mitochondria genome under identical mass concentrations (Figure 3d). Among all groups, only *MitoScript* showed significant suppression of ND6, which is a direct indicator of regulation of LSP-associated mitochondria gene manipulation, according to previous reports.[9] This directly justified the design of the multidomain structures of our *MitoScript* platform. Furthermore, as positive controls, PIPs that target LSP were also treated to HeLa cells at varying concentrations. As expected, high concentrations of (10 μM) solutions of PIPs transfected using dimethyl sulfoxide (DMSO) (2%) resulted in significant suppression of ND6 gene expression, to a similar level from a low concentration (100 nM) of *MitoScript* treatment (Figure 3e). When concentrations of PIPs were lowered to 5 μM, however, their effects on gene expression were decreased, despite that their concentration is still an order higher than *MitoScript*. This is again consistent with the literature on nanoparticle-based nuclear gene transcription regulation that the multivalency of DNA binding motifs can facilitate the recognition and inhibition of target genes during transcription.[58] Remarkably, *MitoScript*-based modulation of mitochondrial gene expression was also successfully reproduced in a human iPSC-NSC line, where only the *MitoScript* induced suppression of ND6 genes after 48 h of *MitoScript* treatment, while the nanocluster alone or nanocluster conjugated with MPP alone did not show any clear effect on the ND6 gene expression (Figure S12). This strongly supports *MitoScript* as a platform for mitochondrial gene

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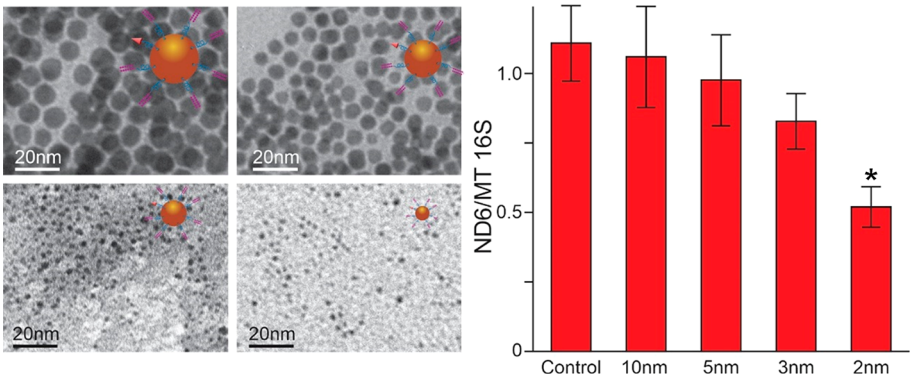

Figure 4. Size-dependent effects of *MitoScript*-based modulation of mitochondria gene expression. The four images on the left panel are representative TEM characterizations of *MitoScripts* constructed from 10, 5, 3, and 2 nm gold nanoparticle/nanoclusters, respectively. 10 and 5 nm gold nanoparticles were initially citrate capped followed by glutathione ligand exchange. The rest of the conjugation procedures are identical across different sizes of *MitoScripts*. The graph on the right panel is qRT-PCR analysis of mitochondrial ND6 gene expression after HeLa cells treated with *MitoScript* at varying sizes. $N = 3$ biological replicates, $^*P < 0.05$ by one-way ANOVA with Tuckey posthoc analysis.

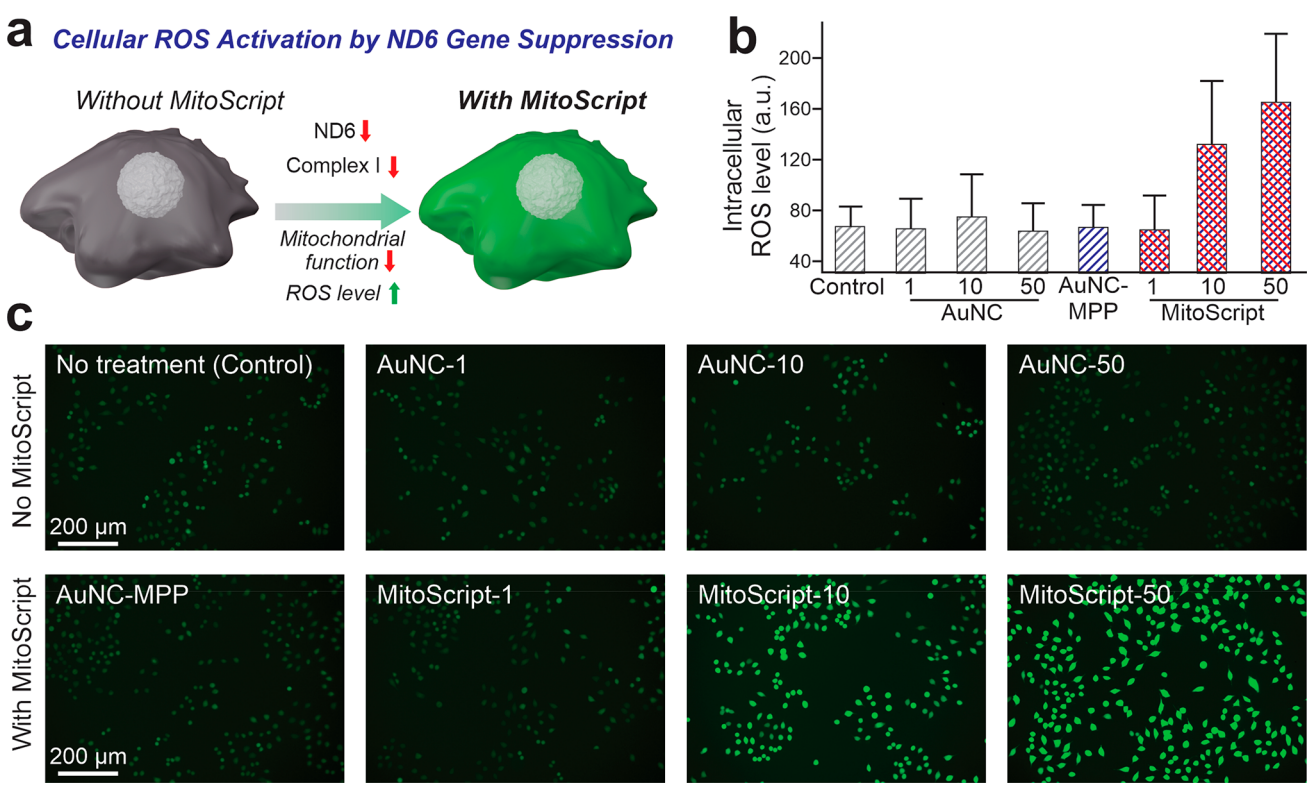

Figure 5. *MitoScript*-mediated ND6 suppression leads to intracellular ROS activation. (a) A schematic diagram showing that *MitoScript*-mediated ND6 suppression leads to alteration of cellular redox status. Specifically, *MitoScript*-based suppression of the ND6 gene would lead to the defects in Complex I in the mitochondria membrane, which further leads to reduction of mitochondria function and increases ROS inside the cell. (b,c) A summary graph (b) and representative ROS staining (c, colored in green, from DCF assay) showing the selective and concentration-dependent activation of ROS by *MitoScript*-targeting LSP. Value shown in the graph in b indicates the concentration of *MitoScript* or nanoclusters (unit: ug/mL). $n = 3$ biological replicates.

expression in applications other than the model HeLa cell line. Meanwhile, it is crucial to note that the efficiency of the positive control (PIP molecule alone) is somewhat inconsistent from the previous reports, which could be attributed to the variations in cell line but would require further investigation and optimization of the *MitoScript* platform.

In addition, to study whether the size of nanoclusters in *MitoScript* plays an essential role in gene regulation, we also synthesized *MitoScripts* using gold nanoclusters/nanoparticles with varying sizes of 2, 3, 5, and 10 nm (Figure 4). While 2 and 3 nm gold nanoclusters were synthesized directly from GSH using similar protocols, 5 and 10 nm gold nanoparticles were synthesized from the citrate-based reduction of $HAuCl_4$, followed by ligand exchange with GSH and conjugation with MPPs and PIPs through a carbodiimide cross-linker. By doing so, we could assume similar surfaces across different gold nanoclusters/nanoparticles. Gene analysis of HeLa cells treated by *MitoScript* assembled from varying sizes revealed a clear trend of size-dependent effect on mitochondria gene manipulation (Figure 4). Specifically, a decrease in the

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nanoparticle sizes resulted in a more robust suppression of mitochondria gene transcription. When sizes reach 10 nm, no effects from the MPP- and PIP-conjugated nanoparticles on mitochondria gene transcription were observed. This result is well-aligned with previous reports on protein transportation across the mitochondria membrane, which unlike the nuclear membrane lacks large pores that allow for the transportation of high-molecular-weight proteins.[59] Taken together, by engineering the multidomain structures and sizes, we optimized our *MitoScript* platform and verified its robust regulation of mitochondria gene transcription *in vitro*.

Selective and reliable manipulation of mitochondria gene transcription can enable the regulation of energy production and redox balancing, which are of the utmost importance for cancer migration, muscle contraction, and neuron death.[60] To confirm whether *MitoScript*-based gene regulation can alter cellular activities, we studied *MitoScript*-induced redox manipulation as a proof-of-concept. ND6 is a key subunit of NADH dehydrogenase in the electron transport chain involved in ATP production.[61] Suppression or mutation of ND6 genes in mitochondria results in ROS production (Figure 5a).[62] As such, we hypothesize that our exemplary *MitoScript* that targets LSP and suppresses the ND6 gene could also manipulate cellular redox by inhibiting NADH dehydrogenase. To prove this, we treated HeLa cells with varying concentrations of *MitoScript* and investigated ROS production using a standard 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA)-based assay 48 h after *MitoScript* treatment (Figure 5b,c). As controls, nanoclusters with equivalent concentrations were also treated to cells, and analysis was performed under identical conditions. Our hypothesis was directly supported by observing a concentration-dependent increase of ROS levels in *MitoScript*-treated HeLa cells, indicated by the increased intensities of green fluorescence, while minimal changes were observed by bare nanocluster treatment across all concentrations (Figure 5b,c). Interestingly, *MitoScript* assembled from different-sized gold nanoclusters/nanoparticles can also show an apparent decrease in ROS production when sizes were increased, which provides additional support for our observation in the size-dependent study on mitochondria gene regulation (Figure S13). Our data indicate that *MitoScript*-based regulation of mitochondrial gene transcription can modify cellular functions, which has huge potential for improving the knowledge and treatment of mitochondrial gene-related diseases and disorders.

In summary, inspired by the multidomain structure of natural transcription factors, we designed and synthesized an ultrasmall nanoparticle-based *MitoScript* platform to manipulate mitochondrial gene transcription effectively. Since there have been only a few methods to regulate the mitochondrial genome, *MitoScript* offers a promising alternative that provides a few benefits. These benefits include intrinsic NIR fluorescence for cellular tracking, multivalency effects for more efficient gene manipulation, and the avoidance of toxic organic solvents and immunogenic viral vectors. By optimizing and engineering the versatile *MitoScript* platform, we further validate its capability for altering cellular redox states by regulating mitochondria gene transcription, which is important for various biological applications. As we move forward, it would be essential to understand the specific interactions between *MitoScript* and the mitochondrial transcriptional machinery utilizing more advanced techniques such as cryo-TEM and to design more effective and selective *MitoScript* platforms based on the in-depth understanding gained from these studies. Using novel nanocluster cores with NIR excitation could significantly help with *in vitro* and *in vivo* studies.[63] Additionally, although prior work by Dervan and coworkers has provided insights into PIP selectivity, it is essential for future applications to conduct a more detailed analysis comparing *MitoScript* with and without single base pair mutations for gene manipulation. This comparison could be studied using methods such as DNase I footprint titration.[64] The detailed cellular uptake mechanisms of *MitoScript* with varying sizes should be studied to better design the future *MitoScript* platform. To advance *MitoScript* for more selective and robust manipulation of mitochondrial genes, it is thus essential to further optimize the platform by varying the ratio of functionalization with different ligands. Furthermore, since both suppression and activation of mitochondria genes have shown relevance in the treatment of mitochondrial diseases, it would be a crucial next step to study whether *MitoScript*, once conjugated with an activation domain, can also allow gene activation in mitochondria and whether it could regulate genes beyond ND6 genes. Finally, because *MitoScript* has various advantages for *in vivo* applications, it would be interesting to investigate the pharmacokinetics of *MitoScript* with different linkers (e.g., polyethylene glycol) other than GSH and compare it to free drugs without conjugation to nanoparticles. *MitoScript* could thus be developed further as a general platform technology and a possible alternative toolset to existing small chemical- and viral-based mitochondrial gene editing technologies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.nanolett.2c03958.

> Synthesis of pyrrole-imidazole polyamide (PIP) ligands, synthesis of *MitoScript* and control nanoparticles, nanoparticle and *MitoScript* characterization, cell culture, *MitoScript* delivery and cell imaging, qRT-PCR analysis of mitochondria gene expression, cell viability and ROS assay, MALDI-TOF and HPLC characterization of LSP-NH_2 PIP ligand and HSP-NH_2 PIP ligand, cellular uptake of bare gold nanoclusters, cell viability after treatment by PIP, nanoclusters and *MitoScript*, UV−vis absorption spectrum of MitoScript and high cellular uptake of *MitoScript*, *MitoScript* size-dependent effects on mitochondrial gene suppression, mitochondrial gene regulation by MitoScript in a hiPSC-NSC line, *MitoScript* size-dependent effects on mitochondria ROS activation, and primer sequences for the gene analysis (PDF)

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Author Contributions

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

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Notes

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■ ABBREVIATIONS

MitoScript, Nanoparticle-based mitochondrial transcription regulator; DNA, Deoxyribonucleic acid; mtDNA, Mitochondrial DNA; PIP, Pyrrole imidazole polyamide; TFAM, Mitochondrial transcription factor A; TALEN, Transcription activator-like ef-factor nucleases; DdCBE, DddA-derived cytosine base editors; ROS, Reactive oxygen species; MPP, Mitochondria-penetrating peptide; GSH, Glutathione; TEM, Transmission electron microscope; UV−vis, Ultraviolet visible; NADH, Nicotinamide adenine dinucleotide; ND6, NADH-ubiquinone oxidoreductase chain 6 protein; TF, Transcription factor; NIR, Near-infrared; Im, Methylimidazole; Py, *N*-Methylpyrrole; LSP, Light strand promoter; HSP, Heavy strand promoter; Cha, Cyclohexylalanine; Arg, Arginine; PBS, Phosphate-buffered saline; TRITC, Tetramethylrhodamine isocyanate; Cy5, Cyanine 5; DMSO, Dimethyl sulfoxide; DCFH-DA, 2′-7′-Dichlorodihydrofluorescein diacetate

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