Programed Assembly of Nucleoprotein Nanoparticles Using DNA and Zinc Fingers for Targeted Protein Delivery

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With a growing number of intracellular drug targets and the high efficacy of protein therapeutics, the targeted delivery of active proteins with negligible toxicity is a challenging issue in the field of precision medicine. Herein, a programed assembly of nucleoprotein nanoparticles (NNPs) using DNA and zinc fingers (ZnFs) for targeted protein delivery is presented. Two types of ZnFs with different sequence specificities are genetically fused to a targeting moiety and a protein cargo, respectively. Double-stranded DNA with multiple ZnF-binding sequences is grafted onto inorganic nanoparticles, followed by conjugation with the ZnF-fused proteins, generating the assembly of NNPs with a uniform size distribution and high stability. The approach enables controlled loading of a protein cargo on the NNPs, offering a high cytosolic delivery efficiency and target specificity. The utility and potential of the assembly as a versatile protein delivery vehicle is demonstrated based on their remarkable antitumor activity and target specificity with negligible toxicity in a xenograft mice model.

1. Introduction

Over the past decades, an understanding of the molecular mechanisms underlying the progression of various diseases has allowed druggable targets to be identified.[1,2] Small molecule–based drugs have been the primary choice, and notable successes have been reported.[3,4] However, such small-molecule agents still suffer from low efficacy and severe toxicity owing to a relatively low specificity.[5,6] In this regard, protein-based biologics have attracted considerable attention owing to their high efficacy and low toxicity, and a number of protein therapeutics for extracellular targets are being clinically used.[7,8] With a growing number of intracellular drug targets and the high efficacy of protein therapeutics, a cytosolic protein delivery platform with high specificity has been considered crucial.[9,10] Despite significant progress, however, the efficient and cell-selective delivery of proteins remains a challenge in realizing their full therapeutic potential.

Nanostructured materials, including liposomes, polymeric NPs, dendrimers, and micelles, have found wide applications in delivery of diverse therapeutic agents, owing to their multifunctionality.[11–13] However, most of them have been obtained through a chemical synthesis or polymerization under harsh conditions, causing intrinsic limitations in terms of productivity, homogeneity, and toxicity.[14,15] In addition, such approaches have resulted in an uncontrolled loading density of the targeting moiety and protein cargos, leading to a low specificity and delivery efficiency.[16–19] As an approach to overcoming such drawbacks, considerable attention has been paid to the assembly of nanostructured materials through biomolecular interactions.[20–22] In particular, the use of DNA and proteins as the building blocks for the construction of nanostructures with high homogeneity has been of great interest owing to their easy design and self-assembly.[21–22] A biomolecule-mediated assembly enables a fine-tuning of the supramolecular organization of a nanosized material, facilitating the rational design of complex superstructures with well-defined functionality, high homogeneity, reproducibility, and biocompatibility. We previously showed that a sequence-specific DNA-binding zinc finger (ZnF) can be harnessed for the construction of inorganic nanoparticle clusters with predesigned sizes through a DNA-directed assembly.[28]
Herein, we explore a programmed assembly of nucleoprotein nanoparticles using DNA and ZnFs for targeted protein delivery. Two types of ZnF recognizing different DNA sequences were genetically fused to a cellular targeting moiety and a cytotoxic protein cargo, respectively. Double-stranded DNA with multiple ZnF-binding sites was designed and grafted onto the gold nanoparticles using streptavidin–biotin chemistry. The DNA-functionalized nanoparticles were coincubated with the ZnF-fused proteins to generate the assembly of nucleoprotein nanoparticles (Figure 1).

The amount of protein cargos in the assembly was controlled by modulating the number of ZnF-binding sites. We demonstrated the utility and potential of our approach by showing an efficient and tumor-selective cytosolic delivery of the protein cargo, as well as a remarkable tumor regression with negligible toxicity in xenograft mice models. Details are reported herein.

2. Result

2.1. Design of Template DNA and Zinc Finger–Fused Protein Cargos

A schematic for the assembly of nucleoprotein nanoparticles (NNPs) using DNA and ZnF proteins is shown in Figure 1. Gold nanoparticles (AuNPs) of 10 nm in diameter were employed as inorganic core particles. Briefly, a nucleotide modified with biotinylated triethylene glycol (Biotin-TEG) was introduced at the 3′ end of the double-stranded DNAs for the purposes of conjugation with neutravidin (NA)-coated AuNPs and proper spacing between the DNAs and AuNPs. We designed a biotinylated DNA with an array of spatially positioned ZnF-binding sites for the stable formation of NNPs without a steric hindrance (Figure 1a). Grafting of DNA molecules onto the nanoparticle surface was revealed to be highly inefficient at low ionic strengths due to strong electrostatic repulsion between negative charged DNAs. The biotinylated DNA was thus immobilized onto the NA–AuNPs under high salt conditions (1.0 M NaCl), yielding DNA-functionalized AuNPs (DNA–AuNPs). As a result, the DNA grafting efficiency was estimated to be 34% (3.4 DNA molecules per AuNP) at a DNA to AuNP ratio of 10:1 (Table S1, Supporting Information). On the other hand, a relatively low grafting efficiency of 16% was observed under physiological conditions (150 × 10⁻³ M NaCl) without a TEG linker. Based on the result, the former reaction conditions were selected for the assembly of nucleoprotein-decorated AuNPs. To load the functional proteins into the template DNA in a controlled manner, two types of ZnF proteins having different sequence specificities, namely QNK–QNK-RHR (QNK) and Zif268 (Z268), were employed as a DNA-binding motif, and were genetically fused with a protein cargo and a targeting moiety, respectively (Figures S1 and S2, Supporting Information). Both the ZnF proteins were known to have a sub-nanomolar affinity for their target sequences. First, we designed consecutively positioned QNK-binding sites with a tetranucleotide spacer in the center region of the template DNA, and inserted a binding sequence for the Z268 protein near the 5′ end of the template DNA for directing a...
targeting moiety outward from the AuNP surface to achieve an effective recognition of a target (Figure 1b).

2.2. Stepwise Assembly and Characteristics of Nucleoprotein Nanoparticles

We next generated the NNPs by conjugating the ZnF-fused proteins to the DNA–AuNPs in a stepwise manner, and investigated their assemblies and characteristics during each step in terms of the particle size and morphology. Unmodified AuNPs and NA–AuNPs were shown to have an average diameter of 18 and 24 nm, respectively, based on a dynamic light scattering (DLS) analysis (polydispersity index (PDI) of 0.08 and 0.05, respectively) (Figure 2a). As described above, biotinylated DNAs of 268 base pairs (bp) were grafted onto NA–AuNPs (Figure S3, Supporting Information), and the diameter of the DNA–AuNPs increased to 68 nm (PDI of 0.18). Subsequently, a threefold molar excess of the QNK proteins to the total number of QNK binding sites was added to the DNA–AuNPs. The ETA–QNK released from the NNPs assembled with template DNA of 268 bp was also analyzed on PAGE (left). The ETA–QNK released from the NNPs is shown as a rectangle. The table shows the measured concentration of ETA–QNK and the theoretical value derived from the number of conjugated template DNAs and the concentration of NNPs.
assembly of NNP s was observed to have a marginally increased size of 79 nm with a single monodisperse peak (PDI of 0.17), indicating the assembly of the NNP s with high homogeneity in a stepwise manner.

ZnF proteins with a positively charged surface are thought to neutralize the negative charges on the template DNA. We checked whether a ZnF-mediated assembly of NNP s leads to a change in both their surface charge density and molecular weight using a gel electrophoresis mobility shift assay (EMSA). Two types of engineered QNK s were used as the model proteins: Spycatcher-fused QNK (Sc–QNK) and mOrange-fused QNK (mO–QNK). Spycatcher has a molecular mass of 15 kDa, and is known to spontaneously form a isopeptide bond with its peptide partner (Spytag). As a result, distinctly shifted bands were observed in both the assemblies with negligible smeared bands compared to DNA–AuNP s (Figure 2b), which implies that a charge counterbalance by the ZnF proteins led to the assembly of NNP s and an increase in their molecular weight. To characterize the size and morphology of the NNP s in detail, we conducted an atomic force microscopy (AFM) analysis of the NA–AuNP s and mO–QNK-incorporated NNP s (Figure S4, Supporting Information). For a clear observation of their shape and size, we used a long-length DNA template of 944 bp with 4 times the number of QNK-binding sites than a 268 base-paired template DNA. AFM images revealed that the mO–QNK-conjugated NNP s have an elongated and flattened morphology with a significantly increased radius (ΔX = 44 nm) and a slight increase in height (ΔY = 17 nm) as compared to the NA–AuNP s with a spherically symmetric structure (ΔX = 17 nm, ΔY = 10 nm) (Figure S4, Supporting Information). The enlarged structure of the NNP s seems to be mainly due to the direct adsorption of surface-exposed nucleoproteins onto the mica surface under air-dried conditions. Collectively, these results demonstrate that the present strategy based on biomolecular interactions between the ZnF and template DNA enabled the facile assembly of NNP s with high homogeneity in a programmable manner.

It was difficult to clearly visualize the formation of ZnF–DNA complexes on the particle surface through an AFM analysis. We therefore devised an indirect method to confirm the conjugation of cargo-fused ZnFs to DNA–AuNP s by transmission electron microscopy (TEM). In detail, smaller-sized AuNP s (5 nm in diameter) were coated with human serum albumin (HSA), and a HSA-binding domain (ABD) was fused to the QNK. Larger-sized AuNP s (10 nm in diameter) were functionalized with template DNA, followed by conjugation with the ABD-fused ZnF. The resulting NNP s were incubated with the HSA-modified AuNP s to generate the DNA-template gold nanoclusters (Figure S5a, Supporting Information). As shown in Figure 2d, the TEM images revealed core–satellite suprastructures with a size of ~50 nm, which consists of a single core AuNP s (10 nm) surrounded by small satellite AuNP s (5 nm). The observed size of the AuNP clusters coincides well with the DLS result. When a long template DNA of 994 bp was used, the size of the nanostructures increased to ~100 nm (Figure S5b, Supporting Information), which correlates well with the AFM data. Considering the diameter of HSA-coated AuNP s (~10 nm) and the distance between consecutive QNK-binding sites (~4.3 nm), it is reasonable for a maximum of six AuNP s to be associated with a single template DNA of 268 bp, which has 14 QNK-binding sites. Based on this, the total number of small AuNP s per single core–satellite nanocluster was estimated to be 18. As shown in Figure 2d, ~13 smaller-sized AuNP s were observed to be associated with a core AuNP, approaching about 70% of theoretical maximum number of nanoparticles, which seems to be due to the steric hindrance and charge repulsion between conjugated AuNP s within close proximity. This result demonstrates the sequence-specific incorporation of a cargo-fused ZnF through interaction between the template DNA and ZnF.

2.3. Controlled Loading of a Protein Cargo in Nucleoprotein Nanoparticles

Controlling the loading amount of the protein cargo in a nanocarrier is crucial for assessing the therapeutic efficacy and targeted delivery in preclinical and clinical tests. We checked the possibility of a controlled loading of a protein cargo in NNP s through a quantitative protein analysis. Pseudomonas aeruginosa exotoxin A (ETA) without the receptor-binding domain was employed as the protein cargo, and fused to the QNK. This bacterial exotoxin is known to strongly induce cell death through the irreversible inhibition of protein synthesis in the cells.[33] To achieve this, we designed three types of template DNA with different lengths (namely, 50, 117, and 268 bp) as well as varying numbers of ZnF-binding sites (Figure S3, Supporting Information). Each template DNA was grafted onto NA–AuNP s at a predetermined ratio, followed by the subsequent addition of the ZnF-fused ETA (Table S2, Supporting Information). The grafting densities of the respective DNAs were determined to be around 19, 9, and 3 DNA strands per AuNP when the ratio of DNA to AuNP for grafting each template DNA (50, 117, and 268 bp) was fixed at 50, 25, and 10, respectively. Accordingly, the numbers of QNK-binding sites for each NNP were calculated to be 38, 45, and 42, respectively. It is interesting to note that although the template DNAs with different lengths and QNK-binding sites were used, the total number of QNK-binding sites was almost similar, and this seems to be due to a decrease in the number of grafted DNA molecules as the length of template DNA increased. A quantitative analysis revealed that 35, 43, and 41 ETA–QNK molecules were loaded on the respective NNP s assembled using the template DNAs with different lengths (50, 117, and 268 bp) (Figure 2e and Figure S6a,b, Supporting Information). These values correspond to 92%, 95%, and 97% occupation of the theoretical ZnF-binding sites by a protein cargo, respectively. Based on the results, it is plausible that the loading amount of a protein cargo in the NNP s can be modulated by the number of ZnF-binding sites. To further validate the controlled loading of a cargo, we constructed NNP s using a Sc–QNK and template DNA of 268 bp under the same conditions described above, and analyzed the amount of loaded Sc–QNK (Figure S6c, Supporting Information). As a result, the number of loaded Sc–QNK molecules in single NNP s was estimated to be 40 (Figure S6c, right, Supporting Information), showing about 95% occupation of the ZnF-binding sites. Based on the results, it is likely that our approach enabled the controlled loading of a protein cargo in NNP s through the modulation of ZnF-binding sites.
2.4. Target Specificity and Internalization of Nucleoprotein Nanoparticles

We evaluated the target specificity and internalization of NNPs using human epidermal growth factor receptor (hEGFR)-expressing cancer cells. As the targeting moiety, an anti-hEGFR repebody (rEgH9), composed of leucine-rich repeat modules, was employed and fused to the Z268 protein. This repebody was previously developed and shown to have a high specificity and sub-nanomolar affinity for the hEGFR ectodomain\(^{[34]}\). We first examined the target specificity of the NNPs using an enzyme-linked immunosorbent assay. To do so, the repebody-fused Z268 (Rb–Z268) protein and monomeric alkaline phosphatase-fused QNK (mALP–QNK) were conjugated to DNA–AuNPs (Figure 3a), and ZnF-fused form of cargo protein (mALP–QNK) also did not show any attenuated activity compared to free form (mALP) (Figure S7, Supporting Information). As a negative control, nontargeting NNPs without the Rb–Z268 were used. As shown in Figure 3b, the targeting NNPs with the Rb–Z268 were observed to bind specifically to hEGFR, while exhibiting a relatively low binding activity for mouse EGRF (mEGRF). On the other hand, a negligible binding of nontargeting NNPs was observed. We further analyzed the binding strengths of the NNPs and a free repebody (Figure 3c). The midpoint concentrations of the NNPs and free repebody for hEGFR were estimated to be 397 and 498 ng mL\(^{-1}\), respectively, which indicates that both ZnF and the repebody on the NNPs retained their binding capacities for their respective targets even in a fusion form.

To examine the internalization of NNPs into the cells, mOrange was employed as a fluorescent reporter, and fused to the QNK protein for the assembly of NNPs (Figure 3a). The resulting NNPs were incubated with three types of cancer cell lines expressing different EGRF levels for 6 h, and internalization of the NNPs was analyzed using a laser scanning microscopy. As a result, EGRF-overexpressing A431 cells displayed a strong mOrange fluorescent intensity over the cytosol in a time-dependent manner (Figure 3d), whereas a relatively weak fluorescence was observed in HT29 and SW620 cells expressing moderate and low EGRF levels, respectively (Figure 3e). This difference in fluorescence intensity according to the cellular expression level of EGRF was clearly visualized even after long-time treatment (24 h) of NNPs (Figure S8, Supporting Information). These results indicate that the NNPs were internalized into the cells in a receptor-specific manner.

To directly confirm the internalization of the NNPs, we analyzed the amount of accumulated AuNPs in the cells using inductively coupled plasma mass spectrometry (ICP-MS). We used folic acid and K562 cells as a targeting ligand and a model cell line, respectively, in order to show the generality of NNPs as a targeted delivery vehicle. To construct folate-targeting NNPs, maleimide-functionalized folic acid were site-specifically conjugated to the N-terminal cysteine of the Z268 protein. The K562 cells were incubated with the NNPs or nontargeting ones for 4 h, and subjected to ICP-MS analysis after three washings (Figure 3f). The NNPs showed about fivefold higher intracellular gold level (1.6 × 10\(^{10}\) particles per cell) than the nontargeting ones (3.3 × 10\(^{9}\) particles per cell). Moreover, a distinct red color was observed from the cells treated with the NNPs. Taken together, it seems that the NNPs showed a high targeting capability by the ZnF-fused targeting moiety, followed by a subsequent internalization through a receptor-mediated endocytosis.

2.5. Cytosolic Delivery of a Protein Cargo Using Nucleoprotein Nanoparticles

We intended to deliver ETA using NNPs for targeted cancer therapy, because ETA itself cannot induce significant cytotoxicity without a receptor-binding domain\(^{[33,35]}\). The cytotoxic domain of ETA was genetically fused to the QNK protein for the assembly of NNPs using an anti-EGFR repebody-fused ZnF (Rb–Z268), and the cytotoxicity of the resulting NNPs was tested against EGRF-expressing cancer cells. The NNPs were shown to give rise to a significant cytotoxicity against EGRF-overexpressing cancer cells (A431, MDA468, and HCC827) in a dose-dependent manner and gradual cytotoxicity with increasing treatment time, whereas a negligible cytotoxicity was observed for MCF7 and SW620 cells with low EGRF levels (Figure 4a,b and Figure S10a–e (Supporting Information)). As negative controls, both NNPs without ETA and nontargeting NNPs were examined to check the effects of the nonspecific uptake and the nanoparticles themselves on the cell viability, respectively (Figure 4a,b and Figure S10 (Supporting Information)). Both the types of NNPs were shown to have a negligible effect on the cell viability, indicating no significant toxicity or nonspecific uptake.

To verify whether the cytotoxicity of the NNPs resulted from ETA-triggered cell death, annexin V staining was conducted to detect any early stage apoptosis in the NNPs-treated cells. After an 8 h incubation period with the NNPs, the A431 and MCF7 cells were labeled with annexin V–fluorescein isothiocyanate (FITC). As shown in Figure 4c, the NNPs strongly induced receptor-specific apoptotic cell death only in the A431 cells, whereas the nontargeting ones displayed a negligible fluorescent intensity in both the types of cells. This result supports the idea that the NNPs can specifically deliver cytotoxic payloads into the cytosol in a target-specific manner. Next, we tested the effects of a short exposure time on the cytotoxicity of the NNPs through a pulse-chase experiment mimicking in vivo particle clearance from the blood. Following a short duration (30 min) of exposure, the NNPs-treated cells were subjected to further incubation with a NNP-free culture medium for 72 h. Despite the transient incubation, the NNPs resulted in a significant induction of selective tumor cell death in a dose-response manner (Figure S11, Supporting Information). This result strongly implies that the NNPs can recognize the cell-surface target and undergo receptor-mediated endocytosis in an efficient manner, leading to effective action of a protein cargo in vitro.

We attempted to evaluate the effects of the loading amount of a targeting moiety in the NNPs on the cell viability by modulating the number of binding sites for Rb–Z268. It was reported that the density of a targeting moiety in a nanocarrier can affect the targeting capability and consequently the delivery efficiency\(^{[27]}\). We constructed NNPs with various numbers of binding sites for the Rb–Z268 using template DNAs with different lengths (Table S2, Supporting Information), whereas the
loading amount of ETA in the NNPs remained nearly the same (Figure 2e and Figure S6a,b (Supporting Information)). The number of the repebody molecule loaded in a single NNP was estimated to vary from 19 to 3. The respective NNPs were incubated with A431 and HCC827 cells, and the cell viabilities were measured. Interestingly, the NNPs with 19 repebody molecules...
were shown to more effectively trigger cell death than those with three repeatbody molecules (Figure 4d and Figure S12a (Supporting Information)). Based on the results, it is plausible that a modulation of the ZnF-binding sites in the template DNA might lead the NNPs to have different functional properties, including cellular uptake and cytotoxicity.

We tested the long-term stability of the NNPs because they were constructed through a noncovalent interaction, such as neutravidin–biotin and DNA–ZnF proteins. The cell-based cytotoxicity of NNPs was assessed with respect to the incubation time in a physiological buffer (Figure 4e and Figure S12b (Supporting Information)). Notably, the NNPs showed a
negligible change in the cytotoxic activity and specificity even after four weeks, proving their long-term stability. We further evaluated whether the NNPs can be used for delivering other protein cargos. We tested a cytotoxic peptide, a mitochondrial-targeting domain (MTD) composed of amino acid residues, and fused it to the QNK protein. MTD is one of the small domains in Noxa, a mediator of p53-induced apoptosis, and is known to trigger apoptosis through the opening of a mitochondrial permeability transition pore and mitochondrial calcium release.\[36\] The resulting NNPs with MTD peptides also effectively induced selective tumor cell death in a dose-dependent manner (Figure S12c, Supporting Information), which supports the general utility of our approach.

2.6. In Vivo Antitumor Activity of Nucleoprotein Nanoparticles

To assess the therapeutic potential, we tested the antitumor activity of NNPs comprising the ETA-fused QNK (ETA–QNK) and anti-EGFR repebody-fused Z268 (Rb–Z268) as a cytotoxic cargo and a targeting moiety, respectively, in xenograft mouse models (Figure 5). Systemic and local injection of the NNPs were shown to result in a remarkable inhibition of tumor growth with a negligible change in body weight, whereas the nontargeting NNPs gave rise to a moderate level of tumor regression (Figure 5a–c and Figure S13a,b (Supporting Information)). It is noteworthy that two of the mice treated intravenously with the NNPs exhibited no signs of observable tumor masses, as shown in the inset of Figure 5b, reflecting the effectiveness of the NNPs for the targeted delivery of a cytotoxic protein in vivo. Analysis of the cumulative amounts of the AuNPs in the tumors after sacrifice showed higher uptake of NNPs than nontargeting NNPs (nt-NNPs) (Figure S13c, Supporting Information). Because NNPs are thought to be cleared through the liver and kidney, which are the major organs for nanoparticle excretion, we also examined the renal and hepatic toxicity of the NNPs. As shown in Figure 5d, negligible changes were observed in the levels of aspartate and alanine aminotransferase (AST and ALT), as well as in the blood urea nitrogen (BUN) and creatine, which implies a negligible toxicity of the NNPs against both the liver and kidney. This result indicates that the NNPs can efficiently deliver a cytotoxic protein to the tumor site in vivo, leading to remarkable tumor suppression with negligible toxicity.

3. Discussion

We demonstrated the programed assembly of NNPs using DNA and ZnFs as a targeted protein delivery platform. With
a growing number of intracellular drug targets and the high efficacy of protein therapeutics, the in vivo targeted delivery of active proteins with negligible toxicity has been a challenging issue in the field of precision medicine.[9,10] Many approaches have been developed, including cell-penetrating peptides, liposomes, dendrimers, polymeric materials, and metallic NPs.[16–19,37–43] Despite many advances, however, most of these methods suffer from low specificity and efficiency, as well as toxic effects. The present strategy, relying on biomolecular interactions, enables a DNA-directed assembly of supramolecular structures with high homogeneity and stability in a stepwise manner. The use of two types of ZnFs with different sequence specificities allowed an efficient incorporation of an antihuman EGFR repebody and bacterial exotoxin A as a targeting moiety and a cytotoxic protein cargo, respectively, in a sequence-specific manner. Furthermore, the use of DNA-binding ZnF proteins enabled the controlled loading of a protein cargo in NNP by simply modulating the number of ZnF-binding sites. The design principle of NNP was mostly inspired by specific DNA–protein interactions in nature, which realized the rational design of nanoassemblies and controlled loading of cargo molecules in a sequence-guided manner. However, these superior features have yet to be achieved through conventional nanocarriers based on multiple nonspecific interactions.[16,44] The NNP was shown to be highly stable, exhibiting high specificity for EGFR-expressing cells, and underwent internalization through a receptor-mediated endocytosis. The utility and potential of the NNP were demonstrated based on a remarkable tumor suppression effect with negligible side effects. Based on the results, it is likely that the NNP can be effectively used for an efficient and tumor-selective delivery of diverse protein cargos in biomedical applications.

In recent years, numerous approaches for protein delivery have gained attention for precise control of intracellular signaling pathways.[16–19,37–43] Among them, metallic NPs are the primary choice for development of a nanocarrier because of their well-defined nanoscale dimension and morphologies.[19,41] It can facilitate construction of nanocomposites in optimum size and volume for intended use. In addition, distinct biophysical properties enable metallic NPs to be used in photothermal therapy, computed tomography, and magnetic resonance imaging,[45–47] allowing them to develop as therapeutic nanomedicines, as well as drug-delivery vehicles.[48,49] These beneficial features of metallic NPs may be easily adopted into our NNP system with established surface chemistry, and bioconjugation methods. Despite these advantages, nondegradability, toxicity, and uncontrolled synthesis of metallic NP–based approaches remain fundamental challenges for realizing biocompatible and homogenous delivery systems.[50,51] To overcome these limitations, various types of bioassemblies have been developed for protein delivery.[21,44] Especially, protein-based nanoassemblies exploited intrinsic biomolecular interaction, providing biocompatibility and homogeneity.[50] Protein nanoparticles including cage proteins, virus-like particles, protein vaults, and peptide-guided assembly have been successfully developed as nanocarriers.[21,52] These are either naturally assembled protein nanoparticles or artificially designed nanoparticles, highly degradable in the body. Notably, cargo loading strategies such as encapsulation, genetic modification, and noncovalent complexation are based on specific protein–protein interactions, enabling homogenous assembly of nanocarriers with defined size.[43,54,55] Considering the molecular structure of NNP, the system is a hybrid approach that can take advantage of metallic NPs and bioassemblies. Depending on properties of core NPs, NNP may be developed as a nanomedicine for targeted protein delivery and molecular imaging. Core materials can be modulated to biodegradable biomolecules (i.e., DNA and proteins) for reducing nanotoxicity and heterogeneity. Collectively, our NNP system is relatively straightforward and effective for designing a nanocarrier with desired properties, and so it is anticipated that NNP can be easily modified and optimized for particular purposes.

For the assembly of NNP in a programmable manner, we employed two types of ZnF proteins with different sequence specificities as specific molecular glue for conjugating functional proteins to ZnF-binding sites of template DNAs. Our strategy enabled a stepwise assembly of NNP, allowing the controlled loading of a protein cargo in NNP. Controlled loading of a cytotoxic cargo in a nanocarrier is crucial for assessing the therapeutic efficacy and optimal dose of a cytotoxic protein cargo in a clinical application.[56] Our results demonstrated that the loading amount of a protein cargo can be controlled through the design of the number of ZnF-binding sites in template DNA. It is interesting to note that most of the ZnF-binding sites in template DNA were associated with the cargo-fused ZnFs, indicating a high loading efficiency onto the NNP. Design of the ZnF-binding site with a four-nucleotide spacer in template DNA seems to offer sufficient spatial margins for the binding of multiple cargo-fused ZnFs to the template DNA with a negligible steric hindrance. The DNA grafting density on AuNPs seems to be also crucial for high loading efficiency of a cargo. The ZnF-fused anti-EGFR repebody retained its targeting ability, leading the NNP to specifically bind to EGFR on the cells, followed by subsequent internalization through a receptor-mediated endocytosis. It is likely that anti-EGFR repebodies on the NNP were well exposed outwardly, enabling the target-specific binding of the NNP. Dose-dependent cytotoxicity against cancer cells expressing high EGFR levels indicates the efficient cytosolic delivery of a protein cargo in a target-specific manner. Interestingly, it has been reported that ZnF proteins have an intrinsic ability to be directly translocated into the cytosol of the cells with a high endosomal escape efficiency.[57] Considering the delivery efficiency of the NNP, ZnF proteins seem to have a potential endosomalytic property in that they facilitate endosomal escape and reduce a lysosomal degradation of the NNP, and eventually enhance the delivery efficiency of a protein cargo to the cytosol. It is known that the association between the ZnF and DNA weakens due to the protonation of histidine residue under an acidic pH in endosomes.[58] It is therefore likely that a protein cargo is released from the NNP in the endosomes according to a similar mechanism.

Controlling the spatial organization of nanostructures has recently attracted considerable attention for achieving a rational design of nanocomposites in a programmable manner.[21,26,54,59] Because DNA can provide prearranged sites to ZnF proteins with high fidelity, we employed DNA molecules as building blocks for the assembly of a nucleoprotein superstructure in a stepwise manner. The DNA-directed assembly enabled the
reproducible construction of nanostructures with a homogeneous size distribution (diameter of about 80 nm) and high stability. It is noteworthy that the design of the template DNA in terms of the number of ZnF binding sites allowed a controlled incorporation of diverse functionalities, such as a protein cargo and a targeting moiety, into predetermined sites of the NNPs. Based on the well-dispersed core–satellite nanostructures of the NNPs, as shown in the TEM images (Figure 2d), it is likely that our strategy efficiently guides the spatially oriented assembly of ZnF-fused proteins onto the DNA-functionalized AuNPs, while minimizing the steric hindrance, allowing easy control in terms of cargo loading and targeting capability. Conventional nanocarriers have suffered from heterogeneity in cargo loading, as well as poor reproducibility, limiting their clinical translation. On the other hand, the NNPs were shown to be easy to load a protein cargo, and deliver a predicted amount of a cargo to the target site. The functional modality of NNPs can also be easily modulated through the topological rearrangement of ZnF-binding sites on the template DNA and the replacement of core nanoparticles with other metallic or organic particles. Such a unique feature can provide a remarkable degree of flexibility in the design and construction of customized NNPs based on the experimental purpose, expanding their application to precision medicine and other related fields.

4. Experimental Section

Materials: Gold nanoparticles (5 and 10 nm in diameter, stabilized suspension in 0.1 × 10⁻³ m phosphate-buffered saline (PBS), reactant free), HSA, and β-casein from bovine milk were purchased from Sigma (St. Louis, MO). Neutravidin and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were from Thermo Fisher Scientific (Waltham, MA). Folic acid polyethylene glycol (PEG) (molecular weight (MW) 20000) maleimide was from Nanocs (NY, USA). Bovine serum albumin (BSA; IgG, fatty acid, and protease free) was obtained from GenDEPOT (Barker, TX). Enzymes and buffers for gene manipulation were from TAKARA Bio Inc. (Shiga, Japan). All other reagents used were of analytical grade.

Preparation of Template DNA: A double-strand DNA (dsDNA) of 50 bp was obtained through hybridization of two strands of DNA. For DNA of 117 bp, template sequence required at polymerase chain reaction (PCR) state was synthesized (Integrated DNA technologies, USA) and amplified by PCR using primers containing Biotin-TEG at the 3’ end. To obtain the template DNAs of 268 and 944 bp, linear PCR products were first generated with primers containing each Ncol (5’ forward primer) or BamHi (3’ reverse primer) cleavage site and one Zif268-binding sequence at the 5’ end. For this, a template of a previously constructed phagemid, which has either 14 or 56 QNK-binding sites, was used.[28] The resulting PCR products with primers were inserted into a pBEL118N phagemid. Finally, DNA was amplified by PCR using primers containing Biotin-TEG at the 3’ end.

Construction, Expression, and Purification of Zinc Finger–Fused Proteins: ZnF protein and its fusion partners were linked with a 10-amino acid flexible linker (CSGASGAAGG or GGGSGGSG). All ZnF constructs were represented in order of cloned position (QNK–Spycatcher, mOrange–QNK, QNK–albumin-binding domain, monoalkaline phosphatase–QNK, QNK–exotoxin A, QNK–mitochondria targeting domain, and repebody-Zif268). The resulting gene constructs were cloned into a PET21a vector between the NdeI and Xhol sites, followed by transformation into BL 21 (DE3) competent cells using a heat shock method and further incubation at 37 °C overnight on a Luria-Bertani (LB) plate containing 100 μg mL⁻¹ ampicillin. To obtain the ZnF-fused proteins, a single colony was inoculated into a test tube containing 10 mL LB–ampicillin and incubated overnight. The culture broth was diluted 100-fold in a 1 L of LB–ampicillin medium and incubated further at 37 °C for 3 h. Isopropyl-β-D-thiogalactopyranoside (IPTG) and ZnSO₄, a cofactor for ZnFs, were added at final concentrations of 0.5 × 10⁻³ and 0.2 × 10⁻³ m, respectively, when optical density at 600 nm (OD₆₀₀) reached 0.6. For the expression of QNK-exotoxin A, a reduced concentration of IPTG (0.1 × 10⁻³ m) was applied. The cells were further grown at 18 °C for 20 h, then collected by centrifugation at 6000 rpm at 4 °C. The soluble ZnF-fused protein was purified using a Ni–nitrotriacetic acid (NTA) column (Qiagen, Germany) according to the protocol provided by the manufacturer, except for with high salt concentrations and the ZnSO₄ supplement. Briefly, the pellet cells were resuspended in a lysis buffer (20 × 10⁻³ m Tris–Cl (pH 7.5), 500 × 10⁻³ m NaCl, 10 × 10⁻³ m imidazole, and 0.2 × 10⁻³ m ZnSO₄), then disrupted through sonication. The lysate was centrifuged at 13 000 rpm for 1 h at 4 °C, and the supernatant was subjected to filtration using a 0.22 μm syringe filter (Millipore, USA). Ni–NTA column was incubated with the filtrated solution, and washed with five column volumes of a washing buffer (20 × 10⁻³ m Tris–Cl (pH 7.5), 1000 × 10⁻³ m NaCl, 20 × 10⁻³ m imidazole, and 0.2 × 10⁻³ m ZnSO₄) 3 times. Resin-bound proteins were then eluted using an elution buffer (20 × 10⁻³ m Tris–Cl (pH 7.5), 300 × 10⁻³ m NaCl, 250 × 10⁻³ m imidazole, and 0.2 × 10⁻³ m ZnSO₄). ZnF-fused protein was analyzed using polyacrylamide gel electrophoresis (PAGE) for determining the purity. The purified protein solution was stored at 4 °C prior to use.

Construction of Folate-Conjugated Zinc Finger Protein: A 20-fold molar excess of folic acid PEG (MW 20000) maleimide was added to Z268 ZnF protein that had been genetically modified with cysteine at its N-terminal end[28] and incubated at 4 °C overnight with mild mixing in conjugation buffer (50 × 10⁻³ m 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5), 150 × 10⁻³ m NaCl, 0.2 × 10⁻³ m ZnSO₄, and 10 × 10⁻³ m tris(2-carboxyethyl)phosphine (TCEP)). After binding the folate-conjugated protein solution with Ni–NTA resin, free folate was removed by washing the resins 2 times, and folate-conjugated Z268 ZnF protein was finally eluted. The purified protein solution was stored at 4 °C prior to use.

Stepwise Assembly of Nucleoprotein Nanoparticles: Neutravidin-coated gold nanoparticles were first constructed as follows. One milliliter of gold nanoparticles was treated with 100 μL of 2.75 mg mL⁻¹ neutravidin in 50 × 10⁻³ m 3-morpholinopropanesulfonic acid (MOPS) buffer (pH 7) for 30 min at room temperature. Next, 100 μL of 10 mg mL⁻¹ bovine serum albumin in PBS buffer (pH 7.5) was added to the reaction solution in order to block the residual surface of gold nanoparticles. After 20 min of passivation, neutravidin-coated gold nanoparticles were separated through three steps: Ultrascl-100 K centrifugal filter (Millipore, MA), followed by PD-10 column (GE healthcare, USA), and finally with centrifugation (13 000 rpm, 1 h). Next, biotinylated templated DNA was grafted onto neutravidin-coated gold nanoparticles through avidin–biotin chemistry. Briefly, 20 × 10⁻⁵ m neutravidin-coated gold nanoparticles were reacted with a tenfold molar excess of biotinylated DNA (268 bp) in DNA binding buffer (50 × 10⁻³ m Tris–Cl (pH 8), 150 × 10⁻³ m NaCl, and 0.05% Tween-20) overnight at 4 °C. The resulting gold nanoparticles were then washed 3 times by centrifugation at 13 000 rpm for 1 h.

Nucleoprotein nanoparticles were assembled by mixing the DNA-functionalized nanoparticles with ZnF-fused proteins at a ratio of the ZnF-fused protein to the ZnF-binding site to be three in ZnF buffer (20 × 10⁻³ m Tris (pH 7.5), 150 × 10⁻³ m NaCl, 0.2 × 10⁻³ m ZnSO₄, 2 mg mL⁻¹ BSA or β-casein, 10% glycerol, and 1 × 10⁻³ m MgCl₂). A targeting moiety and a cargo were genetically fused to two types of ZnF proteins, Z268 and QNK, respectively. After incubation for 30 min at room temperature, the resulting nucleoprotein nanoparticles were washed 5 times using a NanoSorb-100 K centrifugal filter with ZnF buffer, followed by filtration through polyvinylidene fluoride (PVDF) 0.22 μm centrifugal filters (Millipore, MA).

DLS Measurement: DLS was used to estimate the size distribution of the citrate-, neutravidin-, and DNA-functionalized gold nanoparticles as well as nucleoprotein nanoparticles. For DLS measurements, 0.1 mL of a diluted solution of gold nanoparticles at each construction step was transferred into a cuvette, and hydrodynamic size measurements were obtained using a Zetasizer Nano (Malvern, UK).
TEM Imaging: A 200 kV field-emission source transmission electron microscope (JEOL JEM-2100F, USA) was used to obtain the images of core–satellite gold nanoparticle clusters. A 300-mesh carbon coated grid was put onto a filter paper, and a solution containing nanoparticles was dropped onto the grid (10 × 10^3 m gold nanoparticles). The carbon grids were dried at room temperature for at least 60 min, and subjected to TEM imaging.

AFM Imaging: The suspension of neutravidin-coated gold nanoparticles or nucleoprotein nanoparticles containing mOrange–QNK (20 × 10^3 m) in the presence of 10 × 10⁻⁹ m MgCl₂ was deposited onto freshly cleaved mica (Ted Pella Corp., USA). For a clear observation of their shape and size, we used a long template DNA of 944 bp with 4 times the number of QNK-binding sites than a 268 base-paired template DNA. Following 5 min incubation at room temperature, the mica surface was rinsed with deionized water 3 times and dried using nitrogen gas. The resulting samples were additionally dried in a vacuum desiccator overnight, and subjected to scanning in noncontact mode using a Park NX-10 ADM with an NC-NCH tip (Park System Corp., South Korea).

Gel Shift Assay: Suspension of DNA-functionalized gold nanoparticles or nucleoprotein gold nanoparticles (100 × 10⁻⁸ m, 3 µL) in the presence of 10% glycerol were loaded on 10% glycerol were loaded on 0.5% agarose gel in 0.5× Tris–borate–ethylenediaminetetraacetic acid (EDTA) buffer (TBE buffer) and run in a Mupid-2plus horizontal electrophoresis system (Takara, Japan) at 100 V for 20 min.

Quantification of Grafted DNAs onto Gold Nanoparticles: To quantify the grafted DNAs onto neutravidin-coated gold nanoparticles, biotinylated and 5'-fluorescence 6-carboxyfluorescein (6'FAM) dye-modified DNAs were used. DNA-functionalized gold nanoparticles were prepared by mixing neutravidin-coated gold nanoparticle and DNAs, and supernatant solutions were collected. One hundred microliters of free DNA solution were dispensed in a 96-well plate, and the fluorescence intensity was measured and compared with a standard curve. The number of grafted DNAs was determined by subtracting the measured concentrations of supernatants from that of initial DNA used in reaction, and dividing the calculated DNA concentration by the concentration of gold nanoparticles. During the fluorescence measurement, the fluorophore was excited at 455 nm and the emission was collected at 526 nm.

Quantification of the Protein Loaded on Nucleoprotein Nanoparticles: Following centrifugation of 10 × 10⁻⁴–30 × 10⁻⁵ m of nucleoprotein nanoparticles (100 µL) at 15 000 rpm for 60 min, supernatant was removed and 10 µL of 5× sodium dodecyl sulfate (SDS) loading dye (0.25% w Tris–Cl (pH 6.8), 0.25% bromophenol blue, 0.5% dithiothreitol (DTT), 50% glycerol, and 10% SDS) was added. The mixture was boiled for 5 min and loaded on PAGE gel with protein standards. The amount of released protein was calculated from the standard curve which was established by measuring the density of each gel band using Image J program. The amount of conjugated proteins per nucleoprotein nanoparticles was determined by dividing the calculated protein concentration by gold nanoparticle concentration.

Targeting Ability of Nucleoprotein Nanoparticles: To determine the targeting ability of nucleoprotein nanoparticles by enzyme-linked immunosorbent assay, the EGFR-targeting receptor-fused Z268 protein and mALP-fused QNK protein were used for the assembly of nucleoprotein nanoparticles. A 96-well Maxisorp plate (SPL, South Korea) was coated with 10 µg mL⁻¹ of BSA and EGFR family proteins at 4 °C overnight, followed by blocking using a blocking solution (PBS supplemented with 0.1% Tween 20 and 2% BSA) at room temperature for 1 h and subsequent washing 3 times with PBST (PBS supplemented with 0.1% Tween 20). After incubation with the nucleoprotein nanoparticles (5 × 10⁻⁹ m) for 1 h, p-nitrophenyl phosphate solution (Sigma, USA) was added to generate the signals. Following the addition of a 3 m sodium hydroxide solution to stop the reaction, the absorbance at 405 nm was measured using a microplate reader (Tecan, Swiss).

Cell Cultures: HCC827, MDA-MB-468, HT29, MCF7, and K562 cells (ATCC, USA) were cultured using Roswell Park Memorial Institute (RPMI) 1640 media (HyClone, USA). A431 and SW620 cells (ATCC, USA) were grown in Dulbecco’s modified Eagle medium (DMEM) (HyClone, USA) and Leibovitz’s L-15 Medium (Gibco, USA), respectively. Culture media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, USA). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂. When cell density reached about 80% confluency, cell subculture was typically conducted with a split ratio 1:10.

Cellular Targeting and Uptake by Nucleoprotein Nanoparticles: To determine the selective cellular uptake of nucleoprotein nanoparticles, mO–QNK was employed as a cargo to visualize using a confocal microscope. Various cancer cell lines showing different EGFR expression levels (A431, HT29, and SW620 cells) were plated at the concentration of 10⁴ cells per well on a coverslip in an 8-well slide chamber (SPL, South Korea). After 24 h, the cells were treated with 10 × 10⁻⁸ m of nucleoprotein nanoparticles in serum-free media at 37 °C for 6 h. The cells were washed 3 times with Dulbecco’s phosphate-buffered saline (DPBS), followed by treatment with 4',6-diamidino-2-phenylindole (DAPI) for nucleus staining. Fixed cells were imaged on a LSM 780 confocal microscope system (Zeiss, Germany) at a magnification of 400x. In the case of incubation with a soluble mOrange protein control, 10 µg mL⁻¹ of mOrange was treated in A431 and HCC827 cells for 3, 6, and 12 h in serum-free media.

ICP-MS: To quantify the accumulated gold nanoparticles in the cells or organs, ICP-MS (Agilent ICP-MS 770005, USA) was used. Nucleoprotein nanoparticles were assembled using folate-fused Z268 and mO–QNK proteins as a targeting moiety and a cargo, respectively. K562 cells (2 × 10⁶) were plated on a 48-well plate overnight. The cells were treated with targeting and nontargeting nucleoprotein nanoparticles (10 × 10⁻⁹ m) for 4 h in serum-free media, followed by separation and analysis. Prior to ICP-MS analysis, the cells were treated with a mixture of 7 mL of 70% HNO₃ and 3 mL of 35% HCl at 200 °C for 30 min. For the analysis of the Au ions in mouse organs, excised organs on day 27 after intravenous injection of the nucleoprotein nanoparticles were dissolved in 10 mL of 70% HNO₃ at 200 °C for 30 min and analyzed using ICP-MS.

In Vivo Cytotoxicity of Nucleoprotein Nanoparticles: All cancer cell lines were seeded into a 96-well plate (SPL, South Korea) at a density of 3 × 10⁴ cells per well, followed by incubation at 37 °C under 5% CO₂ for 24 h. Different concentrations of nucleoprotein nanoparticles were added to each well, followed by incubation for 72 h under a 3% serum condition. For pulse-chase experiment, following a short duration (30 min) of exposure, the nucleoprotein nanoparticle-treated cells were subjected to further incubation with a nucleoprotein nanoparticle-free culture medium for 72 h. The cell-based cytotoxicity of the nucleoprotein nanoparticles according to the storage time was assessed with respect to the predetermined times in a physiological buffer at 4 °C. Following removal of the culture medium, fresh serum-free medium supplemented with a 10% cell-counting kit-8 (CCK-8) reagent (Dojingo, Japan) was added to each well and incubated for 1 h at 37 °C. The CCK-8 is designed for detection of the cell viability by measuring the amount of an orange colored product (WST-8 formazan), which is converted from a water-soluble tetrazolium salt (WST-8) by nicotinamide adenine dinucleotide (NADH) dehydrogenases in living cells. The cell viability was determined by measuring the absorbance at 450 nm. For detection of early apoptosis, annexin V staining was conducted. A431 and MCF7 cells (10⁶ cells per well) were plated on an 8-well slide chamber (SPL, South Korea) overnight. Nucleoprotein nanoparticles containing 725 × 10⁻⁹ m of ETA–QNK proteins were incubated with A431 and MCF7 cells for 8 h under 3% serum condition. Treated cells were washed with an annexin V binding buffer (10 × 10⁻³ m HEPES (pH 7.4), 140 × 10⁻³ m NaCl, and 2.5 × 10⁻³ m CaCl₂), and a 40-fold diluted annexin V–FITC (e Bioscience, USA) was added to detect exposed phosphatidyserine according to the manufacturer’s protocol. The cells were washed 3 times with annexin V binding buffer, and treated with DAPI for nucleus staining. Fixed cells were imaged on a LSM 780 confocal microscope system (Zeiss, Germany) at a magnification of 100×.

Xenograft Mice and In Vivo Antitumor Activity: Six week old female athymic nude mice (BALB/c nu/nu) were purchased from Orient Bio, Inc. (Seoul, South Korea). Xenograft mice were generated through subcutaneous injection of HCC827 tumor cells into the shoulder (5 × 10⁶ cells mixed with 100 mL of BD Matrigel Matrix (BD Biosciences, USA). All animals were acclimatized to controlled conditions of temperature (23 ± 2 °C), humidity (55 ± 5%), and light (12 h light/dark cycle) at the Central Animal Research laboratory (KAERI) or the Korea Basic Science Institute (KBSI). The animals were provided with a sterile pellet diet and...
water ad libitum. All experiments were approved by the Institutional Animal Ethical Committee and were performed in strict compliance with the guidelines prescribed by the committee.

After 10–20 days, the engrafted mice were used for an assay of the antitumor activity when the tumor size reached \( \approx 50–200 \text{ mm}^3 \). To evaluate the antitumor activity of nucleoprotein nanoparticles in xenograft mice with HCC827 cells, nucleoprotein nanoparticles (20 \( \times \) \( 10^4 \) m) were injected into mice intravenously every 3 days for 5 times. PBS was used as a vehicle control. Intravenously injected mice were monitored for 27 days. The body weight and tumor size were measured individually once every 3 or 4 days. The tumor volume was measured macroscopically with a digital caliper (Mitutoyo, Japan), and the tumor volume was calculated according to the following formula (\( \text{mm}^3 \)):

\[
\text{Tumor volume} = \frac{4}{3} \pi \times \text{length} \times \text{width}^2 \times 0.5.
\]

Hepatotoxicity and nephrotoxicity of nucleoprotein nanoparticles were assessed as described elsewhere. \[55\]

Statistical Analysis: Experimental data were expressed as means \( \pm \) standard deviation (S.D.) Statistical analyses were conducted using a one-way analysis of variance (ANOVA), and intergroup comparisons were made using a Student’s t-test with a Bonferroni adjustment. A value of \( p < 0.05 \) was considered statistically significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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