The assembly of proteins in a programmable manner provides insight into the creation of novel functional nanomaterials for practical applications. Despite many advances, however, a rational protein assembly with an easy scalability in terms of size and valency remains a challenge. Here, a simple bottom-up approach to the supramolecular protein assembly with a tunable size and valency in a programmable manner is presented. The dendrimer-like protein assembly, simply called a “protein dendrimer,” is constructed through a stepwise and alternate addition of a building block protein. Starting from zeroth-generation protein dendrimer (pG0) of 27 kDa, the protein dendrimer is sequentially grown to pG1, pG2, pG3, to pG4 with a molecular mass of 94, 216, 483, and 959 kDa, respectively. The valency of the protein dendrimers at the periphery increases by a factor of two after each generation, allowing a tunable valency and easy functionalization. The protein dendrimers functionalizes with a targeting moiety and a cytotoxic protein cargo shows a typical feature of multi-valency in the avidity and a highly enhanced cellular cytotoxicity, exemplifying their utility as a protein delivery platform. The present approach can be effectively used in the creation of protein architectures with new functions for biotechnological and medical applications.

1. Introduction

Proteins are the most diverse among functional biomolecules and are building blocks of diverse cellular machineries that perform fundamental cellular tasks. In nature, protein assemblies with higher-order architectures confer remarkable multitudes of new functions that would be impossible when in monomeric forms in terms of functional modulation, allosteric regulation, high structural complexity, and stability.[1,2] In this regard, a protein assembly has attracted considerable attention as a versatile platform for practical applications in wide-ranging fields, including vaccines, drug delivery, biosensors, diagnosis, and therapy of diseases[3–7].

A programmable assembly of proteins can provide crucial insight into the creation of highly sophisticated functional nanostructures with novel properties in a predictable way. It also aids in understanding the underlying mechanisms for protein self-assembly in nature. The programmability of protein assembly is especially critical for medical and biotechnological purposes where the size and valency of the assembly are the key factors.[8–10] Although proteins have been considered as promising building blocks for an assembly owing to their unique structural and biophysical features, the extent of the assembly is highly dependent on the characteristics of the monomeric protein, making precise control of the size and valency difficult. Many strategies for assembling proteins into higher-order structures have been reported, including the use of well-defined coiled-coil and helical bundle interactions,[11,12] the peptide–ligand interactions,[13,14] the formation of disulfide bonds,[5] chemical cross-links,[15–17] metal-directed interactions,[18,19] the use of non-biological templates,[20,21] and the genetic fusion of self-associating protein domains or fragments.[22–27] The computational design of a protein–protein interface has also recently been applied to the generation of self-assembling proteins with defined structures.[28–31] Despite numerous advances, however, the development of a programmable protein assembly with easy scalability in terms of size and valency remains a challenging task owing to the structural diversity, conformational heterogeneity, and high molecular weight of proteins compared to other biomolecules.

Herein, we describe a bottom-up approach to the supramolecular assembly of proteins with a tunable size and valency in a programmable manner through a stepwise addition of a building block protein. With two different orthogonal protein–ligand pairs, a dendrimer-like protein assembly, simply called a “protein dendrimer,” was constructed by using three monomeric proteins, namely a core (pG0) and two building-block proteins (B1 and B2) (Scheme 1). The generations of protein dendrimers were grown by an iterative and alternate addition of a building block to a previous generation protein dendrimer, allowing a higher-generation protein dendrimer with a near mega-dalton size and multi-valency. The protein dendrimers were shown
to confer a tunable valency with the generation, leading to a single-step easy functionalization. The protein dendrimer functionalized with a target-specific protein binder and a cytotoxic protein cargo exhibited a typical feature of multi-valency in the avidity and, consequently, a highly enhanced cellular cytotoxicity, exemplifying their utility as a protein delivery platform. Our approach allows for a simple production of protein assemblies with predictable size and valency in a scalable and programmable manner compared to previous methods. Details are reported herein.

2. Results

2.1. Strategy for the Assembly of Dendrimer-Like Protein Architectures

The general procedure for the dendrimer-like protein assembly, namely a “protein dendrimer,” using a total of three monomeric proteins (a core and two building-block proteins) is depicted in Scheme 1. The core protein (pG0), a zeroth-generation protein dendrimer, consists of a genetically fused dual-tandem SnoopCatcher using a flexible GS linker, whereas the two building blocks (B1 and B2) comprise dual-tandem repeats of SpyCatcher or SnoopCatcher linked with SpyTag or SnoopTag, respectively (Scheme 1a). Such proteins are a set of orthogonal pairs that spontaneously form a covalent isopeptide bond between a Catcher and a Tag (Scheme S1, Supporting Information). [14,32–34]

Because each building block is composed of a dissimilar pair of a Catcher and a Tag, no reconstitution or self-cyclization will occur by the single protein alone.

As shown in Scheme 1b, the growth of a protein dendrimer starts with the addition of the building block B1 containing an inter SnoopTag to the core protein pG0. This results in a first-generation protein dendrimer pG1, with four SpyCatcher proteins at the periphery through the interaction between SnoopCatcher and SnoopTag. The generation of the protein dendrimer is further grown by adding the building block B2 with an inter SpyTag to pG1, yielding a second-generation protein dendrimer pG2, which has a total of eight exposed SnoopCatcher proteins. As the periphery of pG2 is returned to SnoopCatcher, the same as pG0, the building block B1 is added again for the construction of a third-generation protein dendrimer pG3. A fourth-generation protein dendrimer pG4 is produced by adding the building block B2 to pG3, and consequently pG4 has 32 SpyCatcher proteins at the periphery. Similarly, the two building blocks, B1 and B2, are repetitively added alternatively for the production of a next-generation protein dendrimer. From only two small building block proteins, protein dendrimers can be grown to a supramolecular scale in a stepwise manner. Starting from pG0 of 27.3 kDa, the protein dendrimer is sequentially grown to pG1, pG2, pG3, to pG4 with a theoretical molecular mass of 92.5, 212.0, 472.6, and 950.5 kDa, respectively. The valency of the protein dendrimers at the periphery increases by a factor of two after each generation, and consequently pG4 has a valency of 32 starting from pG0 with a valency of two.
2.2. Construction and Characterization of Protein Dendrimers

Protein dendrimers are constructed through a bottom-up approach, and it is crucial for each generation to be highly homogeneous. A single malformation in an earlier generation will cause a much greater heterogeneity in the subsequent growth. We thus purified the core protein (pG0) and two building blocks (B1 and B2) to homogeneity by size exclusion chromatography (SEC) and used them for the construction of protein dendrimers (Figure S1, Supporting Information). The core and two building block proteins were shown to exhibit a distinct single band at near the expected molecular mass on SDS-PAGE (3–12% gradient). Following the Scheme 1b, a previous generation protein dendrimer was purified and used for the construction of a next generation protein dendrimer using the two building blocks. The iterative formation of different protein dendrimer generations was traced through SEC (Figure S2, Supporting Information). The previous generation protein dendrimers were shown to be almost completely grown to the next protein dendrimer generation by alternate addition of a building block. This can be attributed to an efficient step-wise assembly of proteins through specific interactions between orthogonal pairs of SpyCatcher/SpyTag and SnoopCatcher/SnoopTag.

We investigated the biophysical properties of different protein dendrimer generations after eluted fractions from SEC were collected (Figure 1). The protein dendrimers were observed to exhibit a distinct single band at near the expected molecular mass on SDS-PAGE (Figure 1a). The subtle variation in the molecular mass of each generation protein dendrimers on SDS-PAGE seems to be due to their branched structures even after boiling. In particular, the band of pG4 appeared to be slightly smeared mainly owing to the fluid-like low gel percentage at the top. Next, different generations of protein dendrimers were analyzed based on SEC, and clear elution peaks were observed without aggregation patterns (Figure 1b). Dynamic light scattering (DLS) analysis also displayed narrow size distributions of the protein dendrimers, and their average hydrodynamic radii were estimated to range from 5.3 to 20.6 nm (Figure 1c,e). The absolute molecular masses of the protein dendrimers were determined using multi-angle light scattering (MALS) (Figure 1d), which were well matched with the expected values (Figure 1e). Based on the results, it is likely that the present approach enabled supramolecular protein assemblies, that is, protein dendrimers, with a well-defined size and valency in a programmable manner.

2.3. Functionalization of Protein Dendrimers

In order for the protein dendrimers to be used for biotechnological and medical purposes, their functionalization with biomolecules is essential. With either SpyCatcher or SnoopCatcher at the periphery depending on the generation, we reasoned that the protein dendrimers could be easily functionalized with diverse biomolecules through interactions with SpyTag or SnoopTag, respectively. We first intended to functionalize the protein dendrimers for tumor-cell targeting, and employed an epidermal growth factor receptor (EGFR)-specific rebody which was previously developed from a non-antibody protein scaffold.13,14 For a simple and general functionalization process, a conjugation module comprising of a tandem of SpyTag and SnoopTag was genetically fused to the C-terminal of the rebody using a flexible linker for optimal exposure to yield T\textsubscript{mono} (indicating a conjugation module-fused targeting moiety) (Figure 2a). As a proof-of-concept, we functionalized pG0, which has a valency of 2, with T\textsubscript{mono}, and the resulting protein dendrimer was designated as pG0\textsubscript{T}, (standing for a zeroth-generation protein dendrimer functionalized with T\textsubscript{mono}). Similarly, pG1 and pG2 were functionalized, yielding pG1\textsubscript{T} and pG2\textsubscript{T}, respectively. Functionalization of different protein dendrimer generations with a conjugation module-fused EGFR-specific rebody was traced through SEC (Figure S3, Supporting Information). The different generations of protein dendrimers were observed to be efficiently functionalized, which seems to stem from the fact the conjugation module shares the same chemistry. Analysis of the functionalized protein dendrimers on SDS-PAGE showed distinct single bands (Figure 2b). From the monomeric T\textsubscript{mono} with a molecular mass of 32.8 kDa, the molecular mass of pG0\textsubscript{T} increased to 474.6 kDa (Figure 2c). The functionalized protein dendrimers were eluted with high homogeneity though SEC, exhibiting a narrow size distribution when analyzed using DLS with hydrodynamic size ranging from the monomeric 6.2 nm to the octomeric 19.7 nm (Figure S4a,b, Supporting Information). The absolute molecular masses determined through MALS were shown to coincide well with the expected values (Figure 2c; Figure S4c, Supporting Information). These results demonstrate that the protein dendrimers can be functionalized using the conjugation module in a highly specific and efficient manner.

We next checked the binding property of the protein dendrimers functionalized with T\textsubscript{mono} carrying an EGFR-specific rebody. As shown in Figure 2d, the functionalized protein dendrimers exhibited distinct signals for human EGFR ectodomain in enzyme-linked immunosorbent assay (ELISA), whereas negligible signals were observed from the protein dendrimers without a targeting moiety. It is likely that the EGFR-specific rebody conjugated to the protein dendrimers maintains its binding capability for human EGFR ectodomain similar to a free rebody. A protein assembly with multiple valency is expected to provide an enhanced binding capability toward a target mainly through the avidity.17–19 To test this expectation, we measured the binding affinities of the protein dendrimers functionalized with T\textsubscript{mono} carrying the EGFR-specific rebody using surface plasmon resonance (SPR) (Figure 2e). As a result, the binding affinity the protein dendrimers for the EGFR ectodomain was shown to increase by approximately one-order of magnitude with the increasing generation. It is noteworthy that pG0\textsubscript{T} carrying eight rebody molecules showed a K\textsubscript{D} value of 107 pm, which corresponds to a 1,336-fold increase in the binding affinity compared to a free rebody with a K\textsubscript{D} value of 143 nm. This result clearly demonstrates a typical feature of multivalent protein dendrimers in the avidity and cooperativity. The dissociation rates were shown to decrease exponentially with the increasing number of the rebody molecules at the periphery, whereas a linear increase in the association rates was observed (Figure 2f; Figure S4d, Supporting Information). Based on the results, it is plausible that the cooperativity and avidity make a major contribution to an increase in the binding affinity of the protein dendrimers functionalized with an EGFR-specific rebody for the target.
Figure 1. Biophysical characterization of different protein dendrimer generations. a) SDS-PAGE (3–12% gradient) of the purified protein dendrimers from generation 1 (pG1) to generation 4 (pG4), core protein (pG0), and two building block proteins (B1 and B2). M indicates size markers. b) SEC of different protein dendrimer generations. The peaks represent the normalized absorbance of different generations at 280 nm. c) DLS analysis of the protein dendrimers. d) MALS analysis of the absolute molecular mass of the protein dendrimers eluted from SEC. e) Summary of the absolute molecular mass of the protein dendrimers determined by MALS in (d). The hydrodynamic radii were determined from the DLS analysis shown in (c).

We attempted to analyze the protein dendrimers using a transmission electron microscope (TEM), but failed to obtain clear images due to low contrast as the dendrimer-like assemblies may be damaged by the electron beam. We thus functionalized the protein dendrimers with an eGFP instead of the repobody, since TEM images of eGFP with high contrast were reported. The protein dendrimers from a zeroth to a third-generation were functionalized with eGFP using the same method as for the repobody, yielding pG0, indicating a zeroth-generation protein dendrimer functionalized with a fluorescence protein) to pG4, followed by TEM imaging. The resulting protein dendrimers were observed to be highly homogenous in both SDS-PAGE, SEC, and DLS (Figure S5, Supporting Information). Interestingly, TEM images of the functionalized protein dendrimers with eGFP through negative staining showed round dark spots with similar size, despite their highly flexible structures (Figure 2g;
Figure 2. Functionalization of protein dendrimers using a conjugation module. a) Graphical representation of the functionalization of different protein dendrimer generations with a targeting moiety. The conjugation module comprises of a tandem of SpyTag and SnoopTag linked using a GS linker. A targeting moiety is genetically fused to the N-terminal of the conjugation module to produce T<sub>mono</sub>. Using either SpyCatcher or SnoopCatcher at the periphery depending on the generation, the protein dendrimers can be easily functionalized using T<sub>mono</sub> carrying biomolecules such as a target-specific protein binder, yielding pG<sub>n</sub>T<sub>mono</sub> (n = 0, 1, 2) and pG<sub>n</sub>T<sub>mono</sub> with a targeting moiety to produce T<sub>mono</sub> carrying an EGFR-specific repebody as a model targeting moiety. b) Summary of the absolute molecular masses and hydrodynamic radii of the functionalized protein dendrimers by MALS and DLS, respectively (Figure S4b,c, Supporting Information). c) Direct ELISA of the protein dendrimers functionalized with T<sub>mono</sub> toward human EGFR ectodomain. d) SPR sensorgrams of T<sub>mono</sub> and the functionalized protein dendrimers with T<sub>mono</sub> toward human EGFR ectodomain. The legends indicate the concentrations of the protein dendrimer in nM. e) Binding kinetics of the functionalized protein dendrimers based on the sensograms shown in (e). f) Summary of the absolute molecular masses and hydrodynamic radii of the functionalized protein dendrimers by MALS and DLS, respectively (Figure S4b,c, Supporting Information). g) TEM images of different generation protein dendrimers functionalized with eGFP. The scale bar represents 100 nm. h) Average diameter of the functionalized protein dendrimers with eGFP shown in (g) (n = 8).
The molecular mass of the octomeric pG2D increased to 806.6 kDa. As a model cargo, eGFP was employed. An additional domonas aeruginosa exotoxin, a cargo module, and a conjugation module was fused to yield D mono (indicating a monomeric cargo for delivery). The protein dendrimers functionalized with D mono are expected to deliver a cargo protein to the cytosol as depicted in Figure S8, Supporting Information. The EGFR-specific reepathway targets the cell-surface EGFR, and the functionalized protein dendrimers undergo receptor-mediated endocytosis. In the endosome, the TDP and the cargo are released from the receptor-binding domain and the protein dendrimer through the cleavage by TDP furin and cathepsin-B, respectively. The cargo is then translocated to the endoplasmic reticulum (ER) by the KDEL receptor, followed by the release to the cytosol. The resulting construct was expressed as a soluble form in E. coli and purified, followed by conjugation to pG0 and pG2, thereby producing pG0D to pG2D. From a monomeric D mono of 74.3 kDa, the molecular mass of the octomeric pG2D increased to 806.6 kDa (Figure S9a, Supporting Information). The functionalized protein dendrimers with D mono were observed to be highly homogeneous, exhibiting single bands on SDS-PAGE (3–6% gradient), and appeared as clear single peaks in SEC and DLS with hydrodynamic size ranging from the monomeric 6.9 nm to the octomeric 26.9 nm (Figure S9b-e, Supporting Information).

We tested the protein dendrimers functionalized with D mono in terms of the intracellular delivery of the cargo using confocal microscopy. High ECFR-expressing A431 cells were incubated with pG0D and pG2D for 6 h with a total of 1 μM cargo protein. The functionalized protein dendrimers have different numbers of cargo proteins depending on the generation. Thus, to match the total concentration of monomeric D mono of 1 μM, the concentrations of the treated pG0D, pG1D, and pG2D were fixed at 500, 250, and 125 nm, respectively. Although the eGFP fluorescence of D mono was difficult to distinguish, the cells treated with pG0D showed a distinct fluorescence despite a lower total concentration of treated protein dendrimer (Figure 3c). The average fluorescence intensity of pG2D was approximately three times greater than that of D mono (Figure 3d). The increase in fluorescence intensity can be explained by the enhanced binding affinity of pG2D owing to the avidity, as described above. Because pG0D has a much lower dissociation rate, a large portion of pG2D will be in a bound state in comparison to D mono, resulting in a significantly enhanced internalization of a cargo into the cells. The fluorescence signals were observed to be distributed throughout the cytosol with negligible co-localization with lysotrackers (Figure S10, Supporting Information). A similar trend was observed in a western blot analysis of A431 cells (Figure 3e). The enhanced cargo delivery by the protein dendrimers was further examined for another EGFR positive cell line, MDA-MD-468, and a similar result was observed (Figure S11a,b, Supporting Information). The protein dendrimers functionalized with a translocation module carrying an off-target reepobody showed negligible fluorescence in both A431 and MDA-MB-468 cells (Figure S11c,d, Supporting Information). Similarly, no fluorescence signals were observed in the EGFR-negative cell line, MCF7 (Figure S12, Supporting Information). As the results indicate, the protein dendrimers can be effectively used as an intracellular protein delivery platform, enabling highly efficient protein delivery mainly owing to the avidity.

2.4. Protein Dendrimers as an Intracellular Protein Delivery Platform

From the observation that the protein dendrimers showed a highly enhanced binding capability due to the avidity, we thought that the protein dendrimers could be used as a platform for an intracellular protein delivery. For this, a protein delivery system was constructed by combining the protein dendrimers with our previous work.[41,42] As depicted in Figure 3a, the protein delivery system is composed of three protein modules which are genetically fused using a flexible linker: a translocation module comprised of a cell-targeting moiety and a translocation domain (TDP) of Pseudomonas aeruginosa exotoxin, a cargo module and a conjugation module.[43] As a model cargo, eGFP was employed. An additional KDEL and cathepsin-B cleavage site were introduced into the C-terminal of the cargo for a retrograde translocation and release of the cargo in the endosome, respectively.[44,45] Lastly, a conjugation module was fused to yield D mono (indicating a monomeric cargo for delivery). The protein dendrimers functionalized with D mono is expected to deliver a cargo protein to the cytosol as depicted in Figure S8, Supporting Information. The EGFR-specific reepobody targets the cell-surface EGFR, and the functionalized protein dendrimers undergo receptor-mediated endocytosis. In the endosome, the TDP and the cargo are released from the receptor-binding domain and the protein dendrimer through the cleavage by TDP furin and cathepsin-B, respectively. The cargo is then translocated to the endoplasmic reticulum (ER) by the KDEL receptor, followed by the release to the cytosol. The resulting construct was expressed as a soluble form in E. coli and purified, followed by conjugation to pG0 and pG2, thereby producing pG0D to pG2D. From a monomeric D mono of 74.3 kDa, the molecular mass of the octomeric pG2D increased to 806.6 kDa (Figure S9a, Supporting Information). The functionalized protein dendrimers with D mono were observed to be highly homogeneous, exhibiting single bands on SDS-PAGE (3–6% gradient), and appeared as clear single peaks in SEC and DLS with hydrodynamic size ranging from the monomeric 6.9 nm to the octomeric 26.9 nm (Figure S9b-e, Supporting Information).

We tested the protein dendrimers functionalized with D mono in terms of the intracellular delivery of the cargo using confocal microscopy. High EGFR-expressing A431 cells were incubated with pG0D and pG2D for 6 h with a total of 1 μM cargo protein. The functionalized protein dendrimers have different numbers of cargo proteins depending on the generation. Thus, to match the total concentration of monomeric D mono of 1 μM, the concentrations of the treated pG0D, pG1D, and pG2D were fixed at 500, 250, and 125 nm, respectively. Although the eGFP fluorescence of D mono was difficult to distinguish, the cells treated with pG0D showed a distinct fluorescence despite a lower total concentration of treated protein dendrimer (Figure 3c). The average fluorescence intensity of pG2D was approximately three times greater than that of D mono (Figure 3d). The increase in fluorescence intensity can be explained by the enhanced binding affinity of pG2D owing to the avidity, as described above. Because pG0D has a much lower dissociation rate, a large portion of pG2D will be in a bound state in comparison to D mono, resulting in a significantly enhanced internalization of a cargo into the cells. The fluorescence signals were observed to be distributed throughout the cytosol with negligible co-localization with lysotrackers (Figure S10, Supporting Information). A similar trend was observed in a western blot analysis of A431 cells (Figure 3e). The enhanced cargo delivery by the protein dendrimers was further examined for another EGFR positive cell line, MDA-MD-468, and a similar result was observed (Figure S11a,b, Supporting Information). The protein dendrimers functionalized with a translocation module carrying an off-target reepobody showed negligible fluorescence in both A431 and MDA-MB-468 cells (Figure S11c,d, Supporting Information). Similarly, no fluorescence signals were observed in the EGFR-negative cell line, MCF7 (Figure S12, Supporting Information). As the results indicate, the protein dendrimers can be effectively used as an intracellular protein delivery platform, enabling highly efficient protein delivery mainly owing to the avidity.

2.5. A Cytotoxic Protein Delivery Using the Protein Dendrimer

To further assess the utility of the protein dendrimers as a protein delivery platform, we intended to deliver a cytotoxic protein cargo into cells, and used gelonin as a model cytotoxic protein. Gelonin is a plant-derived N-glycosidase, which inactivates the 60S ribosomal subunit and is known to be unable to cross the cell membrane alone.[41,46] For this, eGFP was replaced with gelonin in the previous construct, yielding G mono (indicating a monomeric cargo with gelonin) (Figure 4a). The resulting construct with a size of 75.7 kDa was expressed as a soluble form in E. coli and used for functionalization of the protein dendrimers. From the monomeric G mono, the octomeric pG2G had a molecular mass of 836.8 kDa (Figure S13a, Supporting Information). The functionalized protein dendrimers were observed to be highly homogeneous, exhibiting single bands on SDS-PAGE (3–6% gradient), and appeared as clear single peaks in SEC and DLS with hydrodynamic size ranging from the monomeric 6.9 nm to the octomeric 28.3 nm (Figure 4b; Figure S13b–e, Supporting Information).

We investigated the cytotoxic effects of the protein dendrimers functionalized with G mono carrying the EGFR-specific reepobody against four different cancer cell lines. Two EGFR-positive cell lines (A431 and MDA-MB-468) and two EGFR-negative cell lines (MCF7 and NIH3T3) were tested. To match the total amount of gelonin treated, a lower concentration of the G mono functionalized protein dendrimers was added with the increasing protein dendrimer generations. As expected, the octomeric pG2G showed a much higher cytotoxicity than lower generations of.
Figure 3. Protein dendrimer-based intracellular protein delivery. a) Graphical representation of a construct for intracellular protein delivery. The translocation module is composed of a cell-targeting moiety and a TDP of the exotoxin. A cargo protein, fused to a KDEL sequence, and the translocation module are genetically linked to the conjugation module, yielding D_{mono}. All protein domains are linked with flexible linkers. As a model, an EGFR-specific protein binder and eGFP were used as a cell-targeting moiety and a cargo, respectively. The dotted line with an empty triangle on the TDP and that with a filled triangle at the end of eGFP represent the furin and cathepsin B cleavage site, respectively. Different generations of protein dendrimers are functionalized with D_{mono} to produce pG_{0}D, pG_{1}D, and pG_{2}D, respectively. b) SDS-PAGE (3–6% gradient) of D_{mono} and the functionalized protein dendrimers. The protein dendrimers functionalized with D_{mono} carrying an EGFR-specific repebody were designated as (+), whereas those functionalized with D_{mono} carrying an off-target repebody were represented as (−). c) Confocal images of high EGFR-expressing A431 cells after treatment with the functionalized protein dendrimers for 6 h. Merge (−) indicates the protein dendrimers functionalized with D_{mono}, carrying an off-target repebody. d) Average cell fluorescence intensity of eGFP from A431 cells treated with the same protein dendrimers as in (c) (n = 5). e) Western blot analysis of intracellular eGFP in lysate of A431 cells treated with the D_{mono} and pG_{2}D, respectively. β-Actin was used as a control.
protein dendrimers even though the total amount of gelonin treated was same. For the A431 cell line, pG₂,G exhibited a remarkable cytotoxic effect, resulting in a cell viability of 21%, whereas other protein dendrimers resulted in a cell viability ranging from ≈93 to 60% (Figure 4c). A tenfold higher concentration of monomeric G\textsubscript{mono} was required to obtain the same cytotoxicity as the octomeric pG₂,G (Figure S14, Supporting Information). A negligible change in cytotoxicity was observed for the non-functionalized protein dendrimers (Figure S15a, Supporting Information). Similar results were obtained for the MDA-MB-468 cell line (Figure S15b, Supporting Information), while a negligible cytotoxicity was observed in EGFR-low and EGFR-negative cell lines (Figure 4d; Figure S15c, Supporting Information). The functionalized protein dendrimers with G\textsubscript{mono} carrying an off-target repebody (−) or an off-target repebody (−) exhibited no cytotoxic effect on all the tested cell lines (Figure 4c,d; Figure S15d,e, Supporting Information). Based on the results, it seems that a significantly increased cytotoxic effect by pG₂,G can be attributed to an enhanced intracellular delivery of a protein cargo mainly owing to the avidity of the protein dendrimers with multiple valency. Because higher generation protein dendrimers showed a higher avidity, an increase in the binding affinity of such protein dendrimers functionalized with a cell-targeting moiety is the most likely reason for the higher cytotoxicity.
3. Discussion

The quest for the protein assembly with a tunable size and valency has been increasingly high as a versatile platform for creating novel functional nanostructures with a wide range of applications such as vaccines, drug delivery, biosensors, and disease diagnosis and therapy. We demonstrated an efficient and simple bottom-up approach to constructing a supramolecular protein assembly in a way that allows the size and valency to be controlled using only a core protein and two monomeric protein building blocks. Using an orthogonal system paring both SpyCatcher/SpyTag and SnoopCatcher/SnoopTag, a dendrimer-like protein assembly, namely protein dendrimer, was grown to a mega-dalton scale in a highly efficient and stepwise manner through an iterative and alternate addition of a building block. The largest protein assembly constructed in this study was the fourth-generation protein dendrimer, pG4, with 950.5 kDa and 20.6 nm, the size of which is comparable to various viruses.\(^\text{[3]}\) In principle, a higher generation of protein dendrimers can be constructed through the exact same procedure. A drawback of the assembly might be that each growth in generation requires purification of proteins using SEC, raising the issues regarding the cost, efficiency and scalability. This could be improved by simplifying the purification steps using alternative methods such as solid-phase growth. Unlike the protein assemblies found in nature, the present approach allows the use of diverse building blocks with different sizes and structures, leading to the protein assembly with high homogeneity in a programmable manner. To the best of our knowledge, this is the first to report a programmable supramolecular protein assembly with a tunable size and valency depending on the generation.

In order for the protein assemblies to be used practically, their functionalization with diverse biomolecules is crucial. As the reactive termini of the protein dendrimers increases by a factor of 2 with each increase in generation, the valency is scalable accordingly. This allows for an easy functionalization with a controllable number of biomolecules through the use of a conjugation module composed of a tandem of SpyTag and SnoopTag. While a linear chain of proteins was constructed using the orthogonal Spy/SnoopCatcher, such linear assembly has some limitations as a protein assembly since a single growth step is required for every cargo extension.\(^\text{[14]}\) In contrast, the protein dendrimers can be decorated with diverse number of cargos in a single and efficient way at the periphery, generating protein assemblies with multimodality. When functionalized with a target-specific protein binder, the binding affinity of the protein dendrimers could be modulated and enhanced through the avidity effect. Specifically, the second-generation protein dendrimer functionalized with an EGFR-specific rebody exhibited a 1,336-fold increase in the binding affinity for a human EGFR compared to a monomeric equivalent, confirming the effect of the avidity. It is noteworthy that a protein dendrimer with a higher valency led to an exponential decrease in the dissociation rate, mainly contributing to a high binding affinity for the target. A higher generation protein dendrimer is expected to show a further enhancement in the binding affinity. Similarly, the second-generation protein dendrimers functionalized with a translocation module and a protein cargo were shown to have a significantly enhanced intracellular translocation of the protein cargo compared to a monomeric equivalent mainly owing to the avidity. When gelonin was used as a model cytotoxic cargo, the cytotoxic effect on the cell viability was significantly enhanced, even though the same total concentration of gelonin was used. This result exemplifies the utility of the protein dendrimers as an intracellular protein delivery platform. The controllability of the size and valency of protein dendrimers is also an advantage in in vivo environments because a higher target binding affinity and a longer blood circulation time of the protein dendrimers will lead to a higher therapeutic efficacy. Furthermore, the protein dendrimers are biodegradable and offer a high biocompatibility compared to inorganic nanomaterials.

4. Conclusion

In conclusion, we demonstrated the programmable growth and utility of a dendrimer-like supramolecular assembly of proteins, called “protein dendrimers,” as a novel biocompatible platform for various applications such as vaccines, drug delivery, biosensors, and disease diagnosis and therapy. The supramolecular protein assembly was efficiently constructed through a simple bottom-up approach in a way that allows the size and valency to be controlled using only a core protein and two monomeric protein building blocks. The protein dendrimers with sizes ranging from small proteins to virus capsids were constructed through a stepwise and iterative growth using a core protein and two building blocks in a programmable manner. Furthermore, the functionalization of the protein dendrimers was easy to achieve through a simple process using a conjugation module. The multivalent nature of the protein dendrimers showed a distinct avidity effect, leading to a significantly increased binding affinity for a target compared to a monomeric equivalent. Consequently, the protein dendrimers functionalized with a cytotoxic protein cargo exhibited a higher cytotoxic effect on the cell viability. Taken together, the present approach can be effectively used in the creation of advanced protein assemblies for biotechnological and medical applications.

5. Experimental Section

Detailed description of the materials and instruments used, experimental procedures and methods are provided in the Supporting Information.

Supporting Information

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

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