Incorporation of Thrombin Cleavage Peptide into a Protein Cage for Constructing a Protease-Responsive Multifunctional Delivery Nanoplatform

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ABSTRACT: Protein cages are spherical hollow supramolecules that are attractive nanoscale platforms for constructing cargo delivery vehicles. Using ferritin isolated from the hyperthermophilic archaean Pyrococcus furiosus (Pf Fn), we developed a multifunctional protein cage-based delivery nanoplatform that can hold cargo molecules securely, deliver them to the targeted sites, and release them to the targeted cells. The release is triggered by cleavage induced by the protease, thrombin. The thrombin cleavage peptide (GGLVPR/GSGAS) was inserted into the flexible loop region of Pf Fn, which is located at a 4-fold axis of symmetry exposed on the surface of protein cages (Thr-Pf Fn). Subsequently, the C-terminal glycine, which is situated in the interior cavity, was substituted with cysteine (G173C) to permit site-specific conjugation of cargo molecules. The introduced cysteine (G173C) was labeled with a fluorescent probe (F5M-Thr-Pf Fn) for cell imaging and cargo release monitoring. The surface of F5M-Thr-Pf Fn was further modified with biotins (F5M-Thr-Pf Fn-NPB) as targeting ligands. The specific binding of dual functionalized F5M-Thr-Pf Fn-NPB to the MDA MB 231 cell line, which overexpresses biotin-specific receptors on its surface, was confirmed by fluorescence microscopic analyses. The inserted thrombin cleavage peptides were effectively cleaved by thrombin, resulting in the release of the C-terminal helix in buffer and on the targeted cells without disruption of the cage architecture. Protein cage scaffolds that combine genetic and chemical modifications may serve as stimulus-responsive delivery nanoplatforms and provide opportunities for developing new types of theranostic nanoplatforms.

INTRODUCTION

Colloidal nanoparticles have been extensively studied as nanoscale multifunctional delivery vehicles for disease- or target-specific diagnosis and therapy.1−6 Their nanometer-range size allows enhanced circulation of included materials,7,8 such as drugs and diagnostic probes, unlike that in the case of circulation of free drugs or small molecules in blood. Nanoparticles also undergo passive accumulation in tumor sites through the enhanced permeability and retention (EPR) effect.9 Nanoparticle-based delivery systems also provide a high surface-to-volume ratio that results in high cargo-loading capacity for payload delivery and a multivalent surface that can improve the aqueous solubility of desired cargoes, such as drugs and diagnostic probes,10,11 as well as to attach various types of ligands for targeted delivery.1−6 For early detection and localized treatment of diseases, such as cancers, and to reduce side effects of therapeutics, targeted and stimulus-responsive delivery of cargoes using nanoparticle-based delivery systems has been extensively attempted.12−16 Various types of chemical ligands, short peptides, and antibodies have been used as targeting moieties of nanoparticle-based delivery systems to enhance their efficiency of delivery.1−6 For the localized release of therapeutics and target-specific diagnosis of symptoms, nanoparticle-based delivery systems have been designed to release encapsulated drugs and to exhibit diagnostic signals upon changes in pH, light, and temperature, as well as treatment with ligands and specific proteases.12−17 For example, insulin-encapsulating polymeric vesicular polymersomes were shown to release insulin in the presence of excess glucose through the disruption of the block copolymer conformation.18 Additionally, heat shock proteins that contain a peptide sequence cleaved by caspase remained quenched in the absence of caspase but exhibited fluorescence at the region where caspase was present.19

Nanoparticle-based delivery systems are generally prepared from synthetic polymers,2,20 inorganic nanomaterials,4,6 and biomaterials21−25 derived from various types of biological sources. Protein cages are protein-based supramolecular complexes that have well-defined hollow spherical architectures. Protein cages, including ferritins, viral capsids, and heat shock proteins, are precisely self-assembled from multiple copies of a

Supporting Information

Received: August 27, 2012
Revised: October 30, 2012
Published: November 19, 2012
limited number of subunits, which results in highly symmetric and uniformly sized architectures.\textsuperscript{24,25} The interior surfaces of protein cages have been used for encapsulation, attachment, and synthesis of organic and inorganic materials, whereas the exterior surfaces have been used for multivalent presentations of molecules, including affinity tags, antibodies, fluorophores, carbohydrates, nucleic acids, and peptides, and for molecular targeting and hierarchical structure formation.\textsuperscript{26–33} Their nanometer-range sizes, propensity to self-assemble into monodisperse nanoparticles of discrete shapes and sizes, and high degree of symmetry and polyvalence make protein cages attractive nanoplatforms for the development of multifunctional nanoscale delivery vehicles.\textsuperscript{24,25}

As a model nanoscale delivery platform, we used ferritin, isolated from the hyperthermophilic archaeon \textit{Pyrococcus furiosus} (Pf\_Fn). Pf\_Fn is expressed as a monomer and 24 identical subunits self-assemble into a spherical cage with inner and outer diameters of 8 and 12 nm, respectively (Figure 1).\textsuperscript{34} Each subunit consists of a 4-helix bundle (helices A–D) and a tilted fifth short \(\alpha\)-helix (helix E) connected by a flexible loop region which is exposed at the surface of the 4-fold symmetry axis when they assemble to form a spherical cage (Figure 1).\textsuperscript{22,34} The tilted fifth short \(\alpha\)-helices (helix Es) are located in the interior space and project to the middle of the cage where they are securely isolated from the outside of the cage, when they assemble to form a spherical shell (Figure 1).\textsuperscript{22,34} Therefore, the C-terminal end of helix E is a good candidate site for the attachment of therapeutics and addition of cytotoxic materials including apoptotic peptides. To develop the Pf\_Fn protein cage as a stimulus-responsive delivery nanoplatform in this study, we genetically modified the C-terminal end and inserted a short peptide (GGLVPRGSGAS) that is selectively recognized and cleaved by thrombin.\textsuperscript{35} Thrombin is a trypsin-like serine proteinase that plays a critical role in the blood coagulation system to generate insoluble fibrin from soluble fibrinogen. It is also known that thrombin plays an important role in extravascular disease, cancer, cardiovascular diseases, acute kidney injury, and stroke.\textsuperscript{36}

\section*{EXPERIMENTAL SECTION}

\textbf{Thrombin Cleavage Peptide Insertion and Protein Cage Purification.} The sequence GGLVPRGSGAS was inserted into the 146 position of Pf\_Fn by an established PCR protocol using primers containing extra nucleotides and pET-30b based plasmids containing genes encoding Pf\_Fn protein with one site mutation (S98C) as a template. S98C Pf\_Fn behaves almost identical to the wild type (wt) Pf\_Fn\textsuperscript{22,37} and we used S98C Pf\_Fn as a template protein cages hereafter. The amplified DNAs were used to transform the competent \textit{E. coli} strain BL21 (DE), which resulted in the overexpression in \textit{E. coli} of the Pf\_Fn protein cage containing the inserted thrombin cleavage peptide. The resultant protein was purified as described previously.\textsuperscript{37} We routinely obtained 5 mg of Thr-Pf\_Fn from 1 L culture.

\textbf{Mass Spectrometry.} The subunit masses of untreated, chemically modified, and thrombin-treated Thr-Pf\_Fn protein cages were analyzed using an ESI-TOF mass spectrometer (Xevo G2 TOF, Waters) interfaced to a Waters UPLC and an autosampler. Samples were loaded onto the MassPREP Micro desalting column (Waters) and eluted with a gradient of 5–95\% (v/v) acetonitrile containing 0.1\% formic acid with a flow rate of 300 \(\mu\)L/min.\textsuperscript{22} ESI generally produces a series of multiply charged ions and the charges are generally distributed as a continuous series with a Gaussian intensity distribution and the molecular masses of each species can be determined from the charges and the observed mass-to-charge (m/z) ratio values. Mass spectra were acquired in the range of \(m/z\) 500–3000 and deconvoluted using MaxEnt 1 and MaxEnt 3 from MassLynx version 4.1 to obtain the average mass from multiple charge state distributions.\textsuperscript{22} For clarity, only deconvoluted masses were presented.

\textbf{Thrombin Cleavage of Thr-Pf\_Fn.} Chemically modified or intact Thr-Pf\_Fn protein cages were incubated with 10 units/mL of thrombin, and reactions were sampled at 1, 2, 4, 6, 8, 24, and 72 h. The collected samples were analyzed by performing 13\% acrylamide reducing SDS-PAGE, size exclusion chromatography, and mass spectrometry.
Chemical Modifications of Thr-Pf_Fn. Thr-Pf_Fn protein cages were incubated with 3 mol equiv of fluorescein-5-maleimide (F5M) at room temperature in the presence of 0.1 mM TCEP with vigorous shaking overnight. Reactions were dialyzed against buffer (50 mM sodium phosphate, 100 mM NaCl, pH 6.5) overnight to remove unreacted F5M. F5M conjugated Thr-Pf_Fn protein cages were subsequently incubated with 10 mol equiv of NHS-PEG4-biotin (NPB) at room temperature with vigorous shaking for 1 h, and the reactions were dialyzed against the same buffer (50 mM sodium phosphate, 100 mM NaCl, pH 6.5) overnight to remove unreacted biotins.37

Cell Culture. MDA MB 231 cells were obtained from the Korean cell line bank (KCLB) and cultured in RPMI1640 medium with L-glutamine (300 mg/L), 10% FBS, and antibiotics (100 μg/mL penicillin and 50 μg/mL streptomycin) at 37 °C under 5% CO2.

Preparation and Analysis of Fluorescence Microscopy Samples. MDA MB 231 cells were grown on microscope cover glasses (MARIENFELD, 18 mm Ø) in 12-well cell culture plates (SPL, 30012). The cells were fixed with 4% paraformaldehyde in PBS (USB, 19943) and washed two times with PBS containing 0.1% Tween 20. The fixed cells were blocked with 5% BSA, 5% FBS, and 0.5% Tween 20 in PBS at 4 °C for 1 h. F5M-Thr-Pf_Fn-NPB (final concentration of 100 μM) in blocking buffer was applied to the blocked cells at 4 °C for 12 h and then 10 units/mL of thrombin was added at 4 °C for 12 h. The cells were washed three times with PBS containing 0.1% Tween 20.22

RESULTS AND DISCUSSION

Construction of Pf_Fn Protein Cages with Thrombin Cleavage Peptide. To develop Pf_Fn protein cage as a stimulus-responsive delivery nanoplatform, we genetically inserted a short peptide (GGLVPRGSGAS) into the middle of the flexible loop connecting helices D and E (Thr-Pf_Fn), as described in the Experimental Section (Figure 1). Thrombin recognizes the amino acid sequence of LVPRGS and selectively cleaves the peptide bond between the arginine and glycine residues (LVPR/GS). Previously, we genetically introduced a 26 amino acid Fc-binding peptide into the same position and demonstrated that the loop region is tolerant to the peptide insertion.22 Insertion of a thrombin cleavage peptide with extra residues (GGLVPRGSGAS) into the loop region was confirmed by DNA sequencing. The inserted thrombin cleavage peptides were exposed on the surface of the Pf_Fn protein cages and clustered around the 4-fold axis of symmetry (Figure 1). Therefore, thrombin may not only effectively cleave the loop region but also release the helix E from the spherical Pf_Fn protein cages by producing holes with a diameter of approximately 1.5 nm at the 4-fold symmetry axis (Figure 1). To introduce additional chemical functionality, we substituted the glycine residue at position 173, which is the second last residue of the C-terminus (Figure 1). The interior space and surface of Pf_Fn can be utilized for the storage of various types of materials, such as small organic molecules, inorganic nanomaterials, and cytotoxic peptides.26−33 In particular, cytotoxic peptides and small organic drug-like molecules can be easily incorporated into the C-terminus using genetic and chemical modifications and stored securely without contact with external substances.34 Once the helix E is loaded with various types of cytotoxic molecules, Th-
Pf_Fn can carry these cargo molecules to the target cells and locally release them at the targeted sites upon thrombin treatment.

Thr-Pf_Fn was overexpressed and purified in the same manner, as previously described. Purified Thr-Pf_Fn was examined by size exclusion chromatography (SEC), transmission electron microscopy (TEM), dynamic light scattering (DLS), and mass spectrometry (MS) and compared with S98C Pf_Fn (Figure 2 and Supporting Information (SI), Figure 1). Thr-Pf_Fn was eluted at the same position as S98C Pf_Fn on SEC, indicating that it has a stable 24-mer cage architecture (Figure 2A). TEM image of negatively stained Thr-Pf_Fn confirmed its spherical cage-like structure with a uniform size distribution (Figure 2B), and DLS measurements indicated that the size of thrombin functionalized Pf_Fn (12.8 nm) was almost identical to that of S98C Pf_Fn (12 nm; Figure 2C). The Thr-Pf_Fn subunits had a mass of 21296.0 Da, which agreed with the calculated molecular mass (21294.3 Da; Figure 2D). We therefore concluded that the inserted thrombin cleavage peptide does not alter the integrity and stoichiometry of the Pf_Fn protein cage architecture.

**Thrombin Effectively Cleaves the Loop of Thr-Pf_Fn To Release the Helix E Without Disrupting the Cage Architecture.** To investigate the cleavage efficiency of the inserted thrombin cleavage peptide on the loop of the Thr-Pf_Fn cages upon thrombin treatment, we treated intact Pf_Fn with a fixed amount of thrombin (10 units/mL) for desired time periods and monitored the cleavage of the inserted thrombin cleavage peptide using SDS-PAGE (Figure 3A). The cleavage of the inserted thrombin cleavage peptide on the loop of Thr-Pf_Fn cages progressed as the incubation time passed. While the amount of 21 kDa Thr-Pf_Fn subunits decreased gradually, the new 17 kDa fragments appeared and their amount increased as the incubation time increased (Figure 3A). We did not observe 4 kDa fragments in SDS-PAGE analysis because of its small size. However, mass analyses of the cleavage reaction products confirmed the identity of newly generated 17 kDa fragment as a helix A−D and identified 4 kDa fragments (Figure 3C,D) (see below for details). The cleavage reaction was almost completed after 1 day, and nonspecifically cleaved fragments began to appear at longer incubation times (Figure 3A). Although SDS-PAGE and MS data demonstrated the efficient cleavage of the inserted thrombin cleavage peptide between helix A−D and helix E, it was not clear whether the thrombin-treated Thr-Pf_Fn maintained its cage architecture and, if so, whether the cleaved helix E remained with the protein cage or was released from the protein cage. To address these questions, Thr-Pf_Fn treated with thrombin for a day was applied to SEC column and compared with a protein molecular weight standard (Figure 3B). Two distinctly separated peaks were observed (Figure 3B). The first peak (indicated as *) appeared at the same position as the intact Pf_Fn cage and the second peak (indicated as #) was eluted at the same position as the S98C Pf_Fn cage.
second peak (indicated as #) appeared at the position where 3−10 kDa fragments were eluted (Figure 3B), suggesting that the treatment of Thr-Pf_Fn with thrombin results in not only the cleavage of the loop but also the release of helix E from the protein cage without disturbing the protein cage architecture (Figure 3B). To confirm the specific cleavage of Thr-Pf_Fn at the thrombin cleavage site (LVPR/GS) and to determine the identity of the components in the two separated peaks, two SEC separated fractions (first and second peaks) were analyzed using mass spectrometry (MS). The molecular mass of the components of the first and second peaks were determined to be 17752.0 and 3560.0 Da, respectively, which are in an excellent agreement with the corresponding predicted values of 17751.2 Da (helix A−D) and 3561.2 Da (helix E) for cleavage at the thrombin cleavage site (LVPR/GS) (Figure 3C,D). TEM image of negatively stained sample and DLS measurement of sample in the first peak confirmed its intact cage-like structure with a uniform size distribution (SI, Figure 2). These data suggest that thrombin-mediated cleavage of the thrombin cleavage peptides inserted in the loop of Thr-Pf_Fn may generate 1.5 nm holes at the 4-fold symmetry axis in addition to the release of helix E (Figure 1).

**Interior C-Terminal Modification of Thr-Pf_Fn for Probe Attachment and Exterior Surface Modification for Targeting.** The X-ray crystal structure of Pf_Fn shows that helix E is situated in the interior space of the protein cage and its C-terminal end projects toward the middle of the interior space (Figure 1). We also genetically added six consecutive histidine residues to the C-terminus with a short linker and successfully purified them. While they exhibited almost identical morphology and stability, purified C-terminal histagged Pf_Fn did not bind to the nickel chelated resin, suggesting introduced histaggs are not exposed on the surface (SI, Figure 3). Therefore, the C-terminal end of helix E is a suitable location to attach various types of organic molecules chemically or to add extra residues genetically because it is situated in the interior space of the protein cage being perfectly isolated from the outside. Lysine residues on the surface of protein cages have been used for the attachment of various types of ligands.24,25,31,32 The surface of Thr-Pf_Fn has eight lysines per subunit and they would be easily labeled with various types of N-hydroxysuccinimide-ester (NHS-ester) functionalized molecules.

To adapt Thr-Pf_Fn protein cages as protease-responsive targeted delivery nanoplatforms, we attached a cysteine-reactive fluorescent probe (fluorescein-5-maleimide, F5M) as a model cargo at the end of helix E (G173C), and we conjugated amine reactive targeting moieties (NHS-PEG4-biotin, NPB) on the surface of Thr-Pf_Fn. Each subunit of Thr-Pf_Fn has only one cysteine at position 173 and is located in the interior space, where it is protected from outside but accessible to small
molecules. Thr-Pf_Fn was treated with F5M overnight and applied to SEC column to evaluate the coelution of F5M and Thr-Pf_Fn protein cages and to remove unreacted F5M (SI, Figure 4). F5M-conjugated Thr-Pf_Fn was eluted at the same position as untreated Thr-Pf_Fn with a high absorption at 488 nm, which is the maximum absorption wavelength of fluorescein, suggesting that F5M was effectively labeled and that its labeling did not alter the cage architecture (SI, Figure 4A,B). Mass measurements and SDS-PAGE finding for the SEC fractions confirmed that all the Thr-Pf_Fn subunits were labeled with F5M (SI, Figure 3C,D). Additional surface modification was subsequently achieved using NPB and 1−4 biotins were attached to each Thr-Pf_Fn subunit and two biotins per subunit (48 biotins per protein cage) were attached on average (SI, Figure 4E).

Monitoring the Release of Fluorescent-Probe-Labeled Helix E upon Thrombin Treatment. To test whether fluorescently labeled helix E can be as effectively released from Thr-Pf_Fn protein cages upon thrombin treatment as unlabeled helix E, the F5M-labeled Thr-Pf_Fn (F5M-Thr-Pf_Fn) protein cage was treated with thrombin under the same conditions as unlabeled Thr-Pf_Fn, and the reaction products were analyzed using the same methods. The Thr-Pf_Fn protein cages without helix E and the F5M-labeled helix E was separated on the SEC and their identities were confirmed by UV/vis spectrophotometry and mass spectrometry (Figure 4). Although the Thr-Pf_Fn protein cages with helix E removed were eluted at the same position as that of the cleaved helix E in Figure 3, suggesting F5M-labeled helix E was also effectively cleaved and released upon thrombin treatment, even after F5M conjugation (Figure 4A,B). The first and second peaks represent masses of 17752.0 and 3987.0 Da, respectively, which are well matched with the calculated subunit mass for Thr-Pf_Fn with helix E removed (helix A−D, 17751.2 Da) and the calculated mass of F5M-labeled helix E (3988.5 Da; Figure 4C,D). However, a slight absorption at 488 nm was observed in the first fraction. It may result from the cleaved residual F5M-labeled helix E remained inside of the cleaved Pf_Fn protein cages rather than uncleaved F5M-Thr-Pf_Fn because we could not detect uncleaved F5M-Thr-Pf_Fn subunit with mass spectrometric and SDS-PAGE analyses. However, we can not completely rule out the possibility of the limit of detection of those analytical methods.

Targeted Delivery of F5M-Thr-Pf_Fn-NPB and the Controlled Release of Fluorescent-Probe-Labeled Helix E Triggered by Thrombin Treatment. Biotin is a member of the vitamin family (vitamin B12) and is known to be more abundant in cancer cells than in normal cells.20,38 Excess amounts of biotin are known to facilitate the proliferation of cancer cells.20,38 Cancer cells generally overexpress biotin-specific receptors on their surface to uptake more biotin. Because the interaction between biotin and the biotin receptor is specific and strong, biotin-specific receptors are good candidates for targeting sites for the active delivery of cargo.20,38 To impart a targeting capability to the F5M-labeled Thr-Pf_Fn (F5M-Thr-Pf_Fn) protein cage, we additionally modified the surface of F5M-Thr-Pf_Fn with NPB (F5M-Thr-Pf_Fn-NPB) and two biotins per subunit (48 biotins per protein cage) were attached on average (Supporting Information, Figure 4E). We obtained dual functionalized

**Figure 5.** Fluorescent microscopic images of MDA MB 231 cells incubated with F5M-Thr-Pf_Fn (A, D) or F5M-Thr-Pf_Fn-NPB (B, E). F5M-Thr-Pf_Fn-NPB treated MDA MB cells were subsequently incubated with thrombin, washed, and fluorescently imaged (C, F). Thr-Pf_Fn protein cages and nuclei were visualized as green and blue, respectively, by fluorescence microscopy.
Thr-Pf_Fn protein cages that simultaneously contained a fluorescent probe (F5M) inside the cage and a targeting ligand (NBP) on the surface of the cage. To demonstrate specific binding of the dual functionalized Thr-Pf_Fn (F5M-Thr-Pf_Fn-NPB) to the target cells and the thrombin-responsive release of helix E, we chose MDA MB 231 cells as the target cell line. MDA MB 231 cells are known to overexpress biotin-specific receptors on their surface and selectively catch external biotins. F5M-Thr-Pf_Fn-NPB was incubated with MDA MB 231 cells and visualized by fluorescence microscopy (Figure 5). F5M-Thr-Pf_Fn, which does not have targeting ligands on its surface, was also treated to MDA MB 231 cells in parallel as the control. While F5M-Thr-Pf_Fn did not bind to MDA MB 231 cells at all (Figure 5A), F5M-Thr-Pf_Fn-NPB specifically bound to MDA MB 231 cells (Figure 5B). The binding of F5M-Thr-Pf_Fn-NPB to MDA MB 231 cells was inhibited by free biotins in a dose-dependent manner (SI, Figure 5), suggesting that F5M-Thr-Pf_Fn-NPB binding to the cells is mediated by specific biotin–biotin receptor interactions. However, MDA MB 231 cells treated with F5M-Thr-Pf_Fn-NPB completely lost their fluorescent signals upon thrombin treatment and subsequent washing (Figure 5C). These data suggest that F5M-labeled helix E was effectively cleaved by thrombin and removed from the bound Thr-Pf_Fn even when F5M-Thr-Pf_Fn-NPB was bound tightly to the target cells.

Together, these results indicate that the Thr-Pf_Fn protein cage can serve as a stimuli-responsive delivery nanoplatform through combinations of genetic and chemical modifications followed by protease treatment. Because Thr-Pf_Fn has multiple sites and spaces available to address various types of functionalities, it may constitute a versatile nanoplatform that can be adapted for a variety of functions. The interior cavity and surface of Thr-Pf_Fn can be utilized for covalent attachment and encapsulation of small organic molecules, such as drugs and therapeutic reagents, encapsulation of preformed therapeutic, and diagnostic nanoparticles, or insertion of functional peptides, such as apoptotic and cytotoxic peptides. Peptide-based therapeutics is thought to be superior to antibody therapeutics because it is easy to be synthesized and genetically manipulated, and it requires low cost to produce. In addition to the interior space, Thr-Pf_Fn provides an exterior surface for displaying various types of targeting ligands, including synthetic ligands and targeting peptides that are screened by phage and cell surface display techniques. Mixing-and-matching approaches for simultaneous interior and exterior modifications will expand the possibility of utilizing protein cages as multifunctional nanoscale delivery platforms and provide new opportunities for developing new theranostic nanoplatforms.

**CONCLUSIONS**

We have developed a multifunctional protein cage-based delivery nanoplatform that can hold cargo molecules inside securely, deliver them to the targeted sites, and release them to the targeted cells upon triggering by a protease. Thr-Pf_Fn was prepared by genetically inserting the thrombin cleavage peptide into the flexible loop region of Pf_Fn and substituting C-terminal glycine with cysteine (G173C). The inserted thrombin cleavage peptides were clustered at a 4-fold axis of symmetry exposed on the surface of the protein cage and the introduced cysteine residue was situated in the interior cavity. Thrombin recognized and selectively cleaved the inserted peptide resulting in the release of the C-terminal helix E including cysteine 173 residue as well as the production of 1.5 nm holes at the 4-fold symmetry axes. The introduced cysteine (G173C) was labeled with fluorescent probes (fluorescein) for cell imaging and the lysine residues on the surface of Thr-Pf_Fn were subsequently labeled with targeting ligands (biotin). Fluorescence microscopic analyses demonstrated that dual functionalized F5M-Thr-Pf_Fn-NPB specifically bound to the MDA MB 231 cell line, which overexpresses biotin specific receptors on its surface, and subsequent thrombin treatment completely eliminated the fluorescence signal because of selective removal of fluorescence-probe-labeled C-terminal helix E.

**ASSOCIATED CONTENT**

1. Supporting Information

Additional data of SEC, TEM, and mass spectrometry analyses of genetically and chemically modified protein cages are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was supported by Basic Science Research Program (No. 2012-0001688), Advanced Research Center (No. 2012-0008996) of MEST through NRF of Korea, and the Collaborative Research Program for Convergence Technology (Seed-11-6) of the Korea Research Council of Fundamental Science and Technology (KRCF).

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