Protein Cages as Theranostic Agent Carriers

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Abstract— **Protein cages can be engineered to tailor its function as carriers for therapeutic and diagnostic agents. They are formed by self-assembly of multiple subunits forming hollow spherical cage structures of nanometer size. Due to their proteinaceous nature, the protein cages allow facile modifications on its internal and external surfaces, as well as the subunit interfaces. Modifications on the internal surface allow conjugation of small molecule drugs or contrast agent while modifications on the external surface allow conjugation of various ligands including targeting ligands. The subunit interaction is of special interest in engineering controlled release property onto the protein cage. Two different protein cages, E2 protein and ferritin, are described.**

Keywords— **Drug delivery, controlled release, MRI contrast agent, nanocapsules, nanocages**

I. Introduction

Protein cages, such as viral capsids, E2 protein, and ferritin, have been gaining interests for applications in medicine as drug delivery vehicle and as imaging contrast agents [1]. In contrast to the man-made materials, such as nanoparticles, nature-derived protein cages are synthesized with high precision, narrow size distribution, and are naturally biodegradable. They are composed of multiple subunits that self-assemble and contain various functional groups providing a platform for facile manipulations. Extension to the natural functions or imparting non-natural functions is possible through genetic engineering of the protein cage.

To expand the functions of the protein cages, three surfaces are of interests: internal, external, and subunit interfaces. The internal surface of the protein cage can be engineered to allow loading of cargos for therapeutic or diagnosis purposes. Attachment of targeting ligands for localized delivery will require modifications on the external surface of the protein cage. The subunit interfaces are important in the cage formation from the subunits through self-assembly process. Identification of the subunit portions that are responsible in the self-assembly process elucidates the potential of engineering this interaction in controlling the cargo release. The multi-subunit composition gives an advantage to the protein cage that is modification to a single subunit results in multiple modifications to the cage.

This paper describes two protein cages, E2 protein and ferritin, that have been studied for potential applications as therapeutic and diagnostic agent carriers.

II. E2 protein as therapeutic agent carrier

A. Modification of the internal surface

The protein cage in this study is derived from the E2 core domain of the pyruvate dehydrogenase of *Bacillus stearothermophilus* (PDB ID 1B5S). E2 protein is composed of 60 subunits. The 60 subunits self-assemble to form a 25-nm hollow caged structure. To impart a non-natural function to carry therapeutic agent to the E2 protein, the native aspartic acid at position 381 located on the internal surface of the E2 protein is substituted with thiol-containing amino acid, cysteine (D381C) [2]. The amino acid is identified through molecular visualization software of the crystal structure. Modification of the amino acid to cysteine using site-directed mutagenesis facilitates covalent attachment of small molecules, i.e. cancer drugs containing thiol-reactive moiety [3]. In E2 protein, introduction of one thiol on a subunit results in 60 thiols on the fully assembled protein cage (Fig. 1).

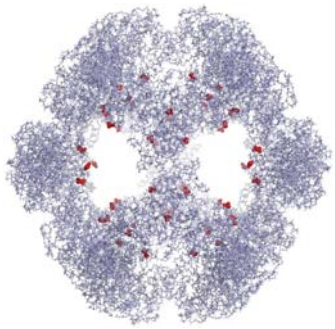

Fig. 1 E2 protein cage model highlighting residue D381 (red) located at the internal cavity. The model was generated and displayed using PyMOL [4].

B. Loading of therapeutic agents into the E2 protein cage

The introduction of thiol functional group through cysteine substitution to the E2 protein cage allows covalent attachment of small molecules with thiol-reactive groups such

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as maleimide. As a proof of concept, model drugs fluorescein-5-maleimide (F5M) and Alexa Fluor 532 C5-maleimide (AF532M) are conjugated to the protein cage [2]. Subsequently, maleimide-modified doxorubicin is incorporated into the protein cage [3]. To load the maleimide-containing molecule into the cysteine-modified protein cage, the thiol groups on the cysteine is reduced in the presence of reducer such as TCEP, β-mercaptoethanol (BME), or DTT. TCEP is preferred over the other two reducers for its resistance to air oxidation resulting in easy handling, besides being odorless. Detailed mechanism of the conjugation can be found elsewhere and the amount of drugs conjugated/cage is determined to range between 84-114 [3].

C. Investigation on cellular uptake

Cellular uptake of the protein cage has been demonstrated [3]. Similar to most nanoparticles in the size range of 20 to 60 nm, the 25-nm E2 protein cage may also be internalized via the endocytosis pathway. The study is conducted on breast cancer cell line MDA-MB-231 incubated with protein-cage-conjugated doxorubicin for 72 hours. Visualized using confocal microscope, the fluorescent doxorubicin co-localizes with the endosomes indicating that the E2 protein cage remains in the endosomes upon internalization. The therapeutic agents are proposed to diffuse from the endosomes to the cytosol and nucleus following release from the protein cage. Further research is required to understand the detailed mechanism of the transport.

III. Controlled Release from E2 Protein Cage

A. pH as a trigger for molecular release

Protein cages have been proposed to be taken up by cells through the endocytosis route. Upon cellular uptake, the protein cage will experience a pH change from 7.4 in the cytosol to 5.0 within the endosome. The pH change can be used as a trigger to release the cargo from the protein cage. To facilitate the pH-sensitive release of from the protein cage, a few strategies have been employed:

Acid-labile linker: For specific release, small molecule drug is synthesized as prodrug and will only be activated upon a particular cellular cue, such as pH change, the presence specific cellular receptor, or cleavage by enzymes. Introducing acid labile linker between the molecular cargo and the protein cage will allow selective release of the cargo within the endosome. Doxorubucin and folate are a couple examples of drugs and targeting ligands that are engineered to be acid-labile [5,6].

Repulsive force between histidine clusters: Histidine is the only amino acid that contains imidazole group on its side chain. With pKa of 6.0, the imidazole group is uncharged at neutral pH while protonated at pH < 6.0. When several histidines are located within the Debye radius and form a cluster at critical positions on a protein cage, the repulsive force at acidic pH is often strong enough to disrupt the stability of the assembly. This inherent characteristic is unique and has been shown to be a viable strategy to impart pH sensitive switch for the cargo release within the endosome.

Structural analysis of the E2 protein cage shows that two clusters of three histidines are stacked on top of each other at the N-terminus. Formation of the clusters is due to the assembly of three subunits referred to as trimer. As illustrated on Fig. 2, the positions of the histidine clusters are relatively buried and protected from solvent access. It is hypothesized that the histidine clusters will impart pH-sensitive characteristic on the E2 protein cage, provided that the clusters are solvent accessible.

To provide solvent access to the histidine clusters, 50 amino acids at the N-terminus of the E2 protein cage are truncated (E2-ΔN). The hydrodynamic size of the E2-ΔN at pH 7.4 is comparable to that of the unmodified E2 protein cage indicating that the truncation does not alter the assembly. However, at pH 5.0 the hydrodynamic size is two orders of magnitude higher indicating the formation of aggregates that are observed to be insoluble [7].

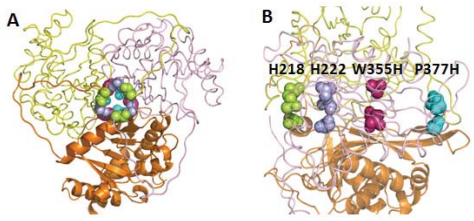

Fig. 2 A trimer model of three E2 subunits highlighting both the native (H218, H222) and non-native (W355H, P377H) histidine clusters viewed along the 3-fold axis (A) and the side (B) [8].

To further confirm that both repulsive interactions between the histidine residues and solvent accessibility cause the disassembly of the E2 protein cage at low pH, the N-terminus is left intact while two non-native histidine clusters are introduced to the intratrimer positions in addition to the two native clusters leaving the clusters buried (E2-(2+2)H, Fig. 2B). In addition, another set of cages was constructed where the histidine cluster was introduced at the intertrimer positions at the C-terminus where the clusters are accessible by solvent (E2-4H). The intratrimer modifications result in E2 protein cage that is correctly assembled at both pH 7.4

and pH 5.0. However, in contrast to E2-ΔN and E2-(2+2)H, the aggregates formed by the intertrimer modified E2-4H at pH 5.0 are soluble. The solubility of the aggregates has important implications in the application of the E2 protein as drug delivery vehicle, where insoluble aggregates can be detrimental to cellular functions.

B. Modulation of self-assembly

Besides utilizing pH as trigger, modulation of the self-assembly has been proposed to be an alternative strategy to control molecular release from the protein cage. In previous section, the interface between trimers has been identified as a departure point for engineering pH-dependent trigger. Further studies on this interface reveal that a particular motif on the C-terminus of E2 protein is responsible for the self-assembly of the cage (unpublished data); molecular dynamic simulation results confirm the role of the motif. The studies suggest that the motif is a promising site onto which other functionalities specific to cellular cues can be engineered to the interface.

IV. Ferritin as Diagnostic Agent Carrier

A. Protein-cage-bound metal nanocore

Ferritin is an iron-storage protein ubiquitous to all living organisms. The ferritin in this study originates from *Archaeoglobus fulgidus* and has been modified (AfFtn-AA; PDB ID 3KX9). AfFtn-AA consists of 24 subunits that self-assemble to form a 15-nm protein cage in high ionic concentration or in the presence of trace amount of metal. It catalyzes the conversion of both Fe(II) and Mn(II) into Fe(III) and Mn(III), respectively, and forms insoluble nanocore within the cavity of the ferritin. The size of the nanocore is confined to the size of the cavity which is ~8 nm, resulting in highly homogeneous nanocore with narrow size distribution. The protein-cage-bound metal nanocore is determined to be magnetic as confirmed by the hysteresis loop obtained by vibrating sample magnetometry [9,10]. The magnetic property of the metal-bound nanocore is further investigated for its potential application as magnetic resonance imaging (MRI) contrast agent.

B. The relaxivity values

Relaxivity is an important measure for a protein-cage-bound nanocore in enhancing contrast during MRI. Higher relaxivity translates to improved contrast enhancement between diseased and healthy tissues, hence increased sensitivity. To determine the relaxivity values, the metal-protein cage conjugate at different concentrations are immobilized onto 0.8% agarose gels and scanned within a 3-Tesla(T) MRI machine. The longitudinal and transverse relaxivities, R1 and R2, are determined from the slope by plotting the inverse of proton relaxation time, T1 and T2, against metal concentration. T1 and T2 are determined from the intensity of the images obtained from the MRI machine using equations 1 and 2.

$$I = A - Be^{\frac{t}{T1}} \quad (1)$$

$$I = Ae^{-\frac{t}{T2}} + B \quad (2)$$

Higher relaxivities of protein-cage-bound iron and manganese nanocores, (Fe)AfFtn-AA and (Mn)AfFtn-AA, respectively, over non-protein-bound nanoparticles have been reported [9,10]. Contribution of the protein shell on the relaxivity improvement will require further research. The high relaxivities also imply that the contrast agents are ultrasensitive; requiring less dosage to achieve the same contrast. The R2 of (Mn)AfFtn-AA in particular is the first to be reported of any manganese-based contrast agent [10]. In clinical applications, manganese-based contrast agent has been traditionally a T1 (bright) contrast. This study has shown the potential of manganese-based contrast agent as a T2 (dark) contrast in addition to T1 contrast to serve as a dual contrast agent.

V. Multiple Encapsulation and Targeted Delivery

A. Multiple encapsulation

Both E2 protein and ferritin can be engineered to carry therapeutic agents and to synthesize nanocores as diagnostic agent, respectively. Encapsulation of both therapeutic and diagnostic agent into a single cage will provide additional versatility as theranostic agent carrier. Nanocore synthesis within the E2 protein cage is possible by substituting several key amino acids (unpublished data) while introduction of thiol moieties to the internal surface of ferritin will provide covalent attachment points to other thiol-reactive small molecules similar to that previously described for E2 protein cage.

B. Targeted delivery

Localized accumulation of the therapeutic/diagnostic agent to a particular diseased tissue/organ makes it possible to reduce undesired side effects and dosage, hence increased efficacy [11]. Most targeting strategies rely on overexpressed protein on the cell surface, such as epidermal growth factor receptor (EGFR). Other cellular functions,

such as cell division and DNA replication, have also been used as target for delivery of nanoparticles. Identifying the interactions between surface protein and antibody specific to the surface protein or between protein/DNA specific to cellular function and the small molecule has become the focus of targeting strategy. Some of the popular cancer targeting ligands are antibody against the EGFR as well as folate which targets folate receptor overexpressed on cancer cells. In addition, peptides have also been gaining interest as an alternative to antibody for its small size and ability to escape the body clearance system [12]. The targeting ligands have been incorporated onto various nanoparticles and increased the amount of nanoparticles localization onto particular cancer cells [11]. Similar approach can also be employed onto the protein cage. Attachment of the targeting ligands is facilitated by covalent conjugation via thiol linkages or by fusing the peptide to the external surface by genetic engineering. The multi-subunit composition of the protein cage makes it possible to attach multiple ligands onto the same protein cage, providing multi-target delivery of the therapeutic and diagnostic agents.

VI. Conclusions

Two protein cages investigated in this work, *B. stearothermophilus* E2 protein and *A. fulgidus* ferritin, have been shown to be promising as a therapeutic and diagnostic agent carriers, respectively. Engineering of the internal, external, and intersubunit interfaces expand the protein cage beyond its natural functions and provide multitude of novel functionalities. Packing of multiple cargos for both therapeutic and diagnosis purposes is possible by engineering internal surface of the cage while targeting ligands can be attached to the external surface. Continuous research in identifying cellular targets will improve the targeting efficiency. The intersubunit interfaces are proving to be important in modulating the self-assembly of the protein cage. Other cell-specific triggers can potentially be engineered onto the interface to achieve the desired release properties.

To reach the actual proposed medical applications, further research on the biocompatibility of the protein cages is required. Currently, the protein cages are being subjected to various experiments to determine its plasma clearance rate, acute and long-term immunological properties, as well as its efficacy. Fundamental understanding on the detailed release mechanism of the therapeutic agents from the endosome to the target cellular compartment as well as the contribution of the protein shells in the contrast enhancement are open for explorations.

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