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Modification of Protein-protein Interaction in a Nanocapsule

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ABSTRACT
The vault nanocapsules are promising carriers in protein-based drug delivery. However, limited studies have been reported on effectively controlling the drug release during delivery. It is hypothesized that the rate of content release is related to the interaction between the major vault protein (MVP) and the interaction domain (INT) of vault poly(ADP-ribose)-polymerase. The MVP domains that specifically interact with INT have been identified and is referred to as iMVP. In this report, a few key amino acids of iMVP on the interaction interface were replaced with histidines to impart pH-sensitive response. In contrast to the wild type soluble iMVP, the mutants are produced as insoluble proteins.
Keywords: vault, nanocapsule, protein-protein interaction, histidine modification

1. INTRODUCTION

Protein nanocapsules, such as Ferritin and E2 protein, have been shown to be promising as carriers for contrast agents and small molecule drugs [1-3]. The nature derived template provides precise spatial control resulting in highly uniform structures while the proteinaceous nature allows for facile modification. Vault, a ribonucleoprotein nanoparticle, is ubiquitous in most living eukaryotic system, with dimensions of 72.5 × 41.0nm [4]. It has been shown to be non-toxic, non-immunogenic, and stable in wide pH and temperature ranges [5]. The large lumen of vault is made of the major vault proteins (MVP) that self-assemble to form a barrel-like nanocapsule. A shuttle peptide named the interaction domain (INT) located at the C-terminus of the vault poly(ADP-ribose)-polymerase (GenBank accession No. AF158255; aa 1563-1724), is known to attach to the inner side of the vault shell through a unique protein-protein interaction. Fusion of therapeutic agents to the INT N-terminus facilitates the encapsulation of the therapeutic agents within the vault[5].Introduction of some functional peptides on the vaults have successfully transport vaults to target cells [6].

Vault has a dynamic structure that involves half-vault exchanges [7]. During the open state, content might be freed from or held within the lumen depending on the association/dissociation between MVP and INT. However, controlling the releaseof therapeutic agents from the vaults interior remains a challenge. Since the tumor microenvironment is mildly acidic (pH 6-7) due to the hypoxic environment and residues from anaerobic metabolism, the ability to control the molecular release at specific pH is favorable. Histidine has been demonstrated to induce repulsive interactions between protein subunits upon pH change from 7.4 to 5.0 [8, 9].The histidine imidazole side-chain has a pKa value of 6.1. At pH 7.4, ~10% of the side chains are positively charged while at lower pH the proportion increases. Upon pH

change, multiple histidines that are strategically located within the Debye radius at the interface will induce enough repulsive forces to trigger disassembly or separation between protein subunits.

In this study, we isolated the MVP domains that specifically interact with INT which is referred to as iMVP. Histidines were subsequently introduced to replace key amino acids on the iMVP located at the interaction interface. The production of the recombinant iMVP and hisditine-substituted iMVP were examined.

2. MATERIALS & METHODS

Histidine-substituted Protein Designs–The interacting amino acids were identified by visualizing the structures using PyMOL. The structure of the INT was predicted using I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) and the interaction with MVP was simulated using online protein docking software (http://vakser.bioinformatics.ku.edu/resources/gramm/grammx). The crystal structure of MVP (PDB No. 2QZV) shows that the interaction surface of iMVP (aa113-276) is mostly negatively charged. To modify the interactions between iMVP and INT, several amino acids were replaced to pH-responsive histidines on iMVP (Table 1). The LDL and VDA on iMVP were selected based on their proper distances to INT residues. The amino acids EE were modified to Ala-Ala as control.

Site-Directed Mutagenesis– Primers with the desired mutation sites were designed (Table 1) and the genes were amplified using PCR. The sequence was confirmed by sequencing and the mutant fragments of iMVP were subclonedintopET-28afor tagging with 6xHisat the N terminus to ease purification. All histidine-tagged proteins from the are denoted by His-, i.e. His-iMVP and His-INT.

Table 1. The mutant constructed at the iMVP interface to investigate the effect of histidine substitution.

Constructions	Oligonucleotides
LDL/3H (L213H/D214H/L215H)	5’-GTC TTT GAA GAG GTC cat catcat GTG GAT GCT GTG ATC-3’ (forward) 5’-GAT CAC AGC ATG CAC atgatgatg GAG CTC TTC AAA GAC-3’ (reverse)
VDA/3H (V216H/D217H/A218H)	5’-GAG GTC CTG GAT CTG cat catcat GTG ATC CTT ACA GAA-3’ (forward) 5’-TTC TGT AAG GAT CAC atgatgatg CAG ATC CAG GAC CTC-3’ (reverse)
EE/AA (E210A/E211A)	5’-CTC CCA GCT GTC TTT gcggcg GTG CTG GAT CTG GTG-3’ (forward) 5’-CAC CAG ATC CAG CAC cgccgcAAA GAC AGC TGG GAG-3’ (reverse)

Protein production and purification–His-INT andHis-iMVPvariants were produced in *E. coli*BL21(DE3) grown in LB broth with 50 mg/L Kanamycin according to the recommended protocol (Stratagene). The production was induced by IPTG at optimum concentration for specific construct. The proteins were purified with affinity chromatography (HisTrap HP column, GE Healthcare).

3. RESULTS AND DISCUSSION

Replacement of native amino acids on the iMVP that are located at the interaction interface with INT is hypothesized to impart pH response providing additional cargo release control to the vault. To identify the amino acid residues on the iMVP that interact closely to the INT, a docking simulation was performed using the online software. The results of the docking simulation were used to evaluate the amino acid residues on the iMVP that are within the Debye radius to the INT residues. Solvent accessibility of the interface is important in the protonation of the histidine residues and was examined as shown by the molecular surface (Fig.1).

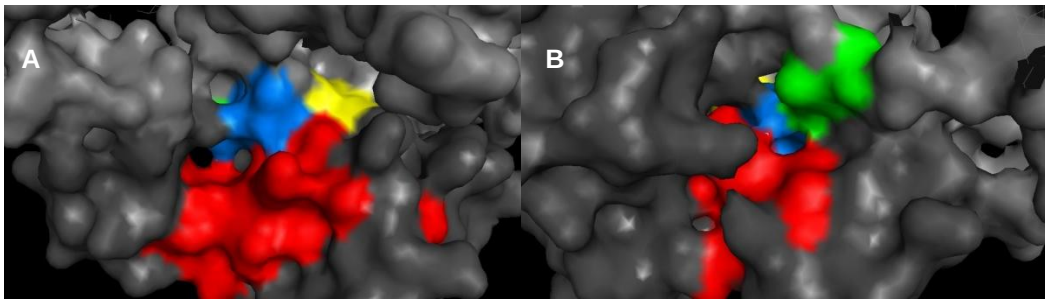

Figure 1. Front (A) and back (B) view of surface structure showing selected iMVP residues for mutation: E210/E211(yellow); L213/D214/L215 (blue);V216/D217/A218 (green); INT residues (red). Figures were generated using in PyMOL [10].

Figure 2 highlights the identified residues on iMVP that are proposed to be interacting with INT within the Debye radius and are solvent accessible.

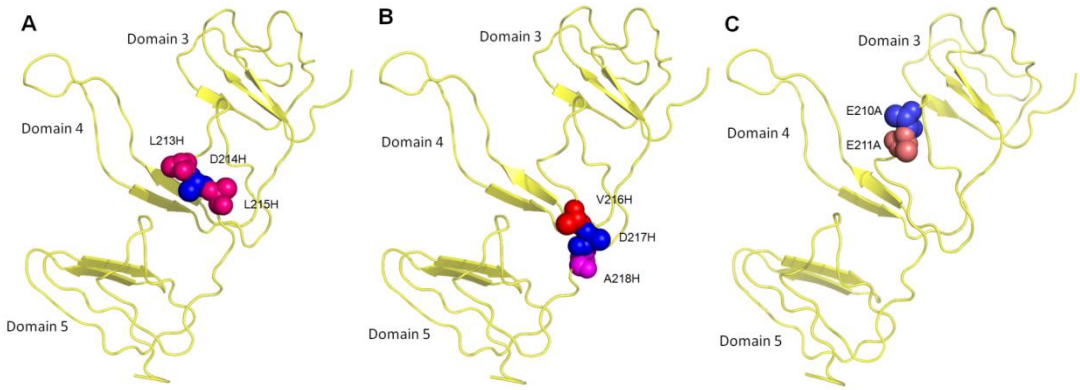

Figure 2. Mutation sites on iMVP domains3, 4 and 5. (A) LDL/3H (L213H/D214H/L215H), (B)VDA/3H(V216H/D217H/A218H),(C) EE/AA(E210A/E211A).

The recombinant protein productions in this study were optimized. His-INT was produced by adding 1 mM IPTG for 3 hours at 37℃. The gene expression of His-iMVP and its mutants were induced by addition of 0.1 mM IPTG for 16 hours at 20℃. Upon harvest, the cells were lysed and the protein production was analyzed using SDS polyacrylamide gel electrophoresis (Fig. 3). Both His-iMVP mutants, LDL/3H and VDA/3H, were produced as insoluble proteins (Fig. 3, lanes 4 and 6) while His-iMVP EE/AA were not produced in either soluble or insoluble fractions (Fig. 3, lanes 1 and 2). The unmodified His-iMVP and His-INT were expressed as soluble proteins and were subsequently purified (Fig. 3, lanes 7 and 8). The summary

of protein production is presented in Table 2.

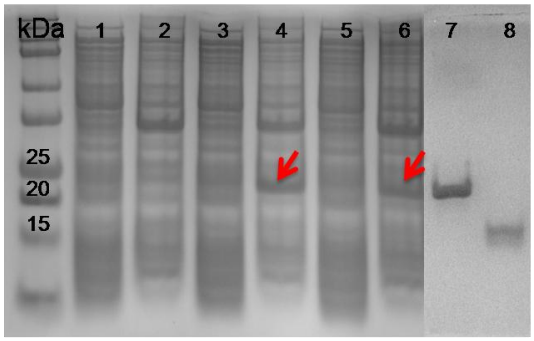

Figure 3. SDS-PAGE gel of proteins production. Lane 1: His-iMVP EE/AA soluble fraction; 2: His-iMVP EE/AA insoluble fraction; 3: His-iMVPLDL/3H soluble fraction; 4:His-iMVPLDL/3Hinsoluble fraction; 5:His-iMVPVDA/3H soluble fraction; 6:His-iMVPVDA/3Hinsoluble fraction; 7: purified His-iMVP, 8: purified His-INT. Red arrows indicate the expected proteins.

Table 2.Summaryof protein productions.

Protein variants	Mutated interface	Expressed in *E. coli*	Soluble for purification
His-iMVP	No	Yes	Yes
His-iMVPLDL/3H	Yes	Yes	No
His-iMVPVDA/3H	Yes	Yes	No
His-iMVP EE/AA	Yes	-	-
His-INT	No	Yes	Yes

The insoluble production of the histidine-substituted His-iMVP mutants is speculated to be due to the change in electrostatic interactions within the protein. To analyze the interactions, the surface potentials of His-iMVP mutants were computed and displayed using PyMOL (Fig. 4). The side-chain charges of the key area of interaction site on iMVP were compared between wild type and mutant on Table 3. As shown in Figure 4 and Table 3, the vicinity of the mutant residues LDL and VDA is negatively charged at pH 5-7.4. Upon introduction of the positively charged histidines, the electrostatic interaction might be disrupted leading to protein misfolding. As a result, His-iMVP LDL/3H and VDA/3H are much less soluble than wild type His-iMVP. Since the gene expression involves many factors, such as control at transcription and translation levels, the non-production of His-iMVP EE/AA is relatively complex to explain. In addition, the alanine replacements might cause interference on the EE interactions which were identified to have both negative and positive charged neighbors in vacuum electrostatics analysis (Fig. 4C).

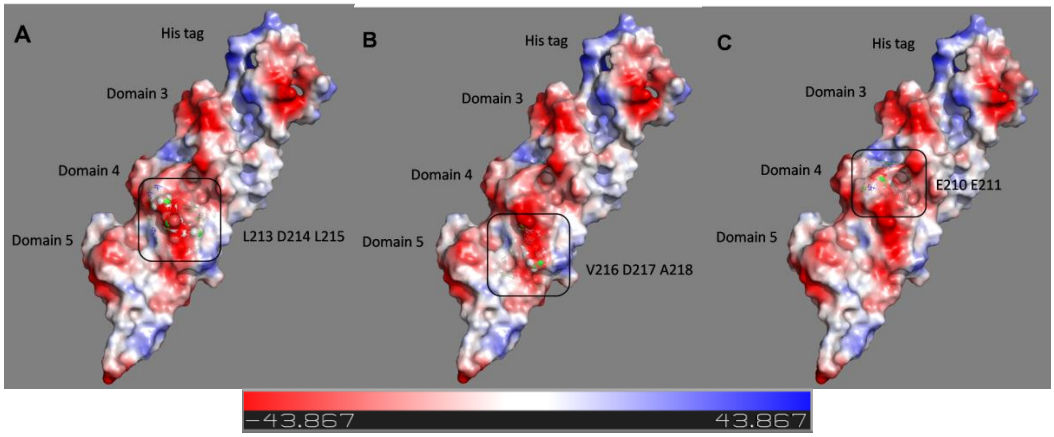

Figure 4.Vacuum electrostatics of His-iMVP drawn by PyMOL. (A) LDL/3H (L213H/D214H/L215H), (B)VDA/3H(V216H/D217H/A218H).

Table 3.Side-chain charges of the key interaction sites on iMVP.

Residue #	iMVP	LDL/3H	VDA/3H	EE/AA
209	F	F	F	F
210	E (-)	E (-)	E (-)	**A**
211	E (-)	E (-)	E (-)	**A**
212	V	V	V	V
213	L	**H (+)**	L	L
214	D (-)	**H (+)**	D (-)	D (-)
215	L	**H (+)**	L	L
216	V	V	**H (+)**	V
217	D (-)	D (-)	**H (+)**	D (-)
218	A	A	**H (+)**	A

For all designed iMVP mutants, attempts to lower the expression temperature to 20℃ and alter inducer concentrations to 0.1 mM did not produce soluble proteins. Subsequent investigations required soluble proteins and could not be performed with these variants.

4. CONCLUSIONS

His-iMVP and His-INT were successfully produced as soluble proteins in *E. coli*. However, attempts to replace amino acids on the iMVP interacting interface with histidines resulted in insoluble proteins. To further investigate the interactions between iMVP and INT, it is important to produce soluble proteins. Modifications on the INT may be possible alternatives to the iMVP modifications.

5. ACKNOWLEDGMENTS

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