Molecular Encapsulation within an Engineered Protein Cage for Drug Delivery

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Helen Chen began her undergraduate research working on alternative, efficient sources of light. From there she moved on to a focus on novel methods of drug delivery, an area of high interest to pharmaceutical companies. Helen has found that her research experience, with its numerous failed experiments leading to a few successes, has helped her develop patience, persistence and optimism. She further demonstrated her passion for research by serving on the UROP Student Editorial Board, assisting with the publication of the 2007 *UCI Undergraduate Research Journal*. Helen is currently attending graduate school.

Key Terms

- Cysteine Mutant
- Drug Delivery Vehicle
- Drug Targeting
- Model Drug Molecules
- Protein Scaffold

Abstract

Most drugs are delivered in the body non-specifically and can act on healthy cells, potentially triggering side effects. The targeting of drug molecules to specific cells to reduce side effects has become the main aim of targeted therapeutics; however, the design of nanoscale drug delivery vehicles remains a challenge. One aspect of this design is the ability to hold and transport pharmaceutically-active molecules. To this end, we developed a protein assembly that can form complexes with drug molecules and essentially house them. The protein assembly is based on the E2 component of pyruvate dehydrogenase from *Bacillus stearothermophilus*. It forms a dodecahedral complex with twelve openings, each 5 nm in diameter, through which small molecules can diffuse. The hollow internal cavity gives the protein scaffold the ability to encapsulate molecules. Genetic modification of the cavity yields a scaffold with 60 cysteine thiol groups available for covalent linkage with guest molecules. Two different fluorescent dye molecules were used as model drugs to assess the reactivity of the thiol side chains. As expected, the cysteine mutant selectively reacted with the maleimide molecules, but not in reactions with the wild-type control. Our results demonstrate the ability of the engineered protein scaffold to encapsulate foreign molecules within its internal cavity and to potentially serve as a drug delivery vehicle.

Faculty Mentor

The delivery of drug molecules to only their intended site of therapy has been a goal in the treatment of diseases but has not yet been fully realized. Such fine control over biodistribution would maximize the efficacy of treatment and minimize side effects. Nanoparticles have been investigated as vehicles for drug delivery, as they exhibit advantageous characteristics for targeting tumor cells. The challenges of traditional nanoparticles, however, are that the required size, size distribution, and chemical functionalization are difficult to achieve. Natural protein scaffolds that form hollow particles can address these issues. In this paper, Helen Chen has shown that the first major requirement, the encapsulation of drug molecules, is achievable. This opens up the possibility of using these scaffolds in therapeutic targeting.

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Introduction

Approximately 40% of all drugs are hydrophobic and consequently have very low aqueous solubility (Chattopadhyay and Shekunov, 2006). Drugs must be soluble in aqueous media in order to have a therapeutic effect in the human body, which is 70% water by weight. Because the number of hydrophobic drugs continues to increase rapidly, strategies are being developed to solubilize hydrophobic molecules to make them therapeutically useful.

In addition to being soluble in aqueous media, drugs also must be able to reach desired sites of action. Unfortunately, most drugs are delivered non-specifically and can act on healthy cells, triggering toxic side effects. Drug targeting strategies localize drug molecules to intended target cells, preventing the occurrence of side effects and improving the therapeutic efficacy of the drug.

The design of drug delivery vehicles remains a challenge, however, due to the limitations that drugs present, such as low solubility and nondiscriminatory action. Emulsions (Shekunov et al., 2006), micelles (Malmsten, 2006), liposomes (Gaucher et al., 2004), and polymer nanoparticles (Tiyaboonchai, 2003) are examples of drug carriers that have been used to overcome these limitations. Our work investigates the feasibility of using a protein nanocapsule as a drug carrier. The focus is to demonstrate a protein nanocapsule's ability to encase model drug molecules.

Background

The Protein Nanocapsule

The protein nanocapsule, named the E2 scaffold, is a component of the pyruvate dehydrogenase multienzyme complex that comes from the *Bacillus stearothermophilus* bacterium. Through genetic engineering, the multienzyme complex has been truncated down to the E2 scaffold, which is a protein cage with icosahedral symmetry.

Native E2 Scaffold. The E2 scaffold self-assembles from 60 identical E2 protein subunits. The subunits are arranged with 532 symmetry, generating a pentagonal dodecahedron with a diameter of 23.7 nm (Izard et al., 1999). Transmission electron microscopy (TEM) confirms that the protein scaffold correctly assembles into a protein cage (Figure 1). Its internal cavity, the region intended to be occupied by drug molecules, is 12 nm in diameter. The cage has 12 pores, which allow drug molecules to diffuse into the scaffold. Some of the cage's pores, each 5 nm wide (Izard et al., 1999), are visible in Figure 2.

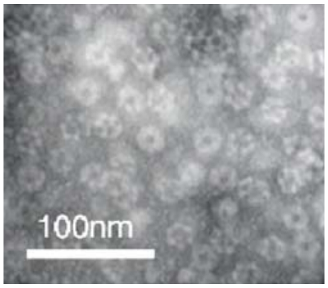

Figure 1

TEM image showing different projections of icosahedral E2 assemblies formed by our system (Image courtesy of C. Lou).

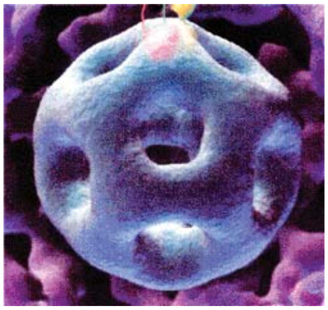

Figure 2

E2 protein complex "1b5s" in Protein Data Bank (Figure from Milne et al., 2002).

Mutated E2 Scaffold. Prior studies in the Wang Lab at UC Irvine indicate that the E2 scaffold can accommodate internal mutations to its cavity. Mutations to the scaffold give it functionality and the ability to react with molecules. Genetic modification of the internal cavity, specifically, will enable the mutant to house or encapsulate guest molecules.

The engineered mutant is generated by making one mutation to a single protein subunit. One mutation results in 60 mutations total per scaffold because each scaffold comprises 60 protein subunits. The mutated site, 381, is chosen because it is surface-accessible within the internal cavity, and is not involved in interactions between protein subunits. Site 381, notated as D381, is originally the amino acid aspartic acid. Position 381 is mutated into an amino acid with a more reactive side chain, cysteine. This side chain contains a thiol group, which is a sulfur atom bonded to a hydrogen atom. The product is the D381C mutant.

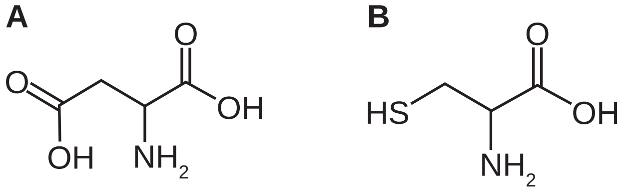

Figure 3

Molecular structures of the amino acids (A) aspartic acid, and (B) cysteine.

Methods and Materials

Preparation of Protein

The native E2 scaffold, referred to as wild-type, is purified in an ion-exchange column and a size-exclusion column to a final concentration of 1 mg/mL in 50 mM potassium phosphate buffer (Fisher Scientific, Pittsburgh, PA) and 100 mM sodium chloride (EMD Chemicals) at pH 7.4. The D381C scaffold, referred to as cysteine mutant, is purified in the same manner. The solvent, potassium phosphate buffer and sodium chloride at pH 7.4, simulates a physiologically-relevant aqueous environment.

Encapsulation Method

Reduce Disulfide Bonds. The cysteine mutant and wild-type, both at 1 mg/mL (35 µM), are treated with 8.5x molar equivalents of tris(2-carboxyethyl)phosphine (TCEP, Pierce, Rockford, IL) (300 µM) for one hour at room temperature prior to labeling to ensure reduction of thiol groups on the interior surfaces of the D381C scaffolds.

Prepare Fluorescent Dye for Conjugation Reactions. Two model drug molecules are used to assess the reactivity of the cysteine mutant. Fluorescent dyes fluorescein-5-maleimide (F5M, Molecular Probes, Eugene, OR) and Alexa Fluor 532 C_5-maleimide (AF532M, Molecular Probes, Eugene, OR) are small, drug-sized molecules that serve as model drugs for conjugation experiments. Their molecular structures are shown in Figure 4. The two different dyes are coupled to the cysteine mutant in separate experiments, and are expected to react with thiols in the same manner because they both contain the maleimide functional group. The scaffold's ability to hold molecules of high molecular weight is measured because AF532M has approximately twice the molecular weight of F5M.

To prepare F5M for coupling reactions, F5M is dissolved in dimethylformamide (DMF, EMD Chemicals). F5M stock solutions are prepared in 100x concentration relative to the desired final concentration for labeling reactions. The concentration of each stock solution is verified using a SpectraMax plate reader, measuring the absorbance of each at 100x dilution in potassium phosphate buffer and calculating the concentration based on the absorbance standard curve of F5M. Absorbance readings of solutions containing F5M are taken at 494 nm. The same procedures are applied to the preparation of stock solutions of AF532M, and absorbance readings are taken at 526 nm.

Conjugation Reaction. Labeling reactions take place at pH 7.4 in potassium phosphate buffer. Two different initial dye concentrations are used for F5M experiments; 1 and 3 molar equivalents of dye per subunit are reacted with the wild-type and D381C scaffolds. One initial dye concentration (ratio of 3 to 1) is used for AF532M experiments. The reactions take place at room temperature for two hours, followed by incubation at 4 °C for 20 hours.

The D381C scaffolds are expected to react with a maleimide by the reaction scheme shown in Figure 4. The reaction begins once the lone pair of the sulfur atom of the thiol group initiates a nucleophilic attack on either carbon of the double bond in the maleimide group. The result is the addition of the thiol to the olefinic double bond. A thioether bond is formed to secure model drug molecules to the inner surface of the cysteine mutant.

Separation Method

The drop dialysis technique is used to separate excess, unbound fluorophore from labeled protein. Fifty microliters of each reaction are dialyzed on membrane filter discs (Millipore) for two hours. During dialysis, each sample is covered with a plastic lid to minimize evaporation. Dialyzed samples are recovered and combined with potassium phosphate buffer, pH 7.4 to restore each sample to its initial volume (50 µL).

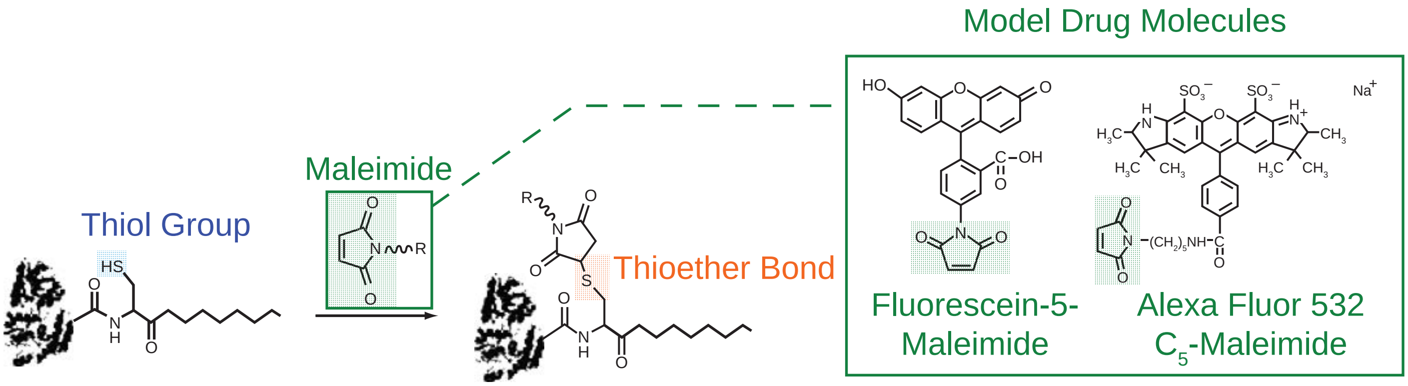

Figure 4

The chemical reaction between a thiol group and maleimide group results in the formation of a thioether bond. The molecular structures of the two dyes are shown to the right of the reaction scheme.

Fluorophore Detection and Quantification

Forty microliters of each dialyzed sample are diluted in potassium phosphate buffer, pH 7.4. The absorbance of the dye is read at 494 nm and 526 nm, for F5M and AF532M, respectively. The absorbance of the protein is read at 562 nm following procedures given in Micro BCA Protein Assay Kit (Pierce, Rockford, IL). A scan of pure wild-type with BCA reagents shows that both dyes' absorbance wavelengths do not interfere with the absorbance of the protein at 562 nm.

Absorbance spectroscopy is used to quantify the amount of dye present in each protein nanocapsule. Dye concentration was calculated for each sample based on the absorbance standard curve that was generated for the dye. The same calculations are performed for both F5M and AF532M experiments, using their respective standard curves.

Results

Extent of Coupling

Coupling experiments of dye to protein were performed on both the native (non-mutated) E2 capsule, referred to as wild-type, and the mutated E2 capsule, referred to as D381C or cysteine mutant. After reacting the dye with the protein and removing excess, unbound dye molecules, the ratio of dye molecules bound to a 60-mer protein capsule was calculated (Figure 5).

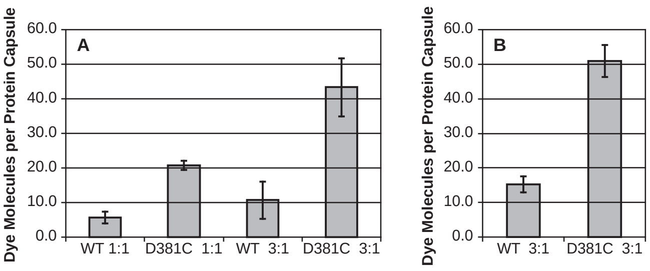

Figure 5

The extent of coupling for (A) F5M conjugation reactions carried out at 1:1 and 3:1 molar ratios of dye to protein subunit, and (B) AF532M conjugation reactions carried out at 3:1 molar ratio of dye to protein subunit.

The cysteine mutant selectively reacts with maleimide molecules, as was expected because the cysteine mutant was engineered to have chemical specificity for the maleimide functional group of the dye, whereas the wild-type nanocapsule served as a negative control. For a 1:1 F5M-to-subunit molar concentration, i.e., a reaction mixture basis of one molecule of F5M to one subunit within the 60-mer scaffold, the cysteine mutant yielded a coupling ratio of 20.8 ± 1.3 molecules per D381C scaffold (Figure 5A). This translates to approximately one-third occupancy of the D381C scaffold because each scaffold theoretically contains 60 thiol groups available for reactivity. The wild-type control was reacted with F5M at the same 1:1 molar concentration, and resulted in a background of 5.7 ± 1.7 molecules of F5M per wild-type scaffold. F5M conjugated to the cysteine mutant more than three times more than the wild-type background, indicating that the cysteine mutant selectively reacted with the dye.

The wild-type is not expected to react chemically with maleimides and should yield a theoretical background of zero. This is because the wild-type scaffold, the non-mutated form of E2, does not possess cysteines on its interior or exterior surfaces. Because two hours of dialysis is assumed to completely remove unbound dye from the reaction mixture, the wild-type background is likely due to nonspecifically-bound dye.

Effect of Varying Dye Concentration. The extent of coupling was controlled by varying the dye-to-subunit molar concentration. This was done by keeping the amount of protein in the reaction mixture constant and varying the initial concentration of dye. When excess dye was added to protein at a 3:1 F5M-to-subunit molar concentration, the cysteine mutant yielded a coupling ratio of 43.3 ± 8.4 molecules of F5M per scaffold (Figure 5A). In comparison to a 1:1 molar concentration, the extent of coupling increased almost two-fold using a 3:1 molar concentration.

Effect of Varying Dye Molecule Size. In addition to reacting the protein with F5M, separate conjugation experiments were performed by reacting the protein with AF532M, a much bulkier fluorescent dye. The reaction scheme of AF532M coupling experiments was assumed to be identical to that of F5M experiments because both dyes contain the same chemically reactive functional group (maleimide). However, AF532M is nearly twice the molecular weight of F5M. Consequently, fewer AF532M than F5M molecules were expected to fit inside and couple to the D381C scaffold. Still, the extent of coupling when using AF532M closely matches that when using F5M. For a 3:1 molar concentration of AF532M-to-subunit, the cysteine mutant yields a coupling ratio of 50.9 ± 4.6 molecules per scaffold (Figure 5B). This is approximately four times the number of AF532M molecules that bound to the wild-type, showing that the cysteine mutant selectively reacts with AF532M.

Formation of Covalent Bond

Dialyzed samples containing dye bound to protein were also analyzed using matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry to confirm the presence of a covalent bond between the dye and the cysteine mutant. A chromatogram of pure D381C scaffolds should show a peak at 28,104.7 g/mol, which is the theoretical molecular weight of a single subunit of the D381C scaffold. If a F5M molecule is covalently bound to the thiol of one subunit, a new peak would be visible on the chromatogram at a theoretical molecular weight of 28,532.07 g/mol; this number was determined by adding the molecular weight of one molecule of F5M to the molecular weight of one D381C subunit. The actual peak was observed at 28,532.0 g/mol, within 0.0002% of the theoretical value, which confirmed that F5M was indeed covalently bound to the cysteine mutant. The same analysis was performed for samples of D381C reacted with AF532M, and the resulting chromatograms contain a peak at 28,873.0 g/mol, which differs from the theoretical value by 0.07%. Mass spectrometry results confirm that the maleimides are able to covalently bind and secure themselves to the cysteine mutant.

Reacted Scaffolds Maintain Caged Structure

If the scaffolds' caged structure were to be compromised, *e.g.* unfolded or denatured, after conjugating with the dye, the mutant would not be able to protect guest molecules from the external environment. This would render the cysteine mutant an unsuitable hydrophobic drug delivery vehicle. Therefore, transmission electron microscopy (TEM) was used to image the protein scaffolds visually to determine whether the scaffolds remain intact after coupling. TEM micrographs of D381C scaffolds that contain dye are shown in Figure 6 and confirm the diameter to be 25 nm, the width of a correctly-assembled scaffold. Images also show the overall shape and architecture to be preserved after conjugation.

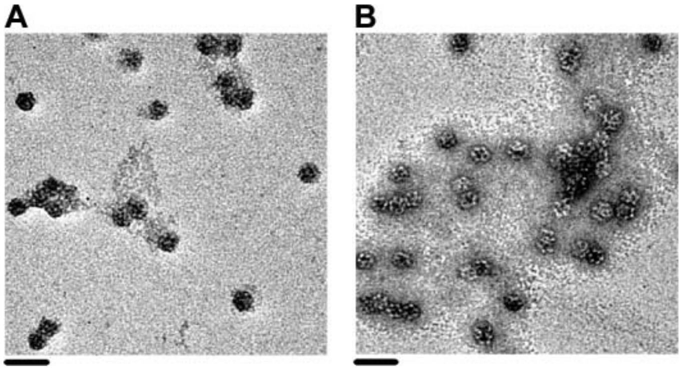

Figure 6

TEM micrographs of D381C scaffolds reacted with (A) F5M and (B) AF532M. Scale bar is 50 nm.

Discussion

Dye Molecule Size Comparison

At first glance, the molecular size of AF532M appears to be twice that of F5M. Ball-and-stick models of the two dyes were generated using MarvinSpace and are shown in Figures 7 and 8. MarvinSpace is software that can be used to visualize molecules in three dimensions and to estimate distances between atoms within a molecule. Based on the distances shown in Figure 7, a single molecule of F5M bound to a thiol group would extend approximately 1 nm from the scaffold's internal surface. In contrast, Figure 8

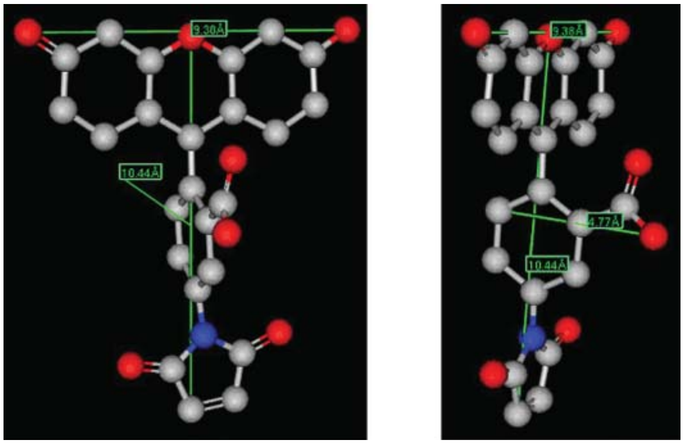

Figure 7

Estimated dimensions of fluorescein-5-maleimide. (A) Front view of the molecule with length and width measurements. (B) Side view of the molecule includes depth measurement (MarvinSpace, 2008).

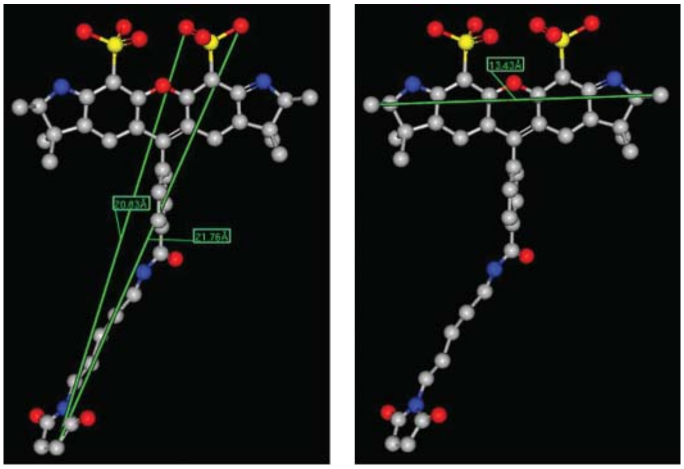

Figure 8

Estimated dimensions of Alexa Fluor 532 C_5-maleimide. (A) Length measurements. (B) Width measurement (MarvinSpace, 2008).

shows that a single molecule of AF532M would protrude approximately 2 nm.

AF532M, the bulkier dye, is expected to occupy more space than F5M in a scaffold and produce a low coupling ratio of dye to protein. However, nearly the same extent of coupling is achieved in experiments using F5M and in experiments using AF532M, despite the latter being twice the molecular weight of the former. A closer look at the molecular structure of AF532M may explain why the scaffold is able to hold as many AF532M molecules as it can F5M molecules.

A large portion of the length of AF532M is its carbon-5 chain, which separates the maleimide group from the bulky portion of the molecule. If the carbon-5 chain of AF532M is flexible, the molecule could potentially curl up and significantly reduce in size. Because the cysteine mutant achieves nearly the same extent of coupling with either dye, the carbon-5 chain of AF532M is likely to be highly flexible and capable of collapsing its bulky portion downward so that it is of similar length to F5M. In addition to a comparison of dye molecule size, the space between neighboring cysteine residues in the hollow cavity can provide further interpretation of the coupling results.

Distance between Cysteine Residues

The icosahedral E2 scaffold comprises 60 subunits arranged with 532 symmetry to generate a pentagonal dodecahedron. Each vertex is composed of three protein subunits and each face of five subunits (Allen and Perham, 1997). A vertex is referred to as a trimer, and a face is referred to as a pentamer. A single scaffold contains 20 trimers, or 12 pentamers. The five subunits of a pentamer are arranged in the shape of a ring. As depicted in Figure 9, one pentamer forms one of the cage's twelve 5-nm wide openings through which dye molecules can diffuse. Neighboring thiols of a pentamer are 3.3 nm apart, which provides enough space for all thiols of the pentamer to be occupied by dye. Dye molecules should extend 1 nm, at most 2 nm, from the surface.

The shortest distance between cysteine residues in the mutant is the distance between two thiols in a trimer, 2.2 nm (Figure 9). Upon reacting with thiols, the dye molecules extend 1 to 2 nm from the internal surface, shielding the third, unoccupied thiol of each trimer from unbound dye. The numerical value of the coupling ratio has significance; because the coupling ratio of dye-to-protein is approximately two thirds, we expect that statistically, two of three cysteine residues of a trimer are occupied by dye.

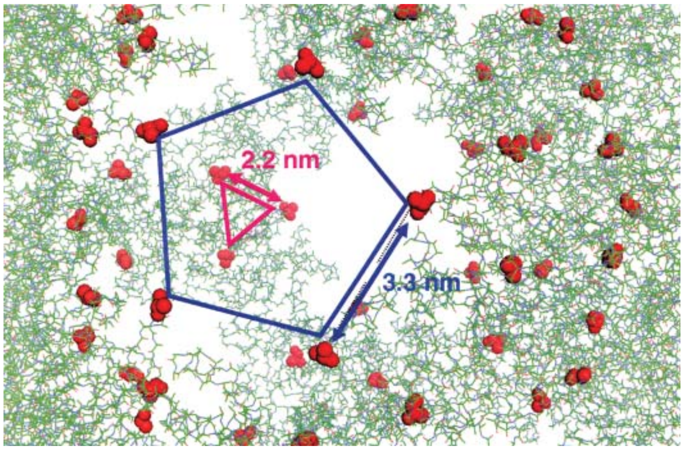

Figure 9

A view of one of twelve openings of the D381C scaffold. Cysteine residues are labeled in red. Cysteine residues of a pentamer are connected with blue lines. A trimer is visible further behind the pentamer and its three cysteine residues are connected with pink lines. The structure is displayed and distances are estimated using PyMOL. The crystallographic file is obtained from the Protein Data Bank (accession code 1b5s).

Implications

The results of the conjugation experiments demonstrate that making mutations to the internal cavity enables the capsule to house foreign molecules. With such ability, the nanocapsule can function as a molecular carrier in nanotechnology applications. The encapsulation of small, drug-sized molecules in particular suggests that the nanocapsule can potentially serve as a drug carrier. In addition to the ability to hold small drug molecules, the results also demonstrate the scaffold's capacity to hold guest molecules of higher molecular weight.

Acknowledgements

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