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Optimizing the design of protein nanoparticles as carriers for vaccine applications

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Abstract

Successful vaccine development remains a huge challenge for infectious diseases such as malaria, HIV and influenza. As a novel way to present antigenic epitopes to the immune system, we have developed icosahedral self-assembling protein nanoparticles (SAPNs) to serve as a prototypical vaccine platform for infectious diseases. Here we examine some biophysical factors that affect the self-assembly of these nanoparticles, which have as basic building blocks coiled-coil oligomerization domains joined by a short linker region. Relying on *in silico* computer modeling predictions, we selected five different linker regions from the RCSB protein database that connect oligomerization domains, and then further studied the self-assembly and stability of *in vitro* produced nanoparticles through biophysical characterization of formed particles. One design in particular, T2i88, revealed excellent self-assembly and homogeneity thus paving the way toward a more optimized nanoparticle for vaccine applications.

From the Clinical Editor: Despite the widespread use of vaccines worldwide, successful development of vaccines against some diseases remains a challenge still. In this article, the authors investigated the physic-chemical and biological properties of icosahedral self-assembling protein nanoparticles (SAPNs), which mimic viral particles, in order to utilize this technology as potential platform for future design of vaccines.

Key words: Protein nanoparticle; Malaria; Vaccine carrier; Protein design; Self-assembly

Inspired by the numerous applications of virus capsids, virus-like particles[1,2] and capsid-like protein cages,[3-6] the self-assembling protein nanoparticles (SAPNs) developed in our laboratory aim to mimic the architecture of viruses and their icosahedral symmetry. Virus capsids are often composed of just one single polypeptide chain that self-assembles into a proteinaceous cover thus protecting the genomic material of the virion. In light of this information, our design has a single polypeptide chain as the building block that self-assembles into a nanoparticle. Icosahedrons have 5-fold, 3-fold and 2-fold symmetry axes. To mimic icosahedral viruses, two of these axes were included in our design by creating a fusion protein of a pentameric coiled coil that is covalently linked to a trimeric coiled coil. Modeling showed that the superposition of these two oligomerization domains onto the corresponding symmetry axes of the icosahedron and application of the symmetry elements gives rise to a nanoparticle with icosahedral symmetry. Under the right conditions, identical copies of the protein chain self-assemble to produce the nanoparticle.

In a nanoparticle with T = 1 icosahedral symmetry, 60 protein chains form the 20 triangular faces and every chain occupies an identical environment. The requirement of a minimal number of 60 asymmetric units is related to the number of symmetry-related asymmetric units in an icosahedron. With a relatively small deviation from the exact symmetry of a T = 1

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Competing interests: PB has an interest in the company Alpha-O Peptides that has patents or patents pending on the technology.

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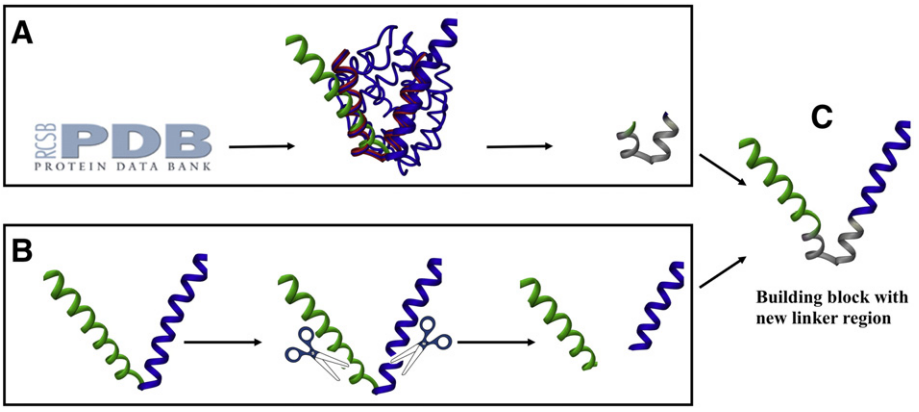

Figure 1. **(A)** PDB search for protein structures with inter-helical angles similar to the mode of the nanoparticle followed by superposition of the helices of the different PDB structures (blue and red) onto the pentamer (green) and trimer (blue) helices of the monomer of the peptide nanoparticle. The residues with angles similar to the angle between the helices of the pentamer and the trimer in the peptide nanoparticle were selected (gray region). **(B)** Schematics of the molecular biology strategy for inserting the new linker region: the original construct with pentameric (green) and trimeric (blue) helices is double digested with restriction enzymes ApaI and XhoI. **(C)** Linker oligonucleotides selected from **(A)** are ligated into double digested vector **(B)** using T4 DNA ligase generating new plasmid which codes for the genetically modified single polypeptide chain.

icosahedron, more protein chains can be incorporated in a viral capsid. Certain multiples (1, 3, 4, etc.) of 60 subunits are allowed, leading to capsids with 60, 180, 240, … protein chains. These multiples are referred to as triangulation numbers T = 1, T = 3, T = 4, etc. Thus, in the case of the nanoparticles, it is theoretically possible to accommodate more than one protein subunit in an asymmetric unit to form nanoparticles with T = 3 or T = 4 icosahedral symmetry. However, to achieve this some of the pentameric coiled coils of the fusion protein would have to switch to form a hexamer, which is rather unlikely. According to the viral tiling theory, which includes surface representations in terms of more than one type of building block per particle,[7] it is more likely that such larger assemblies are non-quasi-equivalent pentamer-only capsids, like the capsids of polyoma or papilloma viruses. For this reason, we refer to them as T = 3-like icosahedra in the context of this manuscript.

Initially, we used solid-phase peptide synthesis to produce a peptide chain composed of two oligomerization domains with different oligomerization states joined by a short linker segment.[8] Biophysical characterization of the synthetic peptide nanoparticles showed that they had a diameter of about 16 nm and that there were 60 peptide chains per nanoparticle, in support of a T = 1 icosahedral model. We also showed that when SAPNs were functionalized with a fragment of the surface protein of severe acute respiratory syndrome (SARS) coronavirus, they were able to elicit conformation-specific antibodies that inhibited infection of cells by SARS viruses *in vitro*.[9] Likewise, we have made nanoparticles for use as repetitive antigen displays for vaccines against malaria,[10-13] HIV,[14] toxoplasmosis[15] and influenza.[16] They can also be used for the design of other tools for nano-biotechnological applications.[17-19]

Given the potential of SAPNs as repetitive antigen display systems for the development of vaccines, we next aimed to rationally modify their building blocks in order to affect epitope density. Our goal was to find out what would happen if we modified the short linker region connecting the pentameric and the trimeric oligomerization domains of the fusion protein (Figure 1). This part of the fusion protein is of great importance as the packing of chains in the nanoparticle is governed by the flexibility of the linker region (Figure 1). By decreasing the angular flexibility between the pentamer and the trimer, nanoparticles with 60 or more chains could be obtained. Researchers have long been investigating the effect of linker length and composition when creating a fusion protein. In 2002, George and Heringa developed a linker database intended for the rational design of linkers for domain fusion,[20] and we have previously noted that one of the factors influencing protein assembly into icosahedral nanoparticles is the length of the linker region that connects the two oligomerization domains.[8] Not only the length of the linker but also its composition is crucial. Indeed, Linhult *et al.* investigated three different linker regions in order to obtain a stable divalent proteinaceous human serum albumin binding ligand under alkaline conditions.[21] They concluded that the length and composition of the linker region was important when connecting two functional domains. In the context of protein design and dynamics, Wriggers *et al.* highlighted the distinction between flexible, glycine-rich linkers and the more rigid, often helical kind.[22] Missirlis *et al.* studied a system of peptide amphiphiles where a bioactive peptide was connected to a hydrophobic segment by a linker.[23] By incorporating different linkers, they showed that linker chemistry influenced peptide folding, which in turn impacted the peptide's biofunction. Arai *et al.*[24] demonstrated the importance of linker rigidity versus flexibility by separating two fluorescent protein domains (EGFP and EBFP) by various types of linkers. Their research showed that FRET was more efficient in flexible, glycine-rich linkers than rigid, helical ones.

The *de novo* design of a protein sequence that will result in a folded protein with the required oligomerization domains connected by the perfect angle for icosahedral symmetry is difficult.[25,26] Therefore, in the course of the present work we used advanced database searching algorithms and molecular visualization tools to obtain promising structures from the Protein Database (PDB). The SAPN T81c-8-pf was selected for the subsequent tests. The construct T81c-8-pf is a prototype malaria vaccine described in detail elsewhere.[11] It contains a