# Computational Design of Protein Nanoparticles Displaying Neuraminidase for Novel Influenza Vaccines

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Abstract

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Influenza virus is responsible for thousands of hospitalizations each year. Glycoproteins on the surface of the influenza virion, haemagglutinin (HA) and neuraminidase (NA), are crucial for viral entry and egress, respectively. Although neutralizing antibodies against HA inhibits infection, HA is a difficult target to effectively immunize against due to its propensity to accumulate escape mutations that evade the pre-existing immunity. NA is gaining attraction as a target for immunization due to its slower rate of mutation, as well as its ability to elicit broad, cross-reactive, and protective antibodies reducing influenza's pathogenicity. Neutralizing antibody responses against NA are typically focused on the catalytic pocket site of NA; however, much of its antigenic landscape is unknown as researchers are still finding new immunologically relevant epitopes. Here, I set out to computationally design nanoparticles capable of displaying stabilized NA to make a vaccine capable of eliciting robust NA-specific neutralizing antibody titers. Successful deployment of this novel platform in mammalian cells may have a substantial

impact in progressing candidate NA immunogens and present a promising avenue towards a universal influenza vaccine.

## Chapter 1: Background

Influenza is responsible for a significant amount of deaths and hospitalizations world wide, claiming nearly 500,000 lives every year (*1*). Influenza's natural reservoir is aquatic waterfowl, but it can spill over to other birds, livestock, and people making its potential global impact multifaceted. Not to be understated, the impact on wildlife can be quite severe in some instances – the US has approved to vaccinate the endangered California-condor with the recent outbreak of the avian H5N1, marking the first highly pathogenic avian influenza (HPAI) vaccination effort of its kind (*2*). In humans, influenza causes increased morbidity and mortality in young (<5 years) and elderly (>65 years) populations at disproportionately high rates, with a mortality rate jumping nearly 20% once individuals reach the age of 65. This is especially evident in years of vaccine mismatch (*3*). Although infection from influenza has a relatively high survival rate in most healthy populations, it mediates the opportunity for secondary infection like bacterial pneumonia and sepsis, a phenomenon that was seen during the pandemics of 1919 and 2009 with disproportionately increased levels of hospitalizations and mortality in certain age groups, but also certain racial minorities and lower socioeconomic status (*4*), (*5*)).

Influenza is a negative sense RNA virus with a genome consisting of eight segments: the three viral RNA-dependent RNA polymerases (PB2, PB1, PA), HA, the viral nucleoprotein (NP), NA, matrix/membrane protein (M1/2), and the nonstructural protein (NS1). HA facilitates viral entry by binding to terminal sialic acids of glycoproteins or lipids on cell surface receptors. Each

monomer of the HA trimer consists of HA1 (the receptor binding site region) and HA2 (stalk region) polypeptide. During the process of viral entry, fusion peptides on the distal tip of HA binds to a-2,3 or a-2,6 sialic acids on the cell surface, or both in the case of certain intermediate hosts like swine and recently cows (*6*). Once the virion is endocytosed, the low-pH environment of the endosome induces a conformational change in HA, allowing the virion to fuse with the endosomal membrane and release viral genes into the cell (*7*), (*8*). NA is a homotetramer that serves to remove sialic acid bound to neighboring HA on the cell surface and mucin decoy receptors on the cytosolic membrane side, preventing aggregation and permitting nascent budding virion to infect new host cells (*9*). The mechanism by which it does this is through hydrolysis of the glycosidic bond between the terminal a-ketosidic link between N-acetyl neuraminic acid (Neu5Ac) and a neighboring saccharide (*10*).

As influenza traverses its various animal reservoirs, HA and NA subtypes accumulate mutations, a phenomenon known as antigenic drift. This drift changes the immunogenicity of the virus, allowing the virus to reinfect an individual despite their previous infection or vaccination history. Antigenic drift is the main driver behind vaccine mismatch and decreasing seasonal vaccine efficacy (*3*). While mutations naturally accumulate over the course of the flu life cycle, for a virus to be officially recognized as having undergone antigenic drift, there needs to be a difference in the antigenic profiles between current available vaccines and circulating strains. The World Health Organization then makes recommendations for what vaccines should be used for the next season (*11*). Mutations in influenza occur frequently and are often not dramatic, but as time progresses fitness “neutral” mutations accumulate and affinity of antibodies elicited by existing vaccines is diminished. Previous antibodies once elicited by influenza infection or

vaccination can no longer bind to their target epitope as potently, allowing viral replication and infection to reoccur.

Commercial vaccines to date (QIV, TIV, Flublok, etc.) target and are federally regulated for proper HA antigenicity due to its crucial role in viral entry and pathogenesis. Successfully matched HA vaccines are powerful tools to diminish the severity of infection and rates of transmission, but due to antigenic drift, HA's immunodominance, current vaccine manufacturing practices, the protection from these vaccines are suboptimal and often require seasonal updates (*12*). Current commercially licensed influenza vaccines poorly elicit protective NA antibodies due to their inability to consistently display key antigenic NA epitopes (*13*). As such, the bulk of the B cell vaccine response disproportionately targets HA. However, during natural influenza infection, it has been shown that induction of NA-reactive B cells occurs and the NA-reactive antibodies that arise are broadly reactive across multiple strains of influenza (*14*). This broad reactivity is due to the more conserved nature of NA sequences across strains, another compelling reason in favor of developing vaccines that target an NA-directed immune response is (cite) NA targeted antibodies typically inhibit the enzymatic function of NA by binding to the catalytic pocket, preventing nascent virion release in an infected host cell (*15*). There have been studies that show H3N2 virus passaged in Madin-Darby canine kidney (MDCK) cells can gain receptor binding capabilities, similarly to the hemagglutinin-neuraminidase (HN) surface protein of viruses such as parainfluenza and measles, and induce hemagglutination, although this strain has yet to been isolated from infection in humans (*16*), (*17*), (*18*). Reporter assays measuring antibody-dependent cell-mediated cytotoxicity (ADCC) have

been activated by NA specific antibodies, and this protection has been found in humans and mice (*19*),(*20*). It has been proposed that antibodies capable of NA inhibition (NAI) weakly induce this protection by binding to the catalytic active site of NA with the effect being weak unless synergistically with antibodies targeting the HA stalk, however the mechanism needs to be studied further (*19*). Recently, 16 novel T-cell epitopes, nine for CD8+ MHC class-I alleles and eight for CD4+ MHC class-II alleles for the NA of the H1N1, have been discovered (*21*) Incorporation of a single shared epitope capable of generating responses for both alleles might elicit robust T-cell help if incorporated into a flu vaccine candidate (*22*). Another study found that NA antibodies elicited from H3N2 infection in humans were able to protect mice from H3N2 lethal dose infection as well as a variety of strains including H6N3, H1N7, H5N1, and others (*23*). This speaks to NA's ability to elicit broadly protective antibodies across multiple strains, and motivate NA-directed immunity as an extremely valuable target with immense potential, with great potential to protect and quicken global immunization against influenza, alongside current available HA-oriented vaccines.

Due to the fact that the NA in licensed vaccines is not held to the same standard of quality or quantity to its counterpart antigen, it is difficult to accurately assess its elicited antibody response (*24*), (*25*). Studies demonstrating the use of soluble recombinant NA (rNA) as a vaccine in mice elicited cross-reactive NA inhibiting (NAI) antibodies, which provided complete protection when challenged with lethal dose of the homologous strain and reduced mortality when challenged with lethal dose of heterologous strains (*26*), (*25*). It's worth noting in that study that mice immunized with H5N1 rNA developed a higher quantity of IgG antibodies against the pH1N1 strain, homologous strains possibly displaying the power of leveraging the conserved sequence of NA despite other changes to the virus strain. Researchers have found that

the immune response in patients naturally infected with H1N1 and H3N2 produced plasmablast populations that reacted to rNA by ELISA antigen at comparable or higher rates to HA (*24*). By live attenuated vaccine, the NA reactive plasmablast frequency was much lower proportionately than to HA or other influenza antigens, revealing significant differences between the immunogenicity of NA presented in whole virus vaccines and NA in authentic viral infection. Interestingly, the immune response elicited from N2 of H3N2 infected individuals seemed to elicit a larger portion of anti-NA antibodies when compared to H3. Recently published work has shown that icosahedral nanoparticles displaying NA (NA-Mi3) on a protein nanoparticle platform does in fact elicit enhanced cross-reactive antibody responses when compared to soluble antigen, despite the symmetry mismatch between the C4 NA and the trimeric protein scaffold used (*27*). These studies inform and encourage the need to develop and include an improved protein nanoparticle optimized for NA, and its potential to supplement current commercially licensed vaccines and improve protection via broadly neutralizing antibodies.

Previous work in the King lab has been done to stabilize neuraminidase using computational RoseTTA metrics as well as homology-directed protein design. Guided by the observation that different NA strains display different conformational preferences, the study showed that making key mutations at various regions near the core and homotetrameric interface skewed NA's conformational dynamics to an open or closed state (*28*). The use of protein nanoparticles as a vaccine platform has developed recently due to their ability to offer control over key aspects like size, shape, and geometry of the immunogen (*29*). The King lab has shown that fixing RSV antigen on a nanoparticle with icosahedral symmetry (I53-dn5) gives 3-fold higher antigen-specific antibody titers than just the antigen alone (*30*). This is due to the fact that

presenting the immune system with structures containing repetitive sequences and geometries helps induce strong B cell activation through the cross linking of B cell receptors (*31*). Also demonstrated in this study is the ability to potentially stabilize an antigen by its fusion to the nanoparticle. Previously, I used a reinforcement learning-based (RL) method to generate nanoparticles successfully in *E. coli*, however the protein scaffold was not sufficient to assemble once fused to the antigen, despite successful NA folding validated by nsEM. This was most likely due to the flexibility and molecular weight disparity between the fused antigen and nanoparticle scaffold. Protein scaffolds generated via RL could not overcome this problem due to lack of diversity in generated outputs. Lastly, the lab has previously used I53-dn5 to display varying strains of HA as a vaccine. This was achieved by fusing different strains to the trimeric component dn5) to create a mosaic array of HA or strains on one particle (*29*). The study found this method to be able to elicit not only equivalent antibodies against neutralizing HA head, but also elicited antibodies against the more conserved stem providing broader protection. In summary, nanoparticles offer a large number of benefits well suited for their use as a vaccine platform displaying NA.

The overarching objective of this project is to investigate the extent to which protein nanoparticles scaffolding NA can tune the antibody response elicited against NA. I hypothesize that administering a combination of vaccination regimes using nanoparticles displaying homosubtypic, heterosubtypic, or a combination of the two in a cocktail will increase cross-reactive antibody titers against conserved epitopes of NA, and subsequently increase the breadth of protection against influenza infection. My thesis work will investigate the capacity of NA protein nanoparticles to elicit higher antibody titers that can potently neutralize NA or cross react, which will potentially improve breadth as NA is under less immune pressure and

undergoes antigenic drift at a discordantly slower rate. Overall, the data collected from this project will enable our understanding of NA-directed immunity, the relationship between immunodominance and vaccine immune pressure, and synergistic functions between antibodies against HA and NA.

## Chapter 1. Computational Design of One-Component Nanoparticle Vaccines Displaying Neuraminidase

I hypothesize that multivalent display of NA on a protein octahedral nanoparticle will enable novel investigation of NA as an immunogen, specifically its ability to elicit higher anti-NA, cross reactive, inhibiting, and more potent antibodies overall in comparison to current licensed vaccines. Displaying NA on a self-assembling protein nanoparticle leverages the efficient recognition of antigen on a repetitive array by the immune system by better activating B cell receptors (BCRs) through antigen-driven cross-linking, lymph node trafficking, and localization. The protein nanoparticle platform enables exchange of antigen components, generation of multivalent display, and increases our ability to standardized manufacturing as *de novo* proteins can be designed to have a higher thermal stability as already exhibited in the design of stabilized NA proteins (sNAps) in previous work. I have:

### *I. Used computational methods to design a one component octahedral protein nanoparticle capable of displaying NA.*

I will utilize sNAps generated from previous work done in the King lab to display on *de novo* monomers generated using RFdiffusion. To generate these monomers, I will align the interface of dimeric proteins on the 2-fold axis of symmetry and the tetrameric bundle of the tetramerization domain of the sNAps to the 4-fold vertices of the octahedral architecture. Using

symmetric RFdiffusion, I will generate a protein backbone to connect the N-termini of the sNAp to the monomer at the C2 interface (**Figure 1**).

Initial set of protein backbones generated by RFdiffusion will be selected by eye (filtering out clashes, checking if secondary structure of a given trajectory are well packed, limited flexibility, etc.), and then assigned residues using ProteinMPNN. AlphaFold2 and RoseTTAFold metrics such as predicted local-difference test (pLDDT) used to calculate the models confidence in the backbone, and the global superposition metric template modeling score (TM-score) will then be used to predict the confidence in the generated structure to order and express genes of designs that pass a sufficient threshold. Typical thresholds that have been shown to correlate with successfully expressed designs are pLDDT scores of > 80, and TM-scores >0.5.

Once designs have been successfully selected and filtered, I will express the designs in *E. coli*. (BL21 strain) using Golden Gate assembly to assemble plasmids from the genes of ordered designs. Genes will be ordered from Genscript. Once expressed, 6xHis-tagged nanoparticles will be Ni-NTA and SEC purified. Purified assemblies will be filtered for successful assembly by native agarose gel to be filtered by molecular weight. To further characterize the diameter of nanoparticles, dynamic light scattering (DLS) will be used to filter by hydrodynamic radius and assess thermostability of antigen. Lastly, negative stain electron microscopy (nsEM) will be used to confirm assembly. Nanoparticles with fused antigen will be expressed in HEK293F cells and nanoparticles will be isolated and validated as previously described.

Successful designs expressed from HEK293F cells will be further characterized for structural presentation of the antigen. To test the presence of the correct epitopes displayed, ELISA and biolayer interferometry (BLI) assays will be performed on the nanoparticles. BLI and ELISA will be done to measure binding of N1-CA09-sNAp-155, A/Michigan/45/15 N1,

B-Victoria B/Colorado/06/2017, and H3N2 A/Wisconsin/67/2005 to monoclonal antibodies 1G01 and CD6. To measure activity of the NA fused to nanoparticles, NA-Fluor Influenza Neuraminidase Assay Kit (ThermoFischer) will be used according to the manufacturer's protocol.

Nanoparticles structurally and functionally validated will be immunized in mice to assess immunogenicity in comparison to commercial (2017-2018) quadrivalent influenza vaccine. Protein dosages of each immunogen will match

***Pitfalls and alternative approaches:***

It is possible that there might be a critical "diffused amino acid limit", in which the search in sequence/structure space given the surrounding structural context and nanoparticle diameter reveals a limitation to symmetrical RFdiffusions ability to find backbone solutions. This problem is highly unlikely given the success rate of experimentally validated cage design in multiple geometries and diameters as shown in the RFdiffusion paper, but is possible given the novelty of these methods and the fact that they are currently still in development. In this scenario, one can imagine constraining the input even further, diffusing to connect smaller stretches of sequence so that the model is tasked with a less complicated trajectory and to minimize the potential of propagation of error to assembly. One can also imagine using a dimeric interface with a different termini orientation relative to the interface on the 2 fold axis so the task of connecting the contigs follows a more one dimensional track and forgoing unnecessary folds or twists around neighboring residues.

***Current progress:***

To build the nanoparticle, I have used multiple computational methods to generate protein backbones. First, I utilized a reinforcement learning (RL) method capable of designing a

symmetric assembly by using a “top-down” approach (*32*). The desired geometry for the design problem, in this case octahedral symmetry, can be specified and guide the design of individual protein subunits. The RL method takes the desired geometry and builds a nanoparticle using smaller fragments or poses of ideal secondary structure, with no amino acid information assigned. To scaffold the NA head, an hVASP tetramerization domain was pre-positioned at the vertices of the assembly (*33*). The inclusion of the hVASP domain gave the protocol an advantage over the traditional use of the method by providing an interface to coordinate assembly of tetramers that properly display NA at the vertices of the nanoparticle.

Once nanoparticles were designed, I filtered the components using Rosetta metrics, looking for proteins that gave scores that suggest good interface shape complementarity score ≥better than 0.6 (sc), a delta-delta G (ddG) ≤ better than -20, and surface accessible surface area score ≥better than 500 (SASA). ProteinMPNN was then used to -design the sequence encoding the backbone of these proteins. PyRosetta was used to relax the backbone into a more idealized position, followed by another round of  proteinMPNN sequence design with the slightly altered structure. This iterative process was arbitrarily repeated three times to determine if backbone adjustments would improve sequence structure or vice versa.

I then ordered and screened 192 octahedral designs by native agarose gel, using a previously designed nanoparticle as a control (KWOCA 4, 675 kDa). Protein shown is O6, marked on the gel by the red arrow **(Figure 2)**. Representative characterization for O6 is shown as it was a leading candidate nanoparticle. Native gel showed 46 hits that migrated near the control. HPLC showed 16 samples eluted at expected volumes near the control (not shown). HPLC hits were taken to SEC to be purified and further characterized.

A total of 6 octahedral nanoparticles were found to elute at the expected volume. Negative stain EM characterization of O6 structure showed nanoparticle assembly of correct architecture, among five other candidates of which O6 was the most promising and therefore characterized and focused on here after. I then expressed nanoparticles genetically fused to stabilized NA protein in mammalian HEK293F cells to test secretion and assembly. I found no cages assembled by DLS or SEC (not shown), although the fused NA was able to assemble and be captured by nsEM **(Figure 3)**. In summary, uUsing the RL method I was able to design nanoparticles with octahedral symmetry in bacteria. These nanoparticles could express and display NA in mammalian cells, however they could not assemble into their nanoparticle form when secreted.

To get cages with more of more diverse structure and a wider range of diameters that assemble and properly display NA when expressed in mammalian cells, I used RFDiffusion, a powerful protein generative protein design AI tool able to create diverse protein backbones and topologies that can scaffold motifs resulting in designable protein:protein interfaces (*34*). Adjacency matrices were also implemented to guide the generation of nanoparticle subunits by specifying what residue positions must come in contact with each other. In doing this, the program was able to better generate appropriate interface contacts. Original input for diffusion was the C2 interface of the expressed O6 cage.

The original interface was taken away and the program was tasked with determining a solution to bridge the gap with the tetramer (**Figure 4**). The number of residues given for the program to use was varied from 20 amino acids to 100 amino acids, with 100 outputs at each iteration as the hVASP helix stayed fixed (shown in red). The most viable looking solutions that

provided realistic looking interfaces (like helical bundles and beta sheets) didn't appear until 90 - 100 were added.

We have generated octahedral nanoparticle designs using symmetric RFdiffusion, iteratively increasing the range of residues as the diameter of the nanoparticles increases. On this pilot design campaign, I diffused 80-140 residues over a diameter range of 20-24 nm. Given the limited initial scope of testing, several designs have already shown enhanced residue packing within the monomer, diversity of secondary structure, and have a higher molecular weight compared to previous scaffolds generated using the RL method. I plan to expand my search to further optimize these parameters to increase structural diversity in the nanoparticles.

**Chapter 2: Future directions: Investigate the immune response to administration of protein nanoparticles displaying a cocktail of homosubtypic NA.**

I hypothesize that administering protein nanoparticles displaying NA will elicit higher titers of broadly neutralizing and cross reactive protective antibodies, with improved potency than current licensed vaccines.

The platform described in the literature uses the SpyTag:SpyCatcher system to conjugate soluble NA antigen onto the trimeric component of an icosahedral nanoparticle originally designed by researchers in the Baker lab. This suboptimal multi-step conjugation method had two issues: the nanoparticle was made at a 1:2 ratio of antigen to nanoparticle and could not saturate valency due to the symmetric mismatch, and the method would be inefficient for scaling up manufacturing due to the potential for aggregation, as observed in previous iterations of their nanoparticle (*35*). With antigens genetically fused to *de novo* protein nanoparticles designed using recently developed methods (RFdiffusion and ProteinMPNN), the nanoparticle sequence

has more potential for redesign in the instance that the scaffold elicits an immunodominant response. Lastly, *de novo* designed protein nanoparticles allows us to display antigen with full valency, which will permit more accurate measurement of the immunogenicity of the nanoparticle while taking full advantage of any dose sparing effects inherent to multivalency. Fusing multiple heterosubtypic NA with control to the nanoparticle is an advantage provided by symmetry matched nanoparticles, and would allow more rigorous investigation of the NA-directed immune response.

Nanoparticle immunogens similar to those I designed in my thesis work could be evaluated in immunogenicity and challenge studies in mice. Specifically, we would administer two doses of nanoparticle immunogens at appropriate intervals and measure antigen-specific antibody responses by ELISA and antibody functionality by MUNANA or NA-STAR. The ability of the nanoparticles to protect against influenza challenge would inform their potential utility as part of next generation influenza vaccines. Such studies would need to include appropriate benchmark immunogens such as current seasonal vaccines and HA nanoparticle vaccines previously published by the King lab in collaboration with the NIAID Vaccine Research Center.

***Pitfalls and alternative approaches***

A major challenge in influenza vaccines is the seasonal reformulation that must be done to keep up with virus evolution. Above, I described the production of protein nanoparticles and their evaluation in immunogenicity studies. However, manufacturing recombinant proteins is slow and likely unable to keep pace with the requirements for seasonal reformulation. Part of the motivation for my thesis work was designing one-component nanoparticle immunogens which can be delivered as mRNA vaccines. Exploring this alternative approach could enable the

translation of “mRNA-launched NA nanoparticle immunogens” as next generation influenza vaccines.

**Significance**

Conformationally stable designed NA has never been put on a nanoparticle before. Historically, the enzyme has been difficult to work with and use due to its dynamic structure and weak interactions between the homotetrameric interfaces, causing aggregation or lack of assembly. Combining stabilized NA with a nanoparticle platform will allow us to leverage the nanoparticle platform's ability to display antigen in such a way that optimizes BCR cross-linking and elicits more robust cross-reactive antibodies towards NA. This in combination with vaccines targeting HA may offer an immunization strategy that will enable minimization of vaccine mismatch, as well as another formidable guard against novel pandemic strains in the future.

The significance of this project lies in the fact that it will empower the further development of the epitope landscape of NA which may offer the discovery of new broadly neutralizing epitopes similar to IG01 and IE04, but may elicit increased titers of pan-influenza antibodies like FNI19 or discover new ones (*23*, *24*).

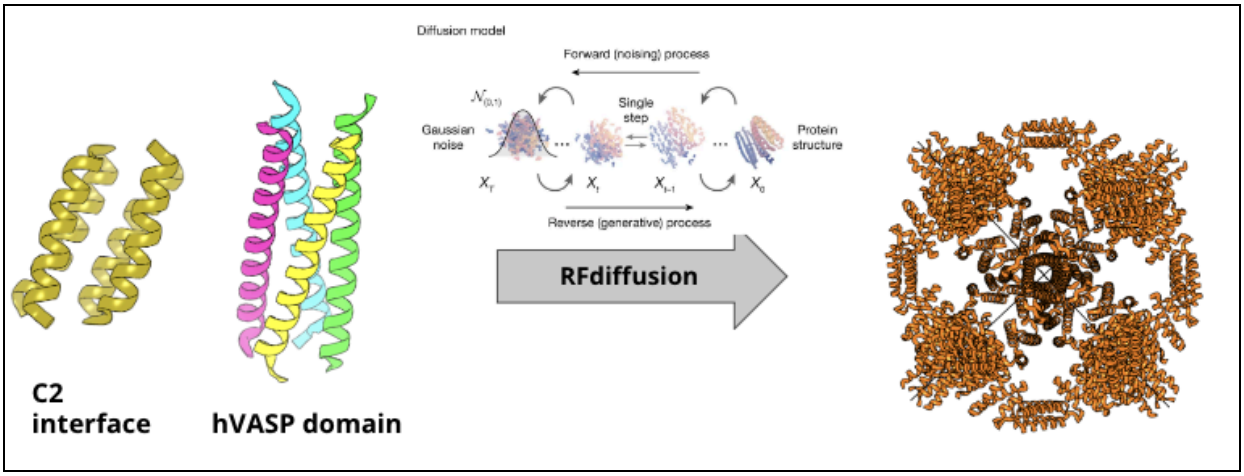


**Figure 1. Symmetric two-template RFdiffusion.** Nanoparticles generated using two motifs as the input for the RFdiffusion software to generate protein backbone connecting specified termini.

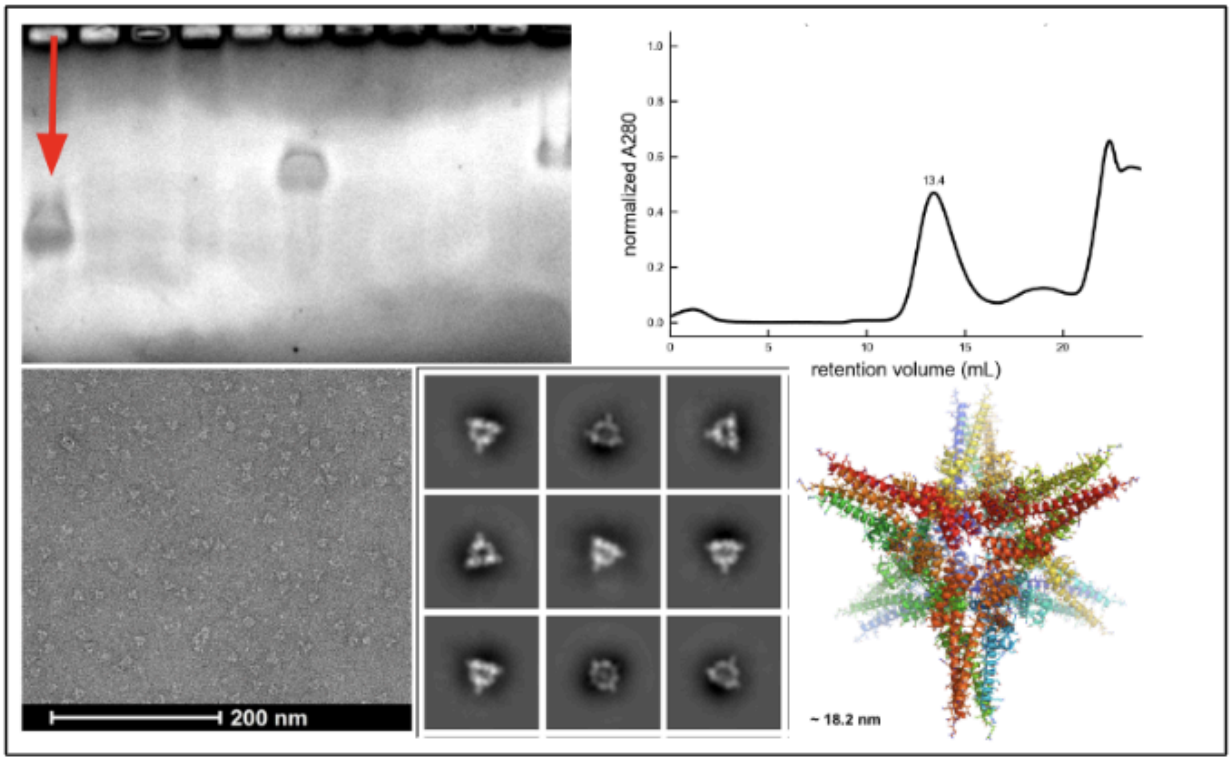


**Figure 2. Experimental validation of nanoparticles by native gel, SEC, and nsEM.**

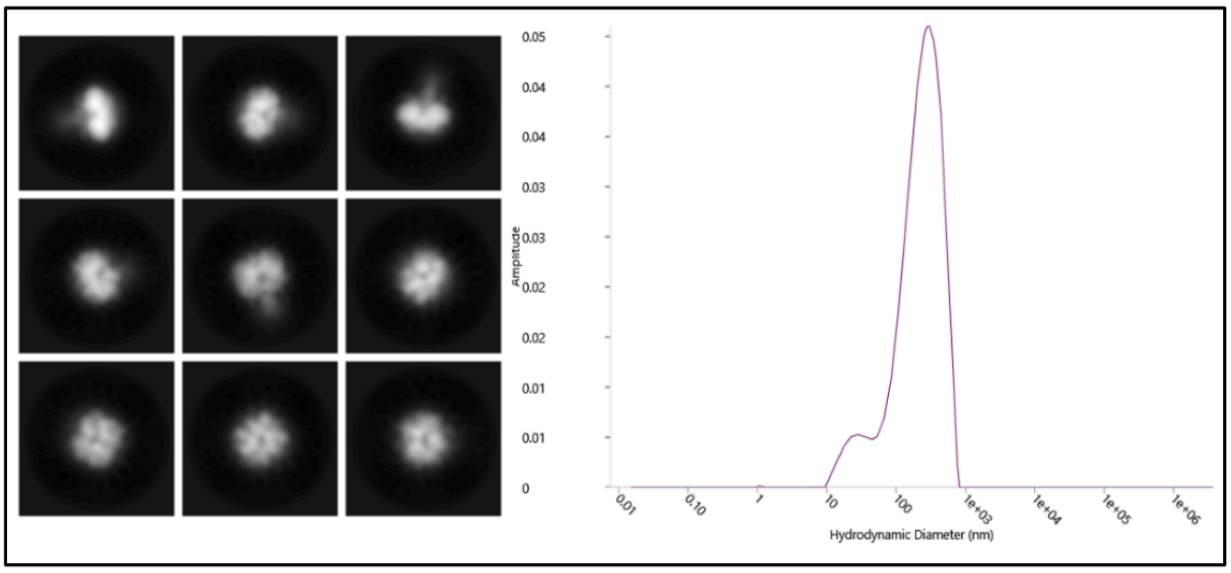


**Figure 3. Nanoparticle-fused tetrameric NA assembly characterized by nsEM and DLS in mammalian cells.** NA fused to nanoparticles were expressed in HEK293F cells. DLS shows slight peak resolving NA assembly. Cage component was not assembled.

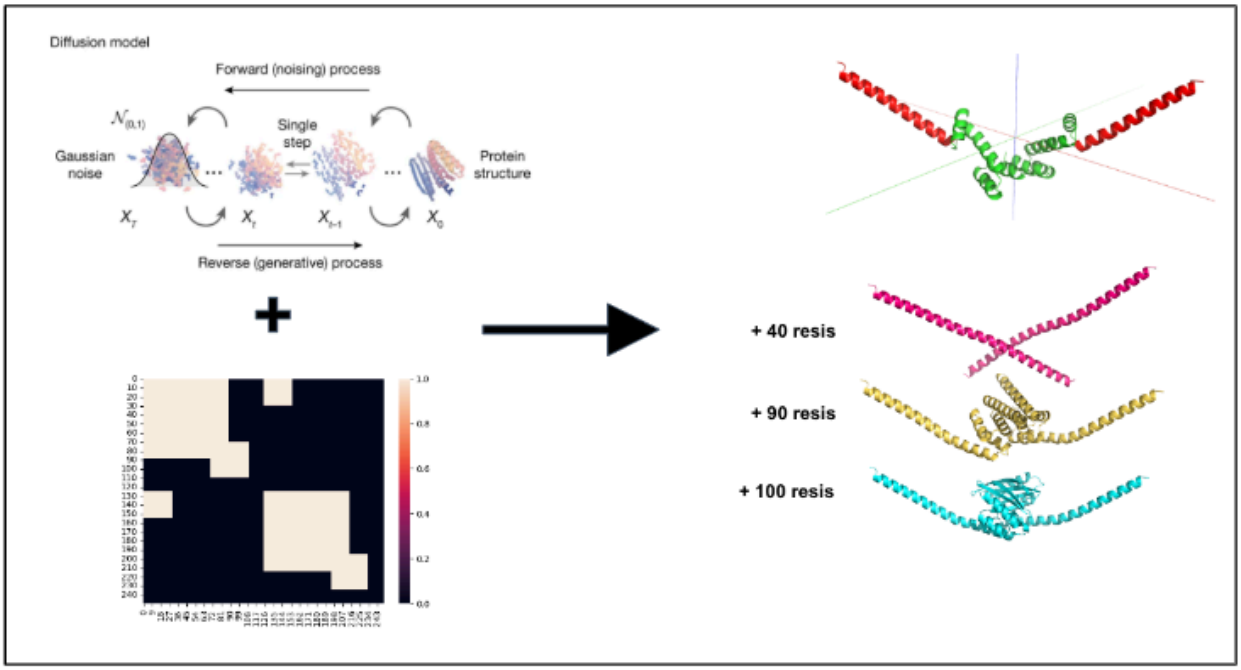


**Figure 4. Adjacency matrix guided diffusion pipeline.** Truncated dimer of previous octahedral designs were guided by adjacency matrices during diffusion to enforce interface contacts.

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