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Polyvalent complexes for vaccine development

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Abstract

Homotypic interaction is a common phenomenon of many proteins, through which they form dimers. We developed a simple approach to turn small dimeric proteins into large polyvalent complexes for increased immunogenicity and functionality. This was achieved via a fusion of two or more dimeric proteins together to induce polyvalent complex formation through intermolecular dimerizations. Two types of polyvalent complexes, linear and network, assembled spontaneously when a dimeric glutathione S-transferase (GST) was fused with one or two protruding (P) domains of norovirus (NoV). Additionally, a monomeric antigen, the peptide epitope M2e of the influenza virus (IV) or the VP8* antigen of rotavirus (RV), can be inserted to the polyvalent complexes. Mouse immunization demonstrated that the polyvalent complexes induced significantly higher antibody and CD4⁺ T cell responses to the complex components than those induced by the free epitope and antigens. Further evaluations indicated that the polyvalent complex vaccines exhibited significantly higher neutralization activity against NoV and RV and stronger protection against IV challenges in a mouse model than those of the monomeric or dimeric vaccines. The binding of NoV P proteins to their HBGA ligands was also significantly increased through the polyvalent complex formation. Therefore, our polyvalent complex system provides a new strategy for novel vaccine development and may find various applications throughout biomedicine.

1. Introduction

Bioengineering and biomaterial have become important fields of modern medicine. Development of recombinant viral subunit vaccines for control and prevention of infectious diseases is a common example. Unlike traditional vaccines, which are either live attenuated or inactivated viruses, the subunit vaccines are recombinant viral proteins made *in vitro* without involvement of infectious viruses, and therefore, are safer vaccines. Successful examples of such recombinant vaccines include the four commercially available virus-like particle (VLP) vaccines: Recombivax HB® (Merck) and Engerix-B® (GlaxoSmithKline) against hepatitis B virus and Gardasil® (Merck) and Cervarix® (GlaxoSmithKline) against human papilloma virus. Additionally, numerous other subviral vaccines, including the norovirus (NoV) VLP [1, 2] and P particle [3-5] vaccines are under intensive development. Hence, recombinant subunit vaccines represent an innovative vaccine strategy complementary to conventional vaccine approaches.

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An important factor for a recombinant viral antigen to become an effective vaccine is its immunogenicity. Most icosahedral VLPs are highly immunogenic because of their large sizes and polyvalent antigenic structures. However, many other monomeric and dimeric viral antigens possess a low immunogenicity due to their smaller sizes and low valences. Traditionally, these smaller antigens need to be presented by a large, multivalent vaccine platform to improve immunogenicity before becoming candidate vaccines [4, 6-11]. For example, the monomeric rotavirus VP8* antigen (159 residues), the outermost portion of the spike protein VP4 of rotavirus, was conjugated to the surface loop of the NoV P particle to increase immunogenicity [4]. Although a number of small viral or bacterial antigens have been successfully presented by different multivalent platforms [11-13], limitations clearly exist due to the structural incompatibility between some antigens and the platforms, preventing wide applications of a given vaccine platform. In the current report, we introduce a simple but effective approach to turn the small dimeric proteins into large polyvalent complexes for enhanced immunogenicity and functionality. This was achieved through fusion of two or more dimeric proteins covalently into one molecule, either homotypically or heterotypically, through recombinant DNA technology. When the fusion proteins were produced in *E. coli*, large polyvalent complexes spontaneously assembled.

The principles of the proposed complex system are presented in Figure 1. Homotypic interactions between two homologous protein components of two fusion proteins, forming a homodimer, are the driving force of the polyvalent complex formation. Thus, a fusion of two dimeric proteins together will form a long, linear complex via intermolecular dimerization of the homologous proteins (Figure 1A). When three such proteins are fused, each of the components will form a dimer with a homologous protein of another fusion protein, resulting in a large network complex (Figure 1B). In both cases, the protein components can be any dimeric proteins. Therefore, polyvalent complexes with different antigens can be obtained, which can be multivalent vaccine candidates. In addition, a monomeric peptide or protein antigen can be inserted into the polyvalent complexes through either an exposed loop or the ends of a protein component (Figure 1C and 1D), for increased complexity and valence for vaccine and/or other purposes. Thus, this complex system may find applications in biomedicine.

In this study, several chimeric linear/network polyvalent complexes with different antigens were designed and constructed to prove the principle of the complex system. The complexes comprising different NoV surface antigens were further examined for their improved humoral and cellular immune responses in mice to each NoV antigen and comparing with those induced by the free antigen alone. The resulting antisera were also tested for their blocking activities on NoV-HBGA ligand interaction, a surrogate neutralization assay of human NoVs, compared with those of sera induced by the free P dimers. Finally, the feasibility of the polyvalent complexes as a vaccine platform for antigen presentation was studied by insertions of the peptide epitope M2e of the influenza virus and a VP8* antigen of rotavirus into the complexes. The resulting chimeric polyvalent vaccines were examined for increased neutralization against rotavirus and NoVs and improved protection efficacy against influenza virus challenges compared with those of the free epitopes and antigens. Our data strongly suggested an application of the polyvalent complexes for vaccine development.

2. Materials and methods

2.1. Expression constructs

All expression constructs were generated with the help of glutathione S-transferase (GST)-gene fusion system (GE Healthcare Life Sciences) using vector pGEX-4T-1. The construct for the GST-HEV P fusion protein was generated by inserting the P2 domain encoding

sequences (residue 452 to 617, GenBank AC#: DQ079627) of hepatitis E virus (HEV) [14, 15] to the vector pGEX-4T-1 between Bam HI and Not I sites. The DNA sequences were chemically synthesized by GenScript. A cysteine-containing tag (CDCRGDCFC) was added to the C-terminus of the HEV P to stabilize the fusion protein. A shortened NoV P domain, designated as NoV P⁻, is the NoV P domain with a deletion of the last four amino acids [16]. The construct GST-NoV P⁻ of NoV VA387 (GII.4) was created previously [16]. Throughout the paper all NoV P⁻s were from GI.4 VA387 unless otherwise indicated.

The construct of GST-NoV P⁻-NoV P⁻ was made by adding one more NoV P⁻ to the end of the GST-NoV P⁻ protein through a linker of 12 glycines. The PCR-amplified NoV P⁻-encoding sequences using two primer pairs (P524/P1617 and P1618/P561) with BsmBI sites (Table 1), were cloned to the end of the GST-NoV P⁻ after digestion with corresponding enzymes. NoV P⁻-NoV P⁻ was a thrombin-cleaved product of the GST-NoV P⁻-NoV P⁻. The constructs of GST-NoV P⁻ (387)-NoV P⁻ (207) and GST-NoV P⁻ (387)-NoV P⁻ (115), complexes containing NoV P⁻ of different strains, were made through similar approach using primer sets of P524-P1617 for VA387 (GII.4), P1957/P1958 for VA207 (GII.9), and P1955/P846 for VA115 (GI.3) (Table 1). The NoV P⁻ (387)-NoV P⁻ (207) and NoV P⁻ (387)-NoV P⁻ (115) proteins were obtained by thrombin digestion of their respective GST-fusion proteins. The constructs of GST-NoV P⁻-VP8* and GST-NoV P⁻-M2e, each with the VP8* or M2e at loop 2 of the P domain [4, 7], were made by cloning the PCR-amplified sequences of NoV P⁻s with M2e/VP8* at loop 2 from previously made constructs [4, 8] using primers P524 and P561 in the vector pGEX-4T-1. cDNA sequences encoding individual NoV P⁻s of VA207 and VA115 were amplified by primer sets P821/P834 and P843/P846, respectively.

2.2. Expression and purification of recombinant proteins

Recombinant proteins were expressed in *E. coli* strain BL21 (DE3) with an induction of 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at room temperature (22°C) overnight as described previously [17, 18, 19]. The GST fusion proteins were purified using resin of Glutathione Sepharose 4 Fast Flow medium (GE Healthcare Life Sciences) according to the manufacturer's instruction. GST was removed from the target proteins by thrombin (GE Healthcare Life Sciences) cleavage either on beads or in phosphate-buffered saline (PBS, pH 7.4).

2.3. Gel filtration chromatography

Gel filtration was performed through an Akta Fast Performance Liquid Chromatography (FPLC) system (model 920, GE Healthcare Life Sciences) using size exclusion columns (Superdex 200, GE Healthcare Life Sciences), as described previously [17, 18, 19]. Two Superdex 200 columns were used: HiLoad 16/60 with 120 ml bed volume and 10/300 GL with 24 ml bed volume. The columns were calibrated using gel filtration calibration kits (GE Healthcare Life Sciences) and the purified NoV P particle (~830 kDa) [18], small P particle [20] and P dimer (~69 kDa) [17] as described previously [4]. The protein identities in the peaks of interest were further analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by a western blot analysis using specific antibody described elsewhere.

2.4. SDS-PAGE and protein concentration determination

Recombinant proteins were analyzed by SDS-PAGE using freshly prepared 10% separating gels. Protein concentrations were determined on SDS-PAGE using diluted bovine serum albumin (BSA, Bio-Rad) as standards [4].

2.5. Western blot analysis

The identity of the NoV P protein was confirmed by Western blot analysis as described previously [4]. Blotted membrane was incubated with homemade guinea pig hyperimmune sera against NoV VLPs (VA387, GII.4; 1:3,000). Then the secondary antibody-horseradish peroxidase (HRP) conjugates (1:5,000; ICN Pharmaceuticals, Costa Mesa, CA) were added. The HRP was detected by enhanced chemiluminescence (ECL) Eastern blotting detection reagents (GE Healthcare, Buckinghamshire, England).

2.6. Electron microscopy (EM)

Negative-stain EM was utilized to visualize the morphology of the complexes as described previously [18, 20]. A 7- μ l drop of protein solution was applied on a parafilm sheet and an EM grid (Electron Microscopy Sciences, Inc) was set on the drop for 10 min in a humidified chamber. The grid was washed for a few seconds on a drop of water and then was stained with the 1% ammonium molybdate solution for 30s. The grid was examined in a Phillips CM10 electron microscope operating at 80 kV.

2.7. Enzyme immunoassay (EIA)

To measure immune reactivity and antibody titers of mouse antisera after immunization with different protein antigens, an EIA was used, as described elsewhere [4]. Different purified recombinant antigens were used for different antisera: 1) gel-filtration purified NoV P^{*} proteins of different strains were used to determine the P domain specific antibody titers of the mouse sera after immunization with the different P domain complexes; 2) free VP8^{*} was used to determine the VP8^{*} specific antibody titer of the mouse sera after immunization with different P complex-VP8 chimeras; and 3) synthesized free M2e peptide was used to determine the M2e specific antibody of the mouse sera induced by different P complex-M2e chimeras. Antigens (1 μ g/ml) were coated on 96-well microtiter plates (Dynex Immulon) at 4°C overnight. Diluted sera were incubated with the coated antigens. The bound antibody was detected by the secondary antibody-HRP conjugate. Antigen-specific antibody titers were defined as the end-point dilutions with a cutoff signal intensity of 0.2. Sera from animals that were immunized with PBS were used as negative controls.

2.8. CD4⁺ T cell responses

Spleens were collected from immunized mice at four weeks after the last immunization. A suspension of splenocytes was prepared by lysing red blood cells with 1 $\times$ RBC lysis buffer (eBioscience) followed by washing with FACS buffer (1 $\times$ PBS with 1% FBS and 0.01% NaN₃). For intracellular cytokine staining, splenocytes were resuspended in RPMI-1640 medium with 1:1000 Brefeldin A (eBioscience) and stimulated with VA387 (HFYQEAAQAQSDVAL), VA207 (ATARSEVALLRFVNP) or VA115 (TLTEAAQLAPPIYPP) CD4⁺ T cell epitopes (synthesized by GenScript) at 5 μ g/ml for 6 hours in a 96-well plate at 37°C. The CD4 T epitope of VA387 was reported previously [21], while those of VA207 and VA115 were predicted by Immune Epitope Database Analysis Resource (IEDB) (<http://tools.immuneepitope.org/main/>). Cells were then washed with FACS buffer and surface markers CD3 and CD4 (Biolegend) were stained for 15 minutes at 4°C. Cells were washed and fixed with fixation buffer at 4°C overnight. For cytokine staining, cells were washed with 1x permeabilization buffer and stained with fluorochrome-conjugated cytokine antibodies (anti-mouse IL-2, IFN- γ , TNF- α , Biolegend) for 30 minutes at 4°C. After being washed with 1x permeabilization buffer, then FACS buffer, cells were resuspended in FACS buffer for acquisition. All samples were analyzed on a BD Accuri™ C6 flow cytometer and data was analyzed by Accuri™ C6 software.

2.9. Histo-blood group antigen (HBGA) binding and blocking assays

The saliva-based binding assays were performed as described elsewhere [22, 23]. Briefly, boiled saliva samples with known HBGA phenotypes were coated on 96-well microtiter plates (Dynex Immulon). Protein complexes GST-NoV P⁻-NoV P⁻ and NoV P⁻-NoV P⁻ and free NoV P⁻ dimer of VA387 were incubated with the coated saliva. The bound NoV P⁻ proteins were detected using guinea pig anti-VA387 VLP antiserum (1:3300), followed by an addition of HRP-conjugated goat anti-guinea pig IgG (ICN Pharmaceuticals). In a blocking assay, the blocking effects of the mouse sera on the binding of the VLP or P particle to saliva were measured by a pre-incubation of the VLP/P particles with diluted sera for 1 h before the VLP/P particles were added to the coated saliva. The blocking rates were calculated as reduction rates by comparing the optical density (OD) with and without blocking by the mouse sera. The blocking titer 50 (BT50) was defined as the dilution of the antisera that produced a 50% reduction on the binding of NoV VLP/P particle to saliva.

2.10. Immunization of mice

Female BALB/c mice (Harlan-Sprague-Dawley, Indianapolis, IN) at 3-4 weeks of age were immunized with different polyvalent complexes: 1) GST-NoV P⁻ (387)-NoV P⁻ (207), 2) GST-NoV P⁻ (387)-NoV P⁻ (115), 3) NoV P⁻ (387)-NoV P⁻ (207), and 4) NoV P⁻ (387)-NoV P⁻ (115). Two 1:1 mixtures of the corresponding free NoV P⁻ dimers [NoV P⁻ (387) + NoV P⁻ (207) and NoV P⁻ (387) + NoV P⁻ (115)] were used as controls, respectively. Equal molar amounts (0.143 nanomole/mouse, e.g. equal to 10 g/mouse for the NoV P⁻-NoV P⁻) of each immunogen were used to ensure the same amount of protein component. Mice ($n = 8$ mice/group) were immunized three times intranasally without any adjuvant in 2-week intervals as described previously [4]. Blood was collected by retro-orbital capillary plexus puncture before each immunization and four weeks after the final immunization. Sera were processed from blood via a standard protocol. Spleens were collected from mice to isolate splenocytes for cellular immune responses.

To determine the immune response induced by the complex-presented VP8*, equal molar amount (0.838 nanomole/mouse, e.g. equal to 15 g/mouse for the free VP8*) of each immunogen (GST-NoV P⁻-VP8*, NoV P⁻-VP8*, and the free VP8*) were used for the same molecular number of VP8*component. Mice ($n = 8$ mice/group) were immunized intranasally without adjuvant three times in 2-week intervals. Mice were bled before and 2 weeks after each immunization.

2.11. Rotavirus plaque assay

This was performed to determine the neutralizing activity of mouse sera after immunization with GST-NoV P⁻-VP8*complexes on rotavirus replication as described elsewhere [4]. Briefly, MA104 cells were cultivated in 6-well plates and tissue culture-adapted rotavirus Wa (G1P[8]) at a titer of ~50 PFU/well was used as the inoculum. Trypsin-treated rotavirus was incubated with mouse sera for 1 h and then was added to the cells. The plates were overlaid with media including trypsin (Invitrogen) and 0.8% agarose. After a 4-day incubation, the plaques were stained and counted. The neutralization (%) of the sera was calculated by the reduction in plaque numbers in the wells treated with antisera relative to the number in untreated control wells.

2.12. Mouse influenza virus challenge model

The model described in a previous study[8] was used to measure the protective efficacy of GST-NoV P⁻-M2e complexes. BALB/c mice ($n = 8$ mice/group) at 6 to 8 weeks of age (Harlan-Sprague-Dawley) were immunized intranasally three times at 2-week intervals with GST-NoV P⁻-M2e, NoV P⁻-M2e, and NoV P⁻ without an adjuvant. Equal molar amounts

(0.556 nanomole/mouse, e.g. equal to 20 $\mu\text{g}/\text{mouse}$ of NoV P⁻-M2e) of each immunogen, GST-NoV P⁻-M2e and NoV P⁻-M2e, were administered to mice. The same amount of NoV P⁻ dimer was used as a negative control. Animals were bled before and 2 weeks after each immunization. Sera antibody titers specific to M2e peptide and NoV P domain were measured by EIA. Two weeks after the third immunization, mice were challenged with mouse adapted influenza virus PR8 strain (H1N1) at a dose of 2×10^6 fluorescent focus forming units (ffu) or approximately $1 \times \text{LD}_{50}$, in 40 l of PBS per mouse. Mice were monitored daily for changes in body weight and mortality.

2.13. Statistical analysis

Using software Minitab, version 15 (Minitab, Inc.), a non-parametric Mann-Whitney test was performed to determine statistically significant differences among data groups. *P*-values were set at 0.05 ($P < 0.05$) for significant difference, and 0.01 ($P < 0.01$) for highly significant difference.

2.14. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (23a) of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Cincinnati Children's Hospital Research Foundation (Animal Welfare Assurance no. A3108-01).

3. Results

3.1. Production of linear polyvalent complexes

Our study started with an observation of large complexes formed by the fusion protein of glutathione S-transferase (GST) of *Schistosoma japonicum* (~26 kDa) with a shortened P domain of NoV (NoV P⁻, 34 kDa), referred as GST-NoV P⁻ protein (60 kDa, Figure 2A to 2D). Both GST [24] and NoV P⁻ [16-17,25-27] are known to form homodimers that were confirmed by gel filtration analysis in this study (Figure 2F and 2G). The fusion protein was expressed well in *E. coli* with high yield of soluble protein (>20 mg/liter bacteria, Figure 2B and Table 2). Formation of large GST-NoV P⁻ complexes was shown by gel filtration, in which the vast majority of the fusion protein was eluted as complexes >800 kDa (Figure 2E). Electron microscopy (EM) demonstrated these complexes as long linear complexes (Figure 2H and 2I) with micrometers in length, with repeated GST and NoV P dimer subunits as clearly recognized round balls.

These linear complexes disappeared completely after a cleavage of the GST-NoV P⁻ fusion proteins with thrombin, resulting in free dimers of GST (~52kDa) and NoV P⁻ (~69 kDa) (Figure 2A, 2F and 2G). EM inspection of the GST and NoV P solutions confirmed the disappearance of the linear complexes (data not shown). To further confirm the observed principle, another fusion protein comprising the GST and a dimeric P domain of hepatitis E virus (HEV P) [14, 15, 24] was made, which also formed linear polyvalent complexes efficiently (Table 2, designated as GST-HEV P). These data demonstrated the feasibility of the formation of linear polyvalent complexes by a fusion of two dimeric proteins together (Figure 1A).

3.2. Production of network polyvalent complexes

This was achieved through fusions of three dimeric proteins together (Figure 1B). Three different fusion proteins were constructed to prove the feasibility. The first one was a fusion of GST with a tandem repeat of NoV P⁻ of VA387 (GII.4), designated as GST-NoV P⁻-NoV P⁻ fusion protein (~92 kDa, Figure 3A to 3C). The fusion protein was expressed well (>7

mg/liter bacteria, Table 2) and can be cleaved into a tandem repeat of NoV P⁻ (68 kDa, designated as NoV P⁻-NoV P⁻) by thrombin (Figure 3B and 3C). As anticipated, the GST-NoV P⁻-NoV P⁻ protein formed large complexes as shown by gel filtration (Figure 3D). EM observation confirmed the fusion proteins from the affinity purification column as network complexes (Figure 3E and 3F), while those after further gel-filtration purification revealed cleaner network complexes under EM (Figure 3G and 3H). As expected, the thrombin released NoV P⁻-NoV P⁻ protein formed linear complexes (Table 2, data not shown).

For better testing the polyvalent complexes as multivalent vaccines, two other fusion proteins, each comprising a GST and two different NoV P⁻s, were also made. They were GST-NoV P⁻ (GII.4 VA387)-NoV P⁻ (GII.9 VA207), designated as GST-NoV P⁻ (387)-NoV P⁻ (207), and GST-NoV P⁻ (GII.4 VA387)-NoV P⁻ (GI.3 VA115), designated as GST-NoV P⁻ (387)-NoV P⁻ (115). These two fusion proteins revealed the same characteristics in production, complex formation and thrombin cleavage (Table 2) as those of the GST-NoV P⁻-NoV P⁻ fusion protein described above. These data further supported the feasibility of the protein complex system.

3.3. Antibody and T cell responses of the polyvalent complexes

The polyvalent complexes increased antibody and T cell responses to individual antigens. This was shown through mouse immunization studies of four polyvalent complexes containing NoV P⁻ antigens: 1) GST-NoV P⁻ (387)-NoV P⁻ (207), 2) GST-NoV P⁻ (387)-NoV P⁻ (115), and 3) NoV P⁻ (387)-NoV P⁻ (207) and 4) NoV P⁻ (387)-NoV P⁻ (115) and their corresponding free NoV P⁻ dimers. Same molar amount of each antigen in the polyvalent complexes and free dimers were used for comparison (see Materials and Methods). Mice (N=8 mice/group) after immunization with the polyvalent complexes revealed significantly higher titers of antibody (IgG, Figure 4A and 4B) and CD4⁺ T cell cytokines (IL-2, IFN- γ , and TNF- α) (Figure 4C and 4D) specific to the individual NoV P⁻ antigens, compared with those induced by the corresponding free NoV P⁻ dimers (P < 0.05, Figure 4). Similar higher antibody responses to GST components induced by the polyvalent complexes than those induced by the free GST dimers were also seen (data not shown). In these experiments, whether GST was removed from the polyvalent immunogens or not did not significantly impact the immune responses (Figure 4A and 4B, P > 0.05). Collectively, these data support the notion that the polyvalent complexes significantly increased the immunogenicity of the antigen component compared with the free antigen alone.

3.4. The chimeric complexes as multivalent vaccines

The mouse antisera were further examined for their abilities to block the binding of NoV VLPs or P particles, the two NoV surrogates, to their HBGA receptors [28-31]. The results showed that the antisera after immunization with the chimeric, polyvalent complexes of GST-NoV P⁻ (387)-NoV P⁻ (207) or NoV P⁻ (387)-NoV P⁻ (207) strongly blocked the attachment of two NoV particles, each representing GII.4 (VA387) and GII.9 (VA207) NoVs, to their carbohydrate receptors with 50 percent blocking titers (BT50s) of ~1:1600 for VA387 (Figure 5A and 5B), or 1:400 for VA207 (Figure 5C), respectively. These BT50s were significantly higher than those of the antisera induced by the free NoV P⁻ dimer, which were ~1:50-1:100 for VA387 and < 1:50 for VA207. These data, together those described above, strongly suggested the chimeric, polyvalent complexes as a good approach for multivalent vaccine development.

3.5. Increased functionality of the polyvalent complexes

NoV P⁻ dimer exhibited weak binding to the HBGA ligands (Figure 6A). Remarkably, the polyvalent complex of NoV P⁻-NoV P⁻ exhibited a radically increased binding activity than that of the NoV P⁻ dimer in a saliva-based binding assay (Figure 6B, P < 0.05). The GST-

NoV P⁻-NoV P⁻ complexes also showed increased binding activity (Figure 6C, $P < 0.05$), but lower than that of the NoV P⁻-NoV P⁻ complexes, indicating a negative impact of the GST on the binding function of the complexes, as reported previously [19]. Thus, formation of a polyvalent complex may be an effective approach to increase the ligands binding function of NoV P domain and similar principle might also apply for other functional protein as well.

3.6. The polyvalent complexes as vaccine platforms

The monomeric VP8* antigen (159 aa) of rotavirus was inserted to the GST-NoV P⁻ complexes through a surface loop (loop 2) of the NoV P⁻ antigen [4, 7], resulting in the GST-NoV P⁻-VP8* fusion protein (Figure 7A). The protein can be produced well in *E. coli* (Figure 7B and 7C, Table 2) and formed linear polyvalent complexes as shown by gel filtration (Figure 7C and 7D) and EM (Figure 7E). In addition, the small peptide M2e epitope (23 aa) of influenza virus [8] was inserted into the same loop of the NoV P⁻ of the GST-NoV P⁻ protein (Figure 7F), resulting in linear polyvalent GST-NoV P⁻-M2e complexes, as shown by gel filtration (Figure 7G, Table 2) and EM (data not shown). The NoV P⁻-M2e dimer was obtained by a thrombin digestion of the GST-NoV P⁻ protein (Figure 7F).

3.7. Immunogenicity of the complex-presented antigen

Immunization of mice (N=8 mice/group) with the polyvalent complexes containing VP8* antigen or M2e epitope resulted in significantly higher titers of VP8*- or M2e-specific antibody than those induced by the free or NoV P⁻ dimer-presented VP8* or M2e ($P < 0.05$) (Figure 8A and 8C). These results indicated significantly improved immunogenicity of the inserted VP8* antigen and M2e epitope through presentation by the polyvalent complexes.

Additionally, neutralization assays revealed that the antisera after immunization with the polyvalent GST-NoV P⁻-VP8* complexes exhibited significantly higher inhibitory activities on rotavirus replication than those of the antisera induced by the dimeric NoV P⁻-VP8* or free VP8* protein ($P < 0.05$) (Figure 8B). Challenge experiments demonstrated that the polyvalent GST-NoV P⁻-M2e vaccine fully protected mice (N=8 mice/group, 100% survival rate) against lethal challenge of the mouse-adapted human influenza virus (PR8, H1N1). This protection was significantly higher than those provided by free M2e (12.5% survival rate) that was measured previously [8] and the dimeric NoV P⁻-M2e vaccine (71% survival rate) (Figure 8D) ($P < 0.05$). It was also noted that both GST-NoV P⁻-M2e and GST-NoV P⁻-VP8* polyvalent complexes induced high titers of NoV-specific antibody (Figure 8E and 8F) that blocked binding of NoV P particles to their HBGA receptors (data not shown). Thus, the polyvalent complexes may serve as useful vaccine platforms for presentation of monomeric antigens for increased immunogenicity for dual vaccine development.

4. Discussion

In this study we proposed and verified a strategy to turn small proteins into large, polyvalent complexes for increased immunogenicity for multivalent vaccine development. This technology takes advantage of the common natures of dimerization of many proteins. Through recombinant technology, two or more such proteins can be fused covalently into one molecule, forcing them to assemble into polyvalent complexes through intermolecular dimerization between the homologous components (Figure 1). Two types of polyvalent complexes, linear and network, have been constructed. Fusion of two dimeric proteins tended to form linear complexes (Figure 1A), while combination of three of such proteins resulted in the network complexes (Figure 1B). Using three dimeric proteins (GST, NoV P⁻, and HEV P) as models, the principle and the feasibility of this strategy have been demonstrated. Since these protein components can be any dimeric proteins, polyvalent

complexes comprising variable antigens can be produced. In addition, a monomeric antigen or epitope can be inserted into the polyvalent complexes (Figure 1C and 1D), which has also been confirmed by the construction of two polyvalent complexes containing rotavirus VP8* antigen and M2e epitope of influenza virus, respectively. As shown in this study, all the polyvalent complexes can be easily produced in *E. coli* and probably in other expression systems as well. All these features suggest that our new technology may find its uses in biomedicine.

The major application of these polyvalent complexes was for improved immunogenicity. This was shown by immunization study in mice using the polyvalent complexes compared with the free protein components. Our data supported the notion that the polyvalent complexes induced significantly higher specific antibody and CD4⁺ T cell responses than those induced by the free protein dimers. More importantly, immunization of a single type of polyvalent complexes of NoV P⁻ (387)-NoV P⁻ (207) to mice resulted in significantly higher titers of antibodies specific to both VA387 and VA207 P domains than those induced by a mixture of the same amount of VA387 NoV P⁻ and VA207 NoV P⁻ dimers. Similar outcomes were also seen for CD4⁺ T cell responses. As a result, the mouse antisera after immunization with the polyvalent complex of NoV P⁻ (387)-NoV P⁻ (207) strongly blocked the attachment of both GII.4 VA387 and GII.9 VA207 NoVs to their HBGA receptors, an surrogate neutralization assay of human NoVs [1]. The increased immunogenicity of the polyvalent complexes compared with their dimers can be well explained by the polyvalent antigenic structures of the large complexes.

The fact that the polyvalent complex increased the immunogenicity of each component strongly suggested the polyvalent complexes as excellent models for multivalent vaccine development. Both polyvalent complexes of GST-NoV P⁻ (387)-NoV P⁻ (207) and NoV P⁻ (387)-NoV P⁻ (207) were demonstrated as promising bivalent vaccines against both GII.4 and GII.9 NoVs, two genetically and antigenically different NoVs. Notably, the two or three components of the polyvalent complexes can be simply replaced with other antigens for new bi- or trivalent vaccines against other pathogens. For human NoVs, GII.4 and GII.3 are the two most predominant genotypes causing the vast majority of NoV epidemics [31, 32]. Thus, a polyvalent complex containing the neutralizing antigen NoV Ps of both types would have a broad protection against most NoV infections. Construction of this potential super vaccine is on-going in our laboratory. In addition, we have successfully constructed the NoV P-HEV P-AstV P (astrovirus P domain) and AstV P-HEV P-VP8* fusion proteins and their polyvalent complexes (Wang and Tan, unpublished data). These new complexes may serve as multivalent vaccine candidates against the four enteric viruses. More importantly, this principle can be applied for vaccine development against many other viral and bacterial pathogens. Thus, our technology provides a new approach to combat infectious diseases. The polyvalent feature of the complexes also makes them potent vaccine platforms and sufficient evidence has been provided for this feasibility. Both the M2e and VP8* antigens were successfully inserted into the polyvalent complexes. The inserted antigen exhibited significantly increased immunogenicity in mice than those induced by the free or the dimer-presented antigens. The increased immunogenicity was further interpreted by the improved neutralizing activity and protective immunity of the chimeric polyvalent complex vaccines against the corresponding pathogens compared with those of the free antigens. Therefore, our study provided models and protocols for future improvement of immunogenicity of many other epitopes or antigens. The fact that monomeric an antigen can be presented by the polyvalent complexes further extends the applications of our technology to a wide range of antigens. In addition, the fact that the complex platform itself remained highly immunogenic provides an additional option for multivalent vaccine development.

Variable levels of antibody responses to NoV P⁻ dimers were seen among immunization experiments with different setups and purposes (Figure 4 and 8). These were most likely due to the different amounts of immunogens (ranging from 0.143 to 0.838 nanomoles/mouse) that were used in these experiments. Increased immunogen amounts tended to increase their antibody responses in general but reduce the differences between the polyvalent and dimeric/monomeric vaccines. In this study, all immunizations were performed intranasally without using any adjuvant to emphasize the differences among various experimental groups, because our preliminary data showed that use of adjuvant reduced such differences. These data suggested that the polyvalent complexes may function as an adjuvant in immune responses of mice.

It was also noted that the polyvalent complexes of NoV P⁻-NoV P⁻ revealed dramatically increased HBGA-binding activity in an EIA-based binding assay than that of the NoV P⁻ dimer. The functional avidity of a complex through an increase valence of the functional motif has been widely reported. For example, the binding activities of the NoV P dimer, the 12mer small P particle, the 24mer P particle and the 180mer VLP increased along with the rise of their P domain valences [20]. Similarly, human HBGAs in milk and saliva exist in two major populations. HBGAs that conjugate to the high molecular weight, polyvalent mucin-like backbones showed significantly higher binding activity to NoV VLPs compared to those HBGAs conjugated to the smaller molecules [35]. The increased HBGA-binding activity in this study may be due to avidity effects of multiple interactions of the P domain to HBGAs or through an increased accessibility of the polyvalent NoV P⁻ molecules by the detection antibody used in the EIA or both. This principle can be extended to other functional proteins to improve the sensitivity of functional assays.

Both protein production and polyvalent complex formation were highly efficient for the examined fusion proteins and similar properties are expected for many other proteins. However, both EM and gel filtration chromatography revealed heterogeneous sizes of the complexes ranging from a few to thousands of subunits. This raises the question of how to control the size of these complexes or, alternatively, how to separate the extremely large complexes from the small ones, because they may be very different in immunogenicity, functionality, and other properties. While separation of the large complexes from the small ones can be done by gel-filtration or other chromatography approaches, the strategy to regulate the sizes of the polyvalent complexes during production remains to be established.

5. Conclusions

We have developed a simple and effective strategy to turn small dimeric antigens into large, polyvalent complexes for improved immunogenicity for vaccine development. Two types of polyvalent complex vaccines, linear and network, have been produced easily based on this strategy. They demonstrated significantly improved immunogenicity, neutralization and protection compared with those of free dimeric vaccines. The polyvalent complexes were also a useful platform for antigen presentation for multivalent vaccine development against different infectious pathogens. Importantly, our study provided sufficient evidence to prove the principle of the polyvalent complex vaccines, which serve as models and procedures for future design, construction, manipulation, and applications of the polyvalent complexes in biomedicine.

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Figure 1.

Principles of polyvalent complex formations. (A) A fusion of two dimeric proteins (left, green and blue half ovals) results in a linear polyvalent complex (right) through intermolecular dimerizations (ovals) between homologous dimeric proteins. (B) A fusion of three dimeric proteins (left, green, purple and blue half ovals) assembles into a network polyvalent complex (right) via intermolecular dimerizations (ovals) between homologous dimeric protein components. (C and D) A monomeric antigen (red balls) can be inserted to the linear (C) or network (D) polyvalent complex through an insertion of the antigen to a complex component.

Figure 2.

Production and characterization of linear polyvalent complexes. (A) Schematic illustration of the GST-NoV P⁻ fusion and its thrombin digestion (+T) into free NoV P⁻ (blue) and GST (green) proteins. The thrombin digestion site (T) between the GST and the NoV P⁻ protein is indicated. (B to D) SDS-PAGE analysis of the GST-NoV P⁻ fusion proteins. (B) The GST-affinity column-purified GST-NoV P⁻ fusion protein. (C) The purified NoV P⁻ protein eluted from the GST-affinity column after a thrombin digestion of the GST-NoV P⁻ fusion protein on the beads. (D) The further purified GST-NoV P⁻ (left panel) and NoV P⁻ (right panel) by gel-filtration chromatography (E and F). Positions of the GST-NoV P⁻ fusion (~60 kDa), NoV P⁻ (~34 kDa), and GST (~26 kDa) proteins are indicated. M represents pre-stained protein markers (Bio-Rad, low range), with bands from top to bottom representing 113, 92, 52, 34, 29, and 21 kDa. (E to G) The elution curves of gel filtration chromatography of the GST-NoV P⁻ fusion (E), free NoV P⁻ (F), and free GST (G) proteins. GST-NoV P⁻ fusion protein revealed a collection of large complexes eluted at and near the void volume of the size-exclusion column Superdex 200 (E), while the free NoV P⁻ (F) and GST (G) proteins

showed a defined peak at ~69 kDa and ~56 kDa, respectively, representing the NoV P⁺ and GST dimers. Gel filtration chromatography was carried out using two size-exclusion columns of Superdex 200 (GE Healthcare Life Sciences): 10/300 GL (E) and HiLoad 16/60 [(F) and (G)]. The gel filtration columns were calibrated by the Gel Filtration Calibration Kit (GE Healthcare Life Sciences) and the recombinant P particle, small P particle, and P dimer of norovirus (VA387). The elution positions of blue Dextran 2000 (~2000 kDa, void), the P particle (~830 kDa), the small P particle (420 kDa), P dimer (~69 kDa), and aprotinin (~6.5kDa) were indicated. (H) and (I) Micrographs of electron microscopy of negatively stained GST-NoV P⁺ protein revealed large linear molecules. The rectangular-labeled region in (H) is enlarged in (I).

Figure 3. Production and characterization of network polyvalent complexes. (A) Schematic illustration of the GST-NoV P-NoV P fusion protein and its thrombin digestion (+T) into free GST (green) and NoV P-NoV P protein (blue), a tandem repeat of NoV P protein. The thrombin digestion site (T) between the GST and the NoV P-NoV P protein is indicated. (B and C) SDS-PAGE analysis of the GST-NoV P-NoV P and NoV P-NoV P proteins (B) and Western blot analysis of the proteins (C) to verify the identity the NoV P protein by NoV P domain-specific antiserum. The bands of GST-NoV P-NoV P (~92 kDa) and NoV P-NoV P (~68 kDa) are indicated. The signals of the lower bands represent degraded products that are often seen in *E. coli* expressed recombinant proteins. M represents pre-stained protein markers (Bio-Rad, low range), with bands from top to bottom representing 113, 92, 52, and 34 kDa. (D) The elution curve of gel filtration chromatography of the GST-

NoV P⁻-NoV P⁻ protein. Majority of the protein was eluted in or near the void volume of the size-exclusion column Superdex 200 (10/300 GL, GE Healthcare Life Sciences). The gel filtration columns were calibrated by the Gel Filtration Calibration Kit (GE Healthcare Life Sciences) and the recombinant P particle, small P particle, and P dimer of norovirus (VA387). The elution positions of blue Dextran 2000 (~2000 kDa, void), the P particle (~830 kDa), the small P particle (420 kDa), the P dimer (~69 kDa), and aprotinin (~6.5kDa) were indicated. (E to H) Micrographs of electron microscopy of negatively stained GST-NoV P⁻-NoV P⁻ from the GST-affinity column (E and F) or from the void volume of the gel filtration of (D) (H and I). In both cases network polyvalent complexes were seen.

Figure 4.

The polyvalent complexes significantly increased the immunogenicity of the individual protein components. (A and B) NoV P domain-specific IgG antibody responses post-immunization. (A) VA387 (GII.4, blue) and VA207 (GII.9, red) P domain-specific IgG antibody titers after immunization with GST-NoV P(387)-NoV P(207) and NoV P(387)-NoV P(207) polyvalent complexes were significantly higher than those induced by the corresponding NoV P dimers. (B) IgG antibody titers against NoV P domains of VA387 (blue) and VA115 (GI.3, purple) after immunization with GST-NoV P(387)-NoV P(115) and NoV P(387)-NoV P(115) polyvalent complexes were significantly higher than those induced by the corresponding NoV P dimers. Mice (N=8 mice/group) were immunized with

equal molar amounts of each immunogen (0.143 nanomoles/mouse, equal to 10 μ g of P(387)-NoV P(207). The statistical significances between the polyvalent complex immunized groups and dimer immunized controls are shown by * symbols (** $P<0.01$). (C and D) NoV P domain-specific CD4⁺ T cell responses post-immunization. (C) VA387 and VA207-specific CD4⁺ T cell responses after immunization with GST-NoV P(387)-NoV P(207) and NoV P(387)-NoV P(207) polyvalent complexes were significantly higher than those induced by the corresponding NoV P dimers. (D) VA387 and VA115-specific CD4⁺ T cell responses after immunization with GST-NoV P(387)-NoV P(115) and NoV P(387)-NoV P(115) polyvalent complexes were significantly higher than those induced by the corresponding NoV P dimers. Mice were immunized with equal molar amounts of each immunogen (N=3 mice/group). After stimulation of the splenocytes with a NoV P domain-specific CD4⁺ T cell epitope, the resulting CD4⁺ T cell cytokines IL-2, IFN- γ , and TNF- α were measured by intracellular cytokine staining. Cytokine-producing T cells from the GST-NoV P(387)-NoV P(207/115), NoV P(387)-NoV P(207/115) and NoV P dimer-immunized mice were shown in red, green, and blue, respectively. Those from unimmunized mice were negative control (Ctrl). Data were presented as cytokine-producing T cell numbers per 10⁶ cells. The statistical significances between the immunized groups and unimmunized control are shown by * symbols (* $P<0.05$, ** $P<0.01$, *** $P<0.001$), while those between the polyvalent complex-immunized groups (red and green) and the NoV P dimer controls (blue) are shown by # signs (# $P<0.05$, ## $P<0.01$, ### $P<0.001$).

Figure 5.

The polyvalent complex-induced antisera exhibited high blocking activities on binding of NoV surrogates to histo-blood group antigen (HBGA) receptors. (A and B) Mouse antisera after immunization with GST-NoV P(387)-NoV P(207) (blue lines) or NoV P(387)-NoV P(207) (red line) polyvalent complexes showed significantly higher blocking activity on binding of NoV P particles of VA387 [GII.4, (A) and (B)] and VLP of VA207 [GII.9, (C)] to type A [(A) and (C)] and type B (B) saliva samples than those (green lines) of the antisera after immunization with NoV P dimers of VA387 (A and B) and VA207 (C) ($P_s < 0.05$ for each indicated dilution). BT50s for every blocking experiment are indicated. Blocking experiments were performed using eight mouse sera independently for each immunization group and the average values are shown.

Figure 6.

Polyvalent complexes of NoV P domain of VA387 (GII.4) exhibited increased binding activity to HBGA ligands. (A) Free NoV P⁻ dimers showed only low binding activity to the saliva samples. (B and C) The polyvalent P complexes NoV P⁻-NoV P⁻ (B) and GST-NoV P⁻-NoV P⁻ (C) exhibited significantly increased binding activity to the saliva samples, respectively. The signal intensities of binding (Y-axis) of the NoV P⁻ polyvalent complexes and dimers at indicated concentrations (X-axis) to type A (blue), type B (red) and type O (green) saliva samples with defined A, B and H antigens, respectively, were shown. All data were average values of triplicate experiments.

Figure 7.

A monomeric antigen can be inserted to the polyvalent complex. (A) Schematic illustration of the GST-NoV P-VP8* fusion and its thrombin digestion (+T) into free GST (green) and NoV P-VP8* (blue) with VP8* (red) antigen (NoV P-VP8*). The thrombin digestion site (T) is indicated. (B) The affinity column purified GST-NoV P-VP8*, NoV P-VP8*, and free VP8* proteins were analyzed by SDS-PAGE. The VP8* protein was inserted to the GST-NoV P-VP8* complex through loop 2 of the NoV P-VP8*. Positions of the GST-NoV P-VP8* fusion protein (~78 kDa), NoV P-VP8* protein (~52 kDa), and free VP8* (~18 kDa) are indicated. M represents prestained protein markers (Bio-Rad, low range), with bands from top to bottom representing 113, 92, 52, 34, 29, and 21 kDa. (C and D) Elution curves of gel

filtration chromatography of the GST-NoV P⁻-VP8* (C) and free NoV P⁻-VP8* dimer (D). GST-NoV P⁻-VP8* proteins revealed a collection of large molecules as shown by the peaks at high molecular weights (C), while the free NoV P⁻-VP8* protein formed dimers (D). The gel filtration columns were calibrated by the Gel Filtration Calibration Kit (GE Healthcare Life Sciences) and the recombinant P particle, small P particle, and P dimer of norovirus (VA387). The elution positions of blue Dextran 2000 (~2000 kDa, void), the P particle (~830 kDa), the small P particle (420 kDa), P dimer (~69 kDa), and aprotinin (~6.5kDa) were indicated. (E) A electron micrograph of negatively stained GST-NoV P⁻-VP8* protein revealed linear polyvalent complexes. (F) SDS PAGE analysis of the GST-NoV P⁻-M2e, and NoV P⁻-M2e. Positions of the GST-NoV P⁻-M2e fusion (~63 kDa) and NoV P⁻-M2e (~36 kDa) proteins are indicated. M represents prestained protein markers as above. (G) The elution curve of gel filtration chromatography of the GST-NoV P⁻-M2e fusion protein, which showed a collection of large molecules eluted at and near the void volume of the size-exclusion column Superdex 200. The proteins of the two major peaks (P1 and P2) were analyzed by SDS PAGE and shown below the elution curves. The gel filtration columns were calibrated as described above.

Figure 8.

The polyvalent complexes increased the immunogenicity of an inserted monomeric antigen. (A) The titers of rotavirus VP8*-specific antibody in mice (N=8 mice/group) induced by the polyvalent complex GST-NoV P⁻-VP8* (red) was significantly higher than those induced by the NoV P⁻-VP8* dimer (green) or the free VP8* monomer (purple). Data were mean values of 8 neutralizing experiments using 8 mouse sera per immunization group. Mice were immunized with equal molar amounts of each immunogen (0.838 nanomoles/mouse, equal to 15 μ g of free VP8*). (B) Mouse antisera (N=8 sera/group) after immunization with the polyvalent complex GST-NoV P⁻-VP8* exhibited significantly higher neutralizing activity (red) against rotavirus replication in a plaque reduction assay than those of sera after immunization with the NoV P⁻-VP8* dimer (green) or the free VP8* monomer (purple). (C) The titers of influenza virus M2e-specific antibody induced by the polyvalent complexes GST-NoV P⁻-M2e (red) was significantly higher than that induced by NoV P⁻-M2e dimer (green). Mouse sera after immunization with NoV P⁻ dimer were used as negative control (purple). Mice (N=8 mice/group) were immunized with equal molar amounts of each immunogen (0.556 nanomoles/mouse, equal to 20 μ g of NoV P⁻-M2e). (D) Survival rates of

mice (N=8 mice/group) after vaccination with polyvalent complex GST-NoV P⁻-M2e (red), NoV P⁻-M2e dimer (green) or NoV P⁻ dimer (purple) followed by challenges with lethal dose influenza virus (PR8). (E and F) The polyvalent complexes of GST-NoV P⁻-M2e (E) and GST-NoV P⁻-VP8* (F) also induced significantly higher titers of NoV P⁻-specific antibody (red) than those induced by NoV P⁻-M2e (green) or NoV P⁻ (purple) dimers (E) and those induced by NoV P⁻-VP8* dimers (green) or free VP8* (purple) (F), respectively. 2nd and 3rd, data after second and third immunization. Statistical differences between data groups are indicated by start symbols (* $P<0.05$, ** $P<0.01$).

Table 1

Primers that were used to generate expression constructs of recombinant proteins

Name	Sequence (5' to 3')	Sense	Enzyme	Locations/Constructs
P524	GCACGGATCCTCAAGAACTAAACCATTACAC	+	BamHI	N-terminus of NoV VA387 P domain
P561	GCGTGCGGCCGCTTACCCCGCTCCATTTCCCATGGG	-	NotI	C-terminus of NoV VA387 P domain without the arginine cluster
P590	GCATGCGGCCGCTTAGCAAAAGCAATCGCCACGGCAATCGCATAATGCACGTCTGCGCCCCGC	-	NotI	C-terminus of NoV VA387 P domain
P821	GCACGGATCCTCAAGACTAAGGCATTAC	+	BamHI	N-terminus of NoV VA207 P domain
P834	AGTCGCGGCCGCTTAGCGCCCACTTCCAGTTCCCAC	-	NotI	C-terminus of NoV VA207 P domain without the arginine cluster
P843	GCACGGATCCAAAAGACTAAACCATTAG	+	BamHI	N-terminus of NoV VA115 P domain
P846	GCATGCGGCCGCTTAACGAGCCGGGCCGGCTGTTC	-	NotI	C-terminus of NoV VA115 P domain without the arginine cluster
P1572	ATATCGTCTCCCCCGCTCCATTTCCCATG	-	BsmBI	C-terminus of NoV VA387 P domain without the arginine cluster
P1617	ATATCGTCTCCCTCCGCCTCCGCCTCCGCCCCCGCTCCATTTC	-	BsmBI	Tandem repeat of NoV VA387 P domain linked with 12G
P1618	TATTCGTCTCCGAGGCGGAGGCGGAGGCGGATCAAGAACTAAACC	+	BsmBI	Tandem repeat of NoV VA387 P domain linked with 12G
P1656	TATTCGTCTCCGGGGATCGAGGGAAGGATTTCATGTCACCGATACTA	+	BsmBI	N-terminus of GST
P1657	GCATGCGGCCGCTTAATCCGATTTTGAGGATGGTCG	-	NotI	C-terminus of GST
P1689	ATACGCGGATCCTGCAACGGCCGTTGCTCAAGAACTAAACCATTACCGT	+	BamHI	N-terminus of NoV VA387 P domain
P1734	ATATCGTCTCCTAATGCACGTCTGCGCCCCGC	-	BsmBI	C-terminus of NoV VA387 P domain with the arginine cluster
P1735	TATTCGTCTCCATTATTAGATGGTCCTTATCAACC	+	BsmBI	N-terminus of human rotavirus VP8
P1736	GCATGCGGCCGCTTATAGACCGTTGTTAATATATTC	-	NotI	C-terminus of human rotavirus VP8
P1955	TATTCGTCTCCGAGGCGGAGGCGGAGGCGGACAAAAGACTAAACCATTAG	+	BsmBI	N-terminus of NoV VA115 P domain
P1957	TATTCGTCTCCGAGGCGGAGGCGGAGGCGGATCAAAGACTAAGGCATTAC	+	BsmBI	N-terminus of NoV VA207 P domain
P1958	GCATGCGGCCGCTTACCCACTTCCAGTTCCCACAG	-	NotI	C-terminus of NoV VA207 P domain
P1985	ATATTATTGCACGGATCCCCGACCCGCTCTCCGGCTC	+	BamHI	N-terminus of HEV P domain
P1986	GCATGCGGCCGCTTAGCAAAAGCAATCGCCACGGCAATCGCACGGGTAGTCAACGGTGTC	-	NotI	C-terminus of HEV P domain

Table 2

Properties of variable protein complexes constructed in this study.

Names of the free and fusion proteins	Molecular weights (kDa)	Assembled complex sizes (kDa) ¹	Structural forms of the resulting complexes in solution	Protein yield in <i>E. coli</i> (mg/liter culture)	Increased immunogenicity to protein components	Increased neutralization and/or protection to pathogens ²
Glutathione S-transferase (GST)	~26	~52	dimer	>20	-	-
Shortened norovirus P domain (NoV P) ³	~34	~68	dimer	>20	as control	as control
P domain of hepatitis E virus (HEV P)	~19	~38	dimer	>20	-	-
GST-NoV P ⁻	~60	>800	linear complexes	>20	-	-
GST-HEV P	~45	>400	linear complexes	>20	-	-
NoV P-NoV P ⁻	~68	>400	linear complexes	~4	yes	yes
GST-NoV P-NoV P ⁻ (387, GIL4 NoV)	~92	>400	network complexes	~5	yes	yes
NoV P(387)-NoV P(207, GIL9 NoV)	~68	>400	linear complexes	~4	yes	yes
GST-NoV P(387)-NoV P(207)	~94	>400	network complexes	~4	yes	yes
NoV P(387)-NoV P(115, GL3)	~68	>400	linear complexes	~4	yes	yes
GST-NoV P(387)-NoV P(115)	~94	>400	network complexes	~4	yes	yes
Rotavirus VP8* (RV VP8*)	~18		monomer	>20	as control	as control
GST-NoV P-RV VP8*	~78	>400	linear complexes	>15	yes	yes
NoV P-RV VP8*	~52	102	dimer	>15	as control	as control
GST-NoV P-M2e	~63	>400	linear complexes	>20	yes	yes
NoV P-M2e	~37	~74	dimer	>15	as control	as control

¹ Determined by gel filtration chromatography.

² Neutralization of norovirus (NoV) is represented by the blocking activity of the mouse sera after immunization with NoV P domain-containing immunogens on the attachment of NoV surrogates to histo-blood group antigen receptors.

³ Shortened NoV P domain is the P domain with a deletion of the C-terminal arginine-cluster.