

Mosaic Q β coats as a new presentation model

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Abstract The new protein carrier was developed on the basis of recombinant RNA phage Q β capsid. C-terminal UGA extension of the short form of Q β coat, so-called A1 extension, served as a target for presentation of foreign peptides on the outer surface of mosaic Q β particles. In conditions of enhanced UGA suppression, the proportion of A1-extended to short coats in mosaic particles dropped from 48% to 14%, with an increase of the length of A1 extension. A model insertion, short preS1 epitope 31-DPAFR-35 of hepatitis B surface antigen, demonstrated superficial location on the mosaic Q β particles and ensured specific antigenicity and immunogenicity.

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Key words: Phage Q β ; Coat protein UGA suppression; A1 extension; Capsid assembly; Hepatitis B virus preS1; Immunogenicity

1. Introduction

Recombinant coats of icosahedral RNA bacteriophages of the *Leviviridae* family are of obvious interest in protein engineering as putative carriers of foreign peptides [1–4]. First of all, owing to their highly-resolved X-ray structures (for review see [5]). *Leviviridae* phages are subdivided into *Levivirus* (serological groups I and II) and *Allolevivirus* (serological groups III and IV), but virions of both genera are icosahedrons with quasi T=3 symmetry, consisting of 180 copies of coat protein enveloping a complex of single-stranded genomic RNA and maturation protein. In contrast to *Levivirus*, *Allolevivirus* virions contain three to five molecules of the prolonged coat, so-called A1 protein, with 196 aa residues of the A1 extension added C-terminally to the short form of coat protein [6]. These extended molecules are formed as a result of natural read-through of the opal (UGA) terminator of the coat protein [7]. Although the biological function of A1 protein is not understood, it is believed to be involved in the *Allolevivirus* infection process [8,9].

Recombinant coats of two representatives of *Levivirus* genus, MS2 and fr, were employed recently as peptide carriers. MS2 capsids accepted 9 aa residues inserted into the external N-terminal hairpin between β -sheets A and B [4], whereas fr coats allowed insertions of a least (i) 19 aa at the N-terminus, (ii) 5 aa at position 51, and (iii) 3 aa at the C-terminus of the molecule [1,3]. However, the extremely rigid structure of icosahedrons of these phages [5] complicated further development of these models. Here, we propose recombinant coats

of Q β phage, a typical representative of *Allolevivirus* genus, as more attractive candidates for peptide presentation, with the use of A1 extensions as targets for foreign insertions. Recent X-ray structure of Q β virions showed that the monomers of coat protein assume three different conformations A, B and C, forming two types of dimers [10], similar to *Levivirus* particles [5]. Nevertheless, Q β dimers, differing from *Levivirus* dimers, are linked in pentamers and hexamers by disulfide bonds [10]. The minority and asymmetric location of A1 protein were reasons why X-ray analysis failed to locate it. The structural similarity of A1 extensions but not the short forms of their coat proteins to superficially located proteins, such as hepatitis B surface antigen (HBsAg) preS1 region, especially in the specific stretch responsible for the attachment of HBV virions to hepatocytes [2], suggested their exterior location on the phage virions. We attempted to mark A1 extensions with a short foreign epitope, the peptide 31-DPAFR-35 from the preS1 region of HBsAg in order to evaluate presentation capabilities of A1 protein. Moreover, we enhanced strongly the number of A1 extensions within the mosaic Q β particles in conditions of strong UGA suppression.

2. Materials and methods

2.1. Antibodies

Polyclonal anti-Q β antibodies were generated by immunization of rabbits with purified Q β capsids encoded by the UAA-terminated short form of Q β C gene [2].

2.2. Construction of plasmids

The plasmid pQ β GGA was created by site-directed mutagenesis. Upstream primers: 5'-CAGCTGAACCCAGCGTACGGAACACTG-3', 5'-TAATACGACTCACTATAGGG-3' and downstream primer: 5'-AACATCAAATTCGCGAGGCTGG-3' were used for the synthesis of mutant PCR-fragment that was reintroduced into the pQ β expression vector at the unique restriction sites *Xba*I, and *Hind*III.

Two sets of plasmids pQ β UGA and pQ β GGA were created by two steps of PCR. The upstream primer (5'-CAGACCGGAATTCGAGCTCGCC-3') was the same in all of the PCR reactions. The downstream primers of the first step of PCR were 5'-GGATCGCTC-GAGCCTGAGCCTCAor(TCC)ATACGCTGGGTTTCAGCT-3' for Q β UGA11 and Q β GGA11; 5'-GCAGGATCGCTCGAGCCTGAGCCACCACCGCAATG-3' for Q β UGA18 and Q β GGA18; 5'-TCGAAAAGCAGGATCCGGAATAACCGGATCGGG-3' for Q β UGA24 and Q β GGA24 construction. The products of the first PCR were used as matrices for the second PCR reactions. The downstream primer 5'-GCGCGAAGCTTATCGGAATGCAGGATCGCTCGAGCCTGAG-3' was used in the second PCR reactions for constructions Q β UGA11, Q β GGA11, Q β UGA18 and Q β GGA18, and the primer 5'-GCGAAGCTTAATTAATTATCGAAAAGCAGGATCGG-3' for construction of Q β UGA24 and Q β GGA24. The products of the second PCR were digested with *Xba*I and *Hind*III, and cloned into the Q β expression vector cleaved by *Xba*I and *Hind*III. Plasmids pQ β UGA203, pQ β GGA203 were created by *Bgl*II digestion of pQ β and its GGA-containing form, with subsequent insertion of a synthetic DNA copy of the preS1 sequence 31-DPAFRA-36 with sticky *Bgl*II ends into the *Bgl*II restriction site.

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2.3. Expression and purification of mosaic Q β particles

E. coli JM109 cells harboring the appropriate plasmids were grown overnight in M9-medium supplemented with 10 g/l Casamino acids (Difco). *E. coli* lysates were prepared by grinding with aluminium oxide (Alcoa A-305, Serva) and sonication of cells incubated for 30 min on ice in lysis buffer (10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.02% Triton X-100, 2 mg/ml lysozyme). After centrifugation, proteins were precipitated from the supernatant with ammonium sulfate at 50% saturation over 0.5 h at 4°C. Pellets were resuspended in 4 ml of PBS. Chimeric Q β particles were purified on Sepharose CL-4B (Pharmacia Biotech) column (1.8 \times 95 cm).

2.4. Electron microscopy

For direct electron microscopy, capsids were stained in 2% phosphotungstic acid. For immunogold detection of epitopes, capsids were incubated with a monoclonal anti-preS1 MA18/7 antibody, developed with gold-labelled goat anti-mouse IgG and negatively stained in 2% uranylacetate.

2.5. SDS-PAGE and Western blotting

Laemmli's electrophoresis on 15% gel, Western blotting, and staining with Coomassie brilliant blue G-250, were performed according to standard protocols.

2.6. Competitive ELISA

Microtiter 96-well plates were coated with synthetic HBV (subtype ayw) preS1 peptide p21–47 (1 μ g/well) and blocked overnight with 0.5% BSA (Pharmacia) at 4°C. Wells were washed with PBS buffer containing 0.5% Tween-20. Aliquots of recombinant capsids and p21–47 peptide as a positive control were added at varying dilutions together with MA18/7 antibody diluted 1:15000. After 1 h incubation at 37°C, the wells were washed, and anti-mouse Ig horseradish peroxidase conjugate (Amersham) was added. Plates were incubated for 1 h at 37°C, then washed, and 100 μ l/well of *o*-phenylenediamine dihydrochloride (Sigma) was added. Inhibition of MA18/7 antibody binding to p21–47 peptide by recombinant capsids or by control peptide p21–47 was calculated according to the following equation: % inhibition = $100 \times (1 - (\text{OD}_{492} \text{ with inhibition} - \text{bkgd}) / (\text{OD}_{492} \text{ without inhibition} - \text{bkgd}))$, where the bkgd was the absorbance from wells coated with BSA only.

2.7. Immunization

Inbred Balb/C mice were immunized by i.p. injection of 20 μ g of chimeric proteins in CFA and boosted with a half-dose of proteins in IFA on days 10, 24. For detection of antibody titers, p21–47 peptide was coated on microtiter plates. Pooled murine sera, collected on day 24 were titrated at different dilutions. The data were expressed as antibody titer representing the highest dilution to yield three times the OD reading of preimmunization sera.

2.8. Computer analysis

The matrix program (BioCan Scientific) was employed for determination of the ratio of A1-extended to short forms of Q β coat protein on Western blots.

3. Results

3.1. Construction of Q β display vectors

Fig. 1 shows the structure of two sets of vectors used for the expression of extended coat proteins of different length. Vector pQ β [11], with the cloned full-length Q β gene C located under the control of *E. coli* trp operon and T7 phage promoters, was used as the initial plasmid for these constructions. One set of vectors (Q β UGA) was designed for the generation of mosaic particles and consisted of modified A1 genes retaining UGA terminator. The other set (Q β GGA) was constructed for the expression of full-length and shortened A1 genes, without any translational read-through. In this case, site-directed mutagenesis was used to change the stop UGA codon of the short form of coat protein to the sense GGA codon.

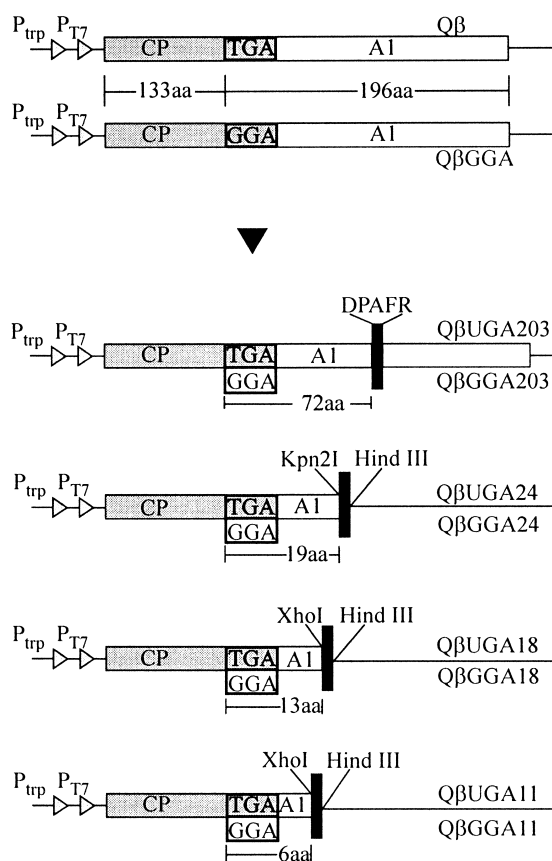


Fig. 1. Two sets of Q β display vectors constructed on the basis of cloned full-length gene C. The structure of the initial pQ β plasmid is shown above. Designations for the appropriate vectors of the first set of UGA-containing read-through genes and of the second set of GGA-containing genes are shown above and under the line, respectively. The numbers of aa residues (including UGA- or GGA-encoded residue from A1 extension) preceding the inserted preS1 epitope 31-DPAFR-35 (shown as black bar) are given. Restriction sites enclosing the preS1 epitope 31-DPAFR-35 are shown.

A synthetic DNA copy of HBV preS1 epitope 31-DPAFR-35, corresponding to the minimal sequence that is necessary and sufficient to be recognized by the monoclonal virus-neutralizing anti-preS1 antibody MA18/7 [12], was introduced into the unique restriction sites at various positions of A1 extension. Deletions of 177 aa (Q β UGA24 and Q β GGA24), 183 aa (Q β UGA18 and Q β GGA18) and 190 aa (Q β UGA11 and Q β GGA11) at the C-terminus of A1 were undertaken with the aim to estimate the role of the size of A1 extensions in capsid-forming ability of modified A1 proteins formed in both sets of vectors.

3.2. Capsid-forming ability of modified A1 proteins

Products encoded by plasmids of the Q β GGA series (Fig. 1) SDS-PAGE, Western blotting and electron microscopy showed that both initial A1 proteins and their full-length form with the DPAFR insertion were unable to form capsids, remained insoluble and formed inclusion bodies, whereas shortened A1 variants with the 24, 18, 11 aa extensions were soluble and appeared as asymmetric particles that differed strongly from icosahedral phage-like shells in size and shape.

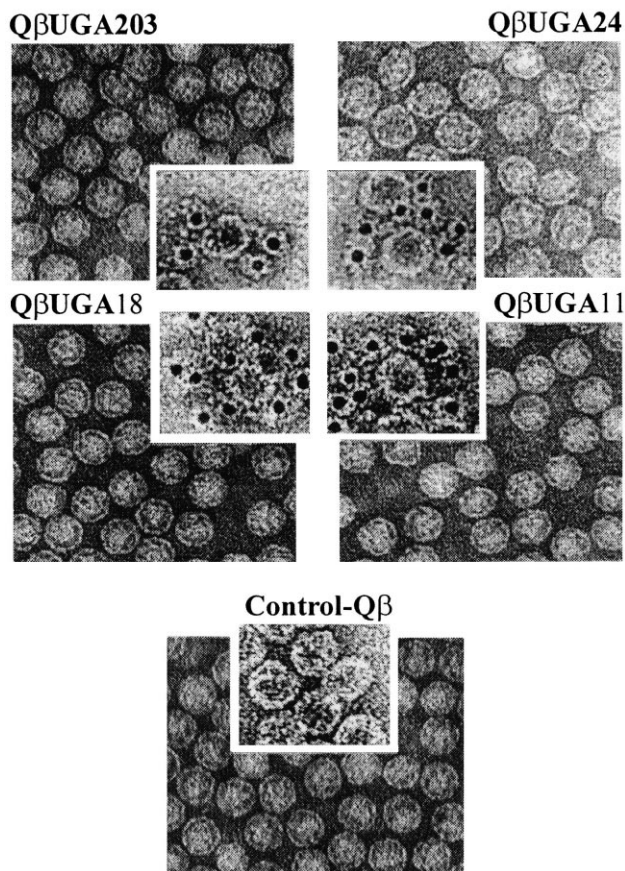


Fig. 2. Negative staining (large photos, magnification 200 000 \times) and immunogold electron microscopy with monoclonal anti-preS1 MA18/7 antibodies (small photos, magnification 300 000 \times) of mosaic particles purified from *E. coli* cells after expression of the appropriate A1-extended genes under strong UGA suppression conditions.

3.3. Capsid-forming ability of modified A1 proteins in the presence of short coats

To enhance the expression of A1-extended read-through genes and, therefore, the content of the appropriate A1-extended proteins in mosaic particles, we used the plasmid pISM3001 which harbors cloned opal tRNA gene under the control of lac promoter and enables translation of UGA codon as a sense tryptophan codon [13]. Quantitative analysis of products encoded by plasmids of the Q β UGA series (Fig. 1) in presence of pISM demonstrated that the UGA suppression level was stable in all of the analyzed subclones, and the synthesis of A1-extended read-through proteins increased up to 40% for Q β UGA203, 48% for Q β UGA24 and Q β UGA18, and 47% for Q β UGA11 (Fig. 4).

The products of all mentioned particulation-competent Q β variants were purified by gel filtration on a CL4B sepharose column. Electron microscopy revealed their icosahedral nature and phenotypic similarity to native Q β virions and recombinant Q β shells (Fig. 2). Quantitative Western blot analysis of the proportions of short and A1-extended forms of coat proteins within the particles (Fig. 3) showed that these particles were in fact mosaic, and the percentages of A1-extended coats reached 14% for a maximal 203 aa extension, but the numbers of A1-extended coats increased to 47–48% for the shorter 11, 18 and 24 aa extensions.

3.4. Antigenicity of mosaic particles

A competitive ELISA test of purified capsid-like particles with monoclonal MA18/7 antibody (Fig. 4) was employed to identify the localization of preS1 epitope on the capsids. Quantification of competitive ELISA indicated that about two third of inserted epitopes are accessible to antibody in the case of 11, 18 and 24, and at least one third of them are antibody-accessible in the case of 203 aa extension. Immunogold electron microscopy of mosaic particles with MA18/7 antibody (Fig. 2) confirmed the superficial localization of preS1 epitope attached to A1 extensions of different length.

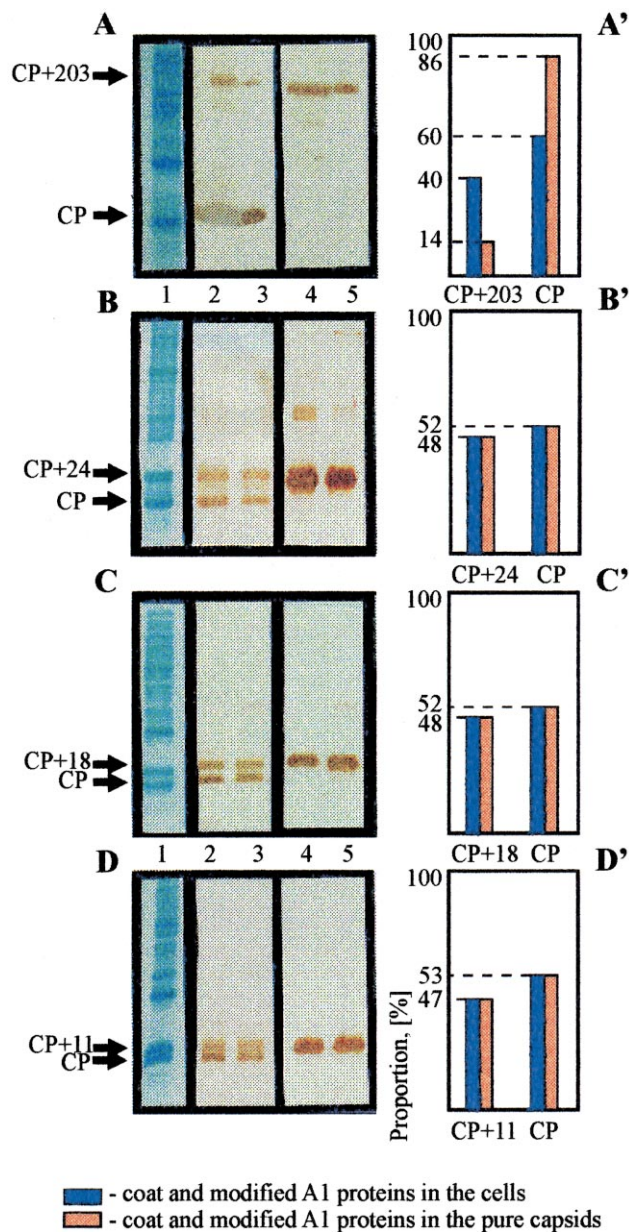


Fig. 3. Proportions of coat (CP) and extended coat (CP+extension[aa]) proteins in the crude *E. coli* cell SDS lysates (lines 1, 2, 4) and pure capsids (lines 3, 5) expressed from plasmids of a Q β UGA series in the *E. coli* cells derived under efficient UGA suppression conditions: SDS-PAGE (line 1), Western blotting with polyclonal Q β antibodies (lines 2, 3) and with monoclonal anti-preS1 MA18/7 antibodies (lines 4, 5), and mathematical alignment (A', B', C', D').

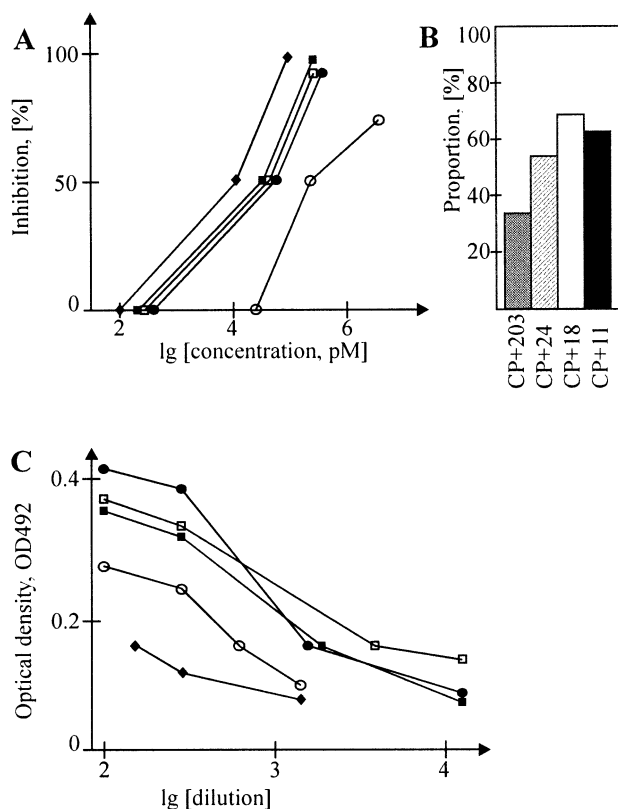


Fig. 4. Antigenical and immunological properties of preS1 epitope exposed on the mosaic Q β particles. A: Competition ELISA with monoclonal anti-preS1 MA18/7 antibody (○, Q β UGA203; ●, Q β UGA24; ■, Q β UGA18; □, Q β UGA11; ◆, peptide preS1 (21–47)). B: Percentage of antibody-accessible preS1 epitope on the outer surface of particles. C: Immunological response of Balb/C mice to preS1 epitope on day 24 (○, Q β UGA203; ●, Q β UGA24; ■, Q β UGA18; □, Q β UGA11; ◆, mice immunized with original Q β capsid).

3.5. Immunogenicity of mosaic particles

The immunogenicity of mosaic particles was tested in Balb/C mice, and their ability to induce anti-preS1 response was detected by standard ELISA test (Fig. 4). Immunogenicity of the preS1 insertion increased with the reduction of the length of A1 extension. The highest immunogenicity was found for mosaic particles harboring A1 proteins with only 11 aa extension (titer 1:3600 of specific anti-preS1 antibodies). The immunogenicity of mosaic particles harboring A1 proteins with 18 and 24 aa extensions was a little bit lower (1:2000), and the product with the longest 203 aa A1 extension showed remarkable level (1:700) of anti-preS1 response.

4. Discussion

Normally the proportion of A1 read-through molecules within Q β virions was not strictly fixed and allowed an increase from 1.6% to 15%, when an UGA suppressor strain was used as a host [6]. Although the low number and irregularity of A1 extensions made them invisible to X-ray analysis, the crystal structure of the Q β particle indicated that the C-termini of the short form of the coat protein, where the read-through A1 extension would be expected, are located on the surface of the capsid. However, the C-termini of Q β CP were

shielded from the exterior to some extent by the N-termini of the co-operating monomer within the dimeric unit [10]. Therefore, spatial limitations may explain why we found that not only full-length A1 proteins are insoluble in the absence of short CP, but also short 11 aa extensions are sufficient to block self-assembly. In the case of Q β UGA series vectors, at enhanced UGA read-through, heterologous dimers may be formed from helper and extended CP. The number of accepted A1-extended proteins depended, however, on the length of extension, and increased from 14% to 48%, with a decrease of its length from 203 to 24 aa residues (Fig. 3). The percentages of A1-extended proteins in the cells and in the purified particles fit well in the case of short extensions (Fig. 3, parts B', C', D'), indicating the absence of any particulation advantage for short CP in these cases. In contrast, long A1-extended protein (Fig. 3, part A') was disadvantageous for self-assembly to short CP by a factor of about 3. The exterior location of A1 extensions was proved experimentally by immunogold decoration of particles (Fig. 2) and by competitive ELISA within specific anti-preS1 monoclonal antibody (Fig. 4, parts A, B). Calculations given in Figs. 3 and 4 showed that most, if not all A1-extended molecules are accessible to antibody within mosaic particles. The inserted model epitope also showed remarkable immunogenicity in mice, in spite of its minimal length (Fig. 4, part C). Historically, filamentous coats of DNA bacteriophages from the *Inoviridae* family and icosahedral coats of cowpea mosaic virus from RNA plant viruses were the first sources for construction of mosaic particles [14,15]. Usage of mosaic technology allowed to enhance capacities of the gene VIII-encoded major protein of filamentous phage fd from 12 to 36 aa residues [16]. Introduction of a modified gene III-encoded minor protein of filamentous phages having a capacity of approximately 57 aa residues may be recognized as a special case of generation of mosaic particles [14]. The number of gene III products within the filamentous phage is constant and does not exceed five copies [17], whereas mosaic Q β particles can include about 25 to 86 copies of A1-extended proteins per shell. The capsid of the popular cowpea mosaic virus can include 50% percent of the small proteins with 27 aa insertion [15]. Another advantage of the proposed model is its non-infectious character. Q β coats are able to self-assemble in the absence of genomic RNA, unlike the filamentous phages and cowpea mosaic virus, where assembly is concomitant with encapsidation of a specific genome [14–17]. Of the existing non-infectious mosaic models, Jonsongrass mosaic virus [18], hepatitis B surface [19] and core [20] antigens are worthy of notice, especially the latter which exploits the same idea of C-terminal UGA extension.

Mosaic Q β particles harboring epitope-marked A1 extensions will open fresh opportunities for detailed structural and functional study of protruding parts of viral coats.

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