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Surface Functionalization of Virus-Like Particles by Direct Conjugation Using Azide–Alkyne Click Chemistry

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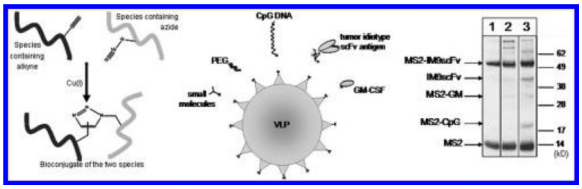
Abstract

We present a cell-free protein synthesis (CFPS) platform and a one-step, direct conjugation scheme for producing virus-like particle (VLP) assemblies that display multiple ligands including proteins, nucleic acids, and other molecules. Using a global methionine replacement approach, we produced bacteriophage MS2 and bacteriophage Qβ VLPs with surface-exposed methionine analogues (azidohomoalanine and homopropargylglycine) containing azide and alkyne side chains. CFPS enabled the production of VLPs with yields of ~300 μg/mL and with 85% incorporation of methionine analogues without requiring a methionine auxotrophic production host. We then directly conjugated azide- and alkyne-containing proteins (including an antibody fragment and the granulocyte-macrophage colony stimulating factor, or GM-CSF), nucleic acids and poly(ethylene glycol) chains to the VLP surface using Cu(I) catalyzed click chemistry. The GM-CSF protein, after conjugation to VLPs, was shown to partially retain its ability to stimulate the proliferation of cells. Conjugation of GM-CSF to VLPs resulted in a 3–5-fold reduction in its bioactivity. The direct attachment scheme facilitated conjugation of three different ligands to the VLPs in a single step, and enabled control of the relative ratios and surface abundance of the attached species. This platform can be used for the production of novel VLP bioconjugates for use as drug delivery vehicles, diagnostics, and vaccines.



INTRODUCTION

We have previously shown that cell-free protein synthesis (CFPS) can be used to produce virus-like particles (VLPs) as symmetrical self-assembled icosahedral nanoscale objects composed of identical protein monomers. The VLPs are noninfectious and intrinsically very

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stable over wide ranges of pH and temperature, making them attractive as the assembly core for vaccines,[1,2] diagnostics,[3] and other therapeutics.[2,4,5] They can potentially serve as polyvalent scaffolds for the display of nucleic acids, proteins, and other chemical moieties, and VLPs are particularly attractive as vaccines as they offer *in vivo* stability, trafficking to lymph nodes,[6] and stimulation of B and T cell responses to displayed epitopes.[1] They can also be filled with cargo to serve as delivery vehicles.[7,8]

We are particularly interested in developing technologies for the presentation of a variety of moieties on the surface of VLPs. We describe the production of MS2 and Qβ VLPs containing surface-exposed non-natural amino acids (nnAAs) to enable conjugation with a variety of biomolecules including nucleic acids and proteins. For these VLPs, individual coat proteins first form dimers which self-assemble into 27 nm diameter icosahedral ($T = 3$) VLPs containing 180 monomers each.[9,10] MS2 and Qβ are known to encapsidate cellular RNAs,[11-13] and this may also offer the opportunity to load alternate cargoes. Both MS2 and Qβ are intrinsically very stable to significant variations in pH, temperature, and the surrounding chemical environment.[14,15] In particular, Qβ VLPs are remarkably stable since they contain multiple intermonomer disulfide bonds arising from cysteine residues at positions 74 and 80 which are present near the 5- and 3-fold axes of symmetry.[10] In this way, each protein dimer is connected to the rest of the capsid by 4 disulfide bonds.

Fusion protein approaches in which foreign peptide sequences are inserted into the coat protein have been successfully used for the presentation of protein sequences on VLPs. However, this method is mostly limited to short peptide sequences consisting of up to 24 amino acids,[16,17] and such fusions often compromise the ability of VLPs to self-assemble. Several methods have been developed for chemical linkage of ligands to exposed amino acids on VLP surfaces.[18,19] Assemblies including nicotine and angiotensin II coupled to Qβ VLPs are in development as vaccines for treating nicotine addiction[20] and hypertension,[21] respectively. In both these examples, chemical coupling of the antigens to VLPs was achieved by means of heterobifunctional linkers using a two-step conjugation scheme. More recently, coupling of proteins and small molecule ligands to VLPs by means of nnAAs was reported.[22] This method utilized a global replacement strategy for substituting methionine residues in VLPs with analogues containing terminal azide (azidohomoalanine: AHA) and alkyne (homoproparglyglycine: HPG) groups (Figure 1A). Next, azide and alkyne functionalized small molecules and proteins were chemically coupled to VLPs using click chemistry. Azide–alkyne click chemistry[23,24] is well-suited for conjugation of biological species since these reactions are fast, relatively efficient, and can be performed under physiological conditions in aqueous buffers. Although small molecules have been directly coupled to the surface of VLPs, protein coupling reported previously in the literature has employed bifunctional linkers.[1,22] To improve attachment efficiency and provide greater control, we sought to develop a one-step chemical coupling method for the direct attachment of proteins to the surface of VLPs (Figure 1).

While VLPs have been produced using a variety of production hosts including *E. coli*, yeast, and mammalian cells, very high volumetric production yields are achieved with our cell-free platform.[25] Cell-free protein synthesis is a versatile platform that has also been used for the production of a variety of proteins including integral membrane proteins,[26] human

transcription factors for nuclear reprogramming,[27] and disulfide bond containing fusion protein vaccines for B cell lymphoma.[28,29] The open cell-free system is well-suited for the production of these complex proteins since it offers direct access to the protein synthesis environment to enable control of parameters such as pH, redox potential, and component concentrations. This system also allows convenient supplementation of desired amounts of additional components including chaperones, disulfide bond isomerases, and components for incorporation of nnAAs.

Our laboratory has previously reported direct conjugation to form GFP dimers using monomers functionalized with azide and alkyne containing tyrosine analogues. In order to introduce a unique nnAA at any desired site in a protein, methods for site-specific incorporation of tyrosine analogues (p-azido-L-phenylalanine, AZF; and *p*-propargyloxyphenylalanine, PPF; Figure 1A) developed by the Schultz group[30,31] were adapted for CFPS.[32,33] However, yields of proteins containing tyrosine analogues have been found to be significantly lower than yields of the corresponding wild-type proteins.[32,33]

In contrast, the global methionine replacement scheme enables the production of proteins containing AHA and HPG with high protein yields using CFPS.[34,35] Also, since proteins typically contain multiple methionine residues, multiple sites for bioconjugation are introduced using this scheme. The open cell-free system provides convenient control of the concentrations of methionine and methionine analogues during CFPS, thereby enabling efficient incorporation of these nnAAs without requiring a methionine auxotrophic *E. coli* production host strain. We also previously reported the use of this approach with the CFPS platform for the production of *Gaussia princeps* luciferase (GLuc)–antibody fragment bioconjugates for the detection of tumor cells.[35] The global methionine replacement scheme enabled the production of GLuc containing multiple HPG residues with high protein production yields. In addition, the site-specific nnAA incorporation scheme enabled the production of antibody fragment fusion proteins with a single azide-containing surface-exposed tyrosine analogue for conjugation to GLuc. The unique azide and alkyne side chains in GLuc and the antibody fragment fusion protein facilitated direct protein conjugation using click chemistry.[35]

In this work, we adopted a similar strategy to produce VLP bioconjugates displaying multiple surface proteins. We are interested in developing tumor idiotype-based vaccines for B cell lymphoma. The unique immunoglobulin idiotype expressed on the surface of B lymphoma cells can be used as an effective antigen in tumor-specific vaccines when fused to immunostimulatory proteins and cytokines. Patient-specific tumor idiotype-based vaccines for B cell lymphoma have shown tremendous promise since they have the potential to create protective memory responses that can help prevent relapse and extend the remission period after treatment.[36-38] These include full-length idiotype antibodies chemically cross-linked to keyhole limpet hemocyanin (KLH)[39] or genetically fused to cytokines including granulocyte macrophage-colony stimulating factor (GM-CSF), Interleukin 2 (IL2), Interleukin 4 (IL4), and Inter-feron gamma.[40] We previously reported the cell-free production of fusion protein vaccines containing idiotype single chain variable fragments (scFvs) fused to GM-CSF and the *E. coli* IM9 protein.[29]

In this work, we use a direct conjugation scheme to produce VLPs displaying idiotypic antibody fragment antigens along with the cytokine GM-CSF and immunostimulatory CpG DNA.[41] We utilized the global replacement scheme for substitution of methionine residues to produce VLPs with high yields and efficient incorporation of AHA and HPG using CFPS. In contrast, introduction of tyrosine analogues in MS2 and Qβ VLPs resulted in significantly reduced soluble monomer protein yields and consequently even lower yields of assembled VLPs. We produced GM-CSF and an idiotype antibody fragment fusion protein, each containing tyrosine analogues at a single site using CFPS. The site-specific incorporation scheme enabled the production of these proteins containing a single nnAA, without introducing any other mutations in the remaining amino acid sequence. GM-CSF and antibody fragment fusion proteins along with alkyne functionalized CpG DNA oligonucleotides were directly and simultaneously coattached to the surface of VLPs in a single reaction using click chemistry.

This direct attachment scheme provides, for the first time, efficient coconjugation of proteins, nucleic acids, and other molecules to VLPs, as well as control over the relative ratios and loading density of the ligands attached to the VLPs. This technology enables the production of custom-designed VLP bioconjugates for use as vaccines, therapeutics, diagnostics, bio-materials, and other nanotechnology products.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids for CFPS

Plasmid pET24a-MS2-cp[25] was used as a template to construct the mutant gene for incorporation of methionine analogues. DNA oligonucleotides GGATGGATCATATGGCATCTAATTTTACTCAGTTCGTTCTGGTTGACAACGGCGGTA**TG**GGCGACGTCACGGTGGCGC and ATCCATCCGCTAGCTTAGTAGATACCAGAGTTCGCGGCAATGGCAGATGGAATCGGGTTACCATCCTTCAGCAGACCCTGCA**G**GGCCTTCACAATCAGTTCGCAGTCGCTATTGGTGGCAAAGATTGGGATCGTCAGCTCCA**G**GTTCAGATAGCTACGCCAGGCCG were used to PCR amplify the gene encoding the MS2 coat protein while introducing 3 mutations (T16M, M89L and M109L; bases used to introduce mutations are underlined in bold font in the extension primer sequences). The PCR product was cloned between the T7 promoter and terminator in pET24a (Novagen, Madison, WI) using NdeI and NheI restriction sites (underlined) contained in the forward and reverse primers, respectively. This plasmid was named pET24a-MS2-cp-T16M-M89L-M109L.

The plasmid for expression of wild-type Qβ VLP coat protein (pET24a-Qβcp, obtained from Dr. Bradley Bundy) contains a gene for the Qβ coat protein. A Qβ K16 M mutant was designed for the introduction of surface exposed methionine analogues on the Qβ VLP. The gene for the Qβ coat protein K16 M mutant was codon-optimized for expression in *E. coli* using DNAworks.[42] The 5′ nucleotide sequence coding for the first 5 amino acids was taken from the gene sequence reported by Strable *et al.*[22] Overlapping oligonucleotides were designed for PCR-based gene synthesis of this DNA sequence using DNAworks. A two-step gene synthesis PCR method[43] was utilized to generate the Qβ coat protein mutant sequence. The Qβ coat protein mutant gene was cloned into a pET24a (Novagen) expression vector

between the T7 promoter and terminator using NdeI and NheI restriction sites. This plasmid was named pET24a-Qβcp-K16M.

The plasmid harboring the murine GM-CSF gene containing an amber stop codon for incorporation of tyrosine analogues was constructed as follows. Plasmid pK7mGM-CSF75–6H (N75TAG) (obtained from Dr. Aaron Goerke) contained the gene for murine GM-CSF with an amber stop codon (TAG) at position N75, a natural glycosylation site.[44] The 5′ coding sequence of this gene had previously been optimized for expression using the CFPS platform,[45] and this sequence also included a C-terminal hexahistidine purification tag. A Strep Tag II affinity purification tag (encoding amino acids WSHPQFEK) was introduced in place of the hexahistidine tag in the mGM-CSF gene by PCR using template plasmid pK7mGM-CSF75–6H (N75TAG) with forward and reverse PCR primers 5′GGATGGATCATATGGCACCAACACGTAGTCCTATCACTGTC and 5′GGCCGGCCGTCGAC*TTATTA***CTTTTCGAACTGCGGGTGGCTCCAGGAGCCACCTCCTCC**TTTTTGGACTGG, respectively. The forward extension primer contained an NdeI restriction site (underlined) and the reverse primer contained a *Sal*I site (underlined) downstream of a peptide spacer consisting of four glycines and a serine (in bold font), a Strep Tag II (underlined, in bold font) and two stop codons (italicized). The PCR product was cloned into expression vector pET24a using NdeI and *Sal*I restriction sites. This plasmid was named pET24a-mGM-75TAG-Strep. The construction of the plasmid pY71-IM9(28TAG)-(38C13)scFv for the expression of the antibody fragment as a fusion with the *E. coli* protein, IM9, containing a site for the incorporation of tyrosine analogues at position S28 in the IM9 domain was previously described.[35]

The above genes in expression plasmids were verified by DNA sequencing. Milligram quantities of plasmids were isolated from *E. coli* cultures grown for 16–20 h at 37 °C in Terrific Broth (Invitrogen) using Maxiprep and Gigaprep kits (Qiagen, Valencia, CA) as per manufacturer instructions.

Cell-Free Protein Synthesis

CFPS was conducted using the PANOx-SP (PEP, amino acids, nicotinamide adenine dinucleo-tide (NAD), oxalic acid, spermidine, and putrescine) cell-free system as described previously[46] with minor changes in component concentrations as specified. CFPS reactions were carried out in 15–30 μL volumes in 1.5 mL microcentrifuge tubes for small-scale process development purposes and at 0.5-1 mL volumes per well in 6 well tissue culture plates (BD Falcon #3046) for preparative purposes. 20 μM L-[U-^{14}C]-Leucine (Amersham Pharmacia, Piscataway, NJ) was added, where specified, for measuring protein yields as described below. All reagents were obtained from Sigma–Aldrich, St. Louis, MO, unless otherwise noted. The standard PANOx-SP CFPS reaction mixture includes: 1.2 mM ATP, 0.85 mM each of GTP, UTP, and CTP, 33 mM phosphoenol pyruvate (Roche Molecular Biochemicals, Mannheim, Germany), 175 mM potassium glutamate, 10 mM ammonium glutamate, 20 mM magnesium glutamate, 1.5 mM spermidine, 1.0 mM putrescine, 0.17 mg/mL folinic acid, 85.3 μg/mL *E. coli* tRNA mixture (Roche Molecular Biochemicals), 13-54 μg/mL plasmid as specified below, 100-300 μg/mL T7 RNAP (formulated in 10 mM KH_2PO_4 and 20% (w/v) sucrose at pH 8.0) as specified below, 2 mM of each of the 20

unlabeled amino acids, 0.33 mM NAD, 0.26 mM Coenzyme A (CoA), 2.7 mM potassium oxalate, and 0.28 volumes of *E. coli* S30 extract.

Cell extracts for CFPS were prepared from cells grown in a B. Braun C-10–2 No. 153 10-L fermentor on defined media with glucose and amino acid feeds using a procedure that promotes logarithmic growth to moderate cell density while minimizing acetate accumulation.[47] The fermentation was harvested at approximately 30 OD_{600}. All cultures were immediately centrifuged at 5000–8000g for 30 min at 4 °C and washed at 4 °C by resuspending in cold S30 buffer (10 mM Tris-acetate pH 8.2, 14 mM magnesium acetate, and 60 mM potassium acetate). The centrifugation and wash procedure was repeated one to two additional times, and the resulting cell paste was stored at −80 °C until it was processed into S30 cell extract. Frozen cell paste was thawed in 1 mL of S30 buffer per 1 g of cell paste and suspended to homogeneity with a model 700 rotary homogenizer (Fisher Scientific). The cells in suspension were lysed by a single pass through an Emulsiflex C-50 high-pressure homogenizer (Avestin, Ottawa, ON, Canada) at 17 500 to 25 000 psi. The homogenate was clarified by centrifugation at 30 000g at 4 °C, twice for 30 min each, and the resulting pellets were discarded. The supernatant was incubated for 80 min at 37 °C in the dark on a rotary shaker at 120 rpm. After this incubation, cell extract was flash–frozen and stored at −80 °C.

Cell extracts for VLP production were prepared from *E. coli* KC6 cells.[48] For global replacement of methionines in VLPs, methionine was left out of cell-free reaction mixtures, and substituted by 6 mM azidohomoalanine (Medchem Source LLP, Federal Way, WA) or homopropargylglycine (Chiralix B.V., Nijmegen, The Netherlands) as specified. CFPS reactions contained 45 μg/mL of VLP expression plasmid and 100 μg/mL of T7 RNA polymerase. Reactions were carried out at 37 °C for 3 h.

E. coli cell extracts for the production of the GM-CSF and IM9scFv proteins containing PPF were prepared from KGK10 cells[49] harboring a plasmid for the constitutive expression of the orthogonal t-RNA from *Methanococcus jannaschii* [pDule-tRNA, described by Goerke *et al*.[52]]. CFPS reactions contained 54 μg/mL or 13 μg/mL of plasmid for expression of GM-CSF or IM9scFv protein, respectively, along with 300 μg/mL of T7 RNA polymerase. The reaction was modified to encourage formation of disulfide bonds.[50,51] First, the cell extract was pretreated at room temperature for 30 min with 50 μM iodoacetamide (IAM). Prior to template DNA addition, a glutathione buffer (4 mM oxidized glutathione, GSSG; and 1 mM reduced glutathione, GSH) was added to the cell-free reaction to stabilize the thiol/disulfide redox potential. Finally, DsbC, a periplasmic disulfide bond isomerase, was prepared as described previously[45] and added to a final concentration of 100 μg/mL. This foldase was stored at 5 mg/mL in a 30% glycerol solution at −80 °C prior to use. 4 mM PPF (synthesized using the procedure described by Deiters et al.[52]) and 500 μg/mL of purified tRNA synthetase (PpaRS-His, prepared as described previously[33]) were added to the cell-free reactions. This tRNA synthetase was produced in *E. coli* BL21(DE3) cells and purified by Ni-NTA chromatography. Purified tRNA synthetase was formulated at approximately 5 mg/ml in 10 mM potassium phosphate buffer, pH 8, with 20% sucrose and stored at −80 °C. Protein synthesis reactions were incubated at 30 °C for 10 h.

Quantification of Protein Yields by Liquid Scintillation Counting

Following the cell-free reaction period, samples were placed on ice to stop the reaction. 3 μL of quenched reaction mixture was spotted on a filter paper and allowed to dry. The remainder of the cell-free reaction solution was immediately centrifuged at 20 800g for 15 min at 4 °C, to isolate the soluble fraction. An equal volume of the supernatant was spotted on filter paper and allowed to dry. The total and soluble protein concentrations were then determined using the trichloroacetic acid procedure described previously to precipitate the synthesized protein.[53] The L-[U-^{14}C]-Leucine radioactivity was quantified by a LS3801 liquid scintillation counter (Beckman Coulter, Brea, CA). The quantity of total and soluble protein produced was then calculated based on incorporated radioactivity and the leucine content of the protein of interest.

Purification of VLPs by Sucrose Density Gradient Ultracentrifugation

Assembled VLPs were purified after CFPS using sucrose gradient ultracentrifugation as described previously.[25] To assess VLP assembly, 700 μL fractions of the sucrose gradient containing the VLP produced with L-[U-^{14}C]-Leucine were collected and the concentration of VLP in each fraction was determined by radioactivity measurement as described. Fractions were analyzed for purity by SDS-PAGE and Coomassie staining (described below).

Confirmation of Incorporation of nnAAs by Electrospray Mass Spectrometry

MS2 VLPs, produced with 6 mM AHA or HPG, were digested with chymotrypsin or AspN (as specified), and resulting peptides were subsequently analyzed by electro-spray mass spectrometry using an Eksigent nano 2D LC and an LCQ Deca XP+ mass spectrometer.

Strep Tag II Affinity Purification of GM-CSF and IM9scFv Containing PPF

Multiple 0.5 mL CFPS reactions for GM-CSF and IM9scFv containing PPF were pooled and dialyzed against 100 volumes of Strep Load Buffer (SLB: 150 mM NaCl, 100 mM Tris-HCl, 1 mM EDTA, pH 8) for 12 h before loading onto a 1 mL StrepTactin Sepharose (IBA Gmbh, Gottingen, Germany) column equilibrated with 10 mL of SLB. Columns were washed three times with 5 mL SLB and eluted with 0.5 and 2 mL of Strep Elution Buffer (SEB: 150 mM NaCl, 100 mM Tris-HCl, 1 mM EDTA, 2.5 mM desthiobiotin, pH 8). The second elution fraction (2 mL) containing purified protein was dialyzed against 2 L 10 mM potassium phosphate buffer, pH 8, for 12 h with three buffer exchanges, the last one containing 20% sucrose. Protein concentrations were quantified using a DC protein assay (BioRad, Hercules, CA) using BSA as a standard. Purified proteins, GM-CSF (N75PPF) and IM9scFv (S37PPF), were stored at −80 °C.

Synthesis of Alkyne-CpG DNA Oligonucleotide

A 20-mer CpG oligonucleotide (CpG 1826: 5′TCCATGA**CG**TTCCTGA**CG**TT)[41] was synthesized by the W. M. Keck Biotechnology Resource Lab at Yale University (New Haven, CT) containing a 3′ alkyne modifier, 5-Octadiynyl-dU (Berry & Associates, Dexter, MI). The immunostimulatory CpG motifs are indicated in bold font.

Azide–Alkyne Click Reactions

The (3 + 2) cycloaddition or "click" reactions were conducted in an anaerobic glovebox (Coy Laboratories, Grass Lake, MI) in the absence of oxygen to preserve the reduced state of the 1 mM tetrakis(acetonitrile)-copper(I)hexafluorophosphate catalyst ($[(CH_3CN)_4Cu]PF_6$) (Sigma Aldrich) or hereafter referred to as the Cu(I) catalyst. 0.5 mM tris(triazolylmethyl) amine Cu ligand (TTMA) enhancer (obtained from the Professor Christopher Chidsey Laboratory at Stanford University, Stanford, CA) was added to improve the rate of the click reactions.[54] The Cu(I) catalyst and TTMA enhancer were added to conjugation reaction mixtures from 10 mM and 2.5 mM stock solutions, respectively. Stock solutions were prepared in 10 mM potassium phosphate buffer (pH 8). Click reaction components, prepared in potassium phosphate buffer (pH 8), were mixed and then deoxygenated by incubation in 1.5 mL microcentrifuge tubes for 45–90 min in the anaerobic chamber before the addition of the reduced copper catalyst. Conjugation reactions were performed in 10–20 μL volumes for process development purposes and in volumes of up to 1 mL for preparative purposes. 200 μL or greater volume reaction mixtures were mixed by axial inversion during the incubation period. Reactions were allowed to proceed under anaerobic conditions for up to 20 h after the addition of the Cu(I) catalyst, as specified.

Size Exclusion Chromatography–High Performation Liquid Chromatography

A 200 μL sample containing 6 μg of either unconjugated Qβ (AHA) VLPs or Qβ (AHA) VLPs after conjugation with 1 kDa alkyne-PEG was separated in a Supelco GFC500 (150 mm length, 4.6 mm internal diameter) column (Sigma-Aldrich) at 0.2 mL/minute with a running buffer consisting of 10 mM Tris-HCl, pH 7.4, and 100 mM sodium chloride. Absorbance at 280 nm was detected in line. Protein-containing fractions were collected separately and concentrated with a 10 kDa MWCO centricon centrifugal filter (Millipore, Billerica, MA). The concentrated fractions were analyzed for Qβ coat protein and Qβ coat protein conjugated to PEG by SDS-PAGE gel electrophoresis and Coomassie staining.

Analysis of Proteins by SDS-PAGE and Autoradiography

Protein samples were loaded onto NuPAGE Bis-Tris gels under reducing conditions (with 50 mM DTT) and were run with MES running buffer (Invitrogen, Carlsbad, CA). Proteins were visualized by Coommassie blue staining. VLPs labeled with L-[U–^{14}C]-Leucine were analyzed by SDS-PAGE and by autoradiography after drying the gels and exposing to a storage phosphor screen (Molecular Dynamics, Sunnyvale, CA) when specified. Screens were scanned using a Typhoon Scanner (GE Healthcare, Piscataway, NJ). Densitometry analysis was conducted with *ImageJ* software (*ImageJ* 1.41o, National Institutes of Health, USA).

Cell Proliferation Assays for Assessing GM-CSF Bioactivity

Cell proliferation assays for assessing GM-CSF bioactivity were performed as described[29] using the GM-CSF dependent cell-line NFS-60. The bioactivities of the GM-CSF (N75PPF) protein and VLP bioconjugates containing GM-CSF (serial diluted from 5 nM down to 0.028 pM, based on final GM-CSF concentrations) were assessed in triplicate. Recombinant murine GM-CSF (R&D systems, Minneapolis, MN) was used as a standard for comparison.

RESULTS AND DISCUSSION

Production of VLPs Containing AHA and HPG Using CFPS

We used a scheme for global replacement of methionine residues to produce VLPs with exposed azide and alkyne reactive groups as described for *in vivo* incorporation using *E. coli*.[22] The genes encoding for MS2 and Qβ were modified such that each contained the ATG codon for methionine in an exposed surface loop region (in place of T16 and K16, respectively) in addition to the start codon. The other wild-type methionine residues were mutated to leucine residues to prevent undesired incorporation of nnAA at these locations. While Qβ has no additional methio-nine residues, the MS2 gene required two methionine to leucine modifications, at positions 89 and 109. These genes were cloned into pET24a expression vectors to enable cell-free expression from the T7 promoter.

We successfully produced MS2 and Qβ coat protein VLPs containing AHA and HPG at high assembled yields using the PANOx-SP CFPS system.[46] 6 mM methionine, AHA, or HPG were each added individually to the appropriate replicate reactions. VLP protein yields were assessed using liquid scintillation counting of incorporated L-[U–^{14}C]-Leucine. 100 μL CFPS reaction products were analyzed using sucrose density ultracentrifugation as described previously[25] to assess the efficiency of VLP assembly (Figure 2). Velocity sedimentation profiles were determined by measuring the radioactivity of collected fractions from a 10–40% continuous sucrose gradient. A total of 24 0.7 mL fractions (#1-24) were collected from the top to the bottom of the gradient. The VLP protein obtained (μg) in each fraction is reported in Figure 2. Fractions 1–5 contain unassessembled monomers and dimers, and fractions 7–19 contain assembled VLPs. CFPS protein production yields and assembly efficiencies for the MS2 and Qβ VLPs containing AHA and HPG are shown in Table 1. Introduction of tyrosine analogues at position 16 in both MS2 and Qβ VLPs using the site-specific nnAA incorporation scheme resulted in significantly reduced soluble monomer protein yields and consequently even lower yields of assembled VLPs (data not shown).

Fractions containing purified assembled VLPs were pooled and concentrated using Amicon Ultra 4 centrifugal concentrators (Millipore, Billerica, MA) and analyzed by SDS-PAGE and Coomassie staining (Figure 2D). In all cases, fractions containing purified VLPs also contained three dominant impurity bands, corresponding to the three subunits (~100 kDa, ~80 kDa, and ~56 kDa in size) of the pyruvate dehydrogenase complex (PDC) (Figure 2C,D). The PDC is a 3750 kDa complex consisting of 16 polypeptide units each of the three different subunits,[55] and is approximately the same size and density as the VLPs. As a result, the PDC proteins co-migrated with the assembled VLPs in the sucrose density gradient. Assembled VLPs were found to appear in fractions 7–17, while the PDC proteins were most abundant in fractions 5–9. As a compromise between maximizing VLP recovery and purity, we chose to pool and retain fractions that contained significant amounts of assembled VLPs (fractions 10–17 for MS2 VLPs and fractions 9–16 for Qβ VLPs) along with only relatively small amounts of the PDC contaminants. Using SDS-PAGE analysis and densitometry, the purity of the MS2 (AHA), MS2 (HPG), Qβ (AHA), and Qβ (HPG) preparations (Figure 2D) were estimated to be approximately 85%, 75%, 75%, and 70%, respectively. In the VLP preparations used in this work, the copurified PDC proteins did not contain nnAAs and

therefore did not participate in the conjugation reactions. We subsequently identified a solution to this contamination problem. Research has shown that the PDC can be dissassembled in the presence of chaotropic agents such as guanidinium–HCl.[56] We have found that the addition of 1 M urea to CFPS reaction mixtures prior to loading onto the sucrose gradient results in little or no co-purification of the PDC proteins along with assembled VLPs (data not shown).

The M89L, M109L mutations along with incorporation of AHA or HPG at the T16 position in MS2 only slightly affected soluble VLP monomer production and VLP assembly as compared to the wild-type sequence (Figure 2A). In the case of the Qβ VLP (Figure 2B), the incorporation of AHA did not deleteriously affect VLP assembly. However, incorporation of HPG reduced the assembly efficiency of Qβ VLPs to ~54% of the soluble protein. Using this scheme, VLPs containing AHA and HPG were produced with high protein yields.

Confirmation of Incorporation of nnAAs by Electrospray Mass Spectrometry

MS2 VLPs, produced with 6 mM HPG, were digested with chymotrypsin or AspN and analyzed by mass spectrometry. Analysis of a peptide containing residue 16 after digestion with chymotrypsin indicated ~83% incorporation of HPG at position 16 (with an instrument error of ±30%). The remaining fraction (~17%) of this peptide containined methionine at position 16. Analysis of the N-terminal peptide after AspN digestion revealed ~11.5% incorporation of HPG at the N-terminus. Only trace amounts (0.9%) of N-terminal peptide that retained the N-terminal methionine were detected. The remaining 87.6% of monomers did not contain an N-terminal methionine or nnAA. The amino acid immediately following the N-terminal methionine is an alanine residue in both MS2 and Qβ. According to the findings reported by Wang *et al.*,[57] the N-terminal amino acid is therefore likely to be efficiently removed by the *E. coli* methionine aminopeptidase, which is consistent with the above observations. MS2 VLPs (produced with 6 mM AHA), digested with chymotrypsin and analyzed by mass spectrometry, were estimated to contain ~85% AHA at position 16 (with an instrument error of ±30%). The remaining fraction (~15%) of the MS2 VLP monomers contained methionine at position 16. Attempts to detect and analyze the N-terminal peptide resulting from AspN digestion of MS2 (AHA) VLPs were unsuccessful.

Conjugation of Functionalized PEG and Nucleic Acids to the VLP Surface

After confirming the incorporation of methio-nine analogues in VLPs produced using CFPS with 6 mM nnAA, we conjugated azide and alkyne functionalized PEG molecules to the surface of the VLPs. 20 μL conjugation reactions were prepared containing 2 μM VLP (VLP concentrations are reported throughout as VLP monomer concentrations) and 200 μM 1 kDa alkyne-PEG (Creative PEG works, Winston-Salem, NC) or 2 kDa azido-PEG (Nanocs, Inc., New York, NY). Conjugation reactions were incubated in an anaerobic chamber for 8 h and analyzed by SDS-PAGE and Coomassie staining (Figure 3A). Densitometry analysis indicated that ~71% and ~95% of the VLP monomers were PEGylated for MS2 (AHA) and Qβ (AHA) VLPs, respectively. Also, approximately 58% PEGylation of MS2 (HPG) VLPs was achieved. These data confirm that the incorporated nnAAs were surface accessible for conjugation by azide–alkyne click chemistry.

We also conjugated a single-stranded DNA oligonucleotide to MS2 (AHA) VLPs (Figure 3B). A 20 nucleotide long CpG oligonucleotide (CpG 1826)[41] was functionalized with a 3′ alkyne modifier. Using a 3-fold and 10-fold excess of alkyne functionalized CpG DNA (alkyne-CpG) relative to VLP monomer, 21% and 15% of VLP monomers were conjugated with DNA, respectively. Increasing CpG DNA concentrations from 60 μM to 200 μM resulted in a reduction in the fraction of MS2 monomers conjugated with CpG DNA. There is evidence to suggest that the presence of chloride ions inhibits the rates of click reactions by competing with azide groups for Cu(I).[58] Perhaps the polyanionic CpG DNA could be interfering with the conjugation reactions in a similar manner.

Verification of VLP Integrity after Attachment of PEG Using Size Exclusion Chromatography.

Qβ (AHA) VLPs, before and after conjugation to 1 kDa alkyne-PEG, were analyzed by size exclusion chromatography–high performance liquid chromatography (SEC-HPLC) to verify VLP integrity after attachment. The absorbance at 280 nm was monitored in-line over a period of 30 min after sample injection (Figure 3B). The flowthrough peak at 9.1-10.4 min (1.82-2.08 mL), the Qβ VLP peak at 10.4-13.0 min (2.08-2.6 mL), and the disassembled Qβ coat protein at 19.1-22.3 min (3.82-4.46 mL) were collected separately and concentrated with a 10 kDa MWCO centricon centrifugal filter (Millipore). The concentrated fractions were analyzed for Qβ coat protein and Qβ coat protein conjugated to PEG by SDS-PAGE gel electrophoresis and Coomassie staining (Figure 3C). A fraction of the Qβ VLPs was found to disassemble after attachment of PEG. Analysis of peak areas indicated that ~70% of VLPs remained assembled after PEGylation (Figure 3), which is in agreement with previous observations with recovery after VLP conjugations.[22]

Direct Attachment of Proteins to the Surface of VLPs

Having demonstrated effective conjugation of PEG and nucleic acids to VLPs, we now sought to develop methods for co-attachment of multiple proteins and other molecules to the surface of VLPs. We produced VLPs containing the methionine analogue, AHA, and two proteins (GM-CSF and the IM9scFv fusion protein) containing the tyrosine analogue, PPF (Figure 1). This scheme was attractive since it avoided the need for AZF, which is sensitive to exposure to light. AHA, an aliphatic azide, is stable to light exposure. Also, we have observed that yields of proteins containing PPF consistently tend to be higher than with AZF. We also observed high assembly efficiencies for both MS2 and Qβ VLPs produced with AHA (Table 1). In addition, higher incorporation efficiencies as well as higher conjugation efficiencies with functionalized poly(ethylene glycol) were observed with MS2 VLPs containing AHA. GM-CSF and the IM9scFv fusion protein containing PPF were produced using CFPS and purified using Strep Tag II affinity chromatography (Figure 4A). Purified yields tended to vary from 10 to 30 μg/mL for GM-CSF (N75PPF) and from 75 to 100 μg/mL for IM9scFv (S37PPF).

To demonstrate direct protein–protein conjugation with VLPs, we conjugated GM-CSF (N75PPF) and IM9scFv (S37PPF) to the surface of MS2 (AHA) VLPs. Conjugation reactions containing 20 μM MS2 (AHA) VLPs and 0.5-5 μM GM-CSF (N75PPF) and IM9scFv (S37PPF) as specified were incubated for 12 h, and the reaction products were

analyzed by SDS-PAGE and autoradiography. For a given concentration of MS2 (AHA), increasing the concentrations of GM-CSF (N75PPF) resulted in an increased surface abundance of GM-CSF conjugated to VLPs (Figure 4A). Also, no unconjugated GM-CSF was detected after 12 h reactions suggesting complete conjugation of GM-CSF to the surface of VLPs. Similarly, with 20 μM MS2 (AHA) and 5 μM IM9scFv (S37PPF), conjugation of ~70–80% of the IM9scFv (S37PPF) protein to the VLP surface was achieved. A relatively small amount of residual IM9scFv (S37PPF) protein was observed after the 12 h incubation period. With Qβ (AHA) VLPs, we observed a significantly lower fraction of IM9scFv (S37PPF) protein conjugated to the VLPs, and the majority of the IM9scFv (S37PPF) protein remained unreacted (data not shown). AHA at position 16 in Qβ VLPs is likely less accessible for conjugation with proteins compared to AHA at position 16 in MS2 VLPs.

Having demonstrated the ability to individually conjugate IM9scFv (S37PPF) and GM-CSF (N75PPF) to the surface of VLPs, we subsequently coattached GM-CSF (N75PPF) and IM9scFv (S37PPF) to MS2 (AHA) VLPs. Figure 4B qualitatively shows that, by varying the relative ratio of GM-CSF (N75PPF) and IM9scFv (S37PPF) in conjugation reactions, we can control their relative VLP surface abundance after conjugation.

Assembly of Custom-Designed VLP Bioconjugates by Direct Conjugation

Using this direct conjugation platform, next we sought to produce VLP bioconjugates displaying IM9-scFv and GM-CSF as well as CpG DNA by means of a single reaction step. VLP bioconjugates were prepared in 0.4 to 0.6 mL volumes with 1, 2, or 3 conjugated adducts (individual component concentrations listed in Table 2). After a reaction period of 8 h, a 3-fold excess of CpG DNA was added to reaction 3 to maximize conjugation of CpG DNA. After 12 h, a 100-fold molar excess of HPG, the alkyne-containing methionine analogue (Figure 1), along with half the initial amount of Cu(I) catalyst was added to all the conjugation mixtures to react with any remaining azide groups in the VLPs, and reactions were allowed to proceed for an additional 4 h. This final step was included to quench the reaction and stabilize any remaining unreacted azide groups on the VLPs.

VLP bioconjugates were separated from unconjugated proteins by centrifugation at 21 000 rcf for 12 h at 4 °C in 1.5 mL microcentrifuge tubes. For each VLP conjugate, supernatant containing unconjugated proteins was removed using a micro-pipet and the pellet containing the VLP bioconjugates was resuspended in PBS buffer, pH 7.4, with 0.01% Tween 20 and 20% sucrose (w/v). Purified VLP bioconjugates were analyzed by SDS-PAGE and Coomassie staining (Figure 5A). All VLP vaccine candidates were designed to contain a higher abundance of conjugated antigen protein (IM9scFv) relative to the immunostimulatory cytokine (GM-CSF) in order to favor the generation of immune responses to the antigen and minimize the risk of autoimmune responses to GM-CSF. SDS-PAGE analysis indicated that a minor fraction of the unconjugated IM9scFv (S37PPF) protein remained in all purified VLP conjugate preparations. Similar co-purification of unconjugated IM9scFv protein was observed with VLP bioconjugates purified using sucrose density ultracentrifugation (data not shown). It is possible that the click reaction conditions resulted in partial IM9scFv denaturation and aggregation with the conjugated protein. Disrupting possible nonspecific interactions of unconjugated IM9scFv protein with

assembled VLP vaccines during the centrifugation at 21 000 rcf for 12 h at 4 °C in 1.5 mL microcentrifuge tubes by means of increasing salt concentrations (0, 100, and 500 mM NaCl), introducing mild denaturing conditions (250 mM, 500 mM, and 1 M urea), and adding a reducing agent (10 mM DTT) did not reduce the amount of co-purified unconjugated IM9scFv protein (data not shown).

The direct protein conjugation scheme enabled the surface modification of VLPs with proteins and nucleic acids as well as poly(ethylene glycol) and provided control over the relative surface abundance of the attached species. Conjugation schemes employing maleimide and NHS reactions do not provide complete control on the conjugation reactions due to the presence of multiple reactive amine and sulfhydryl groups in nearly all proteins. In addition, these methods require careful consideration with regard to the order of conjugation of multiple species and typically require multiple steps, thereby reducing the overall efficiency and convenience. The unique azide and alkyne reactive groups at specific sites on the VLPs enables the convenient one-step conjugation of multiple species in the desired ratios and surface abundance. This site-specific conjugation scheme also takes advantage of precise placement of the unique nnAAs in proteins for subsequent conjugation so as to provide control over the orientation of individual conjugation partners in the resulting conjugates.

Assessment of GM-CSF Bioactivity before and after Conjugation to VLPs

The ability of the GM-CSF (N75PPF) to stimulate the proliferation of cells was assessed before and after conjugation to VLPs in the various vaccines using an established cell proliferation assay. NFS-60 cells, which require GM-CSF for growth, were cultured in the presence of serial dilutions of GMCSF (N75PPF) and the different vaccine candidates. Growth of cells was monitored by the incorporation of tritiated thymidine and was quantified using liquid scintillation counting. The incorporation of tritiated thymidine (in scintillation counts per minute or cpm) in cultures to which were added serial dilutions of samples containing GM-CSF was used to assess the bioactivity of GM-CSF (Figure 5B).

The bioactivity of GM-CSF (N75PPF) before and after conjugation to VLPs was compared to that of a recombinant murine GM-CSF standard. The GM-CSF (N75PPF) protein was produced using CFPS and was purified and formulated in buffers containing 0.01% Tween 20. The bioactivity of GM-CSF (N75PPF) was found to be only marginally lower than that of the recombinant murine GM-CSF standard suggesting that the GMCSF (N75PPF) mutant retained its bioactivity. The MS2/IM9scFv VLPs did not contain any GM-CSF and therefore did not stimulate the proliferation of cells. Bioactivities of GM-CSF in bioconjugates **2** (MS2/IM9scFv/GM-CSF) and **3** (MS2/IM9scFV/GM-CSF/CpG) were found to be 3 to 5-fold lower than that of CFPS GM-CSF (N75PPF). We had previously determined that exposure of GM-CSF (N75PPF) to 1 mM Cu(I) and 0.5 mM TTMA for 12 h in a mock conjugation reaction did not adversely affect the bioactivity (data not shown). The apparent lower specific activity after conjugation may be attributed to a more limited steric availability, where all GM-CSF molecules that are conjugated to the VLP vaccines are not accessible to the cells. In other words, the curvature of the VLP could be preventing access of the cell-surface receptors to all the conjugated GM-CSF molecules on the VLP surface.

Moreover, the crowding of the VLP surface with IM9scFv protein may further prevent access of the cell-surface GM-CSF receptors to the GM-CSF and thereby contribute to reducing the bioactivity of GM-CSF after co-attachment to VLPs. In addition, the site for conjugation could result in an orientation of GM-CSF with less steric availability of the active binding regions of GM-CSF. Encouragingly, all VLP bioconjugates that included GM-CSF were shown to display cytokine-dependent bioactivity in the NFS-60 cell-based assay.

CONCLUSIONS

We successfully developed technologies for the direct conjugation of multiple adducts, including proteins, nucleic acids, and small molecules to the surface of VLPs. CFPS enabled the incorporation of azide and alkyne containing nnAA using 2 different schemes. Global replacement of methionine residues enabled the high-level cell-free production of VLPs containing surface accessible methionine analogues for conjugation. CFPS is well-suited for producing proteins containing methionine analogues because the absence of cell walls allows greater control over the concentrations of both methionine and the nnAA without requiring a methionine auxotrophic *E. coli* production host strain. However, the use of this method is constrained to proteins where mutation of existing methionine residues is not deleterious to protein folding or function. Site-specific incorporation of tyrosine analogues in a fusion protein containing an idiotypic scFv antigen and in GM-CSF, a cytokine, provided a unique reactive group in each of these proteins without introducing additional mutations. The incorporation of unique reactive groups in VLPs and other proteins enabled direct conjugation by azide-alkyne click chemistry. The direct conjugation scheme enabled efficient coupling of 3 different adducts to the surface of VLPs in a single step with control over the ratios and surface abundance of attached species. This technology was used for the single-step assembly of VLPs displaying idiotypic antigens along with an immunostimulatory protein and a stimulatory nucleic acid species to produce novel VLP-based tumor idiotype vaccine candidates for treating B cell lymphoma.

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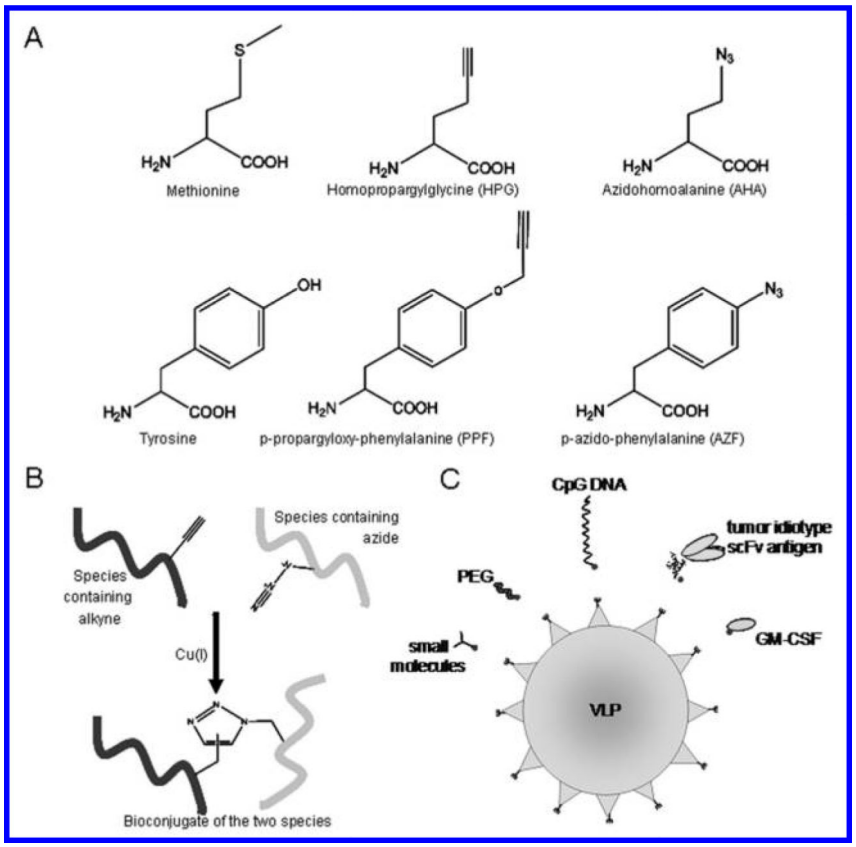

Figure 1.

(A) Azide and alkyne containing non-natural amino acids (analogues of methionine and tyrosine) used in this study. (B) Schematic of direct protein conjugation by click chemistry. Molecules containing azide and alkyne reactive groups can be directly coupled in a single step using Cu(I) catalyzed click chemistry. (C) This scheme is used for the direct coupling of proteins, nucleic acids, and small molecules to the surface of VLPs.

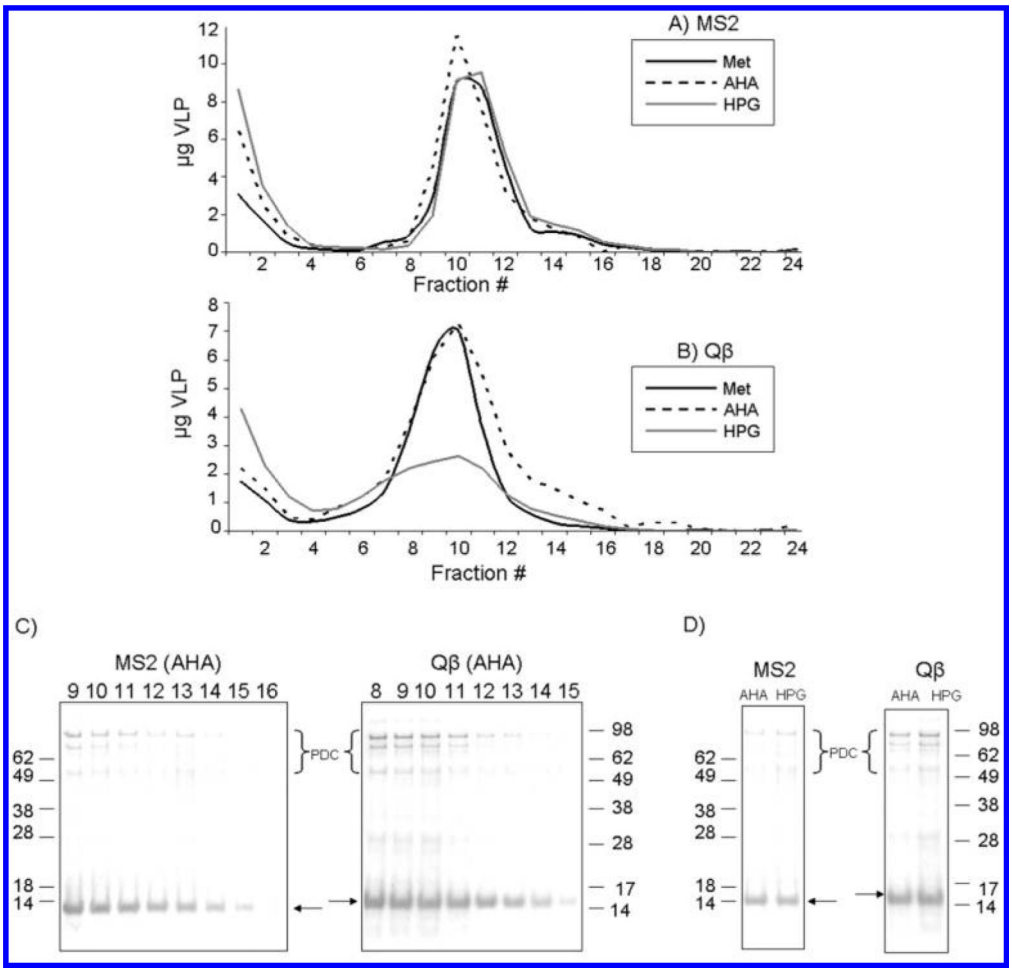

Figure 2.

Sucrose density ultracentrifugation for assessing VLP assembly and for purification of assembled VLPs from crude CFPS reaction mixtures. (A) Wild-type MS2 VLPs produced with methionine (Met) were compared with MS2-T16M-M89L-M109L mutants produced with the methionine analogues, AHA and HPG. (B) Wild-type Qβ VLPs produced with methionine (Met) were compared with Qβ-K16 M mutants produced with methionine analogues, AHA and HPG. (C) SDS-PAGE analysis of sucrose density centrifugation fractions 10–17 for MS2 (AHA) (with the Seeblue molecular weight marker (Invitrogen) as a reference) and fractions 9–16 for Qβ (AHA) (with the Seeblue Plus2 molecular weight marker (Invitrogen) as a reference) indicated that these fractions contained assembled VLPs in relatively pure form. (D) Purified MS2 and Qβ VLPs containing AHA or HPG were analyzed by SDS-PAGE (horizontal arrows indicate VLP monomers). The Seeblue and Seeblue Plus2 molecular weight markers (Invitrogen) were used as a reference in the SDS-PAGE analysis of the purified MS2 and Qβ VLPs, respectively. The 3 subunits of the pyruvate dehydrogenase complex (PDC) co-purified with the assembled VLPs as indicated by brackets.

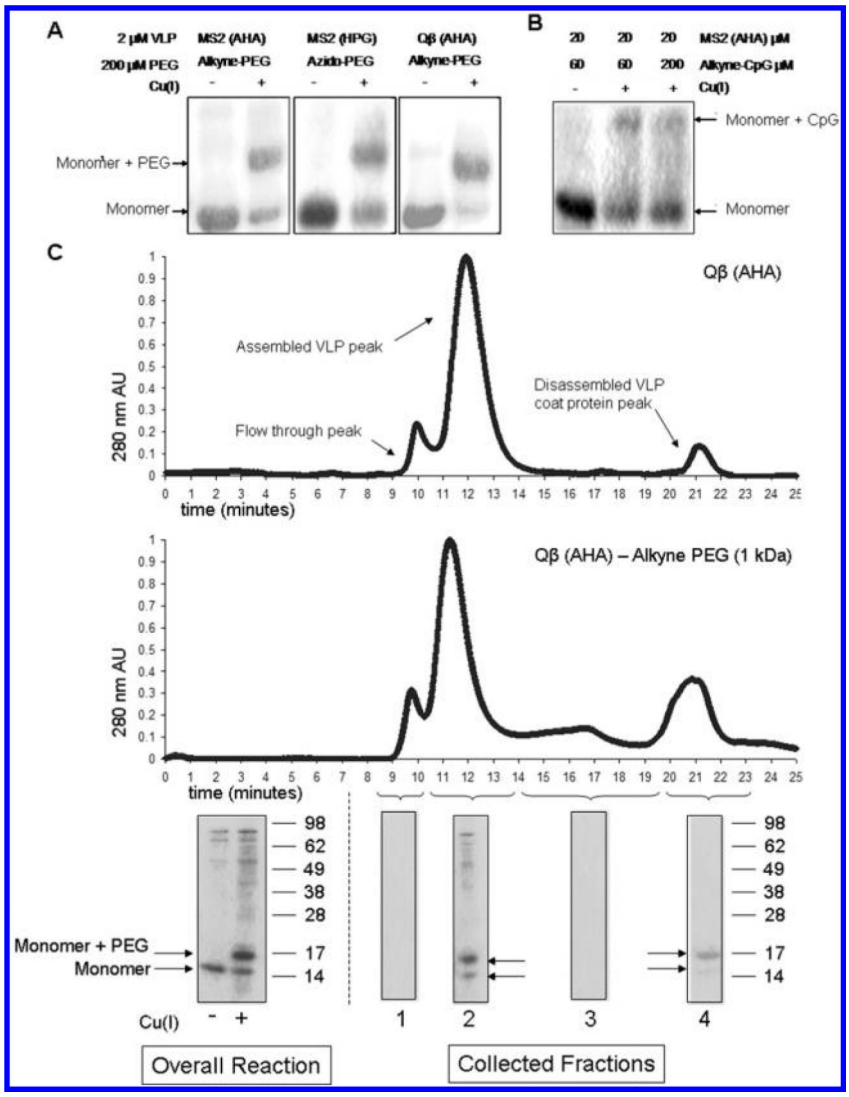

Figure 3.

(A) PEGylation of VLPs containing AHA and HPG. Click reaction products were analyzed by SDS-PAGE and Coomassie staining. Unmodified and modified VLP monomers are indicated with horizontal arrows. Densitometry analysis indicated that ~71% and ~95% of the VLP monomers were PEGylated for MS2 (AHA) and Qβ (AHA) VLPs, respectively. Also, approximately 58% PEGylation of MS2 (HPG) VLPs was achieved. (B) Alkyne-CpG (CpG) was conjugated to MS2 (AHA) VLPs. Unmodified and modified VLP monomers are indicated with horizontal arrows. Using a 3-fold and 10-fold excess of alkyne functionalized CpG DNA (alkyne-CpG) relative to VLP monomer, 21% and 15% of VLP monomers were conjugated with DNA, respectively. (C) Size exclusion HPLC analysis of purified Qβ (AHA) and PEG conjugated Qβ (AHA) VLPs (absorbance at 280 nm over time is shown). Fractions 1, 2, 3, and 4 were collected separately, concentrated, and analyzed along with samples of the conjugation reaction mixtures by SDS-PAGE and Coomassie staining. The Seeblue Plus2 molecular weight marker (Invitrogen) was used as a reference. In the conjugation reaction containing the Cu(I) catalyst (+), ~85% of the VLP monomers appear

to be PEGylated, compared to the negative control lane (−) in which no Cu(I) catalyst was added. Fraction 2 contained assembled PEGylated VLPs, while fraction 4 contained disassembled VLP monomers. Proteins in fraction 1 (flowthrough peak containing aggregates and high molecular weight species) and fraction 3 were not present at a significant abundance to be visualized by SDS-PAGE and Coomassie staining.

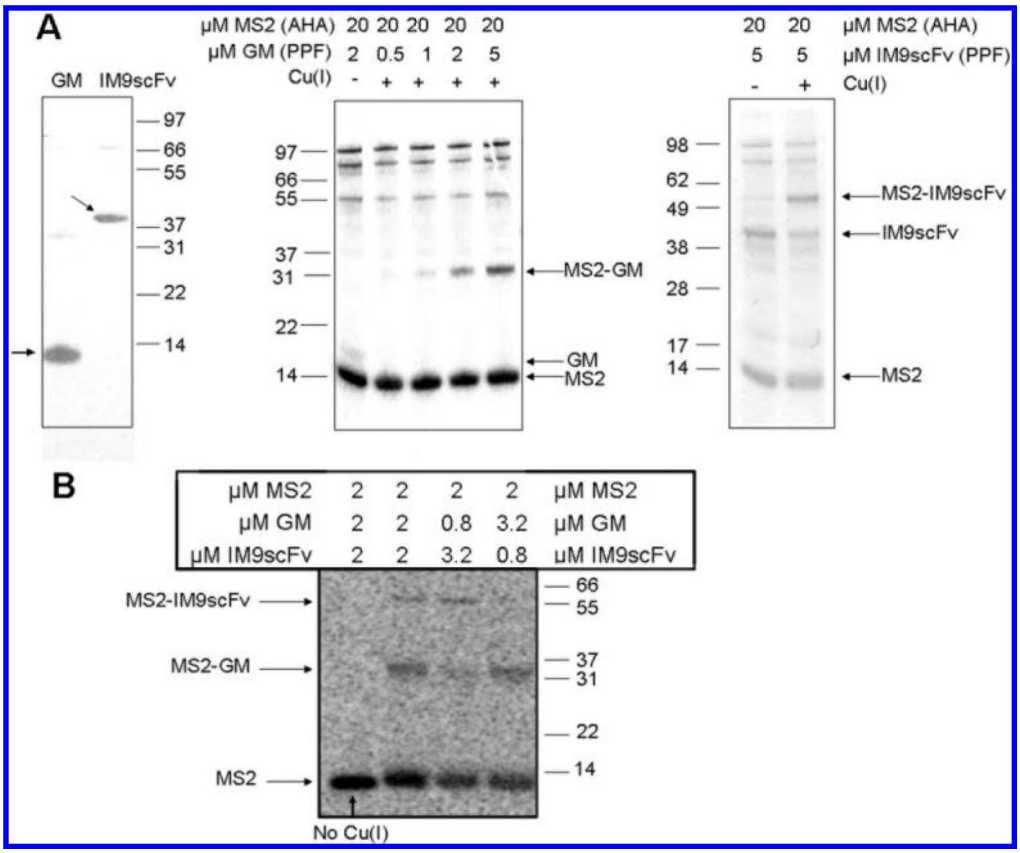

Figure 4.

(A) Purified proteins GM-CSF (N75PPF) and IM9scFv (S37PPF) analyzed by SDS-PAGE and Coomassie staining. The Mark 12 molecular weight marker (Invitrogen) was used as a reference. Arrows indicate the protein bands of interest. GM-CSF (N75PPF) and IM9scFv (S37PPF) were conjugated to the surface of MS2 (AHA) VLPs. SDS-PAGE analysis of the MS2 (AHA) and GM-CSF (N75PPF) conjugation products was performed with the Mark 12 molecular weight marker (Invitrogen) as a reference. Increasing the concentration of GM-CSF (N75PPF) or (GM) relative to MS2 (AHA) VLPs (MS2) results in an increase in intensity of the band corresponding to the MS2-GM conjugate. For all concentrations of GM tested, no detectable unconjugated GM is visible suggesting that conjugation proceeds essentially to completion. SDS-PAGE analysis of the MS2 (AHA) and IM9scFv (S37PPF) conjugation products was performed with the Seeblue Plus2 molecular weight marker (Invitrogen) as a reference. Only a relatively small amount of unreacted IM9scFv (S37PPF) protein is visible, suggesting ~70-80% conjugation of this protein to MS2 VLPs. (B) Autoradiogram to demonstrate coattachment of two proteins to the surface of L-[U-14C]-Leucine labeled MS2 (AHA) VLPs. The Seeblue Plus2 molecular weight marker (Invitrogen) was used as a reference. This autoradiogram qualitatively shows that varying the ratio of the GM-CSF (N75PPF) protein (GM) and the IM9scFv (S37PPF) protein (IM9scFv) in conjugation reactions adjusts the relative ratio of the species conjugated to the MS2 (AHA) VLPs (MS2).

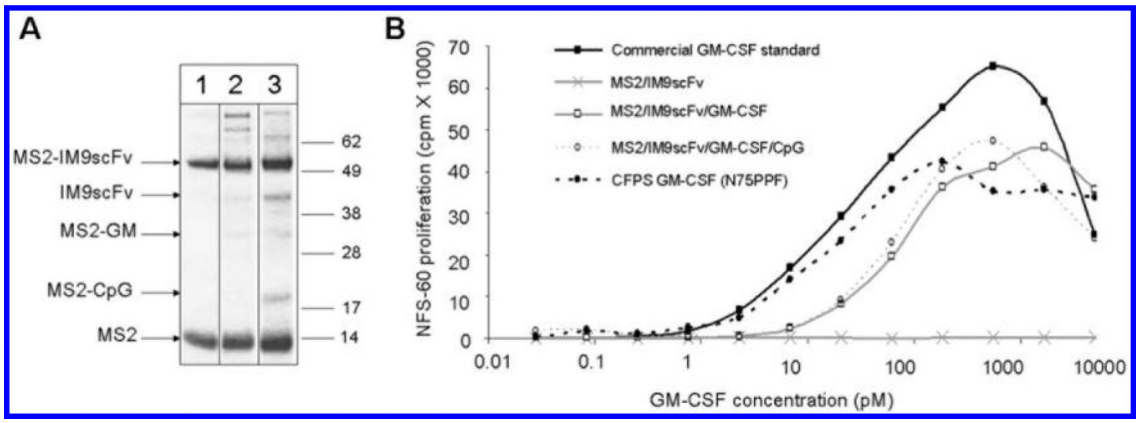

Figure 5.

(A) Purified VLP bioconjugates: MS2 VLPs conjugated with (1) IM9scFv, (2) IM9scFv and GM-CSF, and (3) IM9scFv, GM-CSF, and CpG DNA analyzed by SDS-PAGE and Coomassie staining. The Seeblue Plus2 molecular weight marker (Invitrogen) was used as a reference. On the basis of densitometry, VLP bioconjugates were estimated to include (1) ~50 molecules of IM9scFv, (2) ~50 molecules of IM9scFv and ~6 molecules GM-CSF, and (3) ~50 molecules of IM9scFv, 6 molecules of GM-CSF, and ~20 molecules of CpG DNA per 180-mer VLP. In addition to the expected bands, a relatively minor fraction of co-purified IM9scFv protein is observed in each lane. (B) Assessment of the bioactivity of GM-CSF before and after conjugation to VLPs. GM-CSF (N75PPF) is nearly as active as the commercial GM-CSF standard. The specific activity of GM-CSF (N75PPF) is ~3–4-fold lower after conjugation to VLPs. All VLP bioconjugates that contained GM-CSF displayed cytokine dependent bioactivity.

Table 1

Cell-Free Production Yields and Assembly Efficiencies of VLPs Produced with Methioine (Met) and Methionine Analogues (AHA and HPG)[a]

VLP with (20th amino acid)	total protein (μg/mL)	soluble protein (μg/mL)	% assembled
MS2-WT (Met)	460 ± 45	374 ± 19	83
MS2-T16M-M89L-M109L (AHA)	663 ± 33	427 ± 2	74
MS2-T16M-M89L-M109L (HPG)	611 ± 90	468 ± 44	68
Qβ-WT (Met)	310 ± 41	298 ± 13	85
Qβ-K16 M (AHA)	470 ± 16	400 ± 29	81
Qβ-K16 M (HPG)	360 ± 46	265 ± 2	54

[a]Wild-type MS2 VLPs (MS2-WT) produced with methionine (Met) were compared with MS2-T16M-M89L-M109L mutants produced with the methionine analogues, AHA and HPG. Similarly, wild-type Qβ VLPs (Qβ-WT) produced with methionine (Met) were compared with Qβ-K16M mutants produced with the methionine analogues, AHA and HPG. The average values and 1 standard deviation are reported for total and soluble protein produced in triplicate. Assembled VLPs were separated from VLP monomers by sucrose gradient ultracentrifugation, and the percentage of soluble VLP proteins isolated as assembled VLPs is reported here.

Table 2

Conjugation Reaction Mixtures for the Production of VLP Bioconjugates[a]

	1	2	3	
μM MS2 (AHA)	135	90	90	$t = 0$
μM IM9scFv (PPF)	45	30	30	
μM GM-CSF (PPF)	-	6	6	
μM alkyne-CpG	-	-	10	
μM alkyne-CpG			270	$t = 8$ h
mM HPG	13.5	9	9	$t = 12$ h
reaction volume μL	400	600	600	

[a] 1: MS2/IM9scFv, 2: MS2/IM9scFv/GM-CSF, 3: MS2/IM9scFv/GM-CSF/CpG. VLP bioconjugates were prepared in 0.4–0.6 mL volumes with reactant concentrations listed above. Reactions were prepared containing 0.5 mM TTMA and 1 mM Cu(I) catalyst and were incubated under anaerobic conditions for a total of 16 h. The addition of alkyne-CpG and HPG at 8 and 12 h did not significantly increase the conjugation reaction volumes.