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Escherichia coli-Based Cell-Free Synthesis of Virus-Like Particles

Bradley C. Bundy,[1] Marc J. Franciszkowicz,[1] James R. Swartz[1,2]

[1]Department of Chemical Engineering, Stanford University, Stanford, California 94305-5025; telephone: 650-723-5398; fax: 650-725-0555; e-mail: jswartz@stanford.edu

[2]Department of Bioengineering, Stanford University, Stanford, California, 94305-5444

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ABSTRACT: Virus-like particles (VLP) have received considerable attention for vaccine, drug delivery, gene therapy and material science applications. Although the number of unique VLP and their applications are rapidly growing, the positive impact of VLP applications is limited by the current diverse, expensive, and typically low-yielding production technologies available. These technologies, when scaled, often result in structurally and compositionally inconsistent products. We present *Escherichia coli*-based cell-free protein synthesis as a production technology to overcome many of the limitations of current VLP production processes. Using this technique, the MS2 bacteriophage coat protein VLP was produced at a yield 14 times the best published production yield. Also, a C-terminally truncated Hepatitis B core protein VLP was produced at similarly high yields (6×10^{13} VLP/mL). These VLP were found to have comparable characteristics to those produced in vivo. The scalability of this technology was tested without loss in production yields. To our knowledge, this is the first time a prokaryote-based in vitro transcription/translation system has generated a virus-like particle.

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KEYWORDS: cell-free protein synthesis; VLP; virus-like particles; MS2; hepatitis B

Introduction

Recently viruses have been reported as the most abundant biological entity on the planet (Suttle, 2005). They are so simple in structure and function that they are not classified as living organisms. Yet, viruses have the special ability to protect their cargo from diverse and biologically harsh environments until they find their target cells. This unique capability to protect, deliver, and release valuable molecular cargo, has inspired many researchers to chemically and genetically modify viruses for material science and pharmaceutical applications. For the vast majority of these applications, a genome-free or non-infectious virus-like construct is required. This has led to an explosion of research on the production and modification of virus-like particles (VLP)—genome-free virus capsids formed from the structural proteins of viruses.

These VLP are of great interest for vaccination as they: (1) stimulate strong T cell and B cell responses, due to their repeated and ordered surface (Bachmann and Jennings, 2004), (2) do not cause infection, as they are genome-free, (3) have a long serum half-life due to their size and stability, and (4) are the perfect size for uptake into lymphatic vessels for recognition by the immune system (Reddy et al., 2006). The FDA approved human Papillomavirus vaccine and the human Hepatitis B vaccines are two examples of VLP-based vaccines. While VLP can serve as excellent vaccines against the virus from which they originated, VLP can also serve as vaccine-templates against virtually any disease by surface presentation of exogenous antigens. Although initial attempts to present foreign epitopes fused into the capsid proteins was largely unsuccessful, attachment by chemical linkage to exposed amino acids has been successfully demonstrated for a number of different ligands (Bachmann and Jennings, 2004; Kratz et al., 1999). Examples of ligands attached to the surface of VLP using this technique include: fluorophores (Peabody, 2003), antibodies (Brown et al., 2002), ligands for cell-specific receptor-mediated endocytosis (Wu et al., 1995), poly(ethylene glycol) (Brown et al., 2002), and immunogens for vaccines (Lechner et al., 2002). Cytos Biotechnology has made significant strides in this area with a nicotine coated Qβ phage VLP vaccine that is currently in phase II clinical trials (Maurer and Bachmann, 2006).

The ability to modify VLP has extended VLP applications into material science and drug and gene delivery applications as well. VLP have been used as templates for depositing metal oxide, orienting gold nanoparticles, growing inorganic nanocrystals, and incorporating Gd(III) ions for magnetic resonance imaging (Allen et al., 2005; Blum et al., 2004; Douglas and Young, 2006; Shenton et al., 1999).

Correspondence to: J.R. Swartz

Although there are still many challenges to overcome with VLP-based delivery systems, such as increasing the drug or gene encapsidation efficiency and releasing the cargo upon delivery, the feasibility of cell-specific drug delivery with VLP has been demonstrated (Brown et al., 2002; Wu et al., 1995).

With an ever expanding list of potential applications for VLP, the need for innovative manufacturing technology for the cost effective production of VLP is increasing. Although the human Papillomavirus VLP vaccine *Gardasil* sells at $1,000,000 per gram (Rothengass, 2007), it took a team of scientists and engineers at Merck several years to develop the right conditions to economically express, disassemble, reassemble, and form the correct disulfide bonds to produce uniform, highly immunogenic VLP (Kosinski, 2006).

The *Gardasil* engineering feat is an example of a major problem with VLP production, which is the inability to scale many of the laboratory processes to produce VLP and thus the inability to produce a compositionally and architecturally consistent product (Pattenden et al., 2005). Also, although many laboratory processes claim assembly yields as high as 80%, other studies have shown very low efficiencies especially when disassembly and reassembly is required (Pattenden et al., 2005). Often the reassembly pathway is complicated by the need for chaperones and scaffolding proteins. In a recent review of the technology available to produce VLP, the authors conclude that the current methods for producing VLP are either unscalable or when scaled do not easily provide consistent products. The authors further conclude that, ''New bioprocess technology is needed to shift a larger number of existing and novel VLP from being mere laboratory curiosities to being clinically valuable products'' (Pattenden et al., 2005).

We propose optimized *Escherichia coli*-based cell-free protein synthesis (CFPS) as an excellent bioprocess technology for the production of virus-like particles. The cell-free environment allows direct access to and control over the transcription, translation and VLP assembly environment for production optimization. Of particular interest are optimal VLP assembly conditions that are difficult to establish in vivo such as non-physiological ionic strength, pH, and/or redox potential (Ceres and Zlotnick, 2002). Also of interest when producing viral proteins is the ability of cell-free protein synthesis to produce high yields of proteins that are often cytotoxic or insoluble in vivo (Stiege and Erdmann, 1995). The Cell-free technology used in this study has been scaled from 15 μL to 1 L reaction volumes using conventional fermentation equipment without losses in protein production yield or activity (Voloshin, 2006; Voloshin and Swartz, 2005, 2007). Other advantages of the cell-free system include the potential for single-step purification and recovery (Alimov et al., 2000; Jewett and Swartz, 2004b) and the focus of metabolic resources only toward the transcription and translation of the product. Recent advances to our *E. coli*-based cell-free system have made the technique even more economically competitive by the activation of glycolysis and oxidative phosphorylation (Calhoun and Swartz, 2005; Jewett, 2005). Also, by employing linear DNA templates, the time and labor intensive process of cloning and vector transformation is greatly reduced (Woodrow et al., 2006). Thus, the development and production of a VLP-based vaccine could be expedited for time sensitive applications such as personalized cancer treatment and viral epidemic vaccination. Another advantage is that the cell-free environment is approximately 20-fold more dilute than the cell interior (Underwood et al., 2005) and VLP formed from this system would be expected to be less contaminated with encapsidated protein or nucleic acid. This could potentially eliminate the need for disassembly and reassembly or chemical treatment after VLP production (Pattenden et al., 2005).

To test our *E. coli*-based system, the MS2 bacteriophage coat protein VLP (MS2 VLP) and a C-terminally truncated Hepatitis B core protein VLP (HBV VLP) were chosen as model VLP, as they have been well characterized. The MS2 VLP originates from the icosahedral ssRNA MS2 bacteriophage. Although the virus capsid contains one molecule of protein A and 180 coat protein molecules, the coat protein has been shown to self-assemble into a stable 180mer VLP (triangulation number of $T = 3$) when expressed recombinantly in *E. coli* in the absence of protein A (Mastico et al., 1993; Pickett and Peabody, 1993). The Hepatitis B virus is an enveloped DNA virus that infects hepatocytes in humans. Its capsid forming core antigen has been found to self-assemble when expressed recombinantly in *E. coli* to form both the 180mer ($T = 3$) and 240mer ($T = 4$) icosahedral VLP (Wingfield et al., 1995). A mutant truncated at amino acid 149 has been reported to form predominantly the $T = 4$ VLP (Zlotnick et al., 1996).

By producing the MS2 VLP and HBV VLP, we have demonstrated the first use of an *E. coli*-based cell-free protein synthesis system for the production of efficiently assembled VLP. Furthermore, both the MS2 VLP and the HBV VLP were produced with high assembly efficiency at substantially higher yields than previously reported.

Materials and Methods

Plasmid Construction

The MS2 coat protein gene was optimized for both *E. coli* tRNA relative concentrations and synthesis from oligonucleotides using DNAworks v3.0 (Hoover and Lubkowski, 2002). Oligonucleotides (60 bp average length, Operon Technologies, Huntsville, AL) based on sequences recommended by DNAworks were assembled into the optimized MS2 coat protein gene sequence using two-step PCR. pET24a-MS2cp was generated by ligation (T4 DNA ligase, New England Biolabs, Ipswich, MA) of the optimized MS2 coat protein gene into the pET-24a(+) vector (Novagen, San Diego, CA) at the Nde I and Sal I restriction sites. pET24a-MS2cp was transformed into DH5α cells (One Shot MAXX

Efficiency DH5α-T1^R Competent Cells, Invitrogen, Carlsbad, CA) and the plasmid was purified with Qiagen Plasmid Maxi Kit (Qiagen, Valencia, CA) for use in cell-free protein synthesis (CFPS). The sequence encoding the human Hepatitis B core antigen of subtype adyw (Pasek et al., 1979) with the C-terminus truncated at amino acid 149 was optimized for *E. coli* tRNA concentrations and was synthesized from oligonucleotides designed with DNAworks v3.0. The vector pET24a-HBc149 was prepared using the same procedure as for pET24a-MS2cp with the exception of using the Xho I instead of the Sal I restriction site.

Cell-Free Protein Synthesis

The PANOxSP system (Jewett and Swartz, 2004a) 30 μL cell-free protein synthesis (CFPS) reactions were incubated at 37°C for 3 h in 1.5 mL Eppendorf tubes or in 96-well plates (Fisher Scientific, Waltham, MA). The volume was increased to 1 mL for the scaled-up thin film reactions which were performed in 6-well plates (Fisher Scientific). The reaction includes the following components: 1.2 mM AMP, 0.85 mM each of GMP, UMP, and CMP, 34 μg/mL folinic acid, 170 μg/mL *E. coli* tRNA mixture, 12 nM plasmid (pET24a-HBc149 or pET24a-MS2cp), 100 μg/mL T7 RNA polymerase, 5 μM L-[U-14C] Leucine, 2 mM each of 20 unlabeled amino acids, 0.33 mM nicotinamide adenine dinucleotide (NAD), 0.27 mM coenzyme A (CoA), 33 mM phosphoenolpyruvate, 1.5 mM spermidine, 1 mM putrescine, 170 mM potassium glutamate, 10 mM ammonium glutamate, 20 mM magnesium glutamate, 2.7 mM sodium oxalate, 10 mM phosphate in the form of potassium phosphate dibasic adjusted to pH 7.2 with acetic acid, and 24% v/v of S30 extract prepared as described previously (Liu et al., 2005). T7 RNA polymerase was prepared from *E. coli* strain BL21 (pAR1219) as described by Grodberg and Dunn (1988). L-[U-^{14}C]Leucine was purchased from GE Healthcare (Piscataway, NJ). *E. coli* tRNA mixture was obtained from Roche Molecular Biochemicals (Indianapolis, IN). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO). Total synthesized protein yields were determined by TCA-precipitation and radioactivity measurement using Beckman ReadySafe Scintillation Cocktail and a Beckman LS3801 liquid scintillation counter (Beckman Coulter, Fullerton, CA). Soluble yields were determined by TCA-precipitation and scintillation counting of the supernatants following sample centrifugation at 25°C and 15,000 RCF for 15 min. The detailed procedure has been described previously (Jewett and Swartz, 2004b). Full-length product was verified by SDS–PAGE gel and autoradiography. Mark12 Unstained Standard MW marker, CFPS product, and the soluble fraction of the CFPS product were applied to separate lanes of a NuPAGE 10% Bis-Tris Gel. Samples were run under reduced conditions in the presence of 62.5 mM dithiothreitol (DTT) and NuPAGE LDS Sample Buffer. Gels were stained with SimplyBlue SafeStain and dried with a gel dryer model 583 (Bio-Rad, Richmond, CA), before exposure to Kodak BioMax MR Autoradiography Films (Rochester, NY). SDS–PAGE gels and gel-related reagents were obtained from Invitrogen.

VLP Assembly

To remove unincorporated L-[U-14C] leucine, the cell-free product was immediately dialyzed in 6-8000 MWCO Specra/Pro Molecularporous Membrane Tubing (Spectrum Labs, Rancho Dominguez, CA) against 300 mL of buffer overnight with 2 buffer exchanges. CFPS product from 30 μL reactions producing MS2 VLP were dialyzed against Buffer A (10 mM Tris and 100 mM NaCl adjusted to pH 7.5 with HCl). CFPS product from 30 μL reactions producing HBV VLP were dialyzed against Buffer B (100 mM HEPES and 200 mM NaCl adjusted to pH 7.5 with HCl). The 1 mL CFPS reaction products were dialyzed against Buffer C (10 mM BisTris and 0.385 M NaCl adjusted to pH 5.5 by HCl). The dialyzed cell-free reaction product was subjected to velocity sedimentation. Ten to forty percentage weight per volume continuous sucrose density gradients were prepared in Buffer A, B, or C as indicated in Polyallose 16×102 mm Centrifuge Tubes (Beckman) with the Gradient Master Ver3.05L Gradient Maker (Biocomp Instruments, Inc., Fredericton, Canada). The dialyzed cell-free reaction product (500 μL) was layered on top of the sucrose and centrifuged at 31,000 rpm in a Beckman-Coulter SW-32 swinging bucket rotor (Fullerton, CA) in a Beckman L8-M ultracentrifuge at 4°C for 3.5 h with profile 7 slow acceleration and deceleration. One-half milliliter fractions were collected using a Teledyne ISCO Foxy Jr. Density Gradient Fractionation System (Lincoln, NE) and the concentration of MS2 coat protein or Hepatitis B core antigen in each fraction was determined by radioactivity measurement using Beckman ReadySafe Scintillation Cocktail and a Beckman LS3801 liquid scintillation counter. Collected fractions were analyzed by NuPAGE 10% Bis-Tris Gel under reducing conditions followed by autoradiography as described above.

The extent of VLP assembly was determined by the sum of VLP monomer in the assembled VLP peak fractions divided by the sum of VLP monomer in all the fractions of the gradient. Peak fractions were 6 through 15 for both the MS2 and the HBV VLP. The sedimentation coefficients of the VLP peaks were estimated relative to the location of the 30S, 50S, and 70S ribosomal peaks. The ribosomal profile on the 10–40% sucrose gradient was determined by analyzing CFPS product using the same procedure as described above with the exceptions of loading the gradient immediately after the CFPS reaction, preparing the sucrose gradient in Mg^{2+} containing Buffer D (10 mM Tris, 200 mM NaCl, and 5 mM $MgCl_2$ adjusted to pH 7.5 with HCl) for ribosome stabilization, and recording the 254 nm absorbance profile using the Teledyne ISCO Fractionation System's UA-6 UV detector with a 254 nm filter.

Transmission Electron Microscopy

Sucrose gradient fractions containing CFPS-produced VLP—fractions 7–9 for HBV VLP and fractions 9–12 for MS2 VLP—were concentrated with Amicon Ultra-4 100,000 MWCO Centrifugal Filter Devices. The units were centrifuged for 15 min at 5,500 rpm and 4°C in a Sorvall RC5B Centrifuge with a Fiberlite F13-14 × 15cy rotor and Fiberlight 15 mL adaptors (Piramoon Technology, Santa Clara, CA). A 5 μL sample of concentrated VLP solution (37 nM for MS2 VLP and 181 nM for HBV VLP) was applied to a carbon coated copper/Formvar grid and negatively stained with 1% w/v uranyl acetate, pH 4. Photographs were taken with a Gatan 967 CCD camera in a JEOL 1230 electron microscope at 80 kV acceleration voltage. VLP diameters were determined with ImageJ 1.36b software (Abramoff et al., 2004).

Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) was performed with a Q100 DSC (TA Instruments, New Castle, DE). Sucrose gradient fractions containing CFPS-produced VLP were washed and concentrated to 2.1 mg/mL for HBV VLP and 2.9 mg/mL for MS2 VLP in 0.37 M NaCl with Amicon Ultra-4 100,000 MWCO Centrifugal Filters (Millipore, Billerica, MA) and 10 μL samples were sealed in Aluminum Hermetic Pans (TA Instruments). Samples were equilibrated against a reference Hermetic Pan with 10 μL of 0.37 M NaCl and scanned between 20 and 100°C at 5°C/min. Data was analyzed using Universal Analysis 2000 (TA Instruments).

pH Stability

Sucrose gradient fractions containing CFPS-produced VLP were pooled and aliquoted into separate dialysis containers of 6–8,000 MWCO Specra/Pro Molecularporous Membrane Tubing (Spectrum Labs) and dialyzed for 12 h against 0.5 L of buffers of different pHs ranging from 3 to 12 in 0.385 M sodium chloride. Alkaline buffers contained 10 mM CAPS and acidic buffers contained 10 mM acetic acid which were adjusted to a specific pH with hydrochloric acid or sodium hydroxide. The pH of the buffers was monitored periodically throughout the dialysis period and did not change by more than ±0.2 pH units. The pH measurements were made with a calibrated Orion 9107BN Triode pH Electrode (Thermo Scientific, Waltham, MA). The VLP sample was then subjected to a 10–40% continuous sucrose density gradient sedimentation as described previously with the exception that the gradients were prepared in the same buffer and pH used for dialysis. One-half milliliter fractions were collected and measured for radioactivity as described earlier. The percent VLP recovery was then calculated based on the amount of radiation (radiolabeled VLP) in the VLP containing fractions (fractions 6–15 for both MS2 and HBV VLP).

Nucleic Acid Encapsidation Assays

UV Absorption

Ultraviolet (UV) light absorption spectra are determined for CFPS-produced MS2 and HBV VLP in 0.37 M sodium chloride using a Hewlett Packard Diode Array Spectrophotometer (Model 8452A) and an Ultra-Micro 50 μL cuvette (Agilent Technologies, Santa Clara, CA). VLP were purified and concentrated as described previously by sucrose gradient sedimentation and Amico Ultra-4 filters. Measurements were adjusted to a blank of 0.37 M sodium chloride.

Agarose Gel

For verification of nucleic acid encapsidation, a 1% w/v agarose gel (Invitrogen) was prepared in 50 mM sodium phosphate pH 7.0 (Sigma–Aldrich) with 0.3 μg/mL ethidium bromide (Invitrogen) and loaded with 1.1 μg of purified CFPS-produced MS2 VLP. The gel was subjected to a constant potential of 18 V for 6 h and stained with SimplyBlue SafeStain (Invitrogen).

Polyacrylamide Gels

1.1, 0.11, and 0.011 μg of purified CFPS-produced MS2 VLP and 1.0, 0.1, and 0.01 μg of purified CFPS-produced HBV VLP were incubated in TBE Urea Sample Buffer at 70°C for 15 min before loading on a Novex TBE-Urea gel. Both 10-well 6% polyacrylamide and 15-well 15% polyacrylamide TBE-Urea gels were used. Perfect RNA Markers, 0.2–10 kb (Novagen) at 1 or 0.1 μL loads and 0.1 μL of 10 bp DNA Ladder (Invitrogen) were used as markers. The DNA Ladder was treated with formamide (Sigma–Aldrich) to a final concentration of 49% v/v and all markers were incubated in TBE Urea Sample Buffer at 70°C for 15 min before loading on the gel. Gels were subjected to a constant 180 V for 75 min in TBE running buffer and viewed under UV light following ethidium bromide staining per the manufacture's recommendation. All gel containers were treated with RNAse AWAY Reagent and all buffers and washes were prepared with diethyl pyrocarbonate (DEPC) (Sigma–Aldrich) treated deionized water. Unless specified, all materials were obtained from Invitrogen.

Isoelectric Point Calculation

The Isoelectric points of the VLP monomers were estimated using Protein Calculator v3.3 (Christopher Putnam, Scripps Research Institute). The calculation assumes all residues have the same pK_a values as isolated residues and does not account for interactions in a folded protein.

Results and Discussion

Cell-Free Protein Synthesis of VLP

The MS2 bacteriophage coat protein VLP (MS2 VLP) and a truncated human Hepatitis B core antigen VLP (HBV VLP) were chosen as model VLP as both have been well characterized and studied for VLP applications. The MS2 VLP has remarkable stability in biologically unfriendly environments and has been engineered for drug delivery (Brown et al., 2002; Wu et al., 1995) and foreign antigen presentation (Mastico et al., 1993). The HBV VLP's surface is decorated with an ordered array of projecting alpha helices which have been exploited for successful foreign antigen display in vaccine development (Pumpens and Grens, 2001). The full-length Hepatitis B core antigen VLP is dimorphic forming both the $T=3$ (180 monomers) and $T=4$ (240 monomers) VLP. However, the VLP from the Hepatitis B core antigen truncated at amino acid 149 has been shown to form predominantly (>95%) the $T=4$ VLP (Zlotnick et al., 1996).

Both the MS2 bacteriophage coat protein gene and the C-terminally truncated Hepatitis B core antigen gene (encoding for amino acids 1–149) were assembled from custom oligonucleotides to optimize codon frequency relative to *E. coli* tRNA concentrations and were cloned separately into the pET24a+ plasmid (Novagen). PANO × SP cell-free protein synthesis (CFPS) reactions (Jewett and Swartz, 2004a) were performed to express the MS2 bacteriophage coat protein and truncated Hepatitis B core antigen. The MS2 core protein and the truncated Hepatitis B core antigen were produced in 30 μL reactions at 479 μg/mL (Standard Deviation = 16 μg/mL, $n=3$) and 445 μg/mL (SD = 18 μg/mL, $n=3$), respectively, and in both cases more than 96% of the total synthesized polypeptide was soluble (Fig. 1). Full length product was verified by SDS-PAGE gel autoradiography (Fig. 2). The potential scalability of the CFPS of VLP was tested by increasing the volume from 30 μL to 1 mL using the method of Voloshin and Swartz (2005). The MS2 core protein and truncated Hepatitis B core antigen were produced at yields of 525 μg/mL (SD = 25 μg/mL, $n=3$) and 436 μg/mL (SD = 13 μg/mL, $n=3$), respectively, with ≥92% solubility (Fig. 1). No yield loss was observed for the larger volume reactions.

The assembly of the MS2 and HBV VLP was analyzed by subjecting dialyzed cell-free reaction product to velocity sedimentation. The fractions, collected top to bottom, were analyzed for ^{14}C-labeled MS2 coat protein or truncated Hepatitis B core antigen by measuring the radioactivity in each fraction. Sedimentation coefficients were estimated by comparison to the ribosome and ribosomal subunit profile (Fig. 3). The MS2 coat protein predominantly sedimented to approximately the 70S sedimentation coefficient location and the truncated Hepatitis B core antigen migrated to approximately the previously reported 45S VLP location indicating both MS2 and HBV VLP assembly (Zlotnick et al., 1996). Based on the sucrose gradient profiles, the assembly efficiency for both the MS2 VLP and the HBV VLP produced from 30 μL CFPS reactions was ≥80% resulting in assembled VLP yields of 384 μg/mL (9×10^{13} VLP/mL) and 356 μg/mL (5×10^{13} VLP/mL), respectively. The VLP assembly efficiency at 1 mL reaction volumes was also at

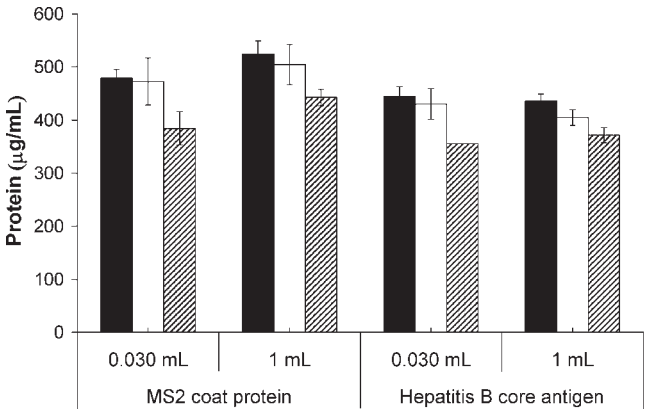

Figure 1. Total (black bars) and soluble (white bars) protein production and VLP assembly yields (stripped bars) from 30 μL and 1 mL cell-free protein synthesis reactions for both the MS2 coat protein and truncated Hepatitis B core antigen. The error bars = 1 Standard Deviation and $n=3$ (with the exception of the HBV VLP assembly yield for the 30 μL reaction where $n=1$).

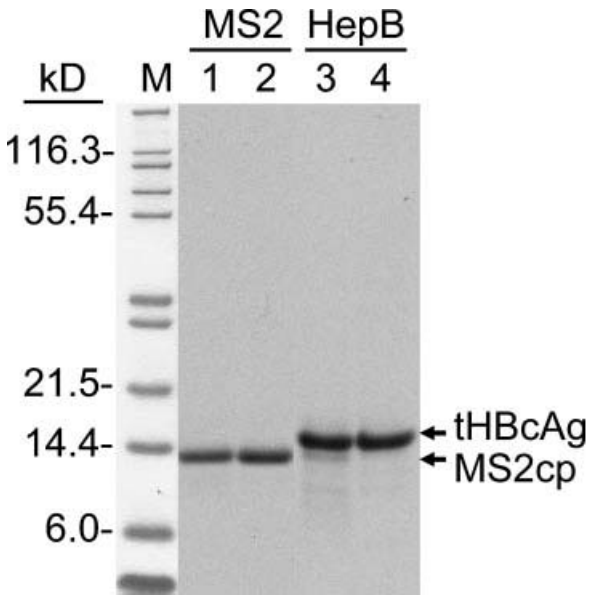

Figure 2. Autoradiogram of SDS–PAGE gel of total and soluble CFPS product when expressing MS2 coat protein (MS2cp) and C-terminally truncated Hepatitis B core antigen (tHBcAg). Lane M: SimplyBlue SafeStain stained SDS–PAGE gel image of Mark12 Unstained Standard aligned with autoradiogram lanes 1–4. **Lane 1**: CFPS MS2 coat protein reaction product. **Lane 2**: Soluble fraction of MS2 coat protein reaction product. **Lane 3**: truncated Hepatitis B core antigen reaction product. **Lane 4**: Soluble fraction of truncated Hepatitis B core antigen reaction product.

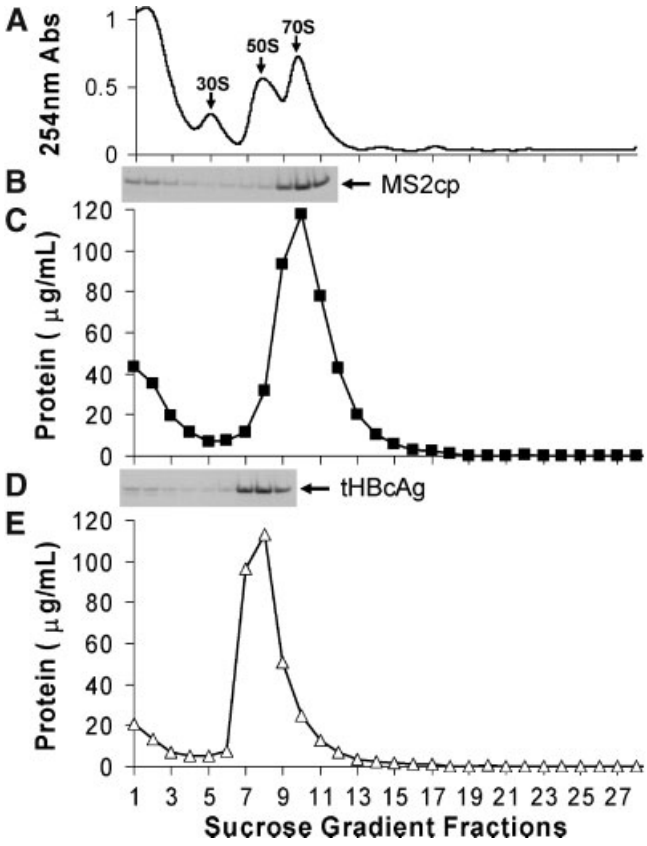

Figure 3. Indication of VLP assembly and estimation of the sedimentation coefficients for CFPS-produced MS2 and HBV VLP. **A**: Ribosome and ribosomal subunit profile (30S, 50S, and 70S) after velocity sedimentation with a 10–40% continuous sucrose gradient as measured by 254 nm absorbance. Autoradiogram (**B**) and velocity sedimentation profile (**C**) for MS2 coat protein (MS2cp) as determined by radioactivity of collected fractions from a 10% to 40% continuous sucrose gradient (filled squares). Autoradiogram (**D**) and velocity sedimentation profile (**E**) for truncated Hepatitis B core antigen (tHBcAg) also determined by radioactivity of collected fractions from a 10% to 40% continuous sucrose gradient (open triangles). Each fraction represents 0.5 mL and fractions 1–28 were collected top to bottom.

≥80% efficiency with VLP yields of 442 μg/mL (1×10^{14} VLP/mL) and 372 μg/mL (6×10^{13} VLP/mL) for the MS2 and HBV VLP (Fig. 1). These are the highest reported yields of MS2 VLP: over 14 times more than the 30 μg/mL reported from MS2 VLP generated by virus infection and pH treatment (Hooker et al., 2004), over 22 times more than the 20 μg/mL MS2 VLP generated from recombinant expression of MS2 coat protein in *E. coli* (Peabody, personal communication), and over 44 times more than the 10 μg/mL MS2 VLP reported for recombinant expression of MS2 coat protein in *Saccharomyces cerevisiae* (Legendre and Fastrez, 2005). The CFPS-produced HBV VLP yield is also the highest reported yield, significantly more than the 200μg/mL reported from recombinant expression of the C-terminally truncated (AA1-149) Hepatitis B core protein in *E. coli* (Zlotnick et al., 1996) and over 90 times greater than the 4 μg/mL reported for full-length HBV VLP expressed with the rabbit reticulocyte-based cell-free system (Lingappa et al., 2005) (Table I).

Characterization by TEM

To verify that the peak fractions containing MS2 coat protein and truncated Hepatitis B core antigen from the sucrose gradients contained correctly formed VLP, transmission electron microscopy with 1% w/v uranyl acetate negative staining was used (Fig. 4). The average diameter was 27.2 nm (SD = 0.9 nm, $n = 100$) for MS2 VLP and 30.7 nm (SD = 1.3 nm, $n = 100$) for HBV VLP which agrees with reported diameters of 27 nm for MS2 VLP and 30 nm for HBV VLP produced using in vivo methods (Hooker et al., 2004; Zlotnick et al., 1996).

Characterization by DSC

The thermal stability (T_m) of the VLP was assessed via differential scanning calorimetry. The CFPS-produced MS2 VLP disassociated at a T_m of 72.3°C (SD = 0.8°C, $n = 3$) which is slightly higher than the reported T_m value of 68.8°C for recombinant MS2 VLP, but within reasonable error considering the T_m range of 55–70°C reported for phage fr VLP (closely related to MS2) (Ashcroft et al., 2005). A T_m of 83.7°C (SD = 0.6°C, $n = 2$) was determined for the CFPS-produced HBV VLP which is considerably different than the 97°C T_m reported for full-length HBV VLP (Wingfield et al., 1995). Part of the 14°C difference can be explained by the reduced state of the HBV VLP produced in CFPS, as indicated by the lack of the C61 intermolecular disulfide linkage visualized with non-reducing SDS–PAGE gel and autoradiography (results not shown). Dithiothreitol treated full-length HBV VLP have been reported to exhibit a lower melting temperature of 93°C. However, most likely, the major reason for the lower T_m is the C-terminal truncation of the Hepatitis B core antigen. An HBV VLP mutant containing eight additional amino acids at the N-terminus, amino acids 3–144 of Hepatitis B core antigen and two additional amino acids at the C-terminus had a T_m of 72°C which was reduced to 67°C in the presence of dithiothreitol (Wingfield et al., 1995).

Characterization of pH Stability

Cell-free synthesized VLP also demonstrated pH stabilities slightly better or comparable to those of in vivo produced VLP. Purified VLP were incubated for 12 h at pHs ranging from 3 to 12 and analyzed by 10–40% continuous sucrose density velocity sedimentation. Remarkably the cell-free synthesized MS2 VLP demonstrated stability over the pH range of 3 through 11 with 78% or greater recovery efficiency (Fig. 5). Hooker et al. (2004) reported 75% or greater recovery over pH 3 through 10 after a similar 12 h incubation with in vivo produced MS2 VLP. The CFPS

Table I. Comparison of assembled VLP production yields for MS2 and HBV VLP.

Production system	MS2 VLP yield (μg/mL)	HBV VLP yield (μg/mL)	Source
30 μL *E. coli*-based CFPS	384	356	Present article
1 mL *E. coli*-based CFPS	442	372	Present article
E. coli infection and pH treatment	30	—	Hooker et al. (2004)
rDNA in *E. coli*	20	200	Peabody (personal communication) and Zlotnick et al. (1996)
rDNA in *S. cerevisiae*	10	—	Legendre and Fastrez (2005)
Eukaryote-based CFPS	—	4[a]	Lingappa et al. (2005)

[a]Full-length HBV VLP.

produced HBV VLP demonstrated greater than 63% recovery of the VLP after a 12 h incubation at pHs 5 through 10. Similarly, recombinant Hepatitis B core antigen VLP produced in *E. coli* have been reported to be unstable at pHs less than 5.0 and greater than 10.5 after 90 min (Nath et al., 1992).

Characterization of Nucleic Acid Encapsidation

Various studies have reported the encapsidation of RNA in MS2 VLP produced in vivo (Peabody, 2003; Pickett and Peabody, 1993). It is suggested that this encapsidation of RNA is due predominantly to the strong affinity of MS2 coat protein for the stem-loop replicase translation operator on the MS2 RNA genome. Such binding domains have also been identified in a number of nucleic acids in *E. coli*. For example, SELEX was used to find multiple endogenous nucleic acid sequences with affinity for the MS2 coat protein, and a few were found to have higher affinity to the MS2 coat protein than the MS2 replicase operator (Shtatland et al., 2000). Pickett and Peabody (1993) have provided convincing evidence that the precursor to 16S rRNA is a predominant source of encapsidated RNA in MS2 VLP produced in *E. coli* and suggested that this occurs due to a motif in the rRNA and its precursors similar to the MS2 replicase operator.

In CFPS-produced MS2 VLP we have also observed encapsidation of nucleic acid. However, this was initially unexpected as (1) the MS2 replicase operator sequence was not expressed in the CFPS reaction, (2) the CFPS reaction is about 20× more dilute than the *E. coli* cytoplasm, and (3) the endogenous *E. coli* mRNA is degraded during extract preparation. However, it has also been suggested that although the MS2 coat protein-replicase operator affinity is thought to facilitate genome packaging, this interaction is not necessary for genome packaging. Peabody (1997) found that although some MS2 translational operator mutants lacked detectable binding to the coat protein, the mutants were still infectious. A similar packaging mechanism could likely be occurring in the CFPS environment.

The initial evidence of nucleic acid encapsidation in CFPS-produced MS2 VLP was its sedimentation to the ~70S location which is close to the 80S coefficient reported for the MS2 bacteriophage instead of at the proposed 43S to 45S location for empty MS2 VLP (Bonner, 1974; Rohrmann

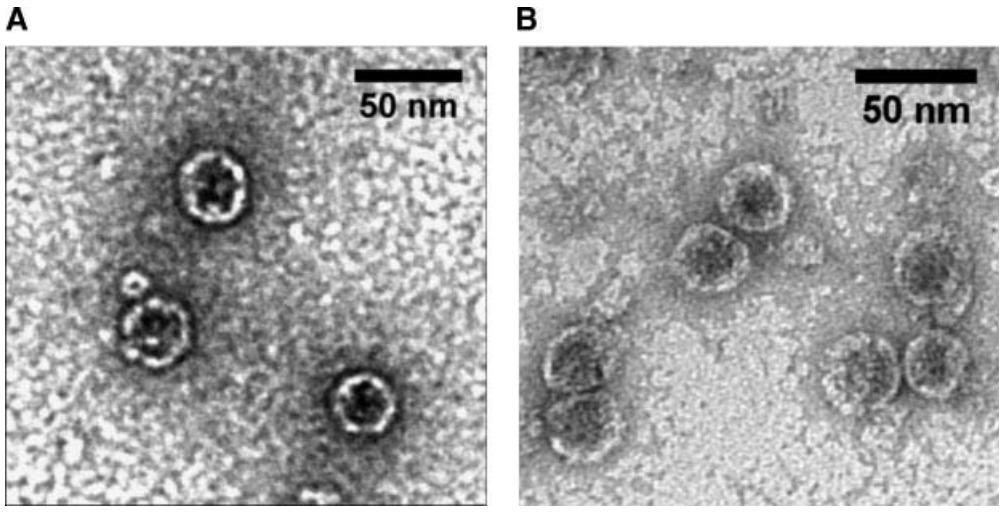

Figure 4. Transmission electron microscope images of CFPS-produced MS2 VLP (**A**) and CFPS-produced HBV VLP (**B**) isolated with sucrose density velocity sedimentation. Concentrated MS2 VLP and HBV VLP isolates were applied to a carbon coated copper/Formvar grid and negatively stained with 1% w/v uranyl acetate at pH 4. Images were taken with a Gatan 967 CCD camera in a JEOL 1230 electron microscope at 80 kV acceleration voltage.

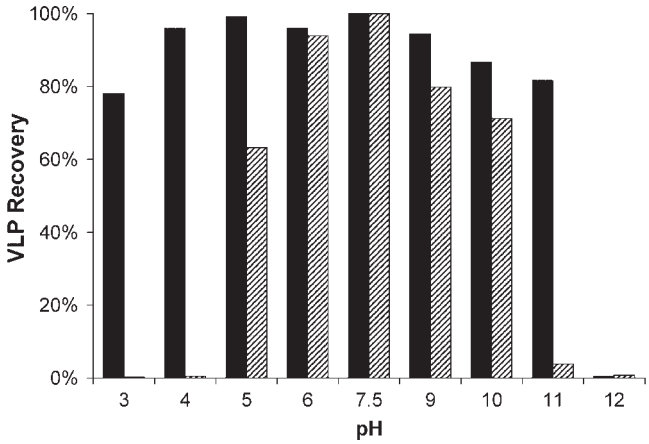

Figure 5. MS2 coat protein VLP (black bars) and C-terminally truncated Hepatitis B core VLP (striped bars) recovery after a 12 h incubation and velocity sedimentation at the indicated pH.

and Krueger, 1970). Nucleic acid encapsidation was verified by the MS2 VLP absorption spectrum which peaked at 260 nm instead of 280 nm (Fig. 6). We further demonstrated nucleic encapsidation by subjecting purified MS2 VLP to 1% agarose gel electrophoresis. In the lane loaded with MS2 VLP, a single protein band (visualized by Coomassie Blue staining) aligned exactly with the sole nucleic acid band (visualized by ethidium bromide fluorescence) (Fig. 7). The presence of co-migrating VLP protein and nucleic acid suggests nucleic acid encapsidation.

The size of nucleic acid encapsidated in CFPS-produced MS2 VLP was determined with denaturing polyacrylamide TBE-Urea gels (Fig. 7). The predominant band was ~600 base pairs (bp) in length which is consistent with packaging of the 583 bp long mRNA that encods the MS2 coat protein.

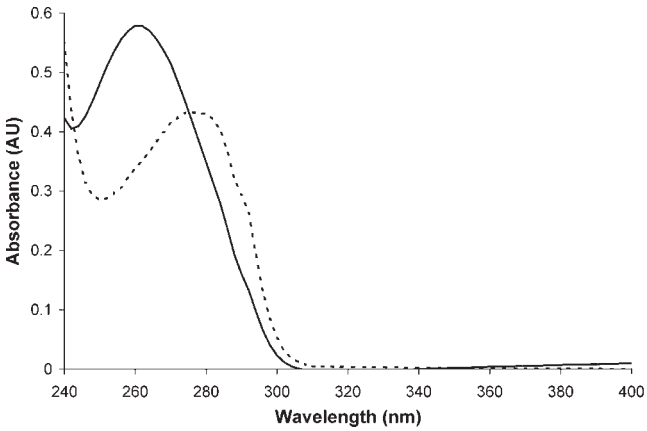

Figure 6. Absorbance spectra of CFPS-produced MS2 and HBV VLP. The HBV VLP absorbance spectrum (dotted line) is dominated by protein absorbance at 280 nm. The MS2 VLP absorbance spectrum (solid line) is dominated by absorbance at 260 nm suggesting nucleic acid encapsidation.

The faint band close to 100 bp is likely tRNA which is added to the CFPS reaction. The predominant encapsidation of MS2 coat protein mRNA may explain why higher than normal plasmid concentrations (12 nM vs. 6 nM) were required to obtain maximal MS2 VLP production (results not shown). As the MS2 coat protein mRNA does not contain the MS2 replicase operator, its encapsidation could possibly be caused by non-specific interactions due to its high concentration in the CFPS reaction. Interestingly, encapsidated nucleic acid was not detected in the CFPS-produced HBV VLP. It sedimented to approximately the 45S location reported for the empty HBV VLP and its absorption spectrum peaked at 280 nm (Fig. 6). Encapsidated nucleic acid was undetectable on the TBE-Urea polyacrylamide gels which spans 40–10,000 bp (Fig. 7). The difference in nucleic acid encapsidation between the HBV VLP and MS2 VLP is not entirely unexpected as the HBV capsid selectively encapsidates its pregenomic RNA only when complexed to the HBV polymerase during infection. However, the difference in encapsidation between the MS2 and HBV VLP does suggest that the concentration of nucleic acid in the cell-free environment is not sufficient for encapsidation. A possible explanation for the differences in MS2 VLP and HBV VLP nucleic acid encapsidation is a difference in charge densities on the interior surface of the VLP at the pH of the CFPS reaction. Using a protein isoelectric point (p*I*) calculation program based on the amino acid sequence, the p*I* of MS2 coat protein is expected to be ~7.9 while the calculated p*I* of truncated Hepatitis B core antigen is ~5.1. This would suggest that the MS2 coat protein is likely positively charged at the pH range of 6.5–7 in the CFPS reaction and would be expected to have strong ion pairing interactions with negatively charged nucleic acid. In contrast the C-terminally truncated Hepatitis B core antigen would be expected to be negatively charged and might repel nucleic acid. To further understand the surface charges on the VLP interior surface, the electrostatic potential along the molecular surfaces of the MS2 VLP and the HBV VLP was visualized using the Coulomb calculation method in Swiss-Pdb Viewer 3.7. A continuous positive charge was shown for MS2 coat protein's interior surface while the exterior surface had pockets of negative charge density. In contrast, a continuous negative charge was visualized on both the interior and exterior surfaces of the Hepatitis B core antigen (results not shown). Although this observation is interesting, further investigation is needed. Regardless of encapsidation differences and mechanisms, we have demonstrated that the HBV VLP and MS2 VLP are produced at high levels with *E. coli*-based cell-free protein synthesis and these VLP have been shown to be similar to in vivo produced VLP by TEM, DSC, and pH stability assays.

Conclusion

We have demonstrated that *E. coli*-based cell-free protein synthesis can produce the MS2 bacteriophage coat protein

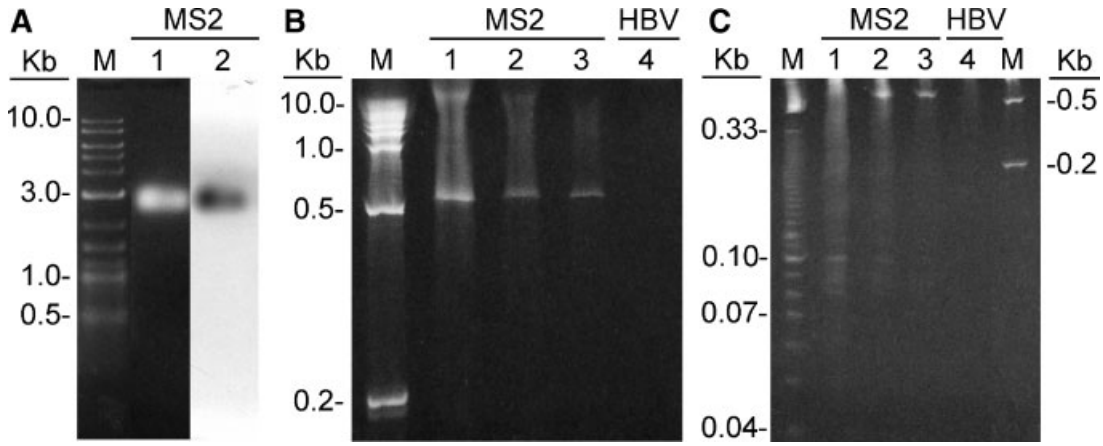

Figure 7. Analysis of nucleic acid encapsidation in CFPS-produced MS2 and HBV VLP with electrophoresis. **A**: 1.1 μg of MS2 VLP was subjected to electrophoresis in a 1% agarose gel and visualized with ethidium bromide (**lane 1**) and Coomassie Blue (**lane 2**). 0.4 μg of 2-log ladder (New England Biolabs, Ipswich, MA) was used as a marker (lane M). MS2 and HBV VLP after denaturation by incubation at 70°C in 3.5 M urea were subjected to electrophoresis in 6% (**B**) and 15% (**C**) polyacrylamide TBE-Urea gels visualized with ethidium bromide. B: Lane M: 1 μg 0.2–10 kb perfect RNA marker; **Lanes 1**, **2**, and **3**: 1.1, 0.11, and 0.011 μg of MS2 VLP; **Lane 4**: 1.0 μg HBV VLP. C: Left lane M: 0.1 μg 10 bp DNA Ladder; **Lanes 1**, **2**, and **3**: 1.1, 0.11, and 0.011 μg MS2 VLP; **Lane 4**: 1.0 μg HBV VLP; Right lane M: 0.1 μg 0.2–10 kb perfect RNA marker.

VLP at yields over 14 times greater than the best previously published yields. Also, the C-terminally truncated Hepatitis B core antigen VLP was produced at significantly greater yields than previously reported using *E. coli* recombinant protein expression techniques. Characterization of the MS2 and HBV VLP by TEM, DSC, and pH stability tests verified that they are similar to previous in vivo produced VLP. To our knowledge, this is the first time a virus-like particle has been generated using a prokaryote-based in vitro transcription/translation system. Although eukaryote-based in vitro systems have been employed, only modest yields of VLP production have been demonstrated (1–20 μg/mL) and assembly efficiencies have typically been low. For example, the Hepatitis B core antigen was produced at 10 μg/mL and 40% or 4 μg/mL assembled into VLP (Lingappa et al., 2005) using a rabbit reticulocyte derived system versus the 356 μg/mL of assembled HBV VLP produced at 80% assembly efficiency with our system. Furthermore, we have demonstrated the potential for reaction scalability by increasing the reaction volume from the μL to the mL scale while maintaining protein production yields and VLP assembly efficiencies. These results demonstrate the feasibility for this production technology to satisfy the demand for a VLP production technology requested by Pattenden et al. (2005) that: (1) is scalable, (2) has high protein production yields, (3) facilitates high assembly efficiencies, and (4) is versatile enough to produce a variety of complex VLP.

For example, this production platform could serve as an excellent technology for vaccine production against a biowarfare threat or for personalized vaccines, as the time between sequence identification and final product delivery would be greatly reduced by (1) the use of linear DNA expression templates instead of plasmids (Woodrow et al., 2006), (2) the high yielding rapid translation/transcription machinery of *E. coli*, (3) the ease of production scale-up (Voloshin, 2006; Voloshin and Swartz, 2007), and (4) simplified purification processes (Alimov et al., 2000; Jewett and Swartz, 2004b). Also, recent and continued advances in reducing the cost of the cell-free system could potentially open the door for the economical production of VLP for nanotechnology applications (Calhoun and Swartz, 2005; Jewett, 2005). The cell-free reaction is an open environment enabling the encapsidation of drugs and/or nucleic acids while the protein is being produced, potentially avoiding the need for disassembly and reassembly steps which are often difficult with complex VLP and result in significant product loss. Finally, site-specific incorporation of unnatural amino acids has been demonstrated with cell-free technology (Kiga et al., 2002) and could be used to produce unnatural amino acid incorporated VLP for the site-specific chemical attachment of antigens or other ligands for the customized design of vaccines, drug delivery carriers, and nanostructure templates.

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