Oxidative coupling of peptides to a virus capsid containing unnatural amino acids†

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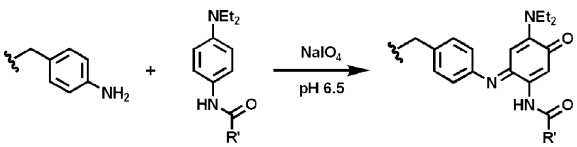
Received (in Austin, TX, USA) 19th November 2007, Accepted 8th January 2008
First published as an Advance Article on the web 29th January 2008
DOI: 10.1039/b717826c

This Communication describes the chemo- and site-selective coupling of cell type-specific targeting peptides to a virus capsid containing aminophenylalanine residues.

Combinatorial synthesis and phage display screens have yielded a number of synthetic peptides that can form well-defined tertiary structures,[1] act as ligands to cellular receptors,[2] and localize in specific tissue types.[3] Through the attachment of multiple copies of these peptides to polymer, vesicle, or protein carriers, tissue-specific delivery vectors can be envisioned for a variety of therapeutic and diagnostic applications. As part of our ongoing efforts[4] to build well-defined, targeted imaging agents from genome-free viral capsids, we sought to append known cancer targeting peptides to the exterior surface of bacteriophage MS2. The coupling of synthetic peptides to protein structures presents a particularly difficult challenge, however, as the reactions used for this purpose must proceed site-selectively in the presence of the full set of amino acid functional groups. Previous approaches to access these conjugates have targeted azide groups, including the use of dipolar cycloadditions with alkynes[5] or the Staudinger ligation.[6] The requisite azide groups have been incorporated into proteins on the translational level using both residue-specific[6] and amber suppression techniques.[7] As an alternative to these methods, we report here an efficient oxidative method that can attach peptides to aniline-containing amino acid side chains introduced into virus capsids using stop codon suppression.

Bacteriophage MS2 is an icosahedral particle that self assembles from 180 copies of a single protein monomer. It can be cultured in Hfr^+ *E. coli* as infectious virions, or the coat protein can be expressed in recombinant systems.[8] Although it has been reported previously that amino acid sequences can be inserted into the capsid monomers on the genetic level,[8] we instead chose to install synthetic targeting peptides through a bioconjugation reaction to minimize the impact on capsid assembly during protein production. This strategy also allows the future incorporation of peptoids and other protease resistant groups that cannot be expressed in natural systems. To do this, we turned to an oxidative coupling reaction recently developed in our lab (eqn (1)).[9] This reaction results in a one-to-one coupling of an aniline and a phenylene diamine derivative under oxidative conditions. It proceeds rapidly and with very high specificity in neutral aqueous solution in the presence of the oxidant sodium periodate. Because this oxidative coupling works at low concentrations and is highly chemoselective, we anticipated that it would be well suited to peptide–protein coupling.



(1)

Based on the success of previous reports,[10] we envisioned installing an aniline functionality into the MS2 capsid through the incorporation of *para*-amino-L-phenylalanine (pAF, Fig. 1(d)). We were also encouraged by the reported incorporation of unnatural amino acids other than pAF into bacteriophage M13 for phage display using amber suppression technology.[11] Preparation of pAF-containing MS2 was accomplished by following the protocol established for the L-4-trifluoromethylphenylalanine amber suppressor system.[12] The MS2 coat protein genetic sequence was cloned into an ampicillin resistant pBAD/*Myc*-his A plasmid containing an arabinose promoter. To optimize surface accessible pAF incorporation, five stop codon variants were constructed: Q6TAG, D11TAG, T15TAG, D17TAG and T19TAG. Next, the tetracyclin resistant plasmid pDULE-pAF, containing the lpp-promoted pAF-specific aminoacyl-tRNA synthetase and its cognate $tRNA_{CUA}$, was co-transformed into DH10B *E. coli* along with one of the pBAD-MS2 amber mutants. One litre cultures were grown in arabinose autoinduction media, and upon reaching an OD_{600} of 0.8, the 1 L was split into two 500 mL cultures.† To one of these 500 mL cultures was added pAF to a final concentration of 1 mM. Fig. 1(c) shows the analysis of the two samples corresponding to the T19pAF mutation after 29 h of culture. Observable MS2 coat protein expression was only found in the presence of pAF.

As the MS2 mutant with pAF at position T19 (MS2-pAF19) produced the highest yield (21 mg L^{-1}) and was found to be a robust oxidative coupling scaffold, it was used for all subsequent experiments. Following production, the capsids

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† Electronic supplementary information (ESI) available. Procedures, experimental and further data. See DOI: 10.1039/b717826c

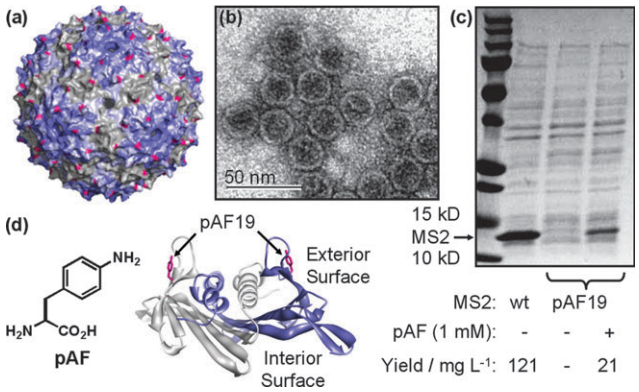
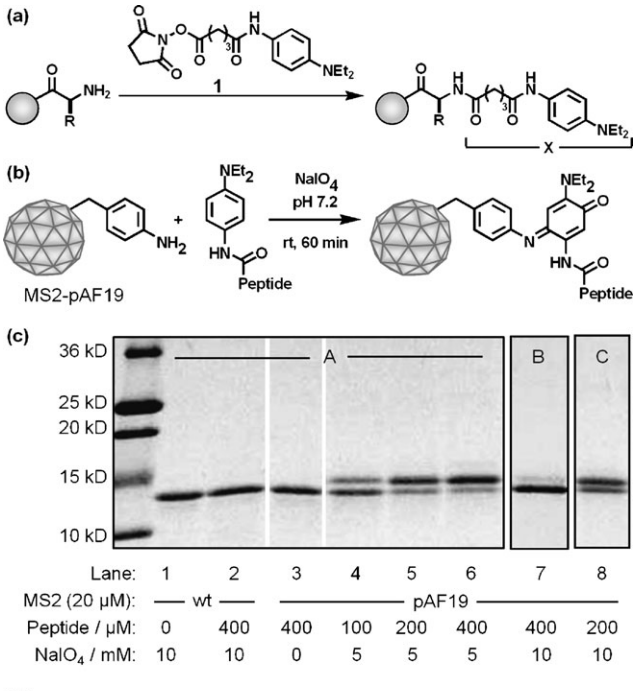


Fig. 1 Expression of MS2 capsids containing *para*-amino-L-phenylalanine (pAF). (a) Rendered MS2 capsid surface showing the location of pAF19 residues in pink. (b) TEM image of MS2-pAF19 capsids stained with $UO_2(OAc)_2$. (c) SDS-PAGE of crude lysates from the expression of wild-type and MS2-pAF19. No overexpression of MS2-pAF19 is observed when pAF is withheld from the growth media. (d) Structure of an MS2 coat protein dimer showing pAF19.

were isolated *via* ion exchange and size exclusion chromatography.† Transmission Electron Microscopy (TEM) showed that the MS2-pAF19 mutant formed capsids (Fig. 1(b)) with no degradation over a period of weeks at 4 °C.

Three peptides known to target specific tissues were chosen for attachment (Fig. 2(d)). Peptide A has been shown to target neuroblastoma and breast cancer cell lines,[13] peptide B binds to matrix metalloproteinases 2 and 9,[14] and peptide C is a derivative of a reported kidney-targeting sequence.[15] The second component of the oxidative coupling, an *N*,*N*-diethyl-*N*′-acylphenylene diamine, was placed at the N-terminus of each peptide during synthesis on the solid phase. After Fmoc-deprotection of the α-amino group on the terminal residue, the resin was incubated with NHS ester **1** (Fig. 2(a)). This yielded a phenylene diamine-containing peptide with essentially quantitative conversion. Following deprotection and cleavage using TFA, the peptides were HPLC-purified.

Oxidative coupling conditions were next screened to determine the optimal reagent stoichiometry of MS2-pAF19, phenylene diamine peptide and sodium periodate (Fig. 2(b)). Dimerization of the aniline groups between MS2-pAF19 capsids was a potential concern, but this was not observed. No coupling occurred with wtMS2 or for MS2-pAF19 in the absence of sodium periodate or peptide (Fig. 2(c), lanes 1–3). Maximum MS2-pAF19–peptide coupling was achieved using 10–20 equivalents of peptide per capsid monomer and 250–500 equivalents of sodium periodate (Fig. 2(c), lanes 6–8). Time-course experiments indicated that maximum conversion was reached after 1 h,† which is consistent with previous studies. Following SDS-PAGE and Coomassie staining, optical densitometry analysis indicated that greater than 75% of the capsid monomers had been modified with a single copy of sequence A—corresponding to more than 135 peptides per capsid and a mass increase of greater than 270 kDa. This level of conversion was not observed for all three peptides, an effect we attribute to secondary structure effects. Peptide A (Fig. 2(d)) has the most linear structure, while the disulfide loops of peptides B and C create a bulkier substrate that may impede



(d)

Sequence	% copy	Copies/capsid	Ref.
A: X-VPWMEPAYQRFLGGG	75	135	12
B: X-GGCTTHWGFTLCG	17	31	13
C: X-CLSGRRVCG	60	108	14

Fig. 2 Modification of MS2-pAF19 using an oxidative coupling strategy. (a) Peptides bearing N-terminal phenylene diamine groups were accessed using solid phase synthesis. (b) Following purification, the peptides were coupled to MS2-pAF19 capsids in the presence of periodate. (c) SDS-PAGE of peptide coupling reactions. (d) Following Coomassie staining, densitometry analysis was used to obtain the levels of peptide coupling. The brackets indicate disulfide bonds. The N-terminal X-substituent is defined in (a).

the coupling reaction. Greater than 60% modification was observed for cyclic peptide C using higher substrate concentrations. Although peptide B coupled to a much lesser extent, the ~31 copies that were installed may already be sufficient to achieve targeting (Fig. 2(d)).

At excessively high concentrations of sodium periodate and peptide, a faint third band was observed by SDS-PAGE. This appears to be the attachment of a second peptide, a product hard to rationalize if it is the result of a second oxidative coupling to the sterically-hindered pAF present after the first oxidative coupling. This band makes up less than 2% of the total protein concentration and is easily avoided by not using unnecessarily high concentrations of the coupling partners. The low abundance of this species has prevented its further characterization.

To determine if the capsids remained intact after the oxidative attachment of the peptides, the products were analyzed by gel filtration chromatography and TEM (Fig. 3). The gel filtration elution times of wtMS2 and modified capsids were identical, and the TEM images showed intact capsids. After modification with peptide A, the TEM image showed an increase in the thickness of the capsid shell. In addition, the modified capsids no longer clustered together, as did

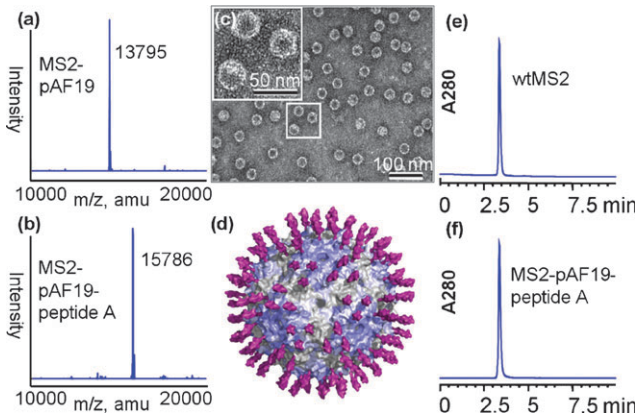


Fig. 3 MS2-pAF19–peptide A structural investigation. (a) ESI-MS reconstruction of MS2-pAF19. Expected mass $[M + H]^+$: m/z = 13795. (b) ESI-MS reconstruction (negative ion mode) of MS2-pAF19–peptide A conjugate. Expected mass $[M - H]^-$: m/z = 15788. (c) TEM image of the capsid with greater than 60% peptide attachment. (d) Rendered MS2-pAF19 depicting one peptide A for every capsid monomer. (e,f) Size exclusion chromatograms (280 nm) of wtMS2 and the MS2-pAF19–peptide A conjugate.

MS2-pAF19 (Fig. 1(b)). These changes in diameter were corroborated by dynamic light scattering (DLS) results: MS2-pAF19 is 27.8 ± 0.15 nm, while MS2-pAF19-peptide A is 30.6 ± 0.07 nm. In addition to this 2.8 ± 0.11 nm increase in the capsid size, the measured polydispersity of 1.192 ± 0.007 indicated a relatively uniform distribution of peptides. Heat-induced denaturation of capsids, with and without peptides attached, was investigated by DLS, showing no loss in thermal stability (the capsids disassembled at ~54 °C).† The oxidative coupling product has previously been shown to be stable from pH 2 to 11,[9] and the peptide conjugates have shown no degradation in solution over a period of weeks.

Mass spectra of the purified conjugates indicated the addition of one peptide and an oxygen atom to each monomer, as would be predicted from the previously observed products of this reaction (Fig. 3(b)). Model coupling studies, using *para*-toluidine as a small molecule analog of the aniline-containing protein, confirmed the expected mass of the adduct.†

Periodate is frequently used in the context of protein activation for either oxidative cleavage of an N-terminal serine or threonine, or for the formation of aldehydes from the sugar residues of glycosylated proteins.[16] In addition, the Kodadek group have used this reagent to effect the crosslinking of 3,4-dihydroxyphenylalanine (DOPA)-labelled moieties to nucleophiles displayed on proteins.[17] Upon incubation with periodate, our methionine (Met)-containing peptides gained 16 mass units, consistent with our expectation that Met side-chains would oxidize to sulfoxides under these conditions. We also found that peptides containing reduced cysteines are oxidized specifically to cyclic disulfides in all the cases we examined.† To the best of our knowledge, disulfide bonds were not further oxidized by the periodate conditions.

It must be noted that the use of sodium periodate in this reaction places certain requirements on sample preparation. Specifically, commonly-used reductants, such as DTT or TCEP, that are present in the solution will inhibit this reaction. Additionally, trace amounts of glycerol that may be present from protein purification steps or introduced by dialysis membranes may lead to reduced yields or the requirement for higher periodate concentrations. Besides these small limitations on sample composition, we have found this reaction and its product to be tolerant of many other functional groups and conditions found in biological samples.[9]

We have developed a highly selective method for the ligation of peptides to a virus capsid containing unnatural amino acids. By incorporating a chemically distinct side chain into the polypeptide sequence, we have ensured that the reaction occurs at only one site on each protein monomer. We are currently developing less sterically-hindered phenylene diamine derivatives as more efficient coupling alternatives. We are also exploring the use of other oxidants that will avoid the oxidation of Met residues. Based on the applicability of amber suppression techniques to the expression of a wide range of proteins, we feel that this method will find many useful applications in the biochemical and materials fields. In the context of MS2, the ability of the attached peptides to target tumors *in vivo* is being investigated.

This work was supported by the NIH (GM072700) and the Berkeley Chemical Biology Graduate Program (Training Grant 1 T32 GMO66698). Portions of this work were performed at the Molecular Foundry, Lawrence Berkeley National Laboratory, which is supported by the Office of Basic Energy Sciences, US DOE contract no. DE-AC02-05CH11231. pDULE-pAF was generously provided by Prof. Peter G. Schultz of the Scripps Research Institute.

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