Strategies and Limitations in Dendrimeric Immunogen Synthesis. The Influenza Virus M2e Epitope as a Case Study

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Dendrimeric platforms such as multiple antigen peptides (MAPs) are regarded as one of the most efficacious approaches for antigenic presentation. Originally described as available by stepwise solid-phase peptide synthesis (SPPS), MAPs have also been prepared by chemical (thioether, oxime, hydrazone) ligation of appropriately functionalized tetra- or octavalent polylysine scaffolds with the peptide antigen to be multiply displayed. In this work, the advantages and limitations of two of the most frequent methods of MAP preparation, namely, chemoselective thioether ligation in solution, and all-solid-phase synthesis, have been tested in the case of a particularly troublesome epitope model, the ectodomain of protein M2 from influenza virus (M2e). The strong tendency of M2e to self-associate is a serious inconvenient for conjugation in solution, which as a result fails to produce the target MAPs with the specified number of M2e copies. In contrast, the fully stepwise SPPS approach is shown to be quite practical, especially when 6-aminohexanoic acid spacer units providing increased internal flexibility are inserted at each branching point.

INTRODUCTION

The usually low immunogenicity of short peptide epitopes, which precludes their use in free form for purposes such as raising antisera or candidate vaccine trials, has been addressed by various strategies, chiefly conjugation to carrier proteins (for an exhaustive treatment, see ref *1*), but also lipidation (*2, 3*) or association to particulate systems such as liposomes (*4, 5*) or immunostimulating complexes (*6*). One particularly successful approach has been multimerization, either by simple polymerization (*7*) or by chemically better defined strategies such as conjugation to sequential oligopeptide carriers (*8, 9*), display on lysine scaffolds (*10, 11*), or, last but not least, incorporation into dendrimeric systems such as the multiple antigenic peptides (MAPs) pioneered by Tam in the 1980s (*12*). MAPs are based on a small immunologically inert core of radially branching lysine dendrites onto which a number of peptide epitopes are attached. The result is a large macromolecule with a high molar ratio of peptide antigen to core molecule that usually does not require further conjugation to a carrier protein (though it may benefit from it; see, e.g., *refs* 13, 14). MAPs were originally described as available by standard stepwise solid-phase peptide synthesis (SPPS) (*12*), but have later also been produced in solution through various forms of chemical ligation (*15*−*17*). As the ligation reactions usually involve the combination of prepurified peptide modules, they can purportedly produce chemically more unambiguous materials (*16*) than SPPS methods, where minute but cumulative synthetic errors (deletions, truncations, etc.) become amplified by multimerization and may predictably lead to relatively heterogeneous immunogens. While the fairly extensive literature on, e.g., the immunological and microbiological applications of MAP and similarly dendrimeric platforms, highlights their value as candidate vaccines (*18*) or antimicrobial agents (*19, 20*), it is not always informative enough on the chemical specifications of the constructs at hand. In particular, and to the best of our knowledge, the literature on immunopeptides, MAPs, and similar bioconjugates lacks comparative studies where the potential merits and flaws of each synthetic (solution or stepwise SPPS) approach are evaluated in adequate chemical detail. In this work, we have investigated these aspects as they apply to a synthetically demanding peptide epitope (see below), then selected and improved the synthetic route leading to MAP platforms with optimal specifications.

The epitope chosen for this study is based on the N-terminal ectodomain (M2e) of influenza type A virus matrix protein 2 (M2). The M2 protein forms a pH-activated proton channel on the surface of the virion that mediates the uncoating of the viral particles in endosomes of the infected cell (*21*). The importance of the M2 protein in viral replication has been demonstrated by the ability of the antiviral drug amantadine and M2 specific antibodies to reduce viral propagation *in vitro* and *in vivo* (*22*−*24*).

M2 is made up of three main regions: the N-terminal, 24-residue ectodomain (M2e), the ensuing 19-amino acid transmembrane region that tetramerizes to form the pore of the proton channel (*25*), and the 54-residue cytoplasmic tail. While M2 is expressed abundantly on infected cell surfaces, in virus particles only a few M2 copies are displayed (*24*), in contrast to the other two major viral surface proteins hemagglutinin (HA) and neuraminidase (NA) (*26*−*29*). M2 is also weakly immunogenic relative to HA and NA (*30*) and highly conserved among human influenza virus strains (*31*). Table 1 lists (nonexhaustively) the M2e sequences from different influenza strains. It can be seen that, despite a high number of outbreaks up to the present 2009 pandemic, M2e has experienced minimal sequence alterations since 1933 (*32*), when the first human influenza strain was isolated. This remarkable lack of genetic variability, plus its relatively small size and the fact that antibodies to M2e protect mice from influenza A mortality, have made M2e a very popular epitope, not only for general immunogenicity studies but also as a potential vaccine candidate (*26, 31, 33*−*37*). M2e-based vaccines have been mostly obtained by either chemical conjugation to carrier proteins such as keyhole limpet hemocyanin, bovine serum albumin, or outer membrane protein complex or

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Table 1. M2 Ectodomain (M2e) Sequences Are Highly Conserved Across Multiple Strains of Influenza

Representative virus	Subtype	Amino acid sequences *
A/Wilson-Smith/1933	H1N1	MSLLTEVETPIRNEWGCRCNDSSD
A/Puerto Rico/8/34	H1N1	MSLLTEVETPIRNEWGCRCNGSSD
A/Wisconsin/3523/88	H1N1	MSLLTEVETPIRNEWGCKCNDSSD
A/California/04/2009	H1N1	MSLLTEVETPTRSEWECRCSDSSD
A/Aichi/470/68	H3N1	MSLLTEVETPIRNEWGCRCNDSSD
A/Hebei/19/95	H3N2	MSLLTEVETPIRNEWECRCNGSSD
A/Viet Nam/1203/2004	H5N1	MSLLTEVETPTRNEWECRCSDSSD
A/Chicken/Nakorn-Patom/Thailand	H5N1	MSLLTEVETPTRNEWECRCSDSSD
A/Thailand/1(KAN-1)/04	H5N1	MSLLTEVETPTRNEWECRCSDSSD
A/Hong Kong/156/97	H5N1	MSLLTEVETLTRNGWGCRCSDSSD
A/Duck/1525/81	H5N1	MSLLTEVETPTRNGWECKCSDSSD
A/Chicken/New York/95	H7N2	MSLLTEVETPTRNGWECKCSDSSD
Consensus		**MSLLTEVETPTRNEWESRSSDSSD**

* Highlighted in bold are variant residues relative to the A/Wilson-Smith/1933 (H1N1) sequence. The two underlined Ser residues in the consensus sequence were introduced as an isosteric replacement for Cys, to avoid synthetic problems.

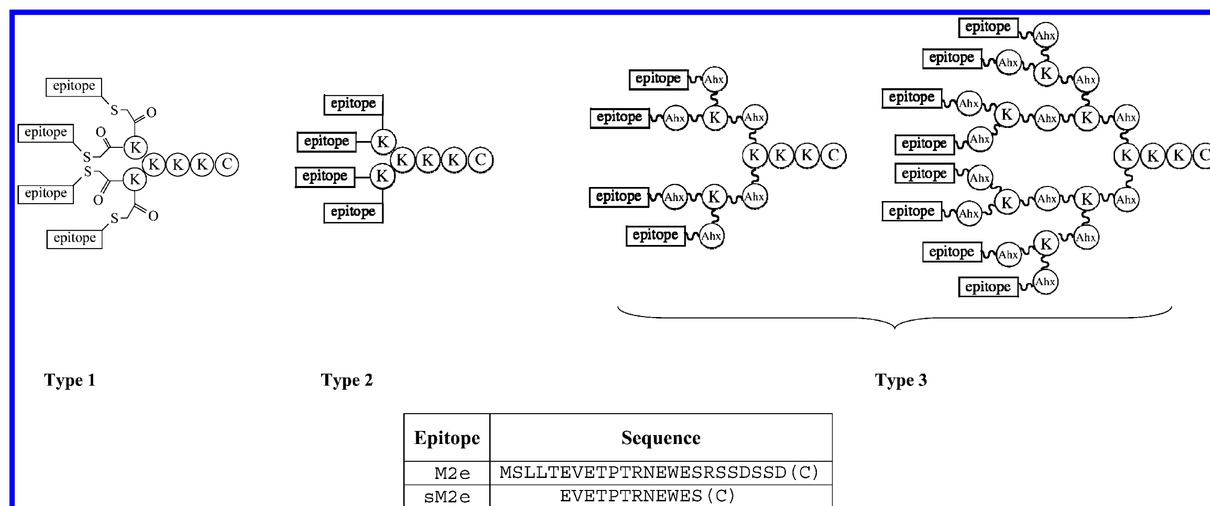


Figure 1. General structures of the different MAPs in this study. For type 1, the tioether linkage between the peptide epitope and the tetra-acetylated Lys core is shown. For type 2, the line between the epitope and the Lys core indicates a peptide bond. For type 3, the wavy lines around every Ahx residue emphasize the flexibility provided by this residue.

by genetic insertion into hepatitis B virus core antigen or papaya mosaic virus (PapMV) vaccine platforms (*26, 31, 38−40*).

As mentioned above, the goal of this study was to evaluate the advantages and limitations of chemical (thioether) ligation vs SPPS approaches for producing MAP dendrimeric peptides, and M2e was judged to be an interesting epitope not only for its above-mentioned biological relevance but also for the fact that despite its apparent (24 amino acid) simplicity it turns out to be synthetically nontrivial. Thus, three different types of MAP constructs (Figure 1) have been designed and prepared by either chemical ligation or SPPS methods, and the product composition of each synthetic trial has been analyzed in considerable detail. The study reveals how some structural peculiarities of the chosen M2e epitope, mainly its tendency to self-associate, entail considerable difficulties for the chemical ligation approach. In such an unfavorable context for conjugation in solution, the SPPS-based MAP synthetic strategy, especially fine-tuned in the present case to prevent sequence aggregation, emerges as the preferred route to MAP platforms displaying the M2e epitope.

EXPERIMENTAL PROCEDURES

Chemicals and Biologicals. Fmoc-protected amino acids were obtained from Senn Chemicals (Dielsdorf, Switzerland). Fmoc-Ser(tBu)-Ser($\Psi^{Me,Me}$pro)OH and Fmoc-Rink-amide (MBHA) resin were from Novabiochem (Läufelfingen, Switzerland). ChemMatrix resin, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and *N*-hydroxybenzotriazole (HOBt) were from Matrix Innovation (Montreal, Canada). HPLC-grade acetonitrile, and peptide synthesis grade *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), *N*,*N*-diisopropylethylamine (DIEA), and trifluoroacetic acid (TFA) were from SDS (Peypin, France). Anti-influenza A virus M2 protein monoclonal antibody 14C2 and rabbit polyclonal antibody to mouse IgG were from Abcam plc (Cambridge, UK). SIGMA*FAST* *p*-nitrophenyl phosphate tablets were from Sigma (Saint Louis, MO, USA). 96 well microplates were from Corning Inc. (Corning, NY, USA). All other reagents were of the highest quality available commercially.

General SPPS Protocols. Automated syntheses were performed in an ABI433 peptide synthesizer (Applied Biosystems, Foster City, CA) running standard Fmoc (FastMoc) protocols (*41*) at 0.1 mmol scale on Fmoc-Rink-amide ChemMatrix resin. Eight-to-10-fold excesses of Fmoc-L-amino acids and HBTU/HOBt, in the presence of double the molar amount of DIEA, were used for the coupling steps, with DMF as solvent. All side-chain functions were protected with TFA labile groups. In the longer M2e epitope, the two pairs of consecutive Ser residues were introduced as the pseudoproline dipeptide Fmoc-Ser(tBu)-Ser($\Psi^{Me,Me}$pro)-OH using a manual coupling with 4-fold excess

Table 2. Chemical Characteristics of Different Dendrimeric Presentations of the M2e Epitopes

MAP type[a]	n° copies	epitope	synthetic approach	composition	MW calculated (mo/av)	MW found
1	1−4	M2e	ligation	$C_{160}H_{263}N_{46}O_{57}S_4Cl_3$	3977.73/3973.71	3977.91
				$C_{275}H_{446}N_{80}O_{104}S_6Cl_2$	6800.32/6794.98	6794.11
				$C_{390}H_{629}N_{114}O_{151}S_8Cl_1$	9622.91/9616.25	9607.10
				$C_{505}H_{812}N_{148}O_{198}S_{10}$	12445.50/12437.53	12460.40
1	1−4	sM2e	ligation	$C_{110}H_{178}N_{31}O_{35}S_3Cl_3$	2697.36/2694.13	2691.51
				$C_{175}H_{276}N_{50}O_{60}S_4Cl_2$	4239.57/4235.84	4237.26
				$C_{240}H_{374}N_{69}O_{85}S_5Cl_1$	5781.79/5777.54	5813.82
				$C_{305}H_{472}N_{88}O_{110}S_6$	7324.00/7319.24	7341.23
2	4	sM2e	SPPS	$C_{281}H_{432}N_{80}O_{102}S$	6591.07/6595.05	6596.28
3	4	sM2e	SPPS[b]	$C_{317}H_{498}N_{86}O_{108}S_1$	7269.58/7273.99	7269.52
3	4	M2e	SPPS[b]	$C_{517}H_{838}N_{146}O_{196}S_5$	12387.86/12395.50	12387.80
3	8	sM2e	SPPS[b]	$C_{637}H_{998}N_{170}O_{216}S_1$	14517.21/14525.97	14515.89
3	8	M2e	SPPS[b]	$C_{1037}H_{1678}N_{290}O_{392}S_9$	24753.78/24768.97	not found

[a] As described in Figure 1. [b] MAPs with flexible spacers.

of dipeptide and HATU in the presence of DIEA (8-fold excess) in DMF. For manual synthesis operations, the peptide resin was placed in a syringe reactor fitted with a porous polyethylene disk. After synthesis completion, peptide resins were N-deblocked with piperidine/DMF (20% v/v) prior to full deprotection and cleavage with TFA/H_2O/EDT/TIS (94:2.5:2.5:1 v/v, 90 min, RT). Peptides were precipitated by addition of chilled methyl *tert*-butyl ether, taken up in aqueous AcOH (10% v/v), and lyophilized.

Analysis and Purification. Analytical reversed-phase HPLC was performed on C_{18} or C_8 columns (4.6 × 50 mm, 3 μm, Phenomenex, Torrance, CA) in a model LC-2010A system (Shimadzu, Kyoto, Japan). Solvent A was 0.045% (v/v) TFA in water, solvent B was 0.036% (v/v) TFA in acetonitrile. Elution was done with linear gradients of solvent B into A over 15 min (see chromatograms for further details) at 1 mL/min flow rate, with UV detection at 220 nm. Preparative HPLC was performed on C_{18} (21.2 × 250 mm, 10 μm, Phenomenex) or on C_8 column (10 × 250 mm, 10 μm, Phenomenex) in a Shimadzu LC-8A instrument. Solvents A and B were 0.1% TFA (v/v) in water and acetonitrile, respectively, and elution was again with linear gradients of solvent B into A over 35 min, at 25 or 5 mL/min flow rate for C_{18} or C_8 separations, respectively, with UV detection at 220 nm. Preparative fractions of satisfactory purity (>95%) by analytical HPLC were pooled and lyophilized. The purified peptides and MAPs were characterized for identity by MALDI-TOF MS in a Voyager DE-STR instrument (Applied Biosystems), using either sinapinic or α-hydroxycinnamic acid matrixes. MS spectra were in the reflector mode for the two linear peptide epitopes and the tetravalent Lys core; for larger-sized analytes, the linear mode was used. For even larger sized MAPs (e.g., the last two entries in Table 2), spectra were acquired in an LTQ-Orbitrap XL instrument (Thermo Fisher, San José, CA).

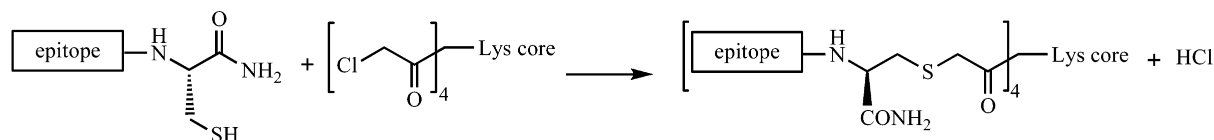
Type-1 MAPs by Thioether Ligation. The peptide epitopes M2e and sM2e to be conjugated to the tetravalent branched Lys core (see Figure 1) were prepared by the above SPPS protocols and, after significant optimization, particularly for M2e (see Results below), were characterized by analytical HPLC and MALDI-TOF MS (see Table S1, Supporting Information). In all cases, the purified peptides were obtained in free thiol form, with no disulfide detected by MS. The tetravalent [$(Lys)_2$LysLysLysCys(S-*t*Bu)] core was also assembled by SPPS in the ABI433 instrument at 0.1 mmol scale. For the first two Lys residues (from the C-terminus), the conventional Fmoc-Lys(tBu) derivative was used; branching was then achieved by introducing Fmoc-Lys(Fmoc) at the following two synthetic cycles. After this, the synthesis was switched to the manual mode (see above) for deprotection with piperidine/DMF (20% v/v) for 5 and 15 min and chloroacetylation with 4 mmol of chloroacetic acid in the presence of an equivalent amount of diisopropylcarbodiimide in dichloromethane. Cleavage with TFA/H_2O/TIS (95:2.5: 2.5 v/v/v, 1 h, RT) and preparative RP-HPLC purification led to the target branched peptide in analytical HPLC-homogeneous form and with the expected mass by MALDI-TOF MS (see Figure S1 and Table S1, Supporting Information).

For the thioether ligation reaction, 1.16 mg (1 μmol) of the tetra-chloroacetylated Lys core was dissolved in 5 mL of 20 mM Tris, 2 M guanidinium chloride buffer, pH 7.6, at 50 °C, then 12 μmol (12-fold molar excess) of either M2e or sM2e peptide epitope were added portionwise to the solution, adjusting the pH as required. Aliquots of the mixture were taken for analytical HPLC and MALDI-TOF MS, and when no further changes in the HPLC profile were observed (usually after 15−20 h), the reaction product was separated by preparative RP-HPLC. Fractions representative of different degrees of epitope incorporation (Table 2, first entry) were characterized by analytical HPLC and MALDI-TOF MS. For further details, see the Results section below.

Full Stepwise SPPS of Type-2 and Type-3 MAPs. The different MAP constructs (see Figure 1 for details) were assembled mostly by automated procedures in an ABI433 synthesizer running the FastMoc protocols discussed above on Fmoc-Rink-amide ChemMatrix resin at 0.02 mmol scale. 10-fold excesses of Fmoc-protected L-amino acids and HBTU/HOBt in the presence of DIEA (20-fold excess) were routinely used throughout. Branching was achieved at the appropriate points by incorporation of the Fmoc-Lys(Fmoc) derivative. As above, the two Ser-Ser dipeptides of the full-length M2e epitope were introduced in the manual mode as the pseudoproline dipeptide Fmoc-Ser(tBu)-Ser($\Psi^{Me,Me}$pro)-OH. Deprotection and cleavage were as described in the general SPPS protocols above. The synthetic crudes were purified by preparative HPLC and characterized by analytical HPLC and MALDI-TOF MS. Analytical data on the different MAPs are given in Table 2.

ELISA. Peptide stock solutions (either the single M2e sequence or tetravalent type-3 MAP) were initially made up at 25 μM concentration in PBS (pH 7.4), then serially diluted so that 0.8, 0.6, 0.4, and 0.2 nmol peptide aliquots in 50 μL PBS were placed in triplicate in the wells of a microtiter plate for overnight coating at 4 °C. Wells were then washed three times with PBS containing 0.05% Tween-20, and the remaining sites on the wells were blocked by 3% BSA in PBS/Tween-20 at 37 °C for 2 h. After another three washes, 200 μL of a 1:10 000 dilution of mouse monoclonal 14C2 was added to wells and incubated for 2 h at 37 °C. The wells were washed three times

Scheme 1. Thioether Ligation Route to Type-1 MAPs



with PBS/Tween-20 to remove unbound antibody, then incubated with a 1:5000 dilution of the alkaline phosphatase conjugate of rabbit polyclonal antimouse IgG in 1%BSA/PBS/Tween-20 for 2 h at 37 °C. Wells were next washed three times with PBS/Tween-20, and 50 μL of a 1.0 mg/mL solution of *p*-nitrophenyl phosphate in 0.2 M Tris, 5 mM $MgCl_2$ was added to each well. After 30 min incubation, the plate was read at 405 nm in an ELx808 microplate reader (Bio-Tek Instruments Inc., Winooski, VT).

RESULTS

Ligation Approach to M2e Dendrimers. The different types of MAP structures produced and examined in this study are shown in Figure 1, along with the sequences of full-length (M2e) and shortened (sM2e) versions of the M2 ectodomain epitope. An influenza H5N1 M2e peptide sequence was used in this study in which the two internal Cys residues were changed to Ser to prevent disulfide bond formation which would complicate the synthetic work (see Table 1). Table 2 provides detailed molecular structures and analytical data to help the reader through the following description and discussion.

The M2e peptide initially used in the ligation approach to type-1 MAP contained a Cys residue in free thiol form required for the thioether ligation reaction with the tetra-chloroacetylated branched lysine core (Scheme 1), which in turn was made with a C-terminal Cys orthogonally protected with a temporary *tert*-butylthio group ensuring temporary protection during the ligation step and eventually enabling, after deprotection, either dimerization of the MAP or further conjugation onto a higher-order carrier. Another feature of this type-1 MAP (see Figure 1) was the two Lys residues adjacent to the first Lys branching point. The purpose of this Lys-Lys motif was to define a cathepsin-like cleavage site (*7*) which might favor the in vivo processing of the dendrimeric moiety.

An unforeseen problem of the ligation approach was that the 25-residue-long M2e peptide turned out to be a relatively challenging synthetic target, requiring considerable optimization. A first synthesis on standard Rink-amide by the typical Fmoc methodology resulted in a complex, unpromising product vis-à-vis its subsequently expected use (Figure 2A). Use of the demonstrably superior Rink-amide ChemMatrix resin (*42*) and double coupling at some positions (Glu residues 16 and 14, plus the entire Glu8-Ser2 stretch, based on MS analysis of the crude from the initial trial, and on prediction of difficult sequences with the Peptide Companion program) ameliorated only partially the situation (Figure 2B). In a third, successful trial, the above improvements plus replacement of the two pairs of consecutive Ser residues in the M2e sequence with the pseudoproline dipeptide Ser(tBu)-Ser($\Psi^{Me,Me}$pro)-OH (*43*−*45*) led to a more acceptable product that could be purified and characterized as the desired M2e peptide in free thiol form (Figure 2C; see also Figure S1 and Table S1, Supporting Information). The chemoselective ligation of this purified, sparingly soluble peptide to the $ClAc_4$-Lys core was carried out in Tris-Cl buffer at pH 7.6 and 50 °C, using a 10−12-fold molar excess of M2e peptide in order to drive the reaction into completion. Addition of chaotropic agents (e.g., guanidinium chloride) to multimer-building reactions of this type has been predicated as preventing aggregation and favoring maximal substitution (*46, 47*), but in our case, it did not have any positive effect. Analytical RP-HPLC of the ligation process revealed quite a complex reaction mixture (Figure 3), which by MALDI-TOF MS analysis (Figure S3,

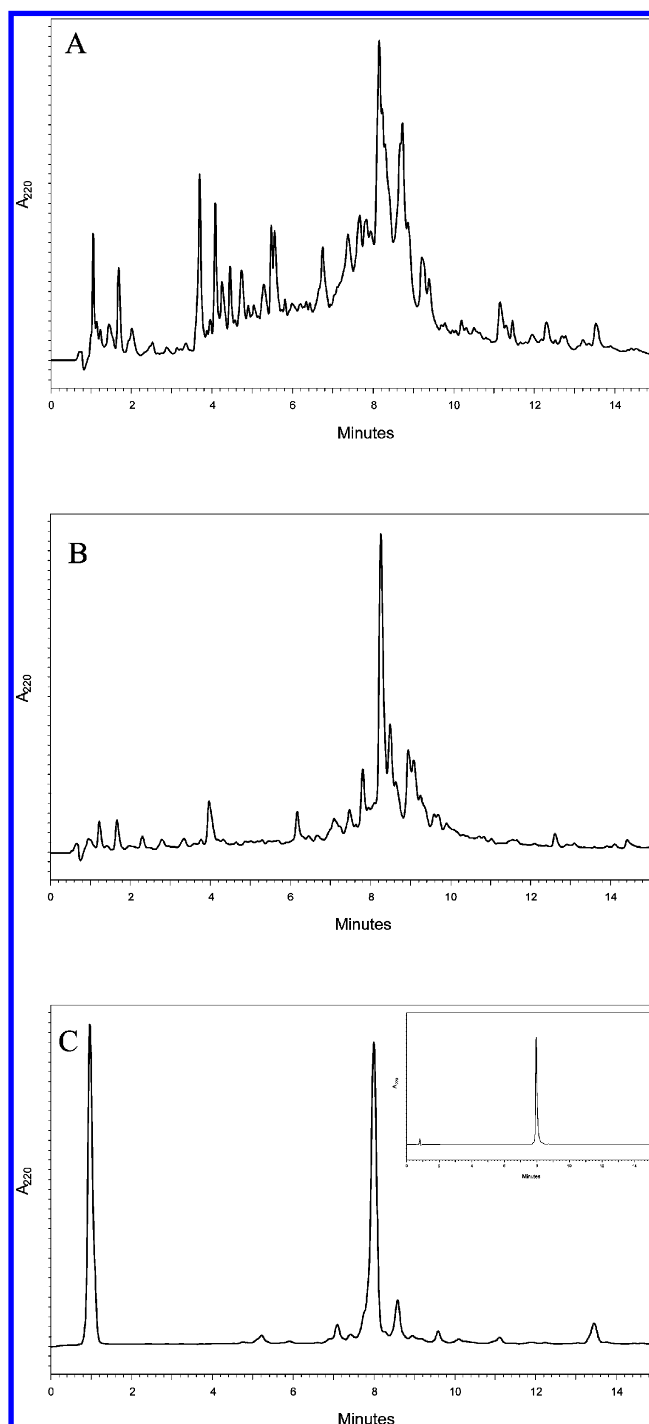


Figure 2. HPLC of crude M2e peptide made by different Fmoc synthesis strategies. A: Rink amide MBHA resin. B: Rink-amide ChemMatrix resin, double couplings at selected positions. C: as in B, with pseudo-Pro dipeptide replacements. 5−50% linear gradient of eluent B into A over 15 min. Analytical C_{18} column. Inset: HPLC of purified M2e, same elution conditions. See Experimental Procedures (Analysis and Purification section) in the main text for further details.

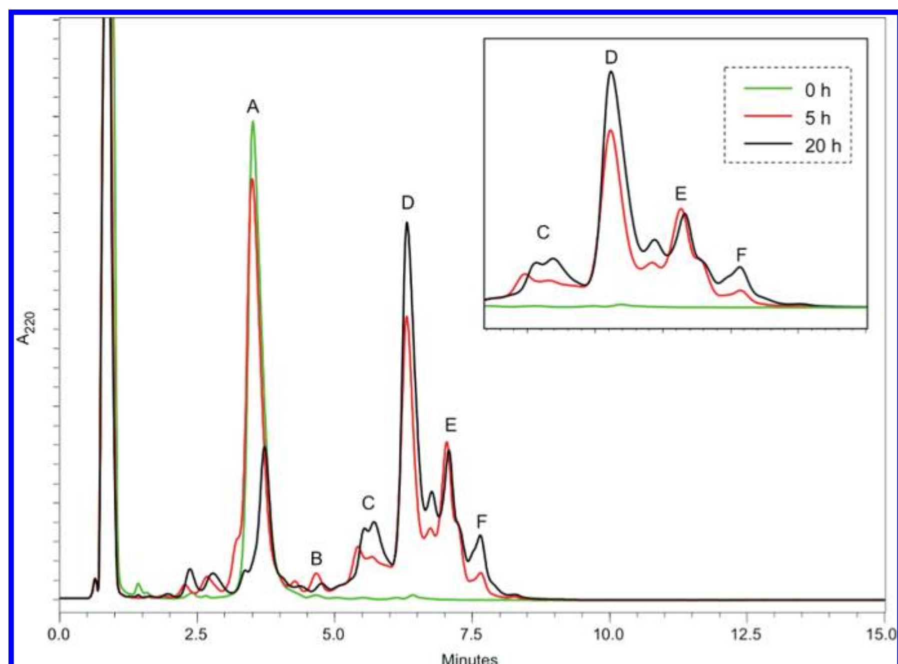


Figure 3. Progress of the chemical ligation of M2e to $ClAc_4$-Lys core monitored by HPLC at 0, 5, and 20 h reaction time (green, red, and black traces, respectively). 20−35% linear gradient of eluent B into A over 15 min. Analytical C_{18} column. Peaks labeled A−F correspond to M2e peptide, 1-copy MAP, mixture of 1- and 2-copy MAP, M2e dimer and tetramer, 3-copy MAP, mixture of 3- and 4-copy MAP, respectively.

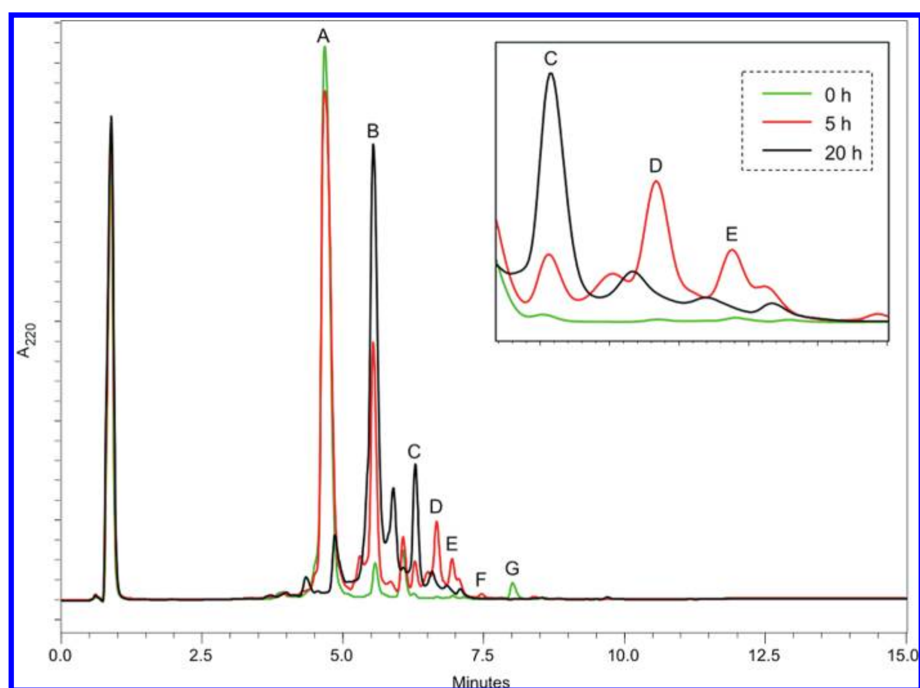


Figure 4. Progress of the chemical ligation of sM2e to $ClAc_4$-Lys core monitored by HPLC at 0, 5, and 20 h reaction time (green, red, and black traces, respectively). 10−40% linear gradient of eluent B into A over 15 min. Analytical C_{18} column. Peaks labeled A−G correspond to sM2e peptide, sM2e dimer, 4-copy MAP, 3-copy MAP, 2-copy MAP, 1-copy MAP, and unreacted $ClAc_4$-Lys core, respectively.

Supporting Information) was shown to include type-1 MAPs containing different numbers of copies of the M2e epitope (Table 2, first entry). HPLC resolution of these MAP products was poor (Figure 3), even using rather shallow gradients. Coeluting with the MAP products, substantial levels of the disulfide dimer of M2e were found (Figure 3), and quite unexpectedly, sizable amounts as well of a species that by MALDI-TOF and MS/MS analysis was identified as a tetramer of M2e (Figures S3A and S4, Supporting Information), i.e., the aggregate of two M2e (disulfide) dimers.

Since the aggregation tendency of the 25-residue M2e epitope posed unanticipated complications in the ligation approach, a shorter version of M2e (sM2e) was prepared (Figure S2 and Table S1, Supporting Information). This peptide was much simpler to make and gave no evidence of multimerization. Using the above reaction conditions, the ligation of the $ClAc_4$-Lys core and sM2e proceeded smoothly, as monitored by analytical RP-HPLC (Figure 4). MALDI-TOF MS analysis (Figure S5, Supporting Information) showed the target dendrimer (four copies of the sM2e peptide) as the main outcome, although byproducts with two or three copies of sM2e were also present in the reaction mixture. Results are summarized in Table 2.

Solid-Phase Approach to M2e Dendrimers. Since the chemoselective ligation described above fell short of expecta-

tions as an efficient route to M2e dendrimeric constructs, we turned our attention to the originally reported SPPS method of MAP synthesis. The two MAP varieties made by this approach, types 2 and 3 (Figure 1), similarly to type 1 contained a Cys residue at the C-terminus, to be used for dimerization, attachment of an additional peptide epitope (e.g., a T-cell epitope), or conjugation to carrier proteins (KLH or BSA), all with a view to further increasing the immunogenicity of the constructs.

Given the difficulties observed during the synthesis of the M2e peptide (see above), the shortened sM2e sequence was initially chosen to optimize the synthetic methodology. As shown in Figure 1, the first MAP attempted by SPPS was of the classical, type 2 variety, displaying four copies of sM2e (Table 2, third entry). This dendrimer was obtained rather straightforwardly by stepwise SPPS on a Rink amide ChemMatrix resin using standard Fmoc chemistry (Figure S6, Supporting Information) with double couplings throughout the sequence of the epitope. Next, and in anticipation of the difficulties that a full-length M2e epitope might pose to MAP synthesis, it was decided to enhance the overall flexibility of the MAP skeleton by introducing 6-aminohexanoic acid (Ahx)[1] spacer units at each branching point. This modification defined a novel architecture, type-3 MAP (Figure 1), first tested in tetravalent fashion with the uncomplicated sM2e epitope (Table 2, fourth entry). Again, this dendrimer was readily accessible, basically trouble-free, through stepwise SPPS (Figure S7, Supporting Information). With such favorable results in hand, it seemed appropriate to take on the potentially challenging synthesis of the MAP with four copies of the full-length M2e epitope (Table 2, fifth entry). The same tactics that had significantly improved the synthesis of the single-chain M2e, namely, replacement of the two Ser-Ser pairs by pseudoproline dipeptides (Fmoc-Ser(tBu)-Ser($\Psi^{Me,Me}$pro)OH), were again applied in a stepwise Fmoc SPPS on Rink amide ChemMatrix resin, again using double couplings throughout the entire M2e epitope sequence. Once more, the SPPS approach turned out a rather clean, easily purifiable and satisfactorily characterized end product (Figures S8 and S9, Supporting Information). Encouraged by this positive result, the strategy was pushed one step further in the synthesis of a type-3 octavalent sM2e MAP (Table 2, sixth entry). Remarkably, the end product was again readily accessible and characterized (Figure S10, Supporting Information). The logical extension of the all-SPPS approach was the octavalent version of the M2e MAP (Table 2, last entry). Unfortunately, application of the so-far successful combination of improvements (i.e., Ahx spacers, ChemMatrix resin, double couplings, pseudoproline dipeptide mimics) completely failed this time to provide any discernible trace of the desired MAP product.

Immunological Evaluation of M2e Presentations. After having proven the feasibility of obtaining a multimeric version of M2e in a relatively trouble-free way, it remained to be ascertained whether the enhanced epitope display provided by the tetravalent MAP platform translated into an equivalent increase in antigenic recognition (*48, 49*). This point was investigated by means of an ELISA where the monovalent M2e peptide and its tetravalent version (Table 2, fifth entry) were tested against the M2e-specific monoclonal 14C2. Lack of specific antibodies prevented a similar evaluation with sM2e-

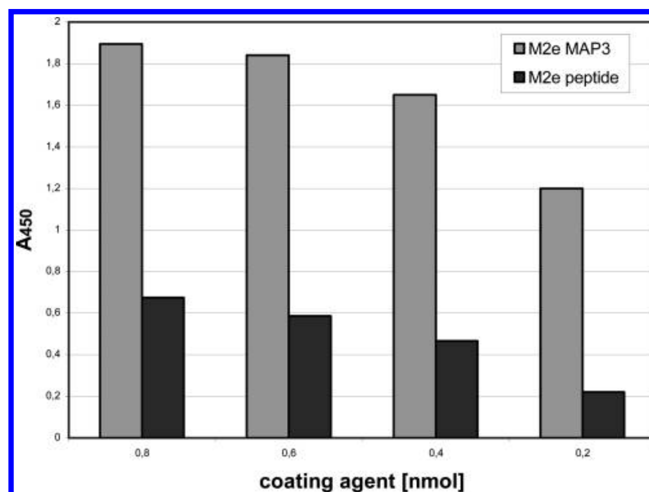


Figure 5. ELISA of M2e peptide in monomer (black columns) and type-3 MAP (gray columns) presentations against 14C2 monoclonal antibody.

displaying MAPs. Figure 5 shows that the antigenic response obtained with the single M2e peptide was indeed amplified with the MAP, with a 3-to-4-fold increased response that was in good agreement with the multimerization level provided by the MAP.

DISCUSSION

The goal of this study was to examine the relative advantages and limitations of the two main approaches to MAP constructs, namely, chemical ligation in solution (*16, 50, 51*) or fully stepwise SPPS (*12*). As a backdrop for such an endeavor, there was an intuition, partially grounded on our own previous experience in the field, that a nontrivial fraction of the synthetic attempts on MAPs by either of the above approaches had met with problems such as insolubility of the final product, sluggish progress of chemical ligation, and so forth, all leading to unsatisfactory results that either went unreported or were published with minimal, if any, chemical documentation.

In principle, the former (ligation) method would appear to be conducive to superior results, since purification of the intermediates (peptide epitope and branched Lys core) would yield an end product devoid of the minute but inevitable impurities (truncations, deletions) accumulated over a stepwise process, where the simultaneous growth of four—or even eight—peptide chains on a single molecular platform may, in some cases, promote aggregation, and in general have an amplifying effect on side reactions and thus the heterogeneity of the final product. These theoretical benefits of the ligation approach, however, can be severely challenged by experimental facts, as the above results on the incomplete conjugation of both M2e and sM2e epitopes on type-1 MAP scaffolds show. The most likely cause for our failure to obtain type-1 MAPs with the maximum number of epitope copies as a major final product is that, as successive epitope units are incorporated onto the $ClAc_4$-Lys scaffold, they generate a certain steric hindrance that may seriously hamper the access of (the Cys nucleophilic sulfur atom of) any incoming peptide epitope to the remaining unreacted ClAc groups. In the particular case of the present study, the nature of one of the peptide epitopes chosen, i.e., the full-length M2e, posed even additional difficulties for the chemoselective ligation approach (see below), exceeding in complexity any other similar reaction within our own expertise (*18*). It is indeed known from structural studies that the ion channel-forming transmembrane domain of M2 protein exists as a homotetramer (*21, 25, 52*−*57*). The present results would appear to suggest that, beyond the transmembrane region, an

[1]Abbreviations: Ahx, 6-aminohexanoic acid (residue); ClAc, chloroacetyl; DIEA, *N*,*N*-diisopropylethylamine; EDT, ethanedithiol; ESI, electrospray ionization; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; LTQ, linear trap quadrupole; MS, mass spectrometry; SPPS, solid phase peptide synthesis; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

aggregation propensity is also inherent (or somehow relayed) to the ectodomain as well.

Yet, even when this latter inconvenient was dealt with appropriately (i.e., by switching to a shorter sM2e epitope), solution ligation remained a less rewarding approach to MAP constructs than originally anticipated. In assessing the evidently complex outcome of this synthetic route (see especially Figure 3, also Figure 4), it is worth noting that, of all the possible products (final and intermediate) of such a ligation reaction, only the 4-copy MAP has a univocal chemical constitution; other intermediates (i.e., those with 1−3 epitope copies) may exist as slightly similar but constitutionally nonequivalent (though isobaric) isomers, depending on which positions in the branched Lys scaffold are occupied by the epitopes. Therefore, and despite very careful collection, HPLC fractions representative of a given M2e epitope multiplicity ($n = 2, 3, 4$) were not strictly homogeneous.

The alternative approach to MAPs, namely, fully stepwise SPPS, was for its part not devoid of difficulties either. While the relatively simple type-2 MAP with four copies of the sM2e epitope was viable, the known propensity to aggregate of the biologically more relevant M2e epitope (see Ligation approach to M2e dendrimers, Results section) might pose synthetic trouble for the corresponding MAP. As a safeguard against interchain association, it seemed to us that augmenting the global flexibility of the growing MAP construct by insertion of Ahx spacing units might be beneficial. This was indeed proven so in the syntheses of the tetravalent type-3 MAPs of both sM2e and M2e, as well as for the octavalent MAP of sM2e. However, extension of the approach to an octavalent M2e MAP did not meet with success, and its synthetic viability remains an open question.

In sum, depending on the targeted level of multiplicity and on the size and aggregation potential of the epitope to be displayed, MAP synthesis can range from facile to outright impracticable. The theoretical advantage that chemoselective ligation of prepurified components offers is seriously offset by slow reaction rates (possibly due to steric hindrance) and, in aggregation-prone peptide epitopes such as M2e, by additional adverse effects. This appears to give an edge to the original SPPS approach, which can be even further improved—especially in the case of potentially problematical epitopes such as M2e—by an enhancement of the internal flexibility of the construct such as that provided by the insertion of Ahx spacing units at the different branching points of the MAP dendrimer. While this appears to have a positive effect on synthetic viability and general quality (e.g., yield, solubility) of the end product, it cannot be regarded as a failsafe solution, as our results on the octavalent M2e show. All in all, our data suggest that, as for other similarly complex biomolecules, careful fine-tuning of synthetic conditions is essential if chemically well-defined MAPs, e.g., suitable for drug registry, are desired. In studies currently underway, we continue to explore the validity of the above and other approaches for other MAP platforms where sequence-specific features of the peptide epitope may again translate into synthetic complications.

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Supporting Information Available: HPLC traces and MALDI-TOF spectra of peptides and MAPs. This material is available free of charge via the Internet at http://pubs.acs.org.

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