Protocol

Journal of PeptideScience

Received: 26 May 2010 Revised: 30 August 2010 Accepted: 12 September 2010 Published online in Wiley Online Library: 30 November 2010

(wileyonlinelibrary.com) DOI 10.1002/psc.1310

Synthesis of multiple antigenic peptides (MAPs) – strategies and limitations

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Dendrimeric platforms such as MAPs can be synthesized either entirely by solid-phase methods (SPPS, direct approach) or by conjugation in solution of preformed, SPPS-made building blocks (indirect approach). Although MAPs and MAP-like constructs have been extensively and successfully used for various biological (mainly immunological) applications, experimental reports are most often lacking in chemical detail about their preparation and characterization. Here, we provide complete accounts of the synthesis and analytical documentation of MAPs and similar dendrimers by either all-SPPS (direct) or chemoselective thioether ligation (indirect) methods. We have chosen as model epitopes a 24-residue sequence of the ectodomain of protein M2 from influenza virus (M2e), which is found to be a rather challenging peptide epitope, and a far more manageable, shortened (12-residue) version of the same peptide. The advantages and shortcomings of both direct and indirect methods are discussed.
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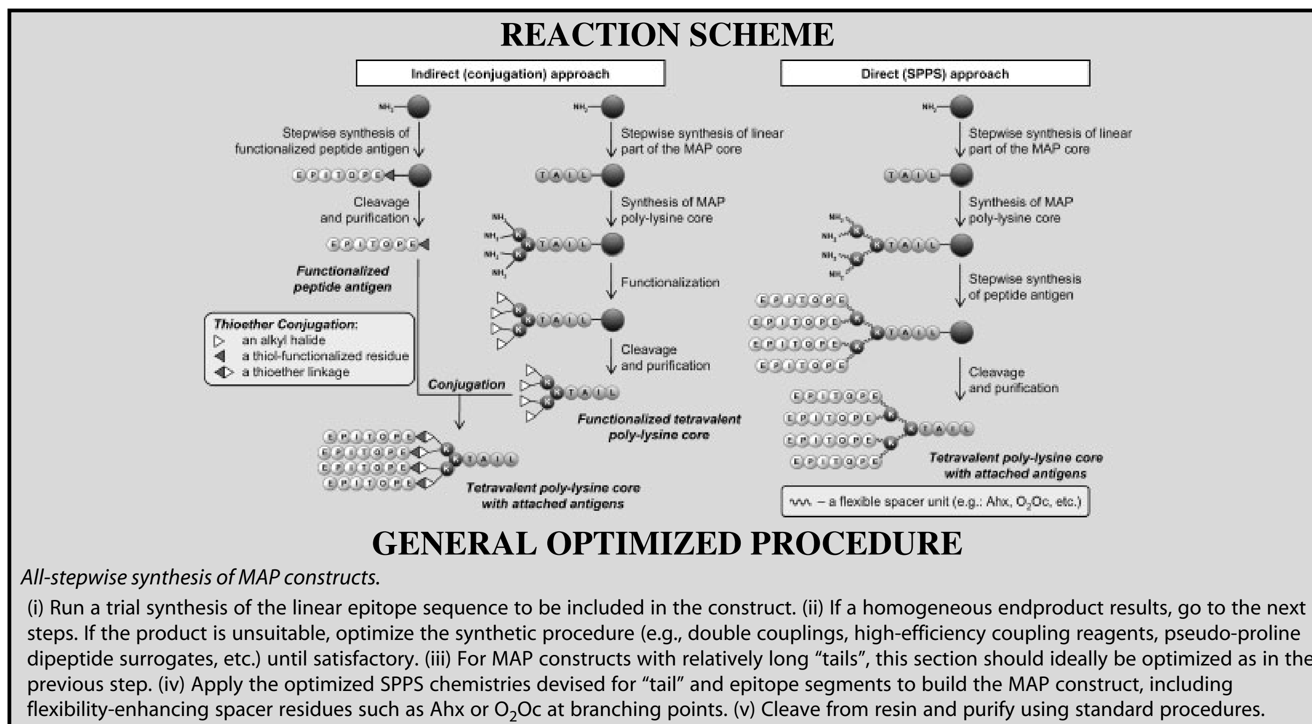
Keywords: multiple antigenic peptides; thioether conjugation; solid-phase peptide synthesis; dendrimers



Scope and Comments

Dendrimers are highly ordered, hyperbranched polymers with potential for a whole range of chemical applications [1]. Both divergent (i.e. stepwise from the core) and convergent methods for their synthesis have been described [2]. MAPs are dendron-like molecular constructs based on a poly-lysine scaffold. With both its

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Abbreviations used: *Ahx, 6-aminohexanoic acid (residue); ClAc, chloroacetyl; LTQ, linear trap quadrupole; O_2Oc, 3,6-dioxaoctanoic acid; TIS, triisopropylsilane.*

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α- and side chain ε-amino groups, lysine is an ideal monomer for creating branching points onto which epitope peptide sequences can be attached. The result is a large peptide macromolecule with a high enough antigen-to-core molar ratio to be used as an immunogen without the need for conjugation to a carrier protein. This type of dendrimer was introduced by J. P. Tam in the 1980s [3] and has found numerous biological applications, e.g. in vaccine and diagnostics research. In its original design, MAP was a simple dendron radiating directly from the lysine core, but the concept can be naturally extended to more tree-like constructs where a single sequence ('tail', see Reaction Scheme) forks out into 2^n branches (n usually ≤ 3). Like other dendrimers, MAPs can be synthetically approached by divergent or convergent methods. The former approach, which we choose to call *direct method*, relies entirely on SPPS, while the latter, referred to also as *indirect method*, uses various forms of chemical conjugation, e.g. thioether, oxime or hydrazone ligations [4]. While both approaches have as common requirement a branched poly-lysine core (see Reaction Scheme), in the direct method each branch is elongated into the corresponding epitope by stepwise SPPS, whereas in the indirect method the core is appropriately functionalized at each branch to give a multivalent platform to which peptide epitopes are chemoselectively tethered. This latter approach has the known theoretical advantage of convergent methods, which allow the pre-purification and characterization of both core and epitope components [5,6], and thus lead to better-defined, more unambiguous end-products than the all-SPPS direct method, where the simultaneous stepwise growth of multiple peptide chains may act as an amplifier of synthetic errors (deletions, truncations, side reactions) and cause microheterogeneity in the final material. These plausible claims in favor of the conjugation method are unfortunately backed by a rather sparse body of reliable chemical documentation, and thus open to question. An additional consideration is that the success of a MAP synthesis is more likely to be sequence-dependent in the indirect method – where solution behavior is an important issue – than on the direct one. Hence, comparing the results of the two methods is risky unless the same epitope is used in both cases. These are non-trivial issues that we have somehow sought to address in the protocols given below for the direct (SPPS) or indirect (conjugation) synthesis of a tetravalent MAP displaying as common model epitope the dodecapeptide EVETPTRNEWES, derived from the ectodomain of protein M2 (M2e) from the influenza virus [7,8]. Evaluation of the experimental results for yield, purity (i.e. content in the tetravalent target), ease of purification and production timeframe clearly rules in favor of the SPPS method (compare Figure 1 with Figure S1(A), Supporting Information), which can be further improved if flexible spacer amino acids such as Ahx [9] are inserted at all branching points (Figure 2). This point is illustrated by the two MAP constructs, both made by the direct method and again displaying the same above epitope, compared in Figures 1 and 2. While the quality of the final products, according to the respective HPLC traces, is very similar, the isolated yield was much better (9 vs 16.5%) in the synthesis where Ahx was used. One can hypothesize that the flexibilizing effect of Ahx helps in keeping peptide chains properly solvated during synthesis, preventing aggregation and hence increasing the amount of viable growing peptide sequences. The validity of this approach has been repeatedly verified in our laboratory (Figure 3) and recently taken a step further by replacing Ahx for 3,6-dioxaoctanoic acid (O_2Oc), which adds PEG-like, solubility-enhancing features to the flexibilizing effect. Figure S2 (Supporting Information) illustrates this point by means of a MAP synthesis that totally failed (due to epitope peptide insolubility) by the indirect (conjugation) method and yet led to a reasonably pure target product by the SPPS approach using O_2Oc spacers.[10] Further illustration of the advantageous use of the SPPS approach in conjunction with the Ahx spacer is provided by the successful synthesis and characterization of an octavalent MAP displaying the sM2e epitope and a tetravalent MAP displaying the epitope sequence FGFPEHLLVDFLQSLS (Figures S3 and S4, Supporting Information).

While the direct (all-SPPS) method may help overcome the difficulties posed by the conjugation approach in terms of yield, lack of a homogeneous MAP composition or sluggish reaction rates (see Figure S1(A), Supporting Information, for a representative example), a further potential source of trouble, namely the intrinsic synthetic difficulty of the epitope sequence itself, must not be forgotten. In this regard, practitioners planning to make MAP constructs should consider it advisable, perhaps almost mandatory, to run a synthesis of the linear epitope as a preliminary exploration of possible pitfalls to be encountered at the multimeric level. A case in point is the full sequence of the M2e domain from influenza virus, a 24-residue peptide (MSLLTEVETPTRNEWESRSSDSSD) that even at the monomer level entailed significant synthetic challenges and thus required considerable fine-tuning, including the use of optimal solid support and of secondary structure-disrupting pseudo-proline dipeptides [10]. These improvements could then be productively applied to the synthesis by the direct SPPS method of a tetravalent MAP displaying this same epitope, as shown in Figure 3. It is worth mentioning that this MAP was only viable when the above improvements, in conjunction with Ahx spacing units, were introduced, and that its convergent (conjugation) alternative was again inefficient and slow [11].

Experimental Procedure

General Procedure for Tetravalent MAP Synthesis in the Solid Phase

The MAP constructs were prepared in automated peptide synthesizers such as the Prelude (Protein Technologies, Tucson, AZ) or the ABI433 (Applied Biosystems, Foster City, CA) running standard Fmoc chemistry protocols [12] on 0.1 g of Fmoc-Rink-amide ChemMatrix resin (Matrix Innovation, Montreal) of 0.2 mmol/g substitution, amounting to 0.02 mmol of amino groups. Resins with higher capacities (e.g. 0.47 mmol/g) did not always lead to satisfactory results. The side chains of trifunctional Fmoc-amino acids were protected with TFA-labile protecting groups as required. Residues corresponding to the monovalent ('tail') part of the construct (see Reaction Scheme), up to the first (bis-Fmoc) Lys residue initiating the dendrimer structure, were incorporated via single couplings. For synthesizers such as the Prelude, with programs that allow adjustment of reagent amounts to individualized synthetic cycles, these single couplings involved 0.2 mmol (tenfold molar excess over amino groups) each of Fmoc-amino acid, HBTU and HOBt, plus 0.4 mmol of DIEA in DMF for 20 min. After Fmoc deprotection (piperidine/DMF, 20% v/v), the following building blocks, i.e. Ahx or O_2Oc spacer units and/or Fmoc-Lys(Fmoc) residues giving rise to the tetravalent dendron, were incorporated via double couplings with 0.4 mmol each (tenfold molar excess over actual amino groups, now double relative to previous stage) of Fmoc-amino acid, HBTU and HOBt and 0.8 mmol of DIEA in DMF for 15 + 10 min. From then on, an amino group content of

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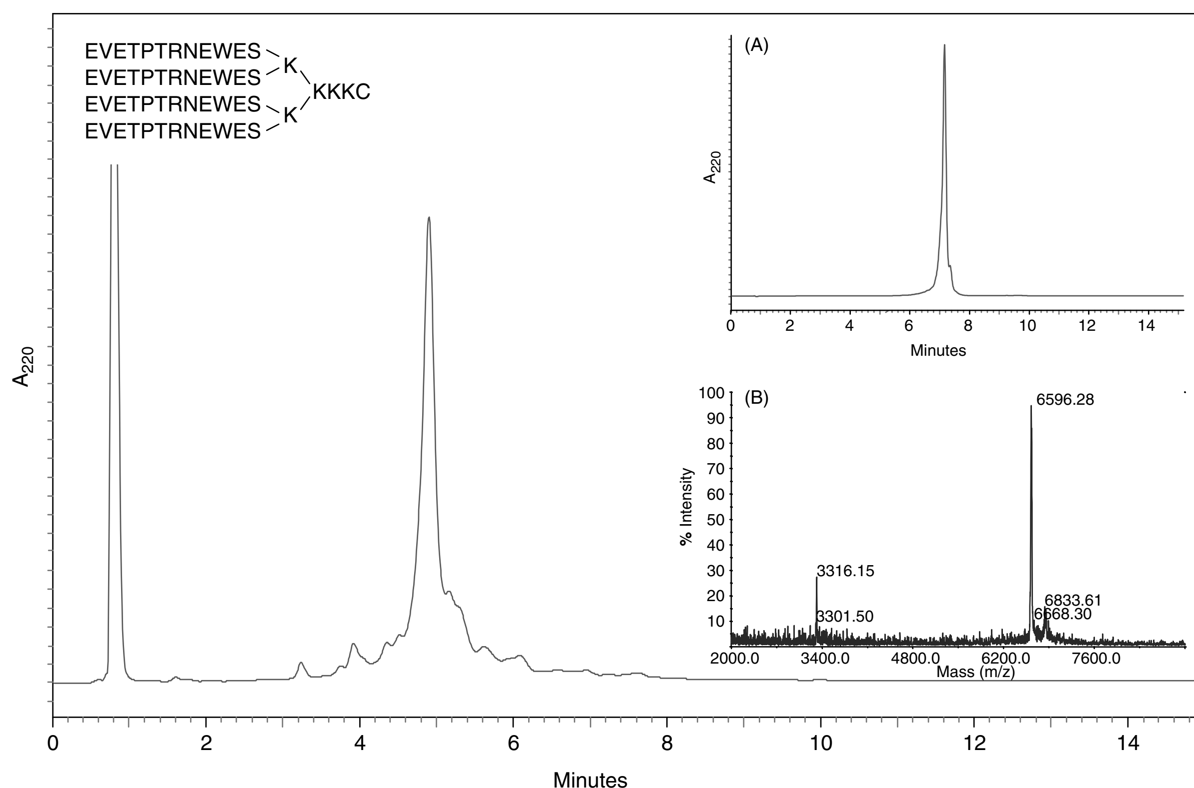

Figure 1. Structure and analytical characterization of the tetravalent construct sM2e-MAP. Main panel: C_{18} RP-HPLC analysis of crude product, 5–50% linear gradient of eluent B into A over 15 min. Inset (A): HPLC of purified product (same elution conditions). Inset (B): MALDI-TOF MS of the product: calculated for $C_{281}H_{432}N_{80}O_{102}S_1$: m/z $[M + H]^+_{av}$: 6,595.05, found: $[M + H]^+_{av}$: 6,596.28, isolated yield 9%.

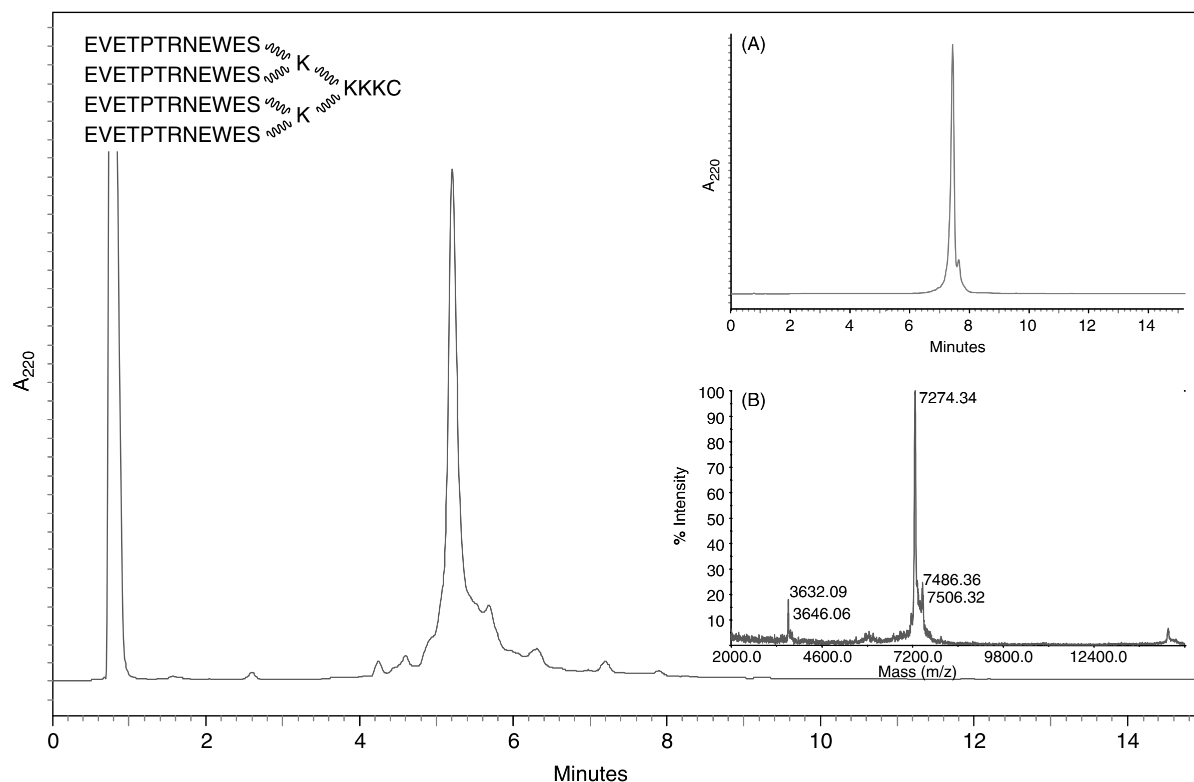

Figure 2. Structure and analytical characterization of the tetravalent construct sM2e-Ahx-MAP (wavy lines indicate a flexible spacer unit Ahx). Main panel: RP-HPLC analysis of crude construct 5–50% linear gradient of eluent B into A over 15 min. Analytical C_{18} column. Inset (A): HPLC of purified product (same elution conditions). Inset (B): MALDI-TOF MS of the product: calculated for $C_{317}H_{498}N_{86}O_{108}S_1$: m/z $[M + H]^+_{av}$: 7 273.99, found: $[M + H]^+_{av}$: 7 274.34, isolated yield 16.5%.

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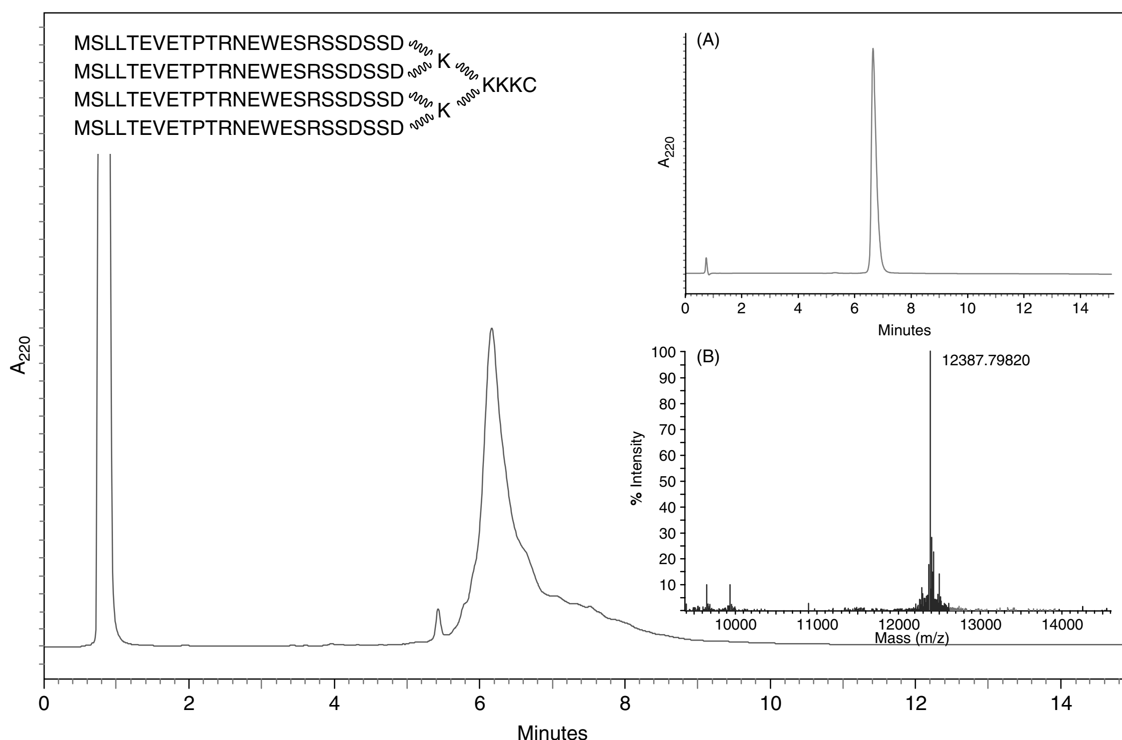

Figure 3. Structure and analytical characterization of the tetravalent construct M2e-Ahx-MAP (wavy lines indicate a flexible spacer unit Ahx). Main panel: RP-HPLC analysis of crude construct 20–40% linear gradient of eluent B into A over 15 min. Analytical C_{18} column. Inset (A): HPLC of purified product (same elution conditions. Inset (B): nano ESMS of the product: calculated for $C_{517}H_{838}N_{146}O_{196}S_5$: m/z $[M + H]^+_{mo}$: 12 387.86, found: $[M + H]^+_{mo}$: 12 387.80 (spectrum recorded in a LTQ-Orbitrap XL instrument), isolated yield 4.8%.

0.08 mmol (4 × the initial one) was assumed for the peptide-resin, and all further building blocks (Ahx or O_2Oc spacers and/or residues of the epitope sequences) were again incorporated via double couplings with tenfold molar excess (0.8 mmol) of Fmoc-amino acid, HBTU and HOBt and twice that amount of DIEA in DMF for 15 + 10 min. For instruments (e.g. the ABI433) not easily allowing individualized reagent delivery at selected synthetic cycles, the maximum amounts of activating species (i.e. 0.8 mmol of Fmoc-amino acid, HBTU and HOBt, and 0.16 mmol of DIEA) were used throughout the entire assembly process.

Once sequence assembly was completed, the Fmoc groups were removed and the peptide-resin was cleaved and fully deprotected with TFA/H_2O/EDT/TIS (94 : 2.5 : 2.5 : 1 v/v, 90 min). The peptide was precipitated by the addition of chilled diethyl ether, centrifuged for 3 × 10 min at 4 °C, and the pellet was taken up in aqueous AcOH (10% v/v) and lyophilized. Purification of the MAP products was done by preparative RP-HPLC on Phenomenex C_{18} (21.2 × 250 mm, 10 µm) or C_8 (10 × 250 mm, 10 µm) columns using linear gradients of solvent B into A (A and B: 0.1% TFA (v/v) in water and acetonitrile, respectively) over 30 min, at 25 or 5 ml/min flow rate for C_{18} or C_8 separations, respectively, with UV detection at 220 nm. Fractions judged to be of adequate purity by analytical HPLC were pooled and lyophilized, and their identity checked by MS (MALDI-TOF or electrospray).

Indirect Method: MAP Synthesis by Conjugation (Thioether) in Solution

Tetravalent functionalized core

This component, preceded or not by a monovalent 'tail' (see Reaction Scheme) section, was assembled by Fmoc-based SPPS as described above, typically at a 0.1-mmol scale on Fmoc-Rink-amide ChemMatrix resin, and using eightfold molar excess (0.8 mmol) of Fmoc-amino acid, HBTU and HOBt in the presence of double that amount of DIEA in DMF as solvent. After deprotection of the two branching Lys residues, chloroacetic acid (4 mmol, tenfold molar excess) was coupled in the presence of an equimolar amount of diisopropylcarbodiimide in dichloromethane. Cleavage with TFA/H_2O/TIS (95 : 2.5 : 2.5 v/v/v, 1 h) followed by preparative RP-HPLC purification led to the target tetravalent scaffold ([ClAc]$_4$-Lys-core) in HPLC-homogeneous form and with the expected mass by MALDI-TOF MS (Figure S1(B), Supporting Information).

Peptide epitopes

The sM2e epitope sequence (EVETPTRNEWES), *C*-terminally elongated with a Cys residue, to be used in the subsequent conjugation step (see Section on Conjugation Reaction), was assembled by Fmoc SPPS on Fmoc-Rink-amide MBHA resin using synthetic protocols similar to above. After cleavage with TFA/H_2O/EDT/TIS (94 : 2.5 : 2.5 : 1 v/v, 90 min, RT) and RP-HPLC preparative purification, the peptide was satisfactorily characterized by MALDI-TOF MS (Figure S1(C), Supporting Information).

Conjugation reaction

The [ClAc]$_4$-Lys-core (1.16 mg, 1 µmol) was dissolved in 5 ml of 20 mM Tris, 2 M guanidinium chloride buffer [13,14], pH 7.6, at 50 °C, then 12 µmol (12-fold molar excess) of the sM2e peptide epitope was added portionwise to the solution, adjusting the pH as required. Aliquots of the mixture were taken for analytical HPLC and MALDI-TOF MS. To monitor the progress of the reaction,

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aliquots were taken from the reaction mixture, neutralized with 10 μl of acetic acid and analyzed by HPLC (20 μl injection) and MALDI-TOF MS (see Figure S1(A), Supporting Information).

Shortcomings and Recommendations

1. Despite the fact that conjugation, by its convergent character, should in theory be superior to stepwise synthesis, its limitations soon become obvious as the size of the epitope to be incorporated increases. Thus, for mid-to-large size epitopes, steric hindrance can hamper access of the Cys thiol to unreacted ClAc groups. The solubility of the peptide epitope can also become an issue and is difficult to predict. In general, an exploratory synthesis of the epitope in linear form is highly recommendable. In the indirect approach, this synthesis will already provide one of the two main conjugation components while, in the all-stepwise method, careful analysis (HPLC, MS) of the resulting synthetic product will provide valuable clues about difficult couplings and other trouble spots that should be adequately addressed (e.g. using extra couplings, pseudo-proline dipeptides [11] or other improvements in chain assembly) in the synthesis of the considerably more complicated MAP structures.
2. In general terms, one will find the conjugation approach considerably more expensive and time-consuming than the all-SPPS approach. For one thing, it requires high excesses of the epitope, which must first be HPLC-purified and then used in high excess (e.g. 12-fold for a tetravalent MAP) to ensure that the process goes to completion. It also tends to generate (in thiol-based conjugations) substantial amounts of disulfide dimer that can be recycled into the thiol form, albeit at an additional cost in time.
3. While the aim of this protocol is to illustrate the feasibility of MAP synthesis (with satisfactory analytical characterization) by either of the two approaches discussed, it seems fair to remind potential users of the non-trivial nature of any MAP synthesis that, even with improvements such as those described above (e.g. flexibility-enhancing spacers, optimized resins, etc.) and other facilitating strategies, may not always meet with success, as illustrated by the difficulties found in one of the octavalent MAPs described in Ref. 10. This emphasizes the need for careful analytical monitoring of the synthetic product, and the advisability of an exploratory synthesis of the epitope sequence as suggested in (1) above.

Acknowledgments

Work supported by MICINN, the Spanish Ministry of Science and Innovation (grants BIO2005-07592-CO2-02 and BIO2008-04487-CO3-02 to D.A.), by the regional government of Catalunya (SGR2005-00494). W.K. is a fellow in the Juan de la Cierva Program of MICINN.

Supporting information

Supporting information may be found in the online version of this article.

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