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Influence of Conjugation Chemistry and B Epitope Orientation on the Immune Response of Branched Peptide Antigens

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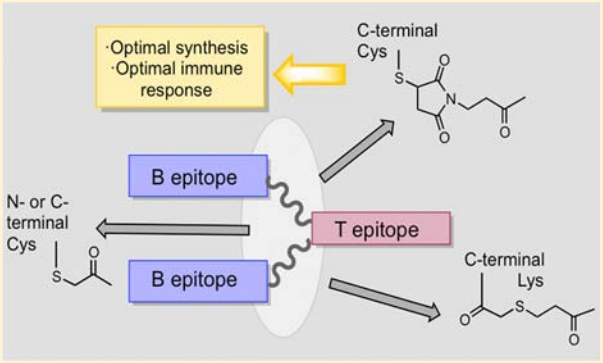
ABSTRACT: Multimeric presentation, a well-proven way of enhancing peptide immunogenicity, has found substantial application in synthetic vaccine design. We have reported that a combination of four copies of a B-cell epitope with one of a T-cell epitope in a single branched construct results in a peptide vaccine conferring total protection against foot-and-mouth disease virus in swine, a natural host (Cubillos et al. (2008) *J. Virol. 82*, 7223−7230). More recently, a downsized version of this prototype with only two copies of the B epitope has proven as effective as the tetravalent one in mice. Here we evaluate three approaches to bivalent platforms of this latter type, involving different chemistries for the conjugation of two B epitope peptides to a branching T epitope. Comparison of classical thioether, "reverse" thioether (Monsó et al. (2012) *Org. Biomol. Chem. 10*, 3116−3121) and thiol−ene conjugation chemistries in terms of synthetic efficiency clearly singles out the latter, maleimide-based strategy as most advantageous. We also examine how minor structural differences among the conjugates—including the N- or C-terminal attachment of the B epitope to the branching T epitope—bear on the immunogenicity of these vaccine candidates, with the maleimide-based conjugate again emerging as the most successful.



INTRODUCTION

Peptide-based vaccines are promising alternatives in the control or therapy of infectious diseases for reasons such as (i) their lack, by definition, of infectious agent, which not only ensures absolute safety but also permits easy differentiation of infected from vaccinated animals (often referred to by the DIVA acronym), (ii) the accurate molecular delineation of the immunogen, which allows to exclude detrimental sequences present in full-length antigens or other pathogen-related molecules, (iii) ease of synthesis and scaleup, or (iv) uncomplicated transport and storage.[1−3] Unfortunately, the ideal realization of these advantages is often hampered by the low immunogenic potential of linear peptides, a drawback that has been addressed in a number of ways,[4] among which multimeric presentation such as that afforded by multiple antigenic peptide (MAP) systems[5−7] stands out for the substantial enhancement of immunogenicity over the monomeric forms it provides. MAPs not only epitomize the aforementioned advantages of peptide-based vaccines, but are far superior to peptide−carrier protein conjugates in terms of chemical definition and offer the additional possibility of combining several epitopes in a single molecular platform.

As originally devised by Tam,[5] a MAP system is a fully peptide-based structure consisting of an immunogenically inert core of Lys residues radially forking into four or eight branches with an epitope copy each. A number of variations on this concept have been reported,[8] including further multimerization[9,10] or appendage of an additional epitope as a C-terminal "tail".[11] Although MAPs were initially reported as available by stepwise solid-phase peptide synthesis (SPPS) methods,[5] the success of this approach is often limited by factors such as the length or the inherent synthetic difficulty of the epitope sequence,[12] usually involving aggregation—favored by the spatially close epitope sequences in the MAP structure—that results in heterogeneous, hard-to-purify products. While some of these problems can be partially ameliorated by tactics such as low resin substitution—precluding interchain interaction, hence decreasing aggregation propensity—and by, in general, a specifically MAP-focused SPPS methodology,[12−14] persistent difficulties with the stepwise approach often make it advisable to consider alternatives such as the convergent approach in which prepurified building blocks (branched Lys core + epitope peptide) are conjugated in solution.[15] Several such strategies have been developed based on thioether,[15,16] oxime,[17] disulfide,[18] thioester,[19] thiazolidine,[20] or hydrazone linkages,[21] as well as on native chemical ligation.[22] A comparative study of several of these conjugation chemistries showed thioether-based conjugation as especially favorable, not only in terms of immunogenicity but also for the its simplicity, good yields and the relative metabolic stability of its products.[23] In its standard formulation,[15] thioether conjugation uses a chloroacetylated version of the branched Lys core to which various copies of the

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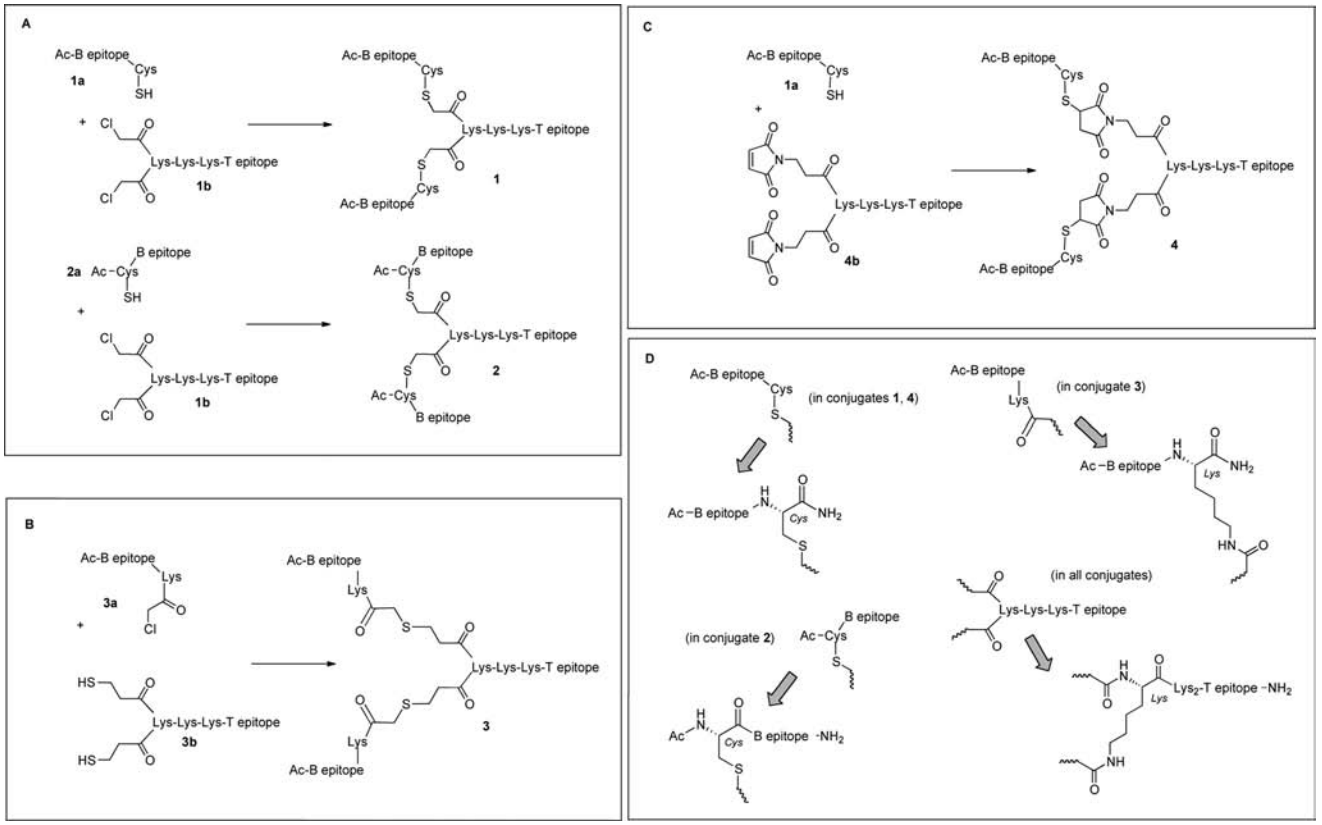

Figure 1. Conjugation approaches to immunogens **1**−**4**: (A) Thioether, C- and N-terminal orientations; (B) reverse thioether; and (C) maleimide. (D) Arrows used to illustrate aspects of the connectivity between the B epitope and the Lys core in further structural/stereochemical detail. B and T epitopes are PVTNVRGDLQVLAQKAART and AAIEFFEGMVHDSIK, respectively. All C-termini are in carboxamide form.

peptide epitope, each with a free-thiol Cys residue, are attached. More recently, a "reverse" thioether method where the functionalities of the building blocks (branched Lys core and the peptide epitope) are swapped has been shown to provide significant advantages over the standard method.[24] In search of further improving not only the efficiency but also the immunogenicity of this type of MAP constructs, in this work we explore two additional related issues, namely, (i) the effect of N- or C-terminal attachment of the epitope to the branched Lys core, and (ii) epitope attachment via thiol-maleimide[25] coupling, in comparison with standard[15] and reverse[24] thioether conjugation. Thiol addition to maleimides,[25−30] an example of nucleophilic conjugate addition to α,β-unsaturated carbonyls, has been used in peptide, DNA, and protein derivatization,[31−33] and also successfully applied to generate nonpeptide dendrimers.[27−30] However, to the best of our knowledge, this chemistry has not been hitherto used to prepare MAP-type vaccines.

The present study builds on the promising properties of B_4T—a MAP-like assembly of four copies of a B epitope and one copy of a T epitope—as a vaccine against foot-and-mouth disease (FMD),[11] the economically most devastating viral disease in animals.[34] A similar design has been applied, with partial success, in vaccines against classical swine fever.[14,35,36] In general, and despite their good immunogenic performance, B_4T vaccine prototypes were hampered by less-than-efficient syntheses[11,13,14] and the laborious purification of the resulting crude end products. This difficulty was pondered in conjunction with recent—and somewhat counterintuitive—evidence that multivalency does not always straightforwardly translate into improved immunogenicity.[7,37,38] In the specific case of FMD, work in our laboratories (Blanco et al., submitted) comparing vaccine candidates with four (B_4T), two (B_2T), or one (linear B-T chimera) B epitope copies shows not only that multivalency is advantageous over simple juxtaposition but also that bivalent constructions elicit similar or even better immune responses than tetravalent ones. In light of this, in the present study we have focused our attention on B_2T-type constructions, with the goal of finding the best possible balance between synthetic expediency and immunogenic profile. Hence, the bivalent platforms **1**−**4** schematized in Figure 1 have been compared on the basis of various synthetic parameters (e.g., reaction time and peptide equivalents per branch required, conversion rate, byproduct levels obtained) and subsequently evaluated for immunogenicity in mice. The B and T epitopes chosen for our study correspond, respectively, to antigenic site A of foot-and-mouth disease virus (FMDV), an immunodominant site comprising residues 141−159 (PVTNVRGDLQVLAQKAART) of capsid protein VP1 (serotype O/UK/01) and to the FMDV-specific T-cell epitope located in residues 21−35 (AAIEFFEGMVHDSIK) of nonstructural protein 3A.[39] Interestingly, the maleimide-based conjugate **4** is shown to be the most advantageous of the four, not only in terms of synthetic practicality but also with regard to immunogenic properties.

EXPERIMENTAL PROCEDURES

Materials and Methods. Fmoc-protected amino acids and HBTU were from Iris Biotech (Marktredwitz, Germany). Fmoc-Rink-amide ChemMatrix resin was from PCAS Bio-

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Matrix, Inc. (Saint-Jean-sur-Richelieu, Quebec, Canada). HPLC-grade CH_3CN and peptide-synthesis-grade DMF, CH_2Cl_2, DIEA, and TFA were from Carlo Erba-SdS (Sabadell, Spain). All other reagents were of the highest quality commercially available from Sigma-Aldrich (Madrid, Spain). Analytical reversed-phase HPLC was performed on C_{18} 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 CH_3CN. Elution was done with linear 20−60% gradients of solvent B into A over 15 min at 1 mL/min flow rate, with UV detection at 220 nm. Preparative HPLC was performed on C_{18} (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 CH_3CN, respectively, and elution was again with linear gradients of solvent B into A over 30 min, at 5 mL/min flow rate with UV detection at 220 nm. Fractions of satisfactory purity (>95%) by analytical HPLC were pooled and lyophilized. Purified peptides and conjugates were characterized for identity by MALDI-TOF MS in a Voyager DE-STR instrument (Applied Biosystems, Foster City, CA), using α-cyano-4-hydroxycinnamic acid as matrix. Spectra were recorded in the reflector mode for linear peptides; for larger-size analytes, the linear mode was used.

General Peptide Synthesis Procedures. Linear peptides were assembled in an ABI433 peptide synthesizer (Applied Biosystems, Foster City, CA) running Fmoc (FastMoc) SPPS protocols at 0.1 mmol scale on Fmoc-Rink-amide ChemMatrix resin. The side chain functionalities were protected with *tert*-butyl (Asp, Glu, Ser, Thr, Tyr), *tert*-butyloxycarbonyl (Lys, Trp), N^G-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Arg), and trityl (Asn, Gln, His) groups. Eightfold excess of Fmoc-L-amino acids and HBTU, in the presence of a double molar amount of DIEA, were used for the coupling steps, with DMF as solvent. Branching, when required, was achieved by the coupling of Fmoc-Lys(Fmoc) at appropriate points, and was followed by incorporation of a derivatizing moiety for each specific conjugation approach (see Figure 1 and next subsection). All peptides were fully deprotected and cleaved from the resin with TFA/H_2O/triisopropylsilane (95:2.5:2.5 v/v, 90 min, r.t.), precipitated by addition of chilled diethyl ether, taken up in aqueous AcOH (10% v/v), and lyophilized. Reverse-phase HPLC purification gave homogeneous materials with the expected mass by MALDI-TOF MS.

Functionalization and Conjugation of the Peptides. *Conjugates 1 and 2 (see Figure 1).* Both were prepared by standard thioether conjugation. Linear peptides **1a** and **2a**, both N-terminally acetylated, simply differed in the C- and N-terminal position of the Cys residue used for conjugation, respectively. Branched Lys core **1b** was synthesized as described above and functionalized by on-resin chloroacetylation at the N-terminal α- and ε-amino groups using 10-fold excess of chloroacetic acid and DIPCI (1:1) in CH_2Cl_2. With **1a**, **1b**, and **2a** components in HPLC-purified form, the conjugation reaction leading to either **1** or **2** was done by dissolving 3.5 mg (1.6 μmol) of **1b** in 10 mL of 0.02 M $NaHCO_3$, pH 7.5 at 50 °C, then adding portion-wise 27.5 mg (8-fold molar excess, 4 equiv/branch) of solid, lyophilized **1a** or **2a**, respectively, then adjusting the pH to 7.5 by addition of dilute NaOH. The reaction was monitored by HPLC and MALDI-TOF at 1 h intervals. When no changes in the HPLC profile were observed, the reaction was stopped with 1 mL of AcOH and the mixture purified by preparative RP-HPLC. Final products **1** and **2** were satisfactorily characterized for purity and identity by analytical HPLC and MALDI-TOF MS, respectively.

Conjugate 3 (see Figure 1). It was made by the reverse thioether approach[24] from **3a** and **3b** (Figure 1). Linear peptide **3a** was synthesized with an Fmoc-Lys(Mmt)-OH residue at its C-terminus to allow subsequent functionalization. After assembling the full sequence in the solid phase and acetylating the N^α group, the Mmt group was selectively removed with 1% TFA in CH_2Cl_2 and the free N^ε group was chloroacetylated as described for **1b**. Functionalization of **3b** as a thiol was carried out by treatment of both α- and ε-amino groups of the resin-bound branched Lys core with 10-fold molar excess of *S*-trityl-3-mercaptopropionic acid and DIPCI (1:1) in CH_2Cl_2. Peptides **3a** and **3b** were obtained after full deprotection and cleavage of the respective peptide resins and purified as previously described. For conjugation, 14.2 mg (4-fold molar excess, 2 equiv/branch) of lyophilized **3a** were added portion-wise to a solution of 3.5 mg (1.6 μmol) of **3b** in 10 mL of 0.02 M $NaHCO_3$, pH 7.5 at 50 °C. The conjugation was monitored and stopped as described above for **1** and **2**. After preparative RP-HPLC purification, **3** was satisfactorily characterized for purity and identity by analytical HPLC and MALDI-TOF MS, respectively.

Conjugate 4 (see Figure 1). Maleimide-functionalized **4b** was obtained by treatment of both α- and ε-amino groups of the resin-bound branched Lys core with 10-fold molar excess of *N*-maleoyl-β-alanine and DIPCI (1:1) in CH_2Cl_2. Deprotection, cleavage, and purification were as for **1b**−**3b** above. For conjugation, 4.5 mg of **4b** was dissolved in 1 mL of 50 mM phosphate buffer, pH 6, and added to a solution of 8.2 mg of **1a** (2-fold molar, stoichiometrical amount) in 5 mL of the same buffer at room temperature. HPLC analysis of an aliquot showed the reaction to be complete within 1 min. Purification and characterization were as above.

Serum Stability of the Conjugates. From a solution of conjugate in water (2.5 mg/500 μL), duplicate aliquots of 200 μL were taken, mixed with 300 μL of serum (Sigma) and incubated at 37 °C in a Thermomixer (Eppendorf, Hamburg, Germany) for up to 4 h. 60 μL samples were extracted at 15, 30, 60, 120, and 240 min incubation and treated each with 300 μL of 4% (v/v) TFA in CH_3CN/H_2O (7:3 v/v), chilled at 0 °C for 30 min and centrifuged at 14 100 rpm in a Minispin Plus (Eppendorf) for 4 min. 80 μL of the supernatant was analyzed in a Shimadzu LCMS-2010EV instrument using an XBridge column (4.6 × 150 mm, 3.5 μm, Waters, Cerdanyola del Vallès, Spain) eluted with a linear 20−60% gradient of solvent A into B (A and B: 0.1% formic acid in H_2O and CH_3CN, respectively) over 15 min at 1 mL/min flow rate. The intensity (total ion counts) of the original peak was monitored over time.

Mice Immunization and Antibody Production. The immune response induced by conjugates **1**−**4** was assessed in outbred Swiss ICR-CD1 mice (Harlan Laboratories, Boxmeer, The Netherlands). Five-to-six-week-old female mice in groups of five were maintained under standard housing conditions in the CISA-INIA Animal Care unit. All experimental procedures were conducted in accordance with protocols approved by the INIA ethical committee. Mice were immunized subcutaneously at days 0 and 20 with 100 μg of each conjugate emulsified in Montanide ISA-50 V2 (Seppic, Puteaux, France) and sacrificed at day 39. A control group of four mice were immunized with Montanide ISA-50 V2 adjuvant alone. Serum samples were collected by tail bleeds before (day 0) and after immunization

(days 15, 20, and 39) and used to study FMDV-specific antibody response by ELISA. To this end, Maxisorp 96-well ELISA plates (Nunc, Roskilde, Denmark) were coated with sucrose gradient-purified (140S) FMDV O/UKG11/2001 particles in PBS overnight at 4 °C. Wells were then washed with PBS and blocked with 5% skimmed milk in PBS for 2 h at 37 °C. Duplicate 3-fold dilution series of each serum sample were made, starting at 1/100, with 50-μL volumes used throughout. Preimmune sera from mice immunized with conjugates **1**–**4** and sera from nonimmunized animals were used as negative controls. Specific antibodies were detected with Zymed horseradish-peroxidase-conjugated goat antimouse IgG (Life Technologies, Alcobendas, Spain), diluted 1/3000. Color development was obtained after addition of 100 μL/well of 3,3′,5,5′-tetramethylbenzidine (Sigma) and stopped by adding an equal volume of 1 M H_2SO_4. Plates were read in an automatic Fluostar Omega microplate reader (BMG Labtech, Ortenberg, Germany) at 450 nm. Antibody titers were expressed as the reciprocal of the last dilution calculated by interpolation to give an absorbance of 1 above background.

IFN-γ Expression. At day 39, Swiss mouse spleen cells were collected and analyzed for specific IFN-γ production by ELISPOT following manufacturer's instructions (Becton Dickinson, Oxford, UK). Briefly, after red blood cell lysis 10^5 and 8 × 10^5 splenocytes were distributed in triplicate wells of Immobilon-P hydrophobic PVDF 96 well plates (Millipore, Billerica, MA, USA) previously coated with 0.5 μg/well of an antimouse IFN-γ antibody (Becton-Dickinson). Cells were stimulated with either autologous peptide at 0.4 μg/well or with sucrose-purified FMDV O/UKG11/2001 at 1 μg/well. Triplicate wells with 8 × 10^5 cells without peptide were used to estimate nonspecific activation. As positive control, triplicate wells with 8 × 10^5 cells were stimulated with phytohemagglutinin (Sigma) at 1 μg/well. After 48 h at 37 °C, 5% CO_2, and 95% relative humidity, plates were washed and incubated with a biotinylated antimouse IFN-γ antibody (Becton-Dickinson) followed by streptavidin-conjugated horseradish peroxidase (Life Technologies). Antibody binding was visualized with the substrate 3-amino-9-ethyl carbazole (Sigma). The frequency of peptide- and virus-specific T cells present in the responding population was expressed as the mean of spot-forming cells in stimulated wells per 10^6 splenocytes.

RESULTS AND DISCUSSION

Bivalent Branched (B_2T) FMD Vaccine Candidates. As the most infectious agent among animal pathogens, FMDV causes a devastating disease affecting millions of animals worldwide and poses a serious sanitary hurdle to global trade in animal products.[40,41] All this emphasizes the need for effective vaccines,[42,43] particularly those providing differentiation between vaccinated and infected animals.[44] The multiepitopic constructions reported in this work can be viewed as derived from a B_4T prototype[11,14,35] that has shown promising results as a vaccine candidate against both FMDV and classical swine fever virus. Subsequent work in our laboratories has shown that some constructs displaying two copies of a B epitope tethered to a T epitope through a branched Lys residue (generically named B_2T) can match or even outperform B_4T in immunogenicity. This rather unexpected finding has also important implications in terms of developing a cost-effective FMDV peptide vaccine, since the not-quite-efficient production of B_4T by thioether conjugation demanded exhaustive purification and yielded a not-quite-homogeneous end product (see refs 11 and 13 for a general view). In contrast, the simpler design of B_2T-type constructions might arguably translate into a significant improvement in synthetic efficacy, hence lower production costs. In setting out to investigate these issues, this work has examined four bivalent platforms,[1−4] available through relatively similar conjugation chemistries, in an attempt to find an optimal approach combining efficiency with immunogenic performance. Three different types of conjugation between the B epitope and the branching T epitope have been examined, as shown in panels A–C of Figure 1 (with further structural details on Figure 1D). Conjugates **1** and **2** (Figure 1A), both generated by standard thioether ligation,[16] simply differ in the C- or N-terminal orientation, respectively, of the B relative to the T epitope. In conjugate **3** (Figure 1B), the B epitope and a Lys core C-terminally elongated with the T epitope are connected by a "reverse thioether" link,[24] the result of switching the electrophile and nucleophile roles of the standard approach[15] to the Lys core-T epitope (**3b**) and B epitope (**3a**) moieties, respectively. Finally, in conjugate **4** (Figure 1C) the two B epitope branches are connected to the Lys core-T epitope via maleimide linkages, accessible through robust conjugation chemistry[26] that uses the same thiol-functionalized version of the B epitope (**1a**) employed for conjugate **1**. While all three approaches share the advantage of allowing purification and characterization of the respective building blocks prior to conjugation—in contrast with similar constructs entirely built by SPPS—in practice, considerable differences are found not only in the efficiency of the various conjugations but also in the performance of the resulting immunogens. With regard to conjugation chemistry, in this study we compare (Table 1) parameters such as reaction time, stoichiometry, byproducts obtained, and yield of isolated product. Finally, the stability of the four different conjugates toward serum is also evaluated.

Table 1. Main Chemical Aspects of Conjugations 1–4

target conjugate	reaction end point[a]	peptide (**1a**, **2a**, **3a**) equivalents/branch	main byproduct	isolated product (%)
1	24 h	4	**1a** dimer	34
2	24 h	4	**2a** dimer	43
3	24 h	2	**3a**-TCEP	46
4	1 min	1	---	65

[a]No further reaction progress by HPLC.

Despite its widespread use as a chemoselective conjugation method,[11,15,16] thioether ligation is not trouble-free. In its more common formulation, based on nucleophilic displacement at a chloroacetyl group by a Cys thiol (Figure 1A), dimerization of the peptide epitope via thiol-to-disulfide oxidation tends to prevail over conjugation[12,13] in the narrow pH range (7–7.5) where thioether formation is practicable. Lower (<7) pH does not help, as the desired substitution reaction becomes fairly sluggish, hence even more disfavored vs dimerization. Thus, the only expedient way to favor thioether formation is to use a large excess of the thiol component, a great deal of which will inevitably be wastefully dimerized. This is illustrated by the 24-h end point HPLC profiles of the conjugation processes leading to both **1** and **2** (Figure 2A and B), where the **1a** and **2a** dimer peaks clearly predominate over those of the target conjugate. The poor efficiency of the process is also evident from the protracted reaction time and by the modest yields in isolated product (Table 1), with minimal differences regardless of

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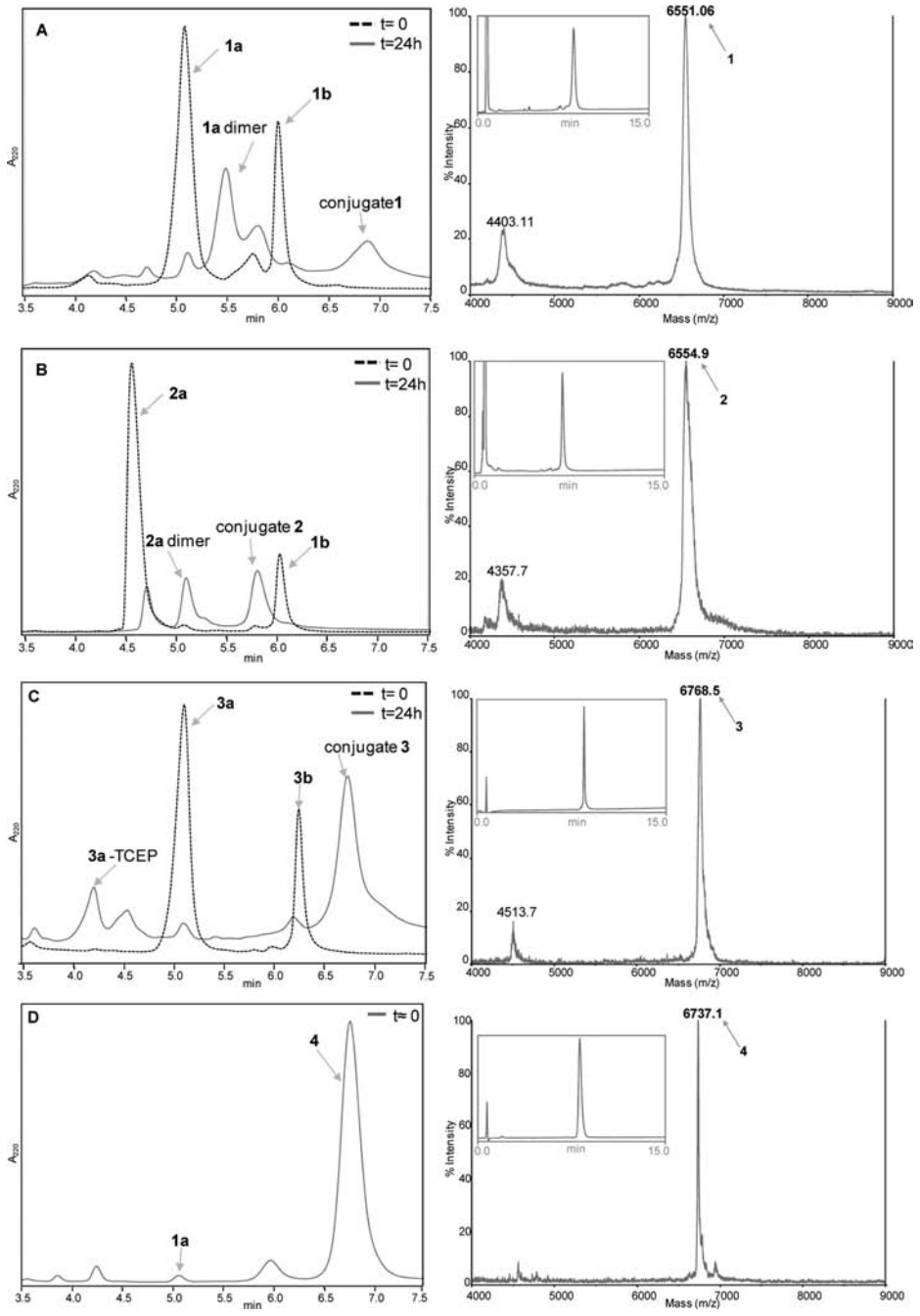

Figure 2. HPLC traces (left panels) of reactions leading to conjugates **1**–**4**: (A) and (B) standard thioether conjugation to give **1** and **2**, respectively; (C) reverse thioether approach to **3**; and (D) maleimide approach to **4**. In each case, the panel on the right shows the MALDI-TOF MS and (insert) HPLC trace of the purified end product.

whether the Cys thiol is located at either C- or N-terminal position of the B epitope peptide (compare panels A and B, Figure 2).

In an attempt to overcome the above problems, our laboratory has recently proposed a reverse version of thioether conjugation (Figure 1B), where a thiol-derivatized Lys core reacts with a peptide epitope bearing an ϵ-amino chloroacetylated Lys residue.[24] This functional group swap has the advantage of allowing *in situ* use of a reducing agent, TCEP, which keeps thiol groups in the Lys core permanently reactive, hence avoiding dimerization and greatly reducing the amount of peptide epitope required to complete the reaction. This is shown in Figure 2C, where the HPLC area of target conjugate **3** is enhanced relative to analogous conjugates produced by the standard thioether method (**1** and **2**, Figure 2A and B). A minor side reaction in this approach is adduct formation between TCEP and the chloroacetylated peptide (Figure 2C). On the whole, the reverse thioether method provides a modest improvement over the standard strategy, in terms of efficiency, yields, and reaction times (Table 1).

Finally, as an alternative to the above thioether-based conjugations, we have explored thiol–ene (maleimide)[45] chemistry (Figure 1C), which is optimally run at pH 6, where the risk of thiol oxidation is essentially averted. In our hands this method has been shown to be ideal, not only in terms of synthetic performance but, quite unexpectedly, with

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regard also to the immunogenic properties of the resulting conjugates (see next section). On the synthetic side, the total absence of dimerization allows a strictly stoichiometric use of thiol-functionalized peptide epitope **1a** (Table 1); this combines with fast reaction times and minimal byproduct formation (Figure 2D) to provide a highly efficient, practical route to B_2T-type immunogens.

Immunogenicity of the Various B_2T Conjugates. The three synthetic procedures described above have been used to produce four types of B_2T conjugates differing in either the attachment of the B epitope sequence relative to the Lys core (C-terminal in all cases except conjugate **2**) or in the structure of the intervening unit between the two (methylenecarbonyl, acetylthiopropionyl, or maleimidopropionyl for **1**−**2**, **3**, and **4**, respectively; Figure 1). Since immunization protocols have been rigorously identical for all four conjugates, any differences in immunogenic behavior must necessarily be related to the above-mentioned variations in either epitope attachment/orientation or connectivity. While a cursory glance at the global similarities of the conjugates might lead to conjecture likewise comparable immunogenic performances, the actual results tell a different story. For instance, an apparent disadvantage is observed for the N-terminal attachment of the B epitope in terms of antibody production, conjugate **2** being the only one for which no immune response is noted after a single dose (Figure 3A). This slow seroconversion rate of **2**, however, does not involve a similar underperformance in terms of T cell response; indeed, as shown by ELISPOT (Figure 3B,C), **2** elicits the highest levels of IFN-γ production of the four conjugates. In contrast with its synthetic appeal, the reverse thioether approach leading to conjugate **3** does not entail obvious benefits in either B or T cell immune response; fairly slow seroconversion (Figure 3A) is accompanied by poor IFN-γ production levels, particularly upon stimulation with native virus (Figure 3C). For its part, conjugate **4** displayed an altogether optimal immune response, combining the fastest and strongest seroconversion of the entire set (Figure 3A) with high levels of IFN-γ production, only slightly inferior to those elicited by **2**, the conjugate providing the best T cell stimulation.

The superior in vivo activity of branched peptides over linear counterparts has been ascribed not only to higher biological response resulting from multiple presentation but also to enhanced resistance to peptidase activity.[46] In this line of argument it would not be unreasonable to expect that the chemical linkage between the branches, e.g., the diverse connectivities tested in this work, might have substantially different effects on the stability of the corresponding conjugates to biological fluids. To this end, we have investigated the serum stability of conjugates **1**−**4** using an LC-MS method (Figure 4). While all four conjugates were reasonably stable, with half-lives >2 h in serum, not unlike those of similar constructs,[47] variations most likely attributable to the connectivity between the B epitopes and the Lys core could be observed. Of those, the most remarkable difference was again unexpectedly for maleimide-based conjugate **4**, with stability to serum ($t_{1/2}$ 210 min) significantly higher than the rest. This increased resistance to proteolysis may partially explain the superior immunogenicity of **4** over the other three constructs; at any rate, it highlights this maleimide-based conjugate as a particularly effective form of epitope presentation.

The above results highlight the oft-neglected relevance of even minor structural details in the immune response[48,49] and

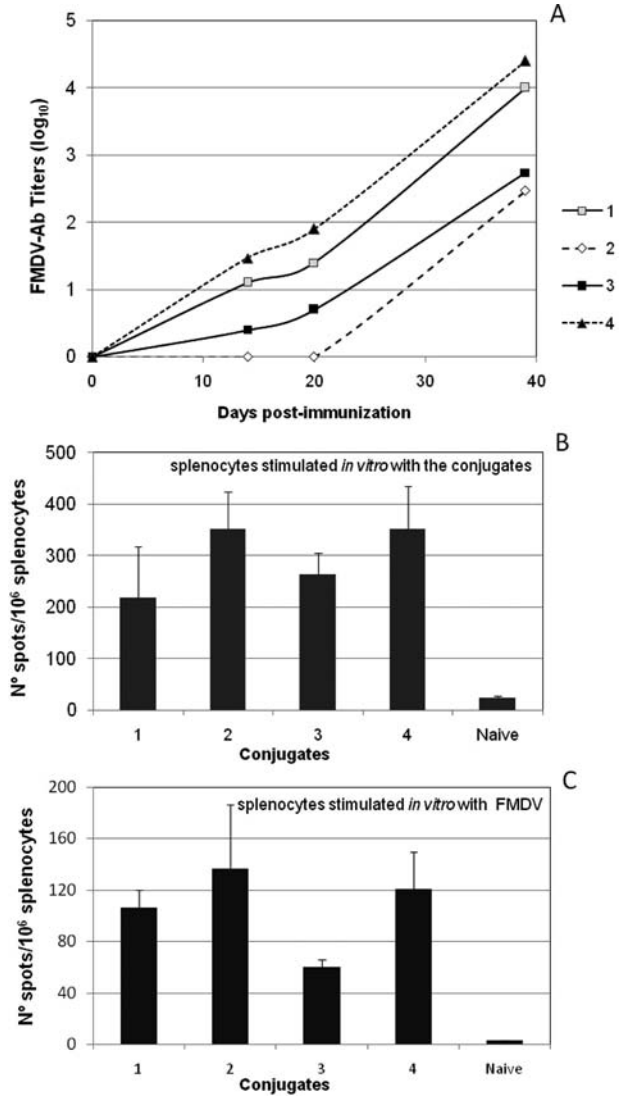

Figure 3. (A) Time course of anti-FMDV antibody responses of mice vaccinated with conjugates **1**−**4**. (B) Levels of FMDV-specific IFN-γ-producing cells (ELISPOT assay) in splenocytes stimulated in vitro with conjugates **1**−**4**. (C) As in (B) but with in vitro stimulation with FMDV.

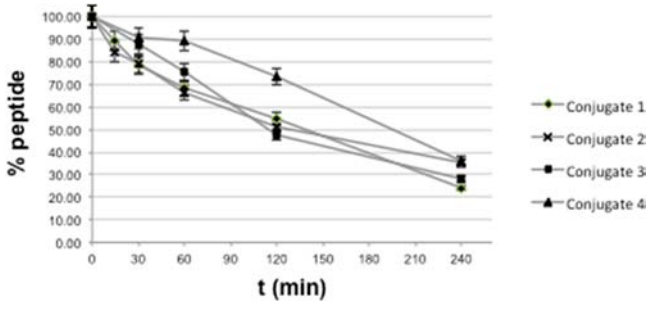

Figure 4. Stability of conjugates **1**−**4** to serum. The amount of conjugate remaining after incubation for the indicated time was determined by LC-MS (see Materials and Methods).

will eventually become significant in our design of FMDV synthetic vaccines. They need, however, to be more conclusively established in vaccination trials currently underway. Until these data are available, a provisional conclusion in

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favor of **4** as the most valuable candidate immunogen is justified on four counts: (1) an essentially trouble-free synthetic process that ensures not only cost-efficient production but also fast adaptability to other serotypes or to variants of a given B epitope resulting from field mutations; (2) superior proteolytic stability, which may be related to (3) powerful antibody response, significantly higher than the rest, and (4) good T cell stimulation. Conjugate **2** admittedly induces slightly better T cell response than **4**, but its sluggish humoral response—significant antibody titers only detectable after a booster—not to mention its less than optimal synthesis and lower proteolytic stability, clearly question its viability.

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Notes

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ABBREVIATIONS:

DIEA, *N*,*N*′-diisopropylethylamine; DIPCI, diisopropylcarbodiimide; DIVA, differentiation of infected from vaccinated animals; FMDV, foot-and-mouth disease virus; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; MAP, multiple antigenic peptide; Mmt, 4-methoxytrityl; Mpa, 3-mercaptopropionic acid; TFA, trifluoroacetic acid; TIS, triisopropylsilane

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