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A technique for the synthesis of highly-pure, mono-epitopic, multi-valent lipid core peptide vaccines

Peter M. Moyle,[a] Yashmeeta Hari,[a] Ning Huang,[b] Colleen Olive,[b] Michael F. Good[b] and Istvan Toth[a,*]

[a]*School of Molecular and Microbial Sciences and School of Pharmacy, The University of Queensland, St. Lucia, QLD 4072, Australia*
[b]*Queensland Institute of Medical Research, Herston, QLD 4029, Australia*

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Abstract—The synthesis of lipid core peptide (LCP) vaccines using stepwise solid-phase peptide synthesis commonly results in products which are difficult to purify to homogeneity. A new technique for synthesizing highly-pure, mono-epitopic, multi-valent LCP-systems using native chemical ligation is presented. Various conditions were assessed for ligating four copies of a thioester-modified 88/30 serotype group A streptococcal peptide antigen onto an LCP-system featuring four cysteine residues. Overall, the vaccine was synthesized in high purity (>99%), and high yield (90%) when the ligation reaction was performed in the presence of 1% sodium dodecyl sulfate and at elevated temperatures (37 °C).
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Traditional vaccine development approaches (e.g., live weakened or killed micro-organisms) have proven to be one of the most effective public health interventions, helping to reduce the morbidity/mortality associated with many infectious diseases, ridding the world of smallpox, and reducing financial strains associated with disease. While these traditional vaccination approaches have proven to be highly effective, new approaches are required to develop vaccines against certain diseases such as cancers and HIV infection. One such approach is the development of peptide vaccines, which contain only the minimal microbial components necessary to elicit appropriate immune responses. Peptide vaccines, however, tend to be poorly immunogenic, requiring their administration with powerful, and potentially toxic adjuvants (e.g., Freund's complete adjuvant (FCA)).

Previously we have described the use of the lipid core peptide (LCP) system[1] for peptide vaccine development. This system incorporates a lipidic adjuvant, synthesized using synthetic racemic lipidic amino acids (lipoamino acids[2]), conjugated through a multiple antigenic peptide (MAP) system[3] to many copies of one or more peptide antigens. Studies have demonstrated the capacity of LCP-systems to elicit serum IgG antibodies against attached peptide antigens when they are administered via parenteral,[4] nasal, or oral routes.[5]

While LCP-systems have yielded excellent results in animal studies, their synthesis by stepwise solid-phase peptide synthesis (SPPS) often results in a product that cannot be easily purified to homogeneity, and cannot be adequately characterized. Vaccines synthesized using the LCP-system are therefore not suitable, in many cases, for use in human studies. For this reason, we have previously attempted to synthesize highly-pure LCP-system analogues using native chemical ligation.[6–8] These analogues have a different structure than traditional LCP-systems. They do not incorporate an MAP-system, and generally incorporate only one copy of each peptide antigen. Previous studies have demonstrated that by increasing the number of copies of a peptide antigen incorporated into a vaccine, the immune response against the antigen can be greatly increased.[9] We therefore aimed to modify the process that was used to synthesize the highly-pure LCP-analogues, in order to enable the synthesis of highly-pure LCP-systems incorporating an MAP-system and four copies of a single peptide antigen.

In this Letter, the synthesis of a tetra-valent, mono-epitopic LCP-system using native chemical ligation is

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* Corresponding author. Tel.: +61 7 3346 9892; fax: +61 7 3365 4273; e-mail: i.toth@uq.edu.au

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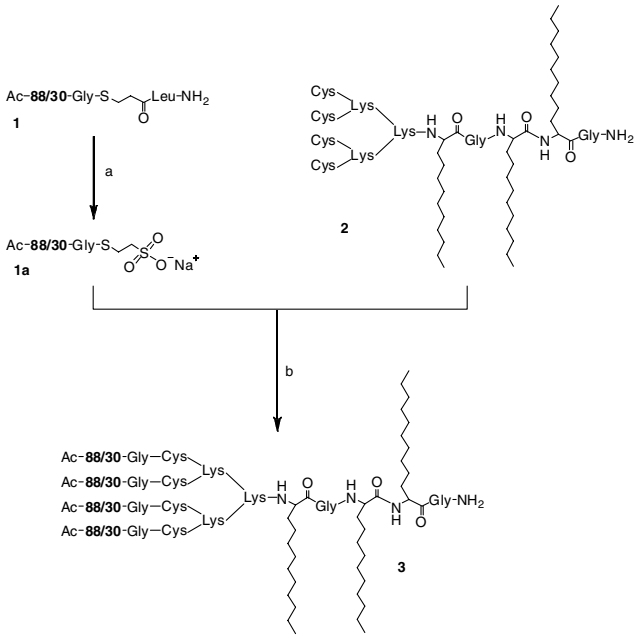
described. In order to achieve our aim, two peptide building blocks were synthesized using stepwise SPPS. Building block **1** (Scheme 1) was designed to incorporate a peptide epitope from 88/30 serotype group A streptococcus (88/30 sequence: DNGKA IYERA RERAL QELGP) conjugated at the carboxyl-terminus, through a glycine residue, to a trityl-associated mercaptopropionic acid leucine[10] linker. The synthesis of similar thioester peptides has previously been described.[8] Building block **2** (Scheme 1) was synthesized to represent an LCP-system with four cysteine residues attached at the amino-terminus instead of peptide antigens, and was synthesized according to previously described protocols for the synthesis of LCP-systems.[11]

The capacity to ligate four copies of **1** (8 equiv/**2**) onto **2** was assessed. In all cases native chemical ligation was performed in 0.1 M phosphate buffer (pH 7.5), in the presence of 2-mercaptoethanesulfonate (MESNA; 10 equiv/**1**) and tris(2-carboxylethyl)phosphine hydrochloride (TCEP; 12 equiv/**2**).

Building block **2** was found to be poorly soluble in 0.1 M phosphate buffer at pH 7.5. In order to solubilize **2**, isopropyl alcohol was added to a 20% (v/v) concentration. While this solubilized **2**, the reaction was slow and incomplete. Analytical RP-HPLC and ESI-MS revealed that the tetra-valent product **3** was not formed, with only small amounts (not quantified) of mono- and di-valent products observed. The addition of sodium dodecyl sulfate (SDS) up to a 1% (w/v) concentration was subsequently assessed. Prior to performing the ligation reaction, **2** was dissolved in water containing an appropriate amount of SDS to yield a 1% (w/v) SDS concentration during ligation. The solution was then subjected to ultrasonication (until clear), frozen and lyophilized to generate a white powder. This powder was subsequently rehydrated with 0.1 M phosphate buffer pH 7.6, to which TCEP (12 equiv/**2**) was added. Another solution containing **1** (8 equiv/**2**) and MESNA (10 equiv/**1**) (left for 1 h to yield **1a**) was then added to this solution to start the ligation reaction (Scheme 1). The reaction was performed at room temperature, or at 37 °C (in an incubator), with monitoring by analytical RP-HPLC and ESI-MS. During the ligation reaction, peaks corresponding to mono-, di- and tri-valent products, as well as the tetra-valent product **3** were observed. The HPLC retention time of these products moved towards a lower retention time as more copies of **1** were ligated to **2**.



Scheme 1. Synthesis of LCP-system **3**. Reagents and conditions: (a) MESNA, 0.1 M phosphate buffer, pH 7.5; (b) TCEP, SDS, MESNA, 0.1 M phosphate buffer, pH 7.5.

Increasing the reaction temperature to 37 °C (from room temperature) greatly increased the ligation rate, and the yield of **3**. The 37 °C reaction was complete within 24 h, with a 90% yield of **3** obtained following purification (by preparative RP-HPLC), and lyophilization. In comparison, the reaction performed at room temperature was not complete after three days, and at this time gave **3** in a 67% yield following purification and lyophilization. Overall, these experiments demonstrated that it is possible to obtain highly-pure LCP-systems using native chemical ligation when the reaction is performed in the presence of 1% SDS, with higher yields obtained when the reaction is performed at 37 °C rather than at room temperature. The product from these reactions was obtained in a highly-pure form (>99%) as demonstrated by RP-HPLC, ESI-MS and SDS-PAGE (Fig. 1).

In order to demonstrate the capacity of **3** to adjuvant the attached 88/30 antigen, 6–8 week old B10.BR mice ($n = 10$/group) were immunized at the tail-base with **3** (30 μg) in phosphate buffer saline (PBS) (50 μL total volume), or with 30 μg of **3** or the 88/30 antigen in a 1:1 emulsion with FCA (protocol previously described[11]). Three weeks after priming, the mice received four boosts at weekly intervals with **3** (3 μg) in PBS. One week following the last immunization, sera was collected, and an ELISA was conducted to quantify serum 88/30-spe-

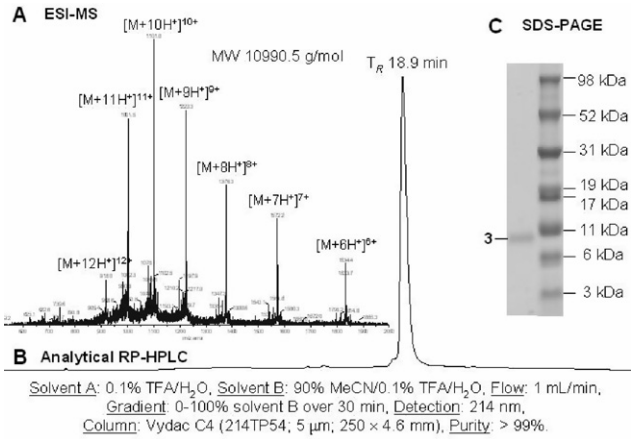


Figure 1. Characterization data (ESI-MS, analytical RP-HPLC and SDS-PAGE) for LCP-system **3**.

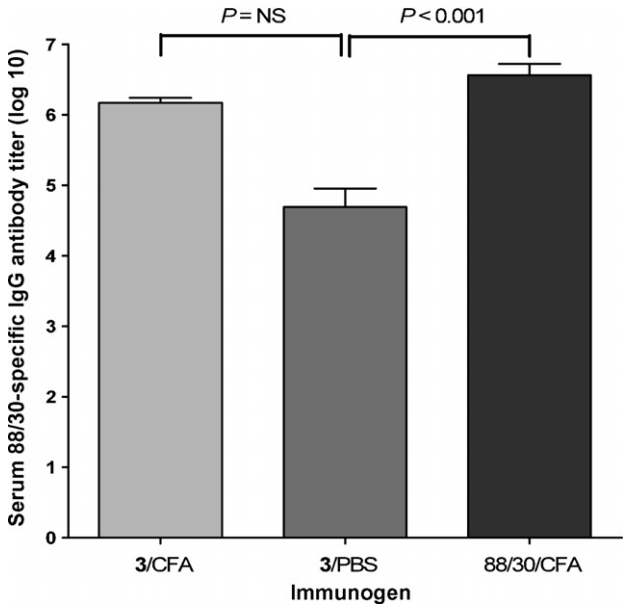


Figure 2. Serum 88/30-specific IgG antibody titers one week after the final boost, in mice immunized with **3** administered in PBS or emulsified in CFA, or with the 88/30 antigen emulsified in CFA. The average IgG antibody titers are reported with the standard deviation indicated. Statistical analysis was performed using a one-way ANOVA followed by Tukey's post-hoc test.

cific IgG antibody titers. This assay demonstrated that serum 88/30-specific IgG antibodies were elicited when mice were administered **3** without any additional adjuvant (Fig. 2).

Overall, we have demonstrated the utility of native chemical ligation for the construction of LCP-systems in a highly-pure state. The technique developed extends the utility of LCP-systems for producing self-adjuvanting peptide vaccines, by enabling the production of vaccines of a suitable purity for human clinical trials (>99%), in high overall yields (90%). The use of SDS for improving the solubility of the lipidic component **2** and to help solubilize the ligation product was essential for the success of this technique.

Acknowledgements

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Supplementary data

The synthesis of **1** and **2**, as well as the details of the ligation reaction, and characterization data are supplied as Supplementary data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2007.05.129.

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