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Synthesis of a Trimeric gp120 Epitope Mimic Conjugated to a T-Helper Peptide To Improve Antigenicity

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Supporting Information

ABSTRACT: A fully synthetic trivalent mimotope of gp120 conjugated to pan allelic HLA DR binding epitope was prepared using solid-phase peptide synthesis and optimized copper-catalyzed azide−alkyne cycloaddition. The methodology efficiently provides chemically uniform heteromultimeric peptide constructs with enhanced binding, avidity, and specificity toward an established HIV-neutralizing human antibody, MAb b12. The versatile synthetic strategy serves as a powerful platform for the development of synthetic peptides as potential HIV-1 vaccine candidates.

Despite the tremendous success brought about by combined antiretroviral therapy in reducing HIV-1-related mortality worldwide, an effective and safe HIV-1 vaccine is still needed for the ultimate eradication of the virus and control of the AIDS pandemic.[1,2] A promising vaccine strategy centers on the design and synthesis of antigenic peptides that mimic HIV envelope protein epitopes.[3−7] The expectation is that these so called mimotopes[8] would be capable of eliciting an immune response leading to the production of broadly neutralizing antibodies. HIV envelope protein has several conserved neutralizing epitopes, which are defined by human monoclonal antibodies (MAbs). We have focused our attention on MAb b12, which recognizes a discontinuous conformational epitope of HIV envelope.[9,10] While phage-display technology has proven effective for the selection of mimotopes, soluble peptide constructs are only weakly immunogenic relative to fusion proteins with the same sequence.[6] The attenuated immunogenicity has been attributed to the intrinsic flexibility, relatively small size, and limited binding interface of monomeric peptides.[11,12] Moreover, phage-encoded peptides are displayed in multiple copies as part of a filamentous protein coat, suggesting that multimeric mimotope constructs may be required for a potent immune response.[3,13−15] Our research efforts have focused on developing a chemical synthesis platform for the construction of chemically uniform multimeric mimotopes with improved antigenicity as part of a larger program directed toward vaccine development.

New mimotopes are continually emerging,[16−19] but many of these peptides are predicted to be hydrophobic, limiting the types of formulations in which they can be used. Our initial studies have focused on a mimotope that is predicted to be water-soluble on the basis of the hydrophobicity score.[20] The 15-mer peptide with the sequence NWPRWWEEFVDKHSS was identified using MAb IgG1 b12-selected phage and gp120 competition.[21] The potent, broadly neutralizing anti-HIV MAb b12[22−24] is known to bind to a discontinuous epitope overlapping the CD4 binding site of the HIV-1 envelope surface protein gp120.[9,10] Because gp120 is known to be trimeric on the viral surface, the design features three copies of the mimotope. In addition to the stimulation of an antigen-specific B-cell response with the trimeric mimotope,[25] an immunogenic T-helper (T_H) epitope is also included. Conjugation to pan allelic HLA DR binding epitope (PADRE), a known T_H epitope with the sequence aKXVAAWTLKAAa, is intended to stimulate T_H cells for a sustained antibody immune response (Figure 1).[26−29]

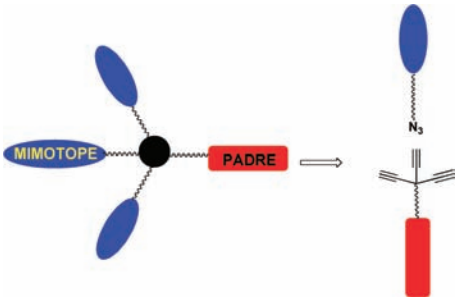

Figure 1. Design of the trimeric mimotope−PADRE immunogen.

There are myriad synthetic approaches available for achieving multivalent presentation of biologically active ligands, including the multiple antigenic peptide (MAP) system.[14] While this methodology allows elaborate synthetic assembly of multiple peptides on the core, difficulties in achieving quantitative couplings and incomplete amino acid side-chain deprotection arise in sterically crowded dendrimer networks, resulting in undefined structures and heterogeneous products.[30] Meijer and coworkers[31] have reported an attractive alternative approach that uses native chemical ligation (NCL) of peptides derived from phage technology by rebuilding the phage's multivalent architecture using well-designed dendritic wedges as synthetic scaffolds. While NCL is one of the most powerful tools for

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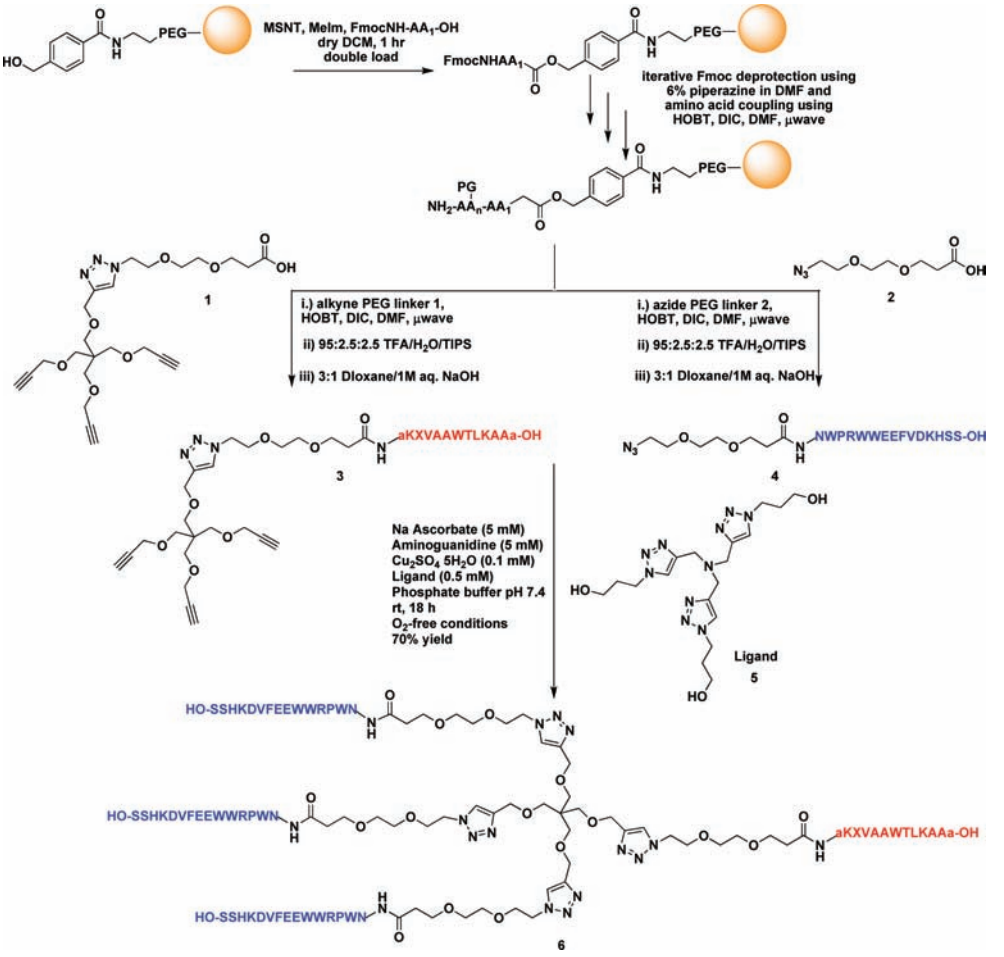
Scheme 1. General Scheme for the Construction of Trimeric Mimotope−PADRE Using SPPS and the CuAAC Reaction



peptide and protein chemical synthesis,[32] one major limitation of this approach is the need for an N-terminal cysteine-containing peptide. Concerns over the presence of cysteine, which may create unpredictable conformational changes of the peptide and/or undesirable conjugates to native peptides, have stimulated the development of alternative synthetic platforms.[33]

Our approach utilizes a well-known bioorthogonal strategy, the copper-catalyzed azide−alkyne 1,3-dipolar cycloaddition (CuAAC) reaction, also called click chemistry.[34−39] The nonnative, highly selective, and exergonic reaction of azide and alkyne functionalities[40−42] and the peptidomimetic nature of the triazole cycloaddition product and its proteolytic stability[43−45] make CuAAC attractive. However, this methodology is not without its limitations, as copper catalysis can lead to unwanted side reactions, particularly in biological settings.[46−48] Nevertheless, our previous success with conjugating the recombinant antibody fragment scFv (~25 kDa protein) to form divalent scFv (di-scFv) led us in this direction. Formation of di-scFv involves the use of an azide and an alkyne poly(ethylene glycol) (PEG) linker to functionalize the protein in preparation for ligation via the cycloaddition reaction.[49] This methodology also allows for the construction of heteromultimers, wherein one arm can be conjugated to a different peptide such as PADRE. Another advantage of this approach is that the azide- and alkyne-functionalized peptides have similar solubility profiles, resulting in a homogeneous reaction medium and higher reaction rates.[50]

We initiated the synthesis of the azide-functionalized mimotope and the trialkyne PADRE using solid-phase peptide synthesis (SPPS) with conventional Fmoc-tBu orthogonal protection and a versatile tentagel resin with a hydroxymethylbenzoic acid (HMBA) linker (Scheme 1). 1-(Mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) and 1-methylimidazole (MeIm) were used as coupling agents to incorporate the first amino acid residue,[51] and this was followed by iterative microwave-assisted Fmoc deprotection and amide coupling using 1-hydroxybenzotriazole (HOBT)/*N*,*N*′-diisopropylcarbodiimide (DIC) as activators.[52] To functionalize the PADRE and the mimotope, the free amino N-termini of the protected peptides on bead were coupled with a trialkyne PEG linker (**1**) and an azide PEG linker (**2**), respectively, using the same microwave irradiation conditions and coupling agents as employed to give the resin-bound functionalized peptides. Amino acid protecting groups were removed by treating the resin with 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIPS), and 2.5% H_2O. Cleavage from the resin was accomplished with 1:3 1 M NaOH/dioxane followed by immediate neutralization using 1 M HCl (Scheme 1). Desalting was achieved using a Sep-Pak C18 cartridge, providing the crude peptides, which were subjected to HPLC purification to give >90% pure trialkyne-functionalized PADRE (**3**) and

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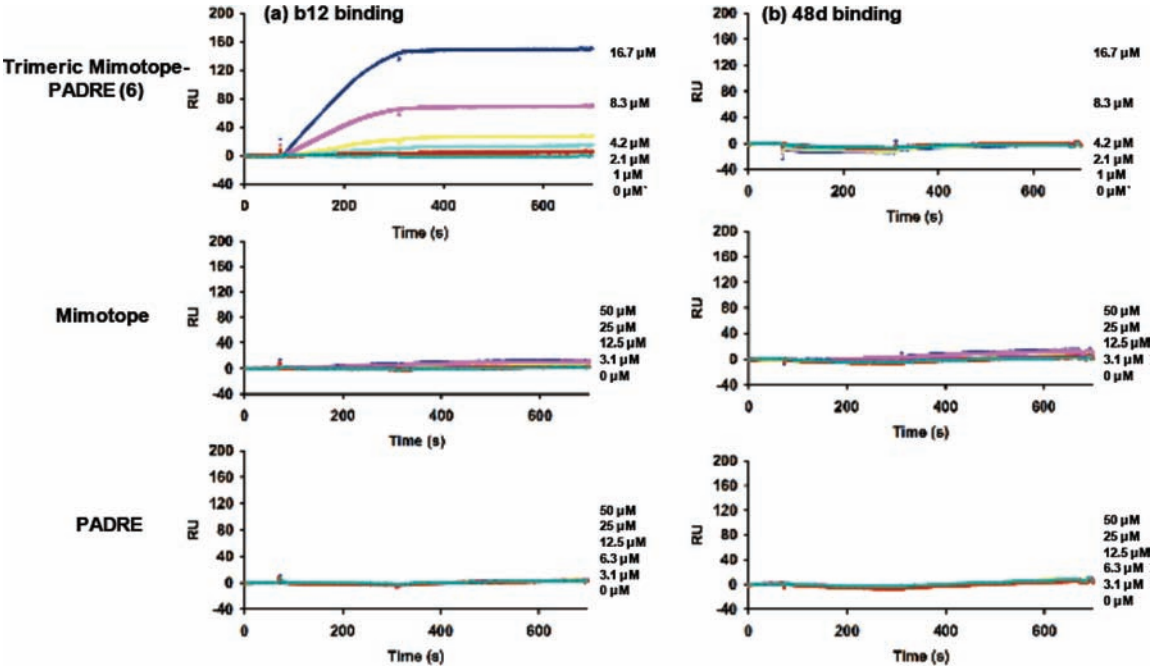

Figure 2. Direct binding of construct **6**, mimotope, and PADRE to (a) MAb b12 and (b) MAb 48d, as shown by SPR. Sensorgram comparisons show enhanced binding, valency effect, and specificity of the trimeric mimotope−PADRE **6** toward MAb b12.

azide-functionalized mimotope (**4**), as confirmed by mass spectrometry (MS) (see the Supporting Information).

The most convenient CuAAC reaction conditions involve the use of $CuSO_4$ and sodium ascorbate as a reductant along with an appropriate chelating ligand that maintains the Cu(I) oxidation state.[53−55] Unfortunately, our initial experiments utilizing bathophenanthrolinedisulfonic acid as a ligand were not fruitful, as only oxidized peptides were retrieved, even under oxygen-free conditions. However, we were fortunate to learn that Finn and coworkers[56] have recently offered a solution to overoxidation that employs tris(3-hydroxypropyltriazoylylmethyl)amine (THPTA) (**5**), which serves as a scavenger for radical species, along with aminoguanidine as an additive to prevent cross-linking and aggregation products. We were hopeful that this approach would be well-suited for mimotope multimerization in view of the high tryptophan content of our peptides.

We followed the Finn protocol by reacting **3** and **4** with 5 mM sodium ascorbate, 5 mM aminoguanidine, 0.1 mM $CuSO_4$, and 0.5 mM ligand **5** in 10 mM phosphate buffer (pH 7.4) containing 5% DMSO for 18 h (Scheme 1). After desalting and buffer-exchange procedures, the lyophilized material gave 3.2 mg (70%) of a white fluffy solid that was analyzed by high-resolution MS. After deconvolution, the spectrum gave an observed monoisotopic mass peak at m/z 8386.9900 (Figure S4 in the Supporting Information), which corresponds to the desired trimeric mimotope−PADRE cycloaddition product **6** (theoretical mass = m/z 8387.0104). The recovered excess starting materials showed minimal oxidation, even after overnight reaction.

We then initiated surface plasmon resonance (SPR) experiments to compare the binding affinity and specificity of the trimeric mimotope−PADRE construct **6** to the HIV broadly neutralizing MAb b12. To validate the effect of valency, molar concentrations were adjusted to equalize the number of ligands or the total binding sites in solution. A blank chip was used as a reference cell for all sensorgrams shown in Figure 2. Detailed information on the SPR experiments can be found in the Supporting Information. As shown in Figure 2a, **6** showed the fastest on rate, featuring enhanced binding toward MAb b12 relative to the monomeric mimotope and PADRE, which served as a negative control. During dissociation (300-660 s), construct **6** exhibited a significant analyte response with a very low off rate, which is a clear indication of increased avidity.[57]

To evaluate the binding specificity for MAb b12, the peptides were screened against a random anti-HIV antibody (MAb 48d), which was captured to equivalent levels (10 000 RU) on a CM5 chip. Comparison of the increased response from the sensorgrams toward MAb b12 binding (Figure 2a) to the lack of any response from the sensorgrams toward MAb 48d binding (Figure 2b) clearly indicates that trimeric mimotope−PADRE **6** binds specifically to MAb b12. Taken together, these data demonstrate that **6** shows increased binding, avidity, and specificity toward MAb b12, which may be attributed to the multivalent presentation of the mimotope.

Presented herein is a versatile synthetic platform for preparing multivalent peptide constructs having two different peptide components. The high efficiency of the 1,3-dipolar cyclization under conditions that alleviate oxidation of susceptible amino acid side chains was critical to the success of this approach. While the hetero-multivalent design presented here includes peptides designed to stimulate both B-cell and T_H-cell immune response, one could conceivably join any combination of peptides using this same strategy, providing a powerful tool for structure−activity relationship analyses. We look forward to reporting the results of such studies in the near future.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details on the synthesis of the peptide and linkers and the multimerization, including SPR experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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