

Peptide crosslinked micelles: a new strategy for the design and synthesis of peptide vaccines

Jihua Hao¹
 Marcin Kwissa²
 Bali Pulendran²
 Niren Murthy¹

¹Georgia Institute of Technology,
 Department of Biomedical
 Engineering, Atlanta, GA, USA;

²Emory Vaccine Center, Department
 of Pathology, Atlanta, GA, USA

Abstract: This report presents a new and simple methodology for the synthesis of multicomponent peptide vaccines, named the peptide crosslinked micelles (PCMs). The PCMs are core shell micelles designed to deliver peptide antigens and immunostimulatory DNA to antigen-presenting cells (APCs). They are composed of immunostimulatory DNA, peptide antigen, and a thiopyridal derived poly(ethylene glycol)-polylysine block copolymer. The peptide antigen acts as a crosslinker in the PCM strategy, which allows the peptide antigen to be efficiently encapsulated into the PCMs and also stabilizes them against degradation by serum components. Cell culture studies demonstrated that the PCMs greatly enhance the uptake of peptide antigens into human dendritic cells.

Keywords: composite, crosslinking, peptide, vaccine

Introduction

Vaccines based on peptide antigens have tremendous therapeutic potential against infectious diseases and tumors, in which peptide epitopes have been defined. Peptide vaccines have been capable of generating protective immunity against infectious diseases in animal models, and numerous clinical trials with peptide vaccines are in progress (Shirai et al 1994; van Endert 2001; Hunziker et al 2002; Purcell et al 2003). However, despite their promise, the first generation of peptide vaccines has performed poorly in human clinical trials, partially due to drug delivery problems (Brander et al 1996; BenMohamed et al 2002). Delivering peptide vaccines has been challenging because they consist of multiple components, such as peptide antigens and immunostimulatory molecules. Although promising results have been obtained with peptide vaccines composed of lipid conjugates and poly(D,L-lactic-co-glycolic acid) microparticles, there is still a great need for the development of new peptide vaccine delivery vehicles (Ertl et al 1996; Jackson et al 1997).

In this report, a new peptide vaccine delivery vehicle is presented that is designed to simultaneously deliver peptide antigens and immunostimulatory molecules to antigen-presenting cells (APCs). They are named peptide crosslinked micelles (PCMs) and are composed of block copolymer micelles that encapsulate immunostimulatory (ISS)-DNA in their cores and are crosslinked by peptide antigens through disulfide linkages (Figure 1). The PCMs are based on the disulfide crosslinking strategy, using thiolated poly(ethylene glycol)(PEG)-peptide block copolymers, pioneered by the Kataoka (Kakizawa et al 1999; Miyata et al 2004) and Rice (Park et al 2002) laboratories for the delivery of DNA.

Methods and results

The PCMs are synthesized by a two-step process that uses the peptide antigen as the crosslinking agent for the micelles. In the first step, the block copolymer I is self-

Correspondence: Niren Murthy¹ and Bali Pulendran²

¹Georgia Institute of Technology,
 Department of Biomedical Engineering,
 315 Ferst Drive, Atlanta, GA 30332 0535,
 USA

Tel +1 404 385 5145

Fax +1 404 894 4243

Email niren.murthy@bme.gatech.edu

²Emory Vaccine Center, Department of
 Pathology, 954 Gatewood Road, Atlanta,
 GA 30329, USA

Tel +1 404 727 8945

Fax +1 404 727 8199

Email bpulend@rmy.emory.edu

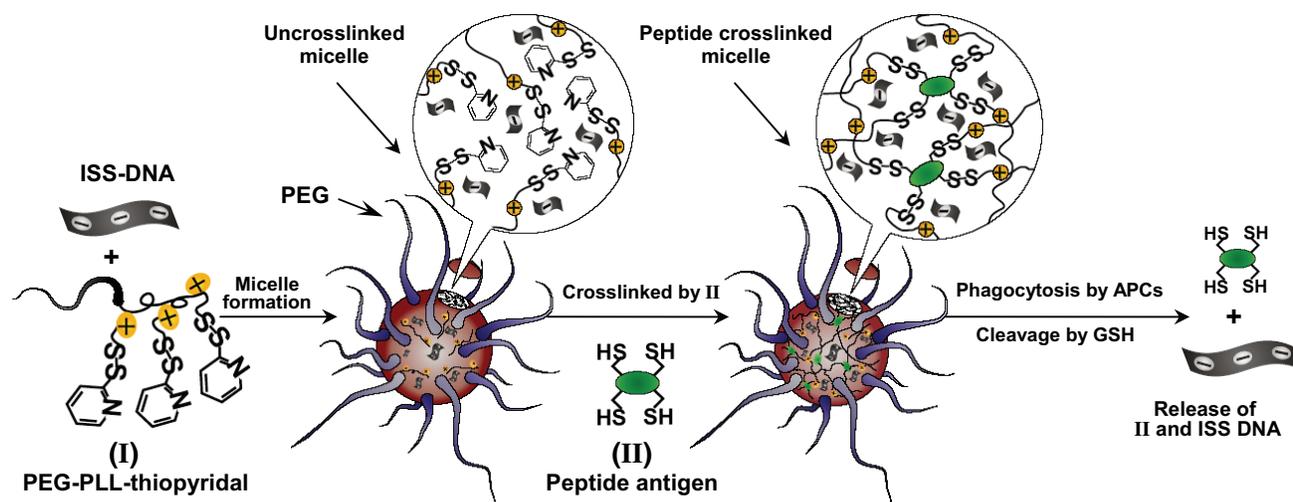


Figure 1 Peptide crosslinked micelle design and synthesis. Step 1: immunostimulatory (ISS)-DNA and I (PEG-PLL, poly(ethylene glycol)-poly-*l*-lysine) are mixed to form micelles (uncrosslinked micelle). Step 2: These micelles are then crosslinked with the peptide antigen (II) to generate a delivery system that can encapsulate both immunostimulatory molecules and peptide antigens. After phagocytosis by antigen-presenting cells (APCs), the peptide crosslinked micelles release their components. **Abbreviations:** GSH, glutathione.

assembled with ISS-DNA to generate core shell micelles, which have a core composed of thiopyridal groups intermixed with ISS-DNA (Figure 1). This step is based on work from the Katoaka laboratory using thiolated PEG-poly-*l*-lysine (PLL) block copolymers and plasmid DNA (Kakizawa et al 1999; Kakizawa et al 2001; Miyata et al 2004). In the second step these micelles are crosslinked by reaction of their thiopyridal groups with a cysteine-modified antigenic peptide to generate a disulfide crosslinked micelle that contains both peptide antigens and ISS-DNA. Thus the antigenic peptide plays a dual role in the PCMs: it acts as a crosslinker to enhance serum stability, but is also the therapeutic that needs to be delivered.

Disulfide linkages are selectively reduced inside cells because of the high glutathione (GSH) concentration (10mM intracellular GSH concentration versus 100mM extracellular concentration) (Kakizawa et al 1999; Park et al 2002; Miyata et al 2004). Therefore, after phagocytosis, the disulfide crosslinks in the PCMs should get reduced, and encapsulated peptide antigens and ISS-DNA will be released. After release, the peptide antigens will be processed

by enzymes in the APCs and presented to T cells (Craiu et al 1997), while the released ISS-DNA will induce the APCs to secrete the cytokines needed for effective T cell activation and proliferation.

The block copolymer I (Figure 2) forms the scaffold of the PCMs and is composed of a PEG chain (5 kDa) connected to a poly(lysine-thiopyridal) block (degree of polymerization = 20). PEG-poly-*l*-lysine (PEG-PLL) block copolymers have been extensively used for the synthesis of disulfide crosslinked micelles. For example, PEG-PLL has been thiolated by the Katoaka laboratory through the reaction of PEG-PLL with either N-succinimidyl 3-(2-pyridyldithio)-propionamido (SPDP) or Trauts reagent (Kakizawa et al 2001; Miyata et al 2004). In this report we chose to synthesize the block copolymer I via a Michael addition reaction between PEG-PLL (III) (Harada et al 1995) and hydroxyl-ethyl thiopyridal acrylate (IV) (Figure 2), because it introduces a thiopyridal group onto the block copolymer while also preserving its positive charges.

A HIV peptide vaccine was synthesized using the PCM strategy with the peptide CGCRIQRGPGRAFVTIGK

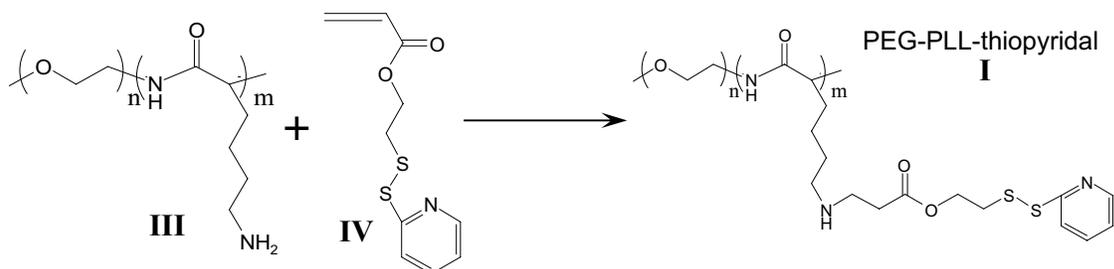


Figure 2 Chemical structure and synthesis of poly(ethylene glycol)-poly-*l*-lysine (PEG-PLL)-thiopyridal (I).

CGCG (II). The peptide II comes from the glycoprotein-120 (GP-120) and contains the sequence RIQRGPGRAFVTIGK, which is both a class I and II antigen (Belyakov et al 1998; Berzofsky et al 1999). First, micelles were formed between I and ISS-DNA by mixing 0.5 mg of I with 0.1 mg of ISS-DNA (5-TCCATGACGTTCCCTGACGTT-3) (charge ratio 1:15 [+/-]) in 0.5 mL of 50 mM phosphate-buffered saline (PBS). Dynamic light scattering of these micelles by the cumulants method indicated that they had an average diameter of 57.0 nm. These micelles were then crosslinked by adding 0.1 mg of II to the micelles (equal molar ratio of cysteines on II to thiopyridal groups on I). The peptide II was incorporated into the micelles through a disulfide exchange reaction.

The kinetics of the disulfide exchange reaction between II and the micelles were quantified by UV absorbance at 342 nm (generated from the released thiopyridone). Figure 3 demonstrates that II reacts rapidly with the micelles. At an equal molar ratio of cysteine to thiopyridal groups, more than 90% of the cysteine groups of the peptide were consumed within 20 minutes. After the peptide crosslinking, the average size of the micelles decreased to 50.0 nm.

The stimuli-responsive release of II from the PCMs due to the presence of GSH was investigated to determine if the PCMs will release peptide antigens after phagocytosis. The PCMs were incubated with different concentrations of GSH for 24 hours in 50 mM pH 7.4 PBS buffer, and then analyzed by high performance liquid chromatography (HPLC) to determine the release of II. Figure 4 demonstrates that the presence of GSH triggers the release of II. Incubation of the PCMs with 10 mM GSH (intracellular levels) induced the release of 71% of II, whereas incubation of the PCMs with just buffer induced the release of only 10% of II.

A key advantage of the PCM strategy is that it generates a crosslinked delivery system. This crosslinking should

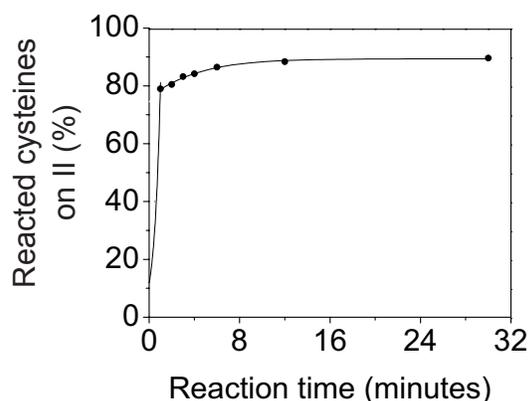


Figure 3 Crosslinking reaction of cysteines on peptide antigen (II) with thiopyridal group in micelles.

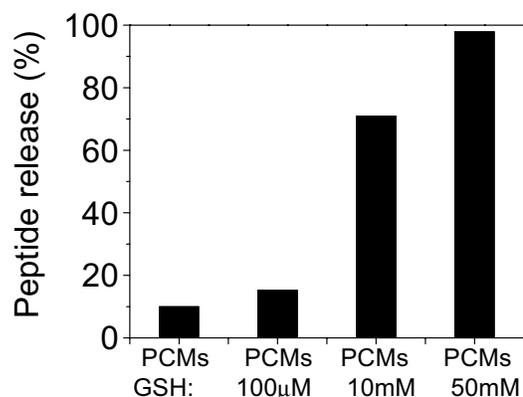


Figure 4 Release of peptide antigen (II) from the peptide crosslinked micelles (PCMs) in the presence of glutathione (GSH). Conditions: 50 mM phosphate buffered saline buffer (pH = 7.4), 24 hours' incubation, room temperature.

stabilize the PCMs *in vivo*. For example, the Katoaka laboratory demonstrated that plasmid DNA, encapsulated in disulfide crosslinked PEG-polylysine micelles, is protected against decomposition induced by polyelectrolyte exchange reactions with charged proteins (Kakizawa et al 1999; Miyata et al 2004). The stability of the PCMs to decomposition was investigated by mixing the negatively charged polymer poly(vinyl sulfate) (PVS) with the PCMs. This mixture was then analyzed by gel electrophoresis to determine the quantity of ISS-DNA displaced by the PVS. As a control, PVS was also incubated with uncrosslinked micelles composed of just I and ISS-DNA. Figure 5a, lane 4, demonstrates that PVS can disrupt uncrosslinked micelles composed of just ISS-DNA and I. In contrast, Figure 5a,

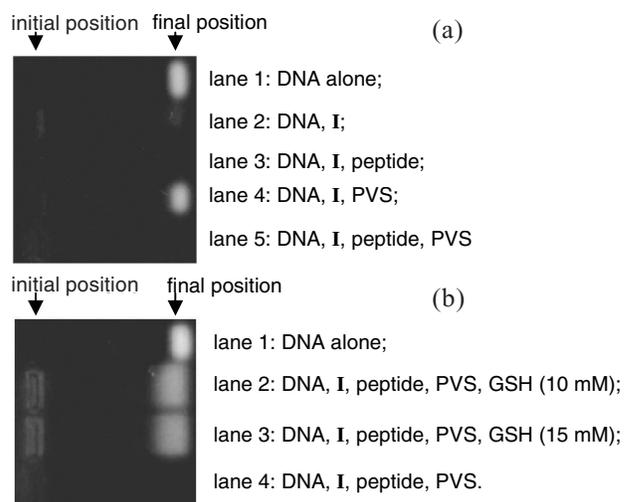


Figure 5 (a) Stability of peptide crosslinked micelles and (b) glutathione (GSH)-induced release of DNA from the peptide crosslinked micelles. Charge ratio of DNA to poly(ethylene glycol)-poly-L-lysine (PEG-PLL)-thiopyridal (I) is 1:15 (-/+); 1 µg of DNA was loaded in each lane; molar ratio of sulfate in poly(vinyl sulfate) (PVS) ($M_w = 17000$) to phosphate in DNA is 6:1; molar ratio of cysteine on peptide to thiopyridal group on I is 1:1. All samples were incubated at room temperature for 24 hours, followed by gel electrophoresis with low range ultra agarose gel in tris/acetic acid/EDTA buffer.

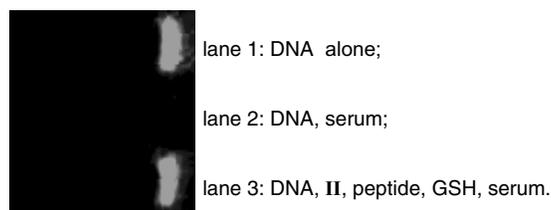


Figure 6 Protection of immunostimulatory (ISS)-DNA in peptide crosslinked micelles against serum nucleases. Charge ratio of ISS-DNA to poly(ethylene glycol)-poly-L-lysine (PEG-PLL)-thiopyridal (I) is 1:15 (-/+); 1 μ g of DNA was loaded in each lane; glutathione (GSH) (100 μ M) was added to lane 3 to induce release of encapsulated ISS-DNA. All samples were incubated with 10% serum at room temperature for 12 hours.

lane 5, demonstrates that PVS cannot displace ISS-DNA from the PCMs, presumably because the peptide crosslinking prevents the PVS from diffusing into the micelles and displacing the ISS-DNA. Importantly, after incubation of the PCMs with intracellular concentrations of GSH, the PCMs release encapsulated ISS-DNA in the presence of PVS, demonstrating that the micelles should release their contents after phagocytosis (Figure 5b, lanes 2 and 3).

The ability of the PCMs to protect encapsulated ISS-DNA from degradation by serum nucleases was also investigated. PCMs were synthesized (as described above) and incubated with 10% serum for 12 hours. These PCMs were then examined by gel electrophoresis to determine the stability of the encapsulated ISS-DNA. As a control, ISS-DNA by itself was incubated with serum. Figure 6 demonstrates that ISS-DNA, by itself, was completely hydrolyzed in 10% serum (lane 2). In contrast, ISS-DNA encapsulated in the PCMs was protected from serum nucleases, presumably because of the effects of the crosslinking (lane 3), which should prevent nucleases from entering the micelle.

The PCMs have a size appropriate for phagocytosis by antigen-presenting cells and should therefore efficiently deliver peptide antigens into antigen-presenting cells. To investigate if the PCMs are efficiently phagocytosed by APCs, we constructed PCMs using a fluorescein (FITC)-labeled crosslinking peptide, FITC-CGSIINFEKLGCG. The PCMs were synthesized by first mixing 1.8 mg of I with 0.3 mg of ISS-DNA (charge ratio 15:1 (+/-)) in 0.5 mL of 50 mM PBS, generating micelles, which were then crosslinked by addition of 2.1 mg of FITC-CGSIINFEKLGCG. These micelles were then incubated with human monocyte-derived dendritic cells (MDDCs), generated as previously described (Agrawal et al 2003), for 4 hours in 10% fetal calf serum (FCS)-containing media. Figure 7 demonstrates that dendritic cells incubated with PCMs

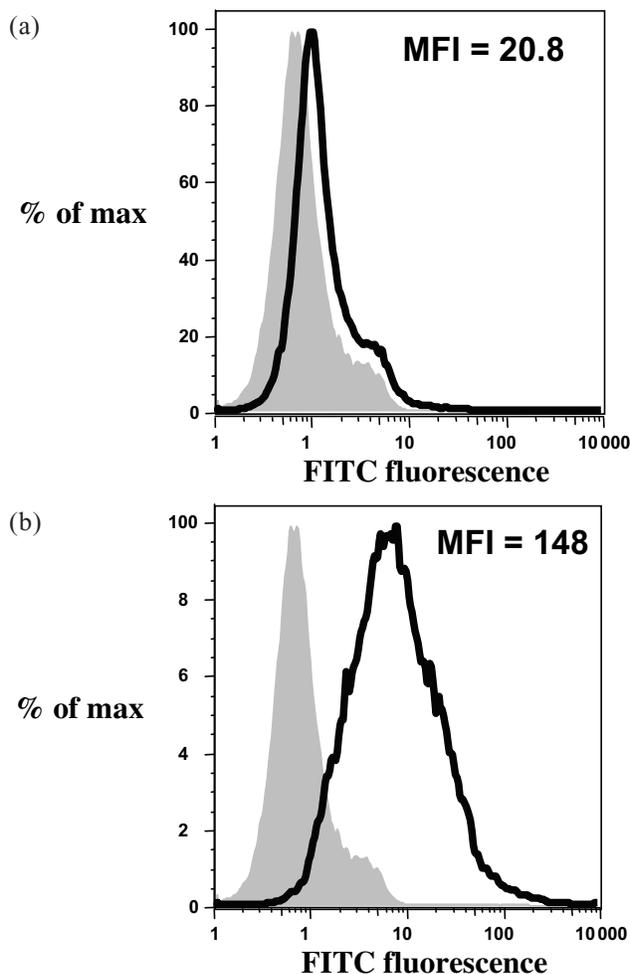


Figure 7 Efficient uptake of peptide crosslinked micelles (PCMs) containing fluorescein (FITC)-CGSIINFEKLGCG by human monocyte-derived dendritic cells (MDDCs). MDDCs were pulsed with either (a) FITC-CGSIINFEKLGCG (10 μ g/ml) or (b) PCMs containing FITC-CGSIINFEKLGCG (10 μ g/ml) for 4 hours in 10% fetal calf serum (FCS)-containing media. Cells were analyzed by flow cytometry for the mean fluorescence intensity (MFI) of fluorescein. Results are representative of three separate experiments. The shaded region is the autofluorescence of MDDCs incubated in just plain FCS-containing media; the dark line is the fluorescence of MDDCs incubated with the fluorescein-containing compounds.

containing FITC-CGSIINFEKLGCG (10 μ g/ml) internalize approximately 7 times more FITC-CGSIINFEKLGCG than dendritic cells incubated only with FITC-CGSIINFEKLGCG (10 μ g/ml). Thus the PCMs are capable of greatly enhancing the delivery of peptide antigens into human dendritic cells.

Conclusions

The PCM strategy is a robust and simple strategy for the development of peptide vaccine delivery vehicles. Any peptide antigen can be potentially incorporated into the PCMs; it simply has to be modified with extra cysteines. The components of the PCMs are also biodegradable and should be easily amenable for human applications. As a

result, the PCMs should find numerous applications in vaccine development.

Acknowledgments

We acknowledge the Georgia Tech/Emory Center for the Engineering of Living Tissues (funded by NSF-EEC-9731643) and the National Institutes of Health UO1 HL80711-01 (NM), U19 AI05726601, RO1 AI048638, RO1 AI056499, RO1 DK057665, R21 AI056957, and U54 AI057157 (BP) for funding.

Appendices

Appendix I: Materials

ϵ -(benzyloxycarbonyl)-*L*-lysine was purchased from Novabiochem (San Diego, CA, USA) and α -methoxy- ω -amino-poly(ethylene glycol) (PEG, $M_w = 5000$) was purchased from NEKTAR Transforming Therapeutics (San Carlos, CA, USA). The immunostimulatory (ISS)-DNA, with the sequence of 5-TCCATGACGTTCTGACGTT-3, was purchased from Integrated DNA Technologies (Coralville, IA, USA). Agarose was purchased from Bio-Rad (Hercules, CA, USA), methanesulfonic acid was

purchased from Acros (Hampton, NH, USA), anisole and triphosgene were purchased from TCI America (Portland, OR, USA), 5,5'-dithio-*bis*(2-nitrobenzoic acid) (Ellman's Reagent) was purchased from Calbiochem (San Diego, CA, USA), and 2,2-dithiodipyridine was purchased from Fluka (SG Switzerland). Sodium hydroxide, potassium hydroxide, sodium phosphate monobasic, and acetonitrile were purchased from Fisher Scientific (Hampton, NH, USA). *L*-glutathione (GSH), dithiothreitol (DTT), poly(vinyl sulfate potassium salt) (PVS, $M_w = 17000$), acetic acid, trifluoroacetic acid, 2-mercaptoethanol, dichloromethylene, tetrahydrofuran (THF), and *N,N*-dimethylformamide (DMF) were purchased from Sigma-Aldrich (St Louis, MO, USA). THF and DMF were doubly distilled before use. The peptide CGCRIQRGPGRAFVTIGKCGCG was synthesized at the Petit Institute for Bioengineering and Bioscience's core facility on a 433A Peptide Synthesizer, Applied Biosystems (Foster City, CA 94404, USA), and was purified by HPLC (Column: Discovery HS C18, made by Supelco; Hewlett Packard Series 1090 Liquid Chromatograph). (5-carboxyfluorescein)-CGSIINFEKLGCG was synthesized by SynPep Corporation (Dublin, CA, USA).

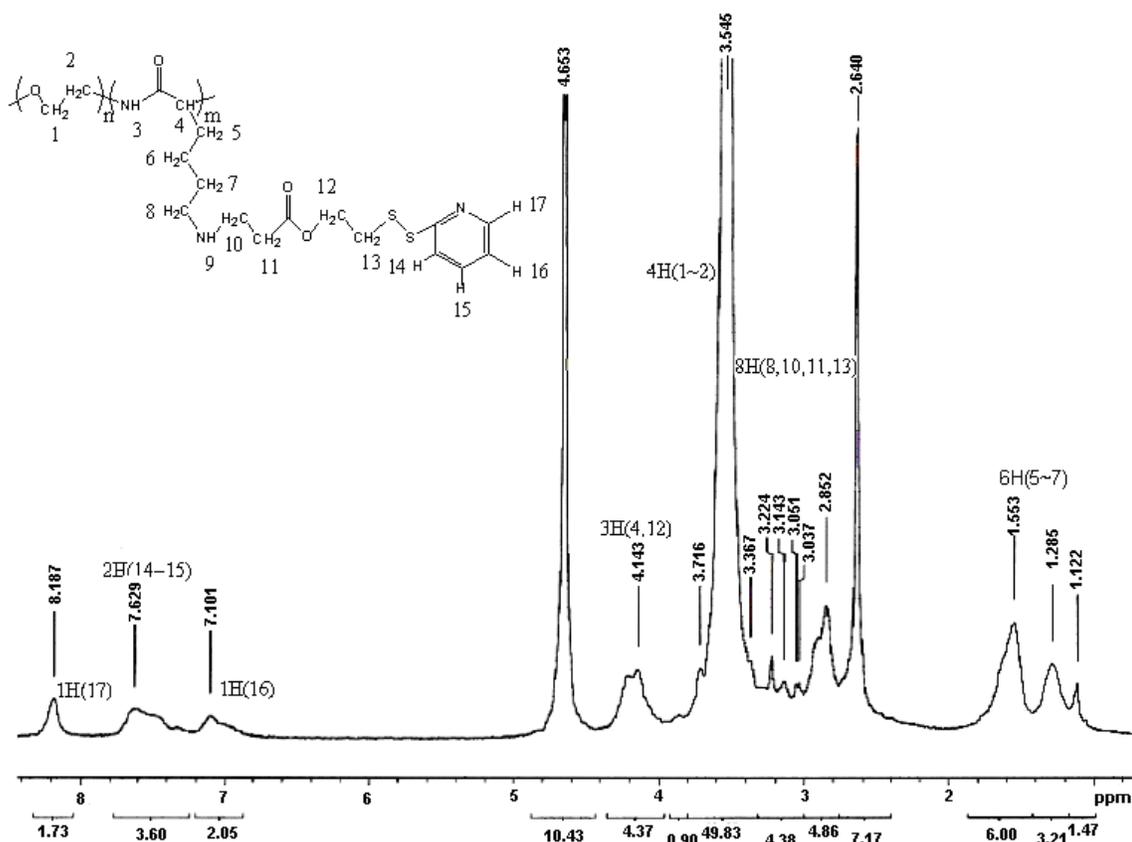


Figure A1 Proton nuclear magnetic resonance (¹H NMR) spectra of poly(ethylene glycol)-poly-*L*-lysine-thiopyridal in D₂O.

Appendix 2: Synthesis of poly(ethylene glycol)-poly-*l*-lysine-thiopyridal (I)

44 μmol of poly(ethylene glycol)-poly-*l*-lysine (PEG-PLL) (III), synthesized according to Harada and Kataoka (1995), with PEG = 5 kDa and poly-*l*-lysine = 5 kDa, was dissolved in 1 mL of *N,N*-dimethylformamide (DMF) in a 5 mL round-bottom flask, fitted with a stir bar (overnight stirring at room temperature was required to completely dissolve the polymer). 415 μmol of hydroxyl-ethyl thiopyridal acrylate (IV) and 58 μl of triethylamine were then added to the PEG-PLL solution and the reaction was allowed to run for 24 hours at room temperature (see Figure 2). The product was isolated by precipitating the reaction solution into 15 mL of ice cold diethyl ether. The yield was 88.2%. The product was analyzed by proton nuclear magnetic resonance (^1H NMR) spectroscopy in D_2O . The spectra are shown in Figure A1. The percentage of amines alkylated was determined by comparing the peak intensity ratio of pyridine protons ($-\text{NC}_5\text{H}_4$: $\delta = 7.101$ ppm, 7.629 ppm, 8.187 ppm) versus α, β, γ -methylene protons of poly-*l*-lysine ($-\text{CH}_2\text{CH}_2\text{CH}_2$: $\delta = 1.122$ ppm, 1.285 ppm, 1.553 ppm). This comparison indicated that 100% of the amines had been reacted.

Appendix 3: Dynamic light scattering analysis of peptide crosslinked micelles

A 50 mM phosphate-buffered saline (PBS) buffer solution, at pH 7.4, containing 0.06 mg/mL of I and 20 $\mu\text{g}/\text{mL}$ of immunostimulatory (ISS)-DNA was made and filtered through a 200 nm syringe filter. This solution was then analyzed by dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments Ltd, Worcestershire, UK), using the cumulant method. The size and the size distribution of the uncrosslinked micelles are shown in Figure A2. This solution was then crosslinked by adding 0.12 mg of II. After 3 hours of reaction the solution was analyzed by DLS as described above. The size and the size distribution of the crosslinked micelles are shown in Figure A3.

Appendix 4: UV analysis of crosslinking reaction between II and block copolymer micelles

Block copolymer micelles were formed between I and ISS-DNA by mixing 0.5 mg of I with 0.1 mg of ISS-DNA

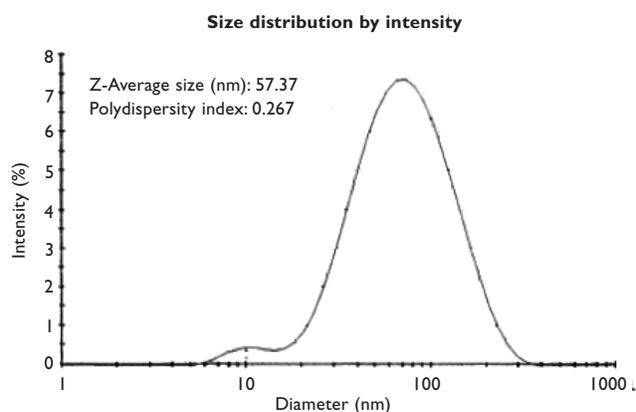


Figure A2 Size distribution of the uncrosslinked micelles.

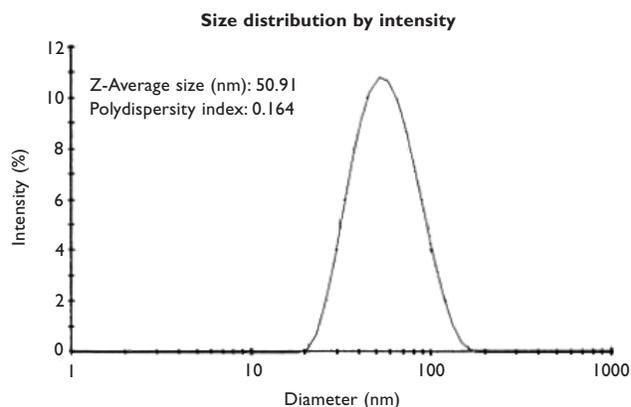


Figure A3 Size distribution of the peptide crosslinked micelles.

(representing a 15:1 amine to phosphate ratio) in 0.5 mL of 50 mM PBS buffer (pH 7.4), in an Eppendorf tube. After incubation for 2 hours at room temperature, 0.1 mg of II (representing a 1:1 cysteine to thiopyridal ratio) was added to the micelles. The crosslinking reaction between the cysteines on II with the thiopyridal groups in the micelles was determined by UV analysis at 342 nm (representing the released thiopyridone). The UV spectra of representative samples are shown in Figure A4. The percentage of cysteine groups reacted was determined by the following formula

$$\text{reacted peptide (\%)} = \frac{\text{ABS}_1 - \text{ABS}_2}{\text{ABS}_0} \times 100\%$$

where: ABS_1 = UV absorption at 342 nm for the peptide-crosslinked micelles reaction (solid circles in Figure A4); ABS_2 = UV absorption at 342 nm for the uncrosslinked micelles, without peptide (open squares in Figure A4); ABS_0 = UV absorption at 342 nm when all of the thiopyridone groups have been reacted (by addition of dithiothreitol) (open circles in Figure A4).

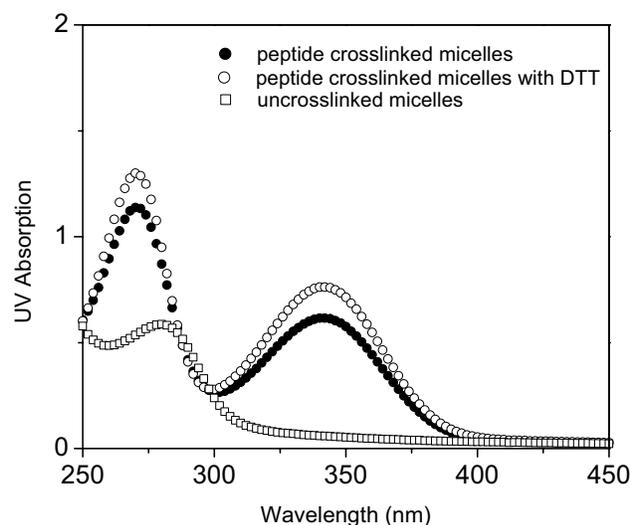


Figure A4 UV analysis for the reaction of cysteine on peptide with thiopyridine in micelles.

Abbreviations: DTT, dithiothreitol.

Appendix 5: Generation of human dendritic cells and dendritic cell uptake

Generation of human monocyte-derived dendritic cells (MDDCs)

CD14⁺ monocytes were enriched from peripheral blood mononuclear cells using an enrichment step, and cultured in six-well plates (1×10^6 per well) for 6 days with recombinant human granulocyte/macrophage colony-stimulating factor at $100 \text{ ng} \cdot \text{ml}^{-1}$ (PeproTech, Rocky Hill, NJ, USA) plus recombinant human IL-4 at $20 \text{ ng} \cdot \text{ml}^{-1}$ (PeproTech). At day 6, the cultures consisted uniformly of CD1a⁺ CD14⁻, HLA-DR⁺ CD11c⁺ cells, which were negative for CD83, the human dendritic cell maturation marker, and expressed intermediate levels of co-stimulatory molecules as CD86.

Pulsing of human MDDCs and analysis

On day 6, 5×10^5 MDDCs were pulsed with $10 \mu\text{g}/\text{mL}$ of FITC-CGSIINFEKLGCG peptide or FITC-CGSIINFEKLGCG crosslinked micelles in DMEM medium supplemented with 10% fetal calf serum for 4 hours at 37°C in 96-well round-bottom plates (Corning, Acton, MA, USA). Cells were washed 3 times and stained with HLA-DR antibody (BD Pharmingen, San Diego, CA, USA). HLA-DR⁺ cells were analyzed on FACScalibur (BD) flow cytometer and the mean fluorescence intensity of fluorescein was estimated using Flow-Jo software (TreeStar Inc, Ashland, OR, USA).

References

- Agrawal S, Agrawal A, Doughty B, et al. 2003. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J Immunol*, 171:4984–9.
- Belyakov IM, Ahlers JD, Brandwein BY, et al. 1998. The importance of local mucosal HIV-specific CD8⁺ cytotoxic T lymphocytes for resistance to mucosal viral transmission in mice and enhancement of resistance by local administration of IL-12. *J Clin Invest*, 102: 2072–81.
- BenMohamed L, Wechsler SL, Nesburn AB. 2002. Lipopeptide vaccines – yesterday, today, and tomorrow. *Lancet Infect Dis*, 2:425–31.
- Berzofsky JA, Ahlers JD, Derby MA, et al. 1999. Approaches to improve engineered vaccines for human immunodeficiency virus and other viruses that cause chronic infections. *Immunol Rev*, 170:151–72.
- Brander C, Corradin G, Hasler T, et al. 1996. Peptide immunization in humans: a combined CD8⁺/CD4⁺ T cell-targeted vaccine restimulates the memory CD4 T cell response but fails to induce cytotoxic T lymphocytes (CTL). *Clin Exp Immunol*, 105:18–25
- Craiu A, Akopian T, Goldberg A, et al. 1997. Two distinct proteolytic processes in the generation of a major histocompatibility complex class I-presented peptide. *Proc Natl Acad Sci U S A*, 94:10850–5.
- Ertl H, Varga I, Xiang Z, et al. 1996. Poly(DL-lactide-co-glycolide) microspheres as carriers for peptide vaccines. *Vaccine*, 14:879–85.
- Harada A, Kataoka K. 1995. Formation of polyion complex micelles in an aqueous milieu from a pair of oppositely-charged block copolymers with poly(ethylene glycol) segments. *Macromolecules*, 28:5294–9.
- Hunziker IP, Grabscheid B, Zurbriggen R, et al. 2002. In vitro studies of core peptide-bearing immunopotentiating reconstituted influenza virosomes as a non-live prototype vaccine against hepatitis C virus. *Int Immunol*, 14:615–26.
- Jackson DC, O'Brien-Simpson N, Ede NJ, et al. 1997. Free radical induced polymerization of synthetic peptides into polymeric immunogens. *Vaccine*, 15:1697–705.
- Kakizawa Y, Harada A, Kataoka K. 1999. Environment-sensitive stabilization of core-shell structured polyion complex micelle by reversible cross-linking of the core through disulfide bond. *J Am Chem Soc*, 121:11247–8.
- Kakizawa Y, Harada A, Kataoka K. 2001. Glutathione-sensitive stabilization of block copolymer micelles composed of antisense DNA and thiolated poly(ethylene glycol)-block-poly(L-lysine): A potential carrier for systemic delivery of antisense DNA. *Biomacromolecules*, 2:491–7.
- Miyata M, Kakizawa Y, Nishiyama N, et al. 2004. Block catiomer polyplexes with regulated densities of charge and disulfide cross-linking directed to enhance gene expression. *J Am Chem Soc*, 126:2355–61.
- Park Y, Kwok KY, Boukarim C, et al. 2002. Synthesis of sulfhydryl cross-linking poly(ethylene glycol)-peptides and glycopeptides as carriers for gene delivery. *Bioconjug Chem*, 13:232–9.
- Purcell AW, Zeng W, Mifsud NA, et al. 2003. Dissecting the role of peptides in the immune response: Theory, practice and the application to vaccine design. *J Pept Sci*, 9:255–81.
- Shirai M, Okada H, Nishioka M, et al. 1994. An epitope in hepatitis C virus core region recognized by cytotoxic T cells in mice and humans. *J Virol*, 68:3334–42.
- van Endert, PM. 2001. Designing peptide vaccines for cellular cross-presentation. *Biologicals*, 29:285–8.

