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Self-Adjuvanting Polymer–Peptide Conjugates As Therapeutic Vaccine Candidates against Cervical Cancer

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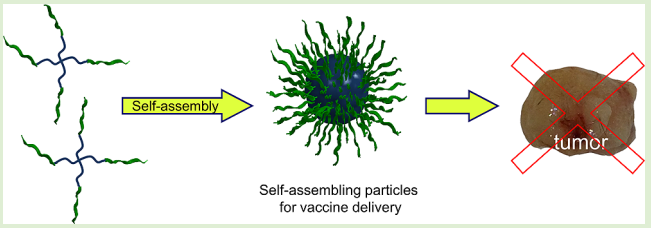
Supporting Information

ABSTRACT: Dendrimers are structurally well-defined, synthetic polymers with sizes and physicochemical properties often resembling those of biomacromolecules (e.g., proteins). As a result, they are promising candidates for peptide-based vaccine delivery platforms. Herein, we established a synthetic pathway to conjugate a human papillomavirus (HPV) E7 protein-derived peptide antigen to a star-polymer to create a macromolecular vaccine candidate to treat HPV-related cancers. These conjugates were able to reduce tumor growth and eradicate E7-expressing TC-1 tumors in mice after a single immunization, without the help of any external adjuvant.



INTRODUCTION

Vaccination is one of the most cost-effective public health interventions, and has been proposed as a promising strategy for the treatment of cancer.[1] Cervical cancer results from the infection of the cervix with human papillomavirus (mainly HPV type 16), which disrupts the cell cycle through interaction between HPV proteins and the host cell.[2] Prophylactic vaccines against HPV have been developed to prevent HPV infection (and by extension, cervical cancer), achieving the best results if administered prior to the commencement of sexual activity.[3] However, a large proportion of the global population is already infected, and many women will continue to become infected with cervical cancer-associated HPV strains until global vaccine coverage is achieved.[4,5] Furthermore, the long-term efficacy of the new HPV vaccines can only be evaluated decades after implementing the existing prophylactic vaccination.[6] Importantly, prophylactic vaccines protect the host against viral infection but are not designed to kill tumor cells. These limitations have prompted research into the development of therapeutic vaccines that treat HPV-related cancer.[2,7−11] Therapeutic vaccine candidates against HPV-related cancers were usually designed to elicit a cytotoxic T-cell response by targeting E7 oncoprotein. This protein is constitutively expressed in cancer cells and is required for the induction and maintenance of tumor growth. T-cells recognize tumor-associated antigen in the form of a defined short peptide sequence (T cell epitope), rapidly and specifically killing target cells that display the antigen.[12] This approach to treating cancer overcomes the many disadvantages of classical chemotherapy, and other therapies like siRNA, which is severely limited by the instability and poor delivery of siRNA molecules into the targeted cells. Therapeutic vaccines use the body's natural fighting system, and have minimal risk of side effects.

All reported peptide-based therapeutic HPV vaccine candidates required formulation with an adjuvant (immunostimulant) to produce the desired immune response.[2] Not only are there a limited number of adjuvants approved for human use, but many of these adjuvants are associated with toxicity and low efficacy.[13] Therefore, the discovery and development of novel adjuvants with potent immunomodulatory activities is of significant importance in the field of cancer immunotherapy. Specifically, the development of potent and well-defined adjuvants without adverse toxicity is crucial to the success of therapeutic vaccines.[14]

Dendrimers are structurally well-defined, synthetic polymers that can be designed to mimic the size and physiochemical properties of biomolecules (e.g., proteins).[15] Their hyperbranched structure allows the attachment and presentation of antigen molecules at the periphery of the dendrimer, resulting in a highly multifunctional biomacromolecule.[15] It was demonstrated that a Multiple Antigenic Peptide (MAP) system with a poly lysine core[16] was able to generate high antibody titers as a prophylactic vaccine, but only when coadministered with an adjuvant. Similarly, linear polymers with the peptide

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epitopes randomly conjugated along the polymer backbone also generated antibody responses when coadministered with the toxic complete Freund's adjuvant (CFA).[17] To overcome these disadvantages, well-defined polymer-based amphiphiles were proposed. Polyacrylate was selected as the most promising candidate because poly(acrylic acid), and its esters were easy to synthesize and had little or no toxicity.[18,19] We recently reported the ability of polyacrylate amphiphilic dendrimers (decorated with B-cell epitopes on the dendrimer's periphery) to act as a self-adjuvanting prophylactic vaccine, and induced a protective (humoral) immunity.[20,21] However, while there are a substantial number of self-adjuvanting systems and adjuvants that induce humoral immunity, the induction of cellular responses for therapeutic vaccination is more challenging. For example, aluminum salts, the only adjuvant that is widely used in humans, are recognized to essentially only stimulate a humoral immunity.[22,23] Thus, there is an urgent need for alternatives that are able to induce cellular immune responses.

In this work, we hypothesized that a star polymer-based delivery systems might have the adjuvanting potency to generate T-cell mediated immunity against cells that produce the E7 protein, and thus could eradicate tumor cells. We designed and synthesized polymer−peptide conjugates with the well-known HPV-16 E7 protein epitope **8Q** (QAEPDRAHYNIVTFCCKCD; $E7_{44-62}$), which contains a CTL epitope ($CD8^+$ cytotoxic T lymphocytes), and T-helper cell ($CD4^+$) and B-cell epitopes (Figure 1).[9] This combination makes **8Q** an ideal candidate to stimulate long-term vaccine efficacy to kill cancer cells. We designed a short series of modified epitopes (Figure 1) to avoid uncontrolled aggregation of the cysteine-rich **8Q** epitope. These epitopes were conjugated to the dendritic structure that resembled a four-arm star-polymer $P(^tBA_{37}\text{-}\equiv)_4$ (**S4**) and self-assembled into particles under aqueous conditions. All conjugates produced microparticles when formulated in phosphate buffer saline (PBS). A conjugate that contained the $\mathbf{8Q_{min}}$ epitope was able to reduce tumor growth and eradicate E7-expressing TC-1 tumors in mice after a single immunization, without help from any external adjuvant.

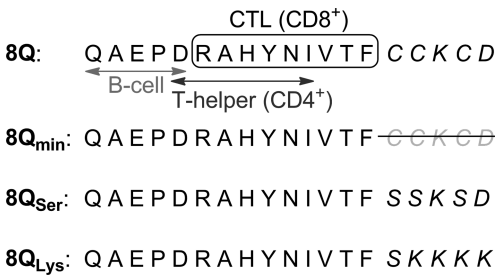

Figure 1. The amino acid sequence of the original **8Q** epitope and its novel derivatives. The CTL epitope ($CD8^+$ cytotoxic T lymphocytes), T-helper cell ($CD4^+$), and B-cell epitope are highlighted within the **8Q** sequence. The immunologically redundant pentapeptide sequence is indicated by italic font.

EXPERIMENTAL SECTION

Materials. Protected L-amino acids were purchased from Novabiochem (Merck Chemicals, Darmstadt, Germany) and Mimotopes (Melbourne, Australia). pMBHA resin was purchased from Peptides International (Kentucky, USA). Rink amide MBHA resin, *N*,*N*′-dimethylformamide (DMF), dichloromethane (DCM), methanol, *N*,*N*′-diisopropylethylamine (DIPEA), piperidine and trifluoroacetic acid (TFA) were obtained from Merck (Hohenbrunn, Germany). Cu wires were purchased from Aldrich (Steinheim, Germany). *t*-Butyl acrylate (^{t}BA, Aldrich, > 99%) was deinhibited before use by passing through a basic alumina column, ethyl 2-bromoisobutyrate (EBiB, Aldrich, 98%), tripropargylamine (TPA, Aldrich, 98%), dimethyl sulfoxide (DMSO, Labscan, AR grade), *N*,*N*,*N*′,*N*′,*N*″-pentamethyldiethylenetri-amine (PMDETA, Aldrich, 99%), copper(I) bromide (Cu(I)Br, MV Laboratories, Inc., 99.999%), copper(II) bromide ($CuBr_2$, Aldrich, 99%) were used as received. Propargyl nitroxide was synthesized according the previous procedure.[24] 1-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 2-(1*H*-7-azabenzotriazol-1-yl-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU) were purchased from Mimotopes (Melbourne, Australia). HPLC grade acetonitrile was obtained from Labscan (Bangkok, Thailand). All other reagents were obtained at the highest available purity from Sigma-Aldrich (Castle Hill, NSW, Australia).

Microwave-assisted Boc-SPPS was carried out using a SPS mode CEM Discovery reactor (CEM Corporation, Matthews, NC, USA). Anhydrous hydrofluoric acid (HF) was supplied by BOC gases (Sydney, NSW, Australia). A Kel-F HF apparatus (Peptide Institute, Osaka, Japan) was used for HF cleavage. Electrospray ionization mass spectrometry (ESI-MS) was performed using a Perkin-Elmer-Sciex API3000 instrument with Analyst 1.4 software (Applied Biosystems/MDS Sciex, Toronto, Canada). ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were recorded with a Bruker Avance 300 MHz spectrometer (Bruker Biospin, Germany). Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was performed using an Agilent instrument with a 1 mL/min flow rate and detection at 214 nm. Separation was achieved using a 0−100% linear gradient of solvent B over 40 min with 0.1% TFA/H_2O as solvent A and 90% MeCN/0.1% TFA/H_2O as solvent B on either a Vydac analytical C4 column (214TP54; 5 μm, 4.6 mm × 250 mm) or a Vydac analytical C18 column (218TP54; 5 μm, 4.6 mm × 250 mm). Preparative RP-HPLC was performed on Shimadzu (Kyoto, Japan) instrumentation (either LC-20AT, SIL-10A, CBM-20A, SPD-20AV, FRC-10A or LC-20AP x 2, CBM-20A, SPD-20A, FRC-10A) in linear gradient mode using a 10−20 mL/min flow rate, with detection at 230 nm. Separations were performed with solvent A and solvent B on either a Vydac preparative C4 column (214TP1022; 10 μm, 22 mm × 250 mm) or a Vydac preparative C18 column (218TP1022; 10 μm, 22 mm × 250 mm). Particle size was measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano Series with DTS software. Sizes were analyzed using a noninvasive backscatter system. Multiplicate measurements were performed at 25 °C with scattering angle of 173° using disposable cuvettes and the number-average hydrodynamic particle diameter are reported. The average particle diameter in PBS was determined with a ZEISS LSM 510 META confocal microscope. The software used for image acquisition was AIM 4.2 (Carl Zeiss, Ltd.) and Carl Zeiss Zen 2009 was used for image analysis.

Synthesis of 8Q Peptide. **8Q** epitope (QAEPDRAHYNIVTFCCKCD; $E7_{44-62}$) was synthesized on pMBHA resin (substitution ratio: 0.45 mmol/g, 0.2 mmol scale, 0.44g) using HBTU/DIPEA Boc-chemistry by Microwave-Assisted Solid-Phase Peptide Synthesis (MW-SPPS). The temperature was set at 70 °C (at 20 W, 10 min) for amino acid coupling except for His, Cys and Asp, which were coupled at 50 °C (at 20 W, 15 min). Each amino acid coupling cycle consisted of Boc-deprotection with neat TFA (2 × 1 min, at room temperature (RT)), a 1 min DMF flow wash, followed by 10 min coupling with the preactivated amino acid. Amino acid activation was achieved by dissolving Boc-amino acid (0.84 mmol, 4.2 equiv), in a 0.5 M HBTU/DMF solution (1.6 mL, 0.8 mmol, 4.0 equiv) followed by the addition of DIPEA (0.22 mL, 1.24 mmol, 6.2 equiv). Amino acids were preactivated for 1 min prior to their addition to the resin. For peptides containing His(DNP) residues, the DNP (2,4-dinitrophenyl) group was cleaved by treating the resin with 20% (v/v) β-mercaptoethanol and 10% (v/v) DIPEA in DMF for 2 × 1 h treatments prior to peptide cleavage. Upon completion of synthesis and removal of the dinitrophenyl (DNP) protecting group, the resin was washed with DMF, DCM, and MeOH, then dried (vacuum desiccator). The peptide was cleaved from the resin using HF, with *p*-

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cresol and *p*-thiocresol as scavengers. The cleaved peptide was precipitated, filtered, and washed thoroughly with ice-cold Et_2O and dissolved in 50% MeCN/0.1% TFA/H_2O. After lyophilization, the crude peptide was obtained as an amorphous powder. The product was purified by preparative RP-HPLC on a C18 column with a solvent gradient of 0−50% solvent B over 50 min. HPLC analysis (C18 column): t_R = 19.18 min, purity >95%. Yield: 12%, ESI-MS: *m/z* 2213.2 (calc 2213.5) $[M+H]^+$; 1107.7 (calc 1107.2) $[M+2H]^{2+}$; 738.5 (calc 738.5) $[M+3H]^{3+}$; MW 2212.49.

Synthesis of Azidoacetic Acid ($N_3CH_2CO_2H$). Azidoacetic acid was synthesized using a similar method to published procedure.[25] Sodium azide (6.0 g, 92.3 mmol, 3.0 equiv) was dissolved in H_2O (10 mL), and bromoacetic acid (4.3 g, 30.8 mmol, 1.0 equiv) was added. The reaction was stirred continuously in an ice bath for 24 h and subsequently acidified with 32% HCl (10 mL). The product was then extracted with Et_2O (4 × 50 mL), dried over anhydrous $MgSO_4$ and the solvent was evaporated under vacuum. The final product was obtained as a colorless oil (2.95 g, 95%) after prolonged evaporation under vacuum to remove organic solvent and the last traces of water. ^1H NMR (300 MHz, $CDCl_3$) δ 10.60 (br s, 1H, OH), 3.98 (s, 2H, CH_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 50.0, 173.7.

Synthesis of Boc-Thr(Fmoc-Val)−OH. The isopeptide unit was synthesized according to a published procedure.[26−28]

Synthesis of 1,3-di(hydroxymethyl)-5-(prop-2-ynyloxy)-benzene. This compound was prepared as previously described.[29,30] ^1H NMR (300 MHz, CD3OD): δ 2.52 (t, *J* = 2.4 Hz, 1H, $CH_2C{\equiv}C\underline{H}$), 4.68 (bs, 4H, 2x$C\underline{H}_2OH$), 4.71 (d, *J* = 2.4 Hz, 2H, $C\underline{H}_2C{\equiv}CH$), 6.92 (bs, 2H, ArH), 7.00−6.98 (m, 1H, ArH).

Synthesis of Polyacrylate P(^{t}BA$_{37}$-≡)$_4$. The alkyne-functionalized 4-arm poly(*t*-butyl acrylate) star (**S4**) was synthesized by atom-transfer radical polymerization. Star-polymer P(^{t}BA$_{37}$-Br)$_4$[21] (M_n = 14900, polydispersity index (PDI) = 1.05; 0.700 g, 3.68 × 10^{-5} mol), propargyl nitroxide (0.078 g, 3.72 × 10^{-4} mol), and PMDETA (0.043 g, 2.48 × 10^{-4} mol) were dissolved in 6 mL of DMSO/toluene mixture (1:2, vol). The mixture was purged with argon for 20 min before the addition of CuBr (0.033 g, 2.32 × 10^{-4} mol). The reaction was stirred at RT for 30 min, then diluted by THF and passed through an activated basic alumina column. The solution was concentrated and the polymer was recovered by precipitation into a MeOH/water (50:50 vol) mixture and then filtered. The polymer was further purified by preparative gel permeation chromatography (GPC). GPC: M_n = 15730, PDI = 1.06. ^1H NMR ($CDCl_3$): δ 1.11−1.21 (m, 15H, methyl group of core and TEMPO ring), 1.45 (b, methyl protons of *t*-BA repeat units), 1.81, 2.21 (b, methylene and methine protons of polymer backbone), 2.40 (s, 4H, $(-OCH_2\text{-}C{\equiv}C\underline{H})_4$), 3.76, (b, 4H, methine proton on a TEMPO ring), 3.80−4.25, (b, 20H, (methylene protons of the core, methine proton of *t*-BA unit close to the alkoxyamine linkage, methylene protons of propargyl group).

Synthesis of N-Terminus Model Azide. N-terminus model azide (N_3CH_2CO-Phe-Cys-NH_2) was synthesized by manual stepwise SPPS on rink amide MBHA resin (substitution ratio: 0.59 mmol/g, 0.2 mmol scale, 0.34 g) using HBTU/DIPEA Fmoc-chemistry. Amino acid activation was achieved by dissolving Fmoc-amino acid (0.84 mmol, 4.2 equiv), in 0.5 M HBTU/DMF solution (1.6 mL, 0.8 mmol, 4.0 equiv) followed by the addition of DIPEA (146 μL, 0.84 mmol, 4.2 equiv). Coupling cycle consisted of Fmoc deprotection with 20% of piperidine in DMF (twice, 10 and 20 min), a 1 min DMF flow-wash, followed by coupling with 4.2 equiv of preactivated Fmoc-amino acids (2 × 2 h). The attachment of azidoacetic acid (4.2 equiv) was achieved using HBTU (3 equiv)/DIPEA (4.2 equiv) at room temperature (2 × 2 h). Upon completion of synthesis, the resin was washed with DMF, DCM, and MeOH, then dried (vacuum desiccator). The cleavage of model azide was carried out by stirring the resin in the solution of TFA (99%)/triisopropylsilane/water (95:2.5:2.5) for 4 h. The cleaved peptide was precipitated, filtered, and washed with ice-cold Et_2O. After lyophilization the crude peptide was obtained as an amorphous powder. The product was purified by preparative RP-HPLC on C18 column with solvent gradient 0−50% solvent B over 50 min. HPLC analysis (C18 column): t_R = 18.4 min, purity >95%. Yield: 96% ESI-MS: *m/z* 702.1 (calc 701.8) $[2M+H]^+$; 351.7 (calc 351.4) $[M+H]^+$; MW 350.40.

Synthesis of N-Terminus 8Q Azide. **8Q** azide peptide epitope (N_3CH_2CO-QAEPDRAHYNIVTFCCKCD-NH_2) was synthesized according to the above procedure. Fmoc deprotection of Thr, Val and Ile were performed with 2% of 1,8-Diazabicycloundec-7-ene (DBU) in DMF (twice, 5 and 10 min) instead of 20% piperidine in DMF. HPLC analysis (C18 column): t_R = 17.5 min, purity >95%. Yield: 17%. ESI-MS: *m/z* 1148.8 (calc 1148.8) $[M+2H]^{2+}$; 766.2 (calc 766.2) $[M+3H]^{3+}$; MW 2295.54.

Synthesis of N-Terminus 8Q$_{min}$ Azide. **8Q$_{min}$** azide peptide epitope (N_3CH_2CO-QAEPDRAHYNIVTF-NH_2) was synthesized according to the above procedure. Fmoc deprotection of Thr, Val, and Ile was performed with 2% DBU in DMF (twice, 5 and 10 min) instead of 20% piperidine in DMF. HPLC analysis (C18 column): t_R = 18.3 min, purity >95% (Figure S1). Yield: 42%. ESI-MS: *m/z* 1743.3 (calc 1743.8) $[M+H]^+$; 872.3 (calc 872.4) $[M+2H]^{2+}$; MW 1742.85.

Synthesis of N-Terminus 8Q$_{Ser}$ Azide. *Method A.* **8Q$_{Ser}$** azide peptide epitope (N_3CH_2CO-QAEPDRAHYNIVTFSSKSD-NH_2) was synthesized according to the above procedure. Fmoc deprotection of Thr, Val, and Ile were performed with 2% DBU in DMF (twice, 5 and 10 min) instead of 20% piperidine in DMF. HPLC analysis (C18 column): t_R = 16.66 min, purity >95%. Yield: 14%. ESI-MS: *m/z* 1124.8 (calc 1124.7) $[M+2H]^{2+}$; 750.2 (calc 750.1) $[M+3H]^{3+}$; MW 2247.34.

Method B. The isopeptide **8Q$_{Ser}$** azide peptide epitope was synthesized according to the above procedure. Boc-Thr(Fmoc-Val)−OH was used to introduce Thr and Val. Fmoc deprotection were performed only with 20% piperidine in DMF (twice, 10 and 20 min). Isopeptide **8Q$_{Ser}$** azide was cleaved from resin (t_R = 15.82 min, C18), lyophilized, and dissolved in 50% aqueous MeCN. Ammonia solution (25%) was added dropwise until the pH was 8−9. The reaction mixture was stirred for 2 h to form the desired migrated **8Q$_{Ser}$** azide. HPLC analysis (C18 column): t_R = 16.66 min, purity >95% (Figure S1). Yield: 55%. ESI-MS: *m/z* 1124.8 (calc 1124.7) $[M+2H]^{2+}$; 750.2 (calc 750.1) $[M+3H]^{3+}$; MW 2247.34.

Synthesis of N-Terminus 8Q$_{Lys}$ Azide. **8Q$_{Lys}$** azide peptide epitope (N_3CH_2CO-QAEPDRAHYNIVTFSKKKK-NH_2) was synthesized according to the above procedure. Fmoc deprotection of Thr, Val, and Ile were performed with 2% DBU in DMF (twice, 5 and 10 min) instead of 20% piperidine in DMF. HPLC analysis (C18 column): t_R = 15.9 min, purity >95% (Figure S1). Yield: 18% ESI-MS: *m/z* 1172.3 (calc 1172.3) $[M+2H]^{2+}$; 781.9 (calc 781.9) $[M+3H]^{3+}$; 586.9 (calc 586.6) $[M+4H]^{4+}$; 469.8 (calc 469.5) $[M+5H]^{5+}$; MW 2342.61.

Copper-Catalyzed Alkyne−Azide Cycloaddition (CuAAC) Reaction: Model Studies. To determine the optimal conditions to avoid disulfide formation during the CuAAC reaction, model azide and 1,3-di(hydroxymethyl)-5-(prop-2-ynyloxy)benzene were employed. The reactions were conducted by using different copper sources (Cu wires, $CuSO_4$, CuI), with or without adding reducing agent (ascorbic acid, tris(2-carboxyethyl)phosphine), different solvents (solvent B (90% MeCN/0.1% TFAH_2O), DMF), and stirring at 50 °C or room temperature. Briefly, model azide (1 equiv) and 1,3-di-(hydroxymethyl)-5-(prop-2-ynyloxy)benzene (1 equiv) were dissolved in DMF (1 mL), and the copper source added. The air in the reaction mixture was removed either by nitrogen or argon bubbling. The reaction mixture was covered and protected from light with aluminum foil and stirred at either 50 °C or room temperature under nitrogen or argon atmosphere. During the reaction, samples (30 μL) were frequently taken and analyzed by analytical HPLC and ESI-MS to monitor the progress of reaction.

Synthesis of Vaccine Candidate P(^{t}BA$_{37}$-≡)$_4$-8Q$_{min}$ (S4−8Q$_{min}$). **8Q$_{min}$** azide peptide epitope (1.8 mg, 1 μmmol, 10 equiv) and P(^{t}BA$_{37}$-≡)$_4$ (1.9 mg, 0.10 μmol, 1.0 equiv) were dissolved in DMF (1 mL). Copper wires (60 mg)—treated with concentrated sulphuric acid (3 min), subsequently washed with distilled water, methanol, and dried under reduced pressure—were added into the mixture. The mixture was bubbled with nitrogen to remove the air. The reaction mixtures were covered and protected from light with

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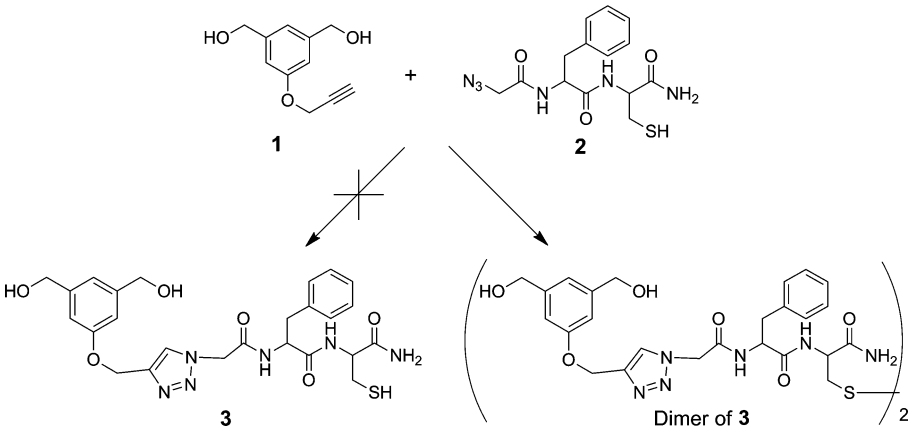
Scheme 1. The CuAAC Reaction Was Examined Using the Model Compounds 1 and 2



aluminum foil and stirred at 50 °C in a temperature controlled oil bath under nitrogen atmosphere for 8 h. The wires were filtered off from the warm solution and washed with 1 mL of DMF. Millipore endotoxin-free water (7 mL) was slowly added to the solution (0.005 mL/min). Particles formed through the self-assembly process were exhaustively dialyzed against endotoxin-free water (pH = 6.8) using presoaked and rinsed dialysis bags (Pierce Snakeskin, MWCO 3K). The resulting particles were self-assembled into particles with diameters above 1 μm as observed by dynamic light scattering.

Synthesis of Vaccine Candidate P(^{t}BA$_{37}$-≡)$_4$-8Q$_{Ser}$ (S4−8Q$_{Ser}$). S4−8Q$_{Ser}$ was synthesized in the same manner as above. 8Q$_{Ser}$ azide peptide epitope (2.5 mg, 1 μmmol, 10 equiv) and star-polymer p(^{t}BA$_{37}$-≡)$_4$ (1.9 mg, 0.10 μmmol, 1.0 equiv) were dissolved in DMF (1 mL). The reaction was terminated after 12 h. The resulting particles were self-assembled into to yield diameters of around 150 and 700 nm as observed by dynamic light scattering.

Synthesis of Vaccine Candidate P(^{t}BA$_{37}$-≡)$_4$-8Q$_{Lys}$ (S4−8Q$_{Lys}$). S4−8Q$_{Lys}$ was synthesized in the same manner as above. 8Q$_{Lys}$ azide peptide epitope (2.9 mg, 1 μmmol, 10 equiv) and star-polymer p(^{t}BA$_{37}$-≡)$_4$ (1.9 mg, 0.10 μmmol, 1.0 equiv) were dissolved in DMF (1 mL). The reaction was terminated after 12 h. The resulting particles were self-assembled into particles with a 26 nm diameter as observed by dynamic light scattering.

Mice and Cell Lines. Female C57BL/6 (6−8 weeks old) mice were used in this study and purchased from Animal Resources Centre (Perth, Western Australia). TC-1 cells (murine C57BL/6 lung epithelial cells transformed with HPV-16 E6/E7 and ras oncogenes) were obtained from TC Wu.[31] TC-1 cells were cultured and maintained at 37 °C/5% CO_2 in RPMI 1640 medium (Gibco) supplemented with 10% heat inactivated fetal bovine serum (Gibco) and 1% nonessential amino acid (Sigma-Aldrich). The animal experiments were approved by the University of Queensland Animal Ethics committee (DI/034/11/NHMRC) in accordance with National Health and Medical research Council (NHMRC) of Australia guidelines.

In Vivo Tumor Treatment Experiments. To test the efficacy of polymer−peptide conjugates as a therapeutic vaccine against established tumors, groups of C57BL/6 mice (5 per group) were first challenged subcutaneously in the right flank with 1×10^5/mouse of TC-1 tumor cells. On the third day after tumor challenge, the mice were injected subcutaneously at the tail base with 100 μg of polymer−peptide conjugates in a total volume of 100 μL of sterile-filtered PBS or control formulations. Polymer-peptide conjugates consisted of S4−8Q$_{min}$, S4−8Q$_{ser}$ and S4−8Q$_{Lys}$. Positive control received 30 μg of 8Q emulsified in a total volume of 100 μL of Montanide ISA51 (Seppic, France)/PBS (1:1, v/v). Three negative control groups were administered PBS, 70 μg of 4-arm star-polymer alone (S4), or 4-arm star-polymer (70 μg) + 8Q (30 μg) physical mixture (S4 + 8Q) in total volume of 100 μL PBS. The mice were given a single immunization only. The size of the tumor was measured by palpation and calipers every two days and reported as the average tumor size across the group of five mice or as tumor size in individual mice.[32,33] Tumor volume was calculated using the formula V (cm^3) = 3.14 × [largest diameter × (perpendicular diameter)^2]/6.[33] The mice were euthanized when tumor reached 1 cm^3 or started bleeding to avoid unnecessary suffering.

Statistical Analysis. All data were analyzed using GraphPad Prism 5 software. Kaplan−Meier survival curves for tumor treatment experiments were applied. Differences in survival treatments were determined using the log-rank (Mantel-Cox) test, with $p < 0.05$ considered statistically significant.

RESULTS AND DISCUSSION

Synthesis and Characterization of the Polymer−Peptide Conjugates. Copper-catalyzed alkyne−azide 1,3-dipolar cycloaddition (CuAAC) "click" reaction[34] was used to synthesize vaccine candidates against HPV-associated cancers. These candidates possessed a hydrophobic polymeric core and multiple copies of a peptide epitope derived from the HPV-16 E7 oncoprotein. The 8Q epitope and its new analogues were applied as antigens, while the polyacrylate 4-arm star-polymer served as a delivery platform. All the peptides contained CTL, T-helper, and B-cell epitopes.

First, the alkyne-functionalized 4-arm poly(*t*-butyl acrylate) star (S4) was synthesized by atom-transfer radical polymerization (ATRP) and end-group functionalization with a very narrow molecular-weight distribution (polydispersity index of less than 1.06). 8Q epitope and its azide (N_3CH_2CO-QAEPDRAHYNIVTFCCKCD-NH_2) were synthesized using microwave-assisted Boc- and Fmoc-SPPS, respectively. During the initial synthesis, a so-called "difficult" sequence[35−37] inside 8Q epitope was identified to be composed of β-branched amino acids (the IVT tripeptide). It was reported that the presence of these sequences predisposed the peptide to aggregate on the resin via β-sheet formation and inhibited completion of Fmoc group deprotection.[38,35] Indeed, a significant amount of amino acid deletion products were detected during 8Q synthesis, resulting from incomplete deprotection of the Fmoc group. Complete deprotection was achieved by replacing piperidine with DBU for the removal of Fmoc groups after coupling isoleucine, valine, and threonine.

To examine the "click" reaction, simple model compounds were designed, in which an alkyne (1) and a dipeptide azide possessing single thiol functionality (2) were coupled using copper-wires as the catalyst (Scheme 1).[21,39−41] Analysis of the reaction showed that instead of the desired "click" product 3, a disulfide bond-mediated dimer of 2 was exclusively produced.

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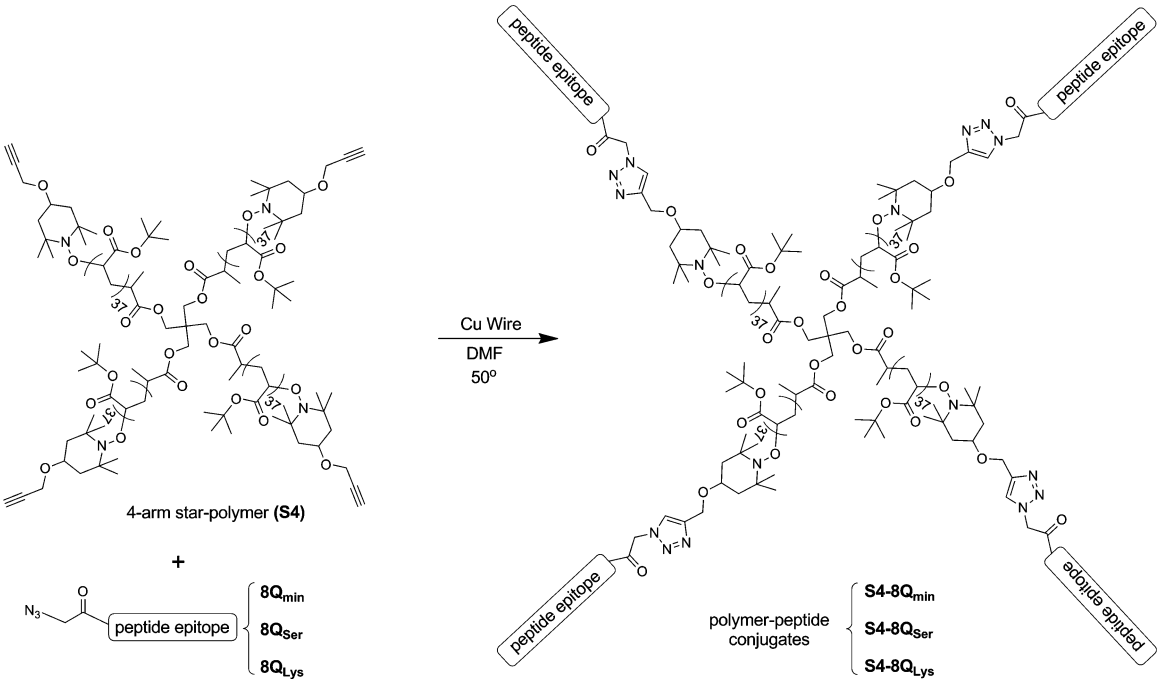
Scheme 2. Synthesis of Polymer−Peptide Conjugates



Subsequently, this dimer reacted with alkyne **1** producing disulfide bond-mediated dimer of compound **3** (Figure S2). Thus, formation of the single product could not be expected during conjugation of **S4** core with **8Q** peptide which possessed multiple thiol groups. In an effort to eliminate disulfide bond formation, we investigated alternative CuAAC "click" reaction conditions by changing the copper source (copper wire, copper sulfate, copper iodide), reducing agents (ascorbic acid, tris(2-carboxyethyl)phosphine), solvents (DMF, acetonitrile/water/trifluoroacetic acid, 90/10/0.1) and temperature (50 °C, RT). Regardless of the experimental conditions, we were unable to obtain compound **3** (Figure S3).

These observations prompted us to modify the **8Q** peptide. Three new epitopes were designed (Figure 1): $\mathbf{8Q_{min}}$ (QAEPDRAHYNIVTF), which lacked the CCKCD sequence; $\mathbf{8Q_{Ser}}$ (QAEPDRAHYNIVTFSSKSD), where the cysteine moieties were replaced with their structural analog, serine; and $\mathbf{8Q_{Lys}}$ (QAEPDRAHYNIVTFSKKKK), in which the whole hydrophilic pentapeptide CCKCD was replaced with an SKKKK sequence derived from a "solubilizing moiety" incorporated in Pam2Cys/Pam3Cys-based vaccine candidates. Pam2Cys/Pam3Cys are popular adjuvants used to stimulate an immune response in peptide-based vaccines.[15]

$\mathbf{8Q_{min}}$ was conjugated to four-arm star-polymer to produce $\mathbf{S4-8Q_{min}}$ (Scheme 2). The product, $\mathbf{S4-8Q_{min}}$, was self-assembled into particles via the solvent replacement method (DMF/water) followed by dialysis against water to remove the organic solvent, excess of unreacted peptide, and copper. Formation of the conjugate was confirmed by elemental analysis,[21] which showed a significant increase in the nitrogen/carbon ratio (N/C = 0.085) compared with that of **S4** (N/C = 0.004), due to presence of a nitrogen-rich peptide. A high conjugation efficacy was obtained (3.5 peptide epitopes per polymer, 88% substitution). The substitution ratio was calculated based on comparison of the observed and theoretical N/C ratio. DLS analysis showed that only large particles with diameters over 1 μm were formed (Figure S4).

$\mathbf{8Q_{Ser}}$ and $\mathbf{8Q_{Lys}}$ azide derivatives were synthesized using the method developed for synthesis of the **8Q** epitope. Despite the use of DBU for removal of the protective Fmoc group, $\mathbf{8Q_{Ser}}$ azide was obtained in relatively poor yield after tedious purification. The isopeptide method developed by Kiso and co-workers was previously applied to the synthesis of peptides containing a "difficult" sequence.[42−45] Thus, isodipeptide unit (Boc-Thr(Fmoc-Val)−OH) was synthesized in a similar manner to the reported procedure,[26−28] and applied to the synthesis of $\mathbf{8Q_{Ser}}$ azide (Scheme S1) to avoid aggregation of the peptide during SPPS.[44] The final compound was obtained after O−N acyl migration of the O-acyl isoform, with significantly improved purity and yield (Figure 2). Both epitopes ($\mathbf{8Q_{Ser}}$ and $\mathbf{8Q_{Lys}}$) were coupled to **S4** to produce the desired conjugates (Scheme 2). The coupling for $\mathbf{S4-8Q_{Ser}}$ and $\mathbf{S4-8Q_{Lys}}$ was calculated to be (on average) 3.0 (75%) and 3.0 (74%) epitopes per **S4**, respectively. Conjugation of peptides to the polymer followed by self-assembly and dialysis was repeated several times, producing virtually identical products.

Both compounds ($\mathbf{S4-8Q_{Ser}}$ and $\mathbf{S4-8Q_{Lys}}$) were self-assembled in DMF/water and dialyzed against water. DLS analysis indicated that $\mathbf{S4-8Q_{Ser}}$ formed large nanoparticles (~530 nm; Figure S4). In contrast, the size distribution of $\mathbf{S4-8Q_{Lys}}$ particles was narrow, and smaller nanoparticles (26 nm) were detected (Figure S4). To prepare samples for in vivo experiments, $\mathbf{S4-8Q_{min}}$, $\mathbf{S4-8Q_{Ser}}$, and $\mathbf{S4-8Q_{Lys}}$ were formulated in PBS. All of the conjugates formed a milky suspension upon addition of the buffer (pH = 7.4). Confocal images were used to visualize microparticles and their aggregates (Figure S5). We were unable to quantitatively determine particles size distribution using DLS, TEM, and analytical ultracentrifugation.

In Vivo Tumor Treatment. To evaluate the therapeutic effect of polymer-peptide conjugates against established HPV tumor using the newly designed epitopes, mice were immunized on day 3 post-tumor implantation[46,47] with $\mathbf{S4-8Q_{min}}$,

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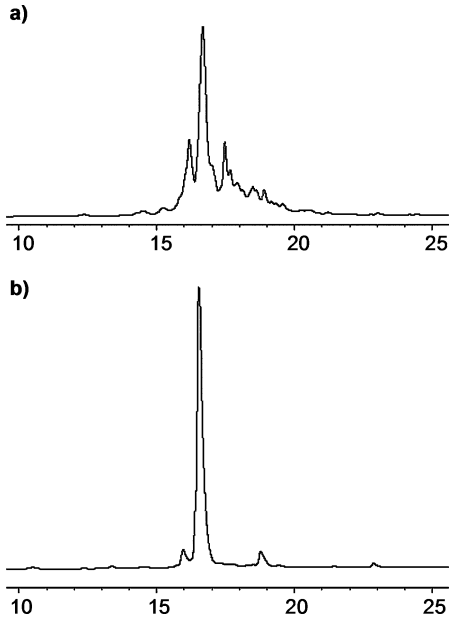

Figure 2. HPLC chart of crude $\mathbf{8Q_{Ser}}$ (a) synthesized by standard SPPS and (b) O-acyl isopeptide methods.

S4−8Q$_{ser}$, **S4−8Q$_{Lys}$** or control groups (**8Q** emulsified with IFA-like adjuvant (Montanide ISA51), physical mixture of **S4** and **8Q**, **S4** alone, and PBS). Mice were monitored every 2 days for evidence of tumor growth by palpation and calipers for 60 days.

The Kaplan−Meier survival curve (Figure 3a) showed that 100% of mice treated with physical mixture (**S4** + **8Q**), **S4** alone and PBS were moribund (i.e., mice were sacrificed due to tumor burden) by day 28, 33 and 33, respectively. In contrast, the survival rate in the mice treated with **S4−8Q$_{min}$**, **S4−8Q$_{ser}$** and **8Q** + ISA51 was 60%, 20%, and 40% over two months, respectively. Among the tested formulations, only **S4−8Q$_{min}$** and positive control showed significantly better survival compared with tumor-bearing mice treated with negative control ($p < 0.0027$).

As shown in Figure 3b, tumor-bearing mice treated with **S4−8Q$_{min}$** showed slower tumor growth over time than the other polymer-peptide conjugates and was similar to the mice treated with positive control. Treatment with **S4−8Q$_{min}$** eradicated tumor by day 21 post-tumor implantation in three mice, while an initial tumor reduction was observed in remaining two mice after 14−17 days (Figure 3c). It is noteworthy that an initial reduction in tumor size was observed in four groups including positive control and all conjugates (see Figure 3c and Supporting Information, Figure S6a−c). Remission of tumor growth was observed after 19 days, especially in the group immunized with **S4−8Q$_{Lys}$**. Thus, **S4−8Q$_{min}$** showed similar efficacy to the positive control while the antitumor efficacy of other conjugates was not statistically significant. No adverse side effects or allergic responses were observed in mice immunized with the conjugates, while one mouse from the group immunized with an adjuvant (**8Q** + ISA51) developed an adverse reaction (scratching resulting in patchy hair loss). Chemical conjugation of epitopes with a polymer core was essential to elicit a therapeutic effect since **S4** + **8Q** failed to induce an antitumor immune response. The three conjugates (**S4−8Q$_{min}$**, **S4−8Q$_{ser}$** and **S4−8Q$_{Lys}$**) demonstrated different antitumor activity, which might be associated with the ability of antigen presenting cells to correctly process and display different epitopes. Correct antigen processing is essential to trigger the desired cellular response. While **8Q$_{min}$** is a native fragment of the HPV-16 E7 protein, both **8Q$_{Ser}$** and **8Q$_{Lys}$** are chemically altered epitopes where the CTL epitope is flanked with a foreign peptide on its C-terminal. This alteration might impair antigen processing and consequently may have reduced the cellular immune response.[48−50] In addition, size can play important role in immune responses, while some groups reported that immune responses were significantly stronger for small nanoparticles (less than 100 nm),[51,52] others claimed that microparticles are required for strong activation of the immune system.[53,54] Small nanoparticles can easily migrate to lymphatic nodes for antigen presentation, whereas large particles are transported to lymphatic nodes by dendritic cells and the immunity induced by large particles is often related to the depot effect.[55] Interestingly, T-cell activation is often facilitated by the extended presence of antigen through depot-forming adjuvants.[54] The three conjugates form microparticles and have the tendency to aggregate further. This observation may suggest that the differences in the conjugates antitumor activity are not related to particle size.

Polymer−peptide conjugate **S4−8Q$_{min}$** demonstrated self-adjuvanting activity because it induced a therapeutic effect against tumor cells without help of any external adjuvant. This delivery system overcomes the poor immunogenicity of peptide-based vaccines and the common toxic side effects associated with external adjuvants. Self-adjuvanting properties of the polymer-based delivery system can be explained by particle-attributed activation of the immune system.[16,56,57] Particulate vaccines potentially cross-present the antigen, and antigen cross-presentation is especially important to induce the priming of the $CD8^+$ T-cell immune responses that naturally play a crucial role for therapeutic vaccines against cancer. This delivery system also exploits the depot effect (i.e., retaining the antigen at the injection site), thus increasing the duration of vaccine exposure to the immune cells. In addition, as particles are covered by multiple copies of the same peptide antigen, they should possess the advantages of a MAP system. Furthermore, formulation of antigens into particles provides some protection against enzymatic degradation, which is important for highly susceptible peptide antigens. Noticeably, in contrast to many previously reported peptide-based vaccine candidates,[33,46,47,58] **S4−8Q$_{min}$** demonstrated a therapeutic effect after a single immunization.

CONCLUSION

In conclusion, we established a synthetic pathway to produce polymer-peptide conjugates as macromolecular vaccine candidates against HPV-related cancers. The synthesis of peptide epitopes was greatly improved by the change of standard SPPS procedure and application of the isopeptide method. Modification of the immunogenic epitope allowed the elimination of undesirable disulfide bond-based aggregation/polymerization of the polymer-peptide conjugates.

Chemical alteration of **8Q** epitope allowed us to find the most effective epitope (**8Q$_{min}$**). Polyacrylate star-polymer conjugated with this epitope produced a robust therapeutic effect against a tumor without the help of any external adjuvant. This delivery system overcomes the poor immunogenicity of peptide-based vaccines. In contrast to many previously reported vaccine candidates, the conjugates demonstrated therapeutic effect after only a single immunization. Thus, we have

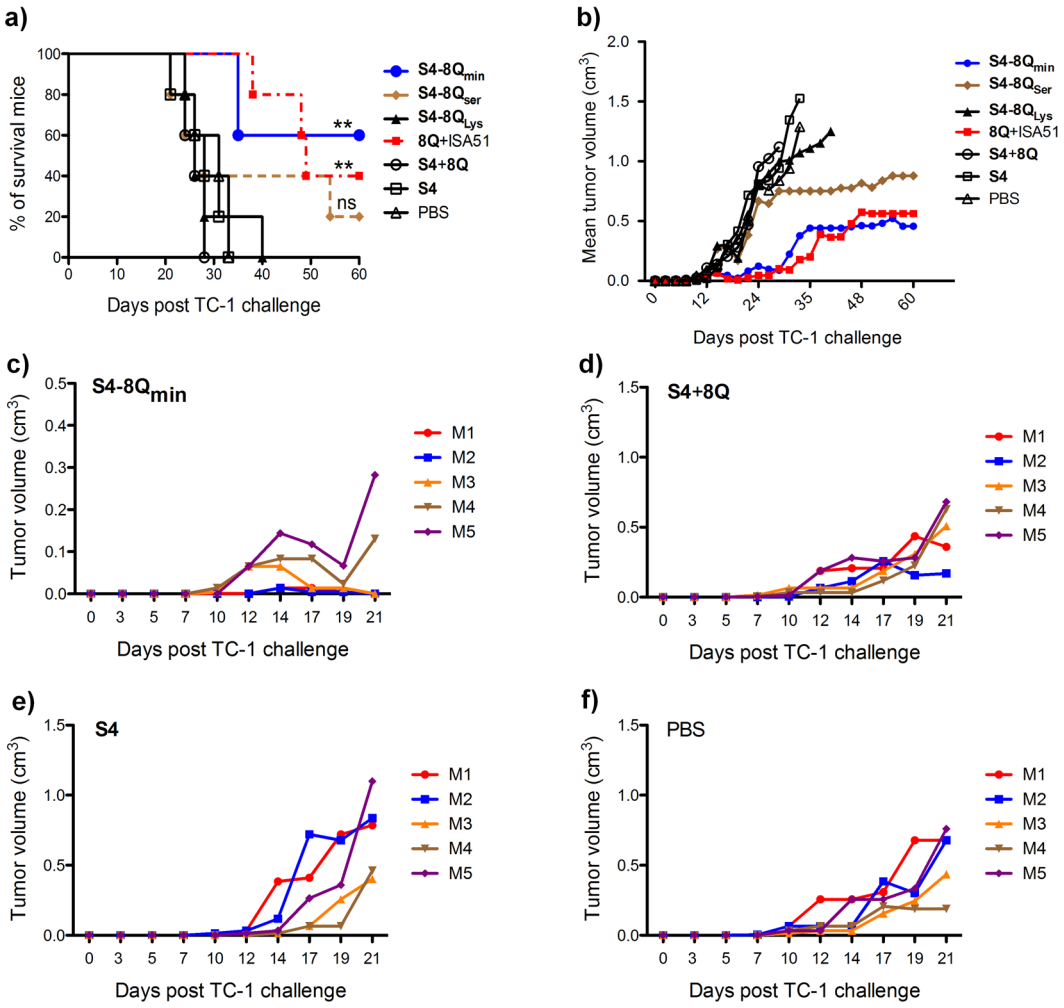

Figure 3. In vivo tumor treatment experiments. C57BL/6 (5 per group) were inoculated subcutaneously in the right flank with 1×10^5/mouse of TC-1 tumor cells (day 0) and vaccinated with different immunogens on day 3 without additional boost. (a) Survival rate monitored over 60 days post implantation and time to death plotted on a Kaplan−Meier survival curve. Mice were euthanized when tumor volume reached 1 cm^3 or started bleeding. The survival rate of each group was compared to the negative control (PBS) and was analyzed using the log-rank (Mantel-Cox) test (**$p < 0.01$). (b) Mean tumor volume (cm^3) in different groups of mice over 60 days post implantation. Tumor volume (cm^3) in individual TC-1 tumor-bearing mice treated with (c) **S4**−**8Q**$_{min}$, (d) **S4** + **8Q** physical mixture, (e) **S4** alone, or (f) PBS shown only up to day 21 of the experiment.

developed the first self-adjuvanting delivery system for a therapeutic peptide-based vaccine against cervical cancer.

■ ASSOCIATED CONTENT

Supporting Information

Additional information related to model study, isopeptide method (reactions schemes and HPCL charts), confocal microscope images and animal study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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