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Multiantigenic peptide-polymer conjugates as therapeutic vaccines against cervical cancer

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KEYWORDS: peptide-based subunit vaccine, multiantigenic, human papillomavirus, therapeutic cancer vaccine, polymer-peptide conjugate, self-adjuvanting, cervical cancer

ABSTRACT

Immunotherapy is one of the most promising strategies for the treatment of cancer. Human papillomavirus (HPV) is responsible for virtually all cases of cervical cancer. The main purpose of a therapeutic HPV vaccine is to stimulate $CD8^+$ cytotoxic T lymphocytes (CTLs) that can eradicate HPV infected cells. HPV oncoproteins E6 and E7 are continuously expressed and are essential for maintaining the growth of HPV-associated tumor cells. We designed polymer-based multi-antigenic formulations/constructs that were comprised of the E6 and E7 peptide epitopes. We developed an N-terminus-based epitope conjugation to conjugate two unprotected peptides to poly *tert*-butyl acrylate. This method allowed for the incorporation of the two antigens into a polymeric dendrimer in a strictly equimolar ratio. The most effective formulations eliminated tumors in up to 50% of treated mice. Tumor recurrence was not observed up to 3 months post initial challenge.

1. Introduction

Human papilloma viruses (HPVs) are the main cause of cervical cancer.[1] There are currently two prophylactic HPV vaccines, Gardasil and Cervarix, that have been developed and commercialized to the global market.[2] However, they are only recommended for naïve females aged from 9 to 26, and not for women already infected with HPVs.[3] For this reason, a new therapeutic vaccine is required for the treatment of the HPV-infected population.

In the last few decades, peptide-based subunit vaccines emerged as promising prophylactic and/or therapeutic medicines against several infectious diseases.[4] The main components of peptide-based subunit vaccines are the small peptides derived from the protein of a targeted pathogen.[5] In contrast to whole-cell or protein vaccines, vaccine non-redundant peptide components are non-toxic and non-infectious, and significantly lower the risks of allergic and/or autoimmune responses in patients.[6] They have high specificity as their peptide epitopes are purposely designed to recognize certain pathogenic targets. The pure peptides are easily produced under simple and economical methods, and microbe culturing is not required. They are

usually water-soluble and stable at room temperature, and do not require special storage conditions. The use of a peptide-based approach in the development of therapeutic anticancer vaccines in contrast to whole oncogenic proteins reduces the risk of vaccine-induced side-effects. However, one of the drawbacks of using peptides is that they require adjuvants as immunostimulant agents to trigger the desired immune responses. Commercially available adjuvants are often weak inducers of anticancer immune responses and/or toxic, and, therefore, new delivery platforms/adjuvants are needed.[6, 7]

To be effective, a therapeutic vaccine must be able to induce antitumor T-lymphocyte responses to directly kill cancer cells and, subsequently, to regress tumor growth.[8] The identification of appropriate peptide epitopes capable of initiating effective antitumor T-lymphocyte responses is critical for the design of a therapeutic vaccine.[9] HPV oncoproteins E6 and E7 are continuously expressed and are essential for maintaining the growth of HPV-associated tumor cells. Therefore, $E6_{43-57}$ (QLLRREVYDFAFRDL)[10] and $E7_{44-57}$ (QAEPDRAHYNIVTF) epitopes were chosen for this study. $E7_{44-57}$ contains a $CD4^+$ T helper cell epitope ($E7_{48-54}$, DRAHYNI) and a $CD8^+$ T cell epitope ($E7_{49-57}$, RAHYNIVTF),[11, 12] similarly $E6_{43-57}$ also includes both $CD4^+$ and $CD8^+$ epitopes.[13] Recently, we showed that $E7_{44-57}$ conjugated to a polymeric delivery system was able to eradicate E7-expressing tumor cells in immunized mice.[11, 12, 14] *tert*-Butyl acrylate polymer was chosen as a delivery platform for the vaccine because of its safety profile[15] and ability to serve as a self-adjuvanting moiety to induce both strong humoral and cellular immune responses.[16-18]

In all of the previous challenge experiments, vaccine candidates were used to treat small tumors, as the vaccines were administered 3 days post tumor implantation. However, ideal therapeutic vaccines should also be able to eradicate large, well-established tumors.

Unfortunately, the trialed therapeutic vaccine candidates often failed to demonstrate this desired efficacy when used for the treatment of advanced cancer, in both mice models and human clinical trials.[19]

Here, we describe the synthesis of vaccine candidates **1-7** (Figure 1) and the biological evaluation of their ability to eradicate TC-1 tumors from female C57Bl/6 mice. In contrast to previous studies, one of the main purposes of this work is to synthesize and test multiantigenic polymer-based vaccine delivery system, carrying both E6 and E7 protein-based epitopes, against 7 day well-established tumors in challenge experiments.

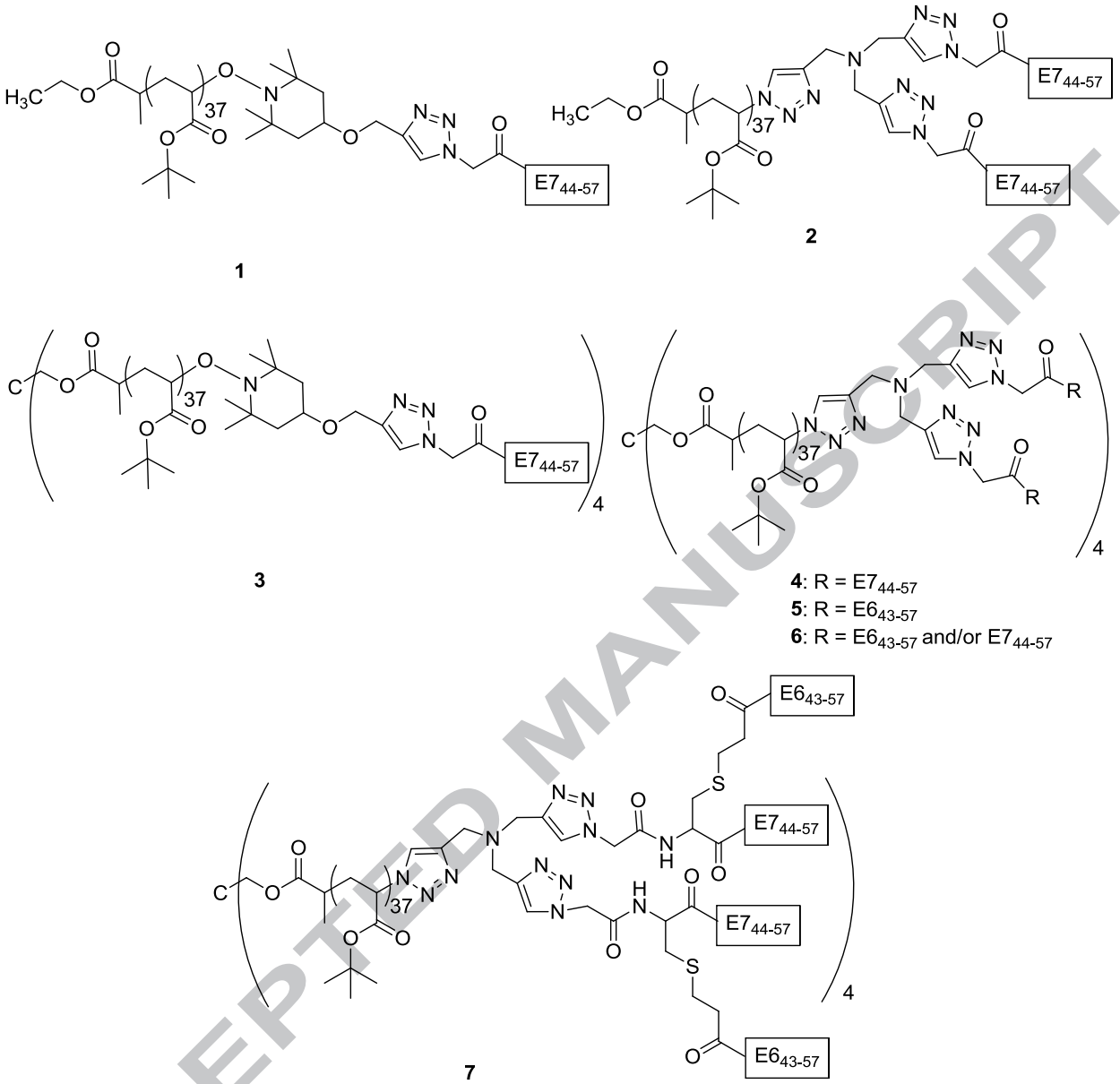


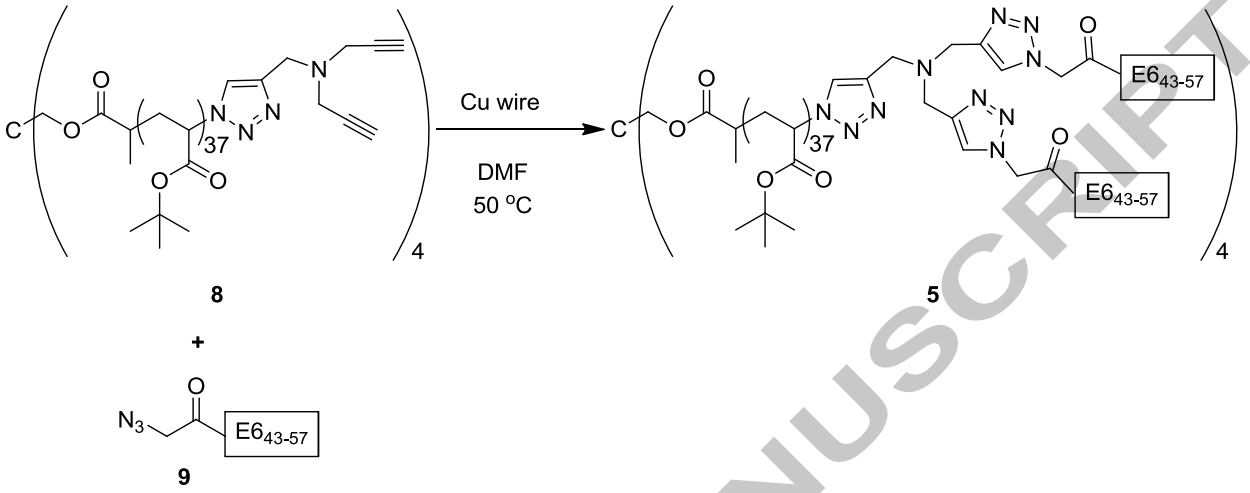
Figure 1. Vaccine candidates **1-7**.

2. Results and Discussion

Synthesis and characterization of polymer-peptide conjugates

Vaccine candidates **1-4** (Figure 1) were synthesized as described previously.[14] Vaccine candidate **5** was synthesized through CuAAC between the alkyne-functionalized poly(*t*-butyl

acrylate) (**8**)[11] and the azido acetic acid derivative of $E6_{43-57}$ epitope (**9**, N_3CH_2CO-QLLRREVYDFAFRDL-NH_2)[20] (Scheme 1).



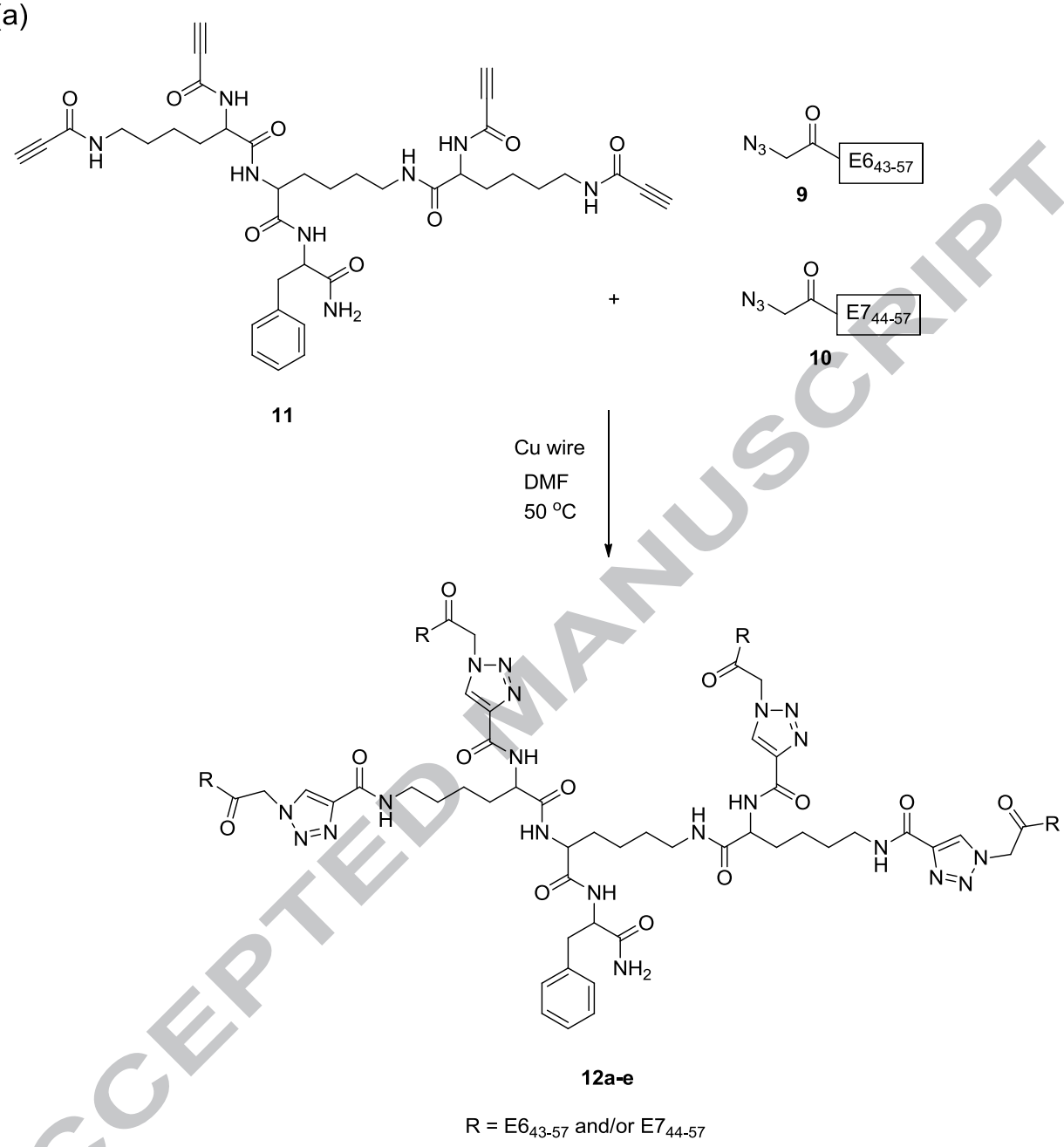
Scheme 1. Synthesis of conjugate **5**.

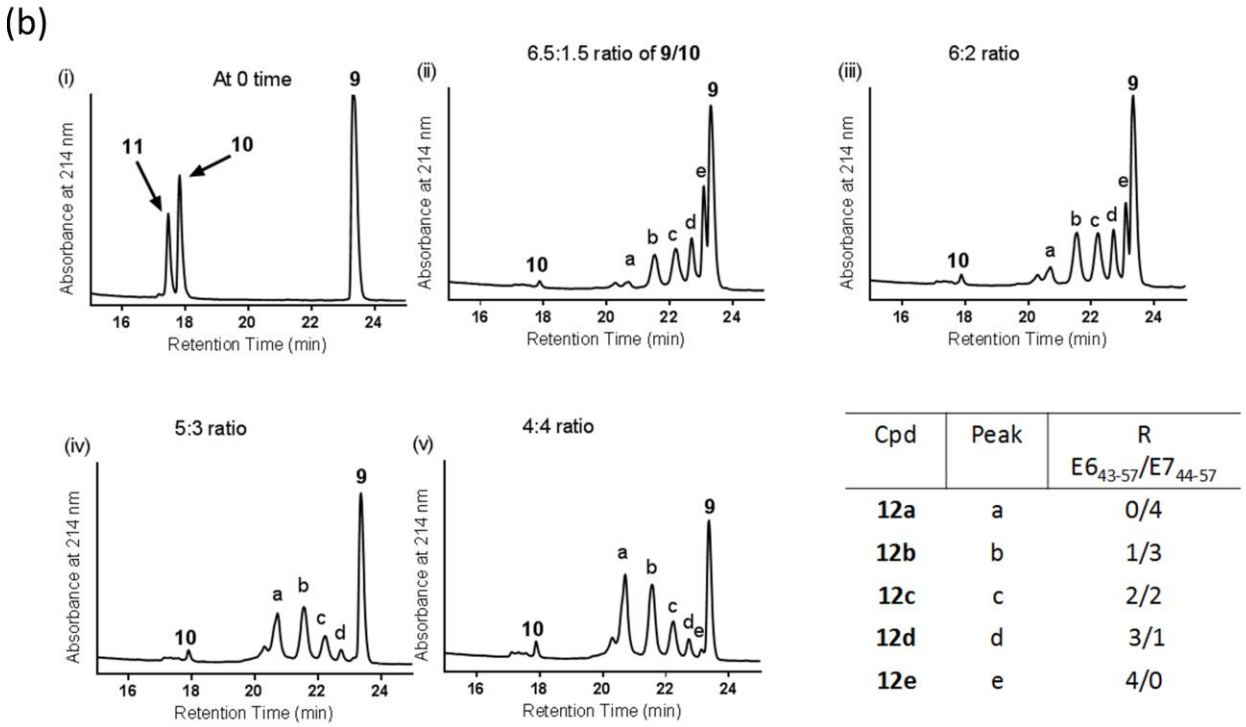
As the synthesis and evaluation of multiantigenic peptide-polymer carrying both E6 and E7 derived epitopes is one of our main purposes in this work, and the N-terminus conjugation of them is required for their activity,[11] the application of appropriate conjugation strategy is crucial for production of desired multicomponent vaccine candidates. Conjugation of peptides, became a popular approach for the synthesis of chemically engineered biomolecules for various biological applications.[21] Peptides ligation is a smart solution to overcome the obstacles in obtaining large homogeneous peptides with more than 50 amino acids by using solid phase peptide synthesis (SPPS).[22] Many peptide ligation techniques were revealed for the conjugation of two peptides via the binding of amino (N) terminal of one of the peptides to the carboxy (C) terminal of the other one.[23] However, a very few number of research focused on peptide conjugation through their N-terminals. Johnson et al. coupled two copies of an unprotected erythropoietin receptor agonist peptide from their N-terminals by employing an amine-reactive difunctional polyethylene glycol

(PEG) molecule, succinimidyl propionate, to form a linear polymer molecule.[24] The presence of an amine group in the side chain of a single lysine within the peptide sequence led to formation of undesired bindings and difficulties in purifications. Szewczuk and co-workers carried out successfully an N-terminal N-terminal dimerization of a peptide fragment on SPPS by using polyethylene glycols spacer; however, the use of fully protected peptides was required.[25,26] Liskamp and his team were able to conjugate 3 different unprotected cyclic peptides to trialkynes scaffold through copper-catalyzed alkyne-azide cycloaddition (CuAAC) reaction; however, the control of substitution ratio was difficult.[27]

Conjugation of dendrimer **8** with a mixture of azides **9** and **10** (N_3CH_2CO-QAEPDRAHYNIVTF-NH_2)[11, 28] via CuAAC was expected to produce a mixture of constructs with different contents of $E6_{43-57}$ and $E7_{44-57}$ epitopes conjugated to dendrimer, and a model experiment was designed to optimize the conjugation conditions. A mixture of azides **9** and **10** was added in different ratios to a model tetra-alkynes peptide (**11**) in the presence of copper wire (Figure 2a). Treatment of tetra-alkynes (**11**) with 6 equivalents of **9** and 2 equivalents of **10** (ratio 6:2) was found to be optimal to obtain compound **6** possessing the most equal contents of both epitopes (Figure 2b and Table 1). Thus, epitopes **9** and **10** (in molar ratio 6:2) were conjugated to dendrimer **8** to produce multiepitope construct **6** (Scheme 2).

(a)



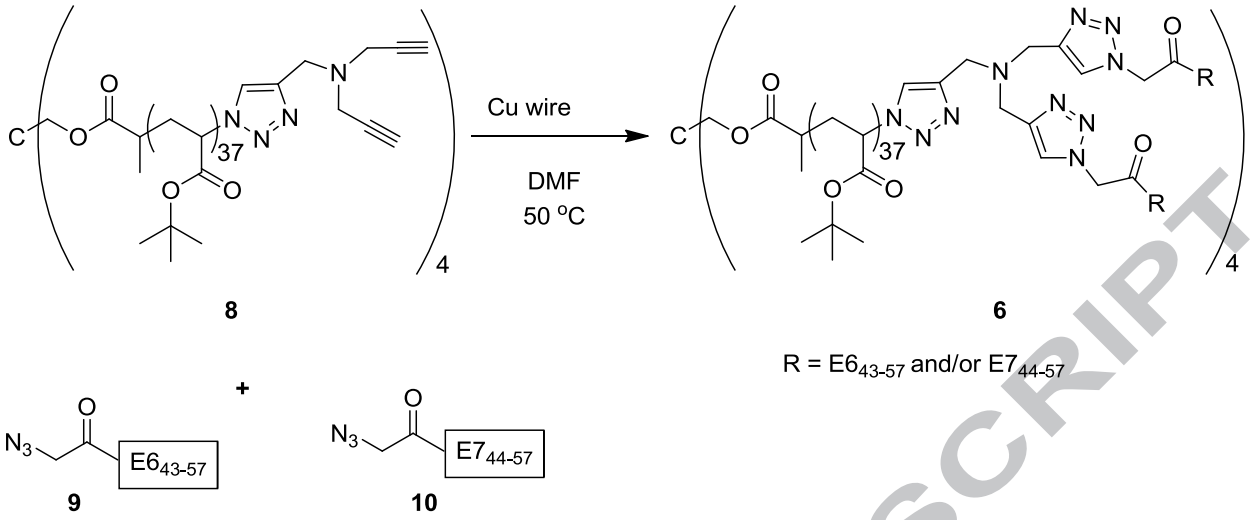


Cpd	Peak	R $E6_{43-57}/E7_{44-57}$
12a	a	0/4
12b	b	1/3
12c	c	2/2
12d	d	3/1
12e	e	4/0

Figure 2. (a) Synthesis of model compound **12**. (b) Results of monitoring the CuAAC reaction between the model tetra-alkynes **11** and azides **9** and **10**. (8 equivalents in total of azides were used for conjugation in different ratio aspects). (i) The reaction at 0 time. The ratio of **9**:**10** was: (ii) 6.5:1.5; (iii) 6:2; (iv) 5:3; (v) 4:4.

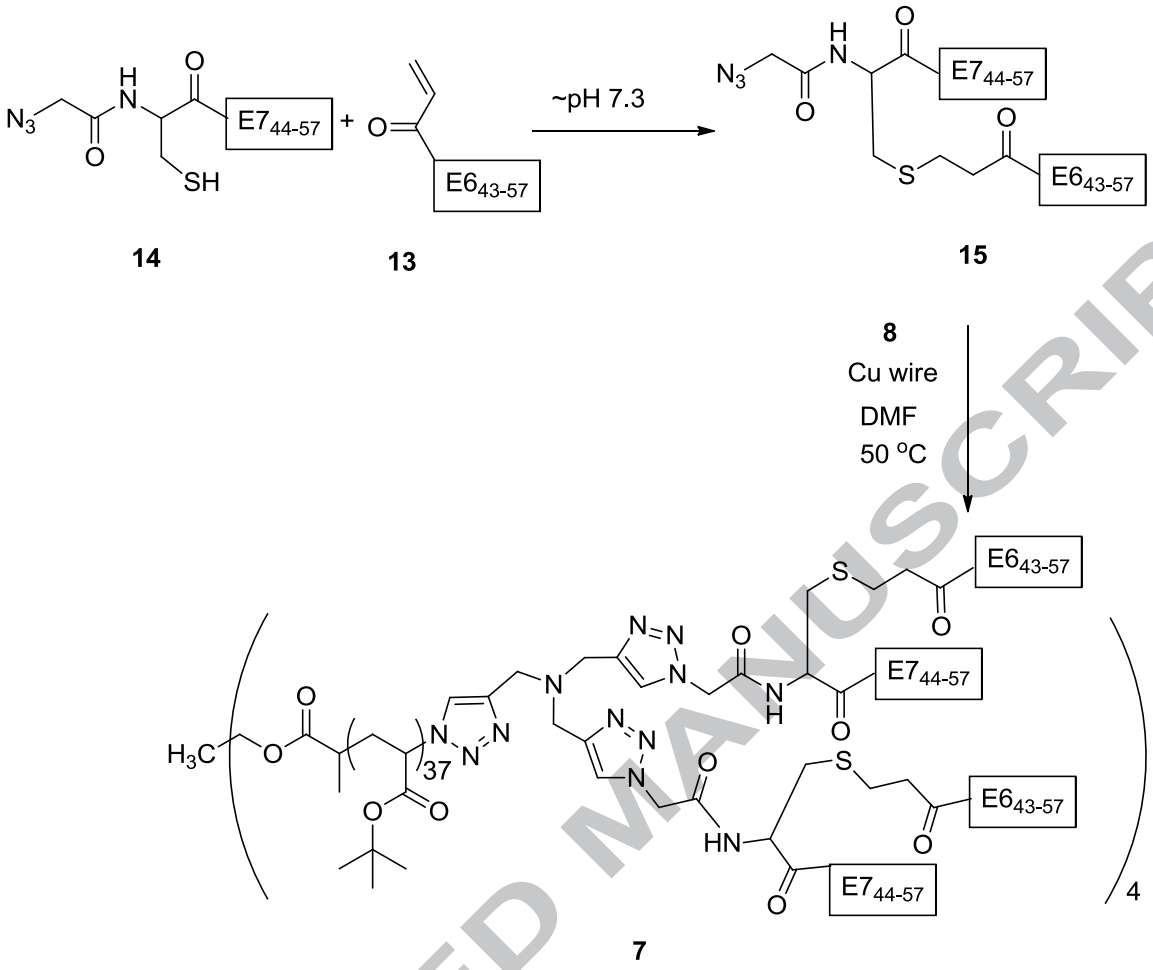
Table 1. The yield of the conjugation between azides **9** and **10** and alkyne **11** based on the HPLC traces integration.

Equivalents ratio of **9** : **10**	Products				
	0/4	1/3	2/2	3/1	4/0
	12a	**12b**	**12c**	**12d**	**12e**
4:4	39%	34%	14%	5%	2%
5:3	33%	39%	16%	5%	1%
6:2	8%	28%	24%	17%	21%
6.5:1.5	3%	21%	23%	18%	33%



Scheme 2. Synthesis of conjugate **6**.

Despite the optimization of the production of compound **6**, this vaccine candidate produced a rather poorly controlled mixture of several conjugates. Thus, to achieve a well-defined vaccine candidate in a reproducible manner, a recently developed double conjugation strategy was proposed.[20] First, an acryloyl derivative of E6$_{43-57}$ (**13**) was conjugated to the mercapto-azide derivative of E7$_{44-57}$ (**14**) at pH ~7.3 to produce compound **15** in 90% yield (Scheme 3).[20] The azide **15** was then conjugated to the dendrimer **8** in the presence of Cu wire to give **7** (Scheme 3).



Scheme 3. Synthesis of conjugate **7**.

Vaccine candidates **1-7** were all self-assembled into particles via the solvent replacement method (DMF/water).[11] Excess unreacted peptides, copper, and organic solvents were removed by extensive dialysis against water.

Conjugation efficiency was proven by elemental analysis, as previously reported.[11, 16] A significant increase in the nitrogen/carbon ratio (N/C) (Table 2), compared to that of polymer (N/C = 0.004 for **L1** (one arm polymer) and **S4** (4 arms polymer); N/C = 0.02 for **L2** (one arm polymer with two alkyne groups) and **D8** (4 arms polymer with 8 alkyne groups)), was apparent, due to the presence of nitrogen-rich peptide in the conjugates. The calculation of the substitution

ratio of either $E6_{43-57}$ and/or $E7_{44-57}$ conjugated to each polymer was based on the comparison of the observed and theoretical N/C ratio for the conjugates, as reported previously.[11, 14] The obtained substitution ratio was high to excellent (Table 2).

Table 2. Substitution ratio for conjugates **1-7** calculated based on nitrogen/carbon ratio (N/C, found by elemental analysis).

Compound	N/C	Substitution Ratio
1	0.082	96%
2	0.143	98%
3	0.078	88%
4	0.124	76%
5	0.136	79%
6	0.141	87%
7	0.196	90%

To prepare samples for *in vivo* experiments, all polymer-peptide conjugates were formulated in phosphate buffered saline (PBS). At pH 7.4, conjugates **1-7** formed a milky suspension in PBS. The particle size of each polymer-peptide conjugate was analyzed by laser particle size analysis (Table 3).

Table 3. Average particle size of the final constructs **1-7**.

Compound	Diameter (μm)	Span
1	17	2
2	13	1.7
3	13	1.1
4	14	1.7
5	-[1]	-[1]
6	143	1.5
7	106	1.7

[1] Highly polydisperse precipitating aggregates (laser diffraction could not determine their sizes).

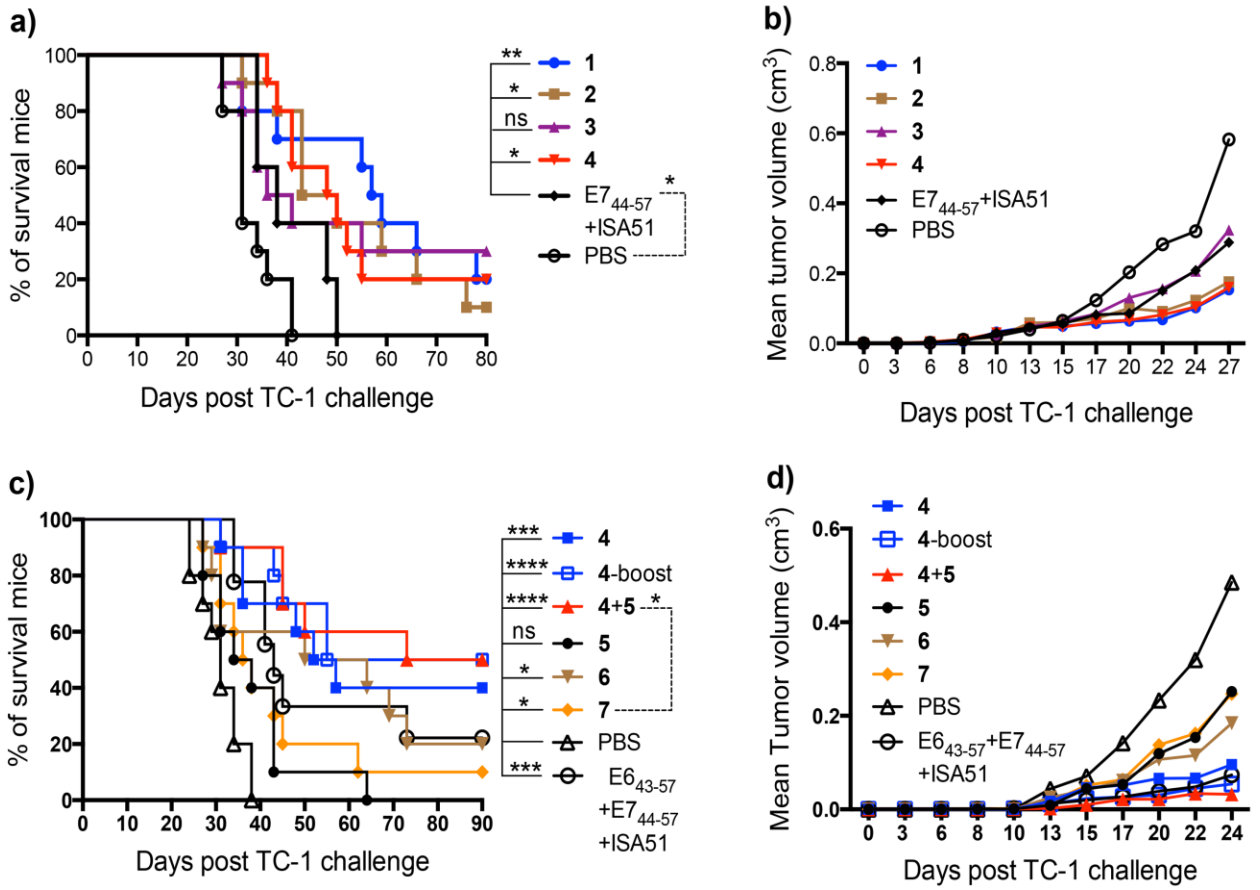


Figure 3. *In vivo* E6/E7-expressing TC-1 tumor treatment experiments. C57Bl/6 mice (10 per group) were inoculated subcutaneously in the flank with 2×10^5 TC-1 cells/mouse (day 0), and injected with (a) compound **1**-**4** and controls on day 7. Survival rate was monitored and time to death plotted on a Kaplan-Meier survival curve. Mice were sacrificed when the tumor volume became greater than 1 cm^3 or started bleeding. The survival rate of each group was compared to the positive control ($E7_{44-57}$ + ISA51), and was analyzed by the long-rank test (ns = $p > 0.05$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$). (b) Mean tumor volume (cm^3) of mice vaccinated with compounds **1**-**4** and controls was monitored and plotted until the first mouse from any group reached the endpoint (day 27). (c) Kaplan-Meier survival curve for mice vaccinated with compounds **4**-**7** and controls. The survival rate of each group was compared to the negative control (PBS). (d) Mean tumor volume (cm^3) of mice vaccinated with compound **4**-**7** and controls was monitored and plotted until the first mouse from any group reached the endpoint (day 24).

In vivo tumor treatments

Previous studies have shown that the peptide epitope $E7_{44-57}$ conjugated with four different polyacrylate polymer analogs (**1-4**) was able to generate a robust therapeutic immunity against HPV-16 E6/E7-expressing TC-1 tumors after a single-dose immunization on day 3 post tumor

challenge.[12, 14] To further test the antitumor efficacy of polymer-peptide conjugates **1**-**4** to control well-established, solid tumors, mice (10 per group) were vaccinated on day 7 (instead of day 3) after tumor cell implantation. All mice received a single-dose treatment without additional boosts. The positive control group received $E7_{44-57}$ emulsified in 1:1 Montanide ISA51/PBS (IFA-like adjuvant) ($E7_{44-57}$ + ISA51).[29] The untreated group was administered PBS. Tumor-bearing mice immunized with compounds **1**-**4** and the positive control showed significantly prolonged survival and slower tumor growth when compared with the untreated PBS group (Figure 3a; b; S1). The antitumor activity elicited by compound **3** was similar to that of positive controls (as observed in previous studies).[11, 14] In contrast, mice treated with compounds **1**, **2** and **4** demonstrated a significantly enhanced therapeutic effect compared to positive controls ($p < 0.05$ for compounds **2** and **4**; $p < 0.01$ for compound **1**). Furthermore, at day 27 post tumor challenge, the average tumor size in mice immunized with vaccine candidates **1**, **2** and **4** were smaller than those in mice immunized with either vaccine candidate **3** or the positive control (Figure 3b). Interestingly, in all groups treated with compounds **1-4**, at least one mouse survived 80 days post tumor implantation, while all mice were moribund in the group treated with commercial adjuvant (positive control) by day 50. Unfortunately, the efficacy of vaccine candidates **1-4** was significantly reduced in established tumors compared to the results from small, "young" tumor challenges, as reported previously.[14]

In order to enhance the therapeutic efficacy of these polyacrylate-peptide conjugates, additional epitope $E6_{43-57}$ derived from HPV-16 E6 oncoprotein was conjugated to 8-arm dendritic polymer to produce compounds **5**-**7** and administered into mice alone (**6**, **7**) or as a physical mixture (i.e. **4** + **5**). Compound **4** was administered using both single-dose and boost regimes. Peptides $E6_{43-57}$ and $E7_{44-57}$ emulsified in 1:1 Montanide ISA51/PBS ($E6_{43-57}$ + $E7_{44-57}$ + ISA51) were

used as positive controls. Mice were immunized with a single injection on day 7 post tumor challenge, except for compound **4**, where mice received additional boosts on day 14 and 21.

The Kaplan-Meier survival curve (Figure 3c) indicates that tumor-bearing mice vaccinated with compounds **6** and **7** had an improved survival rate ($p < 0.05$) compared to the untreated PBS group, while compounds **4**, **4**-boost, **4** + **5** and Montanide-adjuvanted epitopes exhibited an enhanced therapeutic efficacy ($p < 0.001$). Particularly, **4**-boost and the physical mixture **4** + **5** induced the most significant antitumor activity ($p < 0.0001$) of the above vaccine formulations, leading to five and four tumor-free mice, respectively, at the end of the challenge in each group (Figure S2), and ongoing as at 3 months after the initial challenge. Interestingly, the physical mixture of compounds **4** + **5**, which contained $E6_{43-57}$ and $E7_{44-57}$ epitopes, was more efficient than the same epitopes presented in conjugated form in compound **7**.

As shown in the tumor growth curve (Figure 3d), the mice that received a single-dose or boost regiment of compound **4** showed similar efficacy in tumor suppression on day 24. Furthermore, no significant difference was found in survival rate comparisons of single-dose versus boost regimes. Interestingly, although these polymer-based delivery systems were highly efficient in inducing humoral and cellular immunity after single dose administration,[11, 14, 16, 18] multiple administration of vaccine candidate **4**-boost did not significantly improve the efficacy of cellular immune responses.

Compound **5** alone did not induce a significant improvement in the survival rate (Figure 3c) or slow tumor growth in mice (Figure S2c), suggesting that the formation of large aggregates of compound **5** in solution may have diminished its potency to induce an immune response. Compounds **6** and **7**, which were the constructs possessing both E6/E7 epitopes conjugated to the

same polyacrylate dendrimer, produced much weaker anti-tumor activity compared to the physical mixture of two dendritic constructs containing E7 and E6 epitopes (**4** + **5** physical mixture). It has been considered that the uptake of particles by antigen presenting cells (APCs) plays a crucial role in the induction of immune responses for particle-based vaccines.[30, 31,32] Large particles usually have an efficient depot effect for continuous antigen release.[31, 33] We have proven that polyacrylate polymers conjugated to the E7 epitope formed microparticles (12-17 μm), which were able to be efficiently taken up by APCs (including macrophages and dendritic cells), as well as to activate $CD8^+$ and $CD4^+$ cells to elicit adequate cellular immune responses against E7-expressing tumors.[14] However, it has been reported that very large-sized particles ranging from 50-100 μm (which is larger than a typical APC) were least likely to be taken up by APCs, resulting in the generation of a low immune response.[31] Conjugates **6** and **7** formed large microparticles (143 μm and 106 μm, respectively), while construct **5** formed large, highly polydisperse aggregates (laser diffraction could not determine their sizes). Therefore, limited improvement in the efficacy upon incorporation of the E6 epitope into polymer-based vaccine might be related to the hydrophobic properties of the epitope, which cause extensive aggregation of the conjugates bearing it. Thus, the presence of $E7_{44-57}$ epitope was essential to produce a robust CTL response and these cellular immune responses were dependent on the size of particles; smaller particles induced stronger CTL responses than larger.

3. CONCLUSION

Considering the heterogeneity of the human immune response against any one given antigenic epitope, the incorporation of more than one epitope in a proposed vaccine delivery system is more likely to achieve consistent clinical success. A polymer-peptide conjugate

delivery system can bear multiple copies of the same peptide epitope in one construct and is able to generate potent therapeutic immunity when formulating conjugates in a simple physical mixture, without adding external adjuvant after a single-dose vaccination. Importantly, this delivery system resulted in the elimination of tumors in mice and tumor recurrence was not observed for 3 months after the initial challenge. Our finding suggests that this novel self-adjuvanting delivery system would provide a safe, efficient therapeutic vaccine that is effective for the broader population. However, it is important to note that polar properties of the epitopes may significantly influence the efficacy of such polymer-based vaccines, as hydrophobic epitopes may trigger the formation of large aggregates with a limited potency to deliver desired immune responses. To overcome formation of such aggregates, hydrophilic moieties, for instance polyethylene glycol or polyglutamic acid, could be incorporated to increase the hydrophilic character of the epitopes and consequently reduce the particle size of the final constructs.

4. Experimentals

Protected L-amino acids were purchased from Novabiochem (Läufelfingen, Switzerland) and Mimotopes (Melbourne, Australia). *p*MBHA 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). (Dimethylamino)-N,N-dimethyl(3H-[1,2,3]triazolo[4,5-b]pyridin-3-yloxy)-methanim-inium hexafluorophosphate (HATU) was purchased from Mimotopes (Melbourne, Australia). HPLC grade acetonitrile was obtained from Labscan (Bangkok, Thailand). 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 %), sodium azide (NaN_3, Aldrich, ≥ 99.5 %), 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 nitroxde was synthesized according the previously reported procedure.[34] All other reagents were obtained at the highest available purity from Sigma-Aldrich (Castle Hill, NSW, Australia). 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. ESI-MS was performed using a Perkin-Elmer-Sciex API3000 instrument with Analyst 1.4 software (Applied Biosystems/MDS Sciex, Toronto, Canada). Analytical RP-HPLC was performed using Shimadzu (Kyoto, Japan) instrumentation (DGU-20A5, LC-20AB, SIL-20ACHT, SPD-M10AVP) with a 1 mL min^{-1} flow rate and detection at 214 nm and/or evaporative light scattering detector (ELSD). 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 x 250 mm) or a Vydac analytical C18 column (218TP54; 5 μm, 4.6 mm x 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 5-20 mL/min flow rate, with detection at 230 nm. Separations were performed with solvent A and solvent B on a Vydac preparative C18 column (218TP1022; 10 μm, 22 mm x 250 mm), Vydac semi-preparative C18 column (218TP510; 5 μm, 10 mm x 250 mm) or Vydac semi-preparative C4 column (214TP510; 5 μm, 10 mm x 250 mm). The particle size distribution and measurement of the average particle

size were analyzed using a laser particle size analyzer Mastersizer 2000 (Malvern Instruments, England, UK). Multiplicate measurements were performed, and the average particle size was represented. The level of copper traces presented in the polymer-peptide conjugates was precisely determined by inductively-coupled plasma optical emission spectrometer (Perkin-Elmer Optima 8300DV (Dual View), USA). The copper content of conjugates **1-7** was below 100 ppb (the recommended health standard level of copper is ≤ 15 ppm).[35]

Synthesis of $E7_{44-57}$ Peptide[11]

$E7_{44-57}$ epitope (QAEPDRAHYNIVTF) was synthesized by manual stepwise SPPS on *p*MBHA resin (substitution ratio: 0.45 mmol/g, 0.2 mmol scale, 0.44g) using HATU/DIPEA Boc-chemistry. Boc-amino acids were preactivated for 1 min prior to their addition to the resin. The activation of amino acids was achieved by dissolving Boc-amino acid (0.84 mmol, 4.2 equiv.), in 0.5 M HATU/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.). Coupling cycle consisted of Boc deprotection with neat TFA (2 × 1 min, at rt), a 1 min DMF flow-wash, followed by coupling with 4.2 equiv. of preactivated Boc-amino acids (2 × 1 h). 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*-cresol as a scavenger. 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 C18 column with a solvent gradient of 15-35% solvent B over 20

min. HPLC analysis (C18 column): t_R = 16.7 min, purity > 95%. Yield: 27%. ESI-MS: *m/z* 1661.1 (calc 1660.8) $[M+H]^+$; 830.8 (calc 830.9) $[M+2H]^{2+}$; MW 1659.8.

General procedure of manual stepwise SPPS on rink amide MBHA resin - Fmoc-chemistry

Peptides were synthesized by manual stepwise SPPS on rink amide MBHA resin (substitution ratio: 0.60 mmol/g, 0.2 mmol scale, 0.33 g) using HATU/DIPEA Fmoc-chemistry. Amino acid activation was achieved by dissolving Fmoc-amino acid (0.84 mmol, 4.2 equiv.), in 0.5 M HATU/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 × 1 h). Upon completion of synthesis, the resin was washed with DMF, DCM, and MeOH, then dried (vacuum desiccator). The cleavage of peptide 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. After purification of peptides, the yields of purification were calculated based on the mass of peptide as a TFA salt.

Synthesis of $E6_{43-57}$ peptide[20]

$E6_{43-57}$ epitope (QLLRREVYDFAFRDL; $E6_{43-57}$) was synthesized following the general procedure Fmoc-SPPS described above. The crude product was purified by preparative RP-HPLC on C-18 column with solvent gradient 25-45% solvent B over 20 min. HPLC analysis (C-18 column): t_R = 19.8 min, purity > 95%. Yield: 84%. ESI-MS: m/z 970.9 (calc 971.1) $[M+2H]^{2+}$; 647.8 (calc 647.7) $[M+3H]^{3+}$; MW 1940.2.

Synthesis of N-terminus $E6_{43-57}$-azide (9)[20]

N-terminus $E6_{43-57}$-azide peptide epitope (**9**, N_3CH_2CO-QLLRREVYDFAFRDL) was synthesized following the Fmoc-SPPS procedure. The attachment of azidoacetic acid (4.2 equiv.) was achieved using HATU (3 equiv.)/DIPEA (4.2 equiv.) at room temperature (2 × 1 h) and the reaction mixture was covered and protected from light with aluminum foil. The crude product was purified by preparative RP-HPLC on C-18 column with solvent gradient 35-55% solvent B over 20 min. HPLC analysis (C-18 column): t_R = 23.3 min, purity > 95%. Yield: 50%. ESI-MS: m/z 1012.8 (calc 1012.6) $[M+2H]^{2+}$; 675.7 (calc 675.4) $[M+3H]^{3+}$; MW 2023.

Synthesis of N-terminus $E7_{44-57}$-azide (10)[11]

N-terminus $E7_{44-57}$-azide peptide epitope (**10**, N_3CH_2CO-QAEPDRAHYNIVTF) was synthesized following the general procedure by Fmoc-SPPS. 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. The attachment of azidoacetic acid (4.2 equiv.) was achieved using HATU (3 equiv.)/DIPEA (4.2 equiv.) at room temperature (2 × 1 h) and the reaction mixture was covered and protected from light with aluminum foil. The crude product was purified by preparative RP-HPLC on C-18 column with solvent gradient 15-35% solvent B over 20 min. HPLC analysis (C-18 column): t_R = 17.9 min, purity > 95%. Yield: 80%. ESI-MS: m/z 1744.4 (calc 1743.9) $[M+H]^+$; 872.2 (calc 872.9) $[M+2H]^{2+}$; MW 1742.85.

Synthesis of Dendrimer- $E6_{43-57}$ (5)

A mixture of **9** (11.4 mg, 4.8 μmol, 16 equiv.) and **8**[16] (5.8 mg, 0.3 μmol, 1 equiv.) was dissolved in DMF (1 mL), and a copper wire (60 mg) was added. The air in the reaction mixture was removed by nitrogen bubbling. The reaction mixture was covered and protected from light with aluminum foil and stirred at 50 °C under nitrogen for 11 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 using presoaked and rinsed dialysis bags. The final formulation was self-assembled into big particles. The laser diffraction method was not able to determine their size after formulating the conjugate in PBS.

Synthesis of model tetra-alkynes (11)

The Model tetra-alkynes (**11**) was synthesized following the general Fmoc-SPPS procedure. The attachment of propiolic acid (8 equiv.) was achieved using EEDQ (8 equiv.) at room temperature (2 × 2 h). The crude product was purified by preparative RP-HPLC on C-18 column with solvent gradient 15-35% solvent B over 20 min. HPLC analysis (C-18 column): t_R = 17.4 min, purity > 95%. Yield: 35%. ESI-MS: m/z 757.6 (calc 757.9) $[M+H]^+$; MW 756.8.

Synthesis of random multiantigenic-model tetra-alkynes (12a-e)

A mixture of **9** (2.1 mg, 0.9 μmol, 6 equiv.), **10** (0.6 mg, 0.3 μmol, 2 equiv.) and the model tetra-alkynes (**11**, 0.1 mg, 0.15 μmol, 1 equiv.) was dissolved in DMF (0.5 mL), and a copper wire (40 mg) was added. The air in the reaction mixture was removed by nitrogen bubbling. The reaction mixture was covered and protected from light with aluminum foil and stirred at 50 °C under nitrogen. The progress of reaction was monitored by analytical HPLC (C-18 column) and ESI-MS until the tetra-alkynes **11** was completely consumed after 1 h. Compounds **12a-e** were analyzed by ESI-MS.

12a: m/z 1547.2 (calc 1546.7) $[M+5H]^{5+}$; 1289.4 (calc 1289.1) $[M+6H]^{6+}$; 1105.4 (calc 1105.0) $[M+7H]^{7+}$; MW 7728.3.

12b: m/z 2003.7 (calc 2003.2) $[M+4H]^{4+}$; 1603.1 (calc 1602.7) $[M+5H]^{5+}$; 1336.4 (calc 1335.8) $[M+6H]^{6+}$; 1145.3 (calc 1145.1) $[M+7H]^{7+}$; MW 8008.7.

12c: m/z 1659.1 (calc 1658.8) $[M+5H]^{5+}$; 1383.5 (calc 1382.5) $[M+6H]^{6+}$; 1185.6 (calc 1185.2) $[M+7H]^{7+}$; MW 8289.1.

12d: m/z 1715.6 (calc 1714.9) $[M+5H]^{5+}$; 1429.3 (calc 1429.3) $[M+6H]^{6+}$; 1225.6 (calc 1225.2) $[M+7H]^{7+}$; 1072.4 (calc 1072.2) $[M+8H]^{8+}$; MW 8569.5.

12e: m/z 2213.9 (calc 2213.5) $[M+4H]^{4+}$; 1772.0 (calc 1771.0) $[M+5H]^{5+}$; 1476.4 (calc 1476.0) $[M+6H]^{6+}$; 1265.4 (calc 1265.3) $[M+7H]^{7+}$; 1107.7 (calc 1107.2) $[M+8H]^{8+}$; 984.9 (calc 984.3) $[M+9H]^{9+}$ MW 8849.9.

Synthesis of dendrimer- $E6_{43-57}/E7_{44-57}$ (6)

A mixture of **9** (8.5 mg, 3.6 μmol, 12 equiv.), **10** (2.4 mg, 1.2 μmol, 4 equiv.) and **8** (5.8 mg, 0.3 μmol, 1 equiv.) was dissolved in DMF (1 mL), and a copper wire (60 mg) was added. The air in the reaction mixture was removed by nitrogen bubbling. The reaction mixture was covered and protected from light with aluminum foil and stirred at 50 °C under nitrogen for 10 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 using presoaked and rinsed dialysis bags (Pierce Snakeskin, MWCO 3K). The final formulation was self-assembled into particles with diameters above 143 μm as observed by laser diffraction method (Mastersizer 2000, Malvern Instruments, UK) after formulating the conjugate in PBS.

Synthesis of 13[20]

N-terminal acryloyl derivative of $E6_{43-57}$ peptide epitope (**13**, CH_2=CHCO-QLLRREVYDFAFRDL) was synthesized following the general Fmoc-SPPS procedure. The attachment of acrylate (4.2 equiv.) was achieved using HATU (4 equiv.)/DIPEA (4.2 equiv.) at room temperature (2 × 1 h). The crude product was purified by preparative RP-HPLC on C-18 column with solvent gradient 35-55% solvent B over 20 min. HPLC analysis (C-18 column): t_R = 22.7 min, purity > 95%. Yield: 33%. ESI-MS: m/z 998.2 (calc 998.1) $[M+2H]^{2+}$; 665.8 (calc 665.8) $[M+3H]^{3+}$; MW 1994.3.

Synthesis of 14[20]

N-terminus $E7_{44-57}$ mercapto-azie peptide epitope (**14**, N_3CH_2CO-CQAEPDRAHYNIVTF-NH_2) was synthesized following the general Fmoc-SPPS 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. The attachment of azidoacetic acid (4.2 equiv.) was achieved using HATU (3 equiv.)/DIPEA (4.2 equiv.) at room temperature (2 × 1 h) and the reaction mixture was covered and protected from light with aluminum foil. The crude product was purified by preparative RP-HPLC on C-18 column with solvent gradient 20-40% solvent B over 20 min. HPLC analysis (C-18 column): t_R = 18.3 min, purity > 95%. Yield: 72%. ESI-MS: m/z 924.0 (calc 924.0) $[M+2H]^{2+}$; MW 1846.

Synthesis of multiantigenic peptide azide (15) through mercapto-acrylate conjugation[20]

A mixture of the two peptide epitopes acryloyl derivative of $E6_{43-57}$ (**13**, 7.2 mg, 3 μmol, 1.0 equiv.) and $E7_{44-57}$ mercapto-azide (**14**, 13.4 mg, 6 μmol, 2 equiv.) was dissolved in a guanidine buffer at ~pH 7.3. The reaction mixture was incubated at 37 °C for 48 h. The progress of reaction was monitored by analytical HPLC until the acryloyl derivative **13** was completely consumed. The reaction mixture was purified by using semi-preparative HPLC C-18 column (20-60% solvent B over 60 min). After lyophilization the pure azide derivative **15** was obtained as an amorphous white powder. The product was detected using analytical HPLC analysis (C-4 column), t_R = 21.8 min, purity > 97% and (C18 column), t_R = 21.4 min, purity > 95%.

Yield: (12.2 mg, 90%). ESI-MS: m/z 1921.5 (calc 1921.1) $[M+2H]^{2+}$; 1281.3 (calc 1281.1) $[M+3H]^{3+}$; 961.2 (calc 961.1) $[M+4H]^{4+}$; 768.9 (calc 769.1) $[M+5H]^{5+}$; MW 3840.3.

Synthesis of dendrimer- $E6_{43-57}$-$E7_{44-57}$ (7)

A mixture of azide derivative **15** (21.2 mg, 4.8 μmol, 16 equiv.) and **8** (5.8 mg, 0.3 μmol, 1

equiv.) was dissolved in DMF (1 mL), and a copper wire (60 mg) was added. The air in the reaction mixture was removed by nitrogen bubbling. The reaction mixture was covered and protected from light with aluminum foil and stirred at 50 °C under nitrogen for 15 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 using presoaked and rinsed dialysis bags. The resulting particles were self-assembled into particles with diameters above 106 μm as observed by laser diffraction method after formulating the conjugate in PBS.

Synthesis of Vaccine Candidates 1-4

Synthesis of polymer-peptide conjugates **1-4** was performed as previously described[14] and produced virtually identical products. Briefly, alkyne-functionalized poly(*t*-butyl acrylate) polymers with different number of alkyne moieties (1, 2, 4 and 8 alkyne moieties) were synthesized by atom-transfer radical polymerization (ATRP) and end-group functionalization.[14, 16] The $E7_{44-57}$ azide (**10**) epitope was conjugated to different polymers via CuAAC "click" reaction to produce polymer-peptide conjugates **1-4**.

Particle size measurement

The particle size and size distribution (span) of conjugate was measured by laser diffraction method (Mastersizer 2000, Malvern Instruments, UK) after formulating the conjugate in PBS. The geometrical diameter was presented as volume median diameter (D50%). The size distribution (span) value was defined by a standard formula: Span = (D90%-D10%)/D50%. Where D10%, D50% and D90% are the particle diameters determined at the 10^{th}, 50^{th} and 90^{th} percentile of the undersized particle distribution curve.[36]

Biological Assay

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.[37] 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

Experiment 1: Groups of C57Bl/6 mice (10 per group) were first challenged subcutaneously with 2×10^5 TC-1 tumor cells/mouse suspended in 100 μL 1 × PBS in the right flank (day 0), and rested for 7 days to allow for tumor formation. On the day 7 after tumor inoculation, the mice were received a single-dose subcutaneous immunization on each side of the tail base with 50 μg (100 μg in total) of polymer-peptide conjugates (**1-4**) in a total volume of 100 μL 1 × PBS or control formulations. Positive control received 30 μg of $E7_{44-57}$ emulsified in a total volume of 100 μL of Montanide ISA51 (Seppic, France)/PBS (1:1, v/v). Negative control was received 100 μL 1 × PBS. The size of the tumor was monitored every second days using calipers (and palpation) for 80 days.[38, 39] The tumor volume was calculated using the formula:[39]

Tumor volume (cm^3) = $\pi \times$ [largest diameter × (perpendicular diameter)^2]/6.

The mice were euthanized when tumor greater than 1 cm^3 or started bleeding to avoid unnecessary suffering.

Experiment 2: Compounds **4-7** were tested in the same manner as described above. The

size of the tumor was monitored every second days for 90 days. For the physical mixture (**4** + **5**), the mice were received a single-dose subcutaneous immunization on each side of the tail base with a mixture of 50 μg of each compound in a total volume of 100 μL 1 × PBS. The physical mixture (**4** + **5**) was prepared as follow: compounds **4** and **5** were self-assembled into particles separately via the solvent replacement method (DMF/water). Excess unreacted peptides, copper, and organic solvents were removed by extensive dialysis against water. Two equal amounts of both aliquots of **4** and **5** were mixed together.

Positive control received 15 μg of $E7_{44-57}$ and 15 μg of $E6_{43-57}$ emulsified in a total volume of 100 μL of Montanide ISA51 (Seppic, France)/PBS (1:1, v/v). Negative control was received 100 μL of PBS. Compound **4** was also administered using "boost" regiment. Thus, mice in the group **4**-boost received first dose on day 7 and additional boosts on day 14 and 21. All doses included 100 μg of compound **4**.

Statistical Analysis

All data were analyzed using GraphPad Prism 6 software. Kaplan-Meier survival curves for tumor treatment experiments were applied. Statistical analysis between groups was determined using the log-rank (Mantel-Cox) test for survival experiments. Differences were considered statistically significant for $p < 0.05$.

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Supporting Information

Supplementary data associated with this article can be found, in the online version, at XXXXXXXXX. These data include figures related to animal study, Structures, HPLC profiles and MS spectra.

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Graphical Abstract