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KEYWORDS: peptide-based subunit vaccine, human papillomavirus, therapeutic cancer vaccine, polyacrylate, self-adjuvanting, cytotoxic T lymphocyte (CTL) response, cervical cancer

ABSTRACT

Vaccination can provides a safe alternative to classical chemotherapy by using the body's natural defense mechanisms to create a potent immune response against tumor cells. Peptide-based therapeutic vaccines against human papillomavirus (HPV)-related cancers are usually designed to elicit cytotoxic T cell responses by targeting the HPV-16 E7 oncoprotein. However, peptides alone lack immunogenicity and an additional adjuvant or external delivery system is required. In this study, we developed new polymer-peptide conjugates to create an efficient self-adjuvanting system for peptide-based therapeutic vaccines. These conjugates reduced tumor growth and eradicated E7-positive TC-1 tumors in mice after a "single shot" immunization, without help from an external adjuvant. The new conjugates had a significantly higher anticancer efficacy than the antigen formulation that included a commercial adjuvant. Furthermore, the polymer-peptide conjugates were promptly taken up by antigen presenting cells, including dendritic cells and macrophages, and efficiently activated $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T lymphocyte cells. Thus, polyacrylate polymer conjugated to synthetic peptide(s) could serve as an efficient and safe self-adjuvanting delivery system for the induction of antigen-specific immune responses with therapeutic anti-cancer activity.

INTRODUCTION

Vaccines are one of the most cost-effective public health interventions, and offer a promising strategy for the treatment of cancer.[1] The development of safe and effective therapeutic cancer vaccines is an urgent and challenging medical need. Cervical cancer is the second most common cause of cancer death in women worldwide and results from infection with human papillomavirus (HPV), most commonly HPV type 16 (HPV-16).[2] The HPV-16 E7 oncoprotein is required for tumorigenesis and maintenance of tumor growth, thus it has been used extensively in the development of therapeutic vaccines to elicit cytotoxic T cell responses (cellular immunity) against HPV-related cancers.[3]

Peptide-based vaccines are highly promising for the development of therapeutic HPV vaccines because of their safety, stability, and ease of production.[4] The use of a whole protein vaccine is unsafe because HPV oncoproteins can induce genomic instability in normal human cells. Instead, peptide-based vaccines use a minimal non-oncogenic epitope to stimulate an antigen-specific immune response. In general, peptides alone are non-immunogenic. In order to induce adequate immune responses for vaccines composed of a synthetic peptide or subunit antigen, an appropriate adjuvant (immunostimulant) is required to produce the desired immune response.[4] To date, a limited number of adjuvants have been approved for human use: alum is the only vaccine adjuvant that is approved worldwide. However, alum only enhances antibody-mediated immune responses and fails to stimulate cell-mediated immunity.[5, 6] In contrast, microorganism-derived experimental adjuvants are often highly immunogenic but toxic.[5] Therefore, the discovery and development of novel adjuvants or self-adjuvanting delivery systems without adverse toxicity is a key focus in the field of peptide-based vaccines.

Previous work in our lab has led to the development of polymer-peptide conjugates as self-adjuvanting delivery systems for peptide antigens. We have demonstrated the ability of B cell epitopes conjugated to the periphery of polyacrylate amphiphilic dendrimers to act as a self-adjuvanting vaccine and induce protective (humoral) immunity against Group A Streptococcus (*Streptococcus pyogenes* or GAS) infection.[7-9] While a large number of experimental self-adjuvanting systems and adjuvants can induce humoral immunity, the induction of cellular immunity with a therapeutic anti-tumor effect is particularly challenging.[10] There has been considerable interest in developing potent and efficient peptide-based delivery systems to stimulate a cellular immune response without the use of an external adjuvant.[11] Recently, we also reported that a 4-arm star polymer-based delivery system could be used in the design of peptide-based vaccine to reduce and/or eradicate E7-positive tumor cells in a mouse model. Among the tested epitopes, only **8Q$_{min}$** ($E7_{44-57}$, QAEPDRAHYNIVTF) conjugated to a 4-arm star polyacrylate polymer was able to reduce tumor growth in all tested mice and eradicate E7-positive TC-1 tumors in 40% of tested animals.[12]

In this study, we aimed to assess the ability of a variety of polyacrylate polymers to induce anti-tumor immunity in mice. All vaccine candidates (Scheme 1) included the peptide epitope **8Q$_{min}$** derived from the HPV-16 E7 oncoprotein as an antigen because it contains a CTL epitope ($CD8^+$, cytotoxic T lymphocytes) and a T-helper cell epitope ($CD4^+$) (Figure 1).[13] The **8Q$_{min}$** antigenic epitope was conjugated to four different delivery systems: a linear poly(*t*-butyl acrylate) polymer **1**, a branched linear polymer **2**, a 4-arm star polymer **3** and a 4-arm dendritic polymer **4** (Scheme 1). In cancer immunotherapy, activation of $CD4^+$ T-helper cells and, in particular, $CD8^+$ cytotoxic T lymphocyte cells have been correlated with ability to reduce tumor growth.[14] Professional antigen presenting cells (APCs), such as dendritic cells (DCs) and

macrophages are also critical components of an anti-tumor immune cascade.[4] Therefore, we have assessed the uptake of all polymer-peptide conjugates by DCs and macrophages, in addition to the activation of $CD4^+$ cells and $CD8^+$ cells by the most potent vaccine candidate.

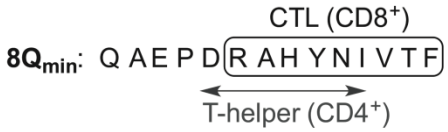


Figure 1. The amino acid sequence of the **8Q$_{min}$** epitope. The CTL epitope ($CD8^+$) and T-helper cell ($CD4^+$) epitope are highlighted within the **8Q$_{min}$** sequence.

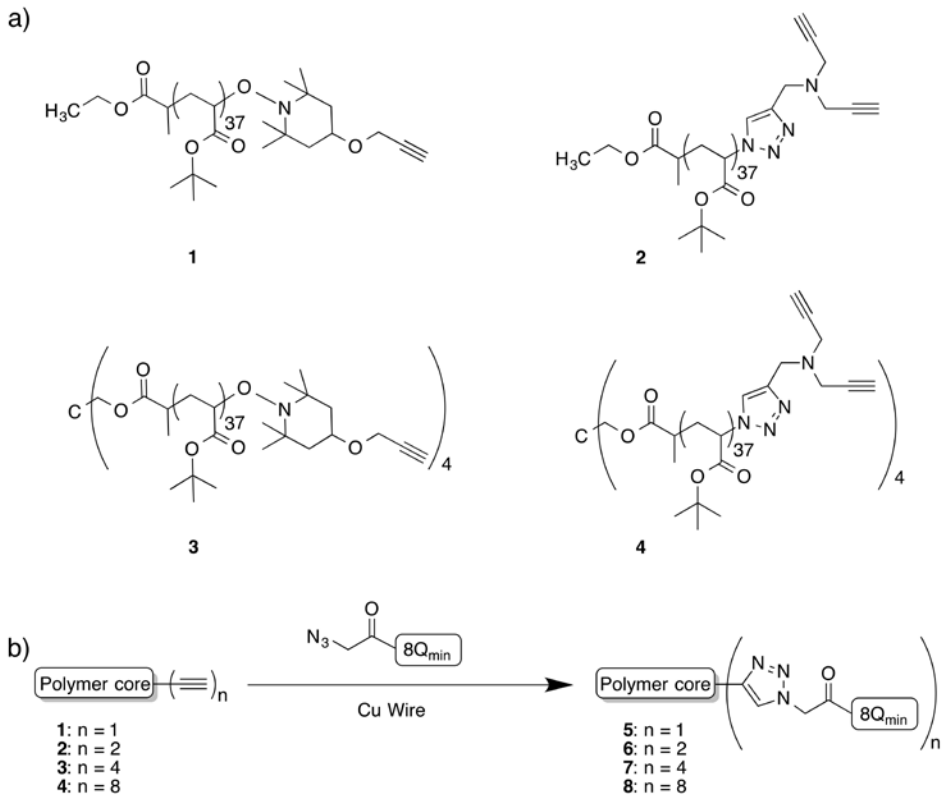
RESULTS

Synthesis and characterization of polymer-peptide conjugates

Alkyne-functionalized poly(*t*-butyl acrylate) polymer with a different number of alkyne moieties **1**-**4** were synthesized by atom-transfer radical polymerization (ATRP) and end-group functionalization. The epitope (**8Q$_{min}$**) and its azide form ($N_3CH_2C(O)$-QAEPDRAHYNIVTF-NH_2) were synthesized in the same manner as reported.[12, 15] Copper (wire)-Catalyzed Alkyne-Azide Cycloaddition (CuAAC)[7, 16] was chosen for the conjugation of **8Q$_{min}$** azide to each polymer to produce the vaccine candidates **5**-**8** (Scheme 1). All the products were self-assembled into particles via the solvent replacement method (DMF/water) followed by dialysis against water to remove the organic solvent, and excess unreacted peptide and copper. The trace level of copper present in the purified polymer-peptide conjugates was precisely determined by

inductively-coupled plasma optical emission spectroscopy. The copper content in all of the conjugates was below 100 ppb (the recommended health standard level of copper is ≤15 ppm).[16] The formation of each polymer-peptide conjugate was confirmed by elemental analysis,[7, 12] which showed a significant increase in the nitrogen/carbon ratio (N/C = 0.082 for **5**, N/C = 0.143 for **6**, N/C = 0.078 for **7** and N/C = 0.124 for **8**) compared with that of polymer (N/C = 0.004 for **1** and **2**; N/C = 0.02 for **3** and **4**), due to the presence of a nitrogen-rich peptide in the conjugates. The substitution ratio of $\mathbf{8Q_{min}}$ conjugated to each polymer was calculated by comparison of the observed and theoretical N/C ratio for the conjugates as previously reported.[7, 12] Compound **5** (96% substitution) and **6** (98% substitution) were obtained and considered to be fully substituted conjugates, while the substitution ratio of **7** and **8** was 88% and 76%, respectively. The conjugation of $\mathbf{8Q_{min}}$ to each polymer followed by self-assembly and dialysis was repeated multiple times, producing virtually identical products (with the same substitution ratio and size).

Samples were prepared for in vivo experiments by formulating all polymer-peptide conjugates in phosphate buffer saline (PBS). All conjugates formed a milky suspension upon addition of the buffer (pH = 7.4). Laser particle size analysis of the polymer-peptide conjugates **5**, **6**, **7** and **8**, was found to be 17 μm, 12 μm, 12 μm and 13 μm in diameter, respectively (Supporting Information Figure S1). The size distribution of all conjugates was narrow (**5**, span = 2.0; **6**, span = 1.7; **7**, span = 1.1; **8**, span = 1.7).



Scheme 1. Synthesis of vaccine candidates. (a) Polymers **1-4** were used in the (b) cycloaddition reaction to form polymer-peptide conjugates **5-8**.

In vivo tumor treatments

The affect of different polymeric structures on the therapeutic potency of polymer-peptide conjugates was examined by conjugating the **8Q$_{min}$** epitope with linear polymer **1** and **2**, star polymer **3**, and dendritic polymer **4** (Scheme 1). Mice (10 per group) were immunized with compounds **5-8** or control groups on the third day after implantation with tumor cells.[17, 18] The positive control group (**8Q$_{min}$**+ISA51) received **8Q$_{min}$** emulsified in Montanide ISA51 (IFA-like adjuvant), while PBS was administered to the negative control group. The Kaplan-Meier survival curve (Figure 2a) showed that 100% of mice treated with PBS were euthanized due to tumor burden by day 43. In contrast, the survival rate in the mice treated with any of the four conjugates

were similar to, or better, than that of positive control (**8Q$_{min}$** + ISA51). Compound **7** showed similar efficacy to positive control group (consistent with previous observations),[12] while **5**, **6** and **8** showed 90% survival rate, and significantly improved survival compared with the positive control. As shown in Figure 2b, tumor-bearing mice treated with any of the four conjugates and positive control showed slower tumor growth than the PBS-only group. Only one mouse treated with **8** developed a tumor, while three mice developed tumors when treated with **5**, and only two mice with **6** (Supporting Information Figure S2). Thus, the dendritic **D8-8Q$_{min}$** construct showed the best therapeutic properties. However, when compared directly, there is no statistically significant difference in mouse survival rate that were treated with linear (**5** and **6**) and dendritic (**8**) vaccine constructs.

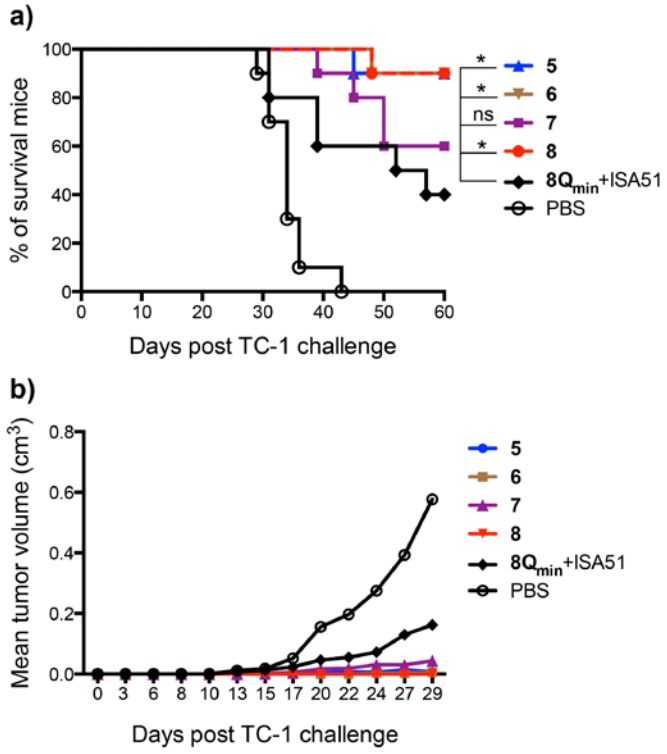


Figure 2. In vivo tumor treatment experiments. C57BL/6 (10 per group) were inoculated subcutaneously in the right flank with 2 × 10^5 TC-1 cells/mouse (day 0), and vaccinated with

different immunogens on day 3 without an 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 positive control (**8Q$_{min}$** + ISA51) (ns $p > 0.05$; * $p < 0.05$). (b) Mean tumor volume (cm^3) in different groups of mice post implantation. Tumor volume is plotted until the first mouse from any group was euthanized (day 29).

Uptake of polymer-peptide conjugates by dendritic cells and macrophages

The uptake of all polymer-peptide conjugates by DCs and macrophages was investigated by incubating murine splenocytes with fluorescently dye (Dil)-labelled conjugates. The resulting cell-associated fluorescence (CD11c labelled with A660, F4/8 labelled with APC-Cy7) was measured by flow cytometry. The DC and macrophage uptake of all four conjugates was significantly higher than negative control (PBS, Figure 3). Of the four conjugates, **7** showed lower uptake by both DCs and macrophages than other polymer-peptide conjugates while **8** showed the highest uptake by APCs. The uptake of **8** by DCs was also confirmed by confocal microscopy (Supporting Information Figure S3). Overall, uptake of the conjugates was biased towards DCs rather than macrophages and the difference in uptake of the four polymeric delivery systems was not statistically significant.

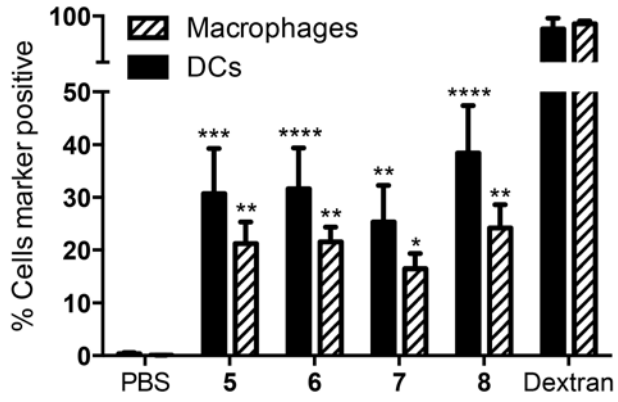


Figure 3. Uptake of **5-8** by mouse splenocyte-derived DCs and macrophages. Dextran was used as a positive control. The experiments were performed in triplicate, and data is presented as percentage of cells double positive for conjugates (labelled with Dil) and CD11c (A660) or F4/80 (APC-Cy7). CD11c (A660) or F4/80 (APC-Cy7) were markers characteristic of DCs and macrophages, respectively. Bars represent the mean ± standard deviation of three experiments. Uptake of each conjugate group was compared to the PBS control group (ns $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

In vivo CD4$^+$ and CD8$^+$ T cell activation

Early activation of the immune system was measured by monitoring CD11a and CD49d expression by T cells[19, 20] seven days after mice were immunized with the lead vaccine candidate **8**. Both **8** and **8Q$_{min}$**+ISA51 achieved significantly higher activation of peripheral CD4$^+$ T cells and CD8$^+$ T cells when compared with PBS (Figure 4).

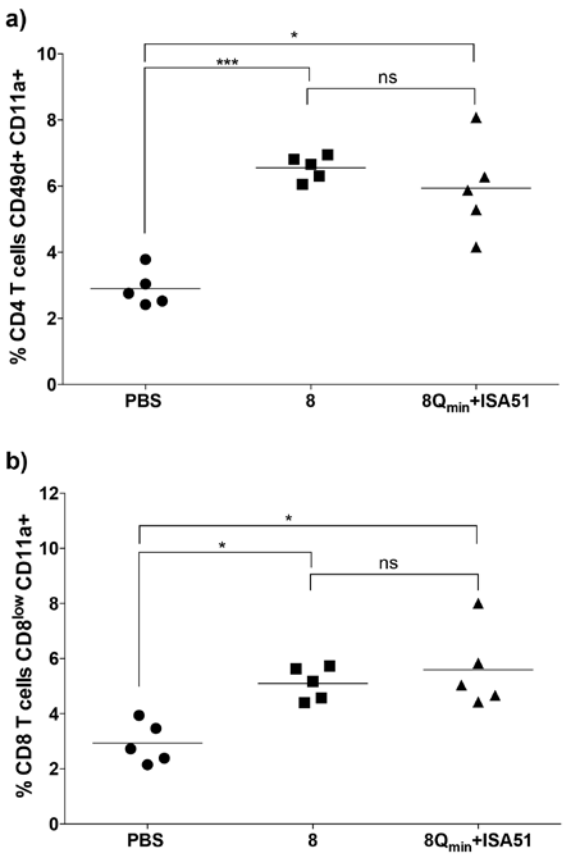


Figure 4. Immune responses induced by polymer-peptide conjugate **8**. Groups of C57BL/6 mice (5 per group) were given a single subcutaneous immunization with **8** or control formulations. Positive control received $\mathbf{8Q_{min}}$ emulsified in Montanide ISA51 ($\mathbf{8Q_{min}}$+ISA51). Negative control was administered PBS. Phenotypes of circulating $CD4^+$ and $CD8^+$ cells were assessed on day 7 after vaccination. Each spot represents one mouse. Horizontal bars represent means. In vivo (a) $CD4^+$ and (b) $CD8^+$ activation of each conjugate group was compared to the PBS control group (ns $p > 0.05$; * $p < 0.05$).

Hemolytic assay

A hemolytic assay was performed to test whether the amphiphilic constructs disrupted biological membranes due to their surfactant–like properties. Even at high concentrations (1

mg/mL), the compounds did not disrupt erythrocyte membranes (Supporting Information Figure S4).

DISCUSSION

Peptide-based vaccines are considered to be a promising candidate for the development of therapeutic vaccines to treat cancer. Poor immunogenicity is a significant challenge in the development of peptide-based vaccines, thus an appropriate adjuvant (immunostimulant) or delivery system is required to elicit the desired immune response. Existing approved adjuvants are ineffective at stimulating the cellular immune response needed for an effective anti-cancer vaccine.[21] Furthermore, most experimental adjuvants are unsuitable for human use because of serious adverse side effects.[4] Safe and robust adjuvants or self-adjuvanting delivery systems are in high demand to overcome obstacles in the field of peptide-based vaccines.

Polymer carriers have been studied for vaccine delivery for many years and a self-assembling amphiphilic polymer has been suggested as a likely platform for the development of peptide-based subunit vaccines.[22] We selected polyacrylate as the most promising antigen carrier because poly(acrylic acid) and its esters were easy to synthesize and are known to possess some adjuvanting activity when physically mixed with inactivated viral antigens.[23] In addition, their safety profile is well established and analogs of polyacrylate have been used for pharmaceutical applications since the 1960s.[24] We recently identified a peptide epitope **8Q$_{min}$** which, upon conjugation to a 4-arm star polyacrylate polymer, reduced tumor growth in mice after a single immunization without the addition of an external adjuvant.[12] It was also demonstrated that the depletion of $CD8^+$ cells diminished **S4-8Q$_{min}$** (**7**) anti-tumor activity, suggesting that **S4-8Q$_{min}$** (**7**) acted through $CD8^+$ T cells, a desirable characteristic for an anti-cancer vaccine.[25]

To improve the ability of this polymer-peptide-based delivery system to induce anti-tumor activity, we designed four different polyacrylate polymer analogs as the delivery platform for peptide antigen **8Q$_{min}$**, each with a different number of alkyne functional groups [12] (Scheme 1). These four polyacrylate polymer analogs were a linear polymer with one alkyne functional group (**1**), a branched linear polymer with two alkyne functional groups (**2**), a 4-arm star polymer with four alkyne functional groups (**3**) and a dendritic polymer with eight alkyne functional groups (**4**). Triprop-2-ynylamine was conjugated to both 4-arm star and linear polymers, resulting in eight or two alkyne functional groups, respectively. The polymer's periphery of alkyne functional groups enabled conjugation to the azide-functionalized **8Q$_{min}$** epitope. The polymer-peptide vaccine candidates **5**-**8** were synthesized via CuAAC "click" reaction using copper wire as a catalyst (Scheme 1). The copper wire was easily removed upon completion of the "click" reaction and residual soluble copper was dialyzed from the solution, leaving only trace copper (below 100 ppb) in the purified conjugates. Copper wire can be easily reused for future reactions [26] and allowed the "click" reaction to proceed without the use of Cu (I) stabilizing and/or Cu (II) reducing agents, minimizing undesirable contaminants in the final product. The hydrophobic polymeric (P^tBA) core and single or multiple copies of the hydrophilic **8Q$_{min}$** epitope resulted in amphiphilic vaccine candidates that self-assembled into 12-17 μm particles when formulated in PBS. All conjugates were also assessed toward their toxicity to erythrocytes because amphiphilic compounds possessing a significant degree of hydrophobicity may have some biological membrane damaging properties. There was no apparent sign of toxicity to erythrocytes for all the conjugates as confirmed by hemolysis assay (Supporting Information Figure S4).

The influence of different polymeric structures on the therapeutic potency of polymer-peptide conjugates was tested by immunizing mice with the test conjugates or control groups on

day 3 after tumor implantation. All conjugates stimulated therapeutic immunity in tumor-bearing mice. Vaccination significantly improved mouse survival and reduced tumor growth when compared with the untreated PBS group (Figure 2). Among the tested formulations, the linear polymer-based conjugates **5** and **6**, and dendritic polymer-based conjugate **8** elicited significantly better survival rates than $\mathbf{8Q_{min}}$ + ISA51, while **7** showed similar anti-tumor efficacy to the adjuvant-based control formulation. In particular, **8** induced an excellent therapeutic response with only one mouse developing a tumor (Supporting Information Figure S2). The role of particle size in inducing an immune response is debated in the literature. Some reports mentioned that immune responses were significantly stronger for small nanoparticles (less than 100 nm),[22, 27] while others claimed that microparticles were necessary for strong activation of the immune system.[28, 29] Neverlethess, all four conjugates formed particles of a similar size (12-17 μm), suggesting that the differences in their anti-tumor activity were not size dependent. The substitution ratio of peptide epitope conjugated to polymer also did not correlate with the anti-tumor efficacy of the constructs.

APCs, including DCs and macrophages, play a crucial role in triggering an immune response, which is initiated by antigen uptake. APCs continuously sample and process antigens in their surroundings and present them via MHC-I and MHC-II molecules to $CD8^+$ and $CD4^+$ T cells, respectively.[4] Efficient particle uptake by DCs was necessary to induce strong antigen-specific $CD8^+$ T cell responses.[22] The depot effect (retention of the antigen at the injection site) is believed to stimulate immune responses against large particles, and similarly depot inducing adjuvants are responsible for T cell activations through uptake by peripheral APCs.[28, 30] Thus, the ability of APCs to take up the conjugates was assessed. All four conjugates were shown to induce significant uptake by DCs and macrophages when compared to PBS (Figure 3 and Figure S3).

Compound **7** showed lower uptake by both DCs and macrophages than the other polymer-peptide conjugates, while **8** showed higher uptake by both DCs and macrophages. Thus, APC uptake of the particles correlated well with the anti-tumor effect stimulated by the vaccine candidates.

In general, research into therapeutic cancer vaccines has aimed to induce antigen-specific activation of both $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T lymphocyte cells. Inclusion of $CD4^+$ T-helper and $CD8^+$ T cell epitopes in the vaccine formulation is important and allows the peptide antigen to elicit potent specific T cell immune responses against tumors.[3, 4] The **8Q$_{min}$** peptide used in this study included a $CD8^+$ T cell epitope ($E7_{49-57}$) and a $CD4^+$ T-helper cell epitope ($E7_{48-54}$) from the E7 protein (Figure 1).[13] The antigen-specific immune responses elicited by the most potent vaccine candidate **8** was evaluated for its ability to activate $CD8^+$ and $CD4^+$ T cells. Compound **8** efficiently activated $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T lymphocyte cells, and showed similar potency to the peptide emulsified in the reference adjuvant (Figure 4). Furthermore, the production of epitope-specific antibodies was not detected (data not shown), consistent with our goal of stimulating cellular immunity to target tumor antigens.

CONCLUSION

The polymer-peptide conjugates produced a robust therapeutic effect against a tumor after a single immunization without the help of an external adjuvant. We also demonstrated that the lead vaccine conjugate **8** induced more potent anti-tumor activity than a formulation that incorporated a classical adjuvant, was efficiently taken up by DCs and macrophages, and stimulated significant $CD8^+$ and $CD4^+$ T cell activation. Interestingly, less sophisticated analogues of the lead compound that possessed a linear polymer moiety (**5** and **6**) induced similar immune responses to **8** and may be used as a simpler and cheaper alternative for further vaccine development. Compound **8** was chosen as the lead vaccine candidate due to its slightly better

efficacy and potential for future conjugation of multiple epitopes to enhance the antigen-specific immune response against the HPV tumor. Our findings suggest that this polymer-peptide conjugate delivery system is a promising strategy for the design of other therapeutic peptide-based vaccines that induce adequate cellular immunity against a target disease. This delivery system also removes the use of incompletely defined and ordinarily toxic immune adjuvants, producing a safe and effective therapeutic vaccine for human use.

EXPERIMENTAL SECTION

Materials

N,N'-dimethylformamide (DMF) and methanol 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%), sodium azide (NaN_3, Aldrich, $\geq$ 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 recieved. Propargyl nitroxde was synthesized according a previously reported procedure.[31] All other reagents were obtained at the highest available purity from Sigma-Aldrich (Castle Hill, NSW, Australia).

Equipment

^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were recorded with a Bruker Avance 300 MHz spectrometer (Bruker Biospin, Germany). 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 using the value of the volume moment. The images of particle uptake by dendritic cells were obtained using 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 for image analysis. The trace level of copper present in the polymer-peptide conjugates was precisely determined by inductively-coupled plasma optical emission spectrometery (Perkin-Elmer Optima 8300DV (Dual View), USA).

Synthesis of linear P(^{t}BA$_{37}$)-Br by ATRP

[^{t}BA]:[EBiB]:[CuBr]:[$CuBr_2$/PMDETA]:[PMDETA] = [80]:[1]:[0.75]:[0.15]:[0.75]. Freshly purified t-BA (12.8 g, 0.10 mol), PMDETA (0.162 g, 9.38 x 10^{-4} mol), pre-formed $CuBr_2$/PMDETA (0.074 g, 1.88 x 10^{-4} mol), EBiB (0.244 g, 1.25 x 10^{-3} mol) and 4 mL acetone were added to a 25 mL Schlenk flask equipped with a magnetic stirrer and purged with Ar for 20 min. CuBr (0.134 g, 9.38 x 10^{-4} mol) was added under positive N_2 flow and purged with N_2 for a further 10 min. The flask was placed in a temperature controlled oil bath at 50 °C for 2 h. The reaction was terminated by quenching in liquid nitrogen followed by exposure to air. The polymerization mixture was diluted with THF, and the copper salts were removed by passage through an activated basic alumina column. The solution was concentrated through evaporation with air flow, and the polymer recovered by precipitation into methanol/water (50:50 vol), filtered and dried for 48 h under high vacuum at 25 °C. The polymer was characterized by GPC. (M_n = 5000, PDI = 1.09, P(t-BA_{37})-Br).

Synthesis of linear P(^{t}BA$_{37}$)-N_3

NaN_3 (0.225 g, 3.46 x 10^{-3} mol) was added to a stirred solution of P^{t}BA_{37}-Br (1.50 g, 3.0 x 10^{-4} mol) in 5 mL DMF. The reaction mixture was stirred for 24 h at R.T. The polymer was recovered by precipitation into methanol/water (50:50 vol), filtered and then dried under high vacuum at 25 ^{o}C.

Synthesis of polyacrylate-based linear polymer 1

P^{t}BA_{37}-Br (0.50 g, 1.0 x 10^{-4} mol), propargyl nitroxide (25.2 mg, 1.2 x 10^{-4} mol), and Me_6TREN (27.6 mg, 1.2 x 10^{-4} mol) were dissolved in a DMSO/Toluene mixture (1.5 mL/2 mL) in a 10 mL Schlenk tube. The mixture was purged by Ar for 20 min and then CuBr (17.2 mg, 1.2 x 10^{-4} mol) was added. The reaction was stirred for 1 h at R.T. and the mixture was diluted with chloroform (10 mL). The solution was washed with deionized water to remove most of the DMSO and Cu(II) complex. The chloroform phase was dried over $MgSO_4$ and passed through a small Al_2O_3 column to remove the copper complex. The solution was blown dry and purified by preparative GPC. GPC: Mn=5270, PDI=1.05. ^{1}H NMR ($CDCl_3$): δ 1.11-1.23 (m, 21H, methyl groups of both initiator residual and TEMPO ring), 1.43 (b, methyl protons of *t*-BA repeat units), 1.82, 2.21 (b, methylene and methine protons of polymer backbone), 2.40 (s, 1H, , (-OCH_2-C≡C<u>H</u>)), 3.76, (b, 1H, methine proton on TEMPO ring), 3.97 (m, 2H, methylene protons of initiator residual), 4.07 (m, 1H, methine proton of *t*-BA unit close to the alkoxyamine linkage).

Synthesis of polyacrylate-based linear polymer 2

P^{t}BA_{37}-N_3 (0.44 g, 8.8 x 10^{-5} mol), TPA (0.23 g, 1.76 x 10^{-3} mol), and PMDETA (7.6 mg, 4.4 x 10^{-5} mol) were dissolved in 2 mL of toluene in a 10 mL schlenk tube. The mixture was purged with Ar for 20 min and then CuBr (6.3 mg, 4.4 x 10^{-5} mol) was added under Ar atmosphere. The reaction was stirred for 1 h at R.T. then diluted with chloroform (10 mL). The solution was passed through a small Al_2O_3 column to remove the copper complex, blown to dry

and purified by preparative GPC. GPC: M_n=5060, PDI=1.09. ^{1}H NMR ($CDCl_3$) : δ 1.11-1.23 (m, 9H, methyl groups of initiator), 1.43 (b, methyl protons of *t*-BA repeat units), 1.82, 2.21 (b, methylene and methine protons of polymer backbone), 2.40 (s, 1H, , (-NCH_2-C≡C<u>H</u>)$_2$), 3.44 (s, 4H, (-$NC\underline{H}_2$-C≡CH)$_2$), 3.85 (s, 2H, methylene protons close to triazole ring), 4.07 (m, 2H, methylene protons of initiator residual), 5.25 (m, 1H, methine proton of *t*-BA unit close to triazole ring), 7.63 (m, 1H, proton of triazole ring).

Synthesis of polyacrylate-based star polymer 3

The star polymer **3** was synthesized according to a published procedure.[12] ^{1}H 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-C≡C<u>H</u>)$_4$), 3.76, (b, 4H, methine proton on 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 polyacrylate-based dendritic polymer 4

The alkyne-functionalized 8-arm poly(*t*-butyl acrylate) dendrimer **4** was synthesized by atom-transfer radical polymerization and copper-catalyzed alkyne-azide 1,3-dipolar cycloaddition (CuAAC) "click" reaction according to a published procedure.[7] ^{1}H NMR ($CDCl_3$) : δ 1.11 (m, 12H, (=CH(C<u>H</u>3))4), 1.45 (b, methyl protons of t-BA repeat units), 1.82, 2.23 (b, methylene and methine protons of polymer backbone), 2.47 (m, 4H, (=C<u>H</u>(CH3))4), 3.48, (s, 16H, (-N(-C<u>H</u>2-C≡CH)2)4), 3.88, (s, 8H, (methylene protons close to the 1,2,3-triazole ring), 3.94-4.22, (m, 8H, C(C<u>H</u>2-O-)4), 5.24, (m, 1H, methine protons of P^{t}BA close to the 1,2,3-triazole ring) 7.72, (m, 4H, methine protons of 1,2,3-triazole ring).

Synthesis of Vaccine Candidate 5

8Q$_{min}$ azide peptide epitope (3.0 mg, 1.6 μmol, 4 equiv.) and polymer **1** (2.1 mg, 0.14 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 to the mixture. Nitrogen was briefly bubbled through the mixture (15 seconds) to remove most of the oxygen from the reaction. The reaction mixture was protected from light with aluminum foil and stirred at 50 °C in a temperature controlled oil bath under nitrogen atmosphere for 4 h. The copper wires were filtered off from the warm solution and washed with 1 mL of DMF. Millipore endotoxin-free water (5 mL) was slowly added to the solution (at 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 formation of the conjugate was confirmed by elemental analysis, which showed a significant increase in the nitrogen/carbon ratio (N/C = 0.082) compared with that of **1** (N/C = 0.004), due to the presence of a nitrogen-rich peptide in **5**. The substitution ratio was calculated by comparing the observed and theoretical N/C ratio for the conjugate and found to be 96%.

Synthesis of Vaccine Candidate 6

Compound **6** was synthesized in the same manner as above. **8Q$_{min}$** azide peptide epitope (3.0 mg, 1.6 μmol, 4 equiv.) and polymer **2** (2.0 mg, 0.14 μmol, 1.0 equiv.) were dissolved in DMF (1 mL). The reaction was stirred for 4 h under nitrogen atmosphere at 50 °C. The formation of the conjugate was confirmed by elemental analysis, which showed a significant increase in the nitrogen/carbon ratio (N/C = 0.143) compared with that of **2** (N/C = 0.02), due to the presence of nitrogen-rich peptide in **6**. The substitution ratio was calculated based on

comparison of the observed and theoretical N/C ratio for the conjugate and found to be 98%.

Synthesis of Vaccine Candidate 7

Compound **7** was synthesized in the same manner as above. **8Q$_{min}$** azide peptide epitope (1.8 mg, 1 μmol, 10 equiv.) and polymer **3** (1.9 mg, 0.10 μmol, 1.0 equiv.) were dissolved in DMF (1 mL). The reaction was stirred for 8 h under nitrogen atmosphere at 50 °C. The formation of the conjugate was confirmed by elemental analysis, which showed a significant increase in the nitrogen/carbon ratio (N/C = 0.078) compared with that of **3** (N/C = 0.004), due to the presence of a nitrogen-rich peptide in **7**. The substitution ratio was calculated based on comparison of the observed and theoretical N/C ratio for the conjugate and found to be 88%.

Synthesis of Vaccine Candidate 8

Compound **8** was synthesized in the same manner as above. **8Q$_{min}$** azide peptide epitope (3.0 mg, 1.6 μmol, 16 equiv.) and polymer **4** (1.9 mg, 0.10 μmol, 1.0 equiv.) were dissolved in DMF (1 mL). The reaction was stirred for 12 h under nitrogen atmosphere at 50 °C. The formation of the conjugate was confirmed by elemental analysis, which showed a significant increase in the nitrogen/carbon ratio (N/C = 0.124) compared with that of **4** (N/C = 0.02), due to the presence of the nitrogen-rich peptide in **8**. The substitution ratio was calculated based on comparison of the observed and theoretical N/C ratio for the conjugate and found to be 76%.

Particle size measurement

The particle size and size distribution (span) of the conjugates was measured by laser diffraction (Mastersizer 2000, Malvern Instruments, UK) after formulation in PBS. The diameter was presented as volume median diameter (D50%). The size distribution (span) value was defined by a standard formula:

Span = (D90%-D10%)/D50%

Where D90%, D10% and D50% are the particle diameters determined from the 90^{th}, 10^{th} and 50^{th} percentile of the undersized particle distribution curve.[32]

Mice and cell lines

Female C57BL/6 (6-8 weeks old) mice were used in this study and purchased from the 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 provided by TC Wu.[33] 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). For injection into mice, TC-1 cells were washed with 1 × PBS, trypsinized, resuspended, and viable cells counted (trypan blue exclusion). The animal experiments were approved by the University of Queensland Animal Ethics committee (DI/034/11/NHMRC) in accordance with the Australian National Health and Medical Research Council (NHMRC) animal handling guidelines.

In vivo tumor treatment experiments

To test the efficacy of each polymer-peptide conjugate as a therapeutic vaccine against established tumors, 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 3 days to allow for tumor formation. On the day 3 after tumor inoculation, the mice were received a single subcutaneous immunization at tail base with 100 μg (50 μg per side) of polymer-peptide conjugates in a total volume of 100 μL 1 × PBS or control formulations. The polymer-peptide conjugates **5-8** were tested while the positive control group received 30 μg of

8Q$_{min}$ emulsified in a total volume of 100 μL of Montanide ISA51 (Seppic, France)/PBS (1:1, v/v) and negative control group received 100 μL 1 × PBS. The size of the tumor was measured every two days using calipers (and palpation) for 60 days.[34, 35] The tumor volume was calculated using the formula:[35]

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

To minimize suffering, mice were euthanized when the tumor reached 1 cm^3 or started bleeding.

Uptake of conjugates by dendritic cells and macrophages

An antigen presenting cell uptake assay was performed using a similar method to one previously described.[36, 37] The uptake of **5-8** by dendritic cells and macrophages was investigated using splenocytes marked with CD11c-A660 (eBioscience) or F4/80-APC-Cy7 (BioLegend, Pacific Heights Blvd, San Diego, CA, USA) antibodies, respectively. Single-cell suspensions of mouse spleens were prepared by passing the organ through a stainless steel mesh. Erythrocytes were lysed with erythrocyte lysis buffer (Sigma-Aldrich) and the remaining cell population was placed in a 6-well plate at a density of 2 x 10^5 cells/well in phenol free IMDM Glutamax medium (Gibco®, Life technologies), supplemented with 10% FBS, 50 μM 2-mercaptoethanol (Gibco®, Life technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco®, Life technologies). All conjugates were labelled with Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) (Vybrant®, Life technologies); 0.5 μL of Dil was added to 100 μL of conjugate solution in PBS and incubated for 30 min (Dil was rapidly adsorbed to the conjugates). Dil-labelled conjugates or Dextran-FITC (positive control) (Chondrex, Inc., USA) were added to the wells and incubated overnight. The adherent cells were scraped from the plate and incubated with Fc-block for 30 min at 4 ^oC, centrifuged and re-

suspended in buffer that contained CD11c-A660 and F4/80-APC-Cy7 antibodies for 30 min at 4 °C. The cells were then centrifuged and resuspended in 0.5 mL of FACS buffer (PBS, 0.02% sodium azide, 0.5% BSA) and analyzed using an LSR II flow cytometry (BD Biosciences). The fluorescent intensities of dendritic cells and macrophages treated with PBS were also measured as a control. The actual uptake was calculated as the percentage of cells double positive for conjugate (Dil) and CD11c (A660), or conjugate (Dil) and F4/80 (APC-Cy7) markers.

In vivo $CD4^+$ and $CD8^+$ T cells activation assay

The activation of $CD4^+$ and $CD8^+$ T cells by polymer-peptide conjugate was assessed using an adaptation of a previously published method.[19, 20] Groups of C57BL/6 mice (5 per group) were given a single subcutaneous injection with 100 μg of **8** in a total volume of 100 μL 1 × PBS or control formulations. Positive control received 30 μg of $\mathbf{8Q_{min}}$ emulsified in a total volume of 100 μL of Montanide ISA51 (Seppic, France)/PBS (1:1, v/v). The negative control group was administered 100 μL 1 × PBS. Seven days after immunization, mouse blood was collected via a submandibular bleed and collected in 5 mM EDTA in PBS. Samples were centrifuged at 400 g for 5 min and the supernatant was discarded. Erythrocytes were lysed by re-suspending in 1 mL ACK lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM $MgNa_2EDTA$) for 5 min at room temperature. A second round of RBC lysis was completed as described above. The resulting cell pellet was re-suspended in 100 μL of MACS buffer (PBS [pH 7.2] without Ca^{++} & Mg^{++}, 0.5% w/v bovine serum albumin (BSA), 2 mM EDTA) and transferred into a 96-well v-bottom plate. The plate was spun at 400 g for 5 minutes and the supernatant discarded. Cells were re-suspended in 50 μL of Fc block (clone 2.4G2), incubated on ice for 10 min and centrifuged as above. The cell pellet was re-suspended in 50 μl of MACS buffer that contained anti-CD4 V500 (Clone RM 4-5), anti-CD8 PerCP-Cy5.5 (Clone 53-6.7), anti-CD11a FITC (Clone 2D7), and

anti-CD49d PE (Clone 9C10) antibodies (all antibodies from BD Biosciences) and incubated on ice (protected from light) for 25 minutes. Compensation controls were prepared in the same way as the cell sample but using anti-rat IgG compensation beads (BD Biosciences) instead of cells (except cells for CD4 V500). Cells were washed three times with 200 μL of MACS and centrifuged as above. Paraformaldehyde (1% aqueous buffered solution, 50 μL) was added and cells were incubated for 5 min on ice. A final wash was done by adding 150 μL MACS and centrifuged as above. The pellet was re-suspended in 200 μL MACS and transferred into 5 mL polystyrene tubes (Falcon) that contained 200 μL MACS. Samples were run on a BD LSR Fortessa and the data was analyzed using FlowJo software version 10.0.7 (Tree Star). The total leukocyte population was gated on $CD4^+$ and $CD8^+$ T cells. Antigen experienced $CD4^+$ T cells were gated as the percentage of $CD4^+$ T cells double positive for CD49d and CD11a. Antigen experienced $CD8^+$ cells were gated as the percentage of $CD8^+$ T cells that are both $CD8^{low}$ and CD11a. Data was graphed using Prism (Graph Pad).

Hemolytic assay

The capacity of polymer-peptide conjugates **5-8** to induce hemolysis was examined using a standard hemolytic assay. Blood was collected from a healthy human volunteer that had given written informed consent (protocol approved by the University of Queensland Ethics Committee, approval number 2009000661). The polymer-peptide conjugates solutions (1 mg/mL in 1 × PBS) were added in 100 μL triplicates to human erythrocytes and incubated at 37 °C for one hour. Sodium dodecyl sulfate (SDS) 100 μg/mL was used as a positive control and PBS as the negative control. After one hour, the plate was centrifuged at 750 × *g* for 15 min and 75 μL of supernatant per well was transferred to a new 96-well plate. The absorbance of each sample was recorded at

540 nm using a Spectramax 250 microplate reader. The data was calculated according to a standard formula:

$$\% \text{ Hemolysis} = [(A_{540}-\text{min}A_{540})/(\text{max}A_{540}-\text{min}A_{540})] \times 100\%$$

where:

-A_{540} is the average absorption of sample at 540 nm

-$\text{min}A_{540}$ is the average absorption of PBS (negative control)

-$\text{max}A_{540}$ is the average absorption of SDS (positive control)

Statistical Analysis

All data was analyzed using GraphPad Prism 6 software. Kaplan-Meier survival curves were applied to the results of the tumor treatment experiments. Statistical analysis between groups was determined using the log-rank (Mantel-Cox) test for survival experiments, two-way ANOVA for uptake assay and the Tukey's multiple comparisons test for CD4/CD8 assay. Differences were considered statistically significant when $p < 0.05$.

Supporting Information

Additional information about the hemolytic assay, particle size, animal study and confocal microscopy images are available free of charge via the Internet at http://pubs.acs.org.

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Notes

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