

Liposomal formulation of polyacrylate-peptide conjugate as a new vaccine candidate against cervical cancer

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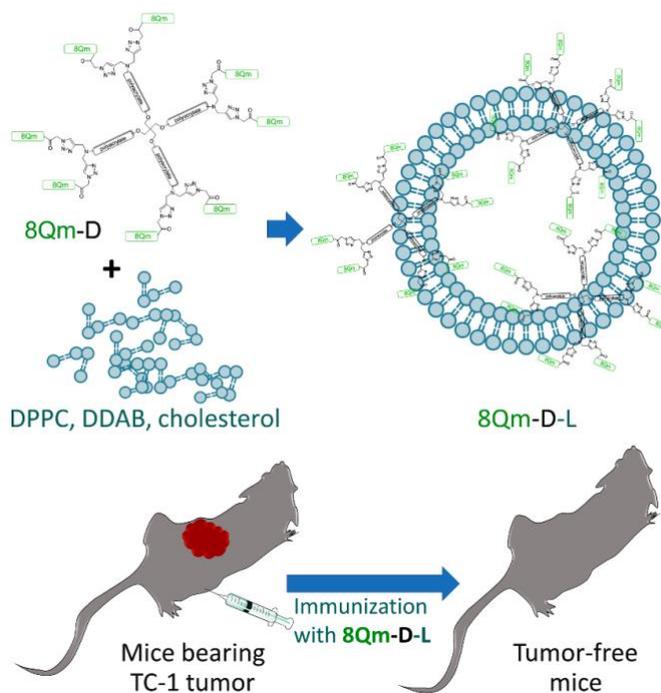
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Graphical abstract:



We demonstrate that a polymer-based delivery system for peptide-based vaccines and liposomes can be incorporated together to greatly improve therapeutic efficacy of the anticancer vaccine.

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Abstract

Peptide-based vaccines have been proposed as a therapeutic strategy for many infectious diseases, including human papilloma virus (HPV)-related cervical cancer. Peptide-based vaccines are a better treatment option than traditional chemotherapeutic agents and surgery, as they rely on the use of the body's immune system to fight cancer cells, resulting in minimal risk of side effects. However, to increase the efficacy of peptide-based vaccines, the application of potent adjuvant and a suitable delivery system is essential. In this study, we developed a self-adjuvating delivery system based on a combination of polymer and liposomes, for a therapeutic vaccine against cervical cancer. Peptide epitope (8Qm) derived from HPV-16 E7 protein was conjugated to dendritic poly(*tert*-butyl acrylate) as a primary delivery system and incorporated into cationic liposomes, which served as a secondary delivery system. Our vaccine candidate was able to kill established HPV-16 E7-positive tumor (TC-1) cells in mice following a single immunization. The immunized mice had 80% survival rate after two months. In contrast, both polymer-8Qm conjugate and liposomes bearing 8Qm failed to eradicate TC-1 tumors. The survival rate of mice was only 20% when immunized with 8Qm formulated with standard incomplete Freund's adjuvant.

Keywords:

- peptide-based vaccine
- liposomes
- polymer-peptide conjugate
- anticancer vaccine
- human papilloma virus

Abbreviations:

- CTL: cytotoxic T lymphocyte (CTL)
- CuAAC: copper-catalyzed alkyne-azide cycloaddition
- DIPEA: *N,N'*-diisopropylethylamine
- DDAB: didodecyldimethyl-ammonium bromide
- DPPC: dipalmitoylphosphatidylcholine
- HATU: (dimethylamino)-*N,N*-dimethyl(3H-[1,2,3]triazolo[4,5-b]pyridin-3-yloxy)-methaninium hexafluorophosphate
- HPV: human papilloma virus
- PDI: polydispersity index

Purpose and Rationale

The purpose of this study was to improve potency of the polymer-peptide conjugate vaccine against HPV associated cancers. The combination of the conjugate with liposomes was expected to not only reduce the size of the anticancer vaccine construct, from micrometers to nanometers range, but to also improve therapeutic efficacy of the vaccine.

Introduction

Cervical cancer is caused by human papilloma virus (HPV) infection. Approximately half a million cases of cervical cancer are reported annually with the disease's death rate reaching a quarter of a million worldwide. While treatments for cervical cancer are available, including

chemotherapy and surgery, most treatments fail due to high disease recurrence and the development of drug resistance.¹ Prophylactic vaccines were recently developed against HPV infections² however, a large proportion of women worldwide are currently infected with the virus and are consequently still at risk of developing cervical cancer. Therefore, the development of a therapeutic vaccine that targets HPV-infected cells is in high demand.³

In contrast to standard vaccines, which induce humoral immune responses (mainly via antibody production), therapeutic anticancer vaccines are designed to elicit cytotoxic T lymphocyte (CTL) responses to eliminate cells bearing specific tumor antigens. As whole HPV or oncoprotein-based vaccines can induce oncogenic changes, a

peptide-based approach has been proposed as a safe alternative.⁴ E7 HPV oncoprotein is unique to cells infected by HPV and is required to maintain HPV-associated tumor cell growth. Therefore, CD8+ peptide epitopes from E7 capable of activating CTLs are commonly used to develop vaccines against cervical cancer.⁵⁻⁹ In general, peptides alone are unable to stimulate the immune system because of their poor immunogenic properties; therefore, their integration with appropriate adjuvants and/or a delivery system is required.¹⁰ Unfortunately, many adjuvants are toxic, unsuitable for human use, or too weak to stimulate immune responses against weak peptide antigens.¹¹

To overcome this problem, the authors developed a self-adjuvating polymer-based delivery system for prophylactic peptide-based vaccines.¹²⁻¹⁵ The system was then adapted for the development of a therapeutic vaccine against cervical cancer.¹⁶ A variety of branched and linear poly(*tert*-butyl acrylate) were conjugated to 8Qm (E7₄₄₋₅₇, QAEPDRAHYNIVTF) peptide derived from HPV-16 E7 oncoprotein (Fig. 1) bearing both CD4+ and CD8+ epitopes.^{17, 18} Of the tested hydrophobic polymers, dendritic polymer (D) conjugated to 8Qm via copper (wires) catalyzed by copper-catalyzed alkyne-azide cyclo-addition (CuAAC) eradicated 3-day-old E7-positive TC-1 tumors in mice after a single immunization. The survival rate for mice

immunized with this vaccine reached 90%, which represented a significantly improvement over immunization via antigen mixed with classic incomplete Freund's adjuvant (IFA), ISA51. It was also shown that 8Qm-D was promptly taken up by antigen presenting cells, and stimulated strong CD8+ cell responses, without activation of TLR2, which is usually responsible for the recognition of hydrophobic ligands.^{19, 20} In addition, depletion experiments with anti-CD4+, anti-CD8+, anti-NK antibodies demonstrated, that vaccine efficacy was strongly dependent on CD8.¹⁹ It was also confirmed that mice did not produce antibodies against 8Qm.¹⁷ However, the survival rate of mice immunized 7 days post tumor implantation dropped to 20-50% after 90 days, even when additional boosts were given.²¹ Therefore, while progressing efforts, this vaccine candidate still required further optimization to improve its efficacy against late stage cervical cancer; an issue we suggest can be addressed via improvement to the vaccine delivery system.

In this study, we developed a new vaccine strategy by integrating a polymer-based delivery system with cationic liposomes. We tested 8Qm-D anchored to liposomes (8Qm-D-L) as a therapeutic vaccine against a model of cervical cancer in mice. 8Qm-D-L greatly reduced the growth of 7-day-old E7-expressing TC-1 tumors and significantly improved mouse survival (4/5) compared to 8Qm adjuvanted with IFA (1/5).

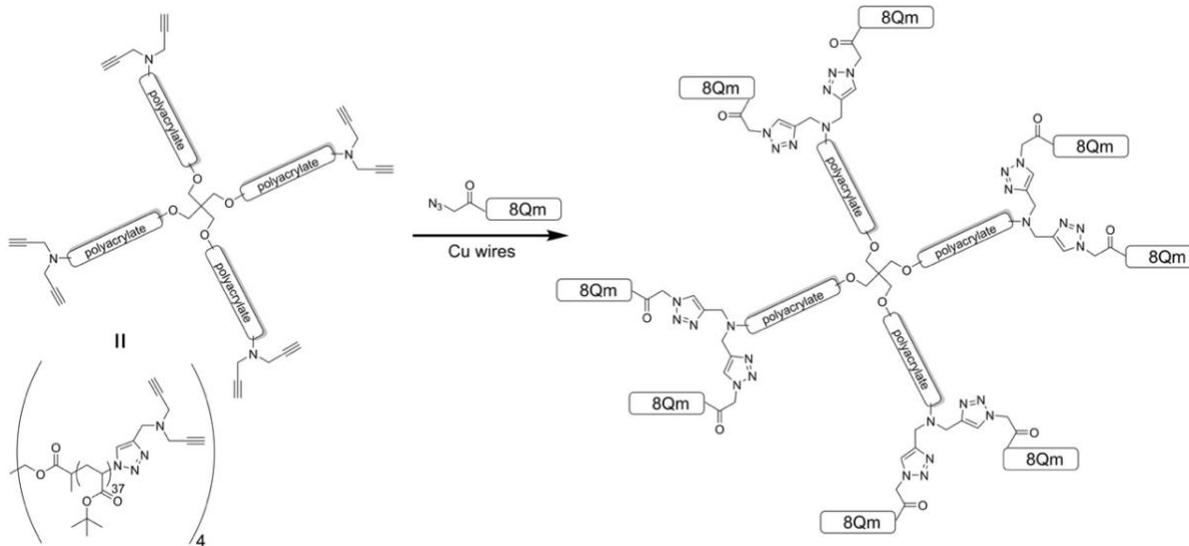


Figure 1. Synthesis of 8Qm-D conjugate by copper-catalyzed alkyne-azide cycloaddition reaction.

Materials and methods

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 were obtained from Merck (Hohenbrunn, Germany). (Dimethylamino)-*N,N'*-dimethyl(3H-[1,2,3]tri-azolo[4,5-b]pyridin-3-yloxy)-methan-iminium hexafluorophosphate (HATU) was purchased from Mimotopes (Melbourne, Australia). Protected L-amino acids were purchased from Novabiochem (Läufelfingen, Switzerland) and Mimotopes (Melbourne, Australia). HPLC grade acetonitrile was obtained from Labscan (Bangkok, Thailand). Copper wires were purchased from Aldrich (Steinheim, Germany). All other reagents were obtained at the highest available purity from Sigma-Aldrich (Castle Hill, Australia). Anhydrous hydrofluoric acid (HF) was supplied by BOC gases (Sydney, 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). Analytical RP-HPLC was performed using Shimadzu (Kyoto, Japan) instrumentation (DGU-20A5, LC-20AB, SIL-20ACHT, SPD-M10AVP) with a 1 mL/min flow rate and detection at 214 nm and/or with an evaporative light scattering detector. Separation was achieved using a 0-100% linear gradient of solvent B over 40 minutes with 0.1% TFA/H₂O as solvent A and 90% MeCN/0.1% TFA/H₂O 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). Dipalmitoyl-phosphatidylcholine (DPPC) and cholesterol (>98%) were purchased from Aventi Polar Lipid, Inc (USA). Didodecyldimethylammonium bromide (98%) (DDAB) was purchased from Sigma Aldrich, USA. Particle size distribution and measurement of the average particle size were taken using a laser particle size analyzer Mastersizer 2000 (Malvern Instruments, England, UK). Multiplicate measurements were performed and the average particle size was recorded.

Experiments

Synthesis of 8Qm.

8Qm epitope (QAEPDRAHYNIVTF;E7₄₄₋₅₇) was synthesized using the previously reported method.¹⁸ Briefly, the peptide was synthesized on pMBHA resin (substitution ratio: 0.59 mmol/g, 0.2 mmol scale, 0.34 g) 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 minute) for amino acid coupling except for His and Asp, which were coupled at 50°C (at 20 W, 15 minute). Each amino acid coupling cycle consisted of Boc-deprotection with 100% TFA (2 × 1 minute, at room temperature (RT)), a 1 min DMF flow wash, followed by two 10 min couplings with the pre-activated amino acid. Amino acid activation was achieved by dissolving Boc-amino acid (0.84 mmol, 4.2 equivalent), in a 0.5 M HBTU/DMF solution (1.6 mL, 0.8 mmol, 4.0 equivalent) followed by the addition of DIPEA (0.22 mL, 1.24 mmol, 6.2 equivalent). Amino acids were pre-activated for 1 min prior to their addition to the resin. Synthesis of 8Qm was finalized with Boc removal, N-acetylation and DNP (2,4-dinitrophenyl) group removal from His by treating the resin with 20% (v/v) β-mercaptoethanol and 10% (v/v) DIPEA in DMF (2 × 1 h) prior to peptide cleavage. Upon synthesis completion, the resin was washed with DMF, DCM, and MeOH, then dried under vacuum. The peptide was cleaved from the resin using HF, with p-cresol as scavengers at -5 °C. The cleaved peptide was precipitated, washed thoroughly with ice-cold Et₂O and dissolved in 50% MeCN/0.1% TFA/H₂O. 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 30–50% solvent B over 40 min. Pure 8Qm peptide took the form of a white powder after lyophilization. HPLC analysis (C18 column): $t_R = 20.92$ min, purity >95%. Yield: 41.6%, ESI-MS: m/z 1702.2 (calc 1702.9) $[M+H]^+$; 851.9 (calc 851.9) $[M+2H]^{2+}$; 635.2 (calc 568.3) $[M+3H]^{3+}$; MW 1701.9.

Synthesis of N₃CH₂C(O)-8Qm.

N₃CH₂C(O)-8Qm was synthesized by manual stepwise SPPS on rink amide MBHA resin (substitution ratio: 0.79 mmol/g, 0.2 mmol scale, 0.25 g) using HBTU/DIPEA Fmoc-chemistry. Amino acid activation was achieved by dissolving Fmoc-amino acid (0.84 mmol, 4.2 equivalent) in 0.5 M HBTU/DMF solution (1.6 mL, 0.8 mmol, 4.0 equivalent.) followed by the addition of DIPEA (146 μ L, 0.84 mmol, 4.2 equivalent.). The coupling cycle consisted of Fmoc deprotection with 20% piperidine in DMF (for 10 min, then again for 20 min), a 1 min DMF flow-wash, followed by coupling with 4.2 equivalent of pre-activated Fmoc-amino acid (for 30 minutes, then again for 1 hour). The attachment of azido acetic acid (4.2 equivalent) was achieved using HBTU (3 equivalent)/DIPEA (4.2 equivalent) at RT for 4 h in the dark. Upon synthesis completion, the resin was washed with DMF, DCM and MeOH, then dried under vacuum. Cleavage of N₃CH₂C(O)-8Qm was carried out by stirring the resin in a 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 Et₂O. 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 10–35% solvent B over 10–40 minute. Pure N₃CH₂C(O)-8Qm was a white powder after lyophilization. HPLC analysis (C18 column): $t_R = 22.5$ min, purity >95%. Yield: 80% ESI-MS: m/z 1742.9 (calc. 1743.9) $[M+H]^+$; 872.2 (calc. 872.5) $[M+2H]^{2+}$; MW 1742.9.

Synthesis of 8Qm-D Conjugate.

8Qm-D (4.8 mg, 0.26 mmol, 10 equivalent) and dendrimer D (5 mg, 0.26 mmol, 1.0 equivalent) were dissolved in DMF (1 mL). Copper wires (55 mg) were added into the mixture. The mixture

was bubbled with nitrogen gas to remove the air for 30s. Reaction mixtures were covered and protected from light with aluminum foil and stirred at 45–50 °C in a temperature-controlled oil bath under nitrogen atmosphere for 12 h. The wires were filtered off from the warm solution and washed with 1 mL of DMF. Particles were self-assembled by slow addition of Millipore endotoxin-free water (7 mL) into the DMF reaction solution (flow rate: 0.05 mL/min) and then exhaustively dialyzed against endotoxin-free water using presoaked and rinsed dialysis bags (Pierce Snakeskin, MWCO 3K) for 3 days. The self-assembled particles were then lyophilized to obtain a white powder form of 8Qm-D conjugate.

Liposome formulation.

All liposomes were formulated using thin-film formation followed by sonication. Dipalmitoylphosphatidylcholine, didodecyldimethylammonium bromide and cholesterol were dissolved in chloroform to final concentrations of 10 mg/mL, 5 mg/mL and 5 mg/mL, respectively. All components were mixed in a round bottom flask with a 5:2:1 weight ratio. One mg of lyophilized 8Qm peptide or 8Qm-D conjugate was dissolved in 1 mL of 1:1 chloroform:MeOH and transferred into the lipid mixture flask. For thin film formation, the organic solvents were gradually evaporated using a rotary evaporator and lyophilized overnight to complete solvent removal. Liposome thin films were rehydrated with 900 μ L of Millipore endotoxin-free water. The suspensions were then sonicated four times with a micro-sonicate probe (40% of power, 20s of pulsing for 2 minutes) to obtain homogenous liposomes. Prior to injection, 100 μ L of 10x PBS was added to the formulation producing 1 mg/mL concentration.

The characterization of liposome containing 8Qm and 8Qm-D.

The hydrodynamic diameter and zeta potential of prepared blank liposome (8Qm-L and 8Qm-D-L) were measured by dynamic light scattering (DLS) using a zetasizer (Nano ZX, Malvern, England). The particle solution was diluted in sterile 1x PBS before being measured. Measurements were performed five times at 25 °C. The particle morphology of 8Qm-L and 8Qm-D-L were examined using transmission electron

microscopy (HT7700 Exalens, HITACHI Ltd., Japan) after vacuum-drying. Briefly, samples were diluted in pure distilled water (1:100) and dropped directly on a glow-discharged carbon coated copper grid and then stained with 2% uranyl acetate. Samples were observed at a magnification of 200,000.

Encapsulation efficacy (%E.E.) of 8Qm into liposomes was determined by addition onto Amicon membrane filter (Amicon Ultra-15, Merck Millipore Ltd., Darmstadt, Germany). Filled amicon tubes were centrifuged at 8000 g for 1 hour. The concentration of un-encapsulated 8Qm in the aqueous phase (supernatant) was measured using a microplate reader and nanodrop with UV-Vis absorption at 280 nm wavelength. %E.E. was calculated as:

$$\%E.E = \frac{C_i - C_f}{C_i} \times 100$$

where C_i represents the initial concentration of peptide added to liposomes and C_f represents the concentration of encapsulated peptide.

Mice and cell lines TC-1.

TC-1 cells (murine C57BL/6 lung epithelial cells transformed with HPV-16 E6/E7 and ras oncogenes) were obtained from TC Wu. TC-1 cells were cultured and maintained at 37°C/5% CO₂ in RPMI 1640 medium (Gibco) supplemented with 10% heat inactivated fetal bovine serum (Gibco). Female C57BL/6 (6–8-week-old) mice purchased from the Animal Resources Centre (Perth, Western Australia) were used. Animal experiments were approved by The University of Queensland Animal Ethics Committee (UQDI/TRI/351/15) in accordance with National Health and Medical Research Council (NHMRC) of Australia guidelines.

In Vivo Tumor Treatment Experiments.

C57BL/6 mice (5 per group) were first challenged subcutaneously in the right flank with 2×10^5 TC-1 tumor cells/mouse. After 7 days, the mice were injected subcutaneously on each side of the tail base (2 injections) with 8Qm-D, 8Qm-

D-L or 8Qm-L (each bearing of 50 µg of 8Qm-D or 15 µg of 8Qm in 50 µL of PBS). Positive control mice received 30 µg of 8Qm (equivalent to the 8Qm content in 100 µg of 8Qm-D) emulsified in a total volume of 100 µL of IFA (Seppic, France)/PBS (1:1, v/v). The negative control group was administered 100 µL of PBS. All mice received a single dose of vaccine. Tumor size was measured by palpation and calipers every second day and reported as the average tumor size across the group of five mice or as tumor size in individual mice. Tumor volume was calculated using the formula: $V(\text{cm}^3) = 3.14 \times [\text{largest diameter} \times (\text{perpendicular diameter})^2] / 6$. Mice were euthanized when tumors reached 1 cm³ or if they started bleeding to avoid unnecessary suffering.

Statistical Analysis.

All data were analyzed using GraphPad Prism 7 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 8Qm-D and 8Qm-D-L

8Qm-D conjugate was synthesized by copper-catalyzed alkyne–azide cycloaddition reaction (Fig. 1) and self-assembled into microparticles (10 µm) via solvent replacement, as reported previously.²¹ Unreacted peptides, copper and organic solvents were removed by extensive dialysis against water for 3 days. The degree of substitution was 85%; thus 7 arms of the 8-arm-dendrimer were substituted with peptide epitopes. The substitution was quantified by elemental analysis based on the observed nitrogen to carbon ratio for 8Qm-D (N/C = 0.134), as compared with that of the polymer (N/C = 0.017),¹⁵ and was slightly higher than reported previously (N/C = 0.124).¹⁷

Table 1. Physicochemical Properties of 8Qm-L and 8Qm-D-L Measured by Dynamic Light scattering.

Liposomes	Size (nm)	PDI (polydispersity index)	Zeta potential (mV)
Blank	145 ± 2	0.21 ± 0.02	39 ± 2
8Qm-L	120 ± 2	0.16 ± 0.01	26 ± 1

8Qm-D-L	136 ± 2	0.30 ± 0.02	23 ± 1
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8Qm-D was anchored to liposomes during thin film formation, followed by hydration with sterile PBS and sonication. The 8Qm peptide was encapsulated into liposomes in a similar manner to form 8Qm-L. Liposome hydro-dynamic size and zeta potential was examined using dynamic light scattering (DLS, Table 1). 8Qm-L and 8Qm-D-L were 120-140 nm in diameter and had similar positive surface charge (23-26 mV). As expected, the incorporation of 8Qm-D in liposomes resulted in increased PDI (0.30) compared to blank liposomes (0.21). TEM photographs demonstrated spherical liposomes with similar size to those measured by DLS. 8Qm-D-L showed a higher tendency to collapse during sample preparation (drying) for TEM analysis

(Fig. 2), similar to what was reported by other groups.^{22, 23} The encapsulation efficacy was indirectly measured using UV-Vis absorption at 280 nm and was found to be approximately 70% for 8Qm-L. As 8Qm-D formed microparticles by itself, the measurement of encapsulation efficacy was not reliable. However, we did not visually observe such particles in the 8Qm-D liposomal formulation through DLS or TEM, suggesting quantitative incorporation of 8Qm-D into liposomes due to the highly hydrophobic nature of poly(*tert*-butyl acrylate). A similar effect was observed when lipids were used to anchor peptides to liposomes.^{24, 25} Taken together, the physicochemical properties of liposomes 8Qm-L and 8Qm-D-L were only slightly different.

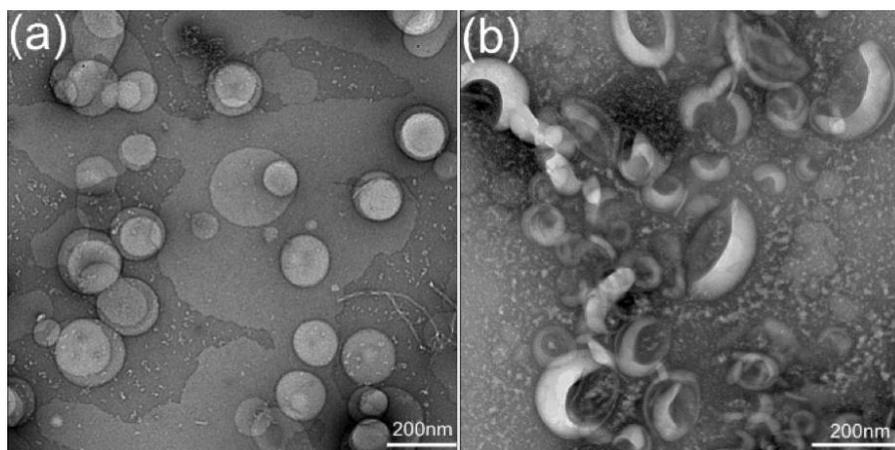


Figure 2. Transmission electron microscopy images of (a) 8Qm-L and (b) 8Qm-D-L stained with 2% uranyl acetate (bar = 200 nm).

In vivo tumor treatment

Previous studies have shown that polymer-peptide conjugates are efficient self-adjuncting delivery systems for anticancer vaccines;^{17, 18} however, once these conjugates were used to treat more advanced tumors (7-day-old), their efficacy was reduced significantly. Following single immunization, the survival rate of mice treated with our most effective conjugate (8Qm-D) dropped from 9/10 (3-day-old tumor)¹⁷ to 2-4/10 (7-day-old tumor).²¹ We recently reported that anchoring peptide epitopes into liposome via hydrophobic moiety (lipid) significantly improved humoral (i.e. antibody-based) immune responses. Importantly, the combination of a

liposomal delivery system with the conjugation strategy improved immune response in a synergistic manner.²⁴ Here we examined whether this strategy can also be applied to polymer-based conjugates designed to induce cellular immune responses. Thus, C57Bl/6 female mice were inoculated subcutaneously in the flank with 2×10^5 TC-1 cells/mouse on day 0, then mice were immunized with 8Qm-D, 8Qm-L, 8Qm-D-L, and controls (PBS, 8Qm + IFA) on day 7. Tumor size and mice survival rate were monitored (**Fig. 3**). Mice were sacrificed when the tumor volume became greater than 1 cm³ or if they started bleeding. In contrast to previous reports on liposomal formulations of E7 peptides or

protein,²⁶⁻²⁸ peptide antigen delivered in liposomes (8Qm-L) was not effective.

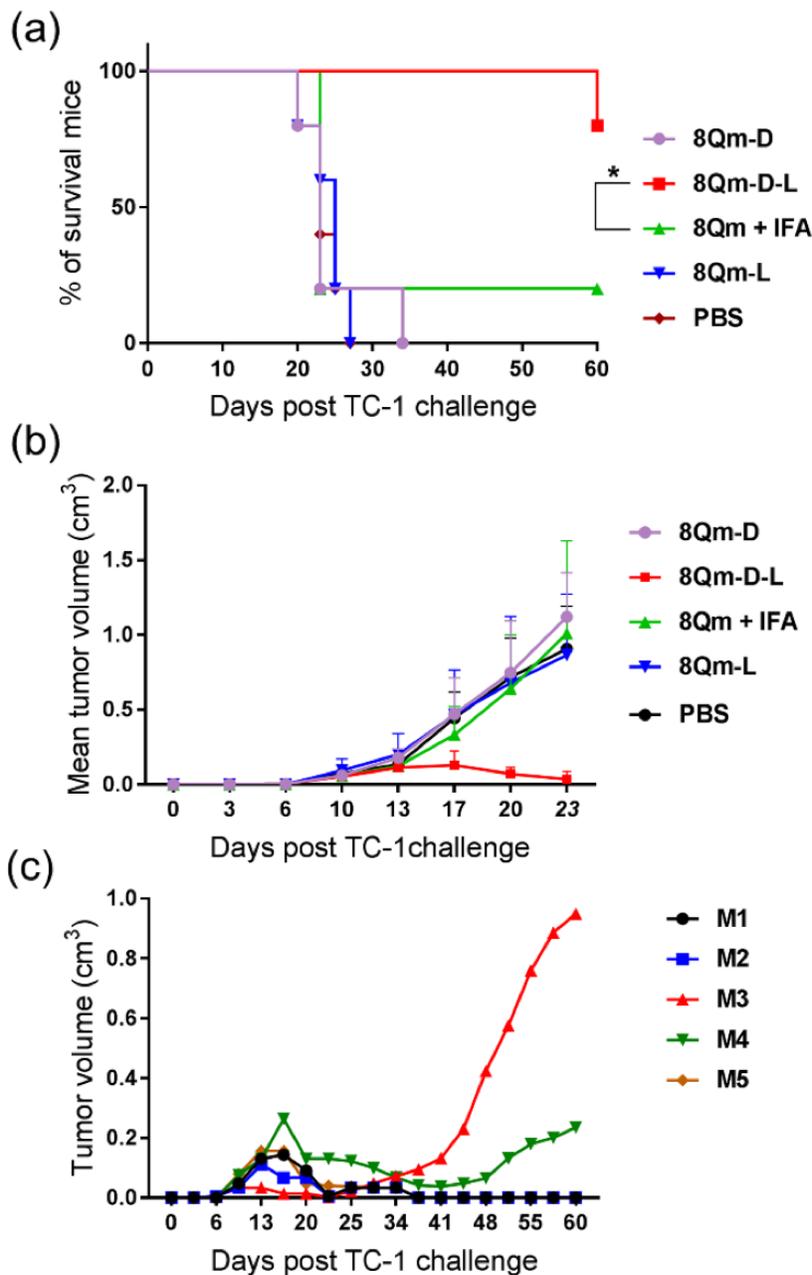


Figure 3. In vivo E7-expressing TC-1 tumor treatment experiments. C57Bl/6 mice (5 per group) were inoculated subcutaneously in the flank with 3×10^5 TC-1 cells/mouse (day 0). On day 7, mice were immunized with a single dose of 8Qm-D, 8Qm-L, 8Qm-D-L, PBS (negative control) or 8Qm + IFA (adjuvanted control). (a) Survival rate was monitored and time to death plotted on a Kaplan–Meier survival curve. (b) Mean tumor volume was monitored and plotted until the first mouse from any group reached the endpoint (day 23). (c) Tumor volume was monitored and plotted for each individual mouse immunized with 8Qm-D-L.

All 8Qm-L-immunized mice exhibited tumors exceeding 1 cm^3 by day 27, which mirrored the negative control group (Fig. 3a). These differences can be explained by the fact that the

present liposomal formulation was not identical to those reported previously; for example, Ossendorp and coworkers used poly(I:C) adjuvant-loaded liposomes to achieve therapeutic

efficacy.²⁷ Interestingly, only one mouse in the 8Qm-D and adjuvanted control (8Qm + IFA) groups survived longer than any of the mice in the negative control group. On average, tumors grew quickly in all above groups (**Fig. 3b**). The weaker efficacy of 8Qm-D than expected could be explained by (a) inconsistency in tumor growth rate between experiments; even relatively small variability in the number of tumor cells used for inoculation had significant influence on tumor growth;^{29, 30} (b) variable sensitivity of C57BL/6 mice to TC-1 tumors;³¹ or (c) (even minor) variation in the properties of 8Qm-D from different synthetic batches (see above). In contrast, the size and polydispersity of liposomal formulation of 8Qm-D can be controlled and well-tuned to a desired size via membrane extrusion or sonication. The small size^{32, 33} of 8Qm-D-L (compared to 8Qm-D), as well as

certain adjuvating properties of positively charged liposomes^{34, 35} were likely responsible for the excellent therapeutic efficacy of 8Qm-D-L. Tumor growth rate was significantly slower in mice immunized with 8Qm-D-L compared to all other groups (**Fig. 3b**). 8Qm-D-L also triggered shrinking of existing tumors in mice (**Fig. 3c**). However, tumors in animal M3 and M4 after initial shrinking regrew, which suggested that booster dose can be used to improve vaccine efficacy. Moreover, survival rate was significantly higher for mice treated with 8Qm-D-L compared to those immunized with classical adjuvant (8Qm + IFA) (**Fig. 3a**). Thus, the liposomal formulation of polymer-peptide conjugate significantly improved the therapeutic properties of the vaccine candidate against HPV-related tumors.

Conclusion

We demonstrated for the first time that a polymer-based delivery system for peptide-based vaccines and liposomes can be incorporated together in a similar fashion to lipid-peptide conjugates anchored to liposomes. Our liposomal formulation was able to greatly enhance the anticancer activity of polyacrylate-8Qm peptide conjugate. It not only reduced tumor growth, but also eradicated tumors from mice when the vaccine was administered 7 days after tumor inoculation. Importantly, the combination of polymer-based and liposome delivery systems was effective without the use of additional adjuvant and with just single dose immunization.

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