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14. ABSTRACT

The main objective of this project is to develop a multistage nanoparticle (NP) platform for both effective systemic and cytosolic delivery of therapeutic proteins into tumors, and to explore the potential of this bioresponsive protein nanotherapy in lung cancer treatment. Over the past year, we have made substantial progress and accomplishments under the proposed tasks. We synthesized and characterized a library of pH-responsive polymers. The NP formation by using these polymers was evaluated by TEM and DLS. To improve the intracellular delivery of proteins, we also synthesized tetra-guanidinium saporin conjugates and tested their anti-tumor efficacy in vitro with A549 and H1299 cells. We have further evaluated the pH-responsive polymeric NPs for PTEN protein delivery in terms of cellular uptake by fluorescence imaging and flow cytometry, and PTEN expression by western blot. The pharmacokinetics of the protein NPs was also examined.

15. SUBJECT TERMS
Nanotechnology, pH-responsive polymer, nanoparticle, protein delivery, saporin, PTEN, lung cancer

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1. INTRODUCTION

This proposal aims to develop a multistage nanoparticle (NP) platform for both effective systemic and cytosolic delivery of therapeutic proteins (such as saporin and PTEN) into tumors, and to explore the potential of this bioresponsive protein nanotherapy in lung cancer treatment. This project has two specific aims: i) engineering of multistage NPs and *in vitro* characterization; and ii) *in vivo* evaluation of multistage protein nanotherapeutics for lung cancer treatment.

2. KEYWORDS

Nanotechnology, pH-responsive polymer, nanoparticle, protein delivery, saporin, PTEN, lung cancer

3. ACCOMPLISHMENTS

➢ What were the major goals of the project?

The project has two specific aims. The major tasks and subtasks in the SOW are shown below.

Specific Aim 1: Engineering of multistage NPs and *in vitro* characterization
Major Task 1. Multistage NP engineering: (i) Synthesis of TME pH-responsive polymer and TCPA component and characterization by ^{1}H-NMR and GPC; (ii) NP formulation and characterization
Major Task 2. In vitro characterization: (i) Cellular uptake of NPs at pH 6.8 vs. 7.4 by flow cytometry measurement; and (ii) NP trafficking and cytotoxicity

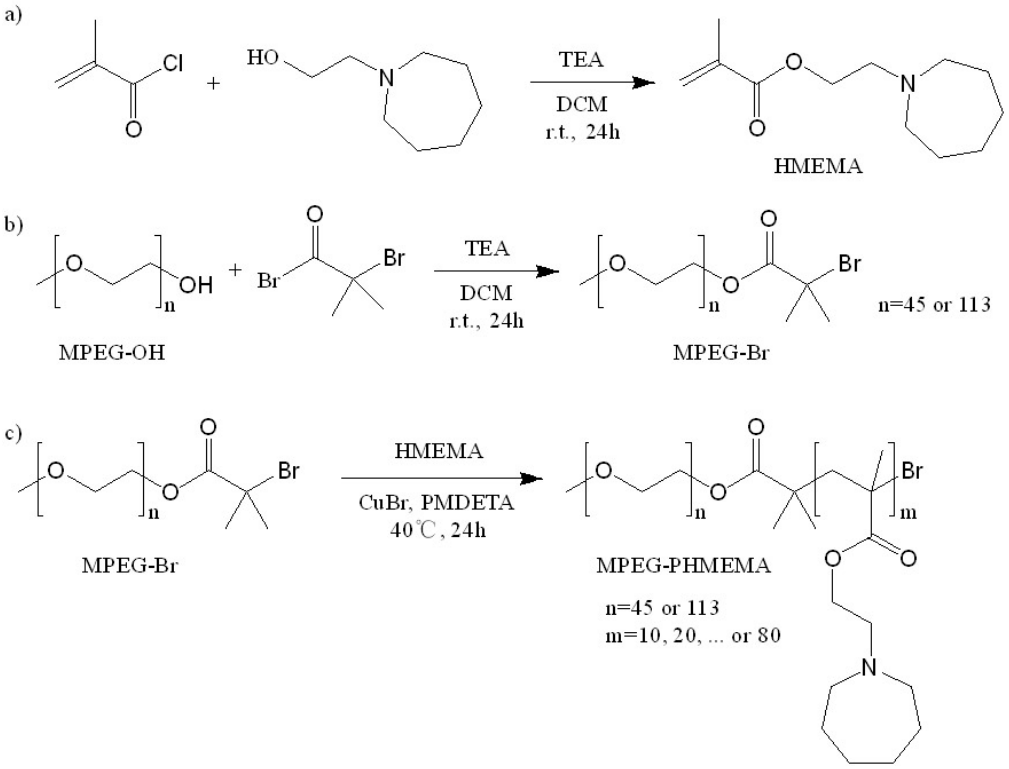
Specific Aim 2: *In vivo* evaluation of multistage protein nanotherapeutics for lung cancer treatment
Major Task 3. In vivo testing of PK, BioD, anti- tumor efficacy and toxicities*:* (i) PK and BioD; (ii) toxicities; and (iii) Anti-tumor efficacy

➢ What was accomplished under these goals?

In Year 1 of this project (8/1/2018 - 7/31/2019), we have made substantial progress and accomplishments for the proposed tasks. We synthesized and characterized a library of pH-responsive polymers. The NP formation by using these polymers was evaluated by TEM and DLS. To improve the intracellular delivery of proteins, we also synthesized tetra-guanidinium saporin conjugates and tested their anti-tumor efficacy in vitro with A549 and H1299 cells. We have further evaluated the pH-responsive polymeric NPs for PTEN protein delivery in terms of cellular uptake by fluorescence imaging and flow cytometry, and PTEN expression by western blot. The pharmacokinetics of the protein NPs was also examined. Below are the achievements for each subtask.

Major Task 1. Multistage NP engineering:
(i) Polymer synthesis and characterization
The pH-responsive polymer consists of the methoxy-terminated poly(ethylene glycol) (MPEG) segment and poly(2-(hexamethyleneimino) ethyl methacrylate (PHMEMA) segment. The protonation of the tertiary amines on the ring under acidic conditions could thus increase the hydrophilicity of the polymer and the rapid disassembly of the MPEG-PHMEMA NPs. As shown in Scheme 1a, the monomer was first synthesized by the amidation of methacryloyl chloride with 2-(hexamethyleneimino) ethanol. The pure HMEMA was obtained by a rapid column chromatography (Figure 1, left). α-bromoisobutyryl bromide was then modified to substitute the hydroxy group at the end of MPEG-OH to obtain the atom transfer radical polymerization (ATRP) initiator (Scheme 1b). As shown in Figure 1 (right), the peak at the chemical shift of 1.93 indicates the entire substitution of Br. Different feeding ratios of HMEMA were reacted with $MPEG_{2K}$-Br or $MPEG_{5K}$-Br separately by the ATRP reaction for 1 day. Eleven polymers were obtained after the dialysis against EDTA solution and pure water to remove cupric ion and unreacted monomer. As shown in Figure 2, the peaks between 1 and 3 indicated

the successful polymerization of PHMEMA. All the ^{1}H-NMR spectrums of the 11 polymers were displayed in Figure 3.



Scheme 1. a) Synthesis route of the pH-responsive monomer HMEMA. b) Synthesis route of the initiator MPEG-Br. c) Polymerization route of the pH-responsive polymer MPEG-PHMEMA with different molecular weights.

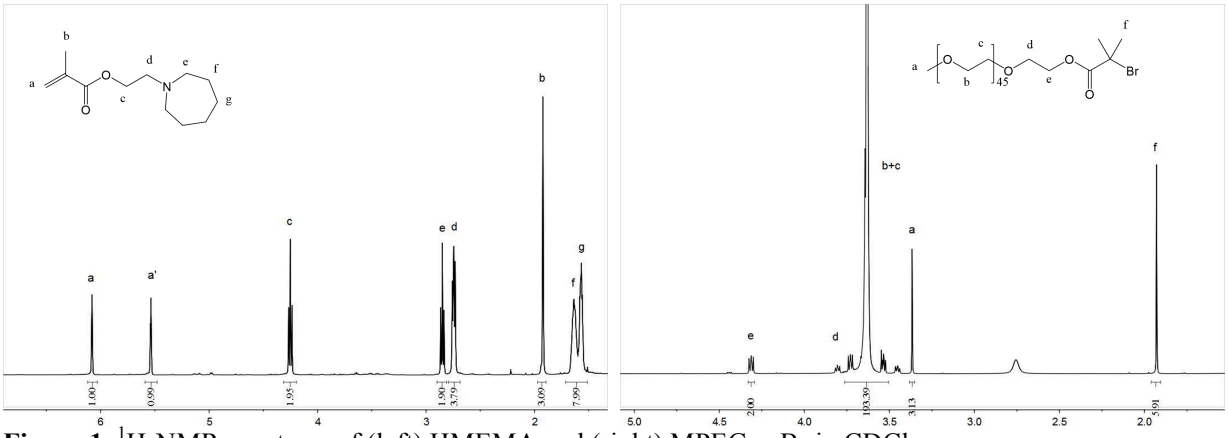


Figure 1. ^{1}H-NMR spectrum of (left) HMEMA and (right) $MPEG_{2K}$-Br in $CDCl_3$.

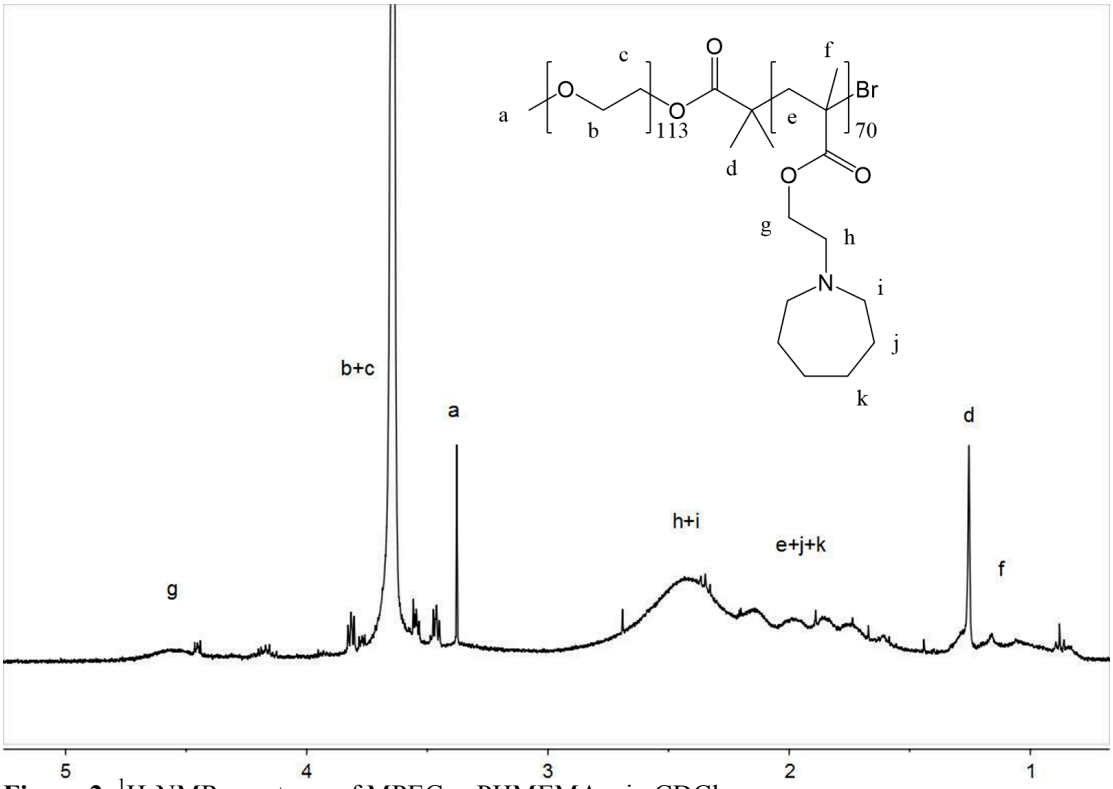


Figure 2. ^{1}H-NMR spectrum of $MPEG_{5K}$-$PHMEMA_{70}$ in $CDCl_3$.

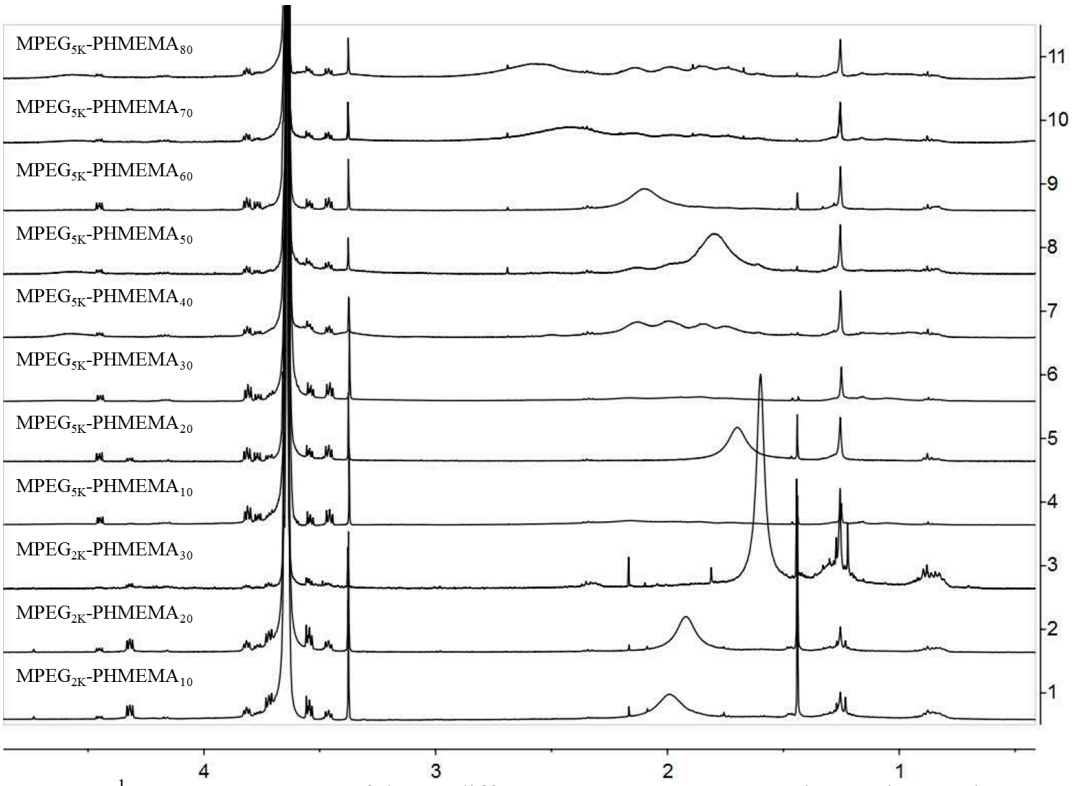


Figure 3. ^{1}H-NMR spectrums of the 11 different MPEG-PHMEMA polymers in $CDCl_3$.

(ii) MPEG-PHMEMA NP formulation and selection

As compared to $MPEG_{2K}$-PHMEMA polymers, $MPEG_{5K}$-PHMEMA polymers showed better and more robust formulation of NPs. Hence, $MPEG_{5K}$-PHMEMA polymers were used for further studies of the morphology and pH-response. A DMF solution of $MPEG_{5K}$-PHMEMA (5 mg/mL) was added dropwise to 5 mL deionized water. After stirred for 5 min, the solutions were collected and purified by using the ultrafiltration device (100 kDa) with phosphate buffered saline (PBS) buffer. The NPs were dispersed in 500 μL PBS at pH 7.4. The size of the NPs was measured by DLS and TEM (Table 1 and Figure 4). All NPs were spherical with the diameter between 40 nm and 130 nm. To further investigate the pH-responsive ability, the pKa of these polymers was measured by acid-base titration in advance. 20 mg $MPEG_{5K}$-PHMEMA polymers were dispersed in deionized water, and 0.1 M HCl aqueous solution was added to adjust the pH to ~ 4.0. Subsequently, 0.1 M NaOH aqueous solution was added in 5-10 μL increments. The pH of the solution was measured by the pH meter after each addition and stir for 2 min. The pKa of the polymers was calculated as the pH that half of the polymer was ionized. As shown in Figure 5, the profiles $MPEG_{5K}$-$PHMEMA_{10}$, $MPEG_{5K}$-$PHMEMA_{20}$ and $MPEG_{5K}$-$PHMEMA_{30}$ didn't have a plateau during the changes of pH. Among the other five polymers, $MPEG_{50}$-$PHMEMA_{70}$ has a pKa of ~ 6.70 (Table 1) which was close to the tumor extracellular pHs (6.5-6.8). The profile also showed that the pH difference from 10 to 90% ionization ($\Delta pH_{10\text{-}90\%}$) was 0.35, suggesting sharp pH-response of this polymer to pH change. Furthermore, the morphology change of $MPEG_{50}$-$PHMEMA_{70}$ NP at an acidic condition was investigated by TEM. As shown in Figure 6a, the NPs expanded to the diameter of ~ 200 nm at pH 6.5. At the intracellular pH of 5.0, the NP disappeared and irregular aggregates formed.

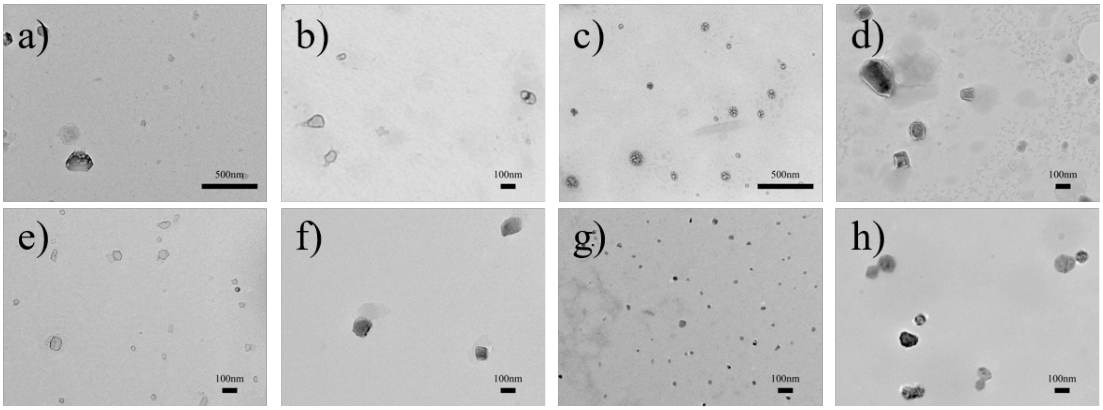


Figure 4. TEM images of a) $MPEG_{50}$-$PHMEMA_{10}$, b) $MPEG_{50}$-$PHMEMA_{20}$, c) $MPEG_{50}$-$PHMEMA_{30}$, d) $MPEG_{50}$-$PHMEMA_{40}$, e) $MPEG_{50}$-$PHMEMA_{50}$, f) $MPEG_{50}$-$PHMEMA_{60}$, g) $MPEG_{50}$-$PHMEMA_{70}$ and h) $MPEG_{50}$-$PHMEMA_{80}$ NPs at pH 7.4.

Table 1. Size and distribution of $MPEG_{5K}$-PHMEMA NPs measured by DLS and TEM, and pKa measured by acid-base titration.

	Diameter measured by DLS (nm)	Diameter measured by TEM (nm)	pKa
$MPEG_{5K}$-$PHMEMA_{10}$	103.0±7.1	84.5±32.6	-
$MPEG_{5K}$-$PHMEMA_{20}$	117.0±5.2	75.3±13.8	-
$MPEG_{5K}$-$PHMEMA_{30}$	130.5±4.7	104.8±12.4	6.72
$MPEG_{5K}$-$PHMEMA_{40}$	122.2±11.6	82.7±17.3	6.42
$MPEG_{5K}$-$PHMEMA_{50}$	73.7±2.5	51.0±11.2	6.47
$MPEG_{5K}$-$PHMEMA_{60}$	128.9±8.7	108.7±20.6	6.49

$MPEG_{5K}$-$PHMEMA_{70}$	88.7±2.6	41.0±2.8	6.70
$MPEG_{5K}$-$PHMEMA_{80}$	90.6±3.9	77.0±19.7	6.48

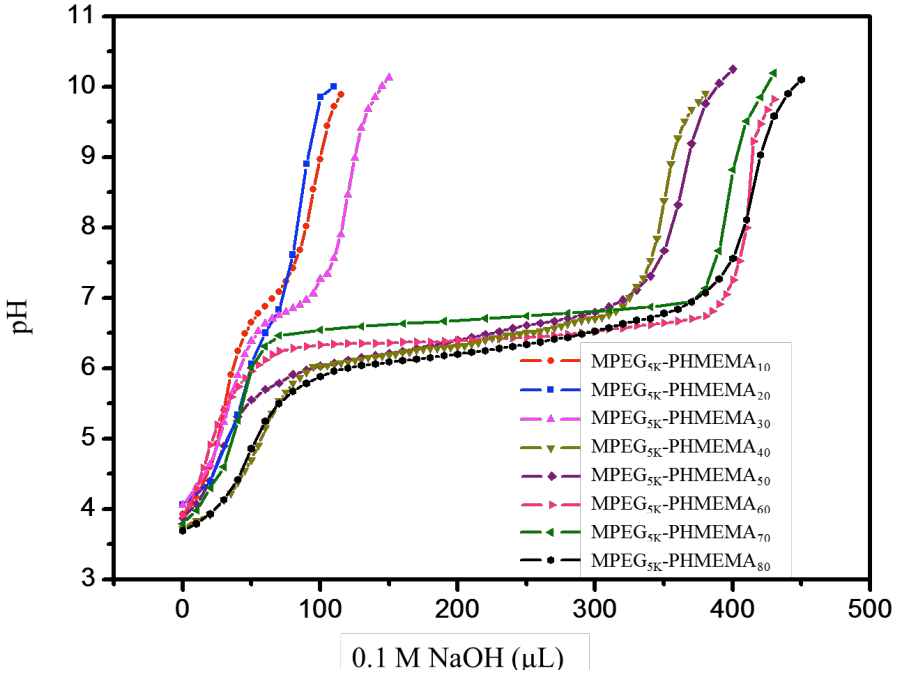


Figure 5. Acid-base titration profiles of the pH-responsive $MPEG_{5k}$-PHMEMA polymers.

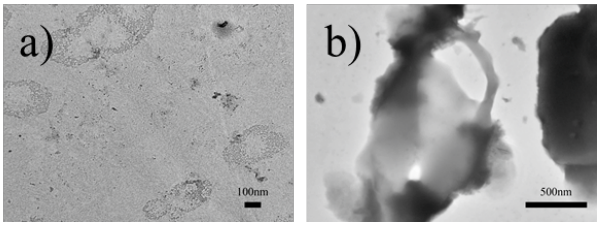
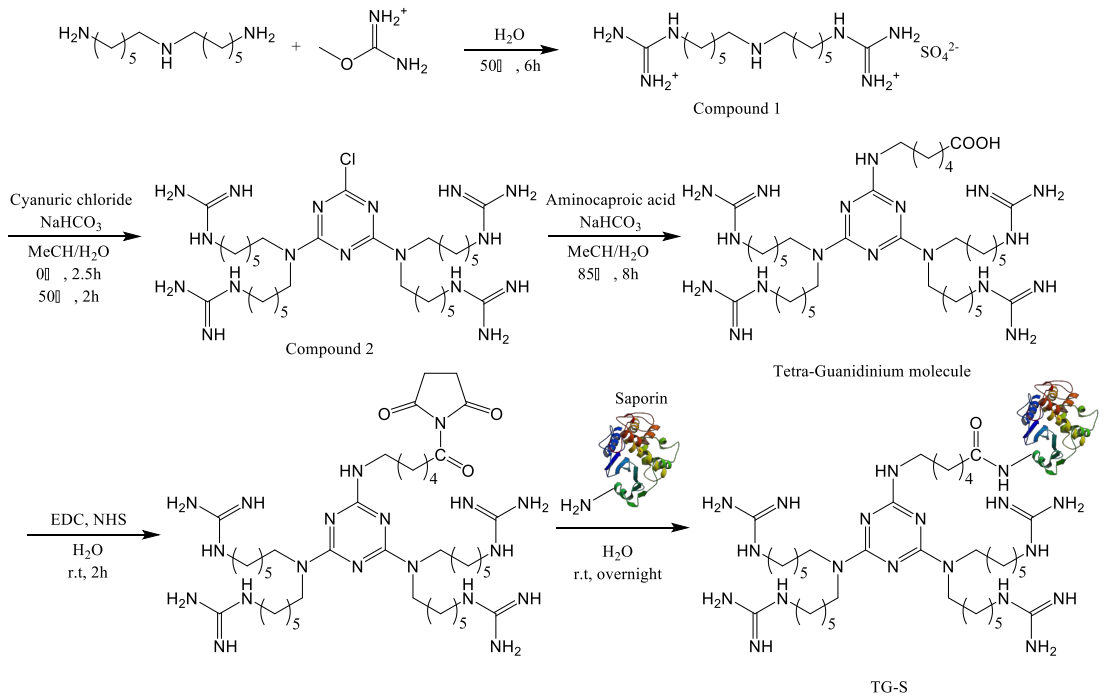


Figure 6. TEM images $MPEG_{50}$-$PHMEMA_{70}$ NPs at pH a) 6.5 and b) 5.0.

(iii) Synthesis and characterization of tetra-guanidinium protein conjugates

Arginine-rich cell-penetrating peptides have shown the capability to cross the cell membrane for intracellular delivery of different therapeutics, but they could also lead inevitable cytotoxicity due to its high positive charge, particularly when there are 6 or more guanidinium groups (Gdms). In addition to the use of tumor cell-targeting and -penetrating peptide-amphiphile (TCPA) containing 8 arginines (shown in *Figure 1C in the proposal*), we here covalently conjugated a dendritic small molecule with only 4 Gdms to proteins (e.g., Saporin). As shown in Scheme 2, the four guanidinium groups were first modified on bis(hexamethylene)triamine. The ^{1}H-NMR spectrum of compound 1 was shown in Figure 7. After reaction in cyanuric chloride, aminocaproic acid, which was used as the linker between the synthesized tetra-guanidinium (TG) molecule and Saporin, was substituted the remaining chlorine. Compound 2 and the TG molecule were obtained without a further purification. After 2 hours activation by NHS and EDC, TG was stirred with Saporin in H_2O overnight. The final TG-Saporin (TG-S) conjugate was purified by ultrafiltration using a centrifugal filter units with 10 kDa cutoff.



Scheme 2. Synthetic route of the Tetra-Guanidinium molecule and Saporin-conjugates (TG-S).

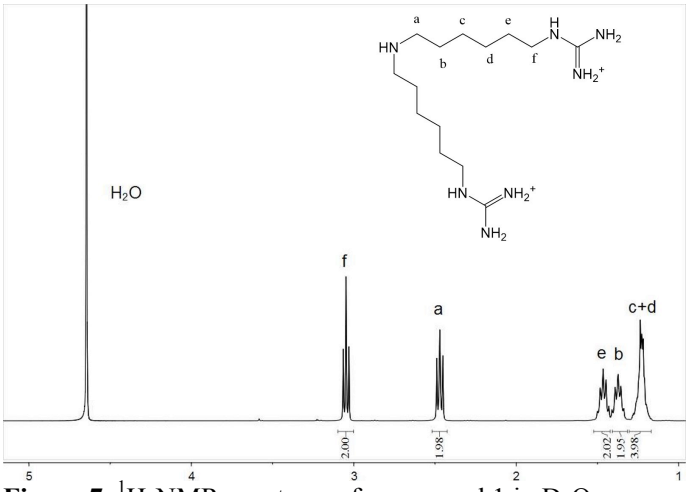


Figure 7. ^{1}H-NMR spectrum of compound 1 in D_2O.

Major Task 2. In vitro characterization:

(i) Cellular uptake of NPs

In this subtask, we first formultated PTEN NPs using the MPEG-PHMEMA and TCPA. TCPA (1 mg/mL) and PTEN (1 mg/mL) were dissolved in water. MPEG-PHMEMA (0.4 mg/mL) were dissolved in DMSO/water (3:7, v/v, pH 3.0) to form a homogenous solution. Then the pH was adjusted to 5.0. 20 μL of PTEN aqueous solution was mixed with 50 μL TCPA solution. Under vigorously stirring (1000 rpm), the mixture was added dropwise to 5 mL of PHMEMA solution and the pH was adjust to 9. The NP dispersion was transferred to an ultrafiltration device (Millipore, MWCO 100 K) and centrifuged to remove the organic solvent and free components. After washing with PBS buffer (3 × 5 mL), the PTEN loaded

NPs (PTEN@NPs) were dispersed in 1 mL of PBS buffer. The NP size was around 113.3 nm with zeta potential ~ 2.04 mV. The encapsulation efficiency (EE%) of PTEN is about 75.6%. For studying the cellular uptake, we used 10% FITC-BSA+90%PTEN to prepare FITC-labeled PTEN@NPs. As can be seen in Figure 8, the fluorescence intensity of PTEN@NPs group was significantly higher than that of free PTEN group in tumor cells. Similar results were obtained in the flow cytometry analysis study to further confirm the much better PTEN uptake as mediated by the NPs.

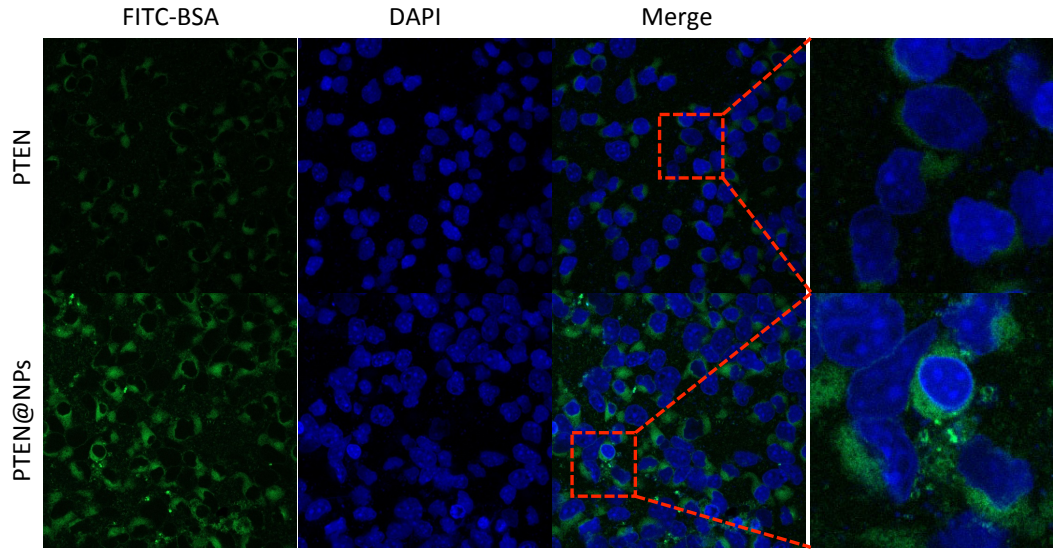


Figure 8. Confocal fluorescence images of cellular uptake of free PTEN vs. PTEN@NPs.

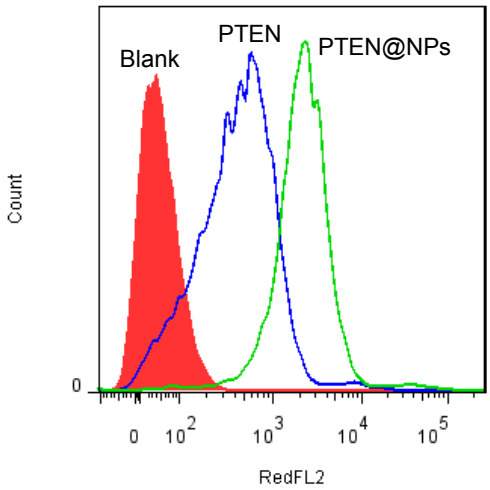


Figure 9. Flow cytometry analysis of cellular uptake of free PTEN vs. PTEN@NPs.

(ii) In vitro anti-tumor efficacy of PTEN NPs

To test the anti-tumor efficacy of the PTEN NPs, we first confirmed PTEN expression in NSCLC cells. A549 and H1650 cells were treated with PTEN@NPs for 4 h and cultured for another 20 h. Cells were then collected and proteins were extracted for PTEN western blotting. Figure 10 shows that the PTEN expression in A549 cells was weak and can be improved by PTEN NPs, while it was more drastic in H1650 cells. In contrast, free PTEN didn't induce much

increase of PTEN expression in tumor cells. This suggests our NPs effectively improved intracellular delivery of PTEN protein.

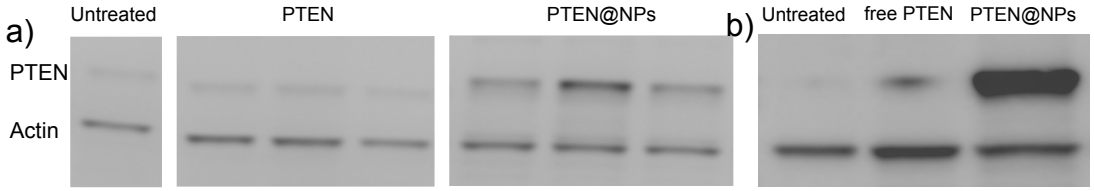


Figure 10. Cell viability of PTEN@NPs as compared to controls (control NPs and free PTEN) in (a) A549 and (b) H1650 cells.

Next, we examined the cell viability of A549 cells after treatment with control NPs, free PTEN, or PTEN@NPs. The cell proliferation was measured by MTT assay and data are presented as mean ± SD (n=3) (Figure 11a). As can be seen, the anti-tumor effect of PTEN@NPs group was significantly higher than the control groups. We also analyzed the cell apoptosis by flow cytometry based on Annexin V-FITC/PI double staining. Results showed that the percentage of apoptotic cells in PTEN@NPs group was ~70%, significantly higher than that of free PTEN group (Figure 11b). Similar results can also be observed with H1650 cells after PTEN@NPs treatment (Figure 12).

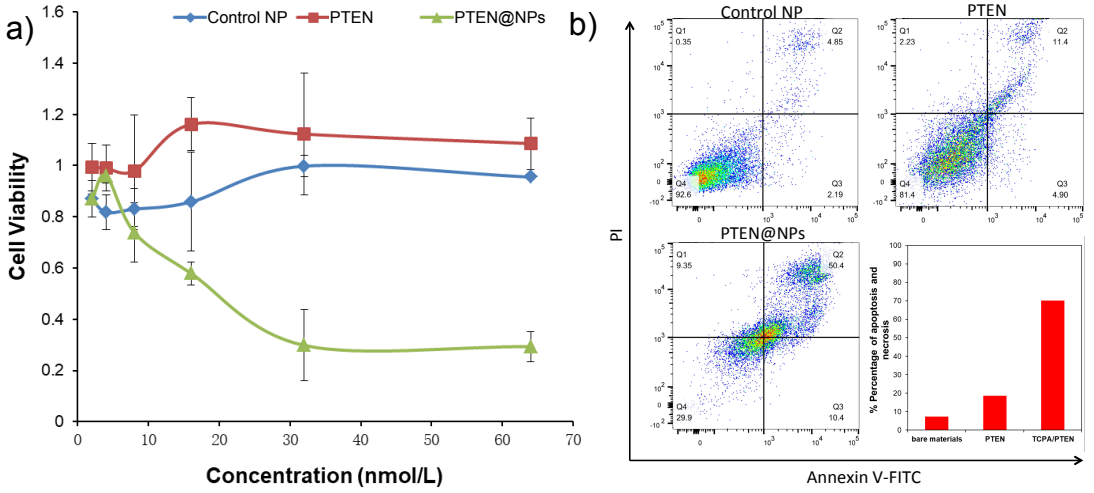


Figure 11. (a) Cell viability and (b) cell apoptosis analysis of PTEN@NPs as compared to controls (control NPs and free PTEN) in A549 cells.

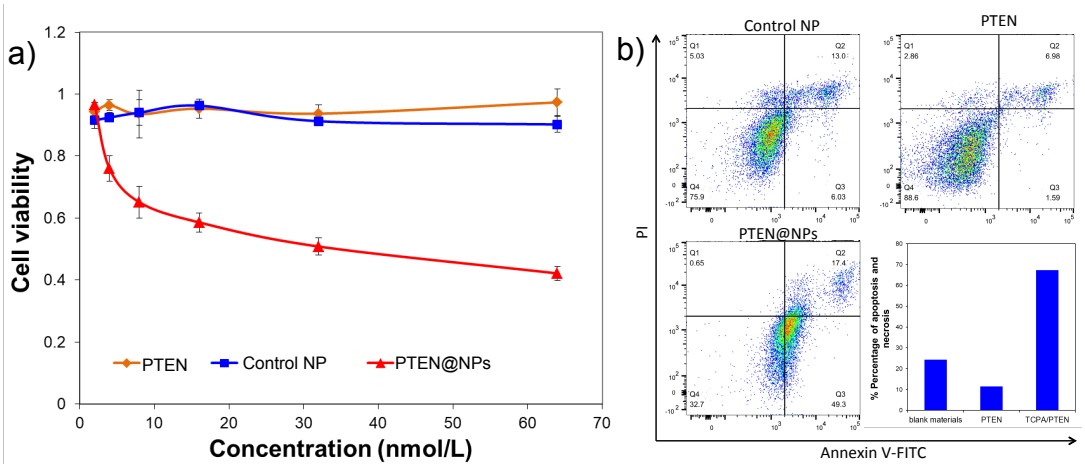


Figure 12. (a) Cell viability and (b) cell apoptosis analysis of PTEN@NPs as compared to controls (control NPs and free PTEN) in H1650 cells.

(iii) In vitro anti-tumor efficacy of TG-Saporin conjugates

The cell proliferation inhibition of Saporin conjugates with different ratios of TG to Saporin was first studied on A549 cells. Different ratios of TG to Saporin (5:1, 2:1 and 1:1) were used to prepare three conjugates (TG5-S, TG2-S and TG-S). A549 cells were incubated with the conjugates for 48 h. As shown in Figure 13a, the TG5-S and TG2-S had similar cell proliferation at each concentration, while TG-S showed best inhibition. The cytotoxicity of TG, Saporin and TG-S were then studied with A549 cells. Figure 13a shows that A549 cells incubated with TG had less than 10% loss in cell viability, indicating its negligible cytotoxicity. Saporin showed less than 20% cell proliferation inhibition on A549 cells. The TG-S had the best cytotoxicity with the IC50 of 15.9 nM. We are now working on the formulation of TG-S@NPs using our selected MPEG-PHMEMA and testing their anti-tumor efficacy in different NSCLC cell lines.

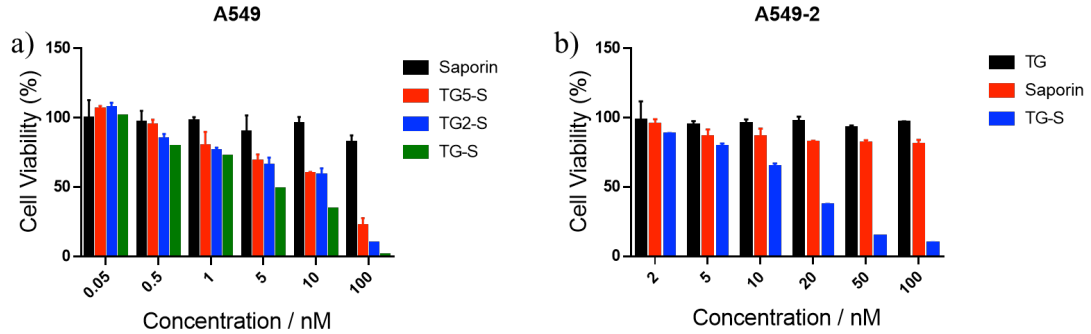


Figure 13. (a) Cell Proliferation of Saporin conjugates with different feeding ratios of TG to saporin in A549 cell line. (b) Cell Proliferation of TG, Saporin and TG-S in A549 cells.

Major Task 3. In vivo testing of PK, BioD, anti- tumor efficacy and toxicities*:*

(i) PK and BioD

In this subtask, we examined the blood circulation of our protein NPs. The pH-responsive polymeric NP were formulated with 10% FITC-BSA and 90% PTEN, and was injected into healthy BALB/c mice through the lateral tail vein. Blood was then collected at different time points (1, 5, 30 min, and 1, 2, 4, 6, 8, 10, 12, 24h) for fluorescence measurement. The PK parameters will be calculated from the circulation profile of the NPs, and compared to the naked Cy5.5-protein.

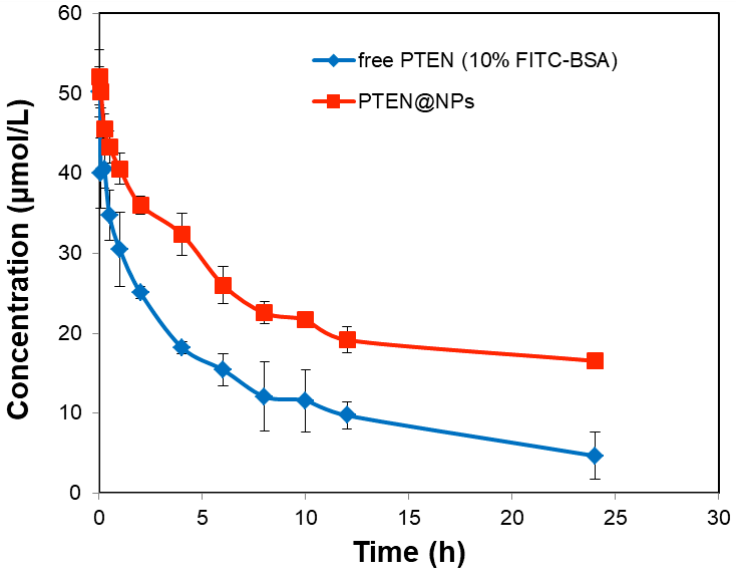


Figure 14. Blood circulation of PTEN@NPs vs. free PTEN by using 10% FITC-BSA as an indicator.

(ii) Toxicities and anti-tumor efficacy
These subtasks will be completed in the non-cost extension year.

- **What opportunities for training and professional development has the project provided?**

While the Brigham and Women's Hospital (BWH) does not have an institutional policy requiring individual development plans for postdoctoral fellows and graduate students, the hospital is very committed to training its students and fellows to meet their research and career goals. The hospital supports a centralized career development office, *Office for Research Careers of BWH Brigham Research Institute*, which offers seminars ranging from career development to responsible conduct of research to how to secure external funding. The office also addresses the specific needs of postdoctoral fellows and faculty investigators in the research community at BWH, and supports BWH researchers across the academic continuum, by providing resources to support career and professional development, by encouraging professional responsibility, enhancing the training experience and fostering effective mentoring. As a teaching affiliate of Harvard Medical School, BWH students and fellows have access to career development and support services offered by Harvard. Within my group, the postdoctoral fellows and students have routine meetings with me to discuss research project, skill and career development, and other needs they may have, and they present research work in the biweekly group meeting. The postdoctoral fellows and students are also encouraged and supported to attend local seminars, workshops, national conferences, and advanced education courses to present their research work, interact with colleagues, and enhance professional knowledge and skills, all of which will be helpful for their career development.

- **How were the results disseminated to communities of interest?**

Nothing to report.

- **What do you plan to do during the next reporting period to accomplish the goals?**

During the period of non-cost extension (8/2019 – 7/2020), we will (i) select the top MPEG-PHMEMA NP formulation for TG-Saporin delivery, by characterizing in vitro uptake and anti-tumor effect; (ii) evaluate the PK and BioD of the TG-Saproin NPs; and (iii) complete the in vivo toxicities and therapeutic efficacy studies as proposed in the xenograft mouse model of lung cancer.

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

While protein therapies have made tremendous contributions to treatment of different diseases including cancer, their current clinical applications are mostly restricted to targets in the vascular or extracellular areas. One major challenge associated with the widespread application of protein therapeutics for cancer treatment is their low membrane permeability and endosomal entrapment. We expect that our multistage NP platform could provide a unique strategy for addressing the challenge of intracellular protein delivery, and may make an impact on the protein therapy field. In addition, we have modified proteins with tetra-guanidinium that demonstrates the capability to facilitate cytosolic transportation of proteins and shows negligible toxicity. The combination of multistage NP delivery with tetra-guanidinium protein conjugates could lead to highly effective intracellular protein delivery to tumor cells in vivo.

- **What was the impact on other disciplines?**

Nothing to report.

- **What was the impact on technology transfer?**

Nothing to report.

- **What was the impact on society beyond science and technology?**

Nothing to report.

5. CHANGES AND PROBLEMS

- **Changes in approach and reasons for change**

Nothing to report.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

During the formulation of protein NPs, we noticed a certain degree of protein denature (which also depends on the protein type), which may be attributable to the use of organic solvent. We have adjusted the formulation method to avoid/minimize the use of organic solvent and will test this new protein NP formulation strategy during the one-year non-cost extension period.

- **Changes that had a significant impact on expenditures**

Nothing to report.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report.

- **Significant changes in use or care of human subjects**

Nothing to report.

- **Significant changes in use or care of vertebrate animals**

Nothing to report.

- **Significant changes in use of biohazards and/or select agents**

Nothing to report.

6. PRODUCTS

- **Publications, conference papers, and presentations**

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

BWH

Name:	*Jinjun Shi*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*0.6*
Contribution to Project:	*Dr. Shi oversees the whole project.*
Funding Support:	

Name:	*Jianxun Ding*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*8*
Contribution to Project:	*Dr. Ding has lead polymer synthesis, protein NP formulation and characterization, and in vitro and PK testing.*
Funding Support:	

Name:	*Liyi Fu*
Project Role:	*Visiting Scholar*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Dr. Fu helped the polymer synthesis, tetra-guanidinium protein conjugation, NP preparation and characterization, and in vitro testing*
Funding Support:	*PMRL Director Education Fund*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report.

- **What other organizations were involved as partners?**

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

➢ COLLABORATIVE AWARDS

Nothing to report.

➢ QUAD CHARTS

Nothing to report.

9. APPENDICES

Nothing to report.