2011

In Vivo Investigations of Polymer Conjugates as Therapeutics

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IN VIVO INVESTIGATIONS OF POLYMER CONJUGATES AS THERAPEUTICS

A Thesis Presented

by

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Submitted to the Graduate School of the

University of Massachusetts Amherst in partial fulfillment

of the requirements for the degree of

MASTER OF SCIENCE

September 2011

Molecular and Cellular Biology

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DEDICATION

To all those who encouraged and supported me,

especially my loving husband David and my sister and mother; most of all to my grandparents, John and Trudie, who never stopped believing in me.

ACKNOWLEDGEMENTS

I would like to acknowledge the tremendous patience, encouragement and support provided to me by my advisor Sallie Smith Schneider and mentor Kelly Gauger, without whom I would never have been able to make it this far. Also, to all the fellow scientists at PVLSI that I was privileged to work with and receive advice and support from during these projects. Finally to Todd Emrick and Xiangji Chen for providing the polymers and performing the HPLC analysis.

ABSTRACT

IN VIVO INVESTIGATIONS OF POLYMER

CONJUGATES AS THERAPEUTICS

SEPTEMBER 2011

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Polymer conjugates offer a way to introduce materials into the body that would normally be rejected or cause toxicity. Two polymers are investigated *in vivo* for uses in chemotherapeutic delivery, protein therapeutics, and DNA transfection. A novel polymer, polyMPC, has the ability to increase doxorubicin loading and its solubility, and is conjugated in a way to release its payload in a low pH environment. Through its conjugation, blood clearance time of doxorubicin is increased, and thus tumor exposure to the drug is increased with a single administration. It can be administered at ten times the concentration of free doxorubicin, and three times the concentration of Doxil®, while decreasing the cardio-toxicity normally associated with doxorubicin administration. These results show that polyMPC has the potential to increase treatment efficacy of doxorubicin. With increased circulation time, MPC polymers have additional potential for protein delivery and variations of its design were tested in linear, branched and grafted states, which show limited affect on tissue weight. An additional polymer for use in DNA transfection, NLS2, demonstrated its lack of tissue toxicity when injected intramuscularly. While continued investigation into these polymers is required, this initial data indicates their promising uses as therapeutics.

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CHAPTER 1

INTRODUCTION

Current chemotherapeutic cancer treatment options are accompanied by adverse side effects that reduce the administrable drug concentration and thus the chances for a successful treatment response. Prodrugs are nanosized, polymer-drug conjugates that offer a way to increase drug dosage concentrations. They prevent toxicity of the drug by releasing it from the polymer following hydrolysis or enzymolysis at the drug to polymer linkage point (Kratz et al. 2008). Polymers can increase a drug's hydrodynamic size, which allows longer circulation time in the blood stream and greater drug accumulation in the tumor environment through the enhanced permeation and retention (EPR) effect (Veronese et al. 2008). The EPR effect describes the more open, or leaky, vasculature of tumor environments, which allows the permeation of larger molecules than normal tissue environments would allow (Greish et al. 2007). The poor lymphatic drainage of tumor environments allows retention of these molecules, increasing exposure time of the tumor environment to the conjugated chemotherapeutic (Greish et al. 2007).

A common drug linkage is the covalent attachment of poly(ethylene glycol) (PEG), which increases a drug's water solubility, leading to an increase in drug dosage concentrations in normally hydrophobic chemotherapeutics such as doxorubicin (Veronese et al. 2008). However, PEGylation alone is not sufficient to reach the high level of drug loading required for increased therapeutic results. A phosphorylcholine (MPC) polymer designed by Dr. Todd Emrick (University of Massachusetts Amherst Polymer Science Department), provides hydrophobic drugs like doxorubicin with

high water solubility as well as increased drug loading (Emrick Lab Data, 2010). A poly(methacryloyloxyethyl phosphorylcholine) (polyMPC)-doxorubicin conjugate of Dr. Emrick's design has shown promise in its use as a chemotherapeutic conjugate (Emrick, 2011). *In vitro* experiments show polyMPC-doxorubicin to be stable at a normal physiological pH of 7.4 and instable, releasing its drug payload, at a low pH indicative of tumor environments due to their release of lactic acid (Emrick Lab Data, 2010; Gerweck *et al*., 2006). This indicates that polyMPC-doxorubicin may be appropriate for tumor site-specific release of doxorubicin.

A polyMPC polymer offers several advantages over other polymers currently under investigation for chemotherapeutic delivery. Dendritic polymers, such as polyamidoamine (PAMAM) and polypropyleneimine, are widely investigated, but require surface modifications to avoid toxicity, while others, like polyaryl ether dendrimers, require modifications to increase water solubility (Gillies *et al*., 2005). In addition, the release of molecules can be difficult to control, not offering the controlled release desirable in drug delivery; along with low hydrodynamic size increases that do not allow utilization of the EPR effect (Jansen *et al*., 1995; Nishikawa *et al*, 1996). Other investigated polymers, such as N-(2-Hydroxypropyl) methacrylamide (HPMA), also carry risk of toxic accumulation. They are not biodegradable and can accumulate in the skin through the reticuloendothelial system (RES) (Duncan *et al.*, 2010). The biggest drawback to both dendritic and HPMA polymers is the complicated synthesis required to create and modify these polymers. PolyMPC has a large advantage over these polymers in this regard, being synthesized in a simple one-pot method.

Protein therapeutics is another area of investigation for cancer treatment, along with other diseases such as Hepatitis C. Obstacles facing protein therapeutic treatment is high blood clearance times, preventing absorption and utilization of the proteins, degradation due to immune response, and protein aggregation (Mero *et al*, 2011). Polymers offer a way to circumvent the natural immune response by masking proteins, and conjugation aids in preventing protein aggregation. The increased circulation time afforded by Dr. Emrick's phosphorylcholine methacrylate polymer over conventional PEGylated polymers makes it a good candidate for protein therapeutics.

A third important area of investigation for both cancers and additional diseases, such as muscular dystrophy, is gene delivery. Efficient gene delivery is highly desirable, yet a continued obstacle for scientists. Gene delivery would allow scientists to correct altered gene expression, whether it be over-expression or lack of expression, which is responsible for disease progression, therefore providing a cure for the disease. Unfortunately, efficient gene delivery and incorporation has continued to elude scientists to date. Polymers offer a way to increase cellular uptake of plasmids and therefore increase the chances of plasmid incorporation and expression. An additional polymer designed by Dr. Emrick has the potential to increase cellular uptake and incorporation of DNA, while decreasing cell death (Parelkar *et al*., 2011).

To further investigate their potential, polyMPC was tested *in vivo* for its maximum tolerated dose, cardio-toxicity, and biodistribution, MPC-protein polymers were tested in three different architectures for *in vivo* tissue toxicity, and a transfection polymer,

NLS2, was tested for its tissue toxicity and transfection efficiency into muscle.

CHAPTER 2

MATERIALS AND METHODS

2.1 Polymers and Chemotherapeutics

The pMPC, pMPC-doxorubicin, and doxorubicin were kindly donated in powder form by the Emrick Laboratory, Polymer Science and Engineering Department, Univeristy of Massachusetts Amherst, stored at 4°C in the dark and kept dry. The powders were weighed, Hank's Balanced Salt Solution (HBSS) was added, and the solutions vortexed until the powders were dissolved. Once dissolved, the solutions were sterilized by filtration through a 0.2 µm pore filter and stored at 4°C until use. Doxil® was also provided by the Emrick Laboratory and stored at 4°C until use. NLS2 and JetPEI™ transfection polymers was provided by the Emrick Lab, stored at -20°C, combined with GFP plasmid, and incubated for 1 hour at room temperature immediately prior to injection. Protein therapeutic polymers (Lysozyme, linear MPC polymer, branched MPC polymer, and grafted MPC polymer) conjugated to Alexa Fluor® 546 (AF546) were supplied by the Emrick Lab and stored at -20°C until use.

2.2 Animal Models

Four to five-week old athymic nude mice (Nu/J) were purchased from Jackson Laboratories (Stock #002019) and housed and treated in accordance with the Institutional Animal Care and Use Committee. PolyMPC treatments were injected into the left or right lateral tail vein and blood was collected from the submandibular vein into 1.5 mL microcentrifuge tubes.

C57BL/6J mice were purchased from Jackson Laboratories (Stock #000664) and used for both the protein polymer and transfection polymer experiments. Protein polymers

were injection into the tails of 6 week old mice, and blood was collected from the tails at 5, 10, 15, 30, 45, 60, and 120 minutes, 4, 6, 12, and 24 hours, and daily for 2 weeks following injection. Transfection polymers were injected into the right anterior tibialis muscle of 5 week old mice.

2.3 Cell Lines

MDA-MB-231 cells were cultured, either from previously frozen laboratory stocks or recently purchased from ATCC (Catalogue #HTB-26), in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1:500 gentamicin (MDA-medium). For *in vivo* experiments, cells were grown to ~80% confluence, harvested with trypsin in log phase, and suspended to a concentration of $1x10^5$ cells/uL in HBSS. Cell suspensions were injected into the right flank of athymic nude mice at a total volume of 100 uL and 1x 10^7 cells. Tumor volume was calculated by $L*W^2*0.52$.

2.4 Blood and Tissue Handling

Blood samples were allowed to clot on ice and then centrifuged at 1500 x g for 15 minutes. PolyMPC serum samples were collected and stored at 4^oC until analyzed for fluorescence excitation at 490 nm and emission at 590 nm, by high performance liquid chromatography (HPLC) by Xiangji Chen. Tissues were collected post-mortem and either fixed in 10% formalin for 24 hours at 4^oC and paraffin embedded or frozen in liquid nitrogen and stored at -80^oC. Frozen tissues were weighed and then homogenized in acidified isopropanol (90% isopropanol, 75mM HCL) and incubated overnight at 4^oC, followed by centrifugation at 1500 x g for 15 minutes. The liquid supernatant was collected and stored at 4^oC until analyzed for fluorescence excitation

at 490 nm and emission at 590 nm, by HPLC by Xiangji Chen. Paraffin embedded tissues were treated with hemotoxylin and eosin (H&E) stain and analyzed for tissue architecture and cell staining patterns, or analyzed for apoptotic cells by TUNEL using ApopTag® Plus Peroxidase *In Situ* Apoptosis Kit according to the provided protocol.

At the end of 2 weeks protein polymer mice were euthanized and spleens, kidneys, intestines, livers, lungs, and hearts were collected, weighed, and homogenized in 1XPBS. Tissue homogenates were centrifuged at 4^{o}C at 1500 x g for 15 minutes and stored at -20^{o}C until analyzed for fluorescence by HPLC.

Transfection polymer tissues were collected after 4 weeks, fixed in 10% formalin for 24 hours at 4^{o}C and paraffin embedded for sectioning. Tissue sections were treated with GFP antibody and analyzed for positive staining or H&E stain and analyzed for tissue architecture and cell staining patterns.

CHAPTER 3

RESULTS

3.1 Maximum Tolerated Dose of polyMPC-Doxorubicin

PolyMPC-drug conjugates containing 30% doxorubicin by weight were tested in doxorubicin doses of 10, 20, 30, 40, and 50 mg of doxorubicin per kg of body weight to determine the maximum dose administrable without eliciting adverse effects. Seven week old animals, three mice per group, received a single, intravenous injection in the lateral tail vein of polyMPC-dox, polyMPC alone, or Hank's Balanced Salt Solution (HBSS). The animals were weighed and examined daily for 30 days. Figure 1 shows the weight changes in mice following treatment. A slight decrease in weight was observed at day five in 30, 40, and 50 mg/kg dose groups, and shows that a single injection at that dose may elicit some toxicity; however the mice were able to recover from such toxicity, indicated by the return to normal weight ranges by day 24. Necrosis around the injection site was observed these doses, most likely caused by extravascular drug administration, and is the likely cause of the weight changes observed in these mice. The results indicate that polyMPC can be used to increase the concentration of doxorubicin without eliciting irreversible side effects.

PolyMPC drug conjugates were then compared to doses of free doxorubicin and Doxil®, a commercially available liposome form of doxorubicin, in ten mice per treatment group. Free doxorubicin was administered at a concentration of 6 mg/kg and Doxil® was administered at a concentration of 10 mg/kg. A dose of 30 mg/kg polyMPC-doxorubicin was chosen as that was the highest doxorubicin concentration with the least observed toxicity in the previous experiment. Doxil®, polyMPC-

doxorubicin, and HBSS were administered on day 0 and day 7, for a total doxorubicin dose of 20 mg/kg in Doxil® and 60 mg/kg in polyMPC-doxorubicin; however free doxorubicin was only administered on day 0 due to observed toxicity prior to and on day 7 of treatment. The animals were weighed and examined daily for up to 54 days (Figure 2). The results indicate that polyMPC-doxorubicin can be given at three times the doxorubicin dose in Doxil®, and ten times the dose of free doxorubicin, while exhibiting less toxicity-induced weight loss. On day 55, or upon presentation of toxicity, hearts were collected, fixed in 10% formalin, and paraffin embedded. Tissue sections were first analyzed for apoptotic cells by TUNEL (Figure 3). No apoptotic were observed in these tissues. Due to lack of apoptotic cells present, cardiac tissues were then treated with hematoxylin and eosin stain. Under 40x magnification, the stained nuclei were counted per field of vision. The hearts of mice treated with free doxorubicin show less nuclear staining per 40x field than the hearts of mice treated with either HBSS, Doxil®, or polyMPC-doxorubicin (Figure 4 & 5). While actively occurring death was not observed, likely due to the length of time following injection, there seems to have been a loss of muscle cells due to doxorubicin, which was prevented by conjugation to the polymers. These results provide evidence of the decreased toxicity with increased drug dosage provided by polyMPC-doxorubicin.

3.2 Biodistribution and Clearance of polyMPC-Doxorubicin

The effect of polymer conjugation on the clearance of doxorubicin in animal serum was tested on five-week old female athymic nude mice. The mice were divided randomly into three groups and injected intravenously through the tail vein with polyMPC-doxorubicin or free doxorubicin at 6 mg/kg, or with HBSS of the same volume. Blood samples of 30-50 uL were taken at 0, 30 minutes, 2 hours, 6 hours, 12

hours, 24 hours, 48 hours, and 72 hours post-treatment and analyzed for doxorubicin fluorescence by HPLC. The results (Figure 6) indicate that polyMPC-doxorubicin has a longer blood circulation time than that of free doxorubicin.

To examine the biodistribution of polyMPC-doxorubicin in comparison to free doxorubicin, kidneys, livers, and hearts were collected after 72 hours from the mice used for the clearance experiment and analyzed for doxorubicin fluorescence by HPLC. The results (Figure 7) indicate there may have been accumulation of polyMPC-doxorubicin in the liver and kidney to a greater extent than free doxorubicin, although the variation between animals was considerably large and there was no statistical difference in accumulation. In addition, H&E staining of livers did not show any discernable differences in tissue architecture (Figure 8).

To observe tumor accumulation of polyMPC-doxorubicin versus free doxorubicin, MDA-MB-231 cells were harvested with trypsin in log phase and suspended in HBSS at a concentration of $1x10^7$ / 100uL and injected into the right flank of six week old athymic nude mice. Once the tumors reached a volume of 300-500 mm^3, mice were divided randomly into three groups of 2 mice and injected intravenously through the tail vein with polyMPC-doxorubicin or free doxorubicin at 6 mg/kg, or with HBSS of the same volume. At 12 hours and 24 hours post-treatment, the tumors were collected, homogenized in acidified isopropanol, and analyzed for doxorubicin fluorescence by HPLC (Figure 9). However, tumors did not grow in the majority of mice injected with MDA-MB-231 cells, and though these results indicate that polyMPC does not increase doxorubicin accumulation in the tumor, there was only one mouse per group and no significant information can be obtained from this data.

3.3 MPC-AF546 Affect on Tissue Weight

Six week old C57BLK/6J mice were injected into the tail with Lysozyme-AF546, Linear MPC-AF546, Branched MPC-AF546, or Grafted MPC-AF546. Blood samples and tissue homogenates were stored at -20^{o}C for future HPLC analysis for AF546 fluorescence. Prior to homogenization, tissues were weighed, and tissue weights were compared to see if there was statistical differences in tissue weight that may indicate toxicity. When compared (Figure 10), there was a significant decrease in lung tissue weight in the grafted polymer group compared to the PBS control (P=0.0038), however there was no significant difference between tissue weights for the other groups or tissues. This decrease in tissue weight may indicate an accumulation and resultant toxicity of the grafted polymer in the lungs.

3.4 NSL2 Muscle Transfection

Five week old C57BLK/6J were injected with 10 uL into the proximal portion of the anterior tibialis muscle, and 10 uL into the distal portion of the anterior tibialis muscle, of either PBS, pEGFP, Jet-PEI-pEGFP (a commercially available linear polyethylenimine tranfection reagent), or NLS2-pEGFP. Four weeks following injection, the anterior tibialis muscles were collected, fixed in 10% formalin, and paraffin embedded. Tissue sections were then treated with GFP antibody to determine protein expression and transfection efficiency. There was not positive GFP staining observed in any of the tissue sections (Figure 11). Sections were then stained with hematoxylin and eosin for tissue architecture (Figure 12). The H&E staining indicates there is no difference in tissue architecture between treatment groups, indicating that NLS2 does not cause tissue toxicity upon direct injection into the muscle.

CHAPTER 4

DISCUSSION & FUTURE DIRECTIONS

By conjugating doxorubicin to polyMPC, the maximum tolerated dose in mice is increased by ten-fold over free doxorubicin and three-fold over Doxil®. Though some weight loss was observed in mice, this was most likely due to experimenter error in administration, not a cause of the polyMPC-doxorubicin, and recovery was observed with return to normal weight ranges. In addition, the cardio-toxicity normally associated with free doxorubicin treatment is circumvented through its conjugation to polyMPC as evidenced through nuclear staining, and is presumably the cause of the increase in the maximum tolerated dose in mice. The conjugation of doxorubicin to polyMPC also increases the blood circulation time of doxorubicin, providing increased exposure time of the drug with a reduced toxicity. We were not able to demonstrate an accumulation of polyMPC-doxorubicin inside the tumor, however, as there were not enough models to determine a lack of accumulation outside of circumstance. Due to difficulty in growing the MDA-MB-231 xenografts, it is possible that an unusual tumor environment was established that deviates in a way to exclude the polyMPC-doxorubicin which would be effective in a normally formed MDA-MB-231 xenograft. There was trend of increased accumulation of polyMPC in the liver and kidneys, however not to a statistically significant amount, and it is possible that this observation was the result of normal excretion of the drug conjugate, and not extratumoral release of doxorubicin from the polymer. Because the tissues were homogenized in acidified isopropanol to release doxorubicin from the polymer for analysis, it is not possible to determine if the accumulated doxorubicin was free to

elicit toxicity in these organs, or if it was still polymer bound and inactive. Since there was no evidence of toxicity in these tissues, it is likely that the doxorubicin was still conjugated and inactive.

The MPC-AF546 polymers showed varying results in tissue weights based on the architecture of the polymers administered, however additional analysis of polymer accumulation in tissues would need to be investigated before conclusions could be drawn from this data. If polymer accumulation coincides with reduced tissue weight, it is possible that the polymer is causing tissue toxicity and tissues should be investigated for apoptosis and altered architecture.

NSL2 was shown not to cause toxicity when injected into the muscle; however transfection of the pEGFP was also not demonstrated. This may be due to the length of time from injection to tissue collection, and not an indication of failed transfection as there was also no positive GFP staining from the JetPEI™ control.

In future, polyMPC-doxorubicin will be tested in vivo for tumor accumulation and treatment efficacy on MDA-MB-231 xenografts to demonstrate its increased efficacy over doxorubicin and Doxil® treatment. Serum samples and tissues from MPC-AF546 treated mice will be analyzed by HPLC to determine blood clearance times and tissue accumulation of the various architectural forms of the polymer to determine its efficacy in protein therapeutics. NSL2 will be tested in highly proliferative cell environments, such as tumor and mammary tissues, to determine its transformation efficiency, as well as being tested in varying polymer:DNA ratios to improve transformation efficiency.

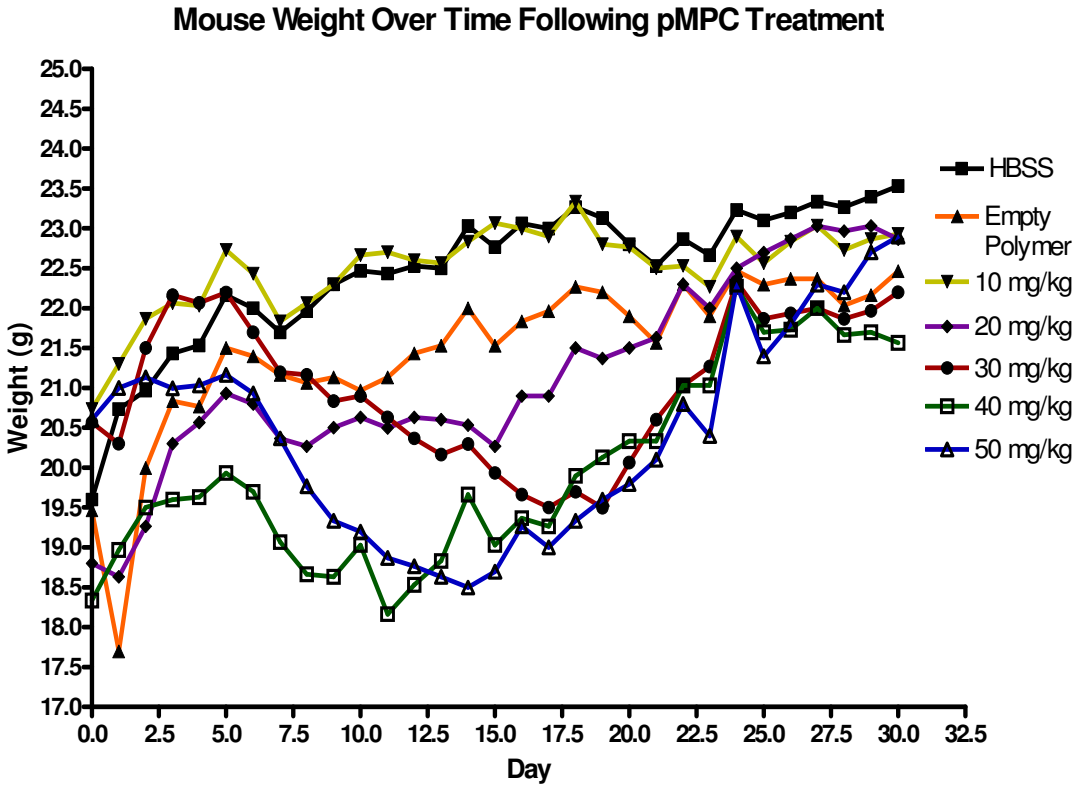

Figure 1 Mouse Weights Following PolyMPC-Doxorubicin Treatment

Three mice per group were injected with 10, 20, 30, 40, or 50 mg of doxorubicin per kg of body weight, or HBSS or empty polyMPC as controls. Mice were weighed and examined for toxicity daily for 30 days. Daily mouse weights were averaged for each group (n=3) and graphed above.

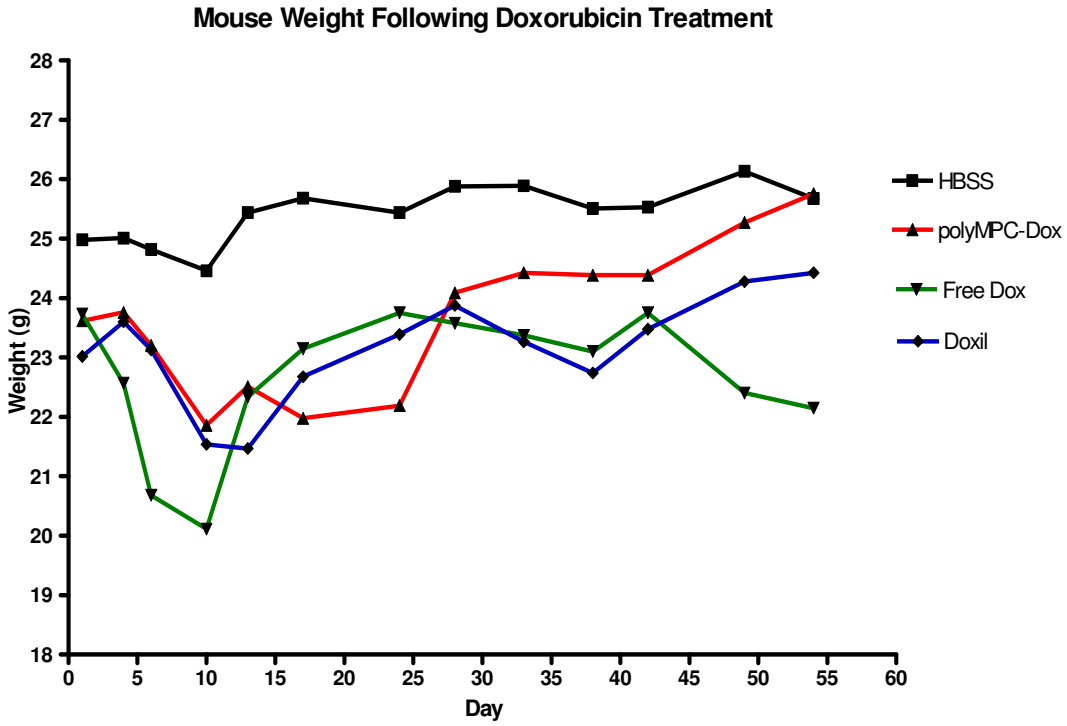

Figure 2 Mouse Weights Following Doxorubicin Treatment

Ten mice per group were injected with the maximum tolerated dose of free doxorubicin (6mg/kg), Doxil® (liposomal doxorubicin, 10 mg/kg), polyMPC-doxorubicin (30 mg/kg), or HBSS as a control. Mice were weighed and examined for toxicity 2 times a week for 54 days. Weights were averaged (starting n=10) and graphed above. Six mice were lost from the free doxorubicin group during the course of the experiment (ending n=4), 3 mice were lost from the polyMPC-doxorubicin group (ending n=7), 2 mice were lost from the Doxil® group (ending n=8), and 1 mouse was lost from the HBSS group (ending n=9).

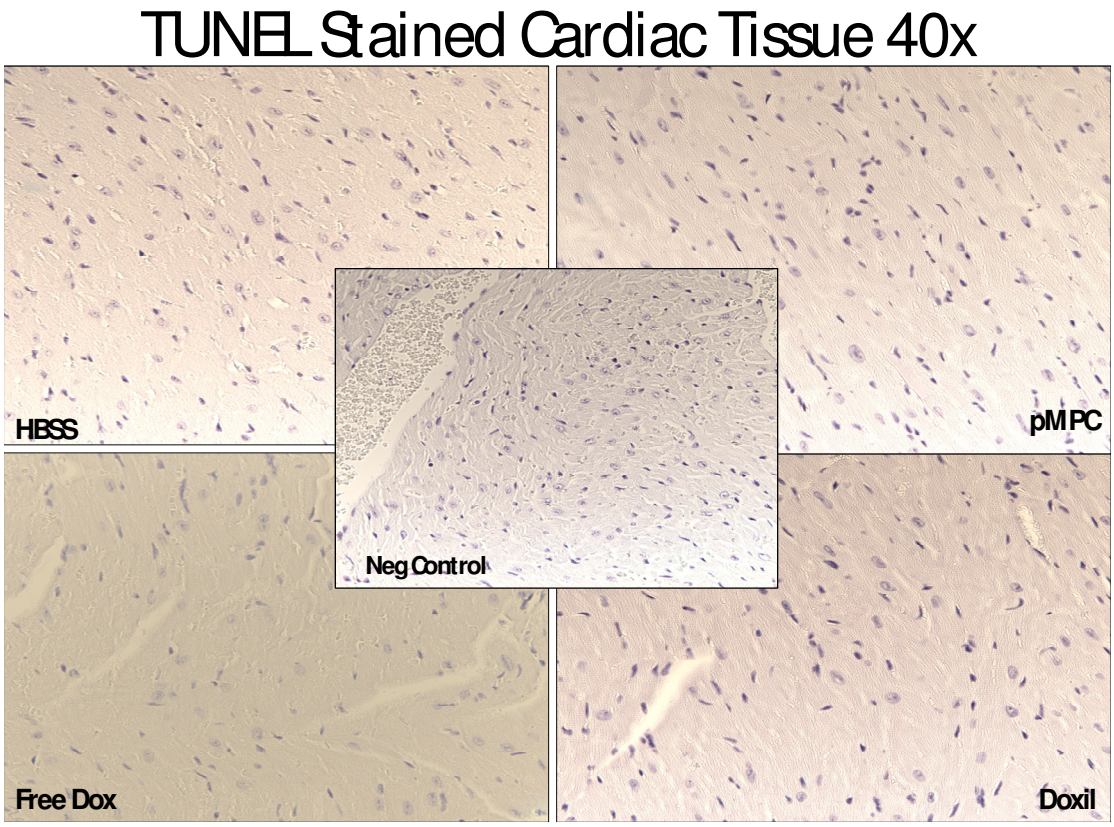

Figure 3 TUNEL Treated Cardiac Tissue of Doxorubicin Treated Mice

Ten mice per group (n=10) were injected with the maximum tolerated dose of free doxorubicin (6mg/kg), Doxil® (10 mg/kg), polyMPC-doxorubicin (30 mg/kg), or HBSS as a control. Following 54 days, or presentation of toxicity, hearts were collected, fixed in 10% formalin, and paraffin embedded. Tissue sections were then labeled with TUNEL for apoptotic cell identification. Positive staining for apoptotic cells was not observed.

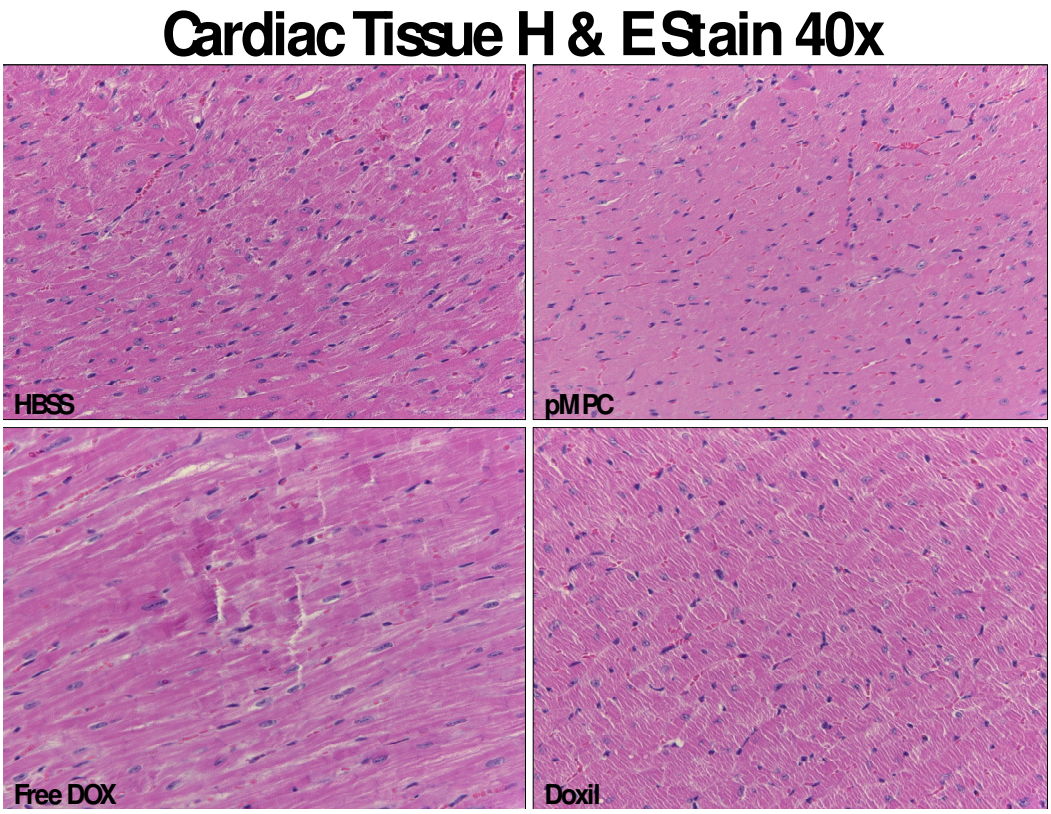

Figure 4 Hematoxylin and Eosin Stained Cardiac Tissue of Doxorubicin Treated Mice

Ten mice per group (n=10) were injected with the maximum tolerated dose of free doxorubicin (6mg/kg), Doxil® (10 mg/kg), polyMPC-doxorubicin (30 mg/kg), or HBSS as a control. Following 54 days, or presentation of toxicity, hearts were collected, fixed in 10% formalin, and paraffin embedded. Tissue sections were then treated with hematoxylin and eosin stain to reveal tissue architecture and cell staining patterns.

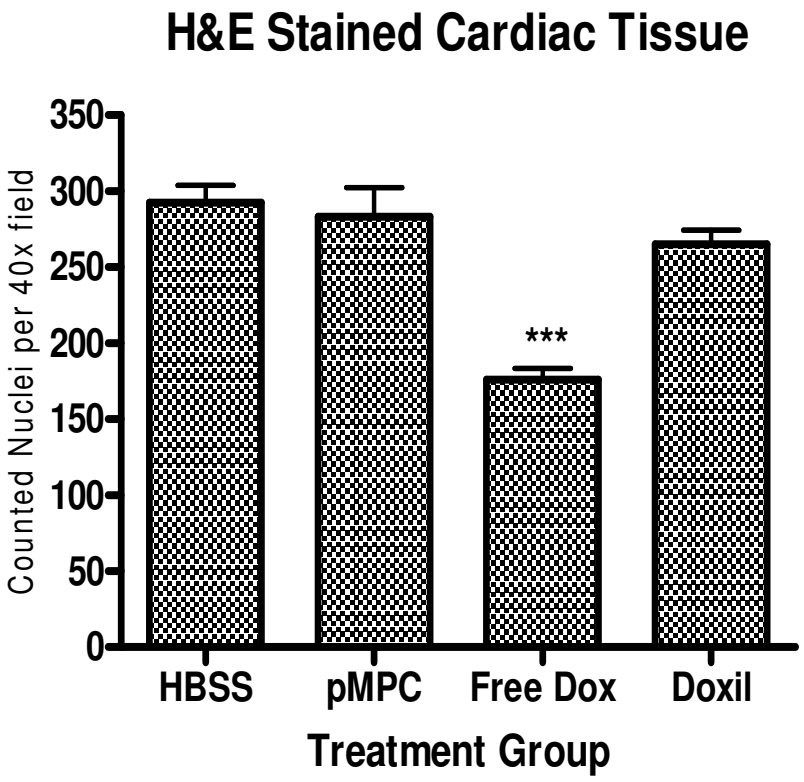

Figure 5 Number of Eosin Stained Nuclei per 40x Field of Vision

Ten mice per group were injected with the maximum tolerated dose of free doxorubicin (6mg/kg), Doxil® (10 mg/kg), polyMPC-doxorubicin (30 mg/kg), or HBSS as a control. Following 54 days, or presentation of toxicity, hearts were collected, fixed in 10% formalin, paraffin embedded, and treated with hematoxylin and eosin stain. Images were collected under 40x magnification bright field microscopy and the total numbers of nuclei in each image were counted and averaged per group (n=10). There was a statistically significant decrease ($P<0.0001$) in nuclei in cardiac tissue collected from free doxorubicin treated mice.

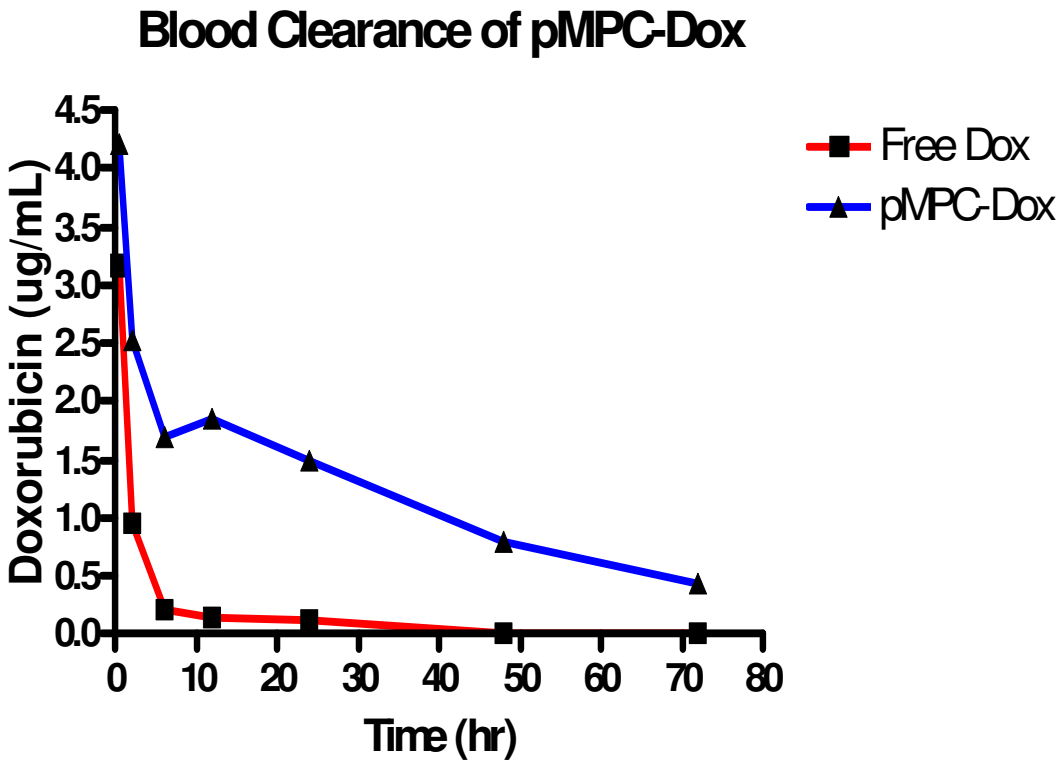

Figure 6 Blood Clearance of PolyMPC-Doxorubicin

Five mice were injected into the tail vein with 6mg/kg of free doxorubicin (n=5), and six mice were injected into the tail vein with 6 mg/kg of polyMPC-doxorubicin (n=6). Blood samples were taken at 30 minutes, 2 hours, 6 hours, 12 hours, 24 hours, 48 hours, and 72 hours post-injection. Serum was collected from the blood and analyzed by HPLC for doxorubicin fluorescence. By 6 hours post-injection, over 93% of free doxorubicin has been eliminated from serum, and doxorubicin levels are not detectable past 48 hours post-injection. At 6 hours post-injection, 40% of PolyMPC-doxorubicin is still present in serum and levels remain detectable at 72 hours post-injection.

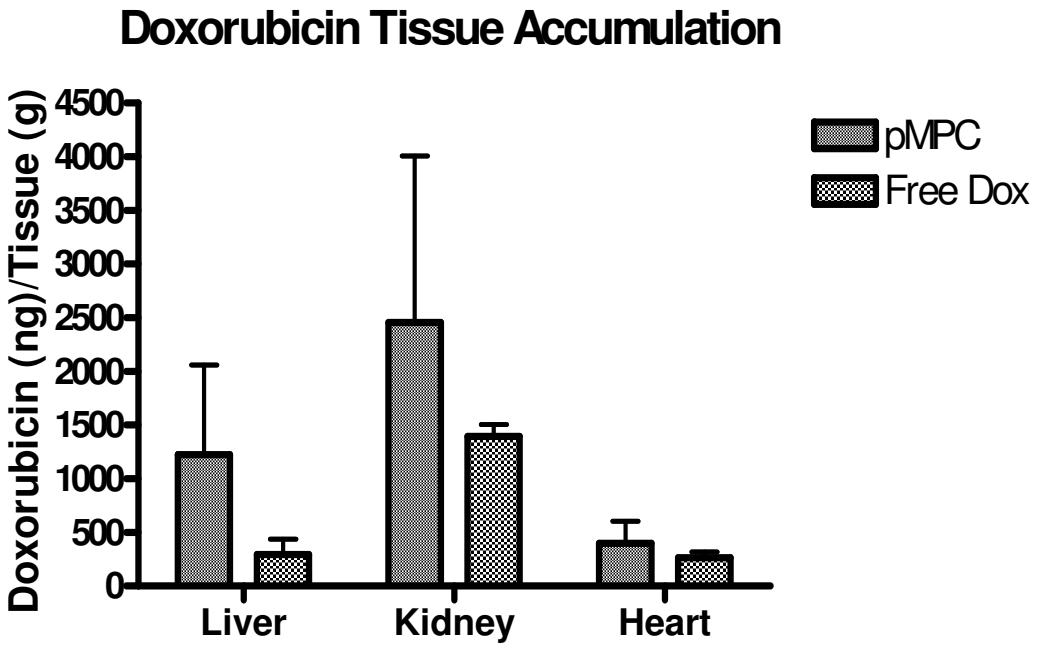

Figure 7 Tissue Accumulation of PolyMPC-Doxorubicin

Five mice were injected into the tail vein with 6mg/kg of free doxorubicin (n=5), and six mice were injected into the tail vein with 6 mg/kg of polyMPC-doxorubicin (n=6). Tissues were collected following 72 hours of treatment, frozen in liquid nitrogen, and tissue homogenates were analyzed by HPLC for doxorubicin fluorescence. Differences in doxorubicin fluorescence in tissues are not statistically significant between treatment groups.

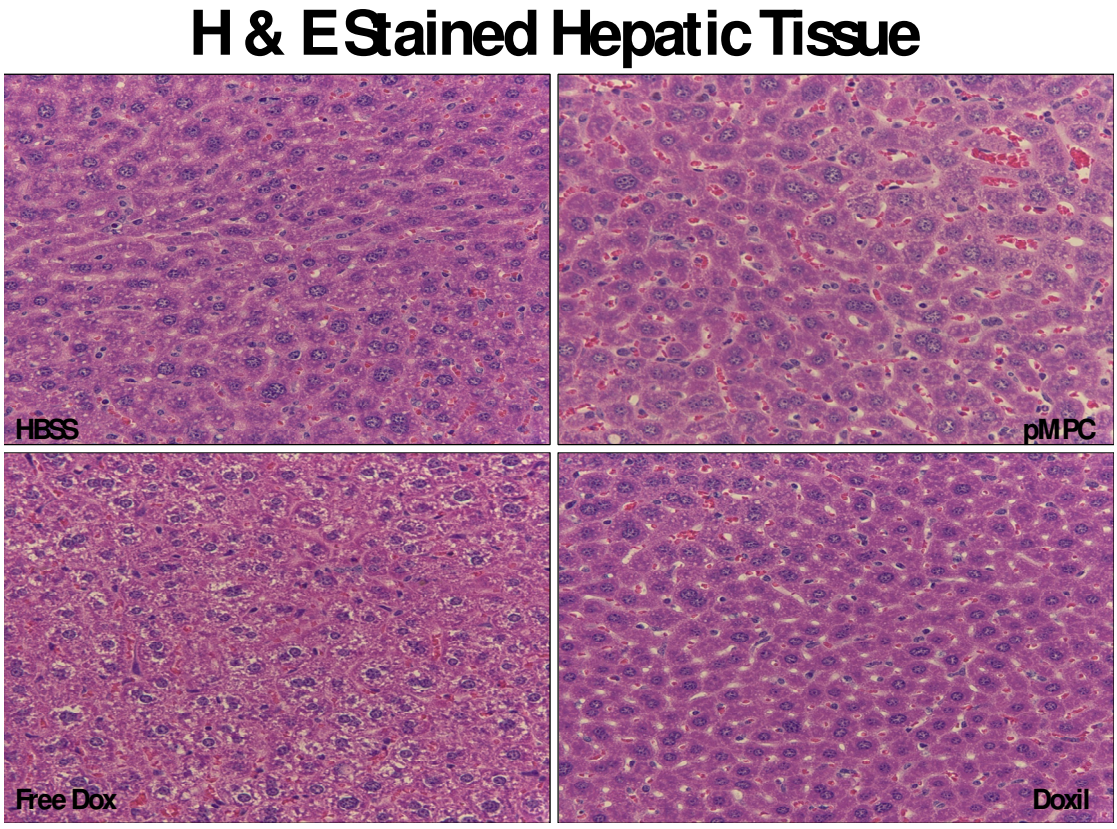

Figure 8 Hematoxylin and Eosin Stained Hepatic Tissue of Doxorubicin Treated Mice Ten mice per group (n=10) were injected with the maximum tolerated dose of free doxorubicin (6mg/kg), Doxil® (10 mg/kg), polyMPC-doxorubicin (30 mg/kg), or HBSS as a control. Following 54 days, or presentation of toxicity, livers were collected, fixed in 10% formalin, and paraffin embedded. Tissue sections were then treated with hematoxylin and eosin stain to reveal tissue architecture and cell staining patterns.

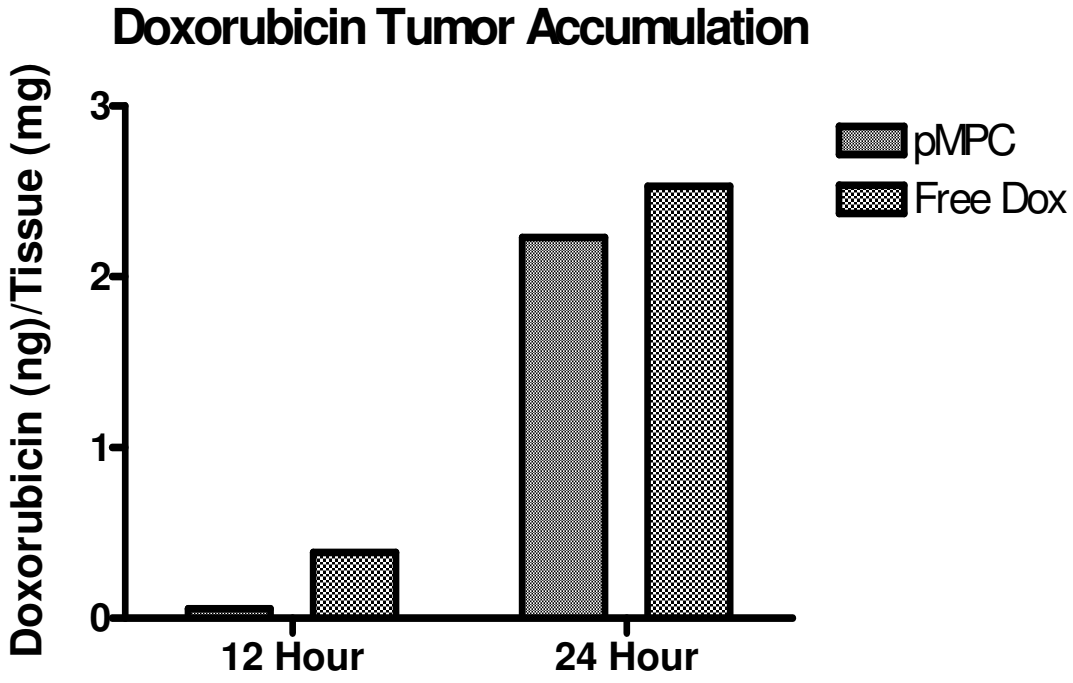

Figure 9 Tumor Accumulation of PolyMPC-Doxorubicin

MDA-MB-231 xenografts were grown in the right flank of six mice to a tumor volume of 100-300 mm^3. Mice were then injected into the tail with 6mg/kg free doxorubicin or polyMPC-doxorubicin, or HBSS as a control. At 12 or 24 hours post-injection, tumors were collected (n=1) and analyzed by HPLC for doxorubicin fluorescence.

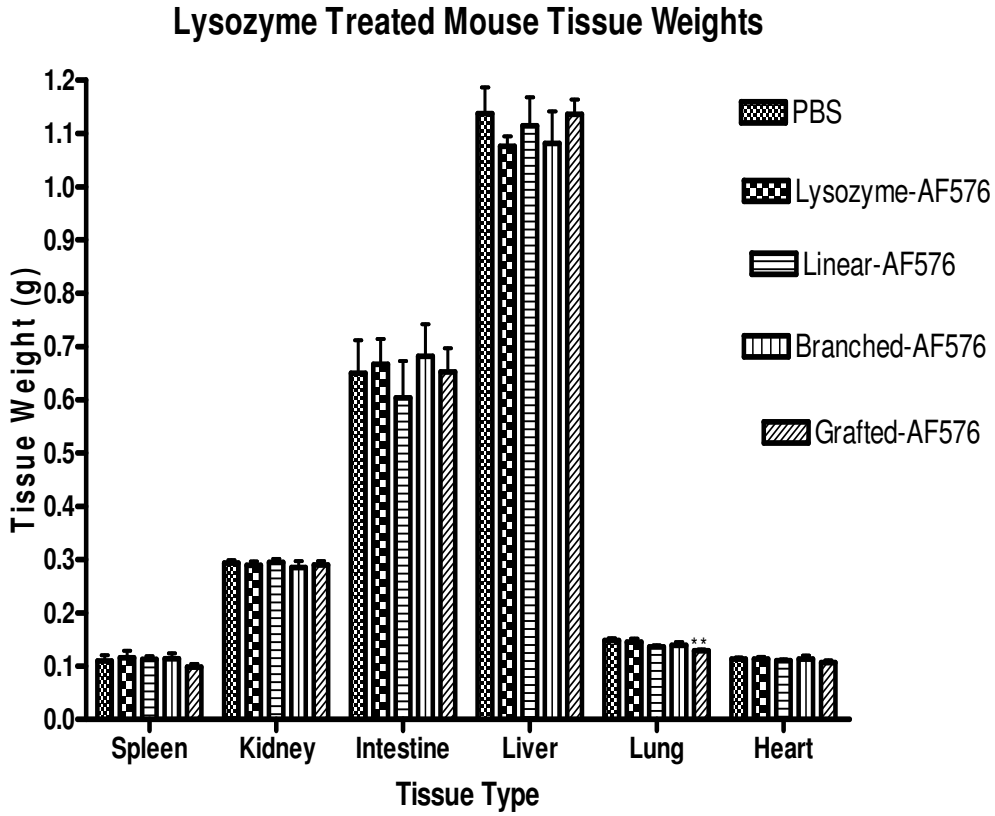

Figure 10 Tissue Weights of Lysozyme Treated Mice

Mice were injected into the tail with three architectural forms of MPC-AF546, or lysozyme-AF546 or PBS as control. Two weeks following treatment, tissues were collected and weighed (n=6). Lungs of mice treated with grafted MPC-AF546 weighed statistically less than lungs of mice treated with PBS (P=0.0038).

All other tissues were not statistically different weights.

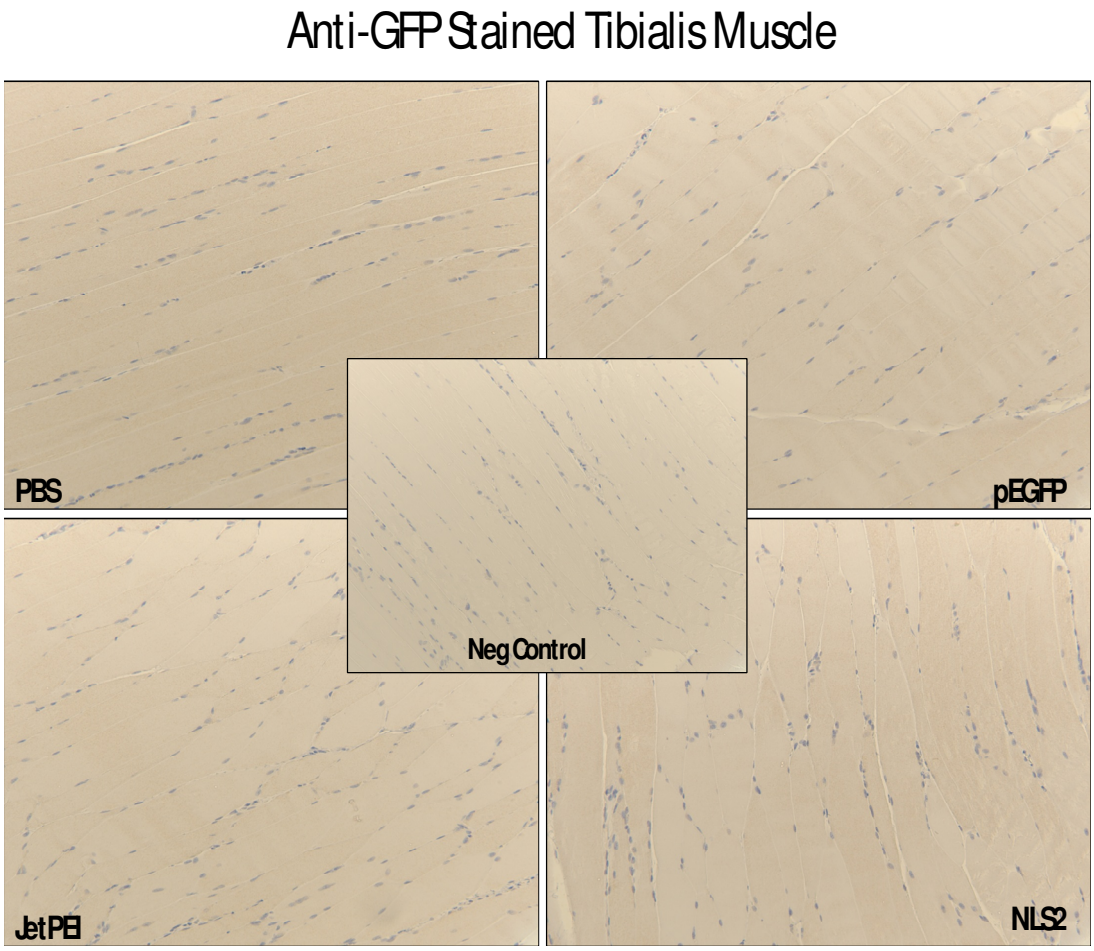

Figure 11 GFP Antibody Staining of Anterior Tibialis Muscles

Mice were injected with 10uL into the proximal anterior tibialis muscle, and 10uL into the distal anterior tibialis muscle, on day 0 and day 7. Tissues were collected 4 weeks post-injection, fixed in formalin, and paraffin embedded. Tissue sections were treated with anti-GFP antibody. No positive antibody staining was observed.

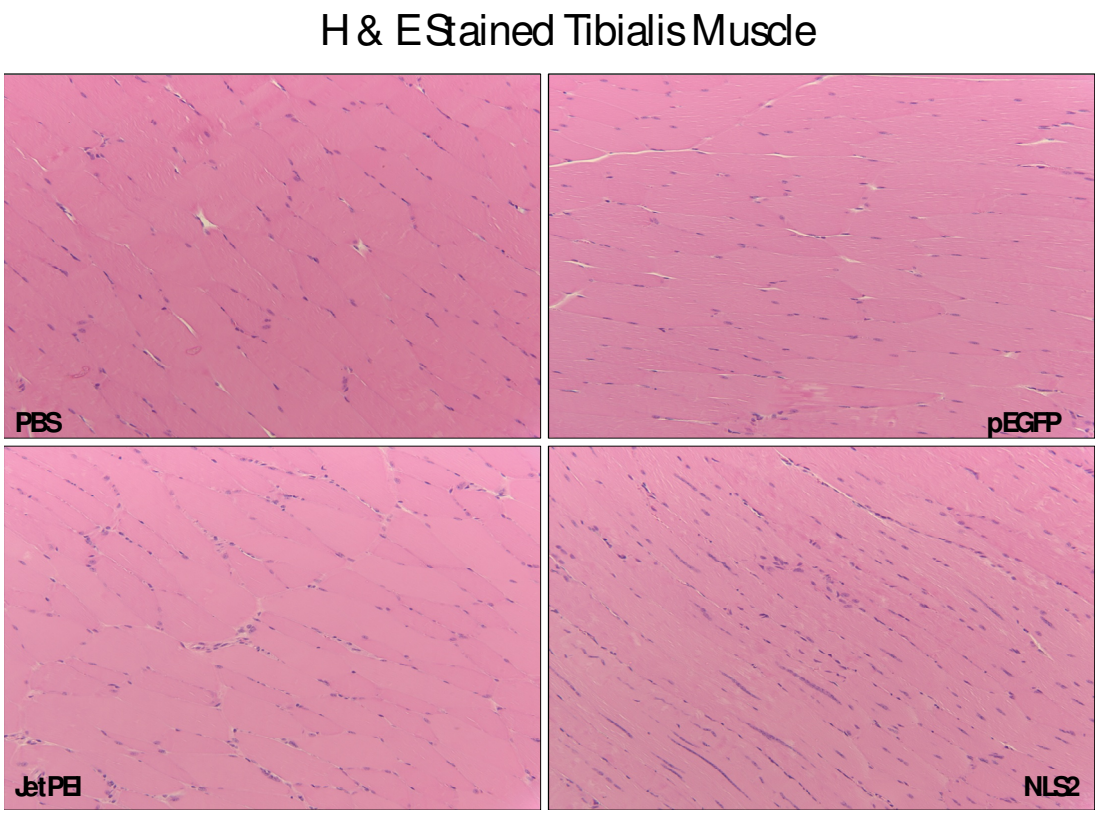

Figure 12 Hematoxylin and Eosin Stained Tibialis Muscles

Mice were injected with 10uL into the proximal anterior tibialis muscle, and 10uL into the distal anterior tibialis muscle, on day 0 and day 7. Tissues were collected 4 weeks post-injection, fixed in formalin, and paraffin embedded. Tissue sections were treated with hematoxylin and eosin stain. No differences in tissue architecture were observed.

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