Development of Targeted Hydrogel Nanoparticles as Delivery Vehicles for Cancer Therapy and Imaging

by

Ming Qin

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Chemistry) in the University of Michigan 2014

Doctoral Committee:

Professor Raoul Kopelman, Chair
Professor Zhan Chen
Professor Adam Matzger
Professor Robert Ziff
To my parents
Acknowledgements

First of all, I would like to give the depth of my gratitude to my advisor, Professor Raoul Kopelman. It has been my great honor to be his student. His great attitude and enthusiasm toward research will always inspire me in my future work as an independent researcher. He has always been most supportive as my teacher and mentor, and has given me the freedom and encouragement to explore new areas of my research. I appreciate all his time, ideas and funding to make my PhD experience fruitful.

I would also like to thank Professor Robert Ziff, who not only helped guide me through my graduate school application process, but also supported me when I experienced difficulty during my second year in Ann Arbor. I still recall with fond memory the time spent in his class of Chemical Engineering 527. I also would like to thank other members on my dissertation committee, Professor Zhan Chen and Professor Adam Matzger, for all their support and valuable insights on my research.

I would like to acknowledge all the current and previous members of the Kopelman group. In particular, I would like to thank Dr. Yong-Eun Koo Lee for her guidance. She helped me on my experimental design, troubleshooting experiments, and proofreading my manuscript. I also wish to thank my friend Teppei Shirakura. We shared many productive discussions on our research and even collaborated on some projects together; he also helped me revise parts of my dissertation. I also thank my friend Dr. Aniruddha...
Ray for the help on microscope experiments, for valuable discussions on research, and for proofreading my dissertation (wish you good luck at NIST). I also appreciate my friends Leshern Karamchand, Hyung Ki Yoon, Remy Elbez, Kristen Herrmann and Akiko Kochi for their camaraderie and valuable comments on my research. I also appreciate all the help from Dr. Hoe Jin Hah, Dr. Shouyan Wang, Dr. Guochao Nie, Dr. Gwangseong Kim and Dr Qinghong Yao. In addition, I also thank Dr. Hong Zong from the Michigan Nanotechnology Institute for Medicine and Biological Sciences, who helped me tremendously on the experiment of peptide conjugation and proofreading my manuscript.

My time at Ann Arbor was made enjoyable in large part due to my dear friends, who became a part of my life. I am grateful for all the time and friendship from Dr. Bo Zhao, Qingjie Zeng, Linsen Li, Dr. Hongliang Xin, Dr. Jian Zhu, Dr. Hao Chen and Dr. Yu Chen.

My greatest appreciation goes toward my family for their love, trust and encouragement. Through thick and thin, they always supported me in all my pursuits. My parents and my sister, I love you all.

Finally, this work could not been finished without the generosity of the funding agencies. I would like to acknowledge the National Institute of Health, the University of Michigan-Shanghai Jiao Tong University, Department of Chemistry, Rackham Graduate School and Gordon Research Conferences for their generous grants and fellowships toward my research and travel to conferences.
# Table of Contents

Dedication .......................................................................................................................... ii
Acknowledgements ........................................................................................................... iii
List of Figures ..................................................................................................................... vii
List of Schemes ................................................................................................................ x
List of Tables ..................................................................................................................... x
Abstract ............................................................................................................................ xiii

Chapter

1. Introduction ......................................................................................................................... 1
   References ....................................................................................................................... 15

2. Methylene Blue covalently loaded Polyacrylamide Nanoparticles for Enhanced Tumor-targeted Photodynamic Therapy ................................................................. 20
   Introduction .................................................................................................................... 20
   Experimental ................................................................................................................. 24
   Results and discussion ................................................................................................. 29
   Conclusion .................................................................................................................... 43
   Acknowledgements ..................................................................................................... 46
   References .................................................................................................................... 47

3. Overcoming Cancer Multidrug Resistance by Codelivery of Doxorubicin and Verapamil with Hydrogel Nanoparticles ................................................................. 49
   Introduction .................................................................................................................... 49
   Experimental ................................................................................................................. 53
   Results and discussion ................................................................................................. 57
   Conclusion .................................................................................................................... 71
   Acknowledgements ..................................................................................................... 74
   References .................................................................................................................... 75

4. Click Conjugation of Peptide to Doxorubicin-loaded Hydrogel Nanoparticles for Targeted Tumor Therapy ................................................................. 77
   Introduction .................................................................................................................... 77
   Experimental ................................................................................................................. 80
   Results and discussion ................................................................................................. 87
   Conclusion .................................................................................................................... 99
   Acknowledgements ..................................................................................................... 103
   References .................................................................................................................... 104
5. Covalent Binding of Coomassie Blue in Polyacrylamide Nanoparticles for in vivo Tumor Delineation..................................................................................106
   Introduction..........................................................................................106
   Experimental .......................................................................................108
   Results and discussion........................................................................112
   Conclusion............................................................................................115
   Acknowledgements................................................................................117
   References ............................................................................................118

6. Summary and Future Directions .............................................................119
   Summary ..............................................................................................122
   Future Directions ................................................................................124
   References ............................................................................................125
List of Figures

Figure 1.1 Schematic diagram of a targeted multifunctional nanoparticle with therapeutic and imaging options for cancer detection and therapy ............................................................16

Figure 2.1 (a) UV-vis spectra of 1 mg mL\(^{-1}\) MBI-PAA NP (MBI loading: 11.0 nmol mg\(^{-1}\)), 1 mg mL\(^{-1}\) MBII-PAA NP (MB loading: 10.4 nmol mg\(^{-1}\)), 11.0 µM MBI and 10.4 µM MBII solutions, when dissolved in DI water. (b) Excitation and emission spectra of 0.3 mg mL\(^{-1}\) MBI-PAA NP (MBI loading: 11.0 nmol mg\(^{-1}\)), 0.3 mg mL\(^{-1}\) MBII-PAA NP (MB loading: 10.4 nmol mg\(^{-1}\)), 3.3 µM MBI and 3.1 µM MBII solutions, when dissolved in PBS buffer (pH 7.4)..........................................................33

Figure 2.2 (a) DLS curves of MBI-PAA NPs (MBI loading: 6.4 nmol mg\(^{-1}\)) and MBII-PAA NPs (MBII loading: 10.4 nmol mg\(^{-1}\)); (b) SEM image of MBI-PAA NPs (MBI loading: 11.0 nmol mg\(^{-1}\)) and (c) SEM image of MBII-PAA NPs (MBII loading: 10.4 nmol mg\(^{-1}\)) ..........................................................33

Figure 2.3 Fluorescence emission spectra of MB in the filtrates of (a) MBI-PAA NP solution (dye loading: 13.8 nmol mg\(^{-1}\)) and (b) MBII-PAA NP solution (dye loading: 12.9 nmol mg\(^{-1}\)) after dye leaching test, showing no measurable leaching of MB from the NPs ......................................................................................................................36

Figure 2.4 (a) Fluorescence spectra of ADPA in a mixed PBS buffer solution (pH 7.4) with 0.3 mg mL\(^{-1}\) MBII-PAA NP (MBII loading: 10.4 nmol mg\(^{-1}\)) solution, after irradiation at 660 nm for 0, 1, 3, 5, 10, and 15 min. (b) Linear fit on the change of ADPA fluorescence intensity at 406 nm to irradiation time, showing the k value of MBII-PAA NPs (MBII loading: 10.4 nmol mg\(^{-1}\)) was 5.1×10\(^{-4}\) s\(^{-1}\)..................................................37

Figure 2.5 k value vs. MB concentration and MB loading of the NPs in MBI-PAA NPs and MBII-PAA NPs solution (0.3 mg mL\(^{-1}\) in PBS buffer) ..........................................................37

Figure 2.6 UV-vis spectra of MBII-PAA NPs (1 mg ml\(^{-1}\) in DI water (a) and fluorescence intensity vs. MB loading in MBII-PAA NPs (0.3 mgml\(^{-1}\) in PBS buffer, excitation / emission: 660 nm/ 680 nm) (b). S1 to S6 in (a) are MBII-PAA NPs with MB loading of 5.5 nmol mg\(^{-1}\), 8.7 nmol mg\(^{-1}\), 10.4 nmol mg\(^{-1}\), 12.0 nmol mg\(^{-1}\), 14.0 nmol mg\(^{-1}\)and 30.1 nmol mg\(^{-1}\) respectively ..........................................................38

Figure 2.7 Enzymatic reduction test results: normalized fluorescence emission intensity at 680 nm of MB (3 µM), MBI (3 µM), MBII (3 µM), MBI-PAA NP (0.3 mg mL\(^{-1}\), dye loading: 11.0 nmol mg\(^{-1}\)) and MBII-PAA NP (0.3 mg mL\(^{-1}\), dye loading: 10.4 nmol mg\(^{-1}\)) solutions. All samples were dissolved in PBS buffer (pH
and mixed with 0.45 µmol NADH and 0.05 mg diaphorase. The photobleaching effect of MB was removed from all the curves.......................... 39

Figure 2.8 Confocal microscopy images of MDA-MB-435 cells after 15 min incubation with (a) F3-MBI-PAA NPs and (b) PEG-MBI-PAA NPs. NP concentration: 0.3 mg mL\(^{-1}\); dye loading: 11.0 nmol mg\(^{-1}\); scale bar: 50 µm ......... 42

Figure 2.9 Confocal microscopy images of MDA-MB-435 cells stained with calcein-AM (green, living cell) and PI (red, dead cell), using a 20× or 40× objective lens, respectively. The cells were incubated with F3-targeted or PEGylated NP solution (0.1 mg mL\(^{-1}\) in PBS buffer (pH 7.4)) for 15 min, respectively: (a) F3- MBI-PAA NPs; (b) PEG-MBI-PAA NPs; (c) F3- MBII-PAA NPs; (d) PEG-MBI-PAA NPs. After incubation, the cells were illuminated at 647 nm with 400 µW of power (ca. 100 J cm\(^{-2}\)) for 1 min. These images were taken before illumination and 20 min after illumination. In the images after illumination (20×), only the cells in the circled central area were illuminated. Scale bar: 50 µm ........................................... 44

Figure 2.10 The viability of MDA-MB-435 cells from MTT assay, after incubation for 1 h with 0.2 mg mL\(^{-1}\) MBI-PAA NP solution (Dye loading: 11.0 nmol mg\(^{-1}\)) and 0.2 mg mL\(^{-1}\) MBII-PAA NP solution (Dye loading: 10.4 nmol mg\(^{-1}\)), respectively. These results showed the dark toxicity of MBI-PAA NPs and MBII-PAA NPs. The average value was obtained from 7 samples. Error bars indicate the standard deviation.......................................................... 45

Figure 3.1 Size distribution of hydrogel NPs from DLS (a) and SEM image (b)..... 59

Figure 3.2 Diameter of hydrogel NPs (from DLS) vs. NaCl concentration in DI water. Error bars indicate the standard deviations................................. 60

Figure 3.3 Degradation of Dox in Dox solution and Dox-loaded NP solution in PBS buffer at 37°C. Dox concentration: 20 µg/mg; Dox/NPs: 2 wt%. Error bars indicate the standard deviations .......................................................... 62

Figure 3.4 Release behaviours of Dox (a) and Vera (b), from hydrogel NPs with varying ratio of CEA in NP matrix. Dox/NPs: 2 wt%; Vera/NPs: 2 wt%; NP concentration: 1 mg/ml. Error bars indicate the standard deviations ................................................. 64

Figure 3.5 (a) Confocal microscopy images of NCI/ADR-RES cells after incubation with free Dox, Dox-NPs, free Dox + free Vera and Dox-NPs + Vera-NPs for 6 h. The nucleus is stained with the Hoechst dye (blue). Top: fluorescence signals from Dox (red); bottom: overlap of the fluorescence signals from Dox (red) and Hoechst dye (blue). (b) Histogram analysis of pixel intensity of Dox in (a). Dox concentration: 5 µM; Vera concentration: 5 µM; scale bar: 25 µm. Error bars indicate the standard deviations .................................................................................. 65

Figure 3.6 Confocal microscopy images of NCI/ADR-RES cells after 6h incubation with Dox-loaded FITC-NPs and Vera-loaded FITC-NPs. (a) Fluorescence signal from Dox (red) in the cells; (b) fluorescence signal from FITC-NPs (green) in the cells; (c) overlap of the fluorescence signal (orange) from Dox and FITC-NPs.
Incubation time: 6 h; Dox concentration: 5 µM; Vera concentration: 5 µM; NP concentration: 1 mg/ml; scale bar: 25 µm

Figure 3.7 Viability of NCI/ADR-RES cells after incubation with blank NPs (1 mg/ml) and Vera (5 µM) for 2 days. Error bars indicate the standard deviations.

Figure 3.8 Viability of NCI/ADR-RES cells after incubation with free Dox, Dox-NPs, free Dox + free Vera, Dox-NPs + free Vera and Dox-NPs + Vera-NPs for 2 days. Dox/NPs: 7.7 wt%; Vera/NPs: 2 wt%; Vera concentration: 5 µM. The total concentration of NPs in the codelivery of Dox-NPs + Vera-NPs is 0.23 mg/ml when the concentration of Dox is 20 µM. Error bars indicate the standard deviations.

Figure 4.1 ¹H NMR spectra of PAA NPs (a), co(CEA-AA) NPs (b) and azide-functional co(CEA-AAm) NPs (c).

Figure 4.2 (a) Mass spectra of F3 peptide (on the top) and alkyne-F3 peptide (on the bottom).

Figure 4.3 HPLC results of F3 and alkyne-F3.

Figure 4.4 FTIR spectra of NPs and azide-NPs.

Figure 4.5 Release profile of Dox from unmodified NPs and F3-targeted NPs in PBS buffer. NP concentration: 0.2 mg/ml; Temperature: 37°C; Loading of Dox/NPs: 8.68±0.16 wt%; loading of Dox/F3-NPs: 8.48±0.15 wt%.

Figure 4.6 Confocal microscopy images of 9L, MCF-7 and NCI/ADR-RES cells after incubation with F3-targeted NPs and nontargeted NPs for 2 h respectively. NPs were labeled with Fluor 488 (green), while the cell nucleus was stained with DAPI (blue). NP concentration: 1.0 mg/ml. scale bar: 25 µm.

Figure 4.7 Blocking (“saturation”) effect of free F3 peptide on nucleolin sites, preventing cell-incorporation of F3-NPs. Pixel intensity analyses of confocal microscopy images of 9L cells. The cells were incubated with varying concentration of F3 peptide for 30 min, after that the cells were incubated with F3-NPs (1 mg/ml) for 2 h. NPs were labeled with Fluor 488. The results showed that with increasing concentration of F3 peptide, the binding of F3-targeted NPs to 9L cells was inhibited significantly. NP concentration: 1.0 mg/ml.

Figure 4.8 Pixel intensity analyses of confocal microscopy images of 9L cells after incubation with F3-targeted NPs (1 mg/ml) or nontargeted-NPs for 2 h at 4 °C or 37 °C. NPs were labeled with Fluor 488. The results showed that the uptake of F3-targeted NPs into 9L cells was affected by the incubation temperature, which may be an energy-dependent process. NP concentration: 1.0 mg/ml.

Figure 4.9 Viability of 9L cells after incubation with Dox-NPs and Dox-F3-NPs (loading of Dox/NPs: 2 % wt). The cells were incubated with NPs for 4 h. After that, the medium was replaced and the cells were left to incubate for 48 h. The NP concentration was 0.05 mg/ml, when the Dox concentration was 2 µM.
Figure 4.10 Viability of 9L cells after incubation with F3-NPs. The cells were incubated with NPs for 4 h. After that, the medium was replaced and the cells were continued to incubate for 48 h ............................................................. 101

Figure 4.11 Viability of NCI/ADR-RES cells after incubation with Dox-NPs and Dox-F3-NPs (loading of Dox/NPs: 10 % wt). The cells were incubated with NPs for 4 h. After that, the medium was replaced and the cells were left to incubate for 48 h. The NP concentration was 0.1 mg/ml, when the Dox concentration was 20 µM .... 102

Figure 4.12 Viability of NCI/ADR-RES cells after incubation with F3-targeted NPs. The cells were incubated with NPs for 4 h. After that, the medium was replaced and the cells were continued to incubate for 48 h ................................................................. 102

Figure 5.1 (a) Size distribution of CB-PAA NPs from dynamic light scattering; (b) SEM image of CB-PAA NPs ........................................................................................................... 111

Figure 5.2 UV-vis absorption spectrum of CB-PAA NPs in DI water (0.1 mg/ml) 114

Figure 5.3 (a) Photographs of representative tumors in BTW models, at 0, 60, and 120 min after injection of F3-targeted CB-linked NPs (A), nontargeted CB-linked NPs (B) and nontargeted CB-loaded NPs (C). The tumor margin in each animal was marked by a white dashed line on the initial (0 min) images. (b) Colorimetric analysis results with ImageJ, to quantify the degree of color change in the tumor. The difference in red hue is the best method to reflect the visual difference between tumor and normal brain tissue........................................................................... 116
List of Schemes

Scheme 2.1 Molecular structure of (a) methylene blue (MB), (b) 3, 7-bisallylmethylene blue (MBI), and (c) 3,7-bismethylacrylamide methylene blue (MBII) ................................................................................................................................. 24

Scheme 2.2 Preparation and F3-targeting of polyacrylamide nanoparticles .......... 26

Scheme 3.1 Molecular structures of acrylamide (AAm), 2-carboxyethyl acrylate (CEA) and 3-(acyloxyloxy)-2-hydroxypropyl methacrylate (AHM) ......................... 58

Scheme 3.2 Delivery into drug-resistant tumor cells of both doxorubicin (derails DNA) and verapamil (blocks efflux pump) with hydrogel NPs ........................................... 73

Scheme 4.1 (a) Synthesis of azide-F3 peptide (TCEP: reducing agent); (b) Synthesis of F3-targeted co(CEA-AAm) NPs via carbodiimide chemistry and click chemistry 82

Scheme 5.1 Synthesis of CB-APMA .................................................................................. 108
List of Tables

Table 2.1 Best k values of MB-encapsulated NPs, MBSE-PAA NPs, MBI-PAA NPs and MBII-PAA NPs in PBS buffer (pH 7.4) .............................................................................................. 39

Table 3.1 Size (diameter) and zeta potential of hydrogel NPs from DLS. Data is shown as mean ± standard deviation ......................................................................................................................... 60

Table 3.2 IC_{50} value of Dox in the formulation of free Dox, Dox-NPs, free Dox + free Vera, Dox-NPs + free Vera and Dox-NPs + Vera-NPs. IC_{50} of Dox is the concentration of Dox required to cause 50% cell killing.................................................. 71

Table 4.1 Size and zeta potential of NPs, azide-NPs and F3-targeted NPs from DLS87

Table 4.2 Pixel intensity analyses of Fluorescence microscopy images of 9L, MCF-7 and NCI/ADR-RES cells after incubation with nontargeted-NPs or F3-targeted NPs for 2 h. NPs were labeled with Fluor 488.................................................................................................................. 96
Abstract

Nanotechnology can provide powerful delivery carriers for cancer therapy. This dissertation addressed some key challenges for the development of nano-drug carriers: targeted therapy, multidrug resistance (MDR) and codelivery of multiple kinds of drugs. A variety of novel hydrogel nanoplatforms were developed for cancer therapy and imaging.

Two kinds of methylene blue-conjugated polyacrylamide (PAA) nanoparticles (NPs) were developed for targeted photodynamic therapy, where methylene blue (MB) was the photosensitizer. The conjugation of MB onto NPs improved the MB loading, prevented any leaching of MB and avoided MB degradation due to the effects of enzymes. After decoration with a tumor-targeting F3 peptide, the MB-PAA NPs showed a selective cell killing ability towards MDA-MB-435 melanoma cells in vitro, under illumination.

A highly engineerable hydrogel NP was developed for the optimal codelivery of a chemodrug, doxorubicin (Dox) and a chemosensitizer, verapamil (Vera), aiming at alleviating tumor MDR. The NP was based on the copolymer of acrylamide (AA) and 2-carboxyethyl acrylate (CEA). Dox and Vera were post-loaded into the respective co(CEA-AA) NPs. We delivered both Dox-NPs and Vera-NPs into the Dox-resistant NCI/ADR-RES cells. This codelivery increased the intracellular accumulation of Dox,
and significantly enhanced the cell killing ability of Dox with respect to NCI/ADR-RES cells in vitro.

For the targeted delivery of Dox, the co(CEA-AA) NP was further conjugated with F3 peptides via a “click reaction”. The F3 peptide targeting moieties dramatically improved the uptake of the NPs by the nucleolin-overexpressing glioma cell line 9L as well as by the drug-resistant cell line NCI/ADR-RES, correlating with nucleolin-mediated endocytosis. In vitro cytotoxicity results show that the Dox-F3-NPs have a stronger cell killing ability, towards both 9L and NCI/ADR-RES cells, than the nontargeted Dox-NPs.

Finally, a tumor-targeted, intensely blue colored, tumor contrast agent was introduced, based on targeted, Coomassie Blue (CB)-linked, PAA NPs. With high loading of CB (7 wt %), the NPs can clearly stain the tumor area, with a tolerable NP concentration. Again, the F3-targeted CB-PAA NPs demonstrated a better contrast delineation effect, as well as a longer retention time in the tumor area, in 9L-glioma bearing rats, compared to nontargeted CB-PAA NPs.