Summer 8-14-2020

Ligand-Installed Polymeric Nanocarriers for Combination Chemotherapy

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LIGAND-INSTALLED POLYMERIC NANOCARRIERS FOR COMBINATION CHEMOTHERAPY

By

Xinyuan Xi

A DISSERTATION

Presented to the Faculty of The Graduate College in the University of Nebraska
In Partial Fulfillment of the Requirements For the Degree of Doctor of Philosophy

Pharmaceutical Sciences Graduate Program

Under the Supervision of Professor Tatiana K. Bronich
University of Nebraska Medical Center
Omaha, Nebraska

June, 2020

Supervisory Committee:

Dr. Joseph Vetro
Dr. Jered Garrison
Dr. Hamid Band
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Pursuing this Ph.D. has been a truly life-changing experience. This would not have been possible without the support and guidance of many people who helped me to shape my graduation. At this special moment, I would sincerely like to appreciate all of them for invaluable support.

First and foremost, my greatest debt of gratitude goes to Dr. Tatiana Bronich for her mentorship and guidance over the course of my Ph.D. training. Dr. Bronich introduced the seed of an idea for research to me and provided all the necessary means for the growth. During the journey to nurture this seed, she was always readily available to discuss and troubleshoot or give feedback if problems arose, such as a plague, hail or drought, despite keeping a busy schedule. Her constant encouragement and motivation helped me to stay productive and focused, especially during tough times. Without her faith in me, I would not have reached the end of my Ph.D. and learned the art of science that allowed me to propose alternative ways in this process. Her qualities of critical thinking and attention to details has changed my way of doing research and handling life changes. I was grateful to be in a training environment provided by Dr. Bronich that was filled with diverse research opportunities and practical experiences that have prepared me to work with unique teams of cross-disciplinary collaborators.

I would extend my gratitude to the members of my advisory committee, which include Dr. Hamid Band, Dr. Jered Garrison, and Dr. Joseph Vetro. They shaped my graduation work effectively with their expert guidance and constructive criticism. Special thanks to Dr. Gautam Nagsen. Dr. Nagsen always made time to help me with pharmacokinetics analysis, no matter how busy his schedule was. I sincerely appreciate his kind help. Furthermore, I want to thank our collaborators, Dr. Adam Karpf, for providing kind help in an animal study of my project, and Dr. Rihe Liu, for sharing crucial materials for my project.
I am also thankful to Xinyue Li in Dr. Jennifer Black’s lab for teaching me some basic biological techniques, including Western Blotting.

During my graduate study, one of the most fortunate things for me was to work under Saul (Dr. Fan Lei) and learn all the technical skills from him that were necessary for my Ph.D. work. After Dr. Bronich, Saul was the one who inspired me the most. I enjoyed each one of our discussions about my research projects. His hard working and dedication to details have set a high standard for us juniors and encouraged me to work harder. No matter how busy he is, he always made time to answer my endless questions and helped me with my experiments. I wouldn’t have finished studies in a timely manner without the help from him. I thank him for not only being a dependable colleague but also a sincere friend who supported me at every stage of my career. I am grateful to Dr. Svetlana Romanova for being a great senior to work with. She always gave me the warmth of family and invited me for her family trip on my first Thanksgiving holiday in United States. I thank her for making me feel at home in this foreign land. I also would like to thank other members (previous and present) of our Nanomedicine group, including Dr. Shaheen Ahmed, Dr. Jinjin Zhang, Dr. Swapnil Desale, Dr. Hangting Hu, Dr. Tong Liu, and Dr. Chantey Morris for being supportive coworkers. A tremendous thank you to administrative staff, including Renee B Kaszynski, Katina Winters, Jamie Arbaugh, Christine Allmon, and Keith Sutton, for their support. A special thanks to Renee for always be there supporting me and giving me solutions to everything I ask for. I wish all of you plenty of success and happiness in life. I would like to acknowledge the technical assistance from UNMC core facilities in my research and financial support from NIH and UNMC Graduate Assistantship.

This journey to seeking the truth would have been very dull without my dearest friends: Xiaoyu Zhang. We have shared the sweetest and the sourest tastes of this journey with each other. I would like to thank all friends that I made in Omaha, especially Saul, Zhen,
Jian, Kai, Zhiyi, Feng, Xiaoxiao, Baiyan, Lingyun, and Xinyue. I do not know how to persist for the last few years without the company of my friends. I appreciate everyone who supported and encouraged me, and I wish them all the best to achieve their goals!

Last but not least, I thank my family for encouraging me in all of my pursuits and inspiring me to follow my dreams. I am extremely grateful to my parents for teaching me to learn, to be happy, to be grateful, and to understand myself and others. Special thanks to my uncle, aunt-in-law, and my little brother Weizhou for always being supportive. I feel so blessed to be part of my family and be the child of my parents. I wish all of them happy and healthy!

Xinyuan Xi

June 2020
Combination chemotherapy remains the mainstay of cancer treatment because such strategy targets different cell signaling pathways to decrease the likelihood of developing protective mechanisms by cancer cells, thereby delaying the onset of recurrence and prolonging the survival. The co-delivery of binary drug combination via a single nanocarrier provides benefits in reducing dose-limiting toxicities, improving the pharmacokinetic properties of the cargo, spatial-temporal synchronization of drug exposure, and synergistic therapeutic effects. Rational design of such regimen is crucial for maximizing the therapeutic effects since only certain drug ratios exposed to the target might be synergistic while other ratios exert additive or even antagonistic effects. Cisplatin-based chemotherapy has shown great responses in several cancer types and has been used as the standard systemic anticancer treatment, such as triple negative breast cancer (TNBC) and ovarian cancer. However, tumor cells are becoming less responsive to cisplatin, which arises from altered signaling patterns of cancer cells, such as activation of pro-survival signal transduction (e.g., epidermal growth factor receptor (EGFR), AKT, PI3-kinase) and DNA-damage repair, etc. EGFR is a member of the HER family of oncogenic receptor tyrosine kinases (RTK), which actively participates in sustaining the growth and the survival of carcinoma cells as well as the pathogenesis and progression of different carcinoma types. It is frequently overexpressed in various cancer types, such as TNBC
and ovarian cancer, making it a major therapeutic target for the development of targeted drug delivery. A combination of EGFR inhibitors with cisplatin have shown strong synergistic effects in EGFR overexpressing cancers both in preclinical and clinical studies. Moreover, the combined regimen significantly outperformed the single treatment as reported. It has been shown preclinically that EGFR inhibitors significantly potentiate the cytotoxic effects of cisplatin in tumors with overexpressed EGFR through blocking oncogenic signal transduction and unfavored pro-survival signals induced by platinum-based therapy. However, inhibiting EGFR could trigger underappreciated resistance and activate the parallel oncogenic signaling pathways through other members of HER family. Using a pan-HER inhibitor is a common strategy to avoid this limitation. To this end, neratinib (NRT) (an FDA-approved pan-HER inhibitor) and cisplatin (CDDP) were selected as the combination regimen to treat EGFR+ cancers: TNBC and ovarian cancer. To incorporate these two molecules with varied physicochemical properties in the same carrier, we designed a biocompatible crosslinked polypeptide-based nanogel (NG) with multifunctional compartments. Such biodegradable platform provides flexibility in adjusting size, loading capacity, surface properties, deformability, softness, and responsive behaviors by tuning the chemical compositions as well as crosslinking levels. For the purpose of the current study, NGs prepared from copolymers poly (ethylene glycol)-block-poly (L-glutamic acid) modified with phenylalanine functionalities ((PEG-P(Glu-Glu/Phe)x150) were developed for simultaneous loading and delivery of binary CDDP and NRT combination. Such NGs have 1) a hydrophilic PEG shell for less RES uptake and extended circulation, 2) an anionic crosslinked core, which incorporates CDDP through coordination with the carboxylic groups of PGlu, 3) hydrophobic regions formed by Phe moieties, which serves as a reservoir for NRT solubilization. By tuning ratio of constituent hydrophilic and hydrophobic moieties, NGs displayed varied dimensions, drug loading capacities, deformability, performance in killing tumor cells, as well as penetrations in
multicellular 3D tumor models. Consistent with properties of chemical composition, NGs with high hydrophobic fraction displayed less swelling ability and more efficient hydrophobic drug loading. Resulting binary drug combination-loaded NGs functionalized with 50 units of hydrophobic Phe were able to encapsulate both CDDP and NRT at a molar ratio of 2:1 and displayed the strongest synergistic effect towards EGFR+ TNBC cells compared with other screened regimens. Such superior synergy was found to be selective and only displayed in the EGFR+ TNBC cell line. Notably, NRT was found to reverse pro-survival signal transduction by CDDP mediated EGFR/Akt/Erk activation as well as increased Cyclin D1 expression, which was believed to be the molecular basis for the synergistic effect of the combination of CDDP and NRT. Drug-loaded carrier system exerted the highest synergy was selected in the following studies targeting EGFR overexpressing ovarian cancer. However, this carrier system solely depends on the EPR effect for drug accumulation in tumor sites, which is limited by heterogeneity among tumor masses as well as vascular density nearby influencing the permeability of nanocarriers. Installing targeting ligands on the surface of carriers to specifically target biomarkers overexpressed on the surface of tumor cells is a well-suited strategy to overcome mentioned limitations and increase retention of payloads in tumors. In the next part of our study, two targeting ligands aiming to bind with EGFR was chosen in our study: L-AE peptide and EGFR-A protein with reported high binding specificity and affinity to EGFR with acceptable stability. Two types of ligand-installed NGs both demonstrated success in significantly increased cellular uptake in EGFR+ ovarian cancer cells as compared to nontargeted NGs. Further optimization was conducted by extending the thickness of PEG stealth layer from 114 monomer units to 228 units for sufficient protection from opsonization. Optimized PEG-based polymeric NGs displayed more favored PK properties, such as remarkably less spleen uptake, higher drug retention in tumor sites, slower clearance from circulation, and more drug exposure. NGs with EGFR targeting
ligands further improved the PK profile by directing remarkably more drugs to the target sites. When tested in vivo, EGFR targeted peptide and protein decorated NGs carrying CDDP and NRT drug combination significantly suppressed the growth of intraperitoneal high-grade serous ovarian tumor xenografts outperforming their nontargeted counterparts without extending their cytotoxicity to the normal tissues. We also confirmed the importance of simultaneous administration of the (CDDP+NRT) via a single NG system which provides more therapeutic benefits than a cocktail of individual drug-loaded NGs administered at equivalent doses. Lastly, our data demonstrated the benefits of local treatment by showing that intraperitoneal (IP) administration of targeted binary drug combination-loaded NGs can be more effective in terms of tumor growth suppression. These data have shown the power of our carrier system in the delivery of a drug combination to treat EGFR overexpressing cancers.
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<td>(^1\text{H} \text{NMR} )</td>
<td>Proton Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>ABS</td>
<td>Acetate Buffered Saline</td>
</tr>
<tr>
<td>ADC</td>
<td>Antibody Drug Conjugates</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine Aminotransferase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BGlu</td>
<td>L-glutamic Acid γ-benzyl Ester</td>
</tr>
<tr>
<td>BUN</td>
<td>Blood Urea Nitrogen</td>
</tr>
<tr>
<td>CA-125</td>
<td>Cancer Antigen 125</td>
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<tr>
<td>CDDP</td>
<td>cis-dichlorodiamminoplatinum (II)</td>
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<tr>
<td>CI</td>
<td>Combination Index</td>
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<tr>
<td>CTB</td>
<td>CellTiter-Blue® Assay</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>D_{\text{eff}}</td>
<td>Effective hydrodynamic diameter</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ED</td>
<td>1,2-ethylenediamine</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced Permeability and Retention</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated Cell Sorting</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
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<tr>
<td>HER2</td>
<td>Human Epidermal Receptor 2</td>
</tr>
<tr>
<td>HGSC</td>
<td>High-grade Serous Carcinoma</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>IC50</td>
<td>Half Maximal Inhibitory Concentration</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively Coupled Plasma-Mass Spectrometer</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
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<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>MDR</td>
<td>Multiple drug resistance</td>
</tr>
<tr>
<td>mPEG</td>
<td>Methoxy Polyethylene Glycol</td>
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<tr>
<td>mTOR</td>
<td>Mechanistic target of rapamycin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>$M_w$</td>
<td>Weight-average Molecular Weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut-off</td>
</tr>
<tr>
<td>NCA</td>
<td>N-carboxyanhydride</td>
</tr>
<tr>
<td>NG</td>
<td>Nanogel</td>
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<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
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<td>NP</td>
<td>Nanoparticle</td>
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<td>Neratinib</td>
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<td>Phosphate Buffered Saline</td>
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<tr>
<td>PDI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>PEG-$b$-PGlu</td>
<td>Polyethylene Glycol-block-poly(L-glutamic acid)</td>
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<td>Platelets</td>
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<td>PME</td>
<td>L-phenylalanine Methyl Ester</td>
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<tr>
<td>PBS</td>
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<tr>
<td>Pt</td>
<td>Platinum</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cells</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate–polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple Negative Breast Cancer</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet–visible Spectroscopy</td>
</tr>
<tr>
<td>WBC</td>
<td>White Blood Cells</td>
</tr>
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LIST OF CONTRIBUTIONS

1. Chapter II – Dr. Fan Lei assisted in polymer synthesis. Dr. Shaheen Ahmed assisted in confocal microscopy analysis and tumor spheroid studies. Victoria B. Smith and Craig Semerad assisted in the analysis of flow cytometry data and data acquisition. Xinyue Li and Dr. Xiaofei Xin assisted in western blot studies. Bin Li and David Moran performed platinum measurements. Dr. Hamid Band from the University of Nebraska Medical Center provided cell line MDA-MB-468.

2. Chapter III – Dr. Fan Lei performed diblock copolymer synthesis for this study and assisted in animal studies. Samantha Wall assisted in flow cytometry data acquisition. Platinum measurements were performed at the core facility of the University of Nebraska Medical Center. Dr. Kirk W. Foster and Dr. Ernesto M. Duarte performed the histopathological analysis of tissue samples. Dr. Jiang Jiang assisted in Ki-67-cleaved-caspase-3 quantifications. Dr. Gautam Nagsen assisted in pharmacokinetics analysis. EGFR-A ligand was provided by Dr. Rihe Liu from the University of North Carolina at Chapel Hill. Dr. Adam Karpf from the University of Nebraska Medical Center provided cell line OVCAR-5/luc and advice for animal studies.

3. Major contributions in all chapters were made by Xinyuan Xi. The overall project was designed under the guidance of Dr. Tatiana K. Bronich. Dr. Hamid Band and Dr. Adam Karpf provided valuable direction to Chapter II and III.

4. This work was supported by NIH grant U01CA198910 (Dr. Tatiana K. Bronich). We acknowledge the assistance of the Nanomaterials Core facility of the Center for Biomedical Research Excellence (CoBRE) Nebraska Center for Nanomedicine supported by the Institutional Development Award (IDeA) from the National
Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM103480 and P30GM127200.
CHAPTER I.

INTRODUCTION

1.1 Anticancer Therapeutic approaches

Despite the availability of improved drugs, cancer is still one of the leading causes of mortality worldwide [1]. Cancer has been defined by the national cancer institute to be a genetic disease that arises from genome changes to cell growth and division affected by the interactions between host and environment [2]. Cancers progress from uncontrolled replication, evasion of apoptosis, sustained angiogenesis, and ultimately to penetration in other tissues [3]. Complexity and heterogeneity, the hallmarks of cancer, make it extremely difficult to have a standard therapy that can be equally effective even within the same type of clinical tumor [4]. The generation of tumor heterogeneity owes to the genomic instability which generally evolves to more diverse with the progression of disease, resulting in a non-uniform distribution of distinct subpopulations across and within tumor sites, and ultimately lead to predispose undesired clinical outcomes [5]. High mutation potential of cancer cells increases the possibility of relapse following initial treatment success, which creates needs for another therapeutic regimen that could be used as later lines of therapy [1]. Altogether, it is difficult to combat cancer using a single therapeutic regimen. Therefore, a variety of combination therapy that reduces drug resistance, while simultaneously providing therapeutic anticancer benefits in a characteristically synergistic or an additive manner has been developed. Traditionally, depending on the tumor location, grade and stage, cancers are treated with a combination of surgery, radiotherapy, chemotherapy and/or immunotherapy [6]. Briefly, 1) surgery is applied to either remove the entire tumor or debulk a tumor to ease disease symptoms [7]. Due to the uncontrolled
post-surgery micrometastases leading to a plateau in curability rates, the use of adjuvant chemotherapy after surgery has been proposed to remove remaining tumor cells and shown promising results in numerous types of tumors [8]. 2) Radiation therapy uses high-energy particles or waves to disease location to damage cancer cells or slows their growth through making breaks in the DNA which keeps cancer cells from growing and dividing [9]. Radiation therapy is given either before surgery to shrink the tumor or after surgery to keep cancer from recurrence. In spite of its potential, current techniques of photon-based radiotherapy are approaching the physical limits of delivering high doses to the target disease volume [10]. 3) Immunotherapy use immune system components to kill tumor cells, including using antibodies to block abnormal proteins in cancer cells, vaccines to trigger the immune system to destroy antigens, and engineered T cells to recognize and destroy cancer cells [11]. It shows potential in promoting long-term anticancer immune responses, although the number of patients who benefit from this treatment is limited, and it sometimes has severe associated side effects [12]. 4) Chemotherapy is the most widely used form of therapy which utilizes a single drug or combination of drugs to interfere with cancer cells’ ability to divide and reproduce. Like radiation, it can be used to shrink a tumor before surgery or applied after surgery or remission to remove remaining cancer cells [13]. Overall, chemotherapy is an invasive treatment while it can sometimes have severe adverse effects due to the systemic administration of drug-mediated off-target toxicities. Apart from the mentioned conventional therapies, gene therapy and nanomedicine are the most promising state-of-the-art therapies. 5) Gene therapy allows doctors to treat diseases by inserting a gene into cells to either removing, inactivating a disease-causing gene or introducing a new gene to fight diseases. Although gene therapy is a promising treatment option for some types of cancer, this technique is risky and still under study for its safety and efficacy [14]. 6) Nanomedicine, an emerging method for treating cancer, is defined as the use of materials between 1 and 100 nm as anticancer medicine [15]. It improves the
balance between the efficacy and toxicity of the active pharmaceutical ingredients carried
within nano-formulations due to favorable drug release profiles, improved biodistribution,
and accumulation at the target site [16]. In preclinical settings, nanomedicines have shown
to inhibit tumor growth and prolong survival as compared to free drugs, while in clinical
practice, patients who received nanomedicine often only benefited from the reduced side
effects. The key challenge for nanomedicine for better clinical translation is to select the
right treatment regimen and apply them in the right patient population [6]. Each type of
treatment mentioned above shows limitations either due to tumor cell heterogeneity, the
intrinsic severe side effects or acquired resistance. Overall, the anticancer therapeutic
regimen is determined based on the stage and type of cancer.

1.2 Combinatorial treatments

1.2.1 Chemotherapy

Due to the increasing knowledge of cancer biology and pharmacology, and the improved
design of clinical trials using this knowledge, chemotherapy has evolved to a part of sound
medical discipline playing an important role in the control of cancer [17]. Chemotherapeutic agents can trigger a series of cellular responses that influence the
tumor cell dividing and survival. It is now believed that the most cytotoxic action of
chemotherapeutic drugs with differing structure and specificity is through the activation,
amplification and execution of the apoptotic process, characterized by morphological and
biochemical changes mediated by a family of caspases [18]. Apoptotic cell death could be
induced through two distant pathways: intrinsic or extrinsic pathways [19]. The extrinsic
pathway could be initiated by ligation of transmembrane death receptors (such as TNF
receptor and TRAIL receptor) to activate membrane-proximal caspases (such as caspase-
8), which in turn cleave and activate effector caspases such as caspase-3 and -7 [20]. The
intrinsic pathway is initiated by the disruption of the mitochondrial membrane and the
release of mitochondrial proteins, including Smac/DIABLO, HtRA2, and cytochrome c [18].

The intrinsic pathway can be activated by “intrinsic stresses”, such as oncoproteins, direct DNA damage, and hypoxia, for which p53 is a critical initiator (Figure 1) [21]. For example, proteins that sense DNA damage phosphorylate and stabilize p53 directly, and inhibit MDM2-mediated ubiquitination of p53 [22]. P53 initiate apoptosis by activating 1) proapoptotic Bcl-2 family members such as Bax and Bak [23], 2) genes that may contribute to apoptosis, including PTEN, Apaf-1, and PERP, 3) genes that lead to increases in reactive oxygen species (ROS), 4) both CD95 and TRAIL receptor 2 to sensitize cells to death-receptor-mediated apoptosis [24] [25].

![Figure 1](image.png)

**Figure 1.** Summary of apoptotic pathways. Adapted from (Johnstone) [18].

1.2.2 Drug resistance

The development of resistance is one of the major drawbacks of monotherapy in cancer therapy, responsible for most relapses in cancer patients following apparent remission
after successful therapy [26]. Understanding why the cell death program fails to be engaged in certain settings could allow us to rethink how the drug resistance may arise and offer a novel approach to overcoming the clinical problem of drug resistance. Although many cancers are susceptible to chemotherapy initially, they can gradually develop resistance [27]. The causes behind the development of resistance are multi-fold and often include different mechanisms (Figure 2), which is usually a cumulative effect of all the targets and pathways that are affected by a particular drug treatment [26].

**Figure 2.** Summary of Categories of mechanisms that promote drug resistance in human cancers. Adapted from (Meghan) [26].
One of the most studied resistance mechanisms might be enhancing drug efflux leading to reduced drug accumulation [27]. The key regulators enabling this efflux are the members of the ATP-binding cassette (ABC) transporter family proteins at the plasma membranes of healthy cells [28]. Three transporters—multidrug resistance protein 1 (MDR1), multidrug resistance-associated protein 1 (MRP1), and breast cancer resistance protein (BCRP)—are found expressed in high level in many drug-resistant cancers associating with poor clinical outcomes [29]. Inhibiting BCRP using the kinase inhibitor, such as Gefitinib, was found to block the transporter function of BCRP and reverse drug resistance [30].

Alteration of drug targets is another common drug resistance reason. As we know that drug efficiency is affected by its molecular target and changes of this target, including mutation or modification of expression level. Alteration of such target in cancer ultimately leads to drug resistance. For example, resistance to camptothecin, a topoisomerase inhibitor, can arise from altered topoisomerase I and II activity which prevents drug-protein binding and promotes the repair of broken strand [31]. Another frequently studied anticancer drug targets are signaling kinases, such as epidermal growth receptor factor, which is constitutively active and/or overexpressed in certain cancers, drives the cell cycle out of control and promotes uncontrolled cell growth. Long-term use of kinase inhibitors targeting those kinases can result in resistances due to the mutation of such kinases [32]. Original dose of the drug is inadequate at sites of diseases eventually. This process might also induce the alteration in the signal transduction process that mediates drug activation or activation in alternative signaling pathways further contributing to the resistance [27]. Many drugs act indirectly or directly to cause DNA damage. Cells can respond to such damage to reverse the drug-induced damage. For example, resistance to the platinum drug, a potent DNA-crosslinking agent, arises from increased expression of proteins responsible for DNA repair [33, 34]. The combined use of agents for inhibiting DNA damage response with DNA damage drugs could sensitize
tumor cells and therefore improve therapeutic efficacy [35]. Another response that tumor cells adapt to resist anticancer drugs is the inhibition of cell death. BCL-2 family proteins, Akt, and other antiapoptotic proteins are key regulators in the cell apoptosis, which are found to be highly expressed and active in many types of cancer [36]. It has been shown that the use of antiapoptotic proteins inhibitors such has histone deacetylase inhibitors (HDACi) could sensitize breast cancer cells to cytotoxic agent TRAIL in a mouse model [37]. Epithelial to mesenchymal transition (EMT) and metastasis are reported to be related closely with resistance and could explain why cancer can relapse at distant sites after apparently successful treatment and remission. During the EMT, cells reduce expression of cell adhesion receptors while increase expression of metalloproteases on the surface of tumor to enable moving outward of tumor cells leading to metastasis. EMT phenotype has been demonstrated in several drug-resistant cancer cell lines, such as gemcitabine/5-FU-resistant pancreatic cancer cells, oxaliplatin-resistant colorectal cancer cells, and paclitaxel-resistant ovarian cancer cells [38] [39] [40]. Although the acquirement of EMT is widely observed to be closely related to drug resistance, the exact mechanism of how EMT is of how EMT regulates drug resistance still remains unanswered. Altogether, cancer cells exposed to monotherapy can develop drug resistance through several mechanisms.

1.2.3 Combination chemotherapy
As discussed above, cancer cells can develop multiple protective mechanisms in response to toxic effects by mono-chemotherapy. It is therefore reasonable to propose the use of combined drugs that work by different mechanisms, thereby decreasing the likelihood that resistant cancer cells will develop. When drugs with different effects are combined, each drug can be applied at its optimal dose, without intolerable side effects. Therefore, this strategy also works for reducing systemic toxicity and increasing
tolerability. The principles of combination chemotherapy development have remained basically the same over the last decades. The general strategies have been to i) use drugs with nonoverlapping toxicities so that each drug can be given at a dose close to maximal; ii) combine agents with different effects and minimal cross-resistance in an effort to inhibit the development of broad-spectrum drug resistance; iii) preferentially use drugs with proven activity as individual drugs and iv) administer the combined treatment at early-stage disease and at a schedule with a minimal treatment-free duration between cycles but enabling the recovery of sensitive healthy tissues [41]. Advantages attributed to combination chemotherapy can be listed as follows: 1) patient compliance can be improved due to the reduced number of administrations, 2) favorable outcomes through various mechanisms, including synergistic effects, additive effects and potentiation effects are achieved (Figure 3) [42], 3) multidrug resistance can be overcome or delayed and drug dose with the consequent diminishing of toxicity to healthy tissues can be reduced.

![Figure 3](image)

**Figure 3.** Summary of the mechanisms underlying combination chemotherapy. Adapted from (Quanyin)

One of the key contributors to drug resistance is the high expression of MDR-related transporters, such as P-glycoprotein (P-gp), on various types of cancers, which increases drug efflux, DNA repair capacity, dysfunctional apoptosis, or activates pro-survival pathway leading to mitigated treatment outcome [43]. Inhibiting such transporters could
inactivate the drug efflux pump and therefore increase the drug accumulation. Yogesh et al. have shown that combining P-gp inhibitors, tariquidar, and cytotoxins, paclitaxel, has shown significantly increased intracellular drug retention and enhanced cytotoxicity in drug-resistance cell lines and mouse models comparing with monotherapy indicating that combination treatment using P-gp modulator and the anticancer drug is a promising strategy to overcome tumor drug resistance [44]. A similar strategy has shown improved therapeutic outcomes in the clinic [45]. Another example is to combine molecularly targeted agents with cytotoxins to disturb or block mutated signal pathways that are crucial in tumorigenesis [46]. The combination of cytotoxins and epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) (e.g., erlotinib and cetuximab) has shown synergistic effects in inhibiting the proliferation of tumor cells. The mechanism of the synergy is that TKIs may help rewire the apoptotic signal pathways, inhibiting P-gp activity of tumor cells, thus sensitize cells to cytotoxins [47] [48]. Wang et al. have shown that DOX uptake was enhanced in the MCF-7 cells with overexpression of drug efflux transporters but not the cells with low-expressing drug efflux transporters when administered DOX and lapatinib (TKI). They also showed that this combination treatment could restore the sensitivity of breast tumors to DOX and reduce the DOX systemic toxicity [49] [46].

1.2.3.1 Combination chemotherapy in the clinical practices

It is very difficult to determine whether a drug combination is working synergistically, additively, or antagonistically in cancer patients [50]. The only way to judge is to see whether a new combination achieves significantly increase in a determined endpoint such as response rate, survival or time to progression. However, preclinical studies focusing on biochemical and molecular mechanisms of drug interaction at the cellular level allow a more rational design of clinical combination chemotherapy. Several factors should be
taken into account when designing such studies, such as drug concentration & exposure, administration schedule & sequence and analytic method [51]. Currently, the most prevalent and widely-accepted method used for quantitative evaluation of drug combinations is the median effect analysis proposed by Chou and Talalay [52] [53] [54]. Although valuable information about drug interaction in in vitro studies can be obtained, in vitro synergistic activity is strongly dependent on the ratio of drugs which has profound implications on clinical practices, since in vivo activity relies on the maintenance of those therapeutic ratios at the tumor sites [55] [56]. Therefore, in order to achieve maximal therapeutic efficacy in vivo, the dosing schedule is essential to expose tumor cells to therapeutic drug concentrations [57]. Combination therapy adopted in clinics generally includes more than one therapeutic agent co-administered simultaneously or a combination of different therapies summarized in Figure 4, such as chemotherapy, hormone therapy, immunotherapy and radiotherapy. Above all mentioned approaches, the co-delivery of various chemotherapeutic agents is the most widely-used combination therapeutic modality in clinical practice [58]. For advanced epithelial ovarian cancer patients, a recent phase II trial using sorafenib, a multi-kinase inhibitor reducing survival/proliferation signals by inhibiting RAS/RAF/MAPK pathway as well as the EGFR signaling pathway, plus topotecan, a well-known chemotherapeutic genome-toxic drug, in platinum-resistant ovarian cancer demonstrated clinically significant improvement in progression-free survival of these patients [59]. However, single sorafenib therapy only showed modest activity in earlier studies [60]. PARP, a validated target in cancer therapy, is an enzyme participating in the repair of single-stranded DNA breakages. Failure to repair single-stranded breaks eventually induce double-stranded DNA break which can be repaired by the homologous recombination (HR) pathway [61] involving functional BRCA1 and BRCA2 [23]. Therefore factors impairing HR or BRCA1/2, such as associated mutations, sensitize tumor cells to the genotoxic effects of PARP inhibitors. Such
combined effect is defined as the term “synthetic lethality” [62]. In a more recent phase II study of combining cediranib (anti-angiogenic agent) and olaparib (PARP inhibitor) versus olaparib in relapsed platinum-sensitive ovarian cancer, Joyce et al. has shown that a combination of cediranib and olaparib increased progression-free survival significantly, when compared with olaparib alone [63]. The enhanced effectiveness of combination might arise from the fact that anti-angiogenic agents can induce the downregulation of genes implicated in HR leading to a creation of an HR-deficient state making affected cells more vulnerable to a PARP inhibitor [64] [65]. Similarly, a combination of PARP inhibitors with chemotherapy has shown great potential in increasing the efficacy of PARP inhibitors and sensitizing triple negative breast cancer patients. Jung et al. reported in a Phase I/II study that the combination of olaparib and carboplatin in BRCA1 or BRCA2 mutation-associated-TNBC induced an overall response rate (ORR) as high as 88% [66]. A combination of radiation therapy and chemotherapy has also been a very common modality in cancer treatment especially for surgically unresectable tumors. Radiotherapy is a major therapeutic option for non–small cell lung cancer (NSCLC) patients with unresectable advanced lung cancer [67]. However, NSCLC cell lines are generally less sensitive to radiation. Pretreating cancer cells with chemotherapy could sensitize cells to radiation therapy in several preclinical studies. Zhang et al. have demonstrated that wortmannin sensitizes NSCLC cells to radiotherapy by inhibiting PI3K/Akt survival signaling and DNA-PKCs, a catalytic subunit required for the non-homologous end joining (NHEJ) pathway of DNA repair [68]. Similarly, gefitinib acts as a radiosensitizer in NSCLC cells by inhibiting ataxia telangiectasia mutated (ATM) activity which is activated by DNA double-strand breaks and thereby inducing mitotic cell death [69]. In a randomized trial, the delivery of cisplatin concurrently with radiotherapy to patients with Stage III inoperable NSCLC has demonstrated improved rates of survival as compared with single radiotherapy [70]. However, the above combination regimen demonstrated increased
toxicity, nausea, and vomiting in this study. Altogether, the co-delivery of various chemotherapeutic drugs has been the most practiced and well-established therapeutic modality among different combination therapies. Therefore, the current perspective in the treatment of cancer has progressively concentrated on developing the delivery of chemotherapeutic drugs in a combination approach with reduced drug-associated side effects and enhanced therapeutic anticancer effects.

**Figure 4.** Types of combination modalities for the treatment of cancer. Adapted from [58].
1.2.3.2 Combination chemotherapy in triple negative breast cancer

Breast cancer represents the second leading cause of death in women after lung cancer and it is the most commonly occurring cancer among females globally [71]. Triple-negative breast cancer (TNBC) accounts for 12-20% of human breast cancers, referring to the breast cancer phenotype diagnosed pathologically by < 1% expression of Human Epidermal Receptor 2 (HER2), estrogen (ER) and progesterone receptor (PgR) [72] [73] [74]. TNBC is the most aggressive histologic subtype with a high potential of disease recurrence and the possibility for distant metastases [75]. Despite optimal systemic chemotherapy, less than 30% of women with metastatic breast cancer survive 5 years after diagnosis, and almost all women with metastatic TNBC will eventually die of their disease. This is due to the relative lack of recognized molecular targets for therapy, poor prognosis and aggressive clinical behavior of the disease [76]. Due to the very limited expression of hormonal and HER2 receptors, conventional targeted therapies (hormonal and anti-HER2) are not effective in TNBC [77]. Currently, cytotoxic chemotherapy, such as anthracycline, gemcitabine and carboplatin, remains the standard established systemic treatment of TNBC showing significant benefits in the either neoadjuvant or adjuvant setting. [76]. Although TNBC patients have a higher response to systemic chemotherapy than those with non-TNBC, the 5-year survival rate is still less than 30% and virtually all women with metastatic TNBC would die of this disease eventually [78]. The limited efficacy could arise from altered signaling patterns of cancer cells leading to modulated effects of treatment, such as activation of pro-survival signal transduction (e.g., epidermal growth factor receptor (EGFR), AKT, PI3-kinase) and damage repair, etc [79]. Many clinical trials have been studied to apply combination chemotherapy regimens to reduce these undesired effects and enhance the survival of patients. Although TNBC is considered a single clinical entity and primarily treated with chemotherapy regardless of the stage of the disease, molecular profiling technologies has revealed that TNBC is highly heterogeneous.
It would be desirable to manage patients through taking both factors into consideration: the current clinical practices and the molecular complexity of TNBC [76]. EGFR are transmembrane glycoproteins belonging to the HER family of receptor tyrosine kinases (RTK), which actively participates in sustaining the growth and the survival of carcinoma cells as well as the pathogenesis and progression of different carcinoma types [81]. Interestingly, up to 78% of TNBC have the EGFR overexpression [82], making it a potential therapeutic target for intervention. Besides, EGFR inhibitors might work synergistically with standard chemotherapy by reducing the unfavored pro-survival signals induced by the latter, which has been shown in several studies [79] [83] [84], further increasing the sensitivity of cancer cells to the designed regimen and enhancing the clinical benefits. FDA approved anti-EGFR agents include monoclonal antibody Cetuximab (Erbitux™; Eli Lilly and Company) and small molecular tyrosine kinases inhibitors (TKIs), such as Erlotinib (Tarceva; Genentech) and Gefitinib (Iressa; AstraZeneca). A major breakthrough of targeting EGFR in TNBC was that in a phase III study, the BALI trial demonstrated a 20% overall response rate in patients with metastatic TNBC who received the cetuximab/cisplatin combination, compared with 10.3% response rate in the cisplatin–alone group [85]. The progression–free survival duration was increased from 1.5 to 3.7 months (HR 0.675, P = 0.032) upon the addition of cetuximab to cisplatin. However, the response rate was considered low since it did not meet the pre–specified primary endpoint. The lack of benefits can arise from the fact that inhibiting EGFR might trigger underappreciated resistance and induce activation of parallel signaling pathways and downstream signaling pathways through other members of HER family [86]. Besides, there is an emerging hypothesis that for TNBC patients whose HER2 expression level even is at the lowest levels, they are not going to get a great pathological complete response rate with standard agents [87]. Given that, eliminating any trace of the HER family of proteins could enhance the response to the standard cytotoxic treatment.
Using pan-HER inhibitors in the treatment for TNBC has been evaluated both in preclinical and clinical studies. I-SPY2 trial [88] has demonstrated that the preoperative inhibition of pan-AKT with the oral agent MK-2206 or inhibition of activated EGFR, HER2 and HER4 with neratinib (NRT) can improve pathological complete response rates (pCR) of TNBC patients achieved with cytotoxic DNA-damaging agents [87]. Furthermore, TNBC patients treated with weekly DNA-damaging agents, with or without NRT, showed 37.5% overall pCR in the NRT arm, but among patients demonstrating phosphorylation of HER2 or EGFR, it rose to 63% which implies clinical benefits might be achieved when applying this strategy to subsets with EGFR or HER2 activation since they might be especially responsive to it. In addition, based on reverse-phase protein array data, only activation of HER2 (p = 0.03) or EGFR (p = 0.009) were found to be positive predictors of pCR [88].

To develop exceptional responsive treatment based on phenotype, several other molecular targets are also being evaluated in clinical studies since TNBC is a highly heterogeneous disease. Glycoprotein non-metastatic b (GPNMB) is overexpressed in 40% to 75% of breast cancer and associated with poor prognosis. In a randomized Phase II Study trial, triple-negative breast cancer patients with GPNMB expression were treated with either an antibody-drug conjugate (ADC) targeting GPNMB, glembatumumab vedotin, or selected chemotherapy. ADC showed better toxicity tolerance in heavily pretreated patients comparing with chemotherapy arm. However, primary endpoint in all enrolled patients with GPNMB-expressing breast cancer was not met [89]. Research is brisk for immune checkpoint inhibitors. Although TNBC is considered a relatively immune-cold disease, clinical trials using inhibitors of programmed cell death protein 1 (PD-1) and its ligand (PD-L1) on TNBC patients are ongoing [90]. In a phase II study, 18.5% of heavily pretreated patients showed response to the PD-1 inhibitor pembrolizumab (Keytruda). In another study, 19% responded to the PD-L1 inhibitor atezolizumab, but when the agent was combined with nab-paclitaxel (Abraxane), the response rate increased to 70% [87,
In spite of encouraging efficacy, whether there will be clinical benefits of this regimen is unknown before more patients are recruited in future clinical trials. For the standard chemotherapy on TNBC, people also would like to seek exceptional responders. In a 2015 Phase II Clinical Trial of platinum monotherapy on 86 patients with metastatic triple-negative breast cancer, response rates were 32.6% to single-agent cisplatin and 18.7% to carboplatin, with highly durable responses observed in 11.7% and 5.7%, respectively. Several cisplatin-treated long-term responders remained alive and progression-free without any therapy after platinum treatment 3 to 6 years later. The phenotype of the exceptional responders was BRCA wild-type diagnostic with breast, lymph node, and parenchymal lung disease, without pleural, bone, or liver disease. Although BRCA1/2 mutation carriers exhibited a significantly higher response rate than noncarriers, there were no differences in median progression-free survival [92].

1.2.3.3 Combination chemotherapy in ovarian cancer

Ovarian cancer (OC) is the most lethal gynecological cancers in the USA with no effective screening strategy. It is estimated by The American Cancer Society that in 2020, there will be about 21,750 new cases of ovarian cancer diagnosed and 13,940 women will die of ovarian cancer in the United States [93]. Although the standard of care of OC patients including surgical cytoreduction followed by platinum-based chemotherapy has shown high response, mortality rates for ovarian cancer have declined only marginally in the forty years emphasizing continued efforts toward better treatments [94]. Most patients present with advanced-stage tumors showing metastasis outside the pelvis at the time of initial diagnosis due to inadequate diagnostic markers and non-specific symptoms [95] [96]. Although ovarian cancer can be divided into several subtypes based on the cell of origin, including germ, epidermoid, stromal, and border cells, epithelial ovarian cancer (EOC) accounts for about 90% of OCs. EOC is associated with high mortality rates due to the
intrinsic aggressive nature and limited early detection techniques. Among all the subtypes of EOC, high-grade serous carcinoma (HGSC) is the most commonly diagnosed form with high sensitivity to current front-line combination platinum- and taxane- based chemotherapy [97, 98]. In spite of high response rates to the standard therapy, EOC frequently relapses and becomes increasingly less sensitive to chemotherapy. We may be approaching the limit of using conventional chemotherapeutics to optimize the treatment of EOC [96]. Delaying the onset of resistance and prolonging the survival of EOC patients will likely occur with progression in the early detection of this disease [99] as well as through the incorporation of biologically targeted therapeutics. Aberrant EGFR expression is reported in up to 60% of malignant ovarian tissue as demonstrated by IHC and occurs in all histologic subtypes [100]. Due to the fact that the increased copy number and overexpression of EGFR were found to be associated with high tumor grade and poor patient outcomes, EGFR expression might be used as an independent prognostic indicator in EOC patients [101-103]. Interestingly, the increased copy number of EGFR was reported to be associated with an increased copy number of HER2 which was also reported to be a negative prognostic indicator in EOC in some reports [101, 104]. Therefore, inhibiting the family of HER might be a promising approach through utilizing biological targets [105, 106]. As mentioned, EOC has been showing high sensitivity towards platinum-based chemotherapy. Cisplatin, a widely used platinum compound, acts by damaging DNA. Once entering the cell through either passive diffusion or active transport, cisplatin undergoes the aquation process and becomes a potent electrophile that reacts with a variety of nucleophiles, including nucleic acids. The formation of cisplatin-DNA adducts disrupts the DNA structure and interferes with DNA replication & transcription leading to either apoptotic cell death or DNA repair with cell survival. The mechanisms of determining the fate of cells remain largely unknown [107]. The declined sensitivity to cisplatin is believed to arise from reduced cellular uptake [108], enhanced
intracellular detoxification, increased DNA repair, tolerance of platinum-DNA adducts and regulation of apoptosis [109, 110]. On the other hand, NRT is an inhibitor of HER family receptor tyrosine kinases, including EGFR, HER2 and HER4. Its mechanism of action involves the irreversibly binding to the intracellular signaling domain of kinases to inhibit their phosphorylation and several HER downstream signaling pathways such as ERK, PI3 kinase/AKT, JAK/STAT, etc. It has been reported that nucleotide excision repair (NER), a primary DNA-damage repair process, may prevent cisplatin-induced apoptosis by activating the ataxia telangiectasia mutated (ATM) pathway [107]. EGFR acts as an upstream effector of ATM by phosphorylating ATM to mediate DNA repair. It was shown that the treatment with EGFR inhibitor successfully blocked ATM activation and subsequently increased tumor cell sensitivity to DNA-damaging treatments [111]. Apart from activating the ATM pathway, EGFR was shown to epigenetically regulate DNA damage signaling pathways by modifying chromatin at critical tyrosine residues [112] and phosphorylating DNA-dependent protein kinase (DNA-PKcs), an enzyme required for DNA-repair [113], to reduce the DNA damage and restored DNA repair activity. As an inhibitor of HER family, NRT might potentiate the therapeutic efficacy of cisplatin by reducing DNA-damage repair through interfering EGFR-mediated Histone H4/DNA-PKcs/ATM pathway and pushing cells towards apoptotic death. On the other hand, EGFR is activated in response to cisplatin in various types of cancer cells that overexpress the receptor, including human breast cancer cells and glioma cells [114], which is believed to be a survival-promoting or cell death-opposing response. Many studies have shown blocking EGFR activation enhances cisplatin-induced cell death [79, 115, 116]. This serves as another strong basis that preventing CDDP-dependent EGFR activation should enhance drug sensitivity and improve clinical efficacy. Another minor mechanism of action of NRT is its ability to provoke cell cycle arrest by downregulating the oncogenic Cyclin D1 level which plays a key role in advancing the cell cycle into the late G1 and S phases
Since tumor cells appear to be maximally sensitive to cisplatin in G1 phase and minimally sensitive in S-phase [118], reducing Cyclin D might keep more cells blocked at the G1 phase during cisplatin treatment and prevent DNA damage repair, which might also explain that the increased cyclin D expression results in less tumor sensitivity to cisplatin in several in vitro models, including fibrosarcoma [119] and pancreatic cancer [120]. Altogether, the above mechanism of actions of both drugs then forms the rational basis for selection of such combined therapy to achieve maximized synergistic clinical effects for treating ovarian cancer. In spite of a strong basis for the synergy of action, this combination was also found to have dose-limiting side effects and therefore offers the potential for the development of nanomedicine-based approaches that can achieve synchronized and ratiometric delivery as well as modulate toxicities associated with free drugs.

1.3 Ligand-installed nanocarriers for combination therapy
Development of drug-delivery systems that selectively target neoplastic cells has been a major goal of nanomedicine in cancer therapy. One major strategy for achieving this goal is to install ligands on the surface of nanocarriers to improves targeting properties of nanocarriers, including overcoming vascular barriers, enabling tumor extravasation, penetrating deeper tumor region and impermeable tissues, selectively increasing retention of payloads in tumors, activating specific molecular signaling, and promoting intracellular delivery [121-125]. These features have allowed ligand-installed nanocarriers to improve the efficacy of both delivered bioactive treatments and diagnosis in various preclinical disease models, including cancer, CNS disorders, and cardiovascular diseases, with potential to enhance the outcome of intractable diseases, such as Alzheimer's disease and drug-resistant tumors, through the development of such novel therapeutic strategies [126]. Such ligand-installed nanocarriers could show substantial advantages over
conventional drugs and nanocarriers in terms of treating small metastases, as well as circulating cancer cells [127]. An ideal strategy to design the targeted nano-drug will be to display targeting moieties on the surface of nanoparticle formulation functioning as a homing signal that allows unique recognition and interaction with biomarkers overexpressed on diseased cells and/or subcellular domains for higher binding chance and specificity, without affecting the nontargeted cells [128]. Despite of success of nanoparticle-guided targeted therapy of cancer in reducing off-target side effects on preclinical platforms, the bench-to-bedside translation has been far from encouraging. A widely used strategy in clinics to develop targeted formulation is to conjugate antibodies with potent drugs [129]. Although not easily adopted in clinics, ligand-installed nanocarriers are able to deliver a concentrated dose of versatile drug payloads (Figure 5), which is a substantial advantage over antibody-drug conjugates (ADC). On top of that, attachment of multiple ligands on the surface of nanocarriers enables multivalent binding to target cells for stronger binding affinity [130], and driving cellular receptors clustering for signaling [131].
Figure 5. Schematic illustration of ligand-installed nanocarriers interacting with cells. Adapted from [126].

Based on targeting ligands being employed for targeted therapy, four types of representative targeting moieties has been categorized [126]: a) antibodies and antibody fragments; b) aptamers; c) peptides or whole proteins; d) small molecules as receptor ligands. Considering a scope of thesis, this dissertation is intended to focus on different ways of delivering chemotherapeutic agents using peptide- or protein-nanoparticle conjugates. Comparing to small molecules, peptides can be designed to fit into a shallow and hydrophobic binding pocket with less challenge. Given that it generally consists less than 50 residues, peptides demonstrate wide application due to the advantages in small
size, stability, and simple synthesis and conjugation [132]. Besides, improving screening techniques for faster ligand–substrate combinations identification has increased the use of peptides as targeting moieties. RGD (arginine–glycine–aspartic acid) peptide family is definitely the most prominent peptide ligand being investigated to target αvβ3 receptors on cancer and angiogenic endothelial cells. To reduce the non-specific binding and improve the targeting efficiency, Colombo et al. investigated that the cyclic RGD conjugated tumor necrosis factor-alpha (TNF) showed more than 10-fold higher antitumor efficiency than its linear counterpart. Such higher targeting efficiency for the cyclic RGD might arise from its thermodynamically favored configuration and higher stability against proteolysis [133]. Besides specifically targeting the biomarkers, peptides could facilitate the penetrating of nanoparticles (NPs) across the cell membrane and into specific cell organelles and eliciting cellular responses. These penetrating peptides usually contain a specific C-terminal sequence, (R/K)XX(R/K) (e.g. LyP-1, iRGD, and F3 peptide) and are capable of interacting with neurophilin-1 to facilitate the cell and tissue transportation [134-136]. On top of that, peptides could stimulate the receptor as agonists or inhibit receptor-induced signaling as antagonists [137, 138], which might work synergistically in combination with nonpeptidic cytotoxic drugs carried in the peptide-nano conjugates while maintaining targeting effect. In spite of wide application, the limitation of peptide ligands may include potential immune responses and influence on the pharmacokinetics [139]. Proteins or protein domains also hold great promise for the targeted delivery of NPs [140]. Due to the difficulty in engineering a full-length antibody with site-specific conjugation and optimizing binding parameters, considerable effort has been fostered to developing smaller binding units with antibody-like specificity and affinity. Transferrin (Tf), an 80-kDa naturally occurring glycoprotein, is one of the most abundantly studied targeting proteins with a high affinity to transferrin-receptor (Tf-R) [132]. NPs decorated with Tf have been developed to target upregulated Tf-R on tumor cells and shown to substantially enhance
the intracellular delivery and gene silencing efficacy of carried siRNA [141]. In addition, synthetic proteins possess advantages over the naturally occurring proteins since they do not have to compete against highly abundant, non-artificial proteins. Affibodies, a class of polypeptide ligands, have been used to develop HER2 targeted paclitaxel-loaded nanocarriers. Affibody-installed carriers demonstrated significantly higher uptake and enhanced cytotoxicity over the nontargeted counterpart in HER2+ cell lines [142]. As listed in Table 1, nanocarriers installed with ligands have been exploited to target cancer cells in various tumors, including breast, ovarian, liver, and lung tumors.

<table>
<thead>
<tr>
<th>Ligand type</th>
<th>Targeting ligands</th>
<th>Targets</th>
<th>Nanocarriers</th>
<th>Cargos</th>
<th>Tumor cells</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins</td>
<td>Transferrin</td>
<td>Transferrin receptor</td>
<td>Thranostic nanoprobe</td>
<td>Indocyanine green</td>
<td>Hela cell</td>
<td>[143]</td>
</tr>
<tr>
<td></td>
<td>Transferrin</td>
<td>Transferrin receptor</td>
<td>NPs</td>
<td>Docetaxel</td>
<td>PC3-LN cell</td>
<td>[144]</td>
</tr>
<tr>
<td></td>
<td>Anti-EGFR affibody</td>
<td>EGFR</td>
<td>Hydrogel PRINT NPs</td>
<td>No drug</td>
<td>A431 cell</td>
<td>[145]</td>
</tr>
<tr>
<td></td>
<td>Ferritin</td>
<td>Transferrin receptor 1 (TfR1)</td>
<td>Ferritin NPs</td>
<td>Doxorubicin</td>
<td>HT29 cell</td>
<td>[146]</td>
</tr>
<tr>
<td></td>
<td>K237 peptide</td>
<td>KDR receptor of tumor EC</td>
<td>Nanoparticle</td>
<td>Paclitaxel</td>
<td>HCT-15 cell</td>
<td>[147]</td>
</tr>
<tr>
<td>Peptides</td>
<td>Recombinant murine epidermal growth factor</td>
<td>EGFR</td>
<td>NPs</td>
<td>Gemcitabine</td>
<td>MDA-MB-468 cell</td>
<td>[149]</td>
</tr>
<tr>
<td></td>
<td>cRGD</td>
<td>αvβ3 integrin receptors</td>
<td>Micelles</td>
<td>Epirubicin</td>
<td>U87MG cell</td>
<td>[122]</td>
</tr>
<tr>
<td></td>
<td>Octreotide</td>
<td>Somatostatin receptors</td>
<td>Micelles</td>
<td>Paclitaxel</td>
<td>MCF-7 cell</td>
<td>[150]</td>
</tr>
</tbody>
</table>

Out of many limitations of traditional chemotherapy that can be remedied by NPs, the most significant one remains increasing solubility and specificity of drugs with low bioavailability. Being able to incorporate more than one type of drugs makes NPs
prominent candidates for combination therapy. Table 2 lists some of the nanoformulation approved for use in clinics, specifically in oncology. Although liposome remains the most popular construct in nanomedicine for clinical use, there are various other nano-architectures being widely studied in bench-side for targeted anticancer therapy, including metal NPs, biomimetic NPs, and polymeric NG.

Table 2. Nano-formulation for clinical application in cancer. Adapted from [151].

<table>
<thead>
<tr>
<th>Name</th>
<th>Particle type/drug</th>
<th>Approved application/indication</th>
<th>Approval (year)</th>
<th>Investigated application/indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>DaunoXome (Galtech)</td>
<td>Liposomal daunorubicin (non-PEGylated)</td>
<td>HIV-associated Kaposi’s sarcoma (primary)</td>
<td>FDA (1996)</td>
<td>Various leukemias</td>
</tr>
<tr>
<td>Myocet (Teva UK)</td>
<td>Liposomal doxorubicin (non-PEGylated)</td>
<td>Treatment of metastatic breast cancer (primary)</td>
<td>EMA (2000)</td>
<td>Various cancers including: breast, lymphoma, or ovarian</td>
</tr>
<tr>
<td>Marqibo (Spectrum)</td>
<td>Liposomal vincristine (non-PEGylated)</td>
<td>Philadelphia chromosome-negative acute lymphoblastic leukemia (tertiary)</td>
<td>FDA (2012)</td>
<td>Various cancers including: lymphoma, breast, leukemia, or melanoma</td>
</tr>
<tr>
<td>MEPACT (Millennium)</td>
<td>Liposomal infusumitide (non-PEGylated)</td>
<td>Treatment for osteosarcoma (primary following surgery)</td>
<td>EMA (2009)</td>
<td>Osteosarcomas</td>
</tr>
<tr>
<td>Onivyde MM-398 (Merrimack)</td>
<td>Liposomal irinotecan (PEGylated)</td>
<td>Metastatic pancreatic cancer (secondary)</td>
<td>FDA (2015)</td>
<td>Various cancers including: solid malignancies, breast, pancreatic, sarcomas, or brain</td>
</tr>
</tbody>
</table>

N/A: No current studies

<table>
<thead>
<tr>
<th>Nanoparticle/microparticle imaging agents</th>
<th>Ultrasound contrast agent</th>
<th>Ultrasound enhancement for: liver or breast or intracranial or pancreatic tumors, pulmonary diseases, heart function, transcranial injuries, strokes, or liver cirrhosis</th>
<th>FDA (2001)</th>
<th>N/A: No current studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonovue (Bracco Imaging)</td>
<td>Phospholipid stabilized microbubble</td>
<td>Ultrasound contrast agent</td>
<td>EMA (2001)</td>
<td>Ultrasound enhancement for: liver neoplasms, prostate or breast or pancreatic cancer, or coronary/pulmonary disease</td>
</tr>
<tr>
<td>Resovist (Bayer Schering Pharma/Clavix)</td>
<td>Iron carboxydextran colloid</td>
<td>Imaging of liver lesions</td>
<td>Some of Europe Discontinued (2009)</td>
<td>N/A: No current studies</td>
</tr>
<tr>
<td>Fenetrean-10/Combodex/Sineron (AMAG)</td>
<td>Iron dextran colloid</td>
<td>Imaging lymph node metastases</td>
<td>Only available in Holland</td>
<td>Imaging lymph node metastases</td>
</tr>
</tbody>
</table>
1.3.1 Liposomes

Liposomes are artificially spherical vesicles consisting lipid bilayers and discrete aqueous spaces, discovered by Alee Bangham in 1963 [152]. Owing to its special structure properties, Liposomes are capable of incorporating hydrophilic drugs in its aqueous spaces and hydrophobic drugs in lipid layers. However, the inefficient loading and rapid release of amphiphilic drugs loaded via passive entrapment mitigate the therapeutic efficacy of liposomes. To improve pharmacokinetics and stability of constructs, PEGylation and inclusion of the highly saturated phospholipid and cholesterol is adopted for effective drug delivery. In addition, stimuli-responsive smart liposomes (inducer: pH, temperature, enzymes, magnetic field, ultrasound, and redox potential) could also be developed for enhancing site-specific drug delivery. Active targeting of liposomes is achieved through meticulous fabrication with selective ligand(s) [153]. For example, a cell-penetrating TAT peptide moiety and cancer-specific monoclonal antibody (mAb) were installed on the surface of liposomes to achieve targeted intracellular drug delivery. To further enhance specificity, liposomes were shielded with a pH-detachable long PEG chain, which could be removed at low pH (typical of solid tumors) to expose the TAT ligand for tumor cell targeting. Such multifunctional immunoliposomal nanocarrier demonstrated enhanced cellular association with tumor cells than normal cells as well as higher cytotoxicity when pre-incubated at lower pH [154]. Showing tremendous potential as a carrier system for therapeutically active agents, several targeted-liposomal formulations have been designed and evaluated in clinical trials as listed in Table 3. EGFR-targeted Doxorubicin liposomal formulations is an example of targeted liposomal delivery in clinical trials under the name of C225-ILS-DOX. Phase I trial showed a good correlation between anticancer efficacy and EGFR overexpression and the clinical Phase II trials are ongoing [155].
1.3.2 Metallic NPs

Metallic NPs are nano-sized entities made of pure metals, including gold, platinum, silver, and iron or their compounds (e.g., oxides, hydroxides, sulfides) [156]. Metallic NPs can be adopted not only as carriers for therapeutic agents, but also as a diagnostic tool through imaging. For example, iron NPs (major two types: superparamagnetic iron oxide (SPIO) and ultra-small superparamagnetic iron oxide (USPIO)) have been used as theranostic agents as contrasting agents for MRI and carriers for targeted drug delivery to the tumor through magnetic guidance [157, 158]. Apart from magnetic drug delivery, SPIOs can also be conjugated with ligands to target specific cells. Zhao et al. demonstrated that labeling a C2 domain of synaptotagmin I (binding to the plasma membrane of apoptotic cells) with SPIOs enabled the imaging of tumor cells which were undergoing apoptosis after chemotherapy [159]. And when the formulation was given intravenously into mice, tumor regions with large numbers of apoptotic cells could be observed due to the decreases in MR image intensities. The magnetic NPs that have been approved by the FDA are listed in Table 4. Another example of ligand-installed metallic NPs is described in a study reported by Kirui et al that biofunctionalization of hybrid NPs (HNP) of gold and iron oxide with tumor antigen specific antibodies resulted in a five times higher cellular uptake of targeted HNP in antigen-expressing cells than normal cells. Besides, such multipurpose agents also provide radiotherapy enhancement and thermal ablation [131].
Table 3. Targeted liposomes currently in clinical trials. Adapted from [155].

<table>
<thead>
<tr>
<th>Product</th>
<th>Cellular target</th>
<th>Targeting ligand</th>
<th>Cargo</th>
<th>Status</th>
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<td>Tf Receptor</td>
<td>Tf</td>
<td>Oxaliplatin</td>
<td>Phase II</td>
</tr>
<tr>
<td>SGT-93</td>
<td>Tf Receptor</td>
<td>Tf R scFv</td>
<td>Plasmid encoding wild-type p53</td>
<td>Phase II</td>
</tr>
<tr>
<td>SGT-94</td>
<td>Tf Receptor</td>
<td>Tf R scFv</td>
<td>Plasmid coding for tumor suppressor RB94 cDNA</td>
<td>Phase I</td>
</tr>
<tr>
<td>MM-302</td>
<td>HER2</td>
<td>scFv</td>
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<td>Phase II</td>
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<td>EGFR</td>
<td>Fab’ fragment of cetuximab</td>
<td>Doxorubicin</td>
<td>Phase II</td>
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<tr>
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<td>scFv</td>
<td>Docetaxel prodrug</td>
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<td>Myosin</td>
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<td>Phase I</td>
</tr>
<tr>
<td>2B3-101</td>
<td>Blood brain barrier (BBB)</td>
<td>Glutathione</td>
<td>Doxorubicin</td>
<td>Phase II</td>
</tr>
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Table 4. FDA-approved SPIOs [160]

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<th>Target organs</th>
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<td>AMI-277</td>
<td>Blood node, lymph node</td>
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<td>AMI-25</td>
<td>Liver/spleen</td>
</tr>
<tr>
<td>SHU 555A</td>
<td>Spleen/liver</td>
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1.3.3 Biomimetic NPs

Biomimetic NPs are an emerging class of NPs that incorporate the functionality of biological materials with the flexibility of artificial materials to mimic biofunctions and achieve effective navigation in complex biological environment. NPs camouflaged in cell membranes carried with imaging reporters and therapeutic cargo are the most representative biomimetic platform. After the discovery of red blood cell (RBC) membrane-coated poly (lactic-co-glycolic acid) (PLGA) NPs, such technology has significantly expanded to the use of membranes from platelets as well as nucleated cells, such as macrophages, neutrophils, and cancer cells. Cancer cell membrane coated NPs (CCMCNPs) synthesized by coating NPs with a lipid bilayer of cancer cell membranes (Figure 6) can carry tumor-specific receptors and antigens for cancer targeting. Such targeting ability of CCMCNPs has been evaluated using PLGA NPs camouflaged with human MDA-MB-435 cancer cell membrane, which demonstrated that CCMCNPs had a much higher affinity with MDA-MB-435 cells compared to RBC-cloaked NPs and bare PLGA cores [161]. The study also showed that such binding was specific to MDA-MB-435 cell indicated by the fact that MDA-MB-435 CCMCNPs exhibited only little increased uptake compared to bare PLGA cores in the negative control cell line. Similar promiscuous binding was observed in CCMCNPs derived from a variety of cancer cells including mouse breast cancer 4T1 cells [162], human squamous carcinoma UM-SCC-7 cells [163], and human breast cancer MDA-MB-231 [164]. In addition, CCMCNPs can be adopted as effective drug delivery nanocarriers when the NP cores are loaded with chemotherapy payloads. In a published study, NP coated with membranes of 4T1 mammary breast cancer cells were loaded with paclitaxel (PTX). The coated NPs showed success preservation of several membrane proteins responsible for cell adhesion and recognition. The 4T1 CCMCNPs treated mice demonstrated selective accumulation of PTX in either the primary tumors or the metastases in pulmonary tissues. The higher accumulation of
drugs resulted in a remarkable antitumor and metastasis efficacy in both orthotopic and advanced metastasis mice models [165]. However, neoantigens on tumor cell membranes might help trigger recognition by immune cells and accelerate the clearance of NPs. A study evaluated the pharmacokinetic profiles of CCMCNPs and uncoated NPs in immunodeficient mice and reported that CCMCNPs were cleared from circulation much faster than bare NPs. Authors believed that this shorter circulation time might arise from the fact that membrane antigens presented on the CCMF-PLGA NPs may trigger tumor-specific immune responses and accelerate clearance resulting in significantly shorter circulation time in the bloodstream [166]. Overall, such bioinspired platforms are still in their infancy. Possible degradation of surface antigens in complex physiological environments and potential adverse effects of immune response need to be addressed. Besides, the mechanisms underlying the targeting and important characteristics need to be further investigated and elucidated.

**Figure 6.** Schematic illustration of synthesis of CCMCNP. Adapted from [167].
1.3.4 Nanogels

Nanogels (NGs) are nano-sized three-dimensional (3D) nonfluid hydrogel NPs made from physically or chemically crosslinked swellable hydrophilic or amphiphilic polymer networks with tunable size and uniformity, which can be composed of naturally occurring polymers, synthetic polymers or a combination thereof [168, 169]. NGs are considered as soft matters because the nanogel composition entails elastic moduli in the range of 0.1–100 kPa [170]. NGs offer a myriad of advantages for drug delivery, including: (i) their characteristics such as size, charge, porosity, amphiphilicity, rigidity, and degradability can be fine-tuned by adjusting the chemical composition [168, 171, 172]; (ii) they can encapsulate a plethora of guest molecules with relatively high drug encapsulation capacity and protect the cargo from degradation; (iii) they undergo physical or chemical changes, including swelling, dissociation in response to stimuli (e.g. acidity, reducing agents, and reactive oxygen species (ROS)); (iv) they show site-specific and markedly increased drug release at the site of action, boosting therapeutic effects of payload drugs and minimizing off-target toxicities; (v) they are endowed with the ability to incorporate entities with very different physical properties within the same carrier which is hard to find in other types of nanoparticulate systems [173, 174]. Due to the great potential in chemotherapy, diagnosis, and site-specific delivery of therapeutic active substances, NGs have emerged as an appealing platform for targeted drug delivery in cancer therapy [175-178]. Ideal NG should deliver payloads sufficiently to sites of action and be able to degrade without causing harm to the healthy tissue of the body. However, no NGs are currently approved by the FDA for \textit{in vivo} use due to several constraints including the one mentioned above. There is scope for more research in the area of development of better and translatable NG platforms.
1.3.4.1 Preparation and properties of polymeric NGs

Current approaches used for preparation of polymeric NGs can be categorized based on two distinct crosslinking methods. The first crosslinking method utilizes covalent bonds to afford robust chemical NGs. Chemical crosslinking points are formed between polymer chains either during polymerization of low molecular weight monomers or after polymerization illustrated in Figure 7 [179]. Developments in controlled radical polymerization techniques have aided in the precision synthesis of robust polymeric building blocks, such as block copolymers, to prepare NGs with diverse composition, dimensions, architectures, and functionalities which further allows multivalent bioconjugation [180]. Other crosslinking approaches, including click reaction, thiol-disulfide exchange, amide crosslinking, photo-induced crosslinking, enzyme-mediated crosslinking etc., have been employed for the preparation of NGs from the polymer precursors [168]. The second crosslinking method employs noncovalent interactions such as ionic bonds, hydrogen bonds, and coordination bonds to make physical NGs which tends to be fragile and collapses easily upon changes in the surrounding environment [181]. One of the challenges in the formation of physical NGs lies in the control over the particle size, which requires fine optimization of the polymer concentrations or environmental parameters such as temperature, pH, and ionic strength [168]. Nielsen and coworkers have reported that these challenges can be tackled by utilizing a microfluidics-based approach [182]. Recently, NGs combined with properties of both chemical and physical NGs have been developed with dynamic covalent bonds that can de-crosslink in response to specific stimuli [179, 183].
Figure 7. Two different synthetic methods toward NG. Adapted from [179].

To characterize the formed NGs, several parameters would be evaluated to confirm the formation of objective NGs. In particular, size and polydispersity, swelling, drug loading and release are important factors for understanding the properties of NGs. The sizes of self-assembled NGs can be controlled by proper selection of the concentration of polymers and external solution conditions, such as pH, electrolyte concentration, and temperature [169]. Besides, adjusting the chemical composition such as the ratio between hydrophilic and hydrophobic block lengths allows the control over the size as well as the uniformity of NGs. Swelling of NGs in an aqueous environment is controlled not only by external solution conditions, but also by nanogel structure (chemical composition, percentage of crosslinking, charge density for polyelectrolyte gels) [177]. Bioactive agents can be incorporated in NGs by (i) physical encapsulation, (ii) chemical conjugation, or (iii) controlled self-assembly. Anionic polyethylene glycol-poly (methyl acrylate) (PEG-b-PMA) NGs could encapsulate hydrophilic drug cisplatin through coordination interactions with COOH functionalities [184]. Cisplatin-loaded NGs displayed sustained pH-sensitive release of Pt(II) species that can be effectively modulated by changing the degree of crosslinking of the crosslinked cores. The released platinum species preserved their
activity and were able to trigger apoptotic cell death through forming Pt adducts with nuclear DNA in the cancer cells. Developed NGs greatly improved drug therapeutic index of cisplatin by improving pharmacokinetic profile, enhancing tumor delivery, boosting antitumor efficacy, and reducing the cisplatin-mediated nephrotoxicity in a mouse model of ovarian cancer [185]. When the same cisplatin-loaded NGs were installed with targeting ligands (e.g., folate or LHRH peptide), tumor growth suppression was even further enhanced [186, 187]. Polyelectrolyte NGs appears to be an appealing platform due to its ability to incorporate small drug molecules with different physicochemical properties via combinations of electrostatic and hydrophobic interactions as well as hydrogen bond formation [169]. Fahmy and coworkers developed biodegradable core–shell NGs of drug-complexed cyclodextrins and cytokine-encapsulating biodegradable polymers that can deliver small hydrophobic molecular TGF-β inhibitor and water-soluble protein cytokine (IL-2) in a sustained fashion to the tumor microenvironment [188]. They demonstrated that he sustained local delivery of these agents, in combination, can induce localized therapeutic immune responses while reducing the immune-resistant nature of the tumor microenvironment. Simultaneous combination delivery showed synergistic therapeutic effects due to the activation of the innate arm of the immune system which ultimately led to delayed tumor growth and enhanced survival.

1.3.4.2 NGs as drug delivery systems

Generally, the reason for observing a relatively higher drug loading for hydrophilic NGs than other nanocarriers such as polymeric micelles and liposomes is that there is a larger cargo space and inner surface area for drug incorporation when NGs is swollen in aqueous media [169]. Furthermore, the high loading in NGs can be achieved in relatively mild conditions compared to other carriers, which is very important for the preservation of biological activity of labile drugs and biomacromolecules, such as proteins and antibodies
As highlighted in previous sections, what makes NGs stand out is that they are able to immobilize desired ratios of multiple bioactive compounds differing in physical properties within the same carrier to form stable colloidal dispersions at physiological pH and ionic strength [190]. Such platform could tackle the challenges in dosing and scheduling of drugs with varied pharmacokinetic and pharmacodynamic profiles. As reported from our group, multicompartment polyelectrolyte NGs [191] prepared from hybrid triblock copolymers poly(ethylene glycol)-block-poly(L-glutamic acid)-block-poly(L-phenylalanine) (PEG–b–PGlu–b–PPhe) have a central hydrophobic core formed by PPhe regions, serving as a non-aqueous reservoir for solubilizing water-insoluble molecules paclitaxel (9% wt.). Its crosslinked anionic layer of PGlu chains entraps hydrophilic chemotherapeutic drug cisplatin with relatively high loading (15% wt.). NG with drug combination exhibited synergistic cytotoxicity against human ovarian A2780 cancer cells and exerted a superior antitumor activity in cancer xenograft models in vivo by comparison to individual drug-loaded NGs or free drugs. The benefits of spatial–temporal synchronization exposure of the platinum-taxane drug combination via a single carrier can be further boosted by targeting NGs to the folate receptor, which are overexpressed in most ovarian cancers [192]. In another study, our group reported an efficient co-encapsulation of DOX and 17-allylaminodemethoxygeldanamycin (17-AAG) into PEG–PGlu NGs with multiple hydrophobic regions that are located within the crosslinked anionic PGlu core [193]. Dual drug-loaded NGs displayed selective synergistic anticancer activity against HER2-overexpressing breast cancer cell lines. This synergistic effect was attributed to the action of 17-AAG, HSP90 inhibitor, which induces degradation of many of the proteins required for DNA-damage response as well as attenuation of oncogenic signaling via degradation of HER2, thereby sensitize cancer cells to cytotoxic effects of DOX. Consistent with the in vitro observations, combination treatment with NG exhibited superior antitumor efficacy, both in terms of tumor inhibition and survival, in a HER2-driven
xenograft model compared to the cocktail of free drugs at equivalent drug concentrations. In another study, a polysaccharide-based cationic nanogel co-delivering oppositely charged plasmid DNA along with phospholipaseA2 (PLA2) was developed [194]. As a lipolytic enzyme, PLA2 catalyzes the hydrolysis of sn-2 ester bond into various different phospholipids promoting membrane permeability. Upon entering the endosome after cell uptake, PLA2 in NGs would function as an endosome escaping agent to disrupt the lipid membrane, and subsequently trigger the release of the co-encapsulated DNA into the cytoplasm. Transfection experiments confirmed that DNA expression level was enhanced when complexed with PLA2. The ability of entrapping a considerable amount of payload with different physical properties provides unprecedented opportunities for combination therapy.

1.3.4.3 In vivo behaviors of NGs
Nanogel systems have the ability to stably encapsulate drug molecules without premature leak and spatially localize the release of potent drugs only at target sites to ensure maximal therapeutic efficacy with minimal side effects [175]. In order for a drug delivery device to achieve these desired benefits it must be present in the circulation long enough to reach or recognize its therapeutic site of action. To achieve this, NGs would have to overcome various barriers depending on the administration routes. Generally, to prolong the circulation time of cargo, NGs are designed to 1) avoid fast clearance especially in the case of small molecules and 2) prevent rapid degradation or metabolism which is more relevant for labile drugs and biomolecules [195]. However, opsonin proteins present in the blood serum is one of the major obstacles to the realization of these goals due to the fact that the binding between opsonin and conventional non-stealth NPs allows macrophages of the mononuclear phagocytic system (MPS) to easily recognize and shuttle them out of the bloodstream to liver, spleen or bone marrow within seconds of intravenous
administration, rendering them ineffective as site-specific drug delivery devices [196, 197].

To address these challenges, several strategies have been developed to mask or camouflage NGs from the MPS. Of these methods, the most preferred is the adsorption or grafting of PEG to the surface of NGs [198]. The addition of PEG and PEG-containing copolymers to the surface of NGs prolongs the blood circulation half-life of the particles significantly. PEGylation of the nanogel surface imparts them with ‘stealth’ properties by creating a hydrophilic protective layer on the surface, shielding a charge that the core might carry and repelling the absorption of opsonin proteins through steric repulsion [195]. The overall shielding effect is highly dependent on the size of nanogel, its shape, molecular weight and surface density of the PEG used [199]. Although this strategy demonstrates success in increasing the blood circulation half-life and reducing MPS uptake, it does not fully block the opsonization process, which eventually still occurs [200]. In addition, PEG is not completely inert, and the generation of PEG-specific IgM antibodies after a single dose of PEGylated NPs might result in their accelerated blood clearance and mitigate the therapeutic efficacy on subsequent dosing [201]. Apart from creating a shielding layer, material mechanical properties should be considered in the critical design to help NGs partially escape the splenic filtration process. Such properties are their primary softness and deformability, which may be relevant in processes such as phagocytosis or endocytosis [202]. For instance, erythrocytes can easily pass through the splenic filtration bed in spite of having a size much larger than the pore size of filtration bed, due to their flexibility and deformability [203, 204]. The biomimetic property of the NGs shows advantageous for their in vivo application. Lyon and coworkers have recently reported that soft spherical acrylamide-based microgel deforms and translocate through cylindrical pores at least tenfold smaller than their hydrodynamic diameter under hydrostatic pressure relevant to renal filtration [205]. Besides, varying Young modulus of similar hydrogel NPs has been shown to affect the uptake rate, the mechanism of
internalization and the concentration of internalized particles in macrophages [202]. Lowering the modulus of hydrogel particles could enable them to bypass \textit{in vivo} physical barriers that would otherwise filter particles with similar size but higher modulus [7]. Another study confirmed the advantages for \textit{in vivo} application by showing that softer PEG-based hydrogel NPs (10 kPa) offered enhanced circulation and subsequently enhanced targeting compared to harder NPs (3000 kPa) \textit{in vivo}. Furthermore, the \textit{in vitro} studies showed that softer NPs exhibit superiorly reduced cellular internalization in various types of cells, including immune cells, endothelial cells, and cancer cells [206]. The inclusion of electrolyte moieties into the polymer network of hydrogel particles to increase the swelling ratio and secure cargos is another straightforward and quite efficient way to decrease the modulus [7]. However, the distribution of charged groups on the surface of a particle can accelerate the clearance of particles. To tackle this challenge, DeSimone group designed an asymmetric particle containing a charged interior and a PEG-quenched surface with near-neutral charge. The strategy was that acidic degradation of the crosslinker allows for swelling of the particles to microgels with a desired size and deformability while neutralized PEGylated surface could contribute to slowing the clearance [7]. A fraction of NPs that are not cleared is then distributed into different organs by the circulating blood. NPs are usually too big to pass through the tight junctions of the normal endothelium but can efficiently extravasate from tumor blood vessels and accumulate in solid tumors or site of infection/inflammation that have unique structural features such as defective vascular architecture and meager lymphatic drainage leading to the well-characterized enhanced permeability and retention effect (EPR) discovered by Maeda and Matsumura [207]. However, targeting of tumor tissues solely through the EPR effect has significant limitations for precision therapy due to its heterogeneity. For instance, variations of EPR effects were observed between the cancer types, between patients with the same tumors, between different tumoral lesions within the same patient, and even
within the same tumor mass [208]. Installing ligands onto the surface of nanocarriers with strong binding affinity to unique cellular markers on the target cell provides a strategy to enhance targeting and internalization of nanocarriers by the target cell regardless of EPR phenotype. As highlighted, numerous targeting ligands have been identified and employed for targeting, including antibodies, proteins, peptides, and small molecules [126]. After extravasation from the vasculature, NPs have to diffuse through the extracellular matrix in the interstitial spaces, reach the targeted cells and exerts its action [209]. Increasing evidence indicates that cellular uptake and intracellular fate of NPs are strongly influenced by size, shape, softness, charge and surface properties, cell types and receptor being targeted [210]. When nanoparticles reach the exterior cell membrane, they mainly enter the cell through endocytosis which is a complex process classified into several types [211]. In general, endocytosis eventually leads to the engulfment of particles into intracellular vesicles, from where they are transported into endosomes and ultimately lysosomes. At each of these stages, NPs are exposed to changing pH of endosomal/lysosomal lumen, degrading enzymes, or reducing environment, which are often utilized as stimuli for the release of cargo carried within the NPs [9]. Nanogel carriers can also be designed to target specific subcellular domains or escape them, depending on the type of cargo that they carry. For example, it is essential for the NPs to facilitate endosomal/lysosomal destabilization so that the encapsulated siRNA or oligonucleotides can be released in their active form in the cytoplasm where they are supposed to exert their therapeutic action [11]. pH-sensitive NGs have shown significantly enhanced endosomal escape. NGs constructed from polyelectrolytes are thought to be able to disrupt endosomes due to the buffering-mediated sponge effect [212]. An increased effort has been devoted to developing NGs that can release bioactive agents in response to external stimuli at the disease site. It is also crucial to create biodegradable NPs to minimize toxicities associated with the retention of the carrier in the body.
1.4 Conclusions

Combination therapy shows confirmed advantages over treatment with single agents to combat most cancers for its ability to target multiple oncogenic pathways at the same time, delay the development of resistance, prolong the survival, and eventually enhance the therapeutic outcome. Cisplatin (CDDP)-based chemotherapy has been the front-line treatment for ovarian cancer since its discovery. However frequent relapse and onset of resistance indicate that new strategic approaches are needed to enhance the outcome. Despite high response rate to the standard therapy, frequently relapse and become increasingly less sensitive to chemotherapy. EGFR is a viable target as aberrant overexpression of EGFR is reported in up to 60% of malignant ovarian tissue. EGFR actively participates in regulating several oncogenic signal transductions promoting cell survival and proliferation. Monotherapy using EGFR inhibitors has shown limited benefits, so it is generally given together with other chemotherapy. Combining EGFR inhibition with cisplatin has shown strong synergistic effects in several cancers in both preclinical and clinical studies. NRT, an FDA-approved potent irreversible pan-HER inhibitor, is proposed here to be combined with cisplatin for treating ovarian cancer. However, the synergy of such therapy is often sequence and dose ratio specific. Besides, administrating two distinct drugs might require repeated dosing and prolonged duration of drug infusion in patients. Moreover, conventional Pt-based chemotherapy generally comes with rapid elimination and severe dose-limiting toxicities including neurotoxicity and nephrotoxicity. Combining such drugs in one delivery carrier is therefore a desirable and convenient strategy for controlling the pharmacokinetics and co-delivery of the desired drug ratio in vivo, to maximize the therapeutic efficacy and minimize drug-associated toxicities.

Crosslinked polypeptide-based nanogel is an appealing platform to achieve this goal. Such biodegradable platform provides flexibility in adjusting size, loading capacity, surface
properties, deformability, softness, and responsive behaviors by tuning the chemical compositions as well as crosslinking levels. Enhanced stability due to the crosslinking adds benefits to prolonging the circulation, which in term enhances drug exposure to tumor by EPR effect. Their mild preparation procedures allow the incorporation of labile drug molecules and biomacromolecules. The most prominent advantage over other nano-sized carriers is their ability to incorporate molecules with different physicochemical properties through versatile interactions. For the purpose of the current study, NGs prepared from copolymers poly (ethylene glycol)-block-poly (L-glutamic acid) modified with phenylalanine functionalities \(((\text{PEG-P(Glu-Glu/Phe)}_x)^{150})\) for simultaneous loading and delivery of binary CDDP and NRT combination were developed. Such core-shell NGs have 1) a hydrophilic PEG stealth shell for less RES uptake and extended circulation, 2) an anionic core, which incorporates CDDP through coordination with the carboxylic groups of PGlu, 3) hydrophobic regions formed by Phe moieties, which serves as a reservoir for NRT solubilization. The crosslinks incorporated into PGlu core keep NGs stable until they are degraded by proteases through cleaving the polypeptide chains of the block copolymers. The developed binary drug NGs formulation showed synergy in EGFR overexpressed cell lines from not only ovarian cancer, but also triple negative breast cancers, while such synergy was not seen in cell lines with low EGFR overexpression. Such drug delivery systems also showed desirable antitumor efficacy in intraperitoneal ovarian cancer mouse model with no observable toxicities while certain toxicities were observed in tumor bearing mice treated with free drugs at the same dose. However, this system solely depends on EPR effect which is limited by heterogeneity among tumor mass as well as vascular density nearby influencing the permeability of nanocarriers. Installing targeting ligands on the surface of carriers to specifically target biomarkers overexpressed on the surface of tumor cells is a well-suited strategy to overcome mentioned limitation and further boosting drug performance. Two targeting ligands aiming to bind with EGFR
was chosen in our study: L-AE peptide and EGFR-A protein. Both ligands are relatively new with reported high binding specificity and affinity to EGFR with acceptable stability. Ligand installed carrier demonstrated success in increasing cellular uptake and further boosting the therapeutic efficacy of targeted binary combination NGs \textit{in vivo}. We also confirmed the importance of simultaneous administration of the (CDDP+NRT) via a single NGs system which provides more therapeutic benefits than a cocktail of individual drug-loaded NGs administered at equivalent doses.
1.5 References


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CHAPTER II.

POLYMERIC NANOGELS FOR COMBINATION DRUG THERAPY IN TRIPLE NEGATIVE BREAST CANCER

2.1 Introduction

Breast cancer represents the second leading cause of death in women after lung cancer and it is the most commonly occurring cancer among females globally [1]. Triple-negative breast cancer (TNBC) accounts for 12-20% of human breast cancers, referring to the breast cancer phenotype diagnosed pathologically by < 1% expression of Human Epidermal Receptor 2 (HER2), estrogen (ER) and progesterone receptor (PgR) [2-4]. Despite optimal systemic chemotherapy, less than 30% of women with metastatic breast cancer survive 5 years after diagnosis, and almost all women with metastatic TNBC will eventually die of their disease. Currently, cytotoxic chemotherapy, such as anthracycline, gemcitabine and carboplatin, remains the standard established systemic treatment of TNBC showing significant benefits in either neoadjuvant or adjuvant setting [5]. Although TNBC patients have a higher response to systemic chemotherapy than those with non-TNBC, 5-year survival rate is still less than 30% and virtually all women with metastatic TNBC would die of this disease eventually [6]. The limited efficacy could arise from altered signaling patterns in cancer cells leading to modulated effects of treatment, such as activation of pro-survival signal transduction (e.g., epidermal growth factor receptor (EGFR), AKT, PI3-kinase) and damage repair, etc. Many clinical trials have been studied to apply combination chemotherapy regimens to reduce these undesired effects and enhance the survival of patients. It would be desirable to manage patients through taking
both factors into consideration: the current clinical practices and the molecular complexity
of TNBC [5]. EGFR are transmembrane glycoproteins belonging to the HER family of
receptor tyrosine kinases (RTK), which actively participates in sustaining the growth and
the survival of carcinoma cells as well as the pathogenesis and progression of different
carcinoma types [7]. Up to 78% of TNBC have the EGFR overexpression [8] making it a
potential therapeutic target for intervention. EGFR inhibitors work synergistically with
standard chemotherapy by blocking oncogenic signal transduction and reducing the
unfavored pro-survival signals induced by those chemotherapies which has been shown
in several studies [9-11], further increasing the sensitivity of cancer cells to the designed
regimen and enhancing the clinical benefits. A major breakthrough of targeting EGFR in
TNBC was that in a phase III study, the BALI trial demonstrated 20% overall response rate
in patients with metastatic TNBC who received the cetuximab (anti-EGFR monoclonal
antibody) / cisplatin combination, compared with 10.3% response rate in the cisplatin–
alone group [12]. However, the response rate for this combination treatment was
considered low which can arise from the fact that inhibiting EGFR might trigger
underappreciated resistance and induce activation of parallel signaling pathways and
downstream signaling pathways through other members of HER family [13]. In another
clinical study, TNBC patients treated with weekly DNA-damaging agents, with or without
neratinib (a pan-HER inhibitor), showed 37.5% overall pCR in the neratinib arm, but
among patients demonstrating phosphorylation of HER2 or EGFR, it rose to 63% which
implies clinical benefits might be achieved when applying this strategy to subsets with
EGFR or HER2 activation [14]. Altogether, it is rational to propose here that combining
neratinib, a pan-HER inhibitor, and cisplatin, a potent DNA-damaging chemotherapeutic
drug, to combat EGFR-overexpressing triple negative breast cancer could enhance the
therapeutic response to the standard cytotoxic treatment by targeting multiple cell-survival
pathways.
However, the synergy of such therapy is often sequence and dose ratio specific. Besides, administrating two distinct drugs might require repeated dosing and prolonged duration of drug infusion in patients. Moreover, conventional Pt-based chemotherapy generally comes with rapid elimination and severe dose-limiting toxicities including neurotoxicity and nephrotoxicity. Combining such drugs in one delivery carrier is therefore a desirable and convenient strategy for controlling the pharmacokinetics and co-delivery of the desired drug ratio in vivo, to maximize the therapeutic efficacy and minimize drug-associated toxicities. Crosslinked polypeptide-based nanogel is an appealing platform to achieve this goal. The most prominent advantage over other nano-sized carriers is their ability to incorporate molecules with different physicochemical properties through versatile interactions. For the purpose of the current study, nanogels (NGs) prepared from crosslinked poly (ethylene glycol)-block-poly (L-glutamic acid) modified with phenylalanine functionalities (PEG-P(Glu-Glu/Phe)) were developed for simultaneous loading and delivery of binary CDDP and NRT combination. Such NGs have 1) a hydrophilic PEG shell for less RES uptake and extended circulation, 2) an anionic crosslinked core, which incorporates CDDP through coordination with the carboxylic groups of PGlu, 3) hydrophobic regions formed by Phe moieties, which serves as a reservoir for NRT solubilization. To maximize the synergistic therapeutic effects, the cargo ratio was tuned by adjusting the ratio of constituent hydrophilic PGlu units and hydrophobic Phe moieties in copolymer chains. The developed binary drug NGs formulation showed synergy in EGFR overexpressed TNBC cell lines, while such synergy was not seen in cell lines with low EGFR overexpression. Such NGs also demonstrated preferable retention in tumor cells while less was seen in the fibroblasts in a multicellular tumor spheroids model containing both cell types.
2.2 Materials and methods

2.2.1 Materials

Poly (ethylene glycol)-b-poly (L-glutamic acid) (PEG-b-PGlu) diblock copolymer (M_w/M_n=1.01, M_w 28,000 g mol^{-1}) was purchased from Alamanda Polymers, Inc. (Madison, AL, USA). The block lengths were 114 and 150 repeating units for PEG and PGlu, respectively. L-phenylalanine methyl ester hydrochloride (Phe), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), ethylenediamine and other chemicals were obtained from Sigma-Aldrich (St Louis, MO) and were used without further purification. Fetal bovine serum (FBS) was purchased from Invitrogen Inc. (Carlsbad, CA). HyClone™ Minimum Essential Medium with alpha modification, EGF Recombinant Human Protein, Pierce™ BCA protein assay kit, CellBrite™ Blue and CellBrite™ Green for cell membrane staining was purchased from Thermo Fisher Scientific (Waltham, MA). CellTiter-Blue® reagent was purchased from Promega (Madison, WI). MammoCult™ human medium kit was purchased from STEMCELL Technologies (Cambridge, MA). Cyanine 5 (Cy5) amine was obtained from Lumiprobe (Hallandale Beach, FL). Cell lysis buffer and protease inhibitor cocktail were purchased from Cell Signaling Technology (Danvers, MA). The following primary antibodies were used in this study: Rabbit anti-EGFR (ab52894) mAb, rabbit anti-phospho-EGFR (phospho Y1068) (ab40815) mAb, rat anti-HSC70 (ab19136) mAb, mouse anti-beta actin (ab8226) mAb used for Western blotting studies, were purchased from Abcam™ (Cambridge, MA); the rabbit anti-Phospho-Akt (D9E) mAb, rabbit anti-phospho-p44/42 MAPK (Erk1/2) (4370T) mAb, rabbit anti-Cyclin D1 (92G2) mAb was kindly provided by Dr. Surinder K. Batra (University of Nebraska Medical Center, Omaha, NE).
2.2.2 Synthesis of NGs

NGs were prepared using a diblock copolymer, PEG-PGlu_{150}, in which part of the Glu units were modified with hydrophobic Phe functionalities via polymer-analogous modification of the PGlu segment with the Phe methyl ester using carbodiimide-chemistry in aqueous media. The targeted degree of modification was set at 25, 50, and 75 Phe units per chain, respectively. The obtained products were characterized by proton nuclear magnetic resonance (^{1}H NMR). Micellar precursors based on PEG-P(Glu-Glu/Phe_{x})_{150} copolymers were prepared by dialysis method using water/organic solvent mixtures followed by direct crosslinking of COOH groups of Glu units with 1,2-ethylenediamine (ED) in the presence of EDC at r.t.. The crosslinking reactions were performed at a molar ratio of [COOH]/[EDC]/[ED]=10/2/1 to obtain a targeted 20% crosslinking degree. The prepared nanogel was abbreviated as NGs (further denotes as rNG_{x/150}). To prepare Cy5 labeled NGs for evaluating penetration in multicellular tumor spheroids, Cy5 amine (0.5 w/w%) was conjugated to cores of NGs using EDC chemistry.

2.2.3 Drug loading

NGs based on PEG-P(Glu-Glu/Phe_{x})_{150} copolymers were used to prepare neratinib (NRT) and cisplatin (CDDP) drug formulations. The primary procedure for loading of NRT in NGs involved the addition of NRT dissolved in ethanol to the aqueous dispersion of NGs upon stirring followed by evaporation of organic solvent and removal of unbound NRT. Then CDDP was loaded through incubation with aqueous dispersions of NRT-loaded NGs for 48h at pH 9 followed by centrifugation to remove unbound CDDP. The amount of CDDP incorporated into NGs was assayed by inductively coupled plasma mass spectrometry (ICP-MS, NexION 300Q, PerkinElmer, Waltham, MA). NRT content was assayed by HPLC under isocratic conditions using an Agilent 1200 HPLC system with a diode array detector set at 266nm. As the stationary phase a Nucleosil C18 column was used (250
mm × 4mm), and a mobile phase of acetonitrile+0.1%TEA/water +0.1%TEA mixture (80/20,v/v) was applied at a flow rate of 1ml/min with a column temperature of 40°C. NGs can entrap considerable amounts of CDDP and NRT, respectively.

2.2.4 Physicochemical characterization of the NGs

The 1H NMR spectra for the copolymers were acquired in TFA-d$_1$ at 25 °C using a Bruker 500 MHz spectrometer. Effective hydrodynamic diameters (D$_{eff}$) and ζ-potential of NGs were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). All measurements were performed in automatic mode at 25°C. Software provided by the manufacturer was used to calculate the size, polydispersity indices (PDI), and ζ-potential of NGs. All measurements were performed at least in triplicate to calculate mean values ± SD. Swelling behavior of NGs in aqueous dispersion was evaluated through recording the D$_{eff}$ and ζ-potential upon pH variation between pH 6-9 using a Zetasizer Nano ZS.

2.2.5 Drug release

Drug release from the NGs was examined in PBS (pH 7.4), and acetate buffered saline (ABS, pH 5.5, 0.14 M NaCl) at 37°C by dialysis method using a membrane with 3,500 Da cutoff. Both media contained 10% FBS. The concentrations of NRT and Pt(II) released were determined by HPLC and inductively coupled plasma mass spectrometry (ICP-MS), respectively, and expressed as a percentage of the total NRT or Pt(II) available vs. time.

2.2.6 Cell culture and cytotoxicity studies

MDA-MB-468 human TNBC cells were provided by Dr. Hamid Band (University of Nebraska Medical Center, NE). Cells were maintained in alpha-MEM medium supplemented with 10% (v/v) FBS in the presence of penicillin and streptomycin (100 U/ml and 0.1 mg/ml, respectively) at 37°C in a humidified atmosphere containing 5% CO$_2$. Cells were harvested with trypsin-EDTA (0.25%) (Life Technologies) after 80% confluence.
Cells seeded in 96-well plates (3,000 cells/well) 24 h prior the experiment were exposed to various doses (0-10 μg/mL on CDDP or NRT basis) of CDDP alone, free binary drugs, CDDP/NG, NRT/NG, a cocktail of individual-drug-loaded NGs and (CDDP+NRT)NGs for 72 h at 37°C. Cytotoxicity of either free drugs or drug-loaded NGs was assessed by a standard CellTiter-Blue® cell viability assay [15] with untreated cells served as 100% cell viability. The IC₅₀ values were calculated using GraphPad Prism software. The combination index (CI) analysis based on Chou and Talalay method [16] was performed using CompuSyn software for CDDP and NRT combinations, determining synergistic, additive, or antagonistic cytotoxic effects against MDA-MB-468 TNBC cells. Values of CI < 1 demonstrate synergism while CI = 1 and CI > 1 values represent additive and antagonistic effects of the drug combination, respectively. Cytotoxicity against TNBC cells with low EGFR expression was conducted on MDA-MB-453 and MDA-MB-231 following the similar protocol mentioned above.

2.2.7 Western blot analysis

Cells were treated with either EGF, EGF + CDDP, EGF + NRT, or EGF + CDDP + NRT for 2h at a concentration of 100 ng/mL EGF, 10 µM/mL CDDP, and 5 µM/mL NRT. Cells were then lysed with cell lysis buffer supplemented with protease inhibitor cocktail. Supernatants were collected after removing cell debris by centrifugation (14,000 g for 20 min), and the protein amount was determined by BCA assay. 5–10 μg of proteins were separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Massachusetts). Non-specific protein binding was blocked in Tris-buffered saline Tween-20 (TBS-T) (50 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.6) containing 5% dry milk for 30 mins at 37°C. Proteins of interest were immunoblotted with the appropriate monoclonal antibodies used at 1:1000 to 1:10000 dilutions at 4°C overnight followed by
the secondary horseradish peroxidase-conjugated antibodies used at 1:1000 dilutions at r.t. for 1 hour. Antibody interactions were visualized with enhanced chemiluminescence (ECL) horseradish peroxidase (HRP) substrate (Thermo fisher scientific, Massachusetts) using iBright Western Blot Imaging Systems (Invitrogen). HSC70 and β-actin has been used as a loading control. Band intensities were analyzed by ImageJ software.

2.2.8 Penetration in tumor spheroids

A suitable 3D composite spheroid model was created using a mixture of the mouse embryonic NIH 3T3 fibroblasts and MDA-MB-468 TNBC cells. Multicellular spheroids were generated using the forced-floating method: 500 cells (MDA-MB-468: 3T3=1:1) were seeded in ultra-low attachment 96-well plates in the presence of MammoCult® medium and incubated at 37°C for 6 days. On day 6, the spheroid morphology was recorded by an inverted microscope. MDA-MB-468/NIH 3T3 spheroids were further used for assessment of penetration of rNGx150 NGs. Cy-5-labeled rNGx150 (0.5 mg/mL) were incubated with 6-day old MDA-MB-468/NIH 3T3 spheroids for various periods of time (2, 4, and 8 h). After the treatment, spheroids were washed with PBS (3 gentle washes) followed with fixing by 4% formaldehyde solution in PBS for 20 mins at r.t. Then the spheroids were transferred to the Lab-Tek® chambered coverglass (NUNC, NY), and stained by 4′,6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, MA) overnight. Overall distribution and penetration of NGs within spheroids were analyzed by confocal microscopy using Z-stack imaging with 10 µm intervals and presented as maximum pixel intensity Z-projections. To further get insight into the cellular composition of co-culture MDA-MB-468/NIH 3T3 spheroids, MDA-MB-468 cells were transfected with F-Luc-GFP Lentivirus (Capital Biosciences, Rockville, MD) which is a lentivirus engineered to constitutively express the green fluorescent protein. Spheroid formation was continuously monitored using Nikon Eclipse E400 microscope. On day 1,3 and 6, spheroids were
dissociated using Accutase (STEMCELL Technologies, MA) and cell composition was quantified by flow cytometry analysis. The % of gated cells were analyzed using BD Biosciences LSR II flow cytometer and FACSDiva software (BD Biosciences, San Jose, CA). At least 10,000 events were acquired in linear mode, gated to exclude debris and dead cells, and visualized in logarithmic mode. The cellular uptake of rNG50/150 in co-culture MDA-MB-468(GFP+)/NIH 3T3 spheroids was further evaluated. On day 6, 48 MDA-MB-468(GFP+)/NIH 3T3 spheroids were incubated with Cy5-labeled rNG50/150 (0.5mg/mL) for 4 hours followed by flow cytometry analysis.

2.2.9 Statistical analysis

Each sample was analyzed based on results that were repeated at least three times. Statistical comparisons were carried out using GraphPad Prism 5 software (GraphPad Software, Inc.). In all cases, differences at P < 0.05 were regarded as statistically significant; ones at P < 0.01 or P < 0.001 were considered higher statistical significances.

2.3 Results and discussion

2.3.1 Synthesis of polymer PEG-P(Glu-Glu/PheX)150

To drive the self-assembly of hydrophilic copolymer PEG-PGlu, hydrophobically modified PEG-b-PGlu derivatives were synthesized via EDC amidation mediated modification of PGlu backbone with L-phenylalanine methyl ester (Phe) moieties. The synthesis of the amphiphilic hybrid polypeptide-based polymer PEG-P(Glu-Glu/PheX)150 is illustrated in Scheme 8.
Figure 8. Scheme for the synthesis of PEG-P(Glu-Glu/Phe\textsubscript{x})\textsubscript{150} polymer via carbodiimide-chemistry.

PEG-\textit{b}-PGlu diblock copolymer (PEG M\textsubscript{w} = 5,000 g mol\textsuperscript{-1}), in which different percentages of Glu units were modified with hydrophobic phenylalanine moieties, were utilized for the synthesis of biodegradable NGs. The Phe grafting degree was varied by adjusting the feed molar ratio of the reaction components, PEG-PGlu:Phe (1:27.5, 1:55, 1:82.5). Followed by the purification of polymers via dialysis against the water using a membrane with 3,500 Da cutoff for 48 hrs, the chemical composition of three resulting PEG-P(Glu-Glu/Phe\textsubscript{x})\textsubscript{150} copolymers was determined by \textsuperscript{1}H NMR analysis (Figure 9) using the peak intensity ratios of the methylene protons of PEG and the methyl protons of the ester of L-phenylalanine (500 MHz in TFA-\textit{d}\textsubscript{1}; \textdelta =3.86 (s, 4H, -OCH\textsubscript{2}CH\textsubscript{2}-), 3.8 (s, 3H, -CH\textsubscript{3})). These copolymers had a constant PEG block (114 repeating units), identical anionic PGlu block (about 150 repeating units), and they were differed in the amount of Phe hydrophobic moieties (26, 51, and 75 units) functionalized to the backbone. They are further denoted as rNG\textsubscript{25/150}, rNG\textsubscript{50/150}, and rNG\textsubscript{75/150}, respectively. The characteristics of synthesized PEG-P(Glu-Glu/Phe\textsubscript{x})\textsubscript{150} copolymers are summarized in Table 5.
Figure 9. $^1$H NMR spectrum for PEG-P(Glu-Glu/Phe)$_{150}$ (TFA-$d_1$, 298K).

Notes: peak assignments are as follows: PEG-P(Glu-Glu/Phe)$_{150}$ $^1$H NMR (TFA-$d_1$): δ ppm= 2.07 (2H, m, -CH$_2$-), 2.55 (2H, s, -CH$_2$-), 3.06 (2H, m, -CH$_2$-), 3.80 (3H, s, -CH$_3$), 3.85 (2H, s, -CH$_2$-), 4.55 (1H, s, -CH-), 4.77 (1H, m, -CH-), 4.94 (1H, m, -CH-); 7.20 (5H, m, C$_6$H$_5$)
### Table 5. Characteristics of the PEG-P\(\text{Glu-Glu/Phe}_x\)\(_{150}\) copolymers.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Feed molar ratio (mmol)</th>
<th>Repeating Units Ratio*</th>
<th>(M_n) (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-b-P(\text{Glu}_150)</td>
<td>1</td>
<td>27.5</td>
<td>32191</td>
</tr>
<tr>
<td>PEG-P((\text{Glu-Glu/Phe}<em>{25}))(</em>{150})</td>
<td>1</td>
<td>55</td>
<td>36221</td>
</tr>
<tr>
<td>PEG-P((\text{Glu-Glu/Phe}<em>{50}))(</em>{150})</td>
<td>1</td>
<td>82.5</td>
<td>40093</td>
</tr>
</tbody>
</table>

*Calculated from \(^1\)H NMR spectrum.

#### 2.3.2 Synthesis of NGs

The amphiphilic nature of resulting copolymers was anticipated to drive the formation of micellar particles in aqueous medium due to the incorporation of hydrophobic Phe moieties. After the polymer synthesis, polymeric micelles were then prepared by mixing the solution of the copolymer in dimethylformamide with water (1: 1 v/v) following the dialysis against water for 48 h. Indeed, the formation of small particles with relatively narrow particle size distribution (PDI < 0.2) and the net negative charge was detected in aqueous solutions for all three types of copolymers (Table 6). However, the formed micellar structures were not stable against pH change. When pH was increased to physiological pH, these micelles could not maintain structural integrity indicated by sharply increased particle size distributions. To solve this issue, PEG-P(\(\text{Glu-Glu/Phe}_x\))\(_{150}\) were further crosslinked to form NGs. The crosslinking of the PEG-P(\(\text{Glu-Glu/Phe}_x\))\(_{150}\) was achieved via condensation reactions between the carboxylic groups of PGlu segments and the amine groups of ethylenediamine in the presence of a water-soluble carbodiimide, EDC. The targeted extent of crosslinking (20%) was controlled by the molar ratio of crosslinker to carboxylic acid groups of the glutamic acid residues. The size of the nanogel
dispersion was similar to that of the precursor micelles at the same pH, confirming that the
micelles retained their integrity and that no observable intermicellar fusion can be
detected. After exhaustive dialysis against water, NGs were characterized at pH 7 (Table
7). The resulting NGs are uniform and possess a net negative charge. Furthermore, the
prepared NGs retained the structural integrity and relatively small particle size distribution
(PDI <0.2) when pH increased up to 9.

Table 6. Physicochemical characteristics of micelles based on PEG-P(Glu-Glu/Phe<sub>x</sub>)<sub>150</sub>
copolymers at pH 5.5

<table>
<thead>
<tr>
<th>Polymers</th>
<th>D&lt;sub&gt;eff&lt;/sub&gt; (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-P(Glu-Glu/Phe&lt;sub&gt;25&lt;/sub&gt;)&lt;sub&gt;150&lt;/sub&gt;</td>
<td>84 ± 3</td>
<td>0.12</td>
</tr>
<tr>
<td>PEG-P(Glu-Glu/Phe&lt;sub&gt;50&lt;/sub&gt;)&lt;sub&gt;150&lt;/sub&gt;</td>
<td>64 ± 2</td>
<td>0.12</td>
</tr>
<tr>
<td>PEG-P(Glu-Glu/Phe&lt;sub&gt;75&lt;/sub&gt;)&lt;sub&gt;150&lt;/sub&gt;</td>
<td>52 ± 3</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Effective hydrodynamic diameters (D<sub>eff</sub>) and polydispersity index (PDI) were determined
by DLS (water, pH 5.5, 25°C). Data are presented as average ± SD from three
experiments.

Table 7. Physicochemical characteristics of NGs based on PEG-P(Glu-Glu/Phe<sub>x</sub>)<sub>150</sub>
copolymers at pH 7

<table>
<thead>
<tr>
<th>rNG&lt;sub&gt;x/150&lt;/sub&gt;</th>
<th>D&lt;sub&gt;eff&lt;/sub&gt; (nm)</th>
<th>PDI</th>
<th>ζ-potential, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>rNG&lt;sub&gt;25/150&lt;/sub&gt;</td>
<td>119 ± 1</td>
<td>0.11</td>
<td>-29.3 ± 2.1</td>
</tr>
<tr>
<td>rNG&lt;sub&gt;50/150&lt;/sub&gt;</td>
<td>83 ± 1</td>
<td>0.10</td>
<td>-21.2 ± 1.0</td>
</tr>
<tr>
<td>rNG&lt;sub&gt;75/150&lt;/sub&gt;</td>
<td>63 ± 2</td>
<td>0.14</td>
<td>-15.0 ± 1.4</td>
</tr>
</tbody>
</table>

Effective hydrodynamic diameters (D<sub>eff</sub>), polydispersity index (PDI), and ζ-potential were
determined by DLS (water, pH 7, 25°C). Data are presented as average ± SD from three
experiments.
The NGs investigated here are composed of poly(L-glutamic acid), a weak polyelectrolyte (pKₐ 4.4). Since ionization degree of PGlu increased at higher pH, dissociation of the glutamic acid carboxylic groups within the NGs induced intramolecular electrostatic repulsions and, thus, caused the overall swelling of the nanogel particles. Furthermore, PGlu chains was reported to undergo a coil-to-helix transition at pH around the pK(a) [17] and these conformational changes can also influence the swelling behavior of NGs containing PGlu block. The pH-induced dimensional changes of the prepared three types of NGs were studied by DLS and electrophoretic mobility measurements, and the results are presented in Figure 10. A decrease of hydrodynamic diameter with a concomitant increase in negative ζ-potential was observed upon the decrease of pH for all three types of NGs. Reduced electrostatic repulsions and transition to an ordered conformation upon protonation of carboxylic acid of the crosslinked PGlu chains led to the collapse of the PGlu network resulting in the shrink of constructs and increase in ζ-potential. However, a sharper change in terms of size and ζ-potential was observed for NGs with less fraction of Phe units in the cores. NGs with higher number of Phe moieties would be anticipated to demonstrate less polyelectrolyte behavior leading to less swelling ability. The observed changes were reversible and the particle size distribution of NGs remained relatively narrow (PDI <0.2).
Figure 10. Swelling behavior of rNG\textsubscript{25/150}, rNG\textsubscript{50/150}, and rNG\textsubscript{75/150} represented by the change of the effective diameter, $D_{\text{eff}}$ (left) and $\zeta$-potential (right) of NGs upon pH change. Data are presented as average ± SD from three experiments.

2.3.3 Drug loading and release profile of loaded drugs

PEG-P(Glu-Glu/Phex\textsubscript{x})\textsubscript{150} NGs have a well-defined structure with a swollen crosslinked polyelectrolyte network functionalized with hydrophobic Phe moieties and external PEG
shell surrounding the network. Each of these three nanoregions contributes to the function of NGs as drug nanocarriers. Phe regions function as a reservoir to encapsulate water-insoluble molecules. PGlu ionic network can incorporate hydrophilic drug molecules through electrostatic interaction or covalent bonding while PEG shell stabilizing NGs in dispersion also minimize the recognition and uptake of the NGs by RES system. Therefore, such NGs have the potential to simultaneously incorporate and deliver multiple chemotherapeutic drugs with very different physical properties and mechanisms of actions via single nanocarrier in an effort to achieve a synergistic effect and improve the treatment outcome. In the present study NGs with a binary drug combination of hydrophilic CDDP and hydrophobic NRT were prepared. At first, NRT was solubilized into the Phe regions using an extraction method. According to this method, NRT-loaded NGs were prepared by adding ethanol solution of NRT (2 mg/mL) dropwise into the aqueous dispersion of NGs (1 mg/mL) upon agitation and the mixture was then stirred at r.t. overnight in an open-air system to allow slow evaporation of ethanol. The residual ethanol was then removed at reduced pressure. Unincorporated NRT was removed by filtration using 0.8 μm syringe filters (Thermo Scientific). Under these conditions NRT loading capacity (the net amount of drug loaded into a carrier) varied from 5 to 12 w/w% weight as was quantified by HPLC. The resulting nanoparticles (NPs) had an average hydrodynamic diameter from 76 to 135 nm and were characterized by narrow particle size distributions as determined by DLS. The size of the NRT-loaded NGs was slightly increased compared to those of empty NGs, while ζ-potential remained comparable indicating that the NRT incorporation into Phe regions did not induce substantial structural rearrangement and significantly affected the macroscopic characteristics of the pre-formed NGs. As the next step, CDDP was loaded into NGs via coordination binding of CDDP with COOH groups of the PGlu units. To this end, the aqueous dispersions of NRT-loaded NGs were incubated with CDDP for 48 h at pH 9.0 at a 1 : 4 molar ratio of CDDP to carboxylate groups of NG [18]. Such conditions
were chosen to maximize the swelling of the polyelectrolyte network, completely ionize carboxylic groups of the NGs and endow higher accessibility of the carboxylate groups to CDDP. Unbound CDDP was then removed by ultrafiltration using Amicon® Ultra-15 centrifugal filter units (MWCO 3,000 Da, Millipore). As expected, the net negative charge and particle size of NGs decreased upon CDDP loading consistent with neutralization of PGLu segments by CDDP and collapse of PGLu network (Table 8).

Table 8. Physicochemical characteristics of NGs before and after drug loading.

<table>
<thead>
<tr>
<th>Samples</th>
<th>pH 7</th>
<th>LC*, w/w% CDDP</th>
<th>Molar Ratio CDDP:NRT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D_{eff}, nm</td>
<td>PDI</td>
<td>ζ-potential, mV</td>
</tr>
<tr>
<td>rNG25/150</td>
<td>119 ± 1</td>
<td>0.11</td>
<td>-29.3 ± 2.1</td>
</tr>
<tr>
<td>NRT/rNG25/150</td>
<td>135 ± 3</td>
<td>0.20</td>
<td>-24.9 ± 1.9</td>
</tr>
<tr>
<td>CDDP/rNG25/150</td>
<td>110 ± 1</td>
<td>0.19</td>
<td>-3.6 ± 1.3</td>
</tr>
<tr>
<td>(CDDP+NRT)/rNG25/150</td>
<td>129 ± 4</td>
<td>0.18</td>
<td>-4.5 ± 1.5</td>
</tr>
<tr>
<td>rNG50/150</td>
<td>81 ± 3</td>
<td>0.10</td>
<td>-21.2 ± 2.0</td>
</tr>
<tr>
<td>NRT/rNG50/150</td>
<td>89 ± 2</td>
<td>0.16</td>
<td>-17.5 ± 0.5</td>
</tr>
<tr>
<td>CDDP/rNG50/150</td>
<td>66 ± 2</td>
<td>0.15</td>
<td>-2.3 ± 1.8</td>
</tr>
<tr>
<td>(CDDP+NRT)/rNG50/150</td>
<td>83 ± 3</td>
<td>0.19</td>
<td>-5.7 ± 0.9</td>
</tr>
<tr>
<td>rNG75/150</td>
<td>63 ± 2</td>
<td>0.14</td>
<td>-15.0 ± 1.4</td>
</tr>
<tr>
<td>NRT/rNG75/150</td>
<td>76 ± 2</td>
<td>0.18</td>
<td>-13.5 ± 0.7</td>
</tr>
<tr>
<td>CDDP/rNG75/150</td>
<td>59 ± 2</td>
<td>0.16</td>
<td>-6.2 ± 2.2</td>
</tr>
<tr>
<td>(CDDP+NRT)/rNG75/150</td>
<td>72 ± 5</td>
<td>0.17</td>
<td>-5.2 ± 1.5</td>
</tr>
</tbody>
</table>

Pt and NRT content were determined by ICP-MS and HPLC, respectively. *: Loading capacity (LC) is expressed as the mass of incorporated drug per mass of drug-loaded NGs (w/w). Effective hydrodynamic diameters (D_{eff}), polydispersity index (PDI) and ζ-potential were determined in water (pH 7). Data are expressed as mean ± SD (n = 3).
As expected, NGs with higher numbers of Phe moieties modified on the PGlu chains demonstrated the highest NRT loading capacity (11 w/w%), while NGs with the lowest number of Phe functionalities were able to encapsulate not more than 5 wt. % of NRT. The loading of hydrophilic molecules of CDDP appears to show a completely opposite trend compared with that of NRT, which is consistent with the number of available Glu units available for binding of CDDP. This study indicates that we could use PEG-P(Glu-Glu/Phe)\_x\_150 as a template to prepare NGs with the ability to vary the ratio of hydrophobic and hydrophilic drugs that they can carry. Determining a suitable drug-loading ratio is necessary because one drug can affect the release kinetics of the other, thus altering the activity [19]. Besides, varied ratios of drugs results in different modes of interaction, which can either be synergy, antagonism, or additivity [20]. NGs developed in this study were granted with the ability to further boost therapeutic efficacy of carrier drugs through screening the drug ratio. Notably, drug-loaded NGs were stable in aqueous dispersions, exhibiting no aggregation or precipitation for a prolonged period of time (weeks).

The release of the drugs from NGs was studied by equilibrium dialysis at 37°C at either pH 7.4 (PBS) or pH 5.5 (ABS) in the presence of 10% (v/v) fetal bovine serum (FBS), which mimics conditions encountered in plasma and in intracellular compartments (lysosomes), respectively. rNG\_25/150 was chosen as a model construct for evaluating the release kinetic of both drugs. The kinetic profiles of drug release from (CDDP+NRT)/rNG\_25/150 are presented in Figure 11. As seen from these data sustained but temporally distinct releases of Pt (II) species and NRT was observed. Notably, NRT release was much faster than that of Pt (II) in both conditions, which is expected since NRT is physically entrapped into the Phe regions through hydrophobic interactions. In contrast, CDDP binds with PGlu chains through electrostatic and coordination interactions and its release usually proceeds via ligand exchange reactions with surrounding
biologically abundant anions, thus delaying its liberation from the NGs. Pt (II) is released from the NGs in a pH-dependent behavior. Specifically, Pt (II) species were liberated from the NGs faster at pH 5.5 than at pH 7.4 (Figure 11B). The protonation of carboxylic groups of PGlu and subsequent weakened electrostatic coupling of CDDP and PGlu might account for this speeded release as was discussed previously [21]. For example, during 24 hrs (CDDP+NRT)/NGs released 27.01 ± 2.0% of loaded Pt (II) at pH 5.5 and only 17.7 ± 1.3% at pH 7.4, respectively. Besides, the release of Pt from NGs in a pure buffer system without FBS was also studied and presented in Figure 11B. Pt (II) liberation from (CDDP+NRT)/NG was further accelerated in the presence of FBS in the media probably due to the consumption of released Pt (II) molecules by protein binding. Notably, pH changes practically did not affect the release of NRT.
Figure 11. *In vitro* release profiles for NRT (A) and CDDP (B) from NGs: (CDDP + NRT) / rNG<sub>20/150</sub> in different buffer systems at 37°C (PBS, pH 7.4; ABS, pH 5.5; PBS + 10% FBS; or ABS + 10% FBS). Data are expressed as mean ± SD (n=3).
2.3.4 Penetration of NGs in tumor spheroids

It is critical to accurately predicting the responsiveness to therapeutic agents through screening pipelines. Among these screening models, multicellular tumor spheroids (MCTS), a three-dimensional multicellular culture system of human cells, is a more physiologically relevant platform providing a high-throughput means to test the efficacy and/or toxicity of new compounds [22]. They have a 3D spatial arrangement with enhanced cell to cell contact and the ability to maintain the specificity and homeostasis of a specific tissue with proliferative gradients, hypoxia, and necrosis [23] as summarized in Figure 12. For example, it was reported that cells from the outer region of the spheroid proliferate and behave healthily while cells in the inner region were apoptotic or injured with chromatin compaction and segregation mimicking the distribution of cells in tumor tissue in vivo [24]. Such platform allows us to study the penetration, distribution, and uptake of NPs due to their potential to form penetration barriers and mimic the heterogenous tumor microenvironment [25-27]. Since stromal fibroblasts have been shown to play an important role in determining tumor cell behavior, as well as conditioning the tumor microenvironment, we created a suitable 3D composite spheroid model containing a mixture of the mouse embryonic NIH 3T3 fibroblasts and MDA-MB-468 TNBC cells. Multicellular spheroids were generated using the forced-floating method: 500 cells (MDA-MB-468: 3T3=1:1) were seeded in ultra-low attachment 96-well plates in the presence of MammoCult® medium and incubated at 37°C for 6 days. On day 6, the spheroid morphology was recorded by an inverted microscope as shown in Figure 13. Spheroids were formed tightly packed in spherical dimension. Within the spheroid, connections between the cells were very close and compact, which is similar to in vivo tumor tissue, but different from 2D monolayer cells. The average diameter of formed spheroids was around 500µm. The forced-floating method enabled us to modulate the spheroid dimension by varying the number of cells in the starting suspension. In particular,
for a mixture of MDA-MB-468 and NIH 3T3 cells at 1:1 ratio, we obtained compact cellular aggregates with a diameter of 700–800 μm starting from a cellular suspension of 1500 cells. However, spheroids prepared from 1500 cells showed high shape variability. To design experiments with more reliability and repeatability, 500 cells was chosen as the starting number of cells.

**Figure 12.** Comprehensive demonstration of the cells and microenvironment inside a MCTS. Adapted from [22]
Figure 13. Representative phase-contrast image of MDA-MB-468/NIH 3T3 co-culture spheroid (Day 6).

We further used MDA-MB-468/NIH 3T3 spheroids for the assessment of penetration of rNGx/150 NGs. Cy5 amine (0.5 w/w%) was conjugated to cores of NGs using EDC chemistry. The particle size of Cy-5-labeled rNGx/150 remained practically the same as those of the unlabeled NGs (Table 9). Cy-5-labeled rNGx/150 (0.5 mg/mL) were incubated with 6-day old MDA-MB-468/NIH 3T3 spheroids for various periods of time (2, 4, and 8 h). Overall distribution and penetration of NGs within spheroids were analyzed by confocal microscopy using Z-stack imaging with 10 µm intervals and presented as maximum pixel intensity Z-projections (Figure 14). A comparative analysis of the fluorescence intensity data obtained from the spheroid images depicts the different pattern of interaction occurring with rNGx/150 having varied content of hydrophobic Phe units: an average fluorescence in the spheroid was increased as fraction of Phe units in the cores of NGs increased and appeared to coincide with the decrease of the size of rNGx/150 (Figure 15-16). This demonstrates the improved efficiency of the rNGx/150 with a higher content of hydrophobic Phe units in terms of penetrating the 3D tumor model and delivering the drug
when compared with rNG

which helps for a further selection of the

appropriate carrier candidate to maximize therapeutic efficacy of the cargo.

**Table 9.** Physicochemical characteristics of Cy5-labeled NGs

<table>
<thead>
<tr>
<th>NGs</th>
<th>D_{eff} (nm)</th>
<th>PDI</th>
<th>ζ-potential, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>rNG_{25/150}</td>
<td>143 ± 8</td>
<td>0.15</td>
<td>-27.7 ± 2.5</td>
</tr>
<tr>
<td>rNG_{50/150}</td>
<td>75 ± 7</td>
<td>0.10</td>
<td>-16.2 ± 1.5</td>
</tr>
<tr>
<td>rNG_{75/150}</td>
<td>61 ± 5</td>
<td>0.14</td>
<td>-12.0 ± 4.4</td>
</tr>
</tbody>
</table>

Effective hydrodynamic diameters (D_{eff}), polydispersity index (PDI), and ζ-potential were determined by DLS (water, pH 7, 25°C). Data are presented as average ± SD from three experiments.
Figure 14. Time-dependent penetration of Cy5-labeled rNGs throughout MDA-MB-468/NIH 3T3 spheroids (Day 6): (A) rNG25/150, (B) rNG50/150, (C) rNG75/150.
Figure 15: 3D surface plot analysis using maximum intensity projection of treated spheroids $a$: rNG$_{25/150}$, $b$: rNG$_{50/150}$, $c$: rNG$_{75/150}$
Figure 16. Qualitative evaluation of the distribution of NGs in MDA-MB-468/NIH 3T3 spheroids. (A) Corrected Cy5 fluorescence intensity vs distance from the periphery quantitates the NG association in spheroids. (B) Fluorescence intensity profile of Cy5-NGs showed the different distributions/penetrations of NGs in spheroids at 50 µm depth.
To further get insight into the cellular composition of co-culture MDA-MB-468/NIH 3T3 spheroids, MDA-MB-468 cells were transfected with a lentivirus engineered to constitutively express the Green Fluorescent Protein. Spheroid formation was continuously monitored using Nikon Eclipse E400 microscope (Figure 17). On days 1, 3 and 6, spheroids were dissociated using Accutase and cell composition was quantified by flow cytometry analysis (Figure 18). As it seen in Figure 17, MDA-MB-468/GFP+ cells were clustering on the edges of the growing spheroids and their number was decreased over the time as evident by the decrease in fluorescent signal (Figure 17), which was further confirmed by cell sorting analysis (Figure 18).

We further studied cellular uptake of rNG50/150 in co-culture MDA-MB-468/NIH 3T3 spheroids. On day 6, 48 MDA-MB-468(GFP+)/NIH 3T3 spheroids were incubated with Cy5-labeled rNG50/150 (0.5 mg/mL) for 4 hours followed by flow cytometry analysis (Figure 19). While all gated cells were associated with Cy5 fluorescent signal, only small cell population (ca. 2%) was GFP/Cy5 positive. Interestingly, median Cy5 fluorescence intensity measured in MDA-MB-468/GFP+ cells (MFI: 259797) was 10-fold higher than in fibroblasts (MFI: 29606.5) suggesting relatively higher accumulation of rNGs in cancer cells. Although we don’t know the exact mechanism behind this finding, there was a study demonstrating a strong cell line dependence of the NPs uptake and excretion dynamics [28]. It was particularly reported that retention of gold NPs coated with dithiolated diethylenetriaminepentaacetic acid was found to be higher in cancer cells than in fibroblasts in a 2D monolayer culture format. The exact mechanism of our observations will be further investigated.
Figure 17. Formation and growth of heterotypic MDA-MB-468/NIH 3T3 spheroids from TNBC MDA-MB-468/GFP+ (green) and murine fibroblasts (3T3). Fluorescence (top) and phase contrast (bottom) images of MDA-MB-468/NIH 3T3 spheroids (top) on days 1, 3 and 6.
Figure 18. Left: Percentage of MDA-MB-468 (GFP+, red) and 3T3 (GFP-, blue) cells in MDA-MB-468/NIH 3T3 spheroids as a function of time. Spheroids (96) were dissociated on days 1, 3 and 6 (n = 2). Right: Flow cytometry data representing cellular compositions of tumor spheroids on D1 (up) and D6 (down).
Figure 19. Cellular uptake of Cy5-labeled rNG_{50/150} in MDA-MB-468-GFP^+ /NIH 3T3 spheroids after incubation for 4h was detected by flow cytometric analysis. GFP^+ cells represent MDA-MB-468 which was transfected to express GFP. GFP^- cells represent NIH 3T3 cells.
2.3.5 \textit{In vitro} cytotoxicity of the drug-loaded NGs

CDDP is a well-known DNA-damaging agent. Its mechanism of action involves the formation of cisplatin-DNA adducts to disrupt DNA structure and interfere with DNA replication & transcription and ultimately induce apoptotic cell death. Herein we studied the synergy of CDDP and NRT, which is an irreversible inhibitor towards EGFR, HER2 and HER4. NRT might potentiate the therapeutic efficacy of cisplatin by reducing DNA-damage repair through interfering EGFR-mediated Histone H4/DNA-PKcs/ATM pathway and pushing cells towards apoptotic death. Besides, NRT could further sensitize cells to CDDP by downregulating oncogenic Cyclin D1 to keep cells blocked at G1 phase during cisplatin treatment since tumor cells appear to be maximally sensitive to cisplatin in G1 phase and minimally sensitive in S-phase [29]. \textit{In vitro} cytotoxicity of either free drugs or single drug-loaded and drug combination in a panel of nanogel formulations were evaluated in human TNBC cell line MDA-MB-468 with high EGFR expression. After 72-hour treatment, the surviving cell population was assessed by a CellTiter Blue assay (CTB), which measures the metabolic activity of viable cells. The half-maximal inhibitory concentrations (IC$_{50}$) of free drugs, single and combination-drug-loaded NGs are compared in Table 10. Indeed, the combination of CDDP and NRT was significantly more efficient in killing EGFR overexpressing cell line MBA-MD-468 than just CDDP treatment indicated by a significant drop in the IC$_{50}$ value of CDDP when administered in combination with NRT in a single carrier for 72 hours. Combination index analysis at IC$_{50}$ using the Chou and Talalay method [30] showed that the strongest synergy between the two drugs was observed at the CDDP:NRT ratio of 2:1 compared with ratio of 7:1 and 1:1, both in the form of free drugs (CI=0.40 ± 0.03) and combination in rNG$_{50/150}$ (CI = 0.40 ± 0.06). When both drugs were administered in the form of a cocktail of single drug-loaded NGs at equivalent doses, the synergy of action was still retained with less prominent effect compared with that seen in case of a combination of dual drug-loaded NGs at the CDDP:
NRT ratio of 7:1 and 2:1. In case of drug ratio at 1:1, the combination of two separate NG formulations (CDDP/rNG75/150 + NRT/rNG75/150) even displayed antagonistic CI value of 1.22 at IC$_{50}$. These data suggest that synchronized delivery of this drug combination in the same nanogel formulation may play an essential role in the cytotoxic activity.

**Table 10.** Comparison of IC$_{50}$ values for various drug-loaded NGs, free NRT and CDDP against EGFR$^+$ MDA-MB-468 as determined by CellTiter-Blue® assay.

<table>
<thead>
<tr>
<th>IC$_{50}$, µM (MDA-MB-468 cells)</th>
<th>Empty CDDP/NGs</th>
<th>CDDP/NGs NRT/NGs</th>
<th>Dual-drug/NGs*</th>
<th>Single drug NGs cocktail*</th>
<th>Free drug cocktail*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDDP</td>
<td>3.17 ± 0.27</td>
<td>0.62 ± 0.04</td>
<td>0.52 ± 0.04</td>
<td>0.91 ± 0.36</td>
<td>1.11 ± 0.37</td>
</tr>
<tr>
<td>NRT</td>
<td>4.45 ± 1.21</td>
<td>0.52 ± 0.04</td>
<td>0.91 ± 0.36</td>
<td>1.11 ± 0.37</td>
<td>0.59 ± 0.19</td>
</tr>
<tr>
<td>rNG$_{25/150}$</td>
<td>4.05 ± 0.51</td>
<td>0.52 ± 0.05</td>
<td>0.36 ± 0.12</td>
<td>0.57 ± 0.30</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td>rNG$_{50/150}$</td>
<td>1.98 ± 0.10</td>
<td>0.40 ± 0.10</td>
<td>0.29 ± 0.04</td>
<td>0.37 ± 0.06</td>
<td>0.25 ± 0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CI** at IC$_{50}$</th>
<th>CDDP:NRT (Molar Ratio)</th>
<th>Dual-drug/NGs</th>
<th>Single drug NGs cocktail</th>
<th>Free drug cocktail</th>
</tr>
</thead>
<tbody>
<tr>
<td>rNG$_{25/150}$</td>
<td>7:1</td>
<td>0.66 ± 0.09</td>
<td>0.74 ± 0.12</td>
<td>0.78 ± 0.32</td>
</tr>
<tr>
<td>rNG$_{50/150}$</td>
<td>2:1</td>
<td>0.40 ± 0.06</td>
<td>0.63 ± 0.02</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>rNG$_{75/150}$</td>
<td>1:1</td>
<td>0.85 ± 0.05</td>
<td>1.22 ± 0.14</td>
<td>0.45 ± 0.10</td>
</tr>
</tbody>
</table>

*calculated with respect to CDDP.

**: Combination index was calculated with respect to CDDP

Data presented as mean ± SD. N=3
Since binary drug combination carried in rNG_{50/150} displayed the highest synergy among three types of nanogel formulations, rNG_{50/150} was selected for the best-suited carrier system in further *in vitro* and *in vivo* studies. Cytotoxicity of the single drug-loaded and combination rNG_{50/150} was further evaluated in a panel of human TNBC cell lines with differential EGFR expression: MDA-MB-468 (EGFR high), MDA-MB-453 (EGFR low), as well as MDA-MB-231 (EGFR low). As described, after 72-hour treatment, the surviving cell population was assessed by CTB assay. The half-maximal inhibitory concentrations (IC_{50}) of free drugs, single and combination drug-loaded NGs are compared in Table 11. We found that (CDDP + NRT)/NGs were significantly more effective in killing EGFR-positive MDA-MB-468 compare to corresponding TNBC cells with low EGFR expression. The combination index (CI) values for drug combination at IC_{50} were less than 1 indicating synergistic cytotoxicity against MDA-MB-468 TNBC cells and thus confirming the benefit of selecting this combination of drugs. The lowest CI value for (CDDP+NRT)/rNGs (CI: 0.4) suggests that this drug combination displays clear synergistic activity at loading CDDP/NRT molar ratio of ca. 2:1. When the same formulations were applied to EGFR low MDA-MB-453 and MDA-MB-231 cells, the drug actions showed a mere additive effect because the CI values were close to 1. Importantly, rNG_{50/150} alone were not toxic at concentrations used for the treatment by CDDP/rNG_{50/150} formulations. CDDP/rNG_{50/150} displayed lower cytotoxic activities than free CDDP which probably arises from the sustained release of CDDP from NGs. These data indicate that the binary drug combination loaded NGs exerted selective synergistic effects in EGFR^{+} TNBC cells while such synergy was not observed in EGFR^{-} TNBC cells.
Table 11. Comparison of IC$_{50}$ values for various drug-loaded NGs and free drugs against panel of TNBC cell lines as determined by CTB assay.

<table>
<thead>
<tr>
<th></th>
<th>MDA-MB-468</th>
<th>MDA-MB-453</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDDP</td>
<td>3.17 ± 0.27</td>
<td>4.11 ± 0.16</td>
<td>4.67 ± 1.06</td>
</tr>
<tr>
<td>NRT</td>
<td>0.62 ± 0.04</td>
<td>0.87 ± 0.30</td>
<td>1.46 ± 0.26</td>
</tr>
<tr>
<td>CDDP/rNG$_{50/150}$</td>
<td>4.05 ± 0.51</td>
<td>7.07 ± 4.89</td>
<td>7.13 ± 0.70</td>
</tr>
<tr>
<td>NRT/rNG$_{50/150}$</td>
<td>0.52 ± 0.05</td>
<td>0.71 ± 0.06</td>
<td>1.68 ± 0.39</td>
</tr>
<tr>
<td>(CDDP + NRT)/rNG$_{50/150}$</td>
<td>(0.40 ± 0.06)</td>
<td>(1.17 ±0.36)</td>
<td>(1.01 ± 0.26)</td>
</tr>
<tr>
<td>CDDP/rNG$<em>{50/150}$ + NRT/rNG$</em>{50/150}$</td>
<td>0.57 ± 0.30</td>
<td>0.92 ± 0.48</td>
<td>3.31 ± 0.47</td>
</tr>
<tr>
<td>Free CDDP + NRT</td>
<td>0.34 ± 0.04</td>
<td>0.78 ± 0.22</td>
<td>2.80 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>(0.40 ± 0.03)</td>
<td>(1.44 ± 0.56)</td>
<td>(1.04 ± 0.11)</td>
</tr>
</tbody>
</table>

*IC$_{50}$ values and combination index (CI, labeled red) for drug combination loaded NGs were calculated with respect to CDDP. Data presented as mean ± SD. N=3*
2.3.6 Mechanism of synergy

To investigate the molecular basis for the synergistic effect of combination of CDDP and NRT at molar ratio of ca. 2:1 observed in the previous cell viability studies, we used western blot analysis to first determine the level of EGFR activation using expression of phosphor-EGFR as the indicator in EGFR+ MDA-MB-468 cell lines under various treatments (Figure 20). Cells were treated with either single CDDP (10µM), NRT (5µM) or the binary drug combination at the same dose for 2h. Then cells were treated with or without EGF for 5mins to stimulate EGFR phosphorylation at a concentration of 100ng/ml. Notably, higher phosphor-EGFR was found in cells treated with CDDP or EGF, a ligand for activation and phosphorylation of EGFR, indicating that CDDP treatment triggered the activation of EGFR which is considered as a pro-survival signal leading to mitigated therapeutic efficacy. No obvious change in terms of the level of phosphor-EGFR was found in cells treated with NRT in the presence of EGF, indicating that NRT successfully suppressed the EGF-mediated activation and phosphorylation of EGFR. Similarly, no observable change in phosphor-EGFR was found in cells treated with combination of CDDP, NRT and EGF, further indicating that NRT was able to suppress EGFR activation induced by CDDP and EGF, suggesting that the CDDP-mediated pro-survival signal could be reversed by NRT, enhancing the cell response to CDDP.
Figure 20. Effect of NRT and CDDP on total EGFR and phosphorylation of EGFR in MDA-MB-468 cells. Cells were incubated in medium containing the indicated concentrations of treatments for 2 hours. At the end of the 2-hour incubation, 100 ng/mL EGF was added for 5 minutes. Total proteins (10 µg) were prepared and separated by 8% SDS-PAGE. After transferring to a PVDF membrane, blots were probed with anti-phospho-EGFR, anti-EGFR, anti-β-actin, respectively. The values underneath the blots represent relative changes of p-EGFR levels present in the treated cells compared to the basal level of those in control cells after normalization to β-actin, quantified by densitometric analysis using software ImageJ. Blots are representative of 3 independent experiments.
HER1/EGFR activates several cytoplasmic signal-transduction pathways such as mitogen-activated protein kinase (MAPK), PI3 kinase/Akt, JAK/STAT [31]. Among them, MAPK and Akt are the major two downstream effectors of EGFR investigated in many studies due to their crucial roles in transporting signals for promoting cell division and survival. We investigated whether NRT would inhibit the activity of these two downstream effectors: MAPK (ERK) and Akt which may correlate with the strong synergy between CDDP and NRT in EGFR+ MDA-MB-468. Similarly, a panel of treatments were given to MDA-MB-468 cells at various concentrations with or without the receptor stimulator EGF (100 ng/mL). As seen in Figure 21, NRT at 5 µM markedly inhibited ERK1/2 (MAPK) as well as Akt activity in EGF-stimulated MDA-MB-468, suggested by more than 2 folds reduction in the level of kinase phosphorylation. Notably, NRT almost completely blocked ERK1/2 activity in MDA-MB-468 cells. Most importantly, CDDP was able to induce almost the same level of Akt/MAPK activation as the stimulator EGF, which was completely reversed by the addition of NRT, further suggests that the reverse of CDDP-induced pro-survival signal by NRT might be the molecular mechanism for strong synergistic effect of the combination in the EGFR+ MDA-MB-468 cell line.
Figure 21. Effect of NRT and CDDP on phosphorylation of MAPK (ERK1/2) and Akt in MDA-MB-468 cells. Cells were incubated in medium containing the indicated concentrations of listed treatments for 2 hours. At the end of the 2-hour incubation, 100 ng/mL EGF was added for 5 minutes. Total proteins (10 µg) were prepared and separated by 8% SDS-PAGE. After transferring to a nitrocellulose membrane, blots were probed with anti-phospho-ERK1/2, anti-phospho-Akt, anti-β-actin, respectively. The values underneath the blots represent the relative changes of p-ERK1/2 and p-Akt levels present in the treated cells compared to the basal level of those in control cells after normalization to β-actin, quantified by densitometric analysis using software ImageJ. Blots are representative of 3 independent experiments.
Cyclin D1 displays proto-oncogenic function due to its contribution in promoting cell cycle progression. Cyclin D1 promotes the cell cycle to transit from G1 to S phase through binding to cyclin-dependent kinase subsequently triggering the transcription of genes required for this progression [32, 33]. Since cells remains most sensitive to CDDP if they are in G1 phase, least sensitive in S phase [29], higher expression of Cyclin D1 would mitigate the therapeutic efficacy of CDDP. Here we further evaluated the changes in the expression level of Cyclin D1 of treated cells. As demonstrated in Figure 22, CDDP treatment or addition of EGF increased the expression of Cyclin D1 up to 2 folds comparing to the basal level which might result in the attenuation of CDDP-induced cell death. Cyclin D1 overexpression was thought to be at least partly dependent on the mitogenic effects of EGF signaling through the EGFR, which is consistent with what was observed in our study [34]. However, the addition of NRT reduced the Cyclin D1 expression back to the basal level. Thus, we believed that the NRT-directed suppression of cyclin D1 expression might help to maintain tumor cells in G1 phase for enhanced susceptibility to CDDP-mediated apoptosis.
Figure 22. Effect of NRT and CDDP on Cyclin D1 expression level in MDA-MB-468 cells. Cells were incubated in medium containing the indicated concentrations of listed treatments for 2 hours. At the end of the 2-hour incubation, 100 ng/mL EGF was added for 5 minutes. Total proteins (10 µg) were prepared and separated by 8% SDS-PAGE. After transferring to a nitrocellulose membrane, blots were probed with anti-Cyclin D1, anti-β-actin, respectively. The values underneath the blots represent the relative changes of Cyclin D1 levels present in the treated cells compared to the basal level of those in control cells after normalization to β-actin, quantified by densitometric analysis using software ImageJ. Blots are representative of 3 independent experiments.
2.4 Conclusion

In the present study, a series of NGs based on amphiphilic PEG_{5K}-P(Glu-Glu/Phe)_{150} copolymers were evaluated as potential vehicles for co-delivery of chemotherapeutic drugs with distinct physicochemical properties and mechanisms of action. These nanostructures were designed to have multifunctional compartments for drug loading and were crosslinked within a polyelectrolyte core of the polymeric NGs to ensure their enhanced stability upon systemic administration. These NGs simultaneously bared a sufficient load of hydrophilic CDDP and hydrophobic NRT, exhibited differential release profiles and synergistic cytotoxic effect against EGFR+ MDA-MB-468 triple negative breast cancer cells. By tuning hydrophilic and hydrophobic fraction ratio, NGs displayed varied dimensions, drug loading capacities, deformability, performance in killing tumor cells, as well as penetrations in multicellular 3D tumor models. Consistent with properties of chemical composition, NG with a high fraction of hydrophobic moieties displayed less swelling ability and more efficient hydrophobic drug loading. Resulting binary-drug-loaded NGs with 50 units of hydrophobic Phe functionalities were able to encapsulate both CDDP and NRT in a molar ratio of 2:1 and displayed the strongest synergistic effect towards MDA-MB-468 cells compared with other screened regimens. Such superior synergy was found to be selective and only displayed in EGFR+ cell line. rNG_{50/150} also displayed preferential uptake by tumor cells in the tumor spheroids model consisting of tumor cells as well as fibroblasts. NRT was found to reverse pro-survival signal transduction by CDDP mediated EGFR/Akt/Erk activation as well as increased Cyclin D1 expression, which was believed to be the molecular basis for the synergistic effect of the combination of CDDP and NRT. Altogether, this study demonstrated a fundamental possibility for the simultaneous delivery of chemotherapeutics via single well-defined and structurally tunable polymeric nanocarrier. Besides, such carriers grant us with flexibility in adjusting cargo ratio as well as dimensions for enhanced therapeutic outcomes of carrier drugs.
Acknowledgments

We acknowledge the assistance of NMR, Electron Microscopy and Nanoimaging Cores, Flow cytometry cores (University of Nebraska Medical Center). The author is grateful to Dr. Shaheen Ahmed for assistance with tumor spheroids, Dr. Kruti Soni for help with cell studies, Dr. Fan Lei for help with nanogel preparation, and Dr. Ram Mahato for providing iBright Western Blot Imaging Systems to perform data acquisition.
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CHAPTER III.

LIGAND-INSTALLED NANOGELS FOR COMBINATION DRUG THERAPY IN OVARIAN CANCER

3.1 Introduction

Ovarian cancer (OC) is the most lethal gynecological cancers in the USA with no effective screening strategy. It is estimated by The American Cancer Society that in 2020, there will be about 21,750 new cases of ovarian cancer diagnosed and 13,940 women will die of ovarian cancer in the United States [1]. Although the standard of care of OC patients including surgical cytoreduction followed by platinum-based chemotherapy has shown high response, mortality rates for ovarian cancer have declined only marginally in the forty years emphasizing continued efforts toward better treatments [2]. Most patients present with advanced-stage tumors showing metastasis outside the pelvis at the time of initial diagnosis due to inadequate diagnostic markers and non-specific symptoms [3, 4]. Although ovarian cancer can be divided into several subtypes based on the cell of origin, including the germ, epidermoid, stromal, and border cells, epithelial ovarian cancer (EOC) accounts for about 90% of OCs. EOC is associated with a high mortality rate due to the intrinsic aggressive nature and limited early detection techniques. Among all the subtypes of EOC, high-grade serous carcinoma (HGSC) is the most commonly diagnosed form with high sensitivity to current front-line combination platinum- and taxane-based chemotherapy. In spite of high response rate to the standard therapy, EOC frequently relapse and become increasingly less sensitive to chemotherapy. We may be approaching the limit of using conventional chemotherapeutics to optimize treatment of EOC [4].
Delaying the onset of resistance and prolonging the survival of EOC patients will likely occur with progression in the early detection of this disease [5] as well as through the incorporation of biologically targeted therapeutics. Aberrant EGFR expression is reported in up to 60% of malignant ovarian tissue as demonstrated by IHC and occurs in all histologic subtypes [6]. Since the increased copy number and overexpression of EGFR were found to be associated with high tumor grade and poor patient outcomes, EGFR expression might be used as an independent prognostic indicator in EOC patients [7-9]. Interestingly, the increased copy number of EGFR was reported to be associated with the increased copy number of HER2 which was also reported to be a negative prognostic indicator in EOC in some reports [7, 10]. Therefore, inhibiting the family of HER might be a promising approach through utilizing biological targets [11, 12]. As mentioned, EOC has been showing high sensitivity towards platinum-based chemotherapy. Cisplatin (CDDP), a widely used platinum compound, acts by damaging DNA. Once entering the cell through either passive diffusion or active transport, cisplatin undergoes the aquation process and becomes a potent electrophile that reacts with a variety of nucleophiles, including nucleic acids. The formation of CDDP-DNA adducts disrupts the DNA structure and interferes with DNA replication & transcription leading to either apoptotic cell death or DNA repair with cell survival. The mechanisms of determining the fate of cells remain largely unknown [13]. The declined sensitivity to cisplatin is believed to arise from reduced cellular uptake [14], enhanced intracellular detoxification, increased DNA repair, tolerance of platinum-DNA adducts and regulation of apoptosis [15, 16]. On the other hand, NRT is an inhibitor of HER family receptor tyrosine kinases, including EGFR, HER2 and HER4. Its mechanism of action involves the irreversibly binding to the intercellular signaling domain of kinases to inhibit their phosphorylation and several HER downstream signaling pathways such as ERK, PI3 kinase/AKT, JAK/STAT, etc. It has been reported that nucleotide excision repair (NER), a primary DNA-damage repair process, may prevent cisplatin-induced apoptosis.
by activating the ataxia telangiectasia mutated (ATM) pathway [13]. EGFR acts as an upstream effector of ATM by phosphorylating ATM to mediate DNA repair. Treatment with EGFR inhibitor successfully blocked ATM activation and subsequently increased tumor cell sensitivity to DNA-damaging treatments [17]. Apart from activating the ATM pathway, EGFR was shown to epigenetically regulate DNA damage signaling pathways by modifying chromatin at critical tyrosine residues [18] and phosphorylating DNA-PKcs, an enzyme required for DNA-repair [19], to reduce the DNA damage and restore DNA repair activity. As an inhibitor of HER family, NRT might potentiate the therapeutic efficacy of CDDP by reducing DNA-damage repair through interfering EGFR-mediated Histone H4/DNA-PKcs/ATM pathway and pushing cells towards apoptotic death. On the other hand, EGFR is activated in response to CDDP in various types of cancer cells that overexpress the receptor including human breast cancer cells and glioma cells [20], which is believed to be a survival-promoting or cell death-opposing response. Many studies have shown blocking EGFR activation enhances CDDP-induced cell death [21-23]. This serves as a strong basis that preventing CDDP-dependent EGFR activation should enhance drug sensitivity and improve clinical efficacy. Another minor mechanism of action of NRT is its ability to downregulate the oncogenic Cyclin D1 level which plays a key role in advancing the cell cycle into the late G1 and S phases [24]. Since tumor cells appear to be maximally sensitive to cisplatin in G1 phase and minimally sensitive in S-phase [25], reducing Cyclin D might keep more cells blocked at the G1 phase during cisplatin treatment and maximize cisplatin efficacy, which might also explain that the increased cyclin D expression results in less tumor sensitivity to cisplatin in several in vitro models, including fibrosarcoma [26] and pancreatic cancer [27]. Altogether, the above mechanism of actions of both drugs then forms the rational basis for the selection of such combined therapy to achieve maximized synergistic clinical effects for treating ovarian cancer. However, the synergy of such therapy is often sequence and dose ratio specific. Besides, administrating two
distinct drugs might require repeated dosing and prolonged duration of drug infusion in patients. Moreover, conventional Pt-based chemotherapy generally comes with rapid elimination and severe dose-limiting toxicities including neurotoxicity and nephrotoxicity [28]. Combining such drugs in one delivery carrier is therefore a desirable and convenient strategy for controlling the pharmacokinetics [29, 30] and co-delivery of the desired drug ratio in vivo, to maximize the therapeutic efficacy and minimize drug-associated toxicities. Crosslinked polypeptide-based nanogel (NG) is an appealing platform to achieve this goal. Such a biodegradable platform provides flexibility in adjusting the size, loading capacity, surface properties, deformability, softness, and responsive behaviors by tuning the chemical compositions as well as crosslinking levels. Enhanced stability due to the crosslinking adds benefits to prolonging the circulation, which in turn enhances drug exposure to tumors by EPR effect. Their mild preparation procedures allow the incorporation of labile drug molecules and biomacromolecules. The most prominent advantage over other nano-sized carriers is their ability to incorporate molecules with different physicochemical properties through versatile interactions. For the purpose of the current study, poly (ethylene glycol)-block-poly (L-glutamic acid) modified with phenylalanine functionalities (PEG-P(Glu-Glu/Phe)) for simultaneous loading and delivery of binary CDDP and NRT combination. Such NGs have 1) a hydrophilic PEG shell which imparts a stealth character against the immune system and extends the systemic circulation, 2) an anionic core, which incorporates CDDP through coordination with the carboxylic groups of PGlu, 3) hydrophobic regions formed by Phe moieties, which serves as a reservoir for NRT solubilization. The crosslinks incorporated into PGlu block layer keep NG stable until they are degraded by proteases through cleaving the polypeptide chains of the block copolymers. The developed binary drug NG formulation showed synergy in several EGFR overexpressed ovarian cancer cell lines, while such synergy was not seen in cell lines with low EGFR overexpression. Such drug delivery systems also
showed desirable antitumor efficacy in an intraperitoneal ovarian cancer mouse model with no observable toxicities while certain toxicities were observed in tumor-bearing mice treated with free drugs at the same dose. However, this system solely depends on the EPR effect which is limited by heterogeneity among tumor mass as well as vascular density nearby influencing the permeability of nanocarriers. Installing targeting ligands on the surface of carriers to specifically target biomarkers overexpressed on the surface of tumor cells is a well-suited strategy to overcome the mentioned limitation and further boosting drug performance. Two targeting ligands aiming to bind with EGFR was chosen in our study: L-AE peptide [31] and EGFR-A protein [32]. Both ligands are relatively new with reported high binding specificity and affinity to EGFR with acceptable stability. Ligand installed carrier demonstrated success in increasing cellular uptake and further boosting the therapeutic efficacy of targeted binary combination NGs in vivo. We also confirmed the importance of simultaneous administration of the (CDDP+NRT) via a single NG system which provides more therapeutic benefits than a cocktail of individual drug-loaded NGs administered at equivalent doses.

3.2 Materials and methods

3.2.1 Materials
Poly (ethylene glycol)-b-poly (L-glutamic acid) (PEG-b-PGlu) diblock copolymer (\( M_w/M_n=1.01, M_w 28,000 \ g \ mol^{-1} \)) was purchased from Alamanda Polymers, Inc. (Madison, AL, USA). The block lengths were 114 and 150 repeating units for PEG and PGlu, respectively. Methoxypolyethylene glycol amine (mPEG-NH₂) polymer (\( M_w/M_n=1.04, M_w 10,000 \ g \ mol^{-1}, 228 \) repeating units), maleimide-PEG-Amine (Mal-PEG-NH₂) polymers with different molecular weights, \( M_w 7,500 \ g \ mol^{-1} (D_M = M_w/M_n = 1.02) \) and \( M_w 15,000 \ g \ mol^{-1} (D_M = 1.04) \) were purchased from JenKem Technology (TX, USA). L-glutamic acid γ-benzyl ester (BGlu), L-phenylalanine methyl ester hydrochloride (Phe), 1-(3-
dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), tris (2-carboxyethyl) phosphine (TCEP), ethylenediamine (ED) and other chemicals were obtained from Sigma-Aldrich (St Louis, MO) and were used without further purification. Fetal bovine serum (FBS) was purchased from Invitrogen Inc. (Carlsbad, CA). RPMI 1640 medium, EGF Recombinant Human Protein, Pierce™ BCA protein assay kit, CA125 Human ELISA kit was purchased from Thermo Fisher Scientific (Waltham, MA). CellTiter-Blue® reagent was purchased from Promega (Madison, WI). Cyanine 5 (Cy5) amine was obtained from Lumiprobe (Hallandale Beach, FL). Cell lysis buffer and protease inhibitor cocktail were purchased from Cell Signaling Technology (Danvers, MA). The following primary antibodies were used in this study: Rabbit anti-EGFR (ab52894) mAb, rabbit anti-phospho-EGFR (phospho Y1068) (ab40815) mAb, rat anti-HSC70 (ab19136) mAb, mouse anti-beta actin (ab8226) mAb used for Western blotting studies, were purchased from Abcam™ (Cambridge, MA); the rabbit anti-Phospho-Akt (D9E) mAb, rabbit anti-phospho-p44/42 MAPK (Erk1/2) (4370T) mAb was kindly provided by Dr. Surinder K. Batra (University of Nebraska Medical Center, Omaha, NE).

3.2.2 Synthesis of NGs

NGs were prepared using a diblock copolymer, PEG-PGLu150, in which part of the Glu units was modified with hydrophobic Phe functionalities via polymer-analogous modification of the PGLu segment with the Phe methyl ester using carbodiimide-chemistry in aqueous media. The targeted degree of modification was set at 50 Phe units per chain since binary-drug-loaded NGs prepared from such polymers showed the highest synergy in the EGFR+ TNBC cell line as we observed in the previous chapter. The obtained products were characterized by proton nuclear magnetic resonance (1H NMR). Micellar precursors based on PEG-P(Glu-Glu/Phe50)150 copolymers were prepared by a dialysis method using water/organic solvent mixtures followed by direct crosslinking of COOH groups of glutamic
acid units with 1,2-ethylenediamine (ED) in the presence of EDC at r.t.. The crosslinking reactions were performed at a molar ratio of \([\text{COOH}]/[\text{EDC}]/[\text{ED}]=10/2/1\) to obtain a targeted 20% crosslinking degree. The prepared nanogel was abbreviated as NG (further denotes as rNG5k). To prepare Cy5 labeled NGs for evaluating cellular uptake, Cy5 amine (0.5 w/w\%) was conjugated to cores of NGs using EDC chemistry.

3.2.3 Synthesis of ligand-installed NGs and drug loading

NGs were functionalized with EGFR-A protein ligands (Mₐ 13,400 Da, provided by Dr. Liu, University of North Carolina at Chapel Hill, NC) that specifically and tightly bind to the extracellular domain of EGFR (further denoted as EGFR-A-NGs). To prepare the protein ligand EGFR-A, a codon-optimized DNA sequence encoding an FN3 domain-based targeting ligand that specifically binds to the extracellular domain of human EGFR (denoted as EGFR-A) was synthesized (by Genscript) and cloned into the pET28b vector according to the previously published procedures (Cite PMID: 30842091). To facilitate affinity purification and site-specific conjugation with NGs, a His×6 tag and the only cysteine residue were genetically engineered at the C-terminus that is far away from the target-binding regions. Once the purified recombinant protein EGFR-A (~13.4 kDa) was obtained and measured by SDS-PAGE, it was conjugated to drug-loaded NGs via PEG spacer having a maleimide group (Mal-PEG₇.₅k-NH₂). Firstly, Mal-PEG-NH₂ (0.013 mmol, 0.0007 eq with respect to the number of carboxylate groups and 1.5 eq with respect to required EGFR-A) was conjugated to the free carboxyl groups (18.57 mmol) of NGs via EDC (0.13 mmol) chemistry. The reaction was allowed to proceed for 4 h followed by ultrafiltration (MWCO 30,000 Da) to remove the byproducts and unreacted PEG linkers. PEG-NGs were then loaded with CDDP through mixing with an aqueous solution of CDDP (1mg/mL) at pH 9.0 at a 0.25 molar ratio of CDDP (0.06mmol) to carboxylate groups (0.24mmol) of the NGs followed by incubation at 37°C for 24h. Unbound CDDP was
removed by Ultracon filter units (MWCO 10,000 Da, Millipore) at 1690 g for 15 min. Activated EGFR-A was further conjugated to maleimide terminus of the PEG linker via thiol groups (PBS, pH 7.4, 2 h). EGFR-A-NGs were purified from unconjugated protein using ultrafiltration (MWCO 100k Da). The amount of conjugated ligand was determined by micro-BCA assay using non-modified PEG-conjugated NGs as a reference. Finally, NRT was encapsulated into the hydrophobic domain of NGs through an extraction method. Briefly, NRT dissolved in ethanol was added dropwise to the aqueous dispersion of NGs upon stirring followed by evaporation of organic solvent [33]. Unbound NRT was removed by filtration with 0.8 μm syringe filters (Thermo Scientific). Single drug-loaded NGs were prepared through the similar procedure. Pt content in NGs ($^{194}\text{Pt} / ^{195}\text{Pt}$) was assayed by inductively coupled plasma mass spectrometry (ICP-MS, NexION 300Q, PerkinElmer, Waltham, MA) calibrated with Pt (0.8 to 500 ng/mL). NRT content was assayed by HPLC under isocratic conditions using an Agilent 1200 HPLC system with a diode array detector set at 266nm. As the stationary phase a Nucleosil C18 column was used (250 mm × 4mm), and a mobile phase of (acetonitrile+0.1%TEA)/(water+0.1%TEA mixture) (80/20,v/v) was applied at a flow rate of 1ml/min with a column temperature of 40°C. NGs can entrap considerable amounts of CDDP and NRT, respectively. L-AE (FALGEA), a novel hexapeptide ligand identified through a mixture-based synthetic combinatorial library, has demonstrated selective binding affinity to EGFR and EGFRvIII as reported [31, 34]. L-AE is another EGFR targeting ligand evaluated in our study as well. We designed cysteine terminated L-AE (sequence: CGGGGFALGEA) to allow site-specific conjugation to NGs. A spacer (GGGG) was added between the cysteine and the peptide to avoid hinderance for more efficient ligand-receptor interaction. The peptide (synthesized by Genscript) was conjugated to NGs through the similar procedure mentioned above (further denoted as LAE-NGs).
3.2.4 Physicochemical characterization of the NGs

The $^1$H NMR spectra for the monomers and copolymers were acquired in CDCl$_3$ or TFA-$d_1$ at 25 °C using a Bruker 500 MHz spectrometer, respectively. Effective hydrodynamic diameters ($D_{	ext{eff}}$) and $\zeta$-potential of NG were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). All measurements were performed in automatic mode at 25°C. Software provided by the manufacturer was used to calculate the diameters, polydispersity index (PDI), and $\zeta$-potential of NGs. All measurements were performed at least in triplicate to calculate mean values ± SD.

3.2.5 Cellular uptake of NGs

Cellular uptake of NGs was assessed using flow cytometry. Cells (EGFR+: SKOV-3 & OVCAR-5, EGFR-: NIH 3T3) were seeded in 6-well plates at a density of $1 \times 10^5$ cells per well, and cultured at 37°C in 5% CO$_2$ for 24 h. Cells grown in RPMI media then were exposed to Cy5-labeled bare NGs or EGFR-A-NGs or LAE-NGs at 4 °C. Following incubation for 1 h, cells were washed three times with PBS, further incubated with fresh medium for 30 mins at 37°C, trypsinized, centrifuged (423 g, 5 min) and resuspended in PBS (pH 7.4). Cells were stored in darkness at 4°C before measurements. The % of gated cells were analyzed using BD Biosciences LSR II flow cytometer and FACSDiva software (BD Biosciences, San Jose, CA). At least 10,000 events were acquired in linear mode, gated to only include non-aggregated live cells, and visualized in logarithmic mode. The results are reported as the percentage of cells associated with fluorescence versus the number of gated cells.

3.2.6 Cell culture and cytotoxicity studies

Cytotoxicity studies were conducted on three human ovarian cancer cell lines: SKOV-3, OVCAR-5 and A2780 (provided by Dr. P.Rogers, Institute of Cancer Research, University of Bristol, UK). First two cell lines overexpressed EGFR while the third one was found to
have negligible expression. Cells were maintained in RPMI medium supplemented with 10% (v/v) FBS in the presence of penicillin and streptomycin (100 U/mL and 0.1 mg/mL, respectively) at 37°C in a humidified atmosphere containing 5% CO₂. Cells were harvested with trypsin-EDTA (Life Technologies) after 80% confluence. Cells seeded in 96-well plates (3,000 cells/well) 24 h prior the experiment were exposed to various doses (0-10 μg/mL on CDDP or NRT basis) of CDDP alone, free binary drugs, CDDP-loaded NGs, NRT-loaded NGs, a cocktail of single drug-loaded NGs, (CDDP+NRT)-loaded NGs and targeted-(CDDP+NRT)-loaded NGs for 48 h at 37°C. Cytotoxicity of either free drugs or drug-loaded NGs was assessed by a standard CellTiter-Blue® cell viability assay [35] with untreated cells served as 100% cell viability. The IC₅₀ values were calculated using GraphPad Prism software. Combination index (CI) analysis based on Chou and Talalay method [36] was performed using CompuSyn software for CDDP and NRT combinations, determining synergistic, additive, or antagonistic cytotoxic effects against ovarian cancer cells. Values of CI < 1 demonstrate synergism while CI = 1 and CI > 1 values represent additive and antagonistic effects of drug combination, respectively.

3.2.7 Western blot analysis

Cells were treated with either EGF, EGF + CDDP, EGF + NRT, or EGF + CDDP + NRT for 2h at a concentration of 100 ng/mL EGF, 10 μM/mL CDDP, and 5 μM/mL NRT. Cells were then lysed with cell lysis buffer supplemented with protease inhibitor cocktail. Supernatants were collected after removing cell debris by centrifugation (14,000 g for 20 min), and the protein amount was determined by BCA assay. 5–10 μg of proteins were separated by 8% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Massachusetts). Non-specific protein binding was blocked in Tris-buffered saline Tween-20 (TBS-T) (50mM Tris–HCl, 150mM NaCl, 0.1% Tween 20, pH 7.6) containing 5% dry milk for 30mins at 37°C. Proteins of interest were immunoblotted with
the appropriate monoclonal antibodies used at 1:1000 to 1:10000 dilutions at 4°C overnight followed by the secondary horseradish peroxide-conjugated antibodies used at 1:1000 dilutions at RT for 1 hour. Antibody interactions were visualized with enhanced chemiluminescence (ECL) horseradish peroxidase (HRP) substrate (Thermo fisher scientific, Massachusetts) using iBright Western Blot Imaging Systems (Invitrogen, kindly provided by Dr. Ram Mahato, University of Nebraska Medical Center, Omaha, NE). HSC70 and β-actin have been used as a loading control. Band intensities were analyzed by ImageJ software.

3.2.8 Optimization of NG by extending the PEG block
A preliminary study of biodistribution of cisplatin loaded into rNG5k after intravenous administration was conducted in Dr. Kabanov’s lab at the University of North Carolina using a TNBC mouse model. Their data suggested that CDDP/ rNG5k mainly accumulated in liver and spleen with very little amount going to the tumor tissue. To better optimize the pharmacokinetic properties of the carrier system and reduce the clearance by the mononuclear phagocyte system (MPS), we developed NGs with a doubled length of PEG block using the same templating method. Instead of 114 of EG repeating units, the new NGs have 228 repeating units with similar numbers of Glu units and Phe moieties.

3.2.8.1 Synthesis of N-carboxyanhydrides (NCA) of BGlu
As described before [37], BGlu (0.020 mole) and anhydrous tetrahydrofuran (THF) were added into a dried glass reactor to form a suspension under inert (nitrogen) atmosphere. Triphosgen (0.023 mole) was then dissolved in fresh anhydrous THF and injected drop wise into the reaction mixture. The reaction was conducted under inert atmosphere with nitrogen bubbled through the mixture. The mixture was heated at about 55°C with constant stirring till the mixture became transparent. The solution was precipitated in n-hexane and then stored at -20°C overnight in order to allow complete precipitation of γ-benzyl L-
glutamate-N-carboxyanhydride (BGlu-NCA). The white solids obtained were collected and purified further by repeated precipitation with n-hexane. The resulting NCA monomers were dried under vacuum for 24 h and characterized by the proton nuclear magnetic resonance (1H NMR).

3.2.8.1 Synthesis of PEG-b-PGlu₁₅₀ diblock copolymer

mPEG₁₀ᵏ-NH₂ (0.02 mmol) was dissolved under stirring in 10 mL of anhydrous dimethylformamide (DMF) under nitrogen atmosphere at 40°C. BGlu-NCA (5 mmol, the feed molar ratio of mPEG-NH₂ to BGlu-NCA was 1:250) dissolved in 5 mL of anhydrous DMF was added dropwise and the solution was stirred for 10 days under a nitrogen atmosphere on a Schlenk line. To monitor the reaction, the aliquot of the reaction mixture was precipitated using excess of diethyl ether and dried in a vacuum. The composition of PEG-PBGlu diblock copolymer was determined by 1H NMR from the peak intensity ratios of the methylene protons of PEG and the phenyl protons of the γ-benzyl groups (500 MHz in TFA-d₁: δ = 3.5 (s, 4H, -OCH₂CH₂-), 4.8 (m, 2H, benzyl), 7.3 (m, 5H, aryl)). Once the target polymerization degree of PGlu block (150 units) was achieved, the reaction mixture was precipitated using excess of diethyl ether, purified by repeated precipitation in diethyl ether, and dried under vacuum. The benzyl groups of PEG-b-PBGlu were removed in the presence of mixture of trifluoroacetic acid (TFA) and hydrogen bromide (HBr) at a ratio of 3:1 (v/v) to obtain PEG-b-PGlu. Polymer sample was dissolved in 9 mL of trifluoroacetic acid followed by dropwise addition of 3 mL of hydrogen bromide solution (33 wt. % in acetic acid). After stirring overnight at 25°C, mixture was precipitated in excess of diethyl ether and purified by repeated precipitation in diethyl ether. Crude product was dissolved in water, neutralized by 1M HCl, and dialyzed using a dialysis membrane (MWCO 3,500 Da) against distilled water for 48 hrs. After dialysis, the produce was lyophilized to obtain the purified product PEG₁₀ᵏ-b-PGlu₁₅₀.
3.2.8.1 Preparation of EGFR-targeted drug-loaded NGs from PEG$_{10k}$-P(Glu-Glu/Phe$_{50}$)$_{150}$

Phe functionalities were introduced in PEG$_{10k}$-b-PGLu$_{150}$ copolymer by polymer-analogous modification of the PGLu segment with the Phe methyl ester using carbodiimide-chemistry as described above. The targeted degree of modification was set at 50 Phe units per chain. The obtained products were characterized by proton nuclear magnetic resonance ($^1$H NMR). Micellar precursors based on PEG-P(Glu-Glu/Phe$_{50}$)$_{150}$ copolymers were prepared by the dialysis method using water/organic solvent mixtures followed by direct crosslinking the COOH groups of glutamic acid units with 1,2-ethylenediamine (ED) in the presence of EDC at RT. The crosslinking reactions were performed at a molar ratio of [COOH]/[EDC]/[ED] = 10/2/1 to obtain a targeted 20% crosslinking degree. The prepared nanogel was abbreviated as NG (further denotes as rNG$_{10k}$). Non-decorated rNG$_{10k}$ and EGFR-targeted drug-loaded rNG$_{10k}$ was prepared through the same process as described for preparing the panel of rNG$_{5k}$. Only the linker between the NG and the ligand was replaced by maleimide-PEG-amine with longer chain length (M$_w$ 15,000 g mol$^{-1}$) to expose the ligand for sufficient interaction with the targeted receptor.

3.2.9 Pharmacokinetic studies

Upon arrival, animals were placed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Food and reverse osmosis water were available ad libitum throughout the study. All animal studies were performed based on the protocols approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Pharmacokinetics of CDDP and CDDP-loaded NGs was conducted on six-week-old female athymic (Ncr-nu/nu) mice purchased from the Charles River Laboratories (Boston, MA). Before the tumor inoculation, mice were quarantined for 7 days to minimize the risk of infection. Mice were xenografted with
human EOC OVCAR-5 cells (2 × 10^6 cells/mice) administered intraperitoneally (i.p.) in sterile PBS. On day 20, 30 animals were randomized into 5 treatment groups and given either free CDDP or CDDP/rNG_{10k} or CDDP/rNG_{5k} or CDDP/PEG-rNG_{10k} or CDDP/LAE-rNG_{10k} by tail vein injection at an equivalent dose of 5 mg CDDP/kg of body weight. About 25 µl of blood of animals in groups of six (n = 6) were collected at each time point (0, 5, 15 and 30 minutes and 1, 1.5, 2, 4, 6, 8, 12, 24, and 48 hours) in heparinized vials through the tail vein. After the final time point, animals were sacrificed and then perfused with about 30 mL saline. Heart, liver, spleen, lungs, kidney and tumors were excised, washed with saline and frozen over dry ice before storage at -80°C. Blood was immediately centrifuged at 2,000 g for 5 minutes to obtain plasma, and frozen at -80°C prior to further analysis. The results were expressed in ng of CDDP accumulated in mg of each organ or mL of plasma. Pharmacokinetic parameters were estimated using non-compartmental analysis in Phoenix WinNonlin software (Certara, Princeton, NJ).

3.2.10 Sample preparation and drug content measurements in tissue samples

Known weights of thawed tissue (about 30 mg) from sacrificed mice were decomposed by wet-ashing in 5 mL Eppendorf tubes with 1.5 mL of mixture of concentrated nitric acid and 30% hydrogen peroxide at ratio of 3:1 (v/v). The mixture was kept at r.t. overnight. An iridium internal standard was added prior to digestion. Platinum concentrations were measured on ICP-MS (NexION 300Q, PerkinElmer, Waltham, MA) using iridium correction. The calibration range for the assay was platinum 0.8–500 ng/mL with extrapolation to platinum 1000 ng/mL. Necessary dilutions were made if the platinum concentration exceeded the calibration range. Assay sensitivity was 0.8 ng Pt/mL, with inter- and intraday assay variability not exceeding 5%. However, this assay did not differentiate between platinum carried in NGs and released from NGs or between free and protein-bound platinum.
3.2.11 Animals bioluminescence imaging procedure

Mice carrying tumors with genetic modifications to express firefly luciferase gene can be injected with luciferin to emit visual bioluminescence (BLI) signals for non-invasive longitudinal monitoring of disease progression. It provides quantitation since the photon flux from the tumor is proportional to the number of light-emitting cells [38]. In this study, bioluminescence imaging was conducted with the IVIS® Spectrum in vivo imaging system (PerkinElmer, Waltham, MA). Before in vivo imaging, animals were anesthetized with isoflurane (Henry Schein, Dublin, OH) and injected IP with D-luciferin (PerkinElmer, Waltham, MA) at the dose of 150 mg/kg (reconstituted in sterile DPBS). Whole-body Imaging was performed 5 min after injection. Total bioluminescence signal in the regions of interest (ROIs) drawn around the whole abdomen region was quantified using Living Image® software (version 4.5; PerkinElmer) and expressed as photons/s/cm²/sr (average radiance).

3.2.12 Evaluation of therapeutic efficacy

Peritoneal high-grade serous ovarian carcinoma was generated by intraperitoneal injection of 2×10⁶ OVCAR-5/Luc cells (kindly provided by Dr. Adam Karpf, University of Nebraska Medical Center, NE) into six-week-old female athymic nude mice. After the development of tumors (10 days after injection) animals were randomized into 10 treatment groups with 13 mice per group. NRT was given as the water-soluble form: neratinib maleate. All treatments were supplemented with dextrose at 5% final concentration and were administered via the tail vein at 4-day intervals. We used one additional group which received IP injection of LAE-(CDDP+NRT)/NG. The treatment groups and the doses of each drug are presented in Table 12. Drug amount was calculated based on the average animal body weight. Animal body weight and tumor progression, as assessed by BLI, were monitored every fourth day. All images were
acquired using identical BLI system settings. Tumors were allowed to grow until ascites developed or animal exhibited body weight loss of 20% or low body condition scoring, then the mice were euthanized via CO₂ asphyxiation. At necropsy, tissues (heart, liver, spleen, lung, kidney, tumor) were collected followed with either fixing in 10% buffered formalin or storing at -80 °C for further analysis. The abdominal cavity was carefully checked, and the spread of the tumor was determined. For evaluating the therapeutic effects on delaying the cancer-induced muscle wasting [39], gastrocnemius and soleus muscles of mice were isolated, weighed for comparison, and stored at -80 °C.

Table 12. Treatment groups and dose of drugs used in antitumor efficacy in vivo studies

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Routes of administration</th>
<th>CDDP (mg/kg)</th>
<th>NRT (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% dextrose</td>
<td>IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR-A-NG</td>
<td>IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAE-NG</td>
<td>IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free CDDP + NRT</td>
<td>IV</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>CDDP/LAE-NG</td>
<td>IV</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>CDDP/NG + NRT/NG</td>
<td>IV</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>(CDDP+NRT)/NG</td>
<td>IV</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>CDDP/LAE-NG + NRT/LAE-NG</td>
<td>IV</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>(CDDP+NRT)/LAE-NG</td>
<td>IV</td>
<td>4</td>
<td>4</td>
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<tr>
<td>(CDDP+NRT)/EGFR-A-NG</td>
<td>IV</td>
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<td>4</td>
</tr>
<tr>
<td>(CDDP+NRT)/LAE-NG</td>
<td>IP</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

3.2.13 Immunohistochemical evaluation of cell proliferation and apoptosis markers

Tumors from mice that received different treatments were excised at day 14 (2-3 mice per group). The tumors were dissected and fixed in 10% neutral buffered formalin. Then, the tissues were processed routinely into paraffin, sectioned at a thickness of 5μm. Proliferating and apoptotic cells were detected using an antibody against Ki-67 and
caspase-3, respectively. Ki-67 and caspase-3 expression was quantified and evaluated by Definiens Tissue Studio 64 with corresponding modules (Definiens, Inc., Cambridge, MA).

3.2.14 Blood chemistry, Cancer antigen (CA)-125 assay and histopathology
Mice (2-3) from each treatment group were sacrificed at 24 hrs after the final dosing. Serum from the blood of sacrificed animals was collected and analyzed for CA-125 levels using the CA-125 ELISA kit (Abcam, MA) according to the manufacturer's instructions. Blood from the sacrificed animals was collected in heparinized tubes and analyzed for blood cell count and liver enzymes using Hematology Analyzer and Vetscan VS2 (Abaxis, CA), respectively. Collected tissues were processed, sectioned, inserted into tissue cassettes, fixed in 10% formalin (Azer scientific, Morgantown, PA) for 48h, dehydrated in 70% ethanol overnight, and paraffin embedded (UNMC Tissue Sciences facility, Omaha, NE). Sectioned tissues (5 μm) were stained with either hematoxylin and eosin (H&E) or by immunohistochemistry (IHC). For histopathological diagnosis, H&E-stained slides were examined by light microscopy and photomicrographs were taken using a Nikon camera mounted on a Nikon Eclipse 600 microscope (both Nikon Instruments, Melville, NY) with Adobe Elements 3.0 software (Adobe Systems, San Jose, CA).

3.2.15 Statistical analysis
Statistical comparisons for in vitro studies were carried out using the Students t-test. Each sample was analyzed based on results that were repeated at least three times. In animal studies, group means bioluminescence signal intensity, body weights and associated data generated in animal studies were analyzed using a one-way analysis of variance [40]. Survival was estimated using Kaplan–Meier analysis and compared using the log-rank test. Analysis of variance and Kaplan–Meier analysis tests were carried out using GraphPad Prism 5 software (GraphPad Software, Inc.). In all cases, differences at P <
0.05 were regarded as statistically significant; ones at $P < 0.01$ or $P < 0.001$ were considered higher statistical significances.

3.3 Results and discussion

3.3.1. Synthesis of PEG-P(Glu-Glu/Phe$_{50}$)$_{150}$

The primary objective of this study was to develop a carrier system for the co-delivery of active molecules with varied physicochemical properties and to use it in cancer therapy. To this end, amphiphilic copolymer PEG-P(Glu-Glu/Phe$_{50}$)$_{150}$ was utilized for the synthesis of such multi-compartment carrier systems. As reported by our group, the introduction of aromatic Phe moieties into hydrophilic PEG-PGlu block copolymers facilitated self-assembly of copolymers in aqueous solution, allowing the carrier systems to solubilize hydrophobic molecules [33, 37]. Besides, carrier systems with incorporated aromatic units were reported to show improved stability, drug retention in carriers and loading capacity, which might be attributed to hydrophobic and $\pi-\pi$ stacking interactions of the aromatic units and the cargos [41, 42]. Therefore, the incorporation of Phe moieties in our carrier system may provide a suitable environment for solubilization of NRT. To drive the self-assembly of hydrophilic copolymer, PEG-PGlu ($M_w/M_n=1.01$, $M_w$ 28,000 g mol$^{-1}$), hydrophobically modified PEG-b-PGlu derivatives were synthesized via EDC amidation mediated grafting of PGlu backbone with L-phenylalanine methyl ester (Phe) moieties. PEG-b-PGlu diblock copolymer (PEG $M_w= 5,000$ g mol$^{-1}$), in which 30% of Glu units were modified with hydrophobic phenylalanine moieties, were utilized for the synthesis of NGs. The synthesis of the amphiphilic hybrid polypeptide-based copolymer PEG-P(Glu-Glu/Phe$_{50}$)$_{150}$ is illustrated in Figure 23.
Figure 23. Scheme for the synthesis of PEG-P(Glu-Glu/Phe\textsubscript{50})\textsubscript{150} polymer via carbodiimide-chemistry.

The chemical composition of PEG-P(Glu-Glu/Phe\textsubscript{50})\textsubscript{150} copolymers was determined by \textsuperscript{1}H NMR analysis (Figure 24) from the peak intensity ratios of the methylene protons besides carboxylic acid of PGlu and the phenyl protons of the benzyl groups from Phe. The characteristics of the PEG-P(Glu-Glu/Phe\textsubscript{50})\textsubscript{150} copolymer are summarized in Table 13. These copolymers had a PEG block (114 repeating units) and anionic PGlu block (about 150 repeating units), in which about 30% of Glu units were modified with hydrophobic Phe moieties.
Figure 24. $^1$H NMR spectrum for PEG-P(Glu-Glu/Phe$_{50}$)$_{150}$ (TFA-$d_1$, 298K).

Notes: peak assignments are as follows: PEG-P(Glu-Glu/Phe$_{50}$)$_{150}$ $^1$H NMR (TFA-$d_1$): δ ppm= 2.23 (2H, m, -CH$_2$-), 2.62 (2H, broad s, -CH$_2$-), 3.21 (2H, m, -CH$_2$-), 3.86 (3H, m, -CH$_3$), 3.91 (2H, s, -CH$_2$-), 4.59 (1H, broad s, -CH-), 4.82 (1H, broad s, -CH-), 5.00 (1H, broad s, -CH-); 7.11 (5H, m, C$_6$H$_5$)

Table 13. Characteristics of the PEG-P(Glu-Glu/Phe$_{50}$)$_{150}$ copolymer.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Feed molar ratio (mmol)</th>
<th>Repeating Units Ratio*</th>
<th>$M_n$* (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-b-PGlu$_{150}$</td>
<td>Phe</td>
<td>PEG</td>
<td>PGlu</td>
</tr>
<tr>
<td>PEG-P(Glu-Glu/Phe$<em>{50}$)$</em>{150}$</td>
<td>1</td>
<td>55</td>
<td>114</td>
</tr>
</tbody>
</table>

*Calculated from $^1$H NMR spectrum.
3.3.2. Synthesis and characterization of ligand-installed NGs

The resulting amphiphilic copolymers were anticipated to self-assemble into micellar particles in aqueous medium due to the incorporation of hydrophobic Phe moieties. After the polymer synthesis, polymeric micelles were then prepared by mixing the solution of polymer precursor in dimethylformamide with water (1: 1 v/v) following the dialysis against water for 48 h. The formation of small particles with relatively narrow particle size distribution (PDI < 0.2) and net negative charge (~-16 mV) was detected in aqueous dispersions. Micelles prepared from PEG-P(Glu-Glu/Phe50)150 were further crosslinked to form NGs. The crosslinking of the PEG-P(Glu-Glu/Phe50)150 was achieved via condensation reactions between the carboxylic groups of PGlu segments and the amine groups of ethylenediamine in the presence of a water-soluble carbodiimide, EDC. The targeted extent of crosslinking (20%) was controlled by the molar ratio of crosslinker to carboxylic acid groups of the glutamic acid residues. After exhaustive dialysis against water, NGs were characterized at pH 7 (Table 15). Detected by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK), the resulting NGs displayed an average diameter of about 75 nm and were uniform (monomodal, narrow size distribution) with net negative charge (ζ-potential = -16.2 mV) which can be explain by the presence and ionization of polyelectrolyte network formed by PGlu chains. NGs were further modified by EGFR targeting ligands via a PEG linker for delivering a drug combination to EGFR-overexpressing ovarian tumors. Briefly, EDC-activated NGs were first reacted with Maleimide-PEG7.5k-NH2 which was chosen to be longer than the PEG stealth layer on NGs to avoid steric interference with receptor binding and display sufficient targeting moieties on the surface for receptor recognition. The hydrodynamic diameter of PEGylated NGs was 80 ± 2 nm, which is marginally larger than that of precursor NGs (75 ± 7 nm). However, the ζ-potential of the PEG-modified NGs significantly increased to -10.6 ± 0.3 mV compared to -16.2 ± 1.3 mV for the unmodified NGs (Table
15), which might be attributed to the consumption of the PGlu carboxylate groups that reacted with the amine-terminated PEG linkers as well as additional shielding of surface charge by tethered PEG chains. Finally, sulfhydryl groups of TCEP (thiol reduction reagent) activated targeting ligands (EGFR-A or L-AE) were reacted to maleimide terminal groups on NG-attached PEG linkers. Such selective and efficient cysteine conjugation using maleimide–thiol reactions is widely used to produce site-specific protein–polymer conjugates [43]. After the conjugation, the particle size and ζ-potential of ligand-installed NGs remained practically the same as those of the PEGylated NGs (Table 14).

3.3.3. Preparation and characterization of drug-loaded polymeric NGs
As mentioned, the carriers are developed to co-deliver two potent anticancer drugs: CDDP and NRT to achieve boosted synergistic therapeutic effects to combat EGFR overexpressing ovarian cancers. To prepare the binary drug combination-loaded NGs, two steps were conducted to achieve the goal. We have previously shown that the attachment of the targeting groups should be carried out after NGs loading with CDDP to avoid inactivation of protein through CDDP binding with its reactive functional groups, such as carboxylic groups [44]. Therefore, in this study to prepare targeted NGs with binary drug combination (further abbreviated as EGFR-A-(CDDP + NRT)/NGs and LAE-(CDDP + NRT)/NGs), CDDP was first loaded into PEGylated NGs through incubation with PEG-NGs aqueous dispersion for 24h at pH 9 followed by conjugation of EGFR targeting ligands to PEG linkers. At the next step, NRT was solubilized into the hydrophobic domain of targeted CDDP/NGs through an extraction method. Scheme for the preparation of EGFR-targeted NGs is presented in Figure 25. Targeted (CDDP+NRT)/NG were small particles (hydrodynamic diameter of approximately 80 nm) with unimodal particle size distribution (PDI < 0.2) as determined by DLS and contained 0.003 μmol EGFR-A or 0.031 μmol L-AE per mg of polymer. As expected, the net negative charge and particle size of
NGs decreased upon CDDP loading consistent with neutralization of PGlu segments by CDDP and collapse of PGlu network (Table 15). The loading capacities for CDDP and NRT, alone or in combination, remained relatively the same as for bare NGs (Table 14). The average molar ratio of the CDDP and NRT incorporated into NGs was close to 2:1, which has been previously shown to provide strong synergistic cytotoxic effects in TNBC cells with high EGFR expression. A similar procedure was used to prepare nontargeted (CDDP + NRT)/NGs from unmodified NG precursors.

Figure 25. Scheme for the preparation of EGFR-targeted NGs
Table 14. Physicochemical characteristics of EGFR-targeted NGs

<table>
<thead>
<tr>
<th>Samples</th>
<th>$D_{\text{eff}}$ (nm)</th>
<th>PDI</th>
<th>$\zeta$-potential, mV</th>
<th>µg of protein or peptide /mg of polymer</th>
<th>µmol of protein /mg of polymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>rNG$_{5k}$</td>
<td>75 ± 7</td>
<td>0.16</td>
<td>-16.2 ± 1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG-rNG$_{5k}$</td>
<td>80 ± 2</td>
<td>0.17</td>
<td>-10.6 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAE-rNG$_{5k}$</td>
<td>81 ± 2</td>
<td>0.18</td>
<td>-11.0 ± 2.1</td>
<td>29 ± 4</td>
<td>0.031</td>
</tr>
<tr>
<td>EGFR-A-rNG$_{5k}$</td>
<td>83 ± 5</td>
<td>0.17</td>
<td>-13.7 ± 0.5</td>
<td>75 ± 10</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Effective hydrodynamic diameter ($D_{\text{eff}}$), polydispersity index (PDI), and $\zeta$-potential were determined by DLS (PBS, pH 7, 25°C). Amount of conjugated protein or peptide was determined by BCA assay. Data are presented as average ± SD from three experiments.

Table 15. Physicochemical characteristics of drug-loaded EGFR-targeted rNG$_{5k}$

<table>
<thead>
<tr>
<th>Samples</th>
<th>$D_{\text{eff}}$ (nm)</th>
<th>PDI</th>
<th>$\zeta$-potential, mV</th>
<th>LC, w/w%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CDDP</td>
</tr>
<tr>
<td>NRT/rNG$_{5k}$</td>
<td>85 ± 2</td>
<td>0.16</td>
<td>-17.5 ± 0.5</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>CDDP/rNG$_{5k}$</td>
<td>66 ± 2</td>
<td>0.15</td>
<td>-2.3 ± 1.8</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>(CDDP+NRT)/rNG$_{5k}$</td>
<td>83 ± 3</td>
<td>0.19</td>
<td>-5.7 ± 0.9</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>NRT/EGFR-A-rNG$_{5k}$</td>
<td>88 ± 4</td>
<td>0.18</td>
<td>-12.4 ± 1.8</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>CDDP/EGFR-A-rNG$_{5k}$</td>
<td>78 ± 3</td>
<td>0.17</td>
<td>-9.5 ± 1.9</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>(CDDP+NRT)/EGFR-A-rNG$_{5k}$</td>
<td>86 ± 3</td>
<td>0.19</td>
<td>-5.4 ± 1.2</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>NRT/LAE-rNG$_{5k}$</td>
<td>87 ± 5</td>
<td>0.19</td>
<td>-13.3 ± 1.1</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>CDDP/LAE-rNG$_{5k}$</td>
<td>74 ± 4</td>
<td>0.16</td>
<td>-7.4 ± 1.6</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>(CDDP+NRT)/LAE-rNG$_{5k}$</td>
<td>82 ± 6</td>
<td>0.18</td>
<td>-9.0 ± 2.4</td>
<td>9 ± 1</td>
</tr>
</tbody>
</table>

Effective hydrodynamic diameters ($D_{\text{eff}}$), polydispersity index (PDI), and $\zeta$-potential were determined by DLS (PBS, pH 7, 25°C). Data are presented as average ± SD from three experiments.
3.3.4. Cellular association

EGFR, a transmembrane receptor promoting cell proliferation and survival, is the receptor of interests being targeted in our studies. To demonstrate the effect of attachment of targeting ligand on cellular association of NGs, we used EGFR⁺ human ovarian carcinoma OVCAR-5 and SKOV-3 cells, MDA-MB-468 TNBC cells as well as a non-EGFR-presenting NIH 3T3 cells. Cultured cells were exposed to fluorescent Cy5-labeled EGFR-A-rNG₅k (0.3 mg/mL) for 2h at 4°C followed by thorough washing with cold PBS to ensure particle removal from the medium. The cells were further incubated with fresh media for an additional 1 h at 37°C. 4°C was chosen as the temperature for exposing cell to NGs to exclude the cellular uptake by energy-dependent vesicular transport since the participating proteins and enzymes are sensitive to temperature. However, the ligand-receptor binding is not inhibited at 4°C; thus the observed cellular association with NGs will be mainly mediated by the interaction between ligand and the corresponding receptor. As shown in Figure 26, the EGFR-A-rNG₅k were effectively associated with EGFR positive MDA-MB-468 cells as compared to NIH 3T3 cells demonstrated by up to 4 folds difference in the percentage of cells associated with EGFR-A-rNG₅k. In contrast, the interaction of non-decorated NGs with both cell types was practically negligible. This selective targeting could be attributed to the complementary binding to the EGFR by the EGFR-A ligands on the surface of NGs. Notably, such enhanced cellular association was also observed in another two EGFR positive cell lines: OVCAR-5 and SKOV3. This further demonstrates that this selective targeting is not cell specific, but more related to the amplified expression of EGFR on the cell surface.

We also compared the cell uptake of NGs when they are applied at different concentrations. As shown in Figure 27, when EGFR-A-rNG₅k concentration was below 0.15 mg/mL, the percentage of cells associated with NGs was almost equal for both EGFR⁺ cells and EGFR⁻ cells. However, when concentration
increased to 0.3 mg/mL, significantly higher cell association with NG was observed in OVCAR-5/EGFR* compared with 3T3/EGFR-. As we know that lowering the culture temperature will affect the diffusion of the NGs, reduced diffusion would result in the hampered NGs uptake in cells [45]. Low temperature hampered the diffusion of NGs which might explain the significantly reduced uptake of EGFR-A-rNG_{5k} in EGFR* cells. For the peptide ligand L-AE, we performed the similar experiment using LAE-rNG_{5k} and also observed that LAE-rNG_{5k} association with EGFR positive OVCAR-5 cells significantly exceeded that of NIH 3T3 cells as shown in Figure 28 (p < 0.01).
Figure 26. Cellular association of Cy-5-labeled EGFR-A-rNG_{5k} (T) and non-decorated rNG_{5k} (NT) in MDA-MB-468/EGFR^+, OVCAR-5/EGFR^+, SKOV-3/EGFR^+ and NIH 3T3/EGFR^- cells as analyzed by flow cytometry. [NGs] = 0.3 mg/mL and 75 μg protein per mg of polymer.
Figure 27. Cellular association of Cy-5-labeled EGFR-A-rNG5k (T) and non-decorated rNG5k (NT) in MDA-MB-468/EGFR⁺ and NIH 3T3/EGFR⁻ cells as analyzed by flow cytometry. [NGs] = 0.3 mg/mL, 0.15 mg/mL and 0.075 mg/mL with 75 μg protein per mg of polymer.
3.3.5. *In vitro* cytotoxicity studies

CDDP is a well-known DNA-damaging agent. Its mechanism of action involves formation of cisplatin-DNA adducts to disrupt DNA structure and interfere with DNA replication and transcription and ultimately induce apoptotic cell death. Herein we studied the synergy of CDDP and NRT, which is an irreversible inhibitor towards EGFR, HER2 and HER4. As an inhibitor of HER family, NRT might potentiate therapeutic efficacy of CDDP by reducing DNA-damage repair through interfering with EGFR-mediated Histone H4/DNA-PKcs/ATM pathway and pushing cells towards apoptotic death. Besides, NRT further sensitizes cells
to CDDP by downregulating oncogenic Cyclin D1 to keep cells blocked at G1 phase during cisplatin treatment since tumor cells appear to be maximally sensitive to cisplatin in G1 phase and minimally sensitive in S-phase [25]. Cytotoxicity of either free drugs or single drug-loaded and combination nanogel formulations were evaluated in human ovarian cancer cell lines SKOV-3 and OVCAR-5 with high EGFR expression as well as in A2780 cells with negligible EGFR expression. After 48-hour treatment, the surviving cell population was assessed by a CellTiter Blue assay (CTB), which measures the metabolic activity of viable cells. The half-maximal inhibitory concentrations (IC50) of free drugs, single and combination-drug-loaded NGs are compared in Table 16. Indeed, the combination of CDDP and NRT was significantly more efficient in killing EGFR overexpressing cell lines than just CDDP treatment indicated by a significant drop in the IC50 value of CDDP when administered in combination with NRT in a single carrier for 48 hours. When the treatment was given to EGFR+ SKOV-3, combination index analysis at IC50 using the Chou and Talalay method [36] showed that the synergistic cytotoxicity (CI<1) between the two drugs was observed both in the form of free drugs (CI=0.43 ± 0.03) and combination in rNG5k (CI = 0.29 ± 0.02). When both drugs were administered in the form of a cocktail of single drug-loaded NGs at equivalent doses, the synergy of action was still retained with less prominent effect compared with that seen in the case of combination of dual-drug-loaded NGs. These data suggest that synchronized delivery of this drug combination in the same nanogel formulation may play an essential role in the cytotoxic activity. We also observed a similar trend in another EGFR overexpressing cell line OVCAR-5, which is a high-grade serous ovarian cancer (HGSOC) cell line, the most commonly diagnosed form of EOC with advanced stage at onset of diagnosis and high mortality rate. However, such strong synergy was not displayed in A2780 with negligible EGFR expression indicated by a CI value close to 1 when cells were given the combination treatments. Importantly, rNG5k alone was not toxic at the range of concentrations used for
the treatment with CDDP/rNG$_{5k}$ formulations (Figure 29). CDDP/rNG$_{5k}$ displayed lower cytotoxic activities than free CDDP which probably arises from the relatively slow release of CDDP from NGs. The functionalization of EGFR-A or L-AE to the NGs didn’t remarkably enhance the cytotoxicity of binary-drug-loaded NGs probably attributed to the fact that the long-term static incubation could enhance non-specific uptake of NGs.

**Figure 29.** *In vitro* toxicity of empty rNG$_{5k}$ in both SKOV-3 and OVCAR-5 cancer cells as determined by CTB assay. Cells (4000 cells/well) were treated with empty NGs at various concentrations for 48 h at 37°C.
Table 16. Comparison of IC$_{50}$ values for various drug-loaded NGs, free NRT and CDDP against EGFR$^+$ SKOV-3 and OVCAR-5 cells and EGFR$^-$ A2780 cells as determined by CellTiter-Blue® assay.

<table>
<thead>
<tr>
<th></th>
<th>SKOV-3</th>
<th>OVCAR-5</th>
<th>A2780</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDDP</td>
<td>3.4 ± 0.8</td>
<td>2.9 ± 0.7</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>NRT</td>
<td>1.6 ± 0.3</td>
<td>2.5 ± 0.4</td>
<td>0.50 ± 0.19</td>
</tr>
<tr>
<td>CDDP/rNG$_{5k}$</td>
<td>12 ± 2.3</td>
<td>29.7 ± 3.7</td>
<td>6.2 ± 2.4</td>
</tr>
<tr>
<td>NRT/rNG$_{5k}$</td>
<td>1.5 ± 0.3</td>
<td>2.9 ± 0.3</td>
<td>0.75 ± 0.14</td>
</tr>
<tr>
<td>*(CDDP+NRT)/rNG$_{5k}$</td>
<td>1.1 ± 0.1</td>
<td>2.8 ± 0.5</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>*(CDDP+NRT)/EGFR$^-$rNG$_{5k}$</td>
<td>Cl$^{**}$ = 0.29 ± 0.02</td>
<td>Cl = 0.41 ± 0.04</td>
<td>Cl = 0.82 ± 0.05</td>
</tr>
<tr>
<td>*(CDDP+NRT)/EGFR-A-rNG$_{5k}$</td>
<td>1.9 ± 0.7</td>
<td>3.4 ± 0.4</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>*(CDDP+NRT)/LAE-rNG$_{5k}$</td>
<td>CI = 0.50 ± 0.17</td>
<td>CI = 0.52 ± 0.19</td>
<td>CI = 0.98 ± 0.13</td>
</tr>
<tr>
<td>*Free CDDP+NRT</td>
<td>1.2 ± 0.5</td>
<td>1.7 ± 0.2</td>
<td>0.50 ± 0.24</td>
</tr>
<tr>
<td>*(CDDP+NRT)/EGFR-A-rNG$_{5k}$</td>
<td>Cl = 0.43 ± 0.03</td>
<td>Cl = 0.61 ± 0.05</td>
<td>Cl = 1.20 ± 0.36</td>
</tr>
<tr>
<td>*(CDDP+NRT)/LAE-rNG$_{5k}$</td>
<td>0.87 ± 0.1</td>
<td>2.4 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>
| *calculated with respect to CDDP. **: Combination index was calculated with respect to CDDP. Data presented as mean ± SD. N=3
3.3.6. Insights in molecular mechanisms of synergistic drug interaction at the cellular level

To investigate the molecular basis for the synergistic effect of combination of CDDP and NRT at a molar ratio of ca. 2:1 observed in the previous cell viability studies, we used western blot analysis to first determine the level of HER1/EGFR expression and phosphor-EGFR level in EGFR+ SKOV-3 and OVCAR-5 cell lines after various treatments (Figure 30 & 31). Cells were treated with either single CDDP (10 µM), NRT (5 µM) or the binary drug combination at the same dose for 2h. Then cells were treated with EGF (100 ng/mL) for 5 mins to stimulate EGFR phosphorylation. Cells without EGF treatment were used as a control. Notably, EGFR expression was relatively the same among a panel of all treated cells, indicating that the treatment with EGF, CDDP or NRT does not change the cell expression level of EGFR. However, higher phosphor-EGFR was found in cells treated with CDDP or EGF, a ligand for activation and phosphorylation of EGFR, indicating that CDDP treatment triggered the activation of EGFR, which is considered as a pro-survival signal leading to mitigated therapeutic efficacy. Such trend was more obvious in SKOV-3 cell line compared to OVCAR-5 cell line. No obvious change in terms of the level of phosphor-EGFR was found in cells treated with NRT in the presence of EGF, indicating that NRT successfully suppressed the EGF-mediated activation and phosphorylation of EGFR. Similarly, no observable change in phosphor-EGFR was found in both cells treated with a combination of CDDP, NRT and EGF, further indicating that NRT was able to suppress EGFR activation induced by CDDP and EGF, suggesting that the CDDP-mediated pro-survival signal could be reversed by NRT, rendering cancer cells more vulnerable to the cytotoxic effects of CDDP when such combination was given to EGFR+ cell lines.
HER1/EGFR activates several cytoplasmic signal-transduction pathways such as mitogen-activated protein kinase (MAPK), PI3 kinase/Akt, JAK/STAT [46]. Among them, MAPK and Akt are the major two downstream effectors of EGFR investigated in many studies due to their crucial roles in transporting signals for promoting cell division and survival. We investigated whether NRT would inhibit the activity of these two downstream effectors, MAPK (ERK) and Akt, which may correlate with the synergistic toxicity between CDDP and NRT in EGFR+ SKOV-3 and OVCAR-5 ovarian cancer cells. Similarly, a panel of treatments was given to both cell lines at various concentrations with or without the receptor stimulator EGF (100 ng/mL). As seen in Figure 32, NRT at 5 µM markedly inhibited ERK1/2 (MAPK) as well as Akt activity in EGF-stimulated OVCAR-5 as evident by more than 7 folds reduction in the level of ERK1/2 kinase phosphorylation and 2 folds reduction in the level of Akt activation. Notably, NRT almost completely blocked ERK1/2 activity in OVCAR-5 cells. Most importantly, CDDP was able to induce almost the same level of Akt/ERK1/2 activation as the stimulator EGF, which was completely reversed by the addition of NRT, further suggesting that the reverse of CDDP-induced pro-survival signal by NRT might be the molecular mechanism for the strong synergistic effect of the drug combination in the EGFR+ OVCAR-5 cell line. Similarly, CDDP induced an increase in the activation of Akt and ERK1/2 in SKOV-3 cells but not so dramatic as OVCAR-5 (Figure 33). Besides, NRT was able to completely reverse the activation of the pro-survival signal induced by CDDP. When the combination treatment was given to SKOV-3, the p-ERK1/2 level was suppressed even below the the basal level. The appearance of the cleaved-PARP band indicates that CDDP treatment successfully triggered the cell apoptosis (Figure 32). Evaluation of the effects of treatment by CDDP and NRT on EGFR signaling in EGFR+ cells was conducted on A2780 cells, a widely investigated ovarian cancer cell line for establishing ovarian cancer xenograft tumor model. As shown in Figure 34, the level of activated EGFR after the treatment with EGF was quite low compared to
EGFR+ SKOV-3 or OVCAR-5 cells. The treatment with CDDP didn’t induce the increase in the activation of EGFR, Akt and ERK1/2. Activation level of kinases in cells was almost the same when given with single drug or binary drug treatments, which might explain the low synergy between CDDP and NRT in A2780 cells.
Figure 30. Effect of NRT and CDDP on total EGFR and phosphorylation of EGFR in OVCAR-5 cells. Cells were incubated in medium containing the indicated concentrations of treatments for 2 hours. At the end of the 2-hour incubation, 100 ng/mL EGF was added for 5 minutes. Total proteins (10 μg aliquot) were prepared and separated by 8% SDS-PAGE. After transferring to a PVDF membrane, blots were probed with anti-phospho-EGFR, anti-EGFR, anti-HSC70, respectively. The values underneath the blots represent relative changes of p-EGFR levels present in the treated cells compared to the basal level of those in control cells after normalization to HSC70, quantified by densitometric analysis using software ImageJ. Blots are representative of 3 independent experiments.
Figure 31. Effect of NRT and CDDP on total EGFR and phosphorylation of EGFR in SKOV-3 cells. Cells were incubated in medium containing the indicated concentrations of treatments for 2 hours. At the end of the 2-hour incubation, 100 ng/mL EGF was added for 5 minutes. Total proteins (10 µg) were prepared and separated by 8% SDS-PAGE. After transferring to a PVDF membrane, blots were probed with anti-phospho-EGFR, anti-EGFR, anti-HSC70, respectively. The values underneath the blots represent relative changes of p-EGFR levels present in the treated cells compared to the basal level of those in control cells after normalization to HSC70, quantified by densitometric analysis using software ImageJ. Blots are representative of 3 independent experiments.
Figure 32. Effect of NRT and CDDP on phosphorylation of MAPK (ERK1/2) and Akt in OVCAR-5 cells. Cells were incubated in medium containing the indicated concentrations of listed treatments for 2 hours. At the end of the 2-hour incubation, 100 ng/mL EGF was added for 5 minutes. Total proteins (10 µg) were prepared and separated by 8% SDS-PAGE. After transferring to a nitrocellulose membrane, blots were probed with anti-phospho-ERK1/2, anti-phospho-Akt, anti-HSC70, respectively. The values underneath the blots represent the relative changes of p-ERK1/2 and p-Akt levels present in the treated cells compared to the basal level of those in control cells after normalization to HSC70, quantified by densitometric analysis using software ImageJ. Blots are representative of 3 independent experiments.
Figure 33. Effect of NRT and CDDP on phosphorylation of MAPK (ERK1/2) and Akt in SKOV-3 cells. Cells were incubated in medium containing the indicated concentrations of listed treatments for 2 hours. At the end of the 2-hour incubation, 100 ng/mL EGF was added for 5 minutes. Total proteins (10 µg) were prepared and separated by 8% SDS-PAGE. After transferring to a nitrocellulose membrane, blots were probed with anti-phospho-ERK1/2, anti-phospho-Akt, anti-HSC70, respectively. The values underneath the blots represent the relative changes of p-ERK1/2 and p-Akt levels present in the treated cells compared to the basal level of those in control cells after normalization to HSC70, quantified by densitometric analysis using software ImageJ. Blots are representative of 3 independent experiments.
Figure 34. Effect of NRT and CDDP on phosphorylation of EGFR, MAPK (ERK1/2) and Akt in A2780 cells. Cells were incubated in medium containing the indicated concentrations of listed treatments for 2 hours. At the end of the 2-hour incubation, 100 ng/mL EGF was added for 5 minutes. Total proteins (10 µg) were prepared and separated by 8% SDS-PAGE. After transferring to a nitrocellulose membrane, blots were probed with anti-phospho-EGFR, anti-phospho-ERK1/2, anti-phospho-Akt, anti-HSC70, respectively. The values underneath the blots represent the relative changes of p-EGFR, p-ERK1/2 and p-Akt levels present in the treated cells compared to the basal level of those in control cells after normalization to HSC70, quantified by densitometric analysis using software ImageJ. Blots are representative of 3 independent experiments.
3.3.7. Optimization of NGs by extending the length of PEG block

PEG$_{10k}$-P(Glu-Glu/Phe$_{50}$)$_{150}$ copolymer was synthesized as illustrated in Figure 35. BGlu-NCA was synthesized first as a monomer. The diblock copolymers PEG-\textit{b}-PBGlue were then synthesized via ring-opening polymerization of BGlu-NCA monomers using amino-terminated PEG (M$_w$ = 10,000 g mol$^{-1}$) as macroinitiator. The length of PBGlue block was set constant by using feed molar ratio of mPEG-NH$_2$ to BGlu-NCA at 1 : 250 to obtain target product: PEG-\textit{b}-PBGlue$_{150}$. To remove the protective benzyl groups from PBGlue block, the deprotection of the glutamate residues was carried out by acidic hydrolysis to obtain PEG-\textit{b}-PGlu. The chemical composition of BGlu-NCA, diblock copolymers before and after deprotection were determined by $^1$H NMR analysis (Figure 36-38). The success in complete deprotection was indicated by the disappearance of benzyl methylene proton signals at δ ppm=5.08 as shown in $^1$H NMR (Figure 38). The polymer precursors PEG-P(Glu-Glu/Phe$_{50}$)$_{150}$ were then synthesized as described previously via polymer-analogous modification of the 30% Glu units with the Phe methyl ester using carbodiimide-chemistry in aqueous media. The resulting copolymer had a PEG block (228 repeating units), anionic PGlu block (150 repeating units), in which about 30% of Glu units were modified with hydrophobic phenylalanine moieties as determined by $^1$H NMR (Figure 38). The polymerization degree of the resulting polymers PEG$_{10k}$-P(Glu-Glu/Phe$_{50}$)$_{150}$ is summarized in Table 17.
Figure 35. Scheme for the synthesis of PEG_{10k}-P(Glu-Glu/Phe_{50})_{150}
Figure 36. BGl-NCA. $^1$H NMR (CDCl$_3$): δ ppm = 2.14 (2H, m, -CH$_2$-), 2.60 (2H, m, -CH$_2$-), 4.37 (1H, m, -CH-), 5.14 (2H, s, -CH$_2$-), 7.35 (5H, m, C$_6$H$_5$)
Figure 37. PEG-b-PBGlue $^1$H NMR (TFA-$d_1$): $\delta$ ppm = 1.95 (2H, m, -CH\text{2}-), 2.17 (2H, m, -CH\text{2}-), 3.88 (4H, s, -CH\text{2CH}2-), 4.67 (1H, m, -CH-), 5.08 (2H, m, -CH\text{2}-); 7.23 (5H, m, C\text{6H}5).
Figure 38. PEG-PGlu $^1$H NMR (TFA-$d_1$): $\delta$ ppm = 2.14 (2H, m, -CH$_2$-), 2.31 (2H, m, -CH$_2$-), 3.88 (4H, s, -CH$_2$CH$_2$-), 4.81 (1H, m, -CH-).
Figure 39. PEG₉₀⁻P(Glu-Glu/Phe₅₀)₁₅₀ ¹H NMR (TFA-d₄): δ ppm = 2.24 (2H, m, -CH₂⁻) 2.69 (2H, m, -CH₂⁻), 3.93 (3H, broad s, -CH₃), 3.97 (4H, s, -CH₂CH₂⁻), 7.18 (5H, m, C₆H₅)

Table 17. Characteristics of the PEG-P(Glu-Glu/Phe₅₀)₁₅₀ copolymer.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Feed molar ratio (mmol)</th>
<th>Repeating Units Ratio*</th>
<th>Mₙ*(g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-b-PGlu₁₅₀</td>
<td>Phe 55</td>
<td>PEG 228</td>
<td>PGLu 150</td>
</tr>
</tbody>
</table>

*Calculated from ¹H NMR spectrum.

The polymer precursors PEG-P(Glu-Glu/Phe₅₀)₁₅₀ were used to prepare NGs through the same procedure as described. The optimized NGs were further denoted as rNG₁₀k. The EGFR-targeted and drug-loaded rNG₁₀k were prepared using the same procedures
described above and their physicochemical characteristics are presented in Table 18. Notably, dimensions as well as ζ-potentials of rNG\textsubscript{10k} were comparable with those of rNG\textsubscript{5k} indicating that the elongation of PEG block didn’t significantly change the microscopic characteristics of NGs. The loading capacities for NRT and CDDP, alone or in combination, remained relatively the same as for rNG\textsubscript{5k}.

Table 18. Physicochemical characteristics of EGFR-targeted rNG\textsubscript{10k}

<table>
<thead>
<tr>
<th>Samples</th>
<th>(D_{\text{eff}}) (nm)</th>
<th>PDI</th>
<th>ζ-potential, mV</th>
<th>µg of Protein/mg of polymer</th>
<th>µmol of Protein/mg of polymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>rNG\textsubscript{10k}</td>
<td>61 ± 4</td>
<td>0.16</td>
<td>-23.1 ± 2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG-rNG\textsubscript{10k}</td>
<td>68 ± 3</td>
<td>0.17</td>
<td>-16.6 ± 1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAE-rNG\textsubscript{10k}</td>
<td>69 ± 4</td>
<td>0.18</td>
<td>-16.0 ± 2.1</td>
<td>26 ± 3</td>
<td>0.027 ± 0.0032</td>
</tr>
<tr>
<td>EGFR-A-rNG\textsubscript{10k}</td>
<td>72 ± 5</td>
<td>0.17</td>
<td>-18.7 ± 0.5</td>
<td>70 ± 7</td>
<td>0.0028 ± 0.00027</td>
</tr>
</tbody>
</table>

Effective hydrodynamic diameters (\(D_{\text{eff}}\)), polydispersity index (PDI), and ζ-potential were determined by DLS (PBS, pH 7, 25°C). Data are presented as average ± SD from three experiments.

Table 19. Physicochemical characteristics of drug-loaded rNG\textsubscript{10k}

<table>
<thead>
<tr>
<th>Samples</th>
<th>(D_{\text{eff}}) (nm)</th>
<th>PDI</th>
<th>ζ-potential, mV</th>
<th>LC, w/w%</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRT/rNG\textsubscript{10k}</td>
<td>71 ± 3</td>
<td>0.18</td>
<td>-24.5 ± 1.5</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>CDDP/rNG\textsubscript{10k}</td>
<td>54 ± 3</td>
<td>0.17</td>
<td>-9.2 ± 2.1</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>(CDDP+NRT)/rNG\textsubscript{10k}</td>
<td>73 ± 5</td>
<td>0.16</td>
<td>-10.7 ± 1.2</td>
<td>9 ± 1 9 ± 2</td>
</tr>
<tr>
<td>NRT/EGFR-A-rNG\textsubscript{10k}</td>
<td>80 ± 4</td>
<td>0.19</td>
<td>-17.3 ± 0.7</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>CDDP/EGFR-A-rNG\textsubscript{10k}</td>
<td>68 ± 4</td>
<td>0.15</td>
<td>-7.3 ± 0.8</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>(CDDP+NRT)/EGFR-A-rNG\textsubscript{10k}</td>
<td>79 ± 3</td>
<td>0.17</td>
<td>-6.9 ± 2.1</td>
<td>10 ± 1 9 ± 1</td>
</tr>
<tr>
<td>NRT/LAE-rNG\textsubscript{10k}</td>
<td>77 ± 6</td>
<td>0.19</td>
<td>-15.8 ± 0.9</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>CDDP/LAE-rNG\textsubscript{10k}</td>
<td>61 ± 3</td>
<td>0.15</td>
<td>-5.6 ± 2.8</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>(CDDP + NRT)/LAЕ-rNG\textsubscript{10k}</td>
<td>78 ± 4</td>
<td>0.16</td>
<td>-8.5 ± 1.5</td>
<td>9 ± 2 9 ± 1</td>
</tr>
</tbody>
</table>
Effective hydrodynamic diameters ($D_{\text{eff}}$), polydispersity index (PDI), and $\zeta$-potential were determined by DLS (PBS, pH 7, 25°C). Data are presented as average ± SD from three experiments.

3.3.8. Pharmacokinetic properties of NGs

Pharmacokinetics (PK) of CDDP and CDDP-loaded NGs after single intravenous administration was determined by measuring platinum concentrations in plasma at different time points over 48 hours and peripheral tissues at the end of study by ICP-MS (Figure 40-41). As frequently observed [47, 48], free CDDP was quickly eliminated from the blood by renal filtration into the urine, with only about 15% of the injected dose detectable in plasma 5 minutes after injection. In contrast to the fast clearance of free CDDP from systemic circulation, up to 80% of the injected CDDP/NGs retained 5 minutes after dosing. Mice treated with CDDP/NGs had a more than the four-fold higher maximum observed platinum concentration ($C_0$) and up to 13-fold higher plasma area-under-the-curve (AUC) compared with that of free CDDP. Both observed volume of distribution ($V_{z}\_\text{obs}$) and observed total body clearance ($C_{l}\_\text{obs}$) were decreased by up to 50-fold and 8-fold, respectively. Importantly, we observed that the plasma Pt concentration in mice treated with CDDP/rNG$_{5k}$ was decreasing faster than that of CDDP/rNG$_{10k}$ especially in the first 6 hrs. The PK profile in the plasma was fitted using a non-compartment model to obtain key PK parameters that quantitatively describe how the mice body handles the drug or NGs (Table 20). Consistent with what was observed in the concentration-time profile of plasma Pt (Figure 40A), Pt plasma exposure of CDDP/rNG$_{10k}$ determined by AUC was 1.5 folds higher than CDDP/rNG$_{5k}$. Both $C_{l}\_\text{obs}$ and $V_{z}\_\text{obs}$ were decreased by 1.5-fold and 4.4-fold, respectively. Further functionalization by PEG and the targeting ligand L-AE did not significantly affect the key PK parameters of CDDP/rNG$_{10k}$. At 48 hours, the last point of evaluation, tumor platinum levels after CDDP/rNG$_{10k}$ treatment remained three-fold higher ($p < 0.01$) compared with free CDDP treatment, and about 1.9-fold higher ($p <$
0.05) compared with CDDP/rNG5k treatment. Notably, CDDP/LAE-rNG10k exhibited a 1.7-fold increase in tumor uptake compared with CDDP/rNG10k treatment indicating that the EGFR targeting ligand successfully enhanced the tumor retention of the decorated carriers due to the EGFR-specific interaction of ligand-installed NGs with cancer cells, which was also consistent with the higher specific cellular uptake observed in in vitro studies. Indeed, the liver and spleen, which are organs responsible for removing opsonized foreign particles through phagocytosis in the liver and splenic filtration, showed a strong accumulation of CDDP/NGs (Figure 41). However, liver and spleen platinum contents were reduced significantly with CDDP/rNG10k treatment compared to CDDP/rNG5k treatment (p < 0.001). The functionalization of CDDP/rNG10k with additional PEG chains further reduced the spleen uptake by 1.9 folds. Since kidney is the major excretory organ for free CDDP, mice treated with free CDDP showed a greater Pt level in kidney compared with all other organs tested which is consistent with previously reported studies [48]. CDDP loaded in NGs displayed comparable renal platinum accumulation as the free CDDP, probably due to the macromolecular nature of NGs which exceeded the glomerular filtration threshold. Overall, these preliminary data suggest that rNG10k showed more favored pharmacokinetic properties than rNG5k by prolonging circulation time of CDDP, slowing its elimination, and distributing more CDDP into tumor tissue. The ligand L-AE further boosted the specific tumor tissue drug accumulation through EGFR-mediated receptor interaction.
Figure 40. Concentration–time profile of platinum in OVCAR-5 intraperitoneal tumors-bearing female nude mice in 48h (A) and first 6h (B). Notes: Platinum levels were measured following a single intravenous dose of 5 mg/kg of cisplatin/NGs or cisplatin.
Table 20. Pharmacokinetic parameter estimates of platinum in plasma for CDDP or CDDP/NGs-treated OVCAR-5 human ovarian cancer peritoneal xenograft-bearing female nude mice

<table>
<thead>
<tr>
<th></th>
<th>Free CDDP</th>
<th>CDDP/rNG_{5k}</th>
<th>CDDP/rNG_{10k}</th>
<th>CDDP/PEG-rNG_{10k}</th>
<th>CDDP/LAE-rNG_{10k}</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_0$, ug/mL</td>
<td>24.72</td>
<td>105.03</td>
<td>98.20</td>
<td>127.59</td>
<td>101.50</td>
</tr>
<tr>
<td>$AUC_{all}$, h*ug/mL</td>
<td>28.6 ± 2.8</td>
<td>209.8 ± 8.2</td>
<td>319.6 ± 22.6</td>
<td>382.4 ± 10.3</td>
<td>366.4 ± 13.9</td>
</tr>
<tr>
<td>$V_z_{obs}$, L/kg</td>
<td>6.60</td>
<td>0.58</td>
<td>0.13</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td>$Cl_{obs}$, L/h/kg</td>
<td>0.11</td>
<td>0.022</td>
<td>0.015</td>
<td>0.013</td>
<td>0.015</td>
</tr>
<tr>
<td>MRT$_{last}$, h</td>
<td>14.51</td>
<td>6.30</td>
<td>4.94</td>
<td>6.08</td>
<td>6.16</td>
</tr>
</tbody>
</table>

**Notes:** The pharmacokinetic parameters were calculated according to noncompartmental analysis (Phoenix WinNonlin). $C_0$ represents the maximum observed concentration at the earliest evaluated time point of 5 min post-injection.

**Abbreviations:** $C_0$, maximum platinum concentration at the earliest evaluated time point; $AUC_{all}$, area under the curve using all the given points including trailing zero concentrations; $Cl_{obs}$, observed total body clearance rate; $V_z_{obs}$, observed volume of distribution; MRT$_{last}$, mean residence time from the time of dosing to the time of last measurable concentration.
Figure 41. Tissue distribution of platinum in different carriers as determined by ICP-MS. Mice were sacrificed at 48h post-injection with free CDDP or CDDP/rNG$_{10k}$ or CDDP/rNG$_{5k}$ or CDDP/PEG-rNG$_{10k}$ or CDDP/LAE-rNG$_{10k}$. Data are presented as mean ± SD (n= 4). * $p < 0.05$. *** $p < 0.001$. 
3.3.1. Evaluation of therapeutic efficacy in vivo

Motivated by the enhanced tumor uptake by targeted formulation in PK studies, we evaluated its antitumor efficacy in a OVCAR-5 HGSOC intraperitoneal xenograft mice model which mimics the most common and deadly form of the ovarian cancer [49]. Tumor model was established by injecting 2 million OVCAR-5/luc cells into the peritoneum of female nude mice to trigger formation of tumor nodules throughout the abdominal peritoneum. Almost 100% tumor take rate was seen following i.p. injection of 2 million OVCAR-5/luc cells. Tumor burden was detectable by BLI as early as 3 days post inoculation. This disease is associated with marked weight loss and often results in mortality within 3-4 weeks. If the mice were left untreated, disseminated tumor nodules would appear on peritoneal surfaces, liver, reproductive tissues, the gastrointestinal tract, particularly the small intestine, which are all typical sites of metastasis clinically observed with advanced stage HGSOC [49]. Although not common, gross metastases to the diaphragm and pancreas were observed. Rare cases of formed ascites fluid in 3-4 weeks were also observed. Treatments were initiated 10 days post OVCAR-5/luc inoculation. IV administrations of each formulation were given 4 times at 4-day intervals at 4 mg/kg CDDP and 4mg/kg NRT equivalents per dose. In this aggressive model, we observed that all treatments were effective in delaying tumor progression in comparison with control group (5% dextrose) as shown in Figure 42 and 43. Of note, the mice in the control group found to be dead even before they reached the euthanization point (Figure 44). In contrast, formulation treated mice were found to survive much longer and remained alive until the euthanization threshold indicating the benefits in extending survival. Importantly, therapy with targeted binary drug combination-loaded NGs, (CDDP+NRT)/LAE-NG, was more effective in inhibiting tumor growth as compared to nontargeted (CDDP+NRT)/NG (p < 0.05) (Figure 42A and B) with significantly longer median survival (50.5 days vs 42 days, p < 0.05) (Table 21). The strong synergy of the binary drug regimen seen in vitro was also
observed in \textit{in vivo}, and targeted binary combination (CDDP+NRT)/LAE-NG showed significant inhibition of tumor growth compared to the targeted single CDDP/LAE-NG (p < 0.05) (\textbf{Figure 42A}) with markedly extended median survival (50.5 days vs 38.5 days, p < 0.05) (\textbf{Table 21}). To compare the ability of the carriers to deliver a cytotoxic payload to the tumor sites as well as whether delivered drugs caused any toxicities in normal tissues, we sacrificed 3 mice per group at 24 hrs after the final dosing. Consistent with what we observed in PK studies, targeted formulations, either targeted single drug NG or binary-drug NG, were able to induce a significantly higher Pt accumulation in the target site compared to nontargeted (CDDP + NRT)/NG (p < 0.05). This observation further reinforced our hypothesis that installation of the EGFR targeting ligand L-AE to the delivery vehicle could enhance drug retention in the target site thereby further boost the cytotoxic activity of (CDDP + NRT) combination chemotherapy in EGFR$^+$ ovarian cancer.

\textbf{Table 21.} Median survival and p-values of different treatment regimens with respect to control group (5% dextrose).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Median Survival (days)</th>
<th>p-values (vs. 5% dextrose control)</th>
<th>% survival at censoring date (80 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Dextrose</td>
<td>31</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>CDDP/LAE-rNG$_{10k}$</td>
<td>38.5</td>
<td>0.0046</td>
<td>0</td>
</tr>
<tr>
<td>(CDDP+NRT)/rNG$_{10k}$</td>
<td>42</td>
<td>0.0009</td>
<td>0</td>
</tr>
<tr>
<td>(CDDP+NRT)/LAE-rNG$_{10k}$</td>
<td>50.5</td>
<td>&lt;0.0001</td>
<td>37.5%</td>
</tr>
</tbody>
</table>
Figure 42. In vivo antitumor efficacy of (CDDP + NRT)/LAE-rNG_10k in OVCAR-5/Luc human ovarian cancer intraperitoneal xenograft-bearing female nude mice. A. Comparison of tumor growth inhibition following IV administration of 5% dextrose (control) or CDDP/LAE-rNG_10k or (CDDP+NRT)/rNG_10k or (CDDP + NRT)/LAE-rNG_10k. Drug formulations were injected in 100 μL at a dose of 4 mg CDDP or 4mg NRT equivalents/kg body weight 4 times at 4-day intervals as indicated by the arrows. Data presented in terms of relative whole-body bioluminescence units (Rt/R0) to day0. Values indicated are means ± SEM (n = 8), *P < 0.05. ****p < 0.0001. B. Kaplan–Meier analysis of overall survival in 5% dextrose (control) or CDDP/LAE-rNG_10k or (CDDP+NRT)/rNG_10k or (CDDP + NRT)/LAE-rNG_10k. Log-rank test was used to determine statistical difference between (CDDP+NRT)/rNG_10k and (CDDP + NRT)/LAE-rNG_10k groups, *P < 0.05, and between 5% dextrose (control) and CDDP/LAE-rNG_10k groups **P <0.01. C. Tissue distribution of platinum in different treatment groups as determined by ICP-MS. Mice were sacrificed at day 14 of the treatment with (CDDP+NRT)/rNG_10k or CDDP/LAE- rNG_10k or (CDDP+NRT)/LAE- rNG_10k. Values indicated are means ± SD (n = 3), * p < 0.05. D. Organ weights of mice sacrificed at day 14 of the treatments. Values indicated are means ± SD (n = 3)
Figure 43. Quantification of tumor progression in individual mouse treated with either 5% dextrose (control) or CDDP/LAE-rNG\textsubscript{10k} or (CDDP+NRT)/rNG\textsubscript{10k} or (CDDP+NRT)/LAE-rNG\textsubscript{10k} based on whole body BLI imaging.
Advanced ovarian cancer patients frequently suffer from worsening nutritional status and cachexia which is a debilitating condition characterized by progressive body weight loss with wasting of skeletal muscle and adipose tissue, accounting for at least 20% of deaths in cancer patients in part due to impaired respiratory or cardiac function [50-53]. Moreover, weight loss has been considered as an indicator of terminal disease, poor prognosis, and high recurrence [54-56]. Thus, reversal of such condition could improve the prognosis of cancer patients and benefit the management of malignancy. Since muscle-wasting process is pathologically reflected by progressive loss of skeletal muscle mass [57], weight
change of skeletal muscle was utilized to evaluate whether the developed treatments would ameliorate ovarian cancer-induced cachexia. Following the treatment, 3 mice were sacrificed on day 10 and 28 to obtain left soleus and gastrocnemius muscles (widely studied cachexia-affected skeletal muscles [53]). As shown in Figure 47A, response to treatment with targeted binary drug combination-loaded NGs was only marginally better than that for nontargeted combination group in terms of soleus muscle loss, while the former treatment was significantly better than the targeted single CDDP loaded NGs group (p < 0.05). Similarly, the most delayed gastrocnemius muscle loss was seen in mice treated with targeted (CDDP+NRT)LAE-NG that outperformed nontargeted (CDDP+NRT)/NG and targeted CDDP/LAE-NG in terms of gastrocnemius muscle mass loss (p < 0.05) (Figure 47B).
Figure 45. Weight change of soleus muscle (up) and gastrocnemius muscle (down) of left triceps surae collected from mice sacrificed on day 13, day 28 and day 33. Data presented in terms of relative weight units (Wt/W0) to day 13 of the treatment. Left: One-way ANOVA was used to determine statistical difference on day 28 between CDDP/LAE-rNG_{10k} and (CDDP + NRT)/LAE-rNG_{10k} groups, *p < 0.05, and between 5% dextrose (control) and (CDDP + NRT)/LAE-rNG_{10k} groups, *p < 0.05. Right: *p < 0.05, between CDDP/LAE-rNG_{10k} and (CDDP + NRT)/LAE-rNG_{10k} groups, and between 5% dextrose and (CDDP + NRT)/LAE-rNG_{10k} groups.
Importantly, neither targeted formulations nor nontargeted formulations caused a significant change in animal body weight, organ weight and blood cell counts (Figure 44, 42D, 45 and Table 22). Although we observed relatively higher exposure of liver and spleen to CDDP in the formulation groups, there was no evidence of liver or splenic toxicity by histopathology (Figure 45) and blood chemistry analysis (Table 22), collectively indicating that the developed formulations are well-tolerated.

Figure 46. Light microscopy images (original magnification 10X) of hematoxylin and osin-stained kidney, liver and spleen sections of mice. Tissue samples were collected at day 14.
Table 22. Clinical chemistry parameters and hematocrit of blood samples collected from animals sacrificed on day 14 across all treatment groups. Data are represented as mean ± SD (n = 3).

<table>
<thead>
<tr>
<th></th>
<th>ALP (IU/L)</th>
<th>ALT (IU/L)</th>
<th>BUN (mg/dL)</th>
<th>RBC (*10¹²/L)</th>
<th>WBC (*10⁹/L)</th>
<th>PLT (*10⁹/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Dextrose</td>
<td>42±6</td>
<td>35±1</td>
<td>19±1</td>
<td>8.2±0.6</td>
<td>2.0±0.8</td>
<td>32±2</td>
</tr>
<tr>
<td>CDDP/LAE-rNG₁₀k</td>
<td>40±7</td>
<td>44±7</td>
<td>20±3</td>
<td>7.5±0.9</td>
<td>3.4±0.1</td>
<td>28±5</td>
</tr>
<tr>
<td>(CDDP+NRT)/rNG₁₀k</td>
<td>45±13</td>
<td>42±3</td>
<td>23±2</td>
<td>7.3±2.7</td>
<td>3.6±0.4</td>
<td>40±5</td>
</tr>
<tr>
<td>(CDDP+NRT)/LAE-rNG₁₀k</td>
<td>40±16</td>
<td>37±2</td>
<td>22±3</td>
<td>8.1±0.2</td>
<td>3.8±0.9</td>
<td>32±10</td>
</tr>
</tbody>
</table>

As further evidence of enhanced antitumor activity of CDDP/LAE-NG or (CDDP+NRT)/NG or (CDDP+NRT)/LAE-NG, we performed histopathological analyses of the tumors excised post-treatment (one day after last injection), using Ki-67 and Cleaved-caspase-3 (CC3) as markers for proliferation and apoptosis, respectively. As expected, treatment with single drug CDDP/LAE-NG caused a significant reduction in Ki67+ cells (p <0.0001) as well as increase in CC3+ cells, consistent with the primarily cytotoxic effects of CDDP [58] (Figure 46). Notably, co-delivery of NRT further enhanced cell responses to CDDP/LAE-NG treatment with increased impact on both parameters (p <0.01 for Ki67+ cells and p <0.05 for CC3+cells) (Figure 46), indicating that the combination is superior and promotes substantial cytotoxic effect. Consistent with the observed significantly higher drug retention achieved by the targeting strategy, targeted (CDDP+NRT)/LAE-NG treatment led to a significant reduction in Ki67+ cells (p < 0.01) and an augmentation in CC3+ cells (p < 0.05) as compared to the nontargeted counterpart. These data reinforced our hypothesis that installation of the EGFR targeting ligand L-AE to the delivery vehicle could enhance drug retention in the target site thereby further boost the cytotoxic activity of CDDP + NRT combination chemotherapy in EGFR+ ovarian cancer.
Figure 47. Quantification of dividing cells marked by Ki-67 staining (A) and apoptotic cells marked by cleaved-caspase-3 (CC3) (B) staining in treated tumors (n = 4). Data are shown as mean ± SEM; **p < 0.01; ****p < 0.0001. Representative immunohistochemical staining of tumors from mice treated with 5% dextrose, CDDP/LAE-NG, (CDDP+NRT)/NG, (CDDP+NRT)LAE-NG at original magnification × 2 (C) and magnification × 20 (D).
In addition to the necessity of incorporating the targeting ligand to the carrier, we further evaluated whether the synchronized and spatial temporal delivery of binary-drug is crucial for exerting its cytotoxic activity. Following the same dosing frequency and timeline as the previous study, each treatment was given intravenously for total of 4 times at 4-day intervals at 4 mg/kg CDDP and 4 mg/kg NRT equivalents per dose. (CDDP+NRT) at 4 mg/kg for each drug was used as a control-free drug combination with the same dosing schedule. The tumor progression, animal body weight and lifespan of animals were monitored and shown in Figure 48 and Table 23. Tumor burden was significantly decreased by free CDDP + NRT treatment (p < 0.05) compared to 5% dextrose control (Figure 48A). However, CDDP-NRT combination administered as single NG formulation was found to be significantly more effective than free drug combination (p < 0.05) (Figure 48A) as measured by BLI. The observed improved efficacy of drug combination treatment in NG format is possibly due to the significantly enhanced drug retention in tumor as was observed in the PK studies. While therapy with targeted (CDDP+NRT)/LAE-NG was marginally more effective in inhibiting tumor growth as compared to a cocktail of targeted single drug-loaded NGs (not statistically significant), the former group extended the median survival time for 4 days suggesting that synchronized delivery of CDDP-NRT combination chemotherapy using NGs plays an crucial role in its cytotoxic activity.

Malignant cells of ovarian cancer tend to spread into peritoneal fluid and disseminate along the abdominal and pelvic peritoneum to form peritoneal metastases. Giving chemotherapeutic agents intraperitoneally by infusion into the peritoneum has been investigated in both bench and bed-side contexts, and demonstrated an improvement in overall and disease-free survival [59, 60]. However, IP administration is associated with higher toxicities due to the tissue exposure to high concentration of drugs. Therefore, we utilize (CDDP+NRT)/LAE-NG to evaluate whether there are therapeutic benefits in delivering targeted IP chemotherapy in ovarian cancer. As seen in Figure 48, the BLI data
shows a trend towards enhanced therapeutic efficacy of (CDDP+NRT)/LAE-NG when administered IP compared to IV regimen. Kaplan–Meier survival analysis also demonstrated that mice given IP (CDDP+NRT)/LAE-NG appear to have the best outcomes. However, the differences between groups were not statistically significant. Overall, major improvement in the antitumor treatment was observed for all co-loaded drug NGs which resulted in a pronounced tumor growth inhibition and an increase of the median lifespan to ~54-68.5 days as compared to the treatments comprised of mixtures of the single drug NGs (Table 23). The longest median survival (68.5 days) was observed in mice treated with (CDDP+NRT)/EGFR-A-NG which was 9 days longer than that in group treated with IP (CDDP + NRT)/LAE-NG.

**Table 23.** Median survival and p-values of different treatment regimens with respect to control group (5% dextrose).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Median Survival (days)</th>
<th>p-values (vs. 5% dextrose control)</th>
<th>% survival at censoring date (90 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Dextrose</td>
<td>36</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>LAE-rNG\textsubscript{10k}</td>
<td>36</td>
<td>0.89</td>
<td>0</td>
</tr>
<tr>
<td>Free CDDP+NRT</td>
<td>40</td>
<td>0.19</td>
<td>0</td>
</tr>
<tr>
<td>CDDP/rNG\textsubscript{10k}+NRT/ rNG\textsubscript{10k}</td>
<td>47</td>
<td>0.016</td>
<td>0</td>
</tr>
<tr>
<td>CDDP/LAE-rNG\textsubscript{10k}+NRT/LAE- rNG\textsubscript{10k}</td>
<td>50.5</td>
<td>0.0009</td>
<td>0</td>
</tr>
<tr>
<td>(CDDP+NRT)/LAE-rNG\textsubscript{10k} IV</td>
<td>54</td>
<td>0.0008</td>
<td>0</td>
</tr>
<tr>
<td>(CDDP+NRT)/LAE-rNG\textsubscript{10k} IP</td>
<td>59</td>
<td>0.0002</td>
<td>14.3</td>
</tr>
<tr>
<td>(CDDP+NRT)/EGFR-A-rNG\textsubscript{10k}</td>
<td>68.5</td>
<td>0.0028</td>
<td>25</td>
</tr>
</tbody>
</table>
Figure 48. In vivo antitumor efficacy of (CDDP + NRT)/LAE-rNG$_{10k}$ and (CDDP + NRT)/EGFR-A-rNG$_{10k}$ in OVCAR-5/Luc human ovarian cancer intraperitoneal xenograft-bearing female nude mice. A. Comparison of tumor growth inhibition following IV administration of 5% dextrose (control) or free CDDP + NRT, CDDP/LAE-rNG$_{10k}$ + NRT/LAE-rNG$_{10k}$ or (CDDP + NRT)/LAE-rNG$_{10k}$ or (CDDP + NRT)/EGFR-A-rNG$_{10k}$. We used one additional group which received IP injection of (CDDP + NRT)/LAE-rNG$_{10k}$. Drug formulations were injected in 100 μL at a dose of 4 mg CDDP or 4mg NRT equivalents/kg body weight 4 times at 4-day intervals as indicated by the arrows. B. Comparison of tumor growth inhibition of various treatments for the first 19 days. Data presented in terms of relative whole-body bioluminescence units (Rt/R0) to the day 0. Values indicated are means ± SEM (n = 8), *p < 0.05, **p < 0.01. C. Kaplan–Meier analysis of overall survival. Log-rank test was used to determine statistical difference between CDDP/rNG$_{10k}$ + NRT/rNG$_{10k}$ and (CDDP + NRT)/LAE-rNG$_{10k}$ IV groups, *p < 0.05, between CDDP/rNG$_{10k}$
+ NRT/rNG10k and (CDDP + NRT)/LAE-rNG10k IP groups, **p < 0.01. D. Relative individual body weight change (W/W₀) were measured following intravenous administration of various formulations. Values indicated are means ± SEM (n = 3).

Assuming that the antitumor efficacy of a drug is related to the amount of this drug delivered to the tumor, we next compared the Pt levels accumulated in the tumor tissue as a result of different treatments (Figure 49A). Animals injected with CDDP/NG + NRT/NG cocktail displayed significantly higher levels of Pt in tumors as compared to free CDDP + NRT treatment group (p < 0.05) which may be attributed to the enhanced permeability and retention (EPR) effect (Figure 49A). Moreover, treatment with cocktail of targeted single drug NGs, CDDP/LAE-NG + NRT/LAE-NG, led to a further significant increase in tumor Pt accumulation (p < 0.05) compared to nontargeted counterpart. It is likely that EGFR-specific interaction of EGFR targeted NGs with cancer cells contributed to this increase. Interestingly, we did not detect significant differences in Pt tumor concentrations between targeted co-loaded drug NGs, (CDDP + NRT)/LAE-NG, and a cocktail of two targeted single drug NGs, CDDP/LAE-NG + NRT/LAE-NG, when administered via IV route. These results are generally consistent with the only marginal improvement in lifespan observed upon treatment with co-loaded drug NGs compared to single drug-loaded NGs cocktail (Table 23). Notably, the Pt tumor concentration for IP (CDDP + NRT)/LAE-NG was increased ~1.5-fold compared to the same dose of drug for IV (CDDP + NRT)/LAE-NG. Such an improvement in drug delivery to the tumor can be related to a more efficient traffic to tumor sites and a slower intraperitoneal clearance. Similar higher local drug retention upon IP route of administration was also reported in previous studies [61-63]. IP group also displayed the higher plasma CDDP concentration at 24 hrs post-injection as compared to IV counterpart (Figure 49B). This increase appears to be due to a delayed absorption from the peritoneal cavity to the circulation.
However, this observation needs further investigation and possibly be dependent on type of the nanocarrier and the cargo. The higher Pt retention in the tumors and plasma might be the reason for the observed better tumor responsiveness to IP (CDDP + NRT)/LAE-NG treatment (Table 23). Consistent with the PK studies, CDDP level in plasma was the lowest in group of free drug cocktail due to the rapid clearance by renal filtration (Figure 49B). In contrast, significantly higher level of CDDP was found in all formulation groups showing their ability to achieve prolongation of drug circulation times and consequently increase tumor exposure that is translating into enhanced antitumor efficacy (Figure 48). CA-125 is a glycoprotein encoded by the MUC16 gene either bound to the cell membrane or released in bodily fluid [64]. Due to the fact that elevated CA125 is detected in most women with advanced ovarian cancer, CA125 is recommended for ovarian cancer screening clinically in the U.S. Practice guidelines [65] with proved value in both detection and disease monitoring [66]. Monitoring CA-125 blood serum levels is also useful for predict the outcome of the therapy and tumor status after the completion of treatment [67]. To further characterize tumor response to the treatments, we analyzed the serum CA-125 levels in mice on day 14 (second day after last injection). As shown in Figure 49C, levels of CA-125 were significantly low in the mice that received nanoformulated drug combinations compared to either control group (p < 0.001) or free CDDP + NRT group (p < 0.05). However, there was no significant difference among drug-loaded NGs groups and CA125 levels in these groups were close to assay detection limit (5 U/mL) suggesting the effectiveness of drug-loaded NGs therapy in reducing tumor burden (Figure 48A).
Figure 49. (A). Pt content in tumor tissues measured 24 hrs post-injection with various treatments (day 14). (B). Organ weights of mice sacrificed on day 14 of the treatments. (C). CA-125 ELISA assay. Quantification of serum CA-125 levels in mice from various treatment groups. (D). Quantification of plasma CDDP levels in mice from various treatment groups on day 14. Values indicated are means ± SEM (n = 3).
It should be emphasized that CDDP and NRT at the designated doses may induce toxicity to the normal tissues as well. However, this was not the case in our studies. Figure 48C shows that either single or binary drug combination-loaded targeted NGs did not induce body weight loss while the same dose of the free CDDP + NRT produced a considerable body weight loss. These effects were reversible after drug discontinuation, which indicated systemic toxicity of free CDDP + NRT. In Mice treated with free CDDP + NRT combination displayed significant decrease of the spleen (Figure 49D, p < 0.01) and kidneys weights (Figure 49D, p < 0.05). We also observed marginal decreases in liver weights in these animals but not statistically significant as compared to the naïve control on day 14. In contrast, such organ weight loss was not observed in any of the drug-loaded NGs groups. Complete Blood Counts (CBC) analysis on day 14 revealed that red blood cells and platelets were within the normal range for all groups (Table 24) while there was evident reduction in the count of white blood cells (WBC) (not statistically significant) in the mice treated with free CDDP + NRT. It was reported that CDDP treatment could trigger apoptosis in the bone marrow, affect cell progression through cell cycle and reduce leukocyte [68]. In contrast, no differences in WBC counts between drug-loaded NG treated and control mice were observed (Table 24). As expected, despite of relatively higher exposure of liver and spleen to CDDP in NGs, there was no evidence of liver or splenic toxicity by histopathology (Figure 50) and related enzyme levels (Table 24). Collectively, these data demonstrate that the developed formulations are well tolerated with reduced toxicity as compared to free drugs at the same dose.
Table 24. Clinical chemistry parameters and hematocrit of blood samples collected from animals sacrificed on day 14 across all treatment groups. Data are represented as mean ± SD (n = 3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ALP (IU/L)</th>
<th>ALT (IU/L)</th>
<th>BUN (mg/dL)</th>
<th>RBC (*10¹²/L)</th>
<th>WBC (*10⁹/L)</th>
<th>PLT (*10⁹/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Dextrose</td>
<td>47 ± 2</td>
<td>48 ± 4</td>
<td>16 ± 2</td>
<td>8.8 ± 0.3</td>
<td>1.6 ± 0.3</td>
<td>357 ± 30</td>
</tr>
<tr>
<td>LAE-rNG10k</td>
<td>41 ± 6</td>
<td>42 ± 8</td>
<td>18 ± 3</td>
<td>8.4 ± 0.3</td>
<td>1.3 ± 0.3</td>
<td>304 ± 48</td>
</tr>
<tr>
<td>Free CDDP+NRT</td>
<td>58 ± 6</td>
<td>51 ± 6</td>
<td>16 ± 4</td>
<td>7.7 ± 0.5</td>
<td>0.9 ± 0.1</td>
<td>325 ± 28</td>
</tr>
<tr>
<td>CDDP/rNG10k+NRT/NG10k</td>
<td>53 ± 4</td>
<td>50 ± 4</td>
<td>19 ± 1</td>
<td>8.6 ± 0.6</td>
<td>1.4 ± 0.4</td>
<td>395 ± 78</td>
</tr>
<tr>
<td>CDDP/LAE-rNG10k +NRT/LAE-rNG10k</td>
<td>47 ± 6</td>
<td>51 ± 4</td>
<td>19 ± 3</td>
<td>8.6 ± 0.5</td>
<td>1.7 ± 0.1</td>
<td>350 ± 64</td>
</tr>
<tr>
<td>(CDDP+NRT)/LAE-rNG10k IV</td>
<td>50 ± 7</td>
<td>51 ± 8</td>
<td>19 ± 4</td>
<td>8.5 ± 0.8</td>
<td>1.9 ± 0.2</td>
<td>322 ± 59</td>
</tr>
<tr>
<td>(CDDP+NRT)/LAE-rNG10k IP</td>
<td>49 ± 2</td>
<td>37 ± 6</td>
<td>17 ± 2</td>
<td>8.5 ± 0.7</td>
<td>1.5 ± 0.2</td>
<td>362 ± 91</td>
</tr>
<tr>
<td>(CDDP+NRT)/EGFR-A-rNG10k</td>
<td>53 ± 3</td>
<td>49 ± 7</td>
<td>18 ± 1</td>
<td>7.7 ± 0.4</td>
<td>1.9 ± 0.1</td>
<td>333 ± 14</td>
</tr>
</tbody>
</table>
Figure 50. (A). Pt content in tissues measured 24 hrs post-injection with various treatments (day 14). Values indicated are means ± SEM (n = 3). (B). Light microscopy images (original magnification 10×) of hematoxylin and eosin-stained kidney, liver and spleen sections of mice. Tissue samples were collected at day 14.

To further determine the molecular changes indicative of effectiveness of various treatments on tumor progression, dividing cells and apoptotic cells in excised tumor tissue sections were evaluated using Ki67 and CC3 biomarkers (Figure 51). The number of proliferating cells were significantly lower in the tumors of animals treated with (CDDP+NRT)/LAE-NG as compared with cocktail of CDDP/LAE-NG + NRT/LAE-NG (p < 0.05) or free (CDDP+NRT) (p < 0.01) (Figure 51A). In parallel, a significant increase in number of apoptotic cells death was also observed in (CDDP+NRT)/LAE-NG treated tumors (Figure 51A). This could explain the delayed tumor growth seen in animals treated with (CDDP+NRT)/LAE-NG even in 2 weeks post- treatment, while the tumors of animals treated with cocktail of CDDP/LAE-NG + NRT/LAE-NG showed a relatively higher rate of
tumor progression (Figure 48A). Overall, these data manifest a strong synergy of anticancer action of the combination of CDDP and NRT co-loaded into a single NG.

To mechanistically link the enhanced antitumor activity of CDDP+NRT combination with the effects of drugs on activation of EGFR, we performed immunohistochemistry (IHC) analyses of phosphor-EGFR levels in tumors of mice treated with various regimens. Indeed, lower phosphor-EGFR levels were seen in tumors of (CDDP+NRT)/LAE-NG treated mice (Figure 51C), correlating with the superior antitumor efficacy of a targeted combination of DNA damaging agent + pan-HER inhibitor loaded NGs strategy.

![Figure 51. Quantification of dividing cells marked by Ki-67 staining (A) and apoptotic cells marked by cleaved-caspase-3 (CC3) (B) staining in treated tumors (n = 3). Data are shown as mean ± SD; *p < 0.05; **p < 0.01. Representative immunohistochemical staining of tumors from mice receiving various treatments at original magnification × 2 (C).](image-url)
Due to the much-extended survival of mice treated with (CDDP+NRT)/EGFR-A-NG, we would like to understand whether the protein ligand itself exerts therapeutic effects. We further evaluated the antitumor efficacy of EGFR-A-NG. To this end, OVCAR-5 cells were treated with either EGFR-A (5 µM) or NRT (5 µM) for 2h. Then cells were stimulated by EGF (100ng/mL) for 5 mins to trigger EGFR phosphorylation. Notably, both EGFR-A and NRT could suppress the EGF-induced EGFR/Akt/ERK (1/2) phosphorylation (Figure 52). However, EGFR-A were not as efficient as NRT in terms of blocking EGFR-mediated signal transduction, indicated by less p-Akt and p-ERK(1/2) reduction.
Figure 52. Effect of EGFR-A on phosphorylation of EGFR, MAPK (ERK1/2) and Akt in OVCAR-5 cells. Cells were incubated in medium containing the 5 µM of either NRT or EGFR-A for 2 hours. At the end of the 2-hour incubation, 100 ng/mL EGF was added for 5 minutes. Total proteins (10 µg) were prepared and separated by 8% SDS-PAGE. After transferring to a nitrocellulose membrane, blots were probed with anti-phospho-EGFR, anti-phospho-ERK1/2, anti-phospho-Akt, anti-HSC70, respectively. The values underneath the blots represent the relative changes of p-EGFR, p-ERK1/2 and p-Akt levels present in the treated cells compared to the basal level of those in control cells after normalization to HSC70, quantified by densitometric analysis using software ImageJ. Blots are representative of 3 independent experiments.

Consistent with the what we observed in western blot assay, EGFR-A-NG exerts certain tumor inhibition effects in mice bearing OVCAR-5 xenograft. On day 10, therapy with EGFR-A-NG without CDDP and NRT was significantly more effective in inhibiting tumor
growth as compared to the control group ($p < 0.05$) (Figure 53A), suggesting that conjugation of protein ligand to NGs does not prevent their binding to EGFR$^+$ cells in vivo and inducing EGFR deactivation. However, the ligand along at the current dose could not sufficiently suppress the tumor progression. On day 17, no statistical difference in tumor growth was detected between EGFR-A-NG treated and control mice (Figure 53A). IHC analyses of phosphor-EGFR levels in tumors of mice demonstrated a lower p-EGFR level in tumors from EGFR-A-NG group as compared to the control group (Figure 56), correlating with its behavior of blocking EGFR activation observed in in vitro setting. And the conjugation of EGFR-A to NG didn’t render this ligand ineffective. Such behavior was translated to its anti-proliferation effects shown in Figure 56C and D that proliferating cells percentage in tumors of mice treated with EGFR-A-NG was lower as compared to the control group (not significant). These data suggest that apart from the targeting ability, the EGFR-A ligand also demonstrated antitumor activity through EGFR inhibition. Moreover, anto-EGFR activity of EGFR-A ligand might contribute into the observed longer lifespan of mice treated with (CDDP+NRT)/EGFR-A-NG as compared to (CDDP+NRT)/LAE-NG. As expected, we didn’t observe any toxicity in terms of CBC results and levels of blood serum enzymes related to liver and kidney functions in all groups (Table 26).
Table 25. Median survival and p-values of different treatment regimens with respect to control (5% dextrose).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Median Survival (days)</th>
<th>p-values (vs. 5% dextrose control)</th>
<th>% survival at censoring date (80 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Dextrose</td>
<td>35</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>EGFR-A-rNG10k</td>
<td>40</td>
<td>0.21</td>
<td>0</td>
</tr>
<tr>
<td>(CDDP+NRT)/EGFR-A-rNG10k</td>
<td>63</td>
<td>0.0007</td>
<td>14.3</td>
</tr>
</tbody>
</table>

Figure 53. In vivo antitumor efficacy of (CDDP + NRT)/EGFR-A-rNG10k in OVCAR-5/Luc human ovarian cancer intraperitoneal xenograft-bearing female nude mice. A. Comparison of tumor growth inhibition following IV administration of 5% dextrose (control) or EGFR-A-rNG10k or (CDDP+NRT)/EGFR-A-rNG10k. Drug formulations were injected in 100 μL at a dose of 4 mg CDDP or 4mg NRT equivalents/kg body weight 4 times at 4-day intervals as indicated by the arrows. Data presented in terms of relative whole-body bioluminescence units (Rt/Ro) to the day0. Values indicated are means ± SEM (n = 7), *P < 0.05. ****p < 0.0001. B. Kaplan–Meier analysis of overall survival in 5% dextrose (control) or EGFR-A-rNG10k or (CDDP+NRT)/EGFR-A-rNG10k. Log-rank test was used to determine statistical difference between (CDDP+NRT)/EGFR-A-rNG10k and EGFR-A-rNG10k groups, **P < 0.01.
Figure 54. Quantification of tumor progression in individual mouse treated with either 5% dextrose (control) or EGFR-A-rNG10k or (CDDP+NRT)/EGFR-A-rNG10k based on whole body bioluminescence imaging.
Figure 55. Relative individual body weight change ($W_t/W_0$) were measured following intravenous administration of either 5% dextrose (control) or EGFR-A-rNG10k or (CDDP+NRT)/EGFR-A-rNG10k. Body weight were measured every 4 days until day 36.

Table 26. Clinical chemistry parameters and hematocrit of blood samples collected from animals sacrificed on day 14 across all treatment groups. Data are represented as mean ± SD ($n = 3$).
Figure 56. Quantification of dividing cells marked by Ki-67 staining (A) and apoptotic cells marked by cleaved-caspase-3 (CC3) (B) staining in tumors from treated mice (n = 3). Data are shown as mean ± SD; **P < 0.01; ***P < 0.001. Representative immunohistochemical staining of tumors from mice treated with 5% dextrose, EGFR-A-NG, or (CDDP+NRT)/EGFR-A-NG at original magnification × 2 (C) and magnification × 20 (D).
It is important to note that in conducted independent animal studies, we repeated several treatments including (CDDP+NRT)/EGFR-A-NG. When the quantification of Ki67+ cells and CC3+ cells was compared side-by-side in a single graph (Figure 57), level of both biomarkers in replicated experiments were quite similar in the same treatment groups among repeated studies, demonstrating the reproducibility of the presented data. Although we did observe the benefit of incorporating the EGFR-A ligand over LAE to (CDDP+NRT)/NG in terms of inducing more apoptotic effects in tumors in repeated experiments, this effect was not significant (p=0.088) possibly due to the limited therapeutic effect of the ligand at the dose used in these studies.

Figure 57. Comparison of level of (A) Ki67+ cells and (B) CC3 positive cells in tumor tissues from mice treated with either dextrose, or (CDDP+NRT)/EGFR-A-NG or (CDDP+NRT)/LAE-NG in two replicated experiments. Data are shown as mean ± SD.
3.4 Conclusions

In this study, we demonstrated that polypeptide-based NG were able to encapsulate both NRT and CDDP in a molar ratio of 1:2. The drug combination at this ratio showed a high level of synergy of cytotoxic action on EGFR overexpressing ovarian cancer cell lines SKOV-3 and OVCAR-5. NRT was found to reverse the pro-survival signals induced by CDDP treatment providing the molecular mechanism for the observed synergy in EGFR⁺ ovarian cancer cell lines. Further optimization of the drug carrier structure by extending the length of PEG chains in the shell of NGs from 114 units to 228 units results in improved PK parameters and biodistribution of NGs. Indeed, optimized PEG-based polymeric NG displayed remarkably less spleen uptake, higher drug retention in tumor sites, and slower clearance from circulation. Modification of NGs with EGFR targeting ligand further improved the PK profile by directing more drugs to the target sites. When tested in an in vivo intraperitoneal xenograft mouse model of high-grade serous EGFR-positive ovarian cancer, EGFR targeted NGs carrying CDDP and NRT drug combination exerted enhanced antitumor efficacy, both in terms of tumor growth inhibition and survival, compared to its nontargeted counterpart. This enhanced efficacy was only observed when CDDP and NRT are simultaneously delivered to the tumor sites. Our data also indicate that IP administration of (CDDP+PTX)/LAE-NG can be more effective in the context of targeted combination therapy without extending its cytotoxicity to the normal tissues.

Acknowledgement:

We acknowledge the assistance of NMR, Flow Cytometry and Tissue Sciences Core Facility (University of Nebraska Medical Center). The authors are grateful to Fan Lei for help in the animal studies, Dr. Foster and Dr. Duarte for help in evaluating tissue pathological toxicities.
3.5 References

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CHAPTER IV.

SUMMARY

Cytotoxic chemotherapy, such as cisplatin-based therapy, remains the standard established systemic treatment of various cancer types, including TNBC and ovarian cancer. However, monotherapy shows limited efficacy which arises from altered signaling patterns of cancer cells, such as activation of pro-survival signal transduction (e.g., epidermal growth factor receptor (EGFR), AKT, PI3-kinase) and damage repair, etc. Therefore, combinatorial chemotherapy remains the major strategy to combat cancer since it works by different mechanisms to decrease the likelihood of developing protective mechanisms by cancer cells. EGFR is one of the HER family of oncogenic receptor tyrosine kinases, which actively participates in sustaining the growth and the survival of carcinoma cells as well as the pathogenesis and progression of different carcinoma types. It is frequently overexpressed in various cancer types making it a major therapeutic target for the development of targeted drug delivery. EGFR inhibitors work synergistically with standard chemotherapy including cisplatin-based standard cytotoxic treatment by blocking oncogenic signal transduction and reducing the unfavored pro-survival signals induced by those chemotherapies as reported. We therefore hypothesized that EGFR-inhibition would potentiate the therapeutic efficacy of cisplatin towards cancers overexpressing EGFR by reducing multiple cell-survival signal transduction and pushing cells towards apoptotic death. To this end, neratinib (an inhibitor of HER family members including EGFR) and cisplatin were selected as the combination regimen to treat EGFR* cancers. However, the administration of the combined drugs sometimes is limited by poor patient compliance due to the inconvenience of repeated or extended duration of drug infusion. Besides, traditional
chemotherapeutic small molecule drugs often show severe dose-limiting toxicities either due to the drug actions or excipients used in the formulation, such as cisplatin that has been linked to significant toxicities including nephrotoxicity, cardiotoxicity, hepatotoxicity and neurotoxicity. Combining such binary drugs in one delivery carrier is therefore a desirable and convenient strategy for controlling the pharmacokinetics and co-delivery of the desired drug ratio in vivo, to maximize the therapeutic efficacy and minimize drug-associated toxicities.

Crosslinked polypeptide-based nanogel is an appealing platform to achieve this goal. Such a biodegradable platform provides flexibility in adjusting size, loading capacity, surface properties, deformability, softness, and responsive behaviors by tuning the chemical compositions as well as crosslinking levels. Enhanced stability due to the crosslinking adds benefits to prolonging the circulation, which in term enhances drug exposure to the tumor by EPR effect. Their mild preparation procedures allow the incorporation of labile drug molecules and biomacromolecules. The most prominent advantage over other nano-sized carriers is their ability to incorporate molecules with different physicochemical properties through versatile interactions, offering advantages for combinatorial encapsulation of drugs. However, not all ratios of the same drug combination would display strong synergistic activity. To exploring systems which allow us to screen ratios of the drug combination for efficacy, in Chapter II we focused on developing a panel of systems using the same polymer template with different fractions of hydrophobic functionalities to achieve the goal. For the purpose of the current study, poly (ethylene glycol)-block-poly (L-glutamic acid) modified with various numbers of phenylalanine units (PEG-P(Glu-Glu/Phe)) for simultaneous loading and delivery of binary CDDP and NRT combination. Such NGs have 1) a hydrophilic PEG shell which imparts a stealth character against the immune system and extends the systemic circulation, 2) an
anionic core, which incorporates CDDP through coordination with the carboxylic groups of PGLu, 3) hydrophobic regions formed by Phe moieties, which serves as a reservoir for NRT solubilization. Consistent with changes of chemical composition, NG with a high content of hydrophobic functionalities displayed less swelling ability and more efficient loading of the hydrophobic drug. Resulting binary drug combination-loaded NGs with 50 units of hydrophobic Phe units per polymer chain were able to encapsulate both CDDP and NRT at a molar ratio of 2:1, and displayed the strongest synergistic effect towards EGFR⁺ TNBC MDA-MB-468 cells compared with other screened regimens. Such superior synergy was found to be selective and only displayed in the EGFR⁺ cell line. NRT was found to reverse pro-survival signal transduction by CDDP mediated EGFR/Akt/Erk activation as well as increased Cyclin D1 expression, which was believed to be the molecular basis for the synergistic effect of a combination of CDDP and NRT. Altogether, this study demonstrates a fundamental possibility for simultaneous delivery of chemotherapeutics via single well-defined and structurally tunable polymeric nanocarrier. Besides, such carriers grant us with flexibility in adjusting cargo ratio as well as dimensions for the enhanced therapeutic outcome of carrier drugs.

However, this system is solely relying on the EPR effect for accumulation in tumor tissues with leaky blood vessels. Such effect showed varied responses due to both inter- and intra-tumor heterogeneity as reported. Installing targeting ligands on the surface of carriers to specifically target biomarkers overexpressed on the surface of tumor cells is a well-suited strategy to overcome delivery barriers including EPR limitations and further boost drug performance. EGFR is one such biomarker of interest that overexpresses in more than 50% of malignant ovarian tissue associated with high tumor grade and poor patient outcomes. In **Chapter III**, two targeting ligands aiming to bind with EGFR was chosen in our study: small L-AE peptide and EGFR-A protein. We designed ligand-installed NGs
incorporating CDDP and NRT combination at a molar ratio of 2:1 and examined whether EGFR-targeted concurrent delivery of synergistic combination of CDDP and NRT can lead to enhanced therapeutic efficacy compared to nontargeted NG system. Two types of ligand-installed drug-loaded NGs both demonstrated success in significantly increased cellular uptake in EGFR+ cancer cells. Further optimization of NG structure was conducted by extending the thickness of PEG stealth layer from 114 monomer units to 228 units for sufficient protection from opsonization. Optimized PEG-based polymeric NG displayed more favored PK properties, such as remarkably less spleen uptake, higher drug retention in tumor sites, and slower clearance from circulation. NGs with EGFR targeting ligands further improved the PK profile by directing more drugs to the target sites. When tested in vivo, EGFR targeted NGs carrying CDDP and NRT drug combination exerted enhanced antitumor efficacy, both in terms of tumor growth inhibition and survival, compared to its nontargeted counterpart in rapidly growing mice model of EGFR-positive ovarian cancer. We also confirmed the importance of simultaneous administration of the (CDDP+NRT) via a single NG system which provides more therapeutic benefits than a cocktail of individual drug-loaded NGs administered at equivalent doses. Besides, our data also indicate that IP administration of (CDDP+PTX)/LAE-NG can be more effective in the context of targeted combination therapy without extending its cytotoxicity to the normal tissues. These data have shown the great potential of our carrier system in the delivery of a drug combination to treat EGFR overexpressing cancers.
LIMITATIONS AND FUTURE DIRECTIONS

Data in the second chapter demonstrated the evaluation of nanocarrier systems encapsulated with CDDP + NRT. Such systems allow for varying the ratio of the two drugs with different physicochemical properties and screening those ratios for efficacy. NPs carried with a 2:1 molar ratio of CDDP and NRT were found to show superior synergistic cytotoxicity activity in the treatment of TNBC \textit{in vitro}. Our future efforts include detailed \textit{in vivo} studies which may help to elucidate whether the observed cytotoxicity would be translated into antitumor efficacy \textit{in vivo} using a TNBC orthotopic mice model. In this chapter, our idea was to deliver the system to tumor sites solely relying on the EPR effect to facilitate the delivery of the drug combination to the tumor site. However, the EPR effect is highly heterogeneous even within the same tumor mass which affects the penetration and accumulation of NPs in the tumor sites. One major strategy for achieving targeted delivery to target tissues is by surface-installation of drug delivery systems with ligands that can target specific biomarkers with a differential expression on the tumor cell surface, which may enhance retention of drug carriers, as well as to improve internalization of nanocarriers by target cells. In our chapter three, we designed ligand-installed carrier systems to compare whether they can specifically deliver CDDP+NRT to EGFR$^+$ ovarian tumors and perform better antitumor efficacy than nontargeted systems. The ligand-installed NPs were made from PEG-based polypeptide and showed great therapeutic efficacy for the co-delivery of CDDP and NRT. This work demonstrated the advantages of nanocarriers that are able to alter the PK properties of a small drug molecule with prolonged circulation, enhanced retention in tumor and improved safety profile as compared to the free drug. The nanocarrier underwent further optimization in terms of PK properties through extending the thickness of the PEG stealth layer by 2 folds. While the optimized NPs displayed remarkably less spleen and liver uptake with more retention in
the target site, NPs still accumulate to a considerable extent in the liver and spleen. There is always a scope to play with the stealth layer to provide sufficient protection for extended circulation and elevated drug exposure. Another limitation is that the developed sub-100 nm NPs are not small enough for efficient tumor accumulation. Some intractable cancers with inherent stromal barriers would prevent such NPs from penetrating into tumor tissues.

Current and future studies planned in our group include the design and fabrication of sub-50 nm nanocarriers with improved stealth properties. One such system under investigation is based on the copolymer of PEG-P(Glu-Glu/Phe)\textsubscript{x,y} utilizing Y-shaped PEG as the stealth layer. Besides extending the length of PEG chains, utilizing Y-shaped PEG might increase the surface brush density of PEG which further minimizes protein binding and RES uptake. This carrier system is being screened with different ratios of hydrophobic and hydrophilic moieties to obtain sub-50nm NPs, which can also be applied in some intractable cancers such as pancreatic cancer and brain cancer. Another limitation is that repeated injections of stealth NPs into mice might provoke the immune system to generate PEG-specific IgM, which would decrease the half-life of the subsequently injected NPs [1]. Such phenomenon might not be seen in nude mice due to the deficient immune system. However, data from other groups have suggested that such accelerated blood clearance phenomenon might not be PEG specific and is dose dependent. Besides, IgM may not be able to opsonize the NPs if there is a sufficient PEG brush functionalized on the surface [2]. To further assess if our systems exert any immunogenicity, a transgenic mouse model could be a more appropriate platform to test. Although the OVCAR-5 intraperitoneal mice model demonstrated good tumor engraftment take, this mice model does not fully recapitulate human disease. Patient derived xenograft (PDX) model genuinely retains the principal histologic and genetic characteristics of the primary tumor, remains stable across passages and mimics the behavior of the primary tumor, such as the response to
chemotherapeutics [3, 4]. Moreover, PDX models have demonstrated success in predicting the clinical outcomes and are being used for preclinical drug evaluation, and biomarker identification [4]. Therefore, the PDX model could be a valuable tool in the prediction of the therapeutic efficacy of our formulations.

References:


