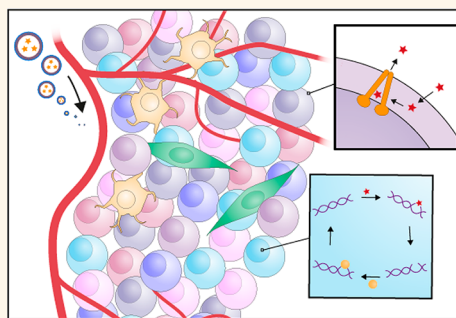


Particle Carriers for Combating Multidrug-Resistant Cancer

Yan Yan,* Mattias Björnholm, and Frank Caruso*

Department of Chemical and Biomolecular Engineering, The University of Melbourne, Victoria 3010, Australia

ABSTRACT Multidrug resistance (MDR) in tumors accounts for significant treatment failure. Particle carriers offer potential benefits for treating cancer, including the ability to target tumors and to deliver multiple cargo, providing opportunities to overcome drug resistance. In this Perspective, we provide a brief introduction to the MDR mechanisms and implications of tumor heterogeneity that contribute to drug resistance. We also highlight recent advances in the design of particles aimed at treating resistant tumors through particle-based codelivery of therapeutics. Finally, we discuss future directions, where an increased understanding of the tumor biology can be leveraged to develop new and improved particle-based cancer therapies.



Cancer is a multifaceted disease characterized by a remarkably high degree of adaptability and resilience.¹ Driven by an improved understanding of cancer biology, the development of chemotherapy has transformed from identification of cytotoxic agents (*e.g.*, paclitaxel, a mitotic inhibitor) to more recent discoveries of targeted therapeutics (*e.g.*, gefitinib, an epidermal growth factor receptor (EGFR) inhibitor).² Despite these advances, a significant fraction of metastatic cancer does not respond to therapeutic agents due to drug resistance. Further, it is often found that cancer cells have simultaneous resistance to multiple drugs with different chemical structures and mechanisms of action. This phenomenon is commonly referred to as the multidrug resistance (MDR) of cancer.³ The development of MDR contributes to significant treatment failure in patients with metastatic cancer.³ Although it is now increasingly realized that MDR, either inherent or acquired, is developed by a variety of mechanisms, MDR has mainly been explained by overexpression of ATP-binding cassette (ABC) transporters in resistant cancer cells.⁴ Therefore, significant effort in combating MDR has been directed toward developing drugs that can inhibit these transporters to sensitize resistant cancer cells. Over the past two decades, several generations of transporter inhibitors have been tested in clinical trials. However, overall, these clinical trials have generated

disappointing outcomes, largely due to the toxicity and low specificity of the inhibitors.⁴ Hence, addressing drug resistance still remains a priority.

The study reported by Hammond and colleagues in this issue of *ACS Nano* provides proof-of-principle evidence that layer-by-layer nanoparticle therapeutics can be used to treat drug-resistant tumors in a xenograft mouse model, suggesting that nanoparticles hold potential for overcoming multidrug resistance in cancer.

Particles that can accommodate multiple drugs and minimize adverse side effects may offer alternative strategies to improve the treatment of MDR in cancer.⁵ In this issue of *ACS Nano*, Hammond and colleagues report the generation and use of layer-by-layer (LbL)-assembled nanoparticles for systemic codelivery of doxorubicin-loaded liposomes and small interfering RNA (siRNA) silencing multidrug resistance

* Address correspondence to
yanyan@unimelb.edu.au,
fcaruso@unimelb.edu.au.

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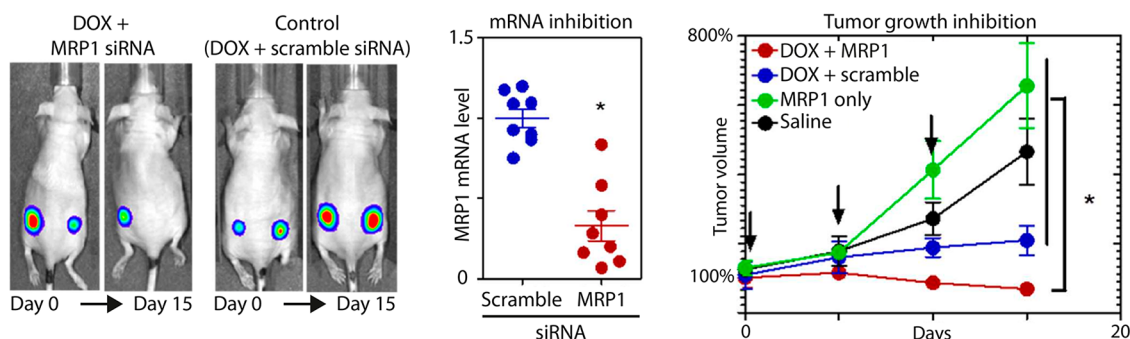


Figure 1. Treatment of luciferase-expressing human breast cancer cells in xenograft mice through codelivery of MRP1 siRNA and DOX using liposome/PLA/siRNA/PLA/HA LbL particles. Three intravenous injections (day 0, 5, and 15) caused significantly lowered levels of MRP1 mRNA levels in the tumor and significant tumor growth inhibition compared to controls. Adapted from ref 6. Copyright 2013 American Chemical Society.

protein 1 (MRP1) to triple-negative breast cancer (TNBC) in an animal model.⁶ This combination therapy via intravenous administration led to significant tumor regression, in contrast to results observed with doxorubicin or siRNA alone (Figure 1). This study provides proof-of-principle evidence that LbL nanoparticle therapeutics can be used to treat drug-resistant tumors in a xenograft mouse model, suggesting that nanoparticles hold potential for overcoming MDR in cancer.

In this Perspective, we provide a brief overview of the underlying mechanisms and recent insights into MDR and highlight several emerging nanoparticle delivery systems that have demonstrated potential to treat drug-resistant cancer, both *in vitro* and *in vivo*. Finally, we discuss challenges and opportunities for further development of nanoparticle-based chemotherapies to circumvent MDR in cancer.

Drug Resistance. By analyzing the genetic background and changes in surviving cancer cells upon exposure to anticancer drugs, a vast array of overlapping mechanisms acting individually or synergistically have been identified as contributing to the emergence of drug resistance.^{3,7} These include alterations to drug uptake and metabolism, enhancement of DNA repair, modification of drug targets, and evasion of apoptotic pathways (Scheme 1).

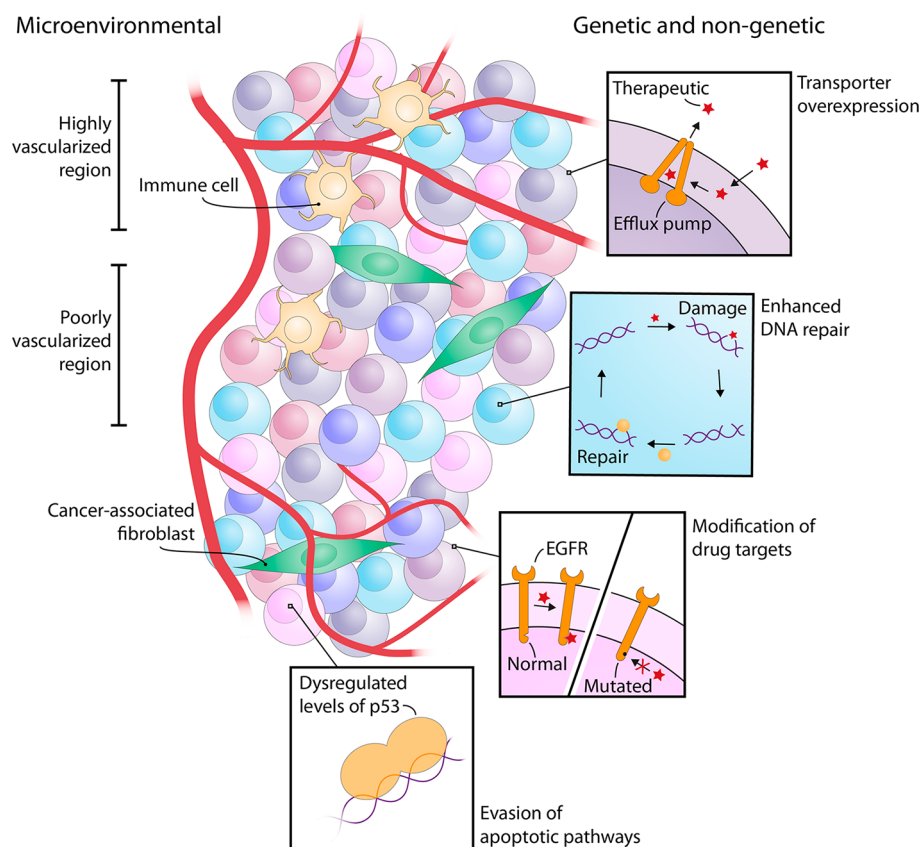
To date, the most studied mode of resistance involves drug transporters on the cell membrane. For example, the ABC transporter family consists of 48 highly conserved members and has an important role in regulating cell and tissue permeability.^{3,4} Due to their exceptionally broad poly-specificity, these transporters are also involved in the efflux of many hydrophobic drugs, such as paclitaxel (PTX) and doxorubicin (DOX). Therefore, alterations in their expression level and activity can prevent drugs from reaching their intracellular targets. It has been found that overexpression of the ABC transporters, particularly P-glycoprotein (P-gp), MRP1, and the breast cancer resistance protein (BCRP), correlates with chemoresistance in many types of cancer.⁴

To exert cytotoxicity more specifically toward cancer cells, many chemotherapeutics are designed to cause extensive DNA damage (*e.g.*, alkylating agents, anthracyclines, and platinum-based therapeutics), which leads to cell cycle arrest and cell death by the intrinsic DNA damage checkpoint.⁸ However, it has been found that by enhancing the ability of DNA repair, cancer cells can evolve to become resistant to these DNA-damaging drugs. As an example, the nucleotide excision repair (NER) pathway is the predominant mechanism that involves repairing platinum-induced DNA damage.⁸ Excision

repair cross-complementing protein 1 (ERCC1) is the key component of the NER pathway. It has been shown that enhanced ERCC1 activity contributes to platinum drug resistance.⁹

With an increasing understanding of cancer biology, new drugs have been designed to target signaling networks that are radically altered in cancer cells to support cancer proliferation. Although many patients have benefited considerably from these new therapies, a subset of patients acquired resistance to these targeted therapeutics. Resistance to targeted drugs can emerge as a result of mutations of drug targets. For example, gefitinib and erlotinib, which attenuate EGFR signal transduction by binding to the c-helix of the tyrosine kinase domain of the EGFR receptor, have been used for treatment of non-small-cell lung carcinoma (NSCLC). When mutations occur at the drug binding sites of EGFR, these inhibitors become ineffective, leading to drug resistance.¹⁰ In addition, disruption of apoptotic pathways, an important hallmark of cancer, can induce *de novo* drug resistance. The most well-known example is p53 mutations, which have been correlated with resistance to a spectrum of drugs.¹¹

Unfortunately, current strategies to combat MDR, including combination therapy and targeted therapy, have been largely ineffective, regardless of the diversity of drugs



Scheme 1. Factors influencing tumor heterogeneity and drug resistance. Genetic, nongenetic, and microenvironmental factors give rise to tumor heterogeneity, which significantly influences the drug sensitivity of cancer cells through an array of cellular mechanisms, such as transporter overexpression.

that have been developed and tested. A reason for this is that an individual MDR phenotype is often mediated by a complex network of cellular pathways built from the aforementioned mechanisms as well as various less common routes. An underlying principle that can be learned is that cancer cannot be considered as one disease.^{12,13} A single tumor comprises heterogeneous populations of cells, which leads to rapid adaptation under selective pressure of a cytotoxic drug.

Novel cellular and molecular insights into MDR have emerged in recent years. It is now widely accepted that the development of human tumors is a complex, multi-stage process that is dependent on the acquisition of multiple oncogenic mutations.¹ Consequently, a tumor comprises cells with divergent subpopulations harboring different mutations.¹² Advances in genomics, proteomics, and systems

A single tumor comprises heterogeneous populations of cells, which leads to rapid adaptation under selective pressure of a cytotoxic drug.

biology have started to reveal the landscape of tumor heterogeneity.¹³ Many genetic, nongenetic, and tumor microenvironmental factors that are continuously shaping cancer cell survival and resistance to therapies have been identified (Scheme 1). For example, genetic heterogeneity, caused largely by genetic instability of cancer cells, enhances the probability of a tumor becoming resistant to therapeutics.

Using next-generation sequencing technology, which systemically quantifies single nucleotide mutations in a fraction of tumor cells, the relative genetic heterogeneity in tumors can be directly measured. It has been shown that estrogen receptor-positive breast cancer that has lower levels of genetic heterogeneity compared with TNBC shows a longer treatment response time and is less likely to develop drug resistance.⁷ High genetic heterogeneity has also been associated with acquired drug resistance in the treatment of gastrointestinal stromal cancer and chronic myeloid leukemia.⁷

Recently, cancer stem cell research has highlighted the importance of nongenetic heterogeneity within a tumor. A variety of different transcriptional network states (*i.e.*, epigenetics) and stochastic fluctuations exist in a tumor.¹⁴ This nongenetic cell-to-cell variability can give rise to

different drug sensitivities. As a result, these nongenetic alterations can drive phenotypic heterogeneity in cancer cells, leading to drug resistance. For example, in treatment with the TNF-related apoptosis-induced ligand (TRAIL), considerable variability occurred in cell death among genetically identical cancer cells.¹⁵

A strong link between tumor microenvironment and chemosensitivity has also been established.¹⁶ Tumor microenvironments are largely shaped by the tumor stroma, which includes cancer-associated fibroblasts, immune cells, endothelial cells, and perivascular cells. These stromal components vary significantly in their distribution and abundance within tumors.¹⁶ Certain traits of the microenvironment have been correlated with poor prognosis. For example, enrichment of reactive myofibroblasts in tumor stroma and high granulin expression are indicative of drug resistance and reduced survival in breast cancer.¹⁷

Combined, heterogeneity arising within tumors as a consequence of genetic, nongenetic, and microenvironmental variability is a key element in both cancer progression and drug resistance, as it allows tumors to evolve during treatment. This evolution also suggests that any single agent alone is unlikely to overcome drug resistance effectively, as cancer is a moving target.

Overcoming Drug Resistance with Particles. Significant effort has been focused on addressing drug resistance with nanoparticle-based therapeutics.¹⁸ Engineered particles may offer alternative strategies to circumvent drug resistance because they can enable preferential accumulation in tumors, deliver drugs *via* endocytosis to evade drug transporters, and combine the synergistic effects of multiple drugs. To date, a number of particle systems have been engineered to evade or to inhibit drug efflux both *in vitro* and *in vivo*. These studies have demonstrated the ability to improve drug

efficacy against resistant tumors. Herein, we highlight several recent examples of particle-based carriers, including liposomes, LbL particles, and mesoporous silica nanoparticles, for combination therapies to overcome drug resistance.

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Liposome-based systems have attracted significant attention, as liposome–drug formulations have already entered the clinic to treat certain forms of cancer.⁵ To reverse drug resistance, efforts have been focused on developing multifunctional liposomes for codelivery of standard chemotherapeutic drugs and siRNA. Huang and colleagues codelivered siRNA targeting c-Myc and DOX in anionic liposome–polycation–DNA (LPD) nanoparticles *via* intravenous injection in a xenograft mouse model using multidrug-resistant human ovarian cancer cells (NCI/ADR-RES).¹⁹ To enhance their specificity toward tumor cells, these LPD nanoparticles were functionalized with poly(ethylene glycol) (PEG) and a targeting moiety, anisamide (AA), which binds to the sigma receptor overexpressed in many human cancer cells. It was shown that c-Myc siRNA delivered by the nanoparticles resulted in significantly reduced expression of both c-Myc

and MDR1 in the NCI/ADR-RES tumor. This, in turn, dramatically increased the uptake of DOX, leading to improved inhibition of tumor growth. By comparison with non-targeted siRNA or DOX alone, it was shown that c-Myc siRNA, DOX, and AA worked in concert to enhance drug accumulation and to promote apoptosis in the resistant tumor. More recently, synergistic effects by codelivering antiangiogenesis and apoptosis agents using targeted lipid/calcium phosphate (LCP) nanoparticles have been demonstrated.²⁰ In that study, the LCP nanoparticles were composed of a solid calcium phosphate precipitate coated with a single lipid bilayer, which was further grafted with a high density of PEG and functionalized with the targeting ligand AA. siRNA specific to vascular endothelial growth factor (VEGF) and gemcitabine monophosphate (GMP) were coencapsulated within the LCP nanoparticles. Through intravenous injections, the combination therapy significantly inhibited tumor growth and decreased the tumor microvasculature density in both subcutaneous and orthotopic xenograft mice models of NSCLC, in comparison to either VEGF siRNA or GMP therapy alone. Similarly, nanostructured lipid carriers (NLCs) have been used for pulmonary delivery to resistant lung cancer in animal models.²¹ The NLCs were prepared by an ultrasonic dispersion method, coated with PEG, and functionalized with a synthetic analogue of luteinizing hormone-releasing hormone (PEG-LHRH). Paclitaxel and two siRNAs (MRP1 and BCL2) inhibiting both drug transporter- and apoptosis-related drug resistance were coencapsulated within the NLCs.²¹ Following administration through inhalation, these multifunctional NLCs substantially enhanced PTX cytotoxicity in the lung cancer cells, resulting in almost complete tumor regression in a mouse orthotopic model of human lung cancer.

Drug delivery to resistant tumors using LbL-assembled particles has

also progressed from bypassing drug transporters *via* endocytosis to inhibiting drug transporters through codelivery of siRNA. Using drug-conjugated poly(glutamic acid) (PGA) particles, evasion of P-gp in drug-resistant human colon cancer cells was demonstrated.²² It was shown that intracellular accumulation of drug in MDR cancer cells was significantly increased in response to particle uptake, whereas free drug was significantly excluded. The enhanced drug accumulation led to partial restoration of drug sensitivity in the MDR cancer cells. These results highlight the need for codelivery to effectively overcome MDR. In this issue of *ACS Nano*, Deng *et al.* report the generation of multifunctional LbL nanoparticles for codelivery of MDR1 siRNA and DOX to a drug-resistant TNBC tumor.⁶ By exploiting the highly modular nature of LbL assembly,²³ three functional modalities were integrated into a single LbL nanoparticle, including a DOX-loaded liposome as the core, poly-L-arginine (PLA) complexed with MRP1 siRNA as multiple layers in the particle walls, and a final outer layer of hyaluronic acid (HA) for targeting breast cancer cells *via* the receptor CD44. Following intravenous injection in a xenograft mouse model, these nanoparticles exhibited an average 4-fold decrease of tumor volume compared with the scrambled siRNA/DOX particle control, with some animals showing complete tumor regression (Figure 1).

Mesoporous silica nanoparticles (MS NPs) are another class of drug carriers that have made substantial advances in basic and preclinical research. Owing to their high surface area and size tunability, many types of anticancer therapeutics have been successfully encapsulated in MS NPs with a high payload.²⁴ In a recent study, 50 nm diameter MS NPs were used to codeliver DOX and siRNA to treat MDR in a xenograft mouse model of human breast cancer.²⁵ The MS NPs were coated with polyethyleneimine—polyethylene glycol for electrostatic

attachment of siRNA. Using a high-throughput screening approach, the synergistic combination of P-gp siRNA and DOX was confirmed in drug-resistant human breast cancer cells (MCF-7/MDR) (Figure 2). Subsequently, the MS NPs containing P-gp siRNA and DOX were intravenously delivered to a MCF-7/MDR tumor. It was shown that P-gp siRNA/DOX-loaded MS NPs provided better inhibition of tumor growth compared with scrambled siRNA/DOX-loaded MS NPs or DOX-loaded MS NPs alone. Interestingly, significant heterogeneity in DOX accumulation and P-gp knockdown were observed within the tumor after the combination therapy. It was shown that low P-gp expression coincided with high levels of DOX and *vice versa* (Figure 2). The heterogeneous vasculature network within a tumor could be a possible reason for such intratumor heterogeneity in response to the MS NP combination therapy, as tumor stroma can influence the accessibility of MS NPs.

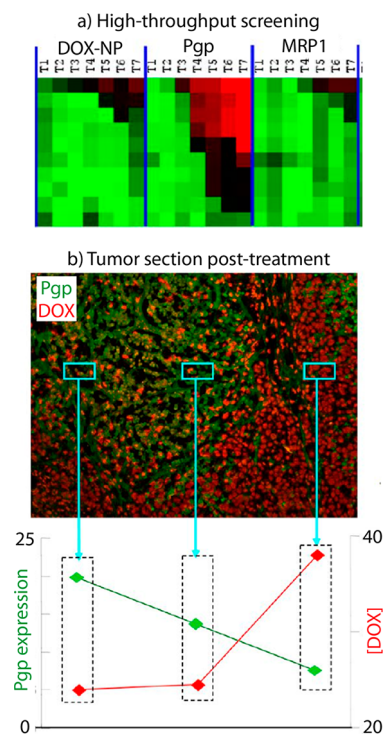


Figure 2. Development and evaluation of DOX-loaded mesoporous silica/PEI/PEG/siRNA. (a) Heatmaps of cytotoxicity from high-throughput screening of siRNA candidates *versus* control. (b) Tumor sections of xenografts from mice post-treatment demonstrate intratumor heterogeneity in P-gp expression and DOX accumulation. Adapted from ref 25. Copyright 2013 American Chemical Society.

OUTLOOK AND FUTURE CHALLENGES

Over the past few decades, cancer chemotherapy has evolved from the discovery of nitrogen mustard treatment in the 1940s to current targeted therapies. The fundamental understanding of cancer biology has revolutionized and will continue to shape cancer therapies. Although effective treatments against some types of cancer have emerged, the development of MDR is still a significant impediment in chemotherapy.

Recent advances in particle–drug formulations have generated exciting platforms toward tumor-specific combination therapies. The studies highlighted in this Perspective are some examples of state-of-the-art endeavors in designing multifunctional particles to overcome MDR in cancer. By coencapsulating multiple drugs in a single particle, synergistic therapeutic effects have been achieved, leading to more effective and longer-term remission

of resistant tumors in animal models. Although current efforts largely concentrate on codelivery of chemotherapeutics and siRNA targeting drug transporters, additional strategies that build synergy based on interdependent traits of tumors, in particular, tumor microenvironment, are expected to emerge.²⁶ To prevent and to treat MDR effectively, it is critical that particles can penetrate deep into solid tumors through the diverse intratumoral microenvironment. Therefore, a better understanding of particle transport in tumor-associated vascular networks will advance the design of particle-based therapeutics to circumvent MDR. Emerging new methodologies, such as three-dimensional cell culture²⁷ and fluidic devices using microengineered *in vitro* tissue models,²⁸ could be valuable in gaining novel insights into these physiological barriers. In addition, particles that are designed to modulate immune responses in the tumor microenvironment, such as inhibiting infiltration of immune cells, can provide a means to enhance immunotherapy and prevent drug resistance. Although treating drug resistance has been challenging, there is optimism that engineered particles can become an effective platform to combat multidrug-resistant cancer through rational particle and drug combinations.

Conflict of Interest: The authors declare no competing financial interest.

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