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Drug and Gene Co-delivery Systems for Cancer Treatment

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Abstract

Cancer remains a major killer and a leading cause of death in the world, so a growing number of new treatments are focused on cancer therapy over the past decades. Chemotherapy, which is thought to be powerful strategy for cancer treatment, has been widely used in clinical therapy in recent years. However, due to the complexity of cancer, a single therapeutic approach is insufficient for the suppression of cancer growth and migration. Therefore, there has been increasing attention to the use of smart multifunctional carriers to combinatorial deliver chemotherapeutic drugs and functional genes to maximize therapeutic efficiency. Combination therapy using selected drugs and genes can not only overcome multidrug-resistance and inhibit the cellular anti-apoptotic process but also can achieve synergistic therapeutic effect. Because multifunctional nanocarriers are important for achieving these goals, this review will illustrate and discuss some advanced biomaterial nanocarriers for co-delivering therapeutic genes and drugs including multifunctional micelles, liposomes, polymeric conjugates and inorganic nanoparticles. In addition, the challenges and future perspectives for co-delivery systems containing therapeutic drugs and genes to achieve better therapeutic effects for cancer treatment will be discussed.

Introduction

Cancer is one of the primary diseases that threaten human lives. Currently, technological advances and comprehensive cognition of this disease have led to more efficient discoveries and development of new therapeutic methods for cancer treatment.[1]

Chemotherapy is the primary therapeutic approach in clinical oncology. However, traditional chemotherapeutic agents, e.g., small molecule anticancer drugs, do not achieve satisfactory therapeutic effects due to their low solubility, poor stability, rapid metabolism and non-targeted body distribution as well as the strong side effects in healthy tissues and poor quality of life in patients.[2] To overcome the above limitations and achieve better therapeutic efficiency, drug delivery systems (DDS) have been designed to transport chemotherapeutic molecules to the tumor site with improved therapeutic effects.[3] Common drug delivery vectors (liposomes, micelles, lipoplexes, inorganic nanoparticles, polymer-drug conjugates etc.) have exhibited respective advantages.[4-6] Liposomes have been used as potential drug carriers due to their ability to protect drugs from degradation, high biocompatibility and a favorable pharmacokinetic profile.[7] However, inherent drawbacks such as low entrapment efficiency for hydrophobic drugs, rapid leakage of water-soluble drugs and poor storage stability inhibit its further clinical application.[8] Another promising drug delivery carrier, polymeric particles, has been extensively investigated because polymers have flexible structures and are easy to chemically modify. Besides, polymeric particles are thought to possess unique advantages over liposomes including enhanced stability of drugs/proteins in the presence of blood components and controlled release of drugs or genes.[7] Currently, a series of polymers have been utilized to encapsulate drugs, such as poly(lactic acid) (PLA), poly(lactide-*co*-glycolide) (PLGA), poly(e-caprolactone) (PCL), proteins, protein-mimicked polypeptides and polysaccharides.[9-15] Specifically, Paclitaxol-loaded polymeric micelle (PEO-PLLA) developed by Samyang Biopharm has already been approved by FDA for clinical trial in cancer treatment. Although

these DDS are effective, some inherent issues also have emerged. Multidrug resistance (MDR), which can defend cancer cells from ectogenic toxic agents, has been discovered to limit the efficiency of well-designed drug delivery systems. Therefore, it is necessary to develop some preferable strategies for improving chemotherapy efficacy.

In recent years, gene therapy has been suggested to be a powerful strategy for cancer treatment as well. It has been demonstrated that a variety of therapeutic genes, which are capable of inhibiting angiogenesis, tumor growth, invasion metastasis and stimulating the immune response against cancer by regulating the molecular processes.[16] To protect and release the genetic cargo at the target site in a timely manner, efforts have been undertaken to design safe and efficient gene delivery systems.[17-20] Polyethylinimine (PEI), one of the earliest cationic polymers used for gene delivery, has been proven to successfully achieve higher gene transfection efficiency, whereas the notable cytotoxicity limits its application in clinical trials.[21, 22] During the past decade, numerous studies on PEI-based gene delivery systems have been performed and various substitutions to PEI including poly(amino esters),[17] poly(amino-acid),[23-28] polyamide,[29] chitosan,[30] etc., have emerged simultaneously, which display higher gene transfection efficiency and lower cytotoxicity. Usually, the vectors prepared using these polymers with positive surface charges and additional modifications electrostatically absorb nucleic acids and improve cellular uptake and assist gene escape from lysosomes during endocytosis. Additionally, diverse targeting moieties can be introduced to the delivery systems to achieve targeted gene delivery. However, because of the complexity of cancer, it appears that it is insufficient to suppress the growth and migration of cancers via a single therapeutic approach.

With better understanding of the properties of both chemotherapy and gene therapy, specifically their respective limitations, using novel multifunctional carriers to combinatorial deliver chemotherapeutic drugs and genes has garnered attention as a method for maximizing therapeutic efficiency. In 2006, Yang's group first designed a co-delivery system to deliver an anticancer drug and a therapeutic gene to the same cells simultaneously for anticancer therapy.[31, 32] Subsequently, the superiority of

co-delivery systems has been gradually exhibited. Currently, co-delivery systems can be primarily divided into the following three strategies according to their therapeutic mechanisms. The first strategy for drug/gene co-delivery is overcoming the membrane transport protein related pump resistance, which is a significant mechanism of cancer multidrug resistance.[33, 34] Membrane transport proteins, particularly two well-known types of P-glycoproteins (P-gp) and multidrug resistance-associated proteins (MRP), are able to efflux anticancer agents from cells or cell organelles, resulting in an obvious decrease of intracellular drug concentration and efficiency.[35] To solve this problem, siRNA against genes encoding efflux pump proteins have been used to sensitize cells to chemotherapeutic drugs and has been demonstrated to be a promising strategy.[36-41] The second co-delivery strategy is the inhibition of cellular anti-apoptotic pathways. To prevent cancer cells from being destroyed by chemotherapy, specifically when pump efflux protein expression is suppressed, cellular anti-apoptotic pathways can be activated to increase drug resistance, defined as non-pump resistance.[42] According to the results from a long-term study of cell apoptosis, the BCL-2 (B-cell lymphoma-2) protein has attracted attention because the BCL-2 protein family (BCL-2, MCL-1 etc.) can inhibits the release of cytochrome *c* from the mitochondrion, which is required to trigger the caspase cascade during apoptosis.[33] Considering the function of BCL-2 protein in anti-apoptosis, a wide variety of sensitization strategies have been used by delivering siRNA to silence BCL-2 genes (siBCL-2), therefore, sensitizing cancer cells to anticancer drugs.[43-48] In addition to overcoming drug resistance, both an anti-proliferative gene and drug can be encapsulated into a single vehicle to achieve a synergetic therapeutic effect, which is the third co-delivery strategy for tumor treatment. Previously, there have been reports that a few therapeutic genes possessing different curative mechanisms have been successfully applied in gene therapy and when combined with anticancer drugs these functions are further enhanced. The p53 gene, which can inhibit cell proliferation through G1-cell cycle arrest in mammalian cells and induce apoptosis, is an ideal tumor suppressor gene for lowering cell viability.[49] Another therapeutic gene, TRAIL, can induce the extrinsic apoptosis

pathway mediated by cell surface death receptors and these receptors have the ability to transmit apoptotic signals after binding with their cognate death ligands.[50] Additionally, VEGF siRNA and survivin shRNA, which suppress protein expression associated with neovascularization and anti-apoptosis, have also been jointly used with chemotherapeutic drugs.[51, 52] These results have demonstrated that co-delivery of specific therapeutic genes and drugs can improve cancer treatment compared with single agent therapy.

Therefore, it is evident that the exquisite co-delivery systems are essential for achieving synergistic therapy. Indeed, numerous vehicles with distinct structures have been reported including micelles,[44, 45] liposomes,[53, 54] complexes,[55-58] inorganic particles, etc. [59, 60] This review summarizes the different types of nanocarriers used for drug and gene co-delivery, combining the superiority of multiform biomaterials, and highlights promising candidates for clinical application.

Materials used as Carriers for Co-Delivery

1. Micelles

Polyethylenimine (PEI) based cationic micelles

Among the numerous cationic polymers for gene delivery, polyethylenimine (PEI) has been demonstrated to be one of the most functional and effective polymers due to its superior capacity to condense DNA/RNA and its unique ability referred to as the 'proton sponge effect'. PEI, possessing high pH-buffering ability, can easily accumulate protons in endosomes along with an inflow of chloride ions across endosome membranes and subsequently increases osmotic pressure, resulting in the physical rupture of the endosomes and the escape of the carrier from the degradative lysosomal trafficking pathway. This process is regarded to be essential for successful gene delivery. Therefore, PEI can be used in drug design as the hydrophilic block of the amphiphilic polymers for self-assembling the co-delivery cationic micelles.[44, 45, 61-64] As depicted in Figure 1A, a diblock copolymer comprising hydrophobic poly(ε-caprolactone) (PCL) and hydrophilic linear PEI was synthesized and used for

self-assembling a biodegradable nanocarrier to combinatorial deliver doxorubicin (DOX) and BCL-2 siRNA.[44] PEGylation of these carriers was conducted via a hierarchical assembly strategy by electrostatic coating of FA-PEG-PGA to the PEI-PCL/siRNA complexes to reduce toxicity and avoid decreasing the DNA-condensing ability. Moreover, to facilitate the internalization of the nanocarriers through a mechanism known as receptor-mediated endocytosis, the tumor-targeting molecule folic acid (FA) was conjugated to the PEG-PGA exposed on the complex surface. Attributed to this well-designed co-delivery system, hepatoma Bel-7402 cell apoptosis was significantly enhanced and DOX-induced cytotoxicity was also greatly potentiated through the synergistic effect of the two therapeutic agents. Further explorations of this multifunctional delivery system in vivo were subsequently performed in a rat brain glioma model.[45] This in vivo study indicated that the tumor size in the animal group receiving the combined siRNA and DOX therapy were decreased and 80% of the animals in this group survived longer than 38 days (Figure 1B and 1C). These results indicate that the combined DOX and BCL-2 siRNA therapy using the folate-targeted multifunctional nanocarrier suppressed cancer growth more effectively than single agent BCL-2 siRNA or DOX therapy for glioma treatment.

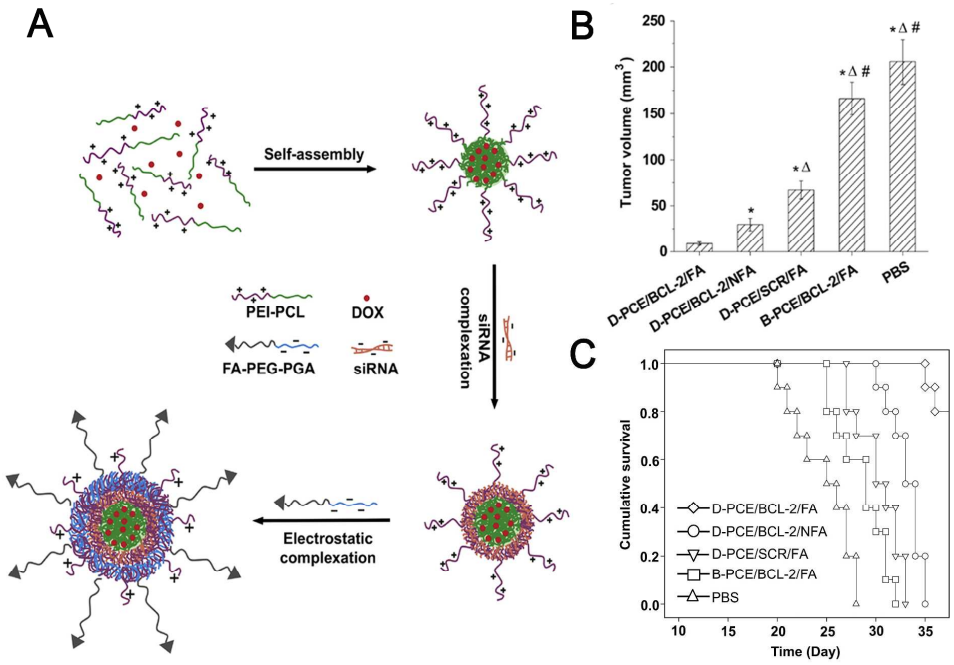

Figure 1. (A) Formation of hierarchical nano-assemblies for combinatorial delivery of siRNA and anticancer drugs; (B) Determination of mean volume of C6 glioma in the brain of rats, using MR imaging 25 days after the first injection; (C) Cumulative survival of rats with C6 glioma in the brain after receiving injections of different formulations. [44, 45]

Another promising multifunctional vehicle for both gene and drug therapy formulated by amphiphilic cationic polymer PEI-SA was designed by Huang's group.[65] Specifically, polyethylenimine (PEI) was modified by grafting stearic acid (SA) for the preparation of polymeric micelles (PEI-SA) with a hydrophobic core and hydrophilic corona for co-delivery. In this research, doxorubicin (DOX) and vascular endothelial growth factor (VEGF) siRNA were selected to observe the synergistic therapeutic effect *in vitro* and *in vivo*. After SA conjugation, PEI-SA polyplexes exhibited low cytotoxicity compared with PEI and maintained high cellular uptake efficiency. In addition, the joint delivery of DOX and VEGF siRNA by the PEI-SA micelles demonstrated a superior combined effect against *in vivo* anti-tumor growth. At day 30 after the injection, the tumor volumes for groups injected with PEI-SA/DOX/siVEGF decreased to 13% compared with the control group.

Poly(2-(N,N-dimethylamino) ethyl mathacrylate) (PDMAEMA) based cationic micelles

As one of the most intriguing temperature and pH sensitive cationic polymers, poly(2-(*N,N*-dimethylamino) ethyl mathacrylate) (PDMAEMA) has superior biocompatibility. In addition, the *tert*-amino groups of PDMAEMA provide a cationic domain for DNA combination through electrovalent bonds, which benefits PDMAEMA as an excellent gene delivery carrier. The cationic micelles formulated by amphiphilic polymer with a hydrophobic segment PCL and hydrophilic block PDMAEMA had been extensively investigated with different architectures. For example, methoxy poly (ethylene glycol)-*b*-poly(ε-caprolactone)-*b*-poly(2-dimethylaminoethyl methacrylate) (mPEG-*b*-PCL-*b*-PDMAEMA) was firstly reported to combinatorial delivery of hydrophobic paclitaxel and pDNA.[66] The results of *in vitro* cell experiment revealed that after introducing hydrophobic PCL segment

into the hydrophilic PEG-PDMAEMA, the gene transfection efficiency of that exhibited a 15-fold enhancement compared with mPEG-*b*-PDMAEMA. In their follow-up study, another type of pH-dependent temperature-sensitive poly(ε-caprolactone)-*graft*-poly(2-(dimethylaminoethyl) methacrylate) (PCL-g-PDMAEMA) was synthesized for drug and gene delivery.[67] With an ultralow critical association concentration, the polymer PCL-*g*-PDMAEMA easily self-assembled into cationic micelles. Notably, because of the particular characters of PDMAEMA, the formulated micelles can respond to the weakly acidic pH environment, coinciding with the acid microenvironment of carcinoma, which may accelerate the carriers taken into cancer cells and decrease the side effects on healthy tissues. Similarly, Zhu's group[68] prepared the biodegradable cationic micelles using PDMAEMA–PCL–PDMAEMA triblock copolymers for the combinatorial delivery of VEGF siRNA and paclitaxel. Due to the enhanced siRNA condensation and endosomal escaping ability of the triblock polymer, the siVEGF knockdown efficiency remarkably enhanced, reaching the high silencing efficiency of 85%.

Another star-shaped polymer consisting of PDMAEMA also has attracted much attention due to distinct properties such as smaller hydrodynamic diameters, lower inherent viscosities as well as better gene transfection. Therefore, a star-shaped four-arm PCL-*b*-PDMAEMA (S-PCL-PDMAEMA) was synthesized to combinatorial deliver DOX and pDNA.[69] With the micelle prepared by a linear PCL-*b*-PDMAEMA copolymer (L-PCL-PDMAEMA) as control, the DOX-loaded S-PCL-PDMAEMA micelles exhibited smaller size (~130 nm), higher drug loading ability (L.E. =16.6 ± 0.2%) and more efficient chemotherapeutic effect. It is worth noting that the S-PCL-PDMAEMA/pDNA polyplex exhibited the similar transfection efficiency with Lipofectamine 2000. Accordingly, this star-shaped S-PCL-PDMAEMA seems to be a promising co-delivery carrier of hydrophobic anticancer drugs and therapeutic pDNA for dual therapy. Similarly, combining the advantage of simultaneous delivery of miR-21i and doxorubicin to enhance the sensitivity of drugs for cancer therapy, a series of amphiphilic star-branched copolymers were synthesized,[70] including PLA-PDMAEMA$_3$, (PLA-PDMAEMA$_3$)$_2$ and (PLA-PDMAEMA$_3$)$_3$, which were

comprised PLA and PDMAEMA. All of these three copolymers displayed low CMC concentration and cytotoxicity. However, the micelles prepared by $(PLA\text{-}PDMAEMA_3)_3$ with a snowflake-like symmetric structure displayed the superior EGFP transfection efficiency compared with another two structures. More importantly, the tumor volume of the combined therapy of D-sCPM3/miR-21i group was 9-fold smaller than that of the DOX or miR-21i loaded in sCPM3 alone treatment, implying that co-delivering the DOX and miR-21i using this nanocarrier resulted in a more obvious stasis of tumor growth. And it is also evidenced that the better therapeutic effect was contribute to the release of DOX to nucleus and especially escaping of miR-21i from lysosome degradation leading to down-regulation of expression of BCL-2 apoptosis by PI3k/AKT signal pathway.

Poly(amino acid) based cationic micelles

In addition to their excellent physicochemical properties such as good biocompatibility and degradability, poly (amino acid) chains can reversibly transition from random coils to α-helix or β-sheet secondary conformations after external stimulation. Therefore, the combination of poly(amino acid) segments endows traditional block copolymers with diversified self-assembling nanostructures to co-deliver genes and drugs. As shown in Figure 2A, Zheng's group[71] prepared polypeptide micelles with a triblock copolymer poly(ethylene glycol)-*b*-poly(L-lysine)-*b*-poly(L-leucine) (PEG-PLL-PLLeu) to co-deliver docetaxel (DTX) and BCL-2 siRNA. In the structure of this polymer PLLeu is the hydrophobic block that forms a core for anticancer drug entrapment; meanwhile, PLL facilitates the delivery of siRNA through electrostatic interactions between the cationic backbone and negatively charged gene. These complexes containing BCL-2 siRNA efficiently inhibit BCL-2 mRNA and protein expression (Figure 2B and 2C). Moreover, simultaneous delivery of the same doses of DTX and BCL-2 siRNA via this carrier exhibited significant inhibition of tumor growth compared with PBS treatment ($p < 0.001$), which demonstrates the synergistic inhibitory effect of the two therapeutic agents on tumor growth shown in Figure 2D. In addition to conventional block

copolymers with linear structures, some novel poly(amino acid) chains have been designed to prepare efficient nanocarriers for combining two or more therapeutic strategies to inhibit tumor growth. Ma's group[72] synthesized a star-shaped copolymer (PP-PLLD-Arg) with a photochemical porphyrin (PP) core and arginine-functionalized poly(L-lysine) dendron (PLLD-Arg) arms. Docetaxel (DTX) and MMP-9 shRNA plasmids, as the therapeutic gene to down-regulate MMP-9 protein expression, were chosen for nasopharyngeal cancer therapy in this research. It was determined that the PP-PLLD-Arg/MMP-9 nanocomplex had photo-enhanced gene transfection efficiency *in vitro* and could mediate a significant reduction of MMP-9 protein expression in HNE-1 cells. After treatment with PP-PLLD-Arg/DTX/MMP-9 complexes, the apoptosis percentage reached 53.3% with 12.9% necrosis, resulting in a decreased invasive capacity of HNE-1 cells.

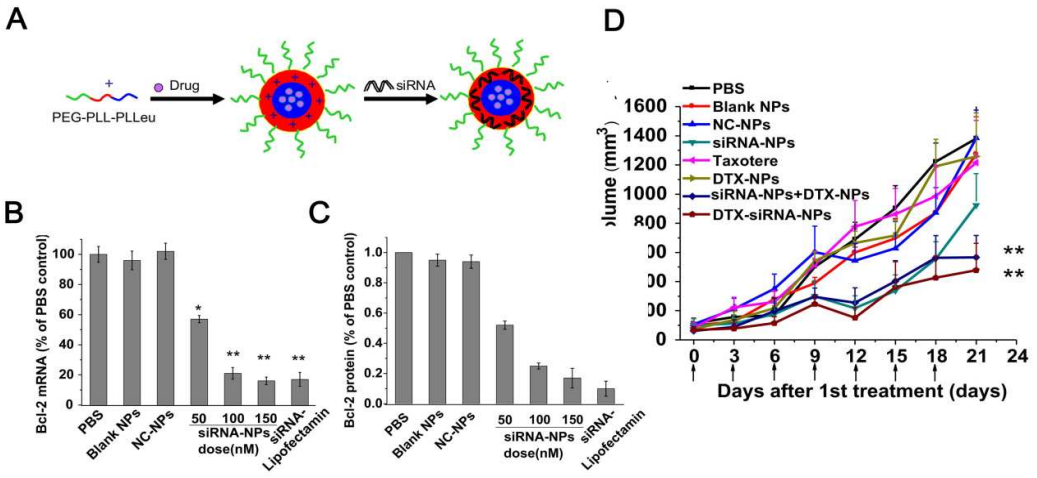

Figure 2. (A) Schematic illustration of a self-assembled cationic micelle and loading of siRNA and drug; (B) Bcl-2 mRNA expression determined using quantitative real-time PCR; (C) Light intensity analysis of Bcl-2 protein expression as the ratio of Bcl-2 to actin from Western blot results; (D) The tumor growth curve illustrates that the treatment groups had significantly inhibited tumor growth compared with control groups. [71]

Liu's group fabricated cationic micellar nanoparticles from ABC-type miktoarm star polypeptide hybrid copolymers consisting of poly(ethylene oxide), poly(L-lysine) and poly(ε-caprolactone) arms and investigated the ability of the carriers to co-deliver chemotherapeutic drugs and plasmid DNA.[73] As shown in reference, in aqueous

media at pH 7.4，PEO-(-*b*-PLL)-*b*-PCL self-assembled into micelles consisting of hydrophobic PCL cores and hydrophilic PEO/PLL hybrid coronas providing domains to carry the model hydrophobic anticancer drug paclitaxel and electrostatically adsorb plasmid DNA, respectively. The *in vitro* gene transfection results demonstrated that the paclitaxel-loaded PEO(-*b*-PLL)-*b*-PCL micelles exhibited improved transfection efficiency compared with pDNA/blank micelles most likely because paclitaxel within the micelle with an anti-mitotic feature could benefit the DNA allowing better uptake into the cell nuclei.

Another strategy for efficient co-delivery of drugs and genes that may offer new opportunities for cancer therapy should be specially mentioned here. Wiradharma's group synthesized oligopeptide amphiphile including three blocks of amino acids, Ac-$(AF)_6$-H_5-K_{15}-NH_2 (FA32) and evaluated whether FA32 had superior properties for the joint delivery of doxorubicin (DOX) and the p53 gene.[74] According to the results in this study, FA32 readily self-assembled into micelles with a small size of approximately 100 nm. Based on the individual advantages of the amino acids in the polypeptide, these micelles had higher capacity for condensing DNA, DOX loading, endosomal escape as well as cellular uptake. More importantly, the co-delivery of DOX and p53-encoding plasmid using FA32 micelles achieved a synergistic effect for suppressing HepG2 cell proliferation compared with the individual DOX and FA32 micelle/p53-encoding plasmid complex treatments.

Poly(amine-*co*-ester) based cationic micelles

Poly(amine-*co*-esters) are particularly promising due to their biodegradability, low cytotoxicity and outstanding transfection efficiency, which mediate the transfer and expression of genes to cells at levels that approach or exceed those using PEI.[17] Yang's group reported a novel amphiphilic material, poly {(N-methyldietheneamine sebacate)-*co*-[(cholesteryl oxocarbonylamido ethyl) methyl bis(ethylene)ammonium bromide] sebacate}(P(MDS-*co*-CES)), which consisted of cholesterol side chains and a cationic main chain[31, 32] as shown in Figure 3A. These self-assembled cationic micelles prepared by P(MDS-*co*-CES) were superior to liposomes because they were

easier to fabricate and more readily subject to size modulation and positive charge degree. More importantly, enhancement of gene transfection efficiency with paclitaxel co-delivery has been demonstrated *in vitro* and *in vivo*. In particular, the paclitaxel co-delivery with Bcl-2-targeted small interfering RNA (siRNA) increased cytotoxicity in MDA-MB-231 human breast cancer cells (Figure 3B), while the tumor growth rate in animals treated with paclitaxel-loaded nanoparticle/IL-12-encoded plasmid complexes was significantly lower than mice treated with a single therapeutic agent (Figure 3C).

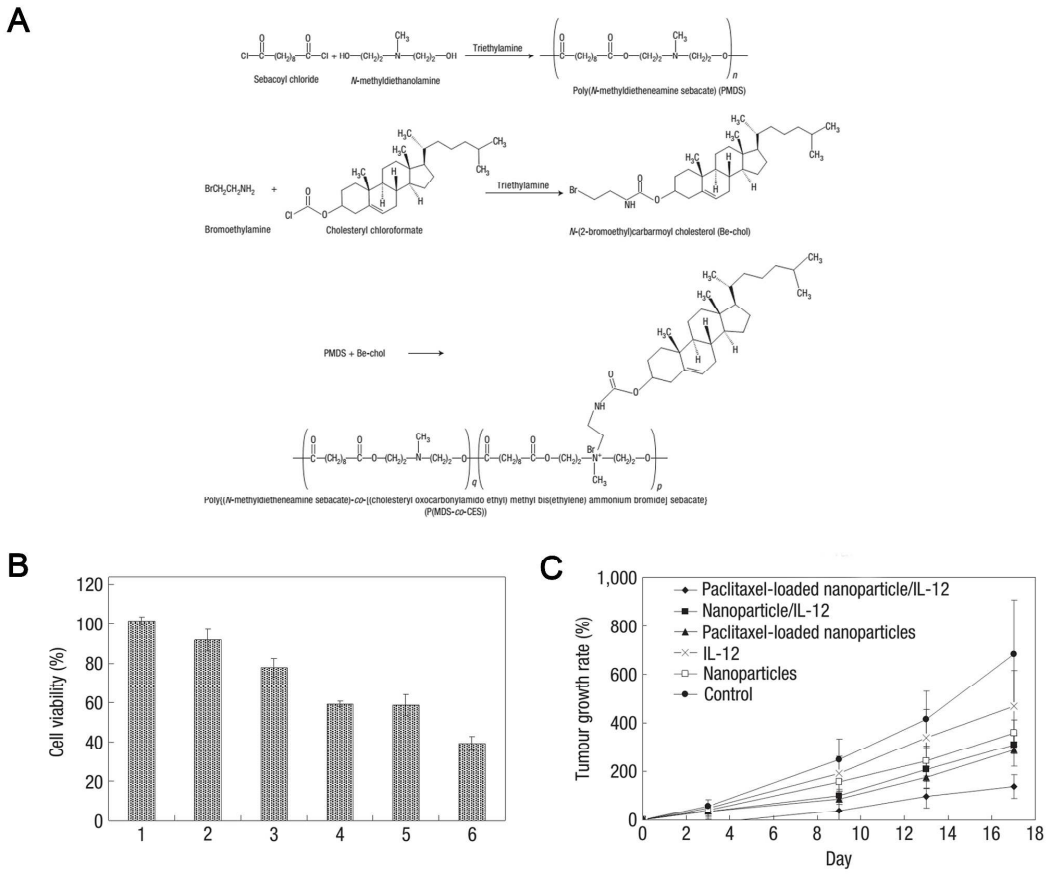

Figure 3. (A) Synthesis of the cationic amphiphilic polymer P(MDS-*co*-CES);(B) and (C) Percentage of GFP expression in 4T1 cells (in triplicate); (B)Viability of MDA-MB-231 cells after treatment with (1) nanoparticles, (2) nanoparticle/siRNA complexes, (3,5) paclitaxel-loaded nanoparticles and (4,6) paclitaxel-loaded nanoparticle/siRNA complexes. (C) Tumor growth rate after treatment with various formulations in a 4T1 mouse breast cancer model. [31]

2. Liposome

Liposomes used as biocompatible drug or gene carriers can enhance the potency and reduce the toxicity of therapeutics. To date, many commercial products consisting of liposomes such as Doxil® and Lipofectamine™ 2000 have been researched and developed for use in basic research or clinical therapy. Based on the advantages of liposomes, some multifunctional liposomes have recently been studied and used for the co-delivery of genes and drugs to potentially improve therapeutic effects in cancer treatments. For instance, an angiopep-2 modified cationic liposome (ANG-CLP) consisting of DC-chol, DOPE, rhodamine-DOPE and COOH-PEG_{2k}-DSPE was used for the co-delivery of a therapeutic gene encoding the human tumor necrosis factor-related apoptosis-inducing ligand (pEGFP-hTRAIL) and paclitaxel (PTX) to glioma and induced apoptosis in 81.99 ± 3.28% U87MG cells, which was 1.98 and 2.8-fold higher than PTX or pEGFP-hTRAIL single agent treatment, respectively.[53] Additionally, the median survival time in brain tumor-bearing mice treated with ANG-CLP/PTX/pEGFP-hTRAIL was 69.5 days, significantly longer than other groups including the commercially available temozolomide treatment group (47 days). Similarly, oligolysine-based cationic lipid derivatives synthesized from oleylamine and various oligolysines with 1–10 lysine residues were formulated into cationic liposomes to combinatorial deliver siRNA and an anticancer drug (Figure 4A).[54] Among various oligolysine-based lipid derivatives, trilysinoyl oleylamide (TLO)-based liposomes (TLOL) exhibited the highest gene transfection efficiency combined with minimal cytotoxicity. In a subsequent study, to enhance the anticancer activity of siMcl1, the anticancer drug suberoylanilide hydroxamic acid (SAHA) was simultaneously encapsulated in pTLOL. After intravenous administration of siMcl1 using SAHA-loaded pTLOL (pSTLOL), an improved therapeutic effect was achieved compared with animals treated with free SAHA or siGL2 (scramble) complexed with pSTLOL (Figure 4B and 4C).

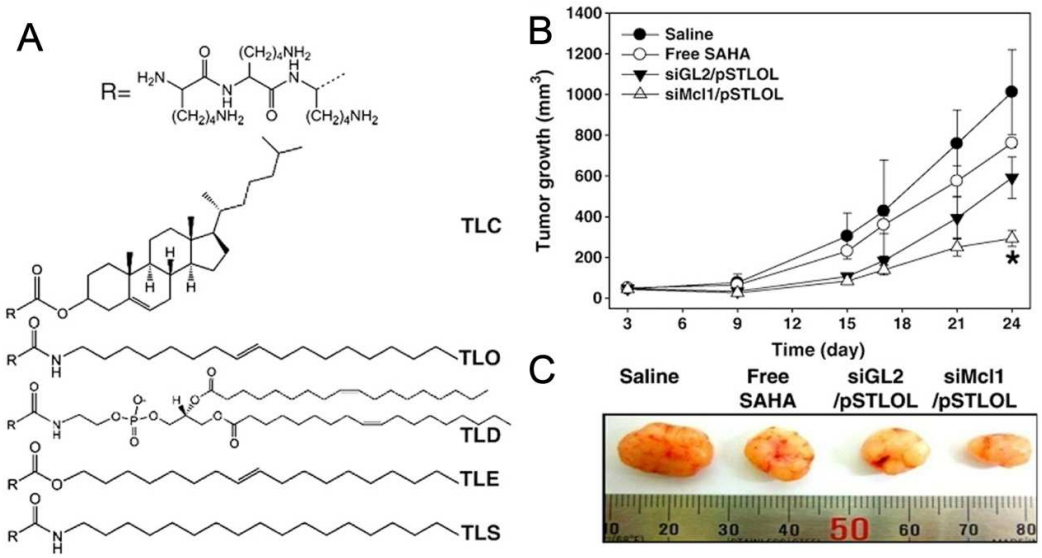

Figure 4. (A) Structures of various trilysinoyl derivatives; (B) and (C) the change in KB tumor volumes after intravenously administered saline, free SAHA, or siRNA complexed to pSTLOL, every other day on seven occasions beginning on day 7.[54]

The simultaneous delivery of drugs and genes in liposome-based carriers also has been developed as an effective approach for overcoming multidrug resistance (MDR).[33, 75, 76] In research to overcome MDR cancer and enhance therapeutic effects, Minko's group recently developed and evaluated in ovarian and breast cancer cells a complex liposomal drug delivery system that encapsulated three main therapeutic agents: (a) antisense oligonucleotides targeted against MDR1 mRNA (to inhibit pump resistance); (b) antisense oligonucleotides targeted to BCL2 mRNA (to inhibit non-pump resistance); and (c) DOX, a traditional anticancer drug (to initiate apoptosis).[33] It was evidenced that simultaneous modulation of multidrug resistance and anti-apoptotic cellular defense by MDR1- and BCL2-targeted antisense oligonucleotides substantially increased DOX cytotoxicity 10-fold when compared with both free and liposomal DOX. This resulted from the inhibition of MRP1 and BCL2 protein synthesis and a substantial increase of DOX anticancer action by stimulating the caspase-dependent apoptosis pathway in multidrug-resistant human lung cancer cells. In a follow-up study, they continued to use this approach to fabricate a targeted a multifunctional nano-structured lipid nanocarrier-based system (NLCS) containing doxorubicin or paclitaxel and siRNA targeting MRP1 and BCL2

mRNA in lung cancer therapy.[75] By examining the NLCS body distribution, it was determined that the total amount of NLCS retained in the lungs after inhalation was higher when compared with i.v. injection of the same nanoparticles. Additionally, compared with non-targeted NLCS, the luteinizing hormone-releasing hormone (LHRH)-targeted NLCS was distributed uniformly through the lungs and predominately accumulated in the lung tumor after inhalation. Therefore, the proposed NLCS effectively performed its multifunctional task providing efficient tumor growth suppression and prevention of adverse side effects in healthy organs.

Another effective delivery system for the simultaneous loading of drugs and genes consists of a lipid layer and a hydrophobic core, which are called hybrid nanoparticles or lipoplexes. The hybrid NPs, which combine the positive attributes of liposomes and solid NPs, have recently received much attention.[7, 77-79] The hydrophobic core in the hybrid construction is capable of encapsulating poorly water-soluble drugs with high loading efficiency. The NP surface is surrounded by cationic lipid molecules that interact with the negatively charged gene. Meanwhile, the target ligands can be conjugated to the lipid end terminals, which promote the specific binding and internalization of targeted nanocarriers by host cells. For instance, Ashley's group established porous nanoparticle-supported lipid bilayers that synergistically combine the properties of liposomes and nanoporous particles.[77] These hybrid nanoparticles are able to encapsulate multiple therapeutics (drugs, small interfering RNA and toxins) and diagnostic agents (quantum dots) as well as promote endosomal escape and nuclear accumulation of selected cargos (Figure 5). Importantly, it was also determined that this delivery system can effectively kill drug-resistant human hepatocellular carcinoma cells, representing a 10^6-fold improvement over comparable liposomes. Furthermore, lipoplexes composed of a hydrophobic PLGA core and a hydrophilic folate coated PEGylated lipid shell (PLGA/FPL NPs) were also fabricated to combinatorial deliver drugs and genes by Chang's group.[78] In this system, hydrophobic drugs (DOX) can be loaded into the core and the cationic shell of the drug-loaded nanoparticles can be used to bind DNA. Moreover, these core-shell nanoparticles achieved simultaneous delivery of drugs and genes to the same cells

with high gene transfection rates and drug delivery efficiency. In a similar study using this core-shell system as co-delivery carriers, Zhang's group described a negative-charge core, where siVEGF was complexed with chondroitin sulfate and was condensed by protamine, that was modified using a cationic lipid shell encapsulating hydrophobic paclitaxel to form the core-shell PLPC/ siVEGF nanoparticle (NP).[79] Additionally, polyethylene glycol phospholipid (DSPE-PEG) and/or vapreotide-modified DSPE-PEG-VAP were inserted into the lipid-shell on the nanoparticle surface, which enhanced their in vivo stability and also targeted to cells with somatostatin receptors (SSTRs) via VAP. Their results also exhibited higher intracellular siRNA accumulation and VEGF down regulation in human breast cancer MCF-7 cells. More importantly, *in vivo* results further demonstrated that the targeted VAP-PLPC/ siVEGF NPs had significantly higher drug distribution in tumor tissues and tumor growth inhibition efficacy via receptor-mediated targeting delivery, accompanied by an obvious inhibition of neovascularization due to VEGF silencing.

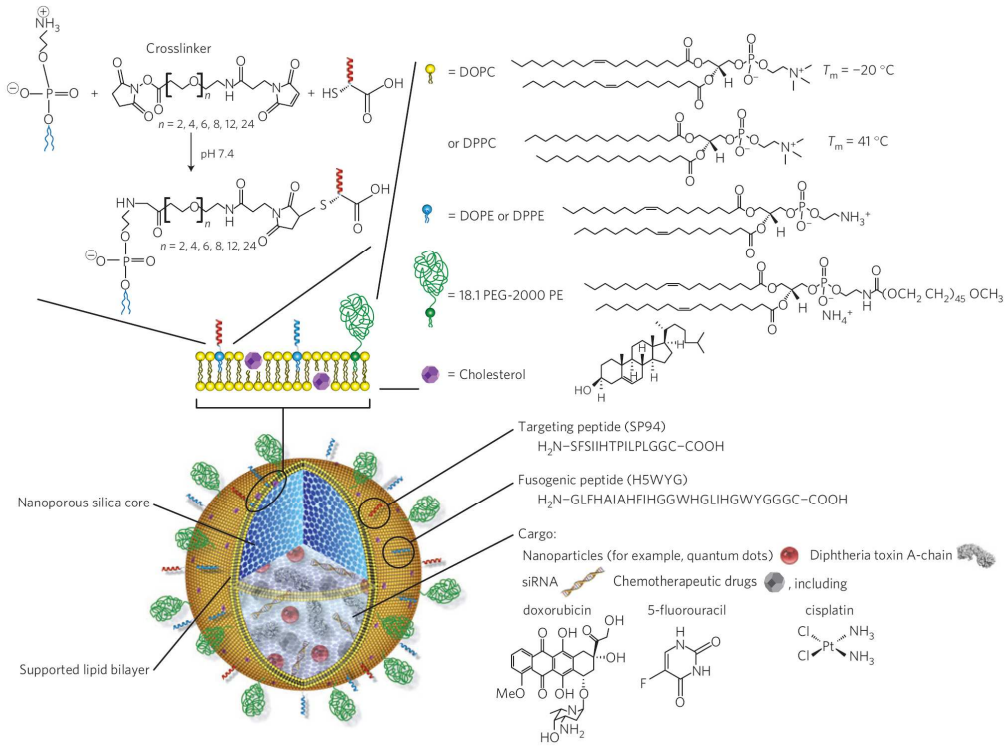

Figure 5. Schematic illustration of the nanoporous particle-supported lipid bilayer, depicting the disparate types of therapeutic and diagnostic agent that can be loaded within the nanoporous silica

core, as well as the ligands that can be displayed on the surface of the SLB. [77]

3. Complexes of gene and polymer-conjugated drugs

In the drug delivery field, polymer-conjugated drugs generally exhibit a prolonged half-life, higher stability, improved water solubility, lower immunogenicity and antigenicity, specific targeting to tissues or cells, etc.[55, 56, 80] Due to these advantages, new polymer-drug conjugates have been designed and synthesized, which are able to also bind with negatively charged DNA via electrostatic interactions to form poly-ion complex nanoparticles. The formation of polyion complex nanoparticles is thought to be an effective method for co-delivering DNA and drugs into various cell lines. For example, novel multifunctional ternary complexes of biotinylated transferrin-avidin-biotin-poly(ethylene glycol)-poly(L-glutamateacid)/ poly(2-(2-aminoethylamino) ethyl methacrylate)/doxorubicin-poly(L-aspartic acid)/pDNA (TAB/PIC-D/pDNA complexes) were fabricated based on polyion complex micelles (PIC), which were also modified with transferrin to target co-delivery of anti-cancer doxorubicin and the gene.[81] In such a system, DOX was conjugated to poly(l-aspartic acid) (PASP) and the genes were condensed via electrostatic interactions. Confocal laser scanning microscopy (CLSM) images determined that doxorubicin and the gene were simultaneously delivered into HepG2 cells by the TAB/PIC-D/pDNA complexes, which also demonstrated that this system provided a facile approach for constructing a multifunctional drug and gene co-delivery system. To maintain lower drug release rates in physiological conditions and accelerate the release rate in an intracellular environment, Liu's group designed a co-delivery system (DGDPT/pORF-hTRAIL) that was loaded with both a therapeutic drug (DOX) and gene (pORF-hTRAIL) (Figure 6A).[82] In this system, DOX was loaded onto the surface of Dendrigraft poly-L-lysine (DGL) using Glu as an acid-sensitive linkage, then the therapeutic gene pORF-hTRAIL was compacted and encapsulated by the DOX conjugated dendrimers. Furthermore, peptide HAIYPRH (T7), a transferrin receptor-specific peptide, was chosen as the ligand to target the

co-delivery system to tumor cells expressing transferrin receptors. The results of in vitro release experiments determined that DOX was rapidly released at pH 5.0 (intracellular environment) while at pH 7.4 (blood) the conjugates were relatively stable. Additionally, an *in vivo* anti-glioma efficacy study confirmed that DGDPT/pORF-hTRAIL displayed anti-glioma activity, but was less toxic to normal tissues (Figure 6B and 6C). Similarly, a micellar system was constructed from degradable poly(ethylene oxide)-block-poly(ε-caprolactone) (PEO-*b*-PCL) block copolymers with functional groups on the side chains.[83] The functional group on the PCL block was used to incorporate short polyamines for complexation with siRNA or to chemically conjugate DOX via a pH-sensitive hydrazone linkage. Additionally, this carrier was also modified with the integrin $\alpha_v\beta_3$-specific ligand (RGD4C) for active cancer targeting and the cell-penetrating peptide TAT for membrane activity. Compared with targeted micelles with scrambled siRNA and bound DOX or targeted micelles with bound DOX only, targeted micelles loaded with MDR1 siRNA and DOX were significantly more cytotoxic in multidrug-resistant MDA-MB-435 human tumor models. Notably, RGD/TAT-micelles with DOX and MDR1 siRNA exhibited a maximum of ~70% cell growth inhibition.

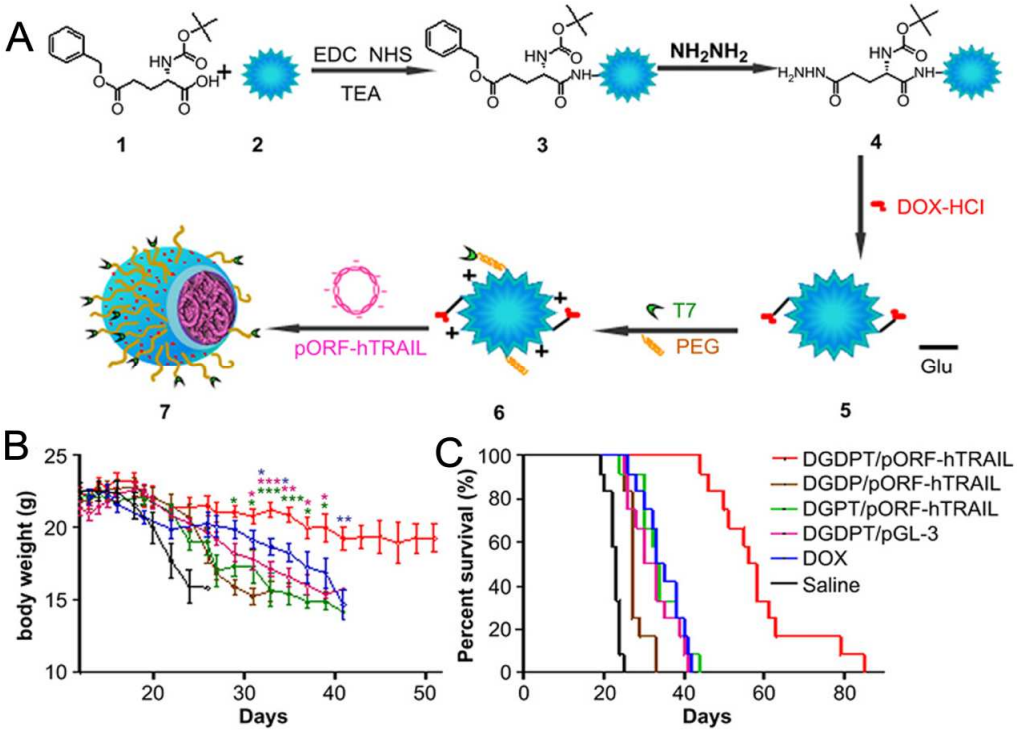

Figure 6. (A) Synthesis of DGDPT/pORF-hTRAIL. 1: OBzl-Glu(Boc); 2: DGL; 3: DGL-Glu; 4: DGL-Glu-$NHNH_2$; 5: DGD. 6: DGDPT; 7: DGDPT/pORF-hTRAIL; (B) the average change in body weight after treatment with DGDPT/pORF-hTRAIL, DGDP/pORF-hTRAIL, DGDPT/pGL-3, DGPT/pORF-hTRAIL with 50 mg DNA and/or 8 mg DOX/mouse, DOX (5 mg/kg) and saline, and (C) Kaplane-Meier survival curves of mice bearing orthotopic U87 tumors. [82]

Furthermore, a unique strategy based on host-guest interactions was also used to co-deliver anticancer drugs and genes. Self-assembly supramolecular nanoparticles (SNPs) are synthesized using polyethylenimine (PEI) crosslinked with cyclodextrins (CyDs), which can easily load anticancer drugs through host-guest interactions and condense therapeutic DNA for cancer therapy with enhanced therapeutic effects.[84] Drug release from the SNPs was also pH-dependent and a 2~3-fold increase in drug release was achieved by lowering the pH value from 7.4 to 5.0 or 3.0. In another example, cationic supramolecular nanoparticles (SPNs), which were produced by â-cyclodextrin-poly-ethylenimine (PEI-CyD) and 2-amineadamantane-conjugated paclitaxel (Ada-PTX), were used to combinatorial deliver PTX and survivin shRNA-encoding plasmids to SKOV-3 cells.[85] Ada-PTX as a core was physically encapsulated into the micelles and survivin shRNA was adsorbed onto the shell of the cationic micelles. *In vivo* antitumor activity experiments determined that the tumor volume of mice treated with co-delivered survivin shRNA and PTX was less than 100 mm^3 and was considerably smaller than the other single therapeutic agent treatment (~150 mm^3) groups, which demonstrated that survivin shRNA and PTX co-delivery suppressed cancer growth more effectively than the delivery of either paclitaxel or shRNA in ovarian cancer therapy. Another interesting approach for a co-delivery system was reported by Zhang's group.[86] This group fabricated novel core-shell structured nanoassemblies assembled through a host-guest interaction by β-cyclodextrin on PEI and the benzyl group on PBLA (Figure 7). The self-assembled nanocarriers provided a hydrophobic container for a highly hydrophobic steroidal anti-inflammatory drug, dexamethasone (DMS), and the cationic shell was used to condense pDNA. Notably, this study demonstrated that these types of assemblies can

combinatorial deliver drugs and pDNA due to the DMS-induced dilation of nuclear pore complexes. Furthermore, a slight increase in transfection efficiency was observed for DMS containing nanocarriers compared with their counterparts without DMS.

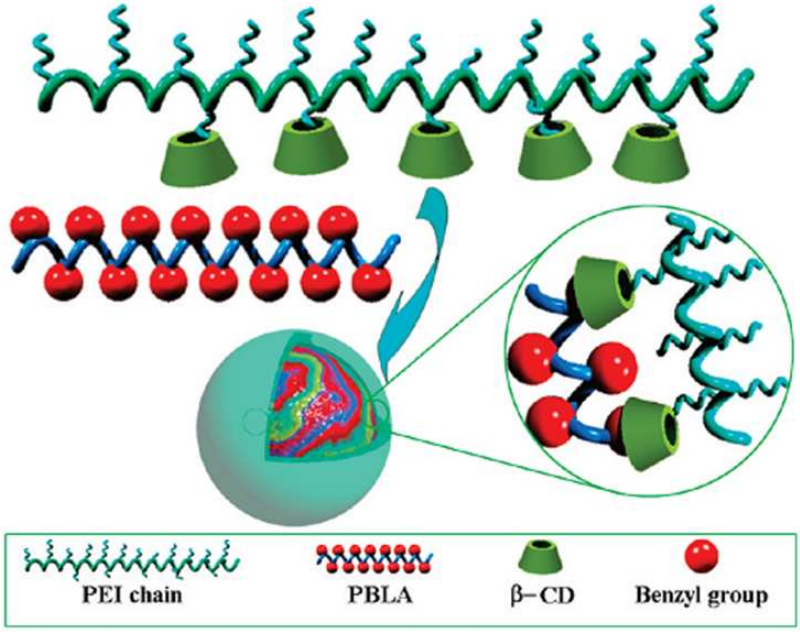

Figure 7. Illustration of the core shell nanoassemblies based on PEI-CD/PBLA using a host guest interaction. [86]

4. Inorganic particles

An additional interesting delivery system for medical and biological application is inorganic particles which have made significant progress due to their extraordinary properties. Drug and gene co-delivery using these vehicles have been extensively evaluated and they have promising applications in cancer treatments. Mesoporous silica nanoparticles (MSNs), with unique structural features such as large surface areas, tunable pore sizes and well-defined surface properties, have been regarded as an ideal carrier for the joint delivery of multiple therapeutic agents.[59, 60, 87, 88] Chen's group[60] utilized MSNs modified with generation 2 (G2) amine-terminated polyamidoamine (PAMAM) dendrimers to combinatorial deliver BCL-2 siRNA and DOX successfully. DOX delivered by MSNs exhibited minimal premature release

during *in vivo* circulation and DOX anticancer efficacy was enhanced 132-fold compared with free DOX, which was attributable to the synergistic effect of the drug and gene. In their follow-up study, another novel inorganic delivery system comprising nanoscale metal-organic frameworks (NMOFs) for cisplatin and siRNA co-delivery was employed to enhance therapeutic efficacy by utilizing the drug and gene synergistic effects to re-sensitize chemoresistant ovarian cancer cells to cisplatin treatment.[89] According to Figure 8, the hexagonal-plate shaped UiO NMOFs were able to encapsulate a cisplatin prodrug and coordinate MDR gene-silencing siRNAs via the absorption between siRNA and the added Zr^{4+}. Meanwhile, the enriched Zr^{4+} were able to bind the negatively charged and phosphate-group enriched membrane to disturb the endosome structure and increase the release of entrapped siRNA resulting in significantly enhanced in vitro chemotherapeutic efficiency (Figure 8B and 8C). It should also be emphasized that the synergistic effects of pooled siRNA targeted at pump and non-pump resistance should be considered when designing analogous delivery systems due to enhanced treatment efficacy compared with single siRNA.

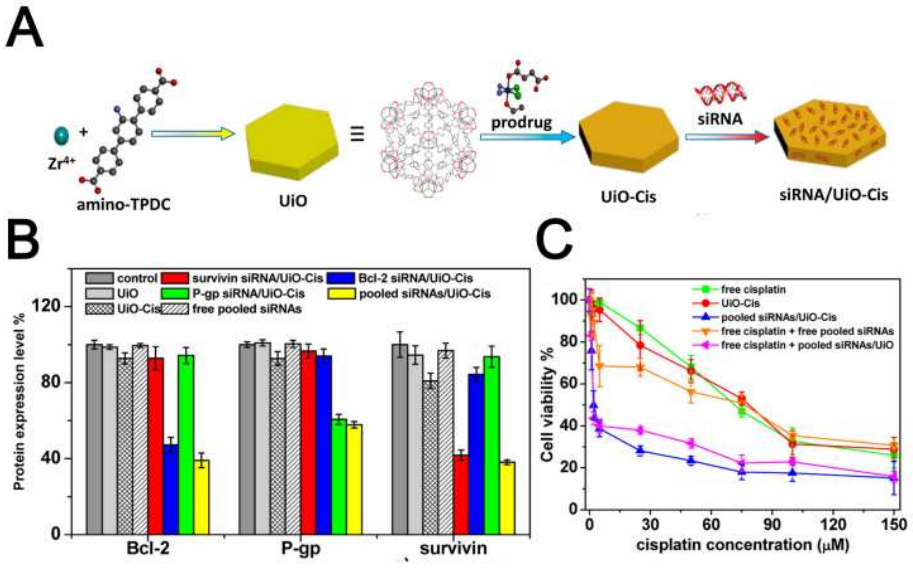

Figure 8. (A) Schematic presentation of siRNA/UiO-Cis synthesis and drug loading; (B) siRNA/UiO-Cis-mediated efficient gene silencing in SKOV-3 cells at a 30 nM siRNA dose. Silencing efficiency was expressed as percentage values of the control group treated with PBS; (C) SKOV-3 cells were incubated with free cisplatin, UiO-Cis, pooled siRNAs/UiO-Cis, free cisplatin plus free pooled siRNAs, and free cisplatin plus pooled siRNAs/UiO at different concentrations

for 72 h and then cytotoxicity was determined using the MTS assay. [89]

It is well-known that the biocompatibility of nanoparticles with positively charged surfaces remain unsatisfactory for clinical applications. To improve delivery system biocompatibility, co-precipitation of Ca^{2+} with a gene in the presence of inorganic anions such as CO_3^{2-} and PO_4^{3-}, has great safety and biocompatibility advantages. Zhao's group[90] investigated alginate modified $CaCO_3$ nanoparticles for joint p53 gene and DOX delivery. With an optimal Ca^{2+}/CO_3^{2-} ratio and alginate amount, both the therapeutic DNA and drug can be efficiently encapsulated in the alginate/$CaCO_3$/DNA/DOX nanoparticles. *In vitro* cell inhibition experiments determined that this system completely inhibited HeLa cell proliferation, indicating that this co-delivery system had promising clinical value for cancer treatment.

Challenges Associated with the Co-Delivery Approach

The preceding review highlighted various types of vehicles for drug and gene co-delivery to treat cancers and these systems may provide effective methods for improving cancer therapeutic outcomes. However, due to the increased complexity of the therapeutic drug and gene co-delivery systems, additional problems and challenges have emerged. These issues must be immediately addressed to design and fabricate more effective co-delivery systems. In this section, several other important considerations will be discussed; however, loading capacity, stability, release kinetics and biocompatibility issues will not be addressed.

To successfully combine drugs and genes within a single platform, the co-delivery system materials should have the ability to simultaneously load therapeutic drugs and genes. Therefore, the materials should have the chemical functionalities or hydrophobic blocks to bind or encapsulate small molecule drugs via covalent or non-covalent interactions and bind genes via electrostatic interactions.[91] Additionally, the lysosome escape capacity, referred to as the 'proton sponge', is often overlooked during the evaluation of gene transfection efficiency. For polycationic chains, the number of secondary and tertiary amines is important for the "proton sponge" effect.[92]

Accordingly, the existence of amine groups in the polymer can condense genes via electrostatic interactions and also aid carriers during lysosome escape. However, excess amines always result in excess charge of the carriers, which primarily leads to high toxicity and instability *in vivo* due to serum components and salt adsorption.[17] And as well known, the successfully delivering of components to targeting site is also a key point to achieve better therapeutic efficacy. If there is no effective accumulation of the therapeutic agents in tumor tissue, no matter how many components in the delivery system will still not alter the present situation of the chemotherapy. However, during the blood circulation, the particles are easily taken by reticuloendothelial system (RES) and rapidly cleared from the circulation.[93] Thus, to shield the excess cationic charge, reduce the toxicity of carriers and prolong the circulation time, PEG ligands and anionic coatings such as poly(γ-glutamic acid) (γ-PGA), hyaluronic acid (HA) and oligonucleotides have been used to modify carriers to facilitate the co-delivery system *in vitro* and *in vivo*.[62, 94-97]Although the accumulation of these particles in the tumor can be improved to some extent by the enhanced permeability and retention (EPR) effect, these hydrophilic modifications still can not lead the drug-loaded carriers to avoid the non-specific taken by the normal tissues[98]. Additionally, the dilemma induced by these modification also inhibits particle interaction with cancer cells and reduce cellular uptake rates, which can also influence their therapeutic efficacy.[98] Thus, to further enhance the tumor targeting efficiency, various strategies were performed and applied in delivery system. Targeting ligands modification has been widely used to improve the tumor targeting efficiency and cellular uptake efficiency.[98, 99] For example, RGD peptides, which were found to specially target to both neovascular endothelial cells and tumor cells, were modified on the surface of delivery carriers, resulting in an enhanced tumor targeting efficiency and a higher cellular internalization.[7, 100] On the other hand, the switchable surface charge from negative to positive is also a promising way to achieve the enhanced tumor accumulation and tumor cell uptake. In the blood circulation, the negative charged particles can efficiently avoid the interaction with the serum components, prolong the circulation time and increase the probability of accumulation at tumor

site.[101, 102] Once entering the tumor tissue, the specific extracellular environment of cancer cell, such as slightly acidic pH, will stimulate the surface charge to revers to be positive, which will be beneficial for the interaction between the particles and cell membrane.[102, 103] Through the above discussions, it is clear that some properties such as loading capacity, lysosome escape capacity, stability, biocompatibility, the tumor targeting efficiency, should be taken into consideration when designing and preparing the co-delivery system. These properties also govern the success of the co-delivery system. Therefore, overcoming these issues is one of the main challenges for preparing a co-delivery system.

The controlled release of genes or drugs from the co-delivery system to achieve maximum therapeutic response is another major challenge. It is well known that the burst drug-release phenomenon often restricts some drug-loaded carriers formulated using amphiphilic block copolymers such as PEG-*b*-PLA. Due to the hydrophobic interaction between the polymer chain and drugs, hydrophobic drugs will either encapsulate into the micelle cores or attach on or near the micelle surface. Through the molecular diffusion mechanism, the drugs near the micelle surface will be rapidly released during blood circulation, which also leads to drug concentrations near or above toxic levels in normal tissues.[104] Conversely, drug molecules entrapped at or close to the center of the micelle core will be released slowly, over several weeks or longer.[45, 105, 106] Both of these scenarios lead to low drug availability inside cancer cells, resulting in insufficient cytotoxicity and drug resistance. In addition, the transcription and translation of exogenous DNA or protein expression down-regulation by siRNA is completed only after 24-72 h, implying that the drugs and genes encapsulated in the co-delivery carriers should be sequentially released.[91] The optimal sequential release of drugs and genes will result in effective synergistic or additive therapeutic effects. However, the release kinetics of drugs and genes during co-delivery remains largely empirical at present and additional systematic studies should be performed to ensure that each therapeutic agent can sufficiently act at the target site in a timely manner.

Conclusions and Outlook

Compared with monotherapy, drug and gene combination therapy is undoubtedly more complex. However, combinatorial delivering therapeutic drugs and genes using synthetic carriers is a highly effective strategy for capitalizing on the favorable therapeutic effects of each agent and reducing chemotherapy toxicity in normal tissues. Therefore, this approach is expected to be an effective strategy for combating cancer in the near future. In this review, various multifunctional carriers for constructing co-delivery systems were examined and discussed. All of the discussed co-delivery carriers can simultaneously encapsulate therapeutic drugs and genes and target them to the desired cellular or tissue compartments with controlled release characteristics. Additionally, several issues such as loading capacity, stability, release kinetics and biocompatibility were also highlighted in this review. These issues are essential for achieving improved therapeutic efficacy and must be thoroughly studied to fully exploit the potential of the co-delivery approach in cancer therapy.

With regards to future trends in the design and synthesis of co-delivery systems, it is believed that detailed systematic investigations for understanding and optimizing the conditions for efficient loading as well as sequential release of the respective therapeutic agents will be performed. By increasing the understanding of the parameters that govern the success of the co-delivery strategy, rational and systematic improvements to drug and gene co-delivery systems will achieve improved therapeutic efficacy in the future.

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