Chapter 4
Application of Nano Drug Delivery Systems in Inhibition of Tumors and Cancer Stem Cells

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Abstract For its high mortality rate, cancer has posed a significant threat to human's lives. Every year, more than 3.4 million people died for cancer all over the world. The main therapeutic methods for cancer include surgery, chemotherapy, and radiotherapy. However, surgery is only conducted for patients with early-stage cancers; chemotherapy and radiotherapy have obvious side effects. In addition, many researches have indicated that cancer stem cells play a crucial role in tumor recurrence and multidrug resistance. Compared with traditional drug carriers, nano drug delivery systems have many advantages in targeting delivery, combination therapy, etc. In recent years, more and more nano drug systems are applied in clinical practice, and various multifunctional nano drug systems are designed to kill cancer stem cells. Our review introduced the main problems in anticancer therapy for cancer stem cells, and the developments of several nano drug delivery systems.

Keywords Cancer · Nano drug delivery systems · Cancer stem cells · Chemotherapy · Targeting therapy · Combination therapy

4.1 Introduction

Today, cancer has produced a great threat to human's lives for its highest mortality rate. Tumor cells tend to metastasize to healthy organs and then cause invasion and end in multiple organ failure to death. Every year, 3.4 million patients die of cancers all over the world [1]. The main treatments for cancers include surgery, chemotherapy, and radiotherapy. Nevertheless, surgery is conducted for patients with early-stage cancer, so metastasized tumors cannot take surgery. Chemotherapy and radiotherapy have toxic side effects, which usually cause serious damage to patients' immune and hematopoietic systems. The combination therapy becomes

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common treatment for malignant tumors. Different drugs unite to exert synergistic effects that can improve anticancer activity and reduce toxic side effect [2]. Nevertheless, drugs usually have different pharmacokinetic properties due to their physicochemical properties, resulting in different distribution ratios in vivo, so in the combination therapy seldom achieve the prospective results. In addition, many researches have reported that cancer stem cells play a crucial role in tumor recurrence and multidrug resistance.

In the past decades, nano drug delivery systems have a dramatic development, some of which has been used in clinical practice. Compared with traditional drug carriers, nano carriers show various advantages, such as better stability, targeting, biocompatibility, etc. In our review, we mainly summarized the advances of nano drug carriers in inhibiting cancer and its stem cells in recent years.

4.2 Cancer Stem Cell

Recent studies have indicated that cancer is difficult to cure because tumor tissues are made up by heterogeneous cell populations, in which there are not only rapidly proliferating tumor cells but also a little number of cancer stem cells (CSCs) with stem cell nature [3]. In 1997, Bonnet et al. [4] first proved the presence of CSCs in a patient with acute myeloid leukemia. Since then, a variety of CSCs has been successfully isolated and cultured [5–8]. The theory of CSCs believes that tumors are heterogeneous cell population. There is a part of cancer cells similar to embryonic stem cells, which can unlimitedly self-renew and divide and regulate the occurrence and development of tumors [5, 9, 10]. Conventional chemoradiotherapy mainly targets ordinary tumor cells, while CSCs are not sensitive to this. On the other hand, with the stimulation of radiotherapy and chemotherapy and the elimination of ordinary tumor cells, the microenvironment for CSCs changes. CSCs are enriched, and their capabilities of proliferation, invasion, and metastasis can be further enhanced. They may show more durable resistance to chemotherapy drugs. Some studies indicate that implanting a small number of CSCs into mice could reshape the phenotype of the tumors, which show that CSCs often lead to tumor recurrence, drug resistance, and metastasis [10, 11].

Just like other ordinary stem cells, CSCs highly express various stem genes, such as OCT4, NANOG, and SOX2, as well as stem signal pathways, such as Wnt/β-catenin, PI3K/Akt, NF-κB, PTEN, and JAK/STAT [12–14], to maintain the stemness of CSCs and regulate the development of tumors. The biological characteristics of CSCs determine that CSCs are different from ordinary tumor cells. CSCs are usually hidden in cancer cells and in a resting state. CSCs can weaken the effect of drug-induced DNA damage, enhance the ability to repair DNA damage, and maintain the stable genetic inheritance. These biological properties of CSCs are controlled by complex intracellular and extracellular regulatory networks.

4.2.1 Cell Cycle Arrest

CSCs are dormant and proliferate inactively in most cases. Many chemotherapeutic drugs tend to target dividing cancer cells. Ordinary tumor cells usually stay in the G2 or S phase, while CSCs tend to stay in the G0/G1 phase, so CSCs react insensitively to chemotherapeutic drugs. Due to the effect of radiotherapy and chemotherapy, ordinary tumor cells are eliminated, and CSCs can be enriched. At the same time, for the stimulating effect of chemotherapeutic drugs, stationary CSCs is quickly activated and enter the G2/S phase to proliferate and divide, resulting in tumor recurrence. Cioffi et al. [15] found that the expression of cyclin-dependent protein kinase inhibitor P21 and tumor suppressor P53 was increased in drug-resistant pancreatic tumors, while the expression of cyclin D1 was decreased, and cells were arrested to the G0/G1 stage. As a result, tumor cells were not sensitive to common chemotherapeutic drugs, and they often led to tumor recurrence. Acetaldehyde dehydrogenase 1 (ALDH1) is often considered as a sorting marker for CSCs. It can oxidize aldehydes to carboxylic acids, resist the damage of alkylating agents, and is highly expressed on the surface of CSCs. Meng et al. [16] found that the sensitivity of CSCs with positive ALDH1 expression was insensitive to chemotherapeutic drugs. ALDH1A regulated cell cycle by regulating KLF4 and P21, and CSCs could rest at the G0/G1 phase. In addition, glycogen synthase kinase-3 (GSK-3) promoted ubiquitination of β-catenin through Wnt/β-catenin signal pathway, blocked the activation of downstream target cyclins-1 and c-Myc, and inhibited the progress of cell cycle.

4.2.2 Drug Efflux

Chemotherapeutic drugs require specific drug concentration to exert their killing effect. Compared with ordinary tumor cells, the concentration of drug is much lower inside CSCs, which is related to high expression of multidrug resistance (MDR) on the surface of CSCs. The ABC transporter family is a type of MDR, namely, ATP-binding cassette protein. It can exert the energy released by ATP hydrolysis to exclude the therapeutic drug out of cells, resulting in low drug concentration inside cells, which finally leads to drug resistance and tumor recurrence. In addition, ABC transporters can also be used as tumor prognostic factors. ABC subfamily C member 2 (ABCC2), ABCC3, and ABCG2 are markers of CSCs, and their expression is related to the prognosis of patients with colon cancer. Because a drug can be excreted by multiple transporters, inhibiting a specific transporter alone cannot hinder the efflux of the drug. Wu et al. [17] found that the tyrosinase inhibitor, tepotinib, could inhibit ABCB1-mediated drug efflux, but could not block the efflux effect of ABCG2 and ABCC1.

4.2.3 DNA Damage Tolerance and DNA Damage Repair

Nowadays, parts of chemotherapeutic drugs kill tumor cells by inducing damage to tumor cell DNA. CSCs are not only in a resting state in most cases but also have a strong resistance to DNA damage and the ability to repair DNA damage. The main mechanism is that damaged DNA, on the one hand, can be removed by excision repair. On the other hand, CSCs with damaged DNA can enhance recombinational repair. Cisplatin inhibits DNA replication and transcription of tumor cells and induces tumor cells to apoptosis. Srivastava et al. [18] found that the highly expressed DNA polymerase Polη in ovarian CSCs could avoid the damage of cisplatin by skipping the damaged DNA replication point to promote DNA synthesis. In addition, DNA damage repair can be accomplished by enhancing the repair of DNA double-strand break. Gold et al. [19] found that the antitumor mechanism of spironolactone was inhibiting the repair of DNA double-strand break of tumor cells. Because spironolactone had no effect on normal CSCs, it only inhibited the DNA damage repair of CSCs where DNA double-strand break occurred. Spironolactone weakened the DNA damage tolerance of CSCs and inhibited the DNA damage repair. It could interfere with CSCs division, resulting in eliminating CSCs and inhibiting tumor recurrence and drug resistance.

4.2.4 Epithelial Mesenchymal Transformation (EMT)

EMT means that epithelial cells abandon the characteristics of epithelial cells and express the nature of interstitial cells and get the ability to invade and metastasize. EMT plays a crucial role in the generation of CSCs and is related to biological characteristics of CSCs and drug resistance. Currently, it is believed that parts of CSCs are generated by dedifferentiation of differentiated tumor cells. Some studies [20] have indicated that the upregulation of EMT transcription factors, such as snail and slug, could induce differentiated tumor cells to dedifferentiate into CSCs and produce chemical resistance. Shuang et al. [21] found that the expression of stem genes was enhanced in the tumor cells with EMT, indicating that the occurrence of EMT could dedifferentiate tumor cells into CSCs and obtain stem characteristics. Meanwhile, CSCs overexpress mesenchymal markers. Wang et al. [22] found that while pancreatic cancer cells highly expressed the stem markers, like CD44 and NANOG, the expression of EMT transcription factors, such as snail, also increased. Gao et al. [23] found that in liver CSCs, $CD44^+$ CSCs highly expressed mesenchymal cell markers, like vimentin and N-cadherin, but lowly expressed epithelial cell markers. Removal of $CD44^+$ CSCs could inhibit the ERK/Snail signaling pathway to weaken the metastasis of liver cancer cells. Many evidences show the direct connection between EMT and CSCs [24, 25]. EMT and CSCs can jointly promote tumor invasion and metastasis and regulate the occurrence and development of tumors. EMT is a dynamic and reversible process. Therefore, by directly targeting one or several EMT-related transcription factors, it is not possible to inhibit the occurrence of EMT.

4.2.5 Tumor Microenvironment

The drug resistance of CSCs is not only related to their stem characteristics but also regulated by tumor microenvironment. CSCs alone cannot survive and require the support of tumor microenvironment. Tumor microenvironment includes not only tumor cells themselves but also tumor-related stromal cells, microvessels, interstitial cells, and cytokines. Tumor microenvironment is dynamically changing and regulated by tumor development. Hypoxia, low pH, and low glucose supply of tumor microenvironment further maintain the stem characteristics of CSCs and improve drug resistance. CSCs in the dormant state can adapt to low energy supply in the microenvironment. Once the environment changes, the energy utilization mode of CSCs will change accordingly and enter the proliferation stage [26]. Therefore, tumor microenvironment of CSCs is an important factor in maintaining stem characteristics, driving tumor development, recurrence, and drug resistance. The same tumor has significant differences in gene expression and biological behavior in different tumor microenvironments, which may be related to the differences among tumor microenvironments.

4.3 Nano Drug Delivery Systems

According to clinicopathological and physiological studies, there are obvious differences in the structure of normal cells and tumor cells. Tumor tissues are characterized by poor vascular integrity due to the porous structure of capillaries (the pore size is 100–780 nm [27]). Furthermore, due to the collapse of lymphatic vessel wall and the loss of lymph circulation in tumor tissues, macromolecules and lipid particles cannot be absorbed back into blood through lymphatic system, so macromolecules and lipid particles are easily taken up and retained by tumor tissues, making them easily play the corresponding biological effect in tumor tissues [28]. This effect is named the enhanced permeability and retention effect (EPR) [29, 30]. Nano drug carriers make use of this effect of tumors and prepare drug delivery systems in the nano-size category. The passive targeting of nanomedicine is based on the EPR, which makes blood circulation time increased in specific tumor tissues, thereby enabling nano drugs to selectively concentrate in tumor tissue and perform better therapeutic effects [31]. Nano drug delivery systems can be roughly divided into liposomes, polymeric micelles, and other nanoparticles. Compared with traditional drugs, nano drug delivery systems have more advantages.

4.3.1 Strengthen Drug Stability

There are various enzymes in the human body, which can destroy and degrade drugs in the process of drug absorption. As a result, drugs are easily lost during blood circulation. Nano drug delivery systems can encapsulate drugs and provide protective effect by external physical barrier, which significantly strengthen the stability of drugs. In the process of implanting nano drug delivery systems into body, not only the loss of encapsulant in blood circulation is avoided, but also the dose dependence of traditional drugs can be changed, which directly improves drug efficacy.

4.3.2 Enhance Drug Targeting

Nano drug delivery systems can change the distribution of drugs to a certain extent. Nano drug carriers can enhance drug targeting and reduce toxicity by decreasing drug leakage to other healthy tissues [32]. During the design process of nano drug carriers, surficial materials can be assigned reasonably and modified according to specific physical and chemical properties, to change drug load, pharmacokinetics, and biocompatibility. At the same time, the targeting of nanoparticles to cells or molecules can be further enhanced, leading to the sustained release and better stability. The occurrence and development of tumors are very rapid. Tumors cannot form integral blood vessel wall; thus, a large number of pores will be formed. The diameter of these pores is nanometer-scale, so that nano drug delivery systems can reach the diseased tissues and organs.

4.3.3 Better Degradability

Due to their large specific surface, small particle size and strong adsorption capacity, nano drug carriers degrade more completely than traditional carriers. Nano carriers can increase the binding time of drugs to the affected part and further increase the absorption rate of drugs. According to pharmacokinetics, if drugs cannot be effectively degraded, there is a risk of toxic effects.

4.3.4 Increase Bioavailability of Drugs

Nano drug carriers can enhance the permeability of encapsulant to biofilms. Nano drug carriers enable drugs pass through blood-brain barrier or biofilm more efficiently, thereby improving the bioavailability of drugs. Many oral macromolecule drugs are hard to take effects for the first pass elimination. Nano drug carriers can

improve the solubility of macromolecular drugs that are difficultly absorbed by oral administration, resulting in higher concentrations of drugs in tumor tissues and better drug utilization and treatment effects.

4.4 Liposomes

Alec Bangham first built the hollow phospholipid structure, which laid the foundation for the liposomal model in 1965 [33, 34]. After that, many phospholipid bilayer structures were designed [35]. Gregory Gregoriadis put forward the idea that liposomes might perform well in drug delivery systems [36, 37]. A part of articles suggested that liposomes had influence on the distribution of encapsulant in vivo [38–40]. On the other hand, some researchers utilized liposomes to deliver the chemotherapeutic agent—cytosine arabinoside—and significantly increased the lifetime of mice bearing L-1210 leukemia [41, 42]. It was the first time for liposomes to enhance the activities of wrapped drugs. Other small molecular therapeutics entrapped in liposomes were also in the attempt and showed improved better effects for animal disease models [43–46].

Liposomes are hollow vesicles encased in the lipid bilayer, whose diameters range from nanometers to a few micrometers. Due to their good biocompatibility, easy modification, and specific targeting, liposomes have been generally applied in fields of drug carrier [47]. Phospholipid and cholesterol are the main compositions of liposomes. The most common phospholipid used in the liposome includes phosphatidylcholine, phosphatidylinositol, phosphatidylglycerol, phosphatidylserine, phosphatidylethanolamine, and phosphatidic acid. The cell membrane is also composed of phospholipid, so liposomes have good biocompatibility and low toxicity. Phospholipid is an amphiphilic molecule, which include nonpolar skeleton and polar head. According to phospholipid's charge, four categories of liposomes are classified, including uncharged liposomes, positively charged liposomes, negatively charged liposomes, and zwitterionic liposomes. The charge of liposomes significantly determines the liposomal property [48]. The most common liposomes are positively charged, and these liposomes tend to attract cell membranes based on electrostatic interaction, leading to an increase in cellular intake of carriers. In addition, the positively charged head assists to achieve lysosomal escape based on "proton sponge effect" and reduce the degradation of drugs in lysosomes. Positively charged liposomes are suitable carriers for nucleotide therapeutics, because DNA or RNA is also negatively charged [49]. Another main component for liposomes is cholesterol. About 30 percent composition of cell membrane is cholesterol, and it is usually neutrally charged. Cholesterol plays a key role in the properties of liposomes. The interaction between fatty acid chain of phospholipid and cholesterol contributes to maintaining the stability of liposomes [50, 51]. Furthermore, cholesterol can control the rigidity of bilayer structures [52] and condense phospholipid molecules to enhance the density [53]. It brings about a more ordered structure in the tail area, along with low polarity [54], increased bilayer viscosity, and enhanced

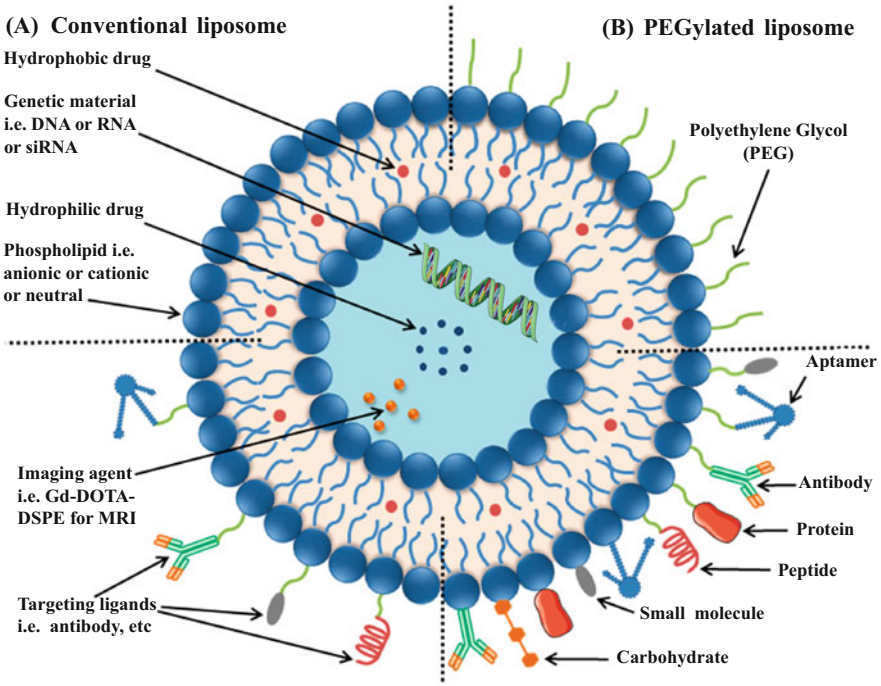

Fig. 4.1 The development of liposomes in different stages. (**a**) Convention liposomes; (**b**) PEGylated liposomes; (**c**) ligand-targeted liposomes modified with aptamer, antibody, protein, peptide, etc.; (**d**) multifunctional liposomes. Copied with permission [57]. Copyright 2018, International Journal of Molecular Sciences

membrane rigidity [55]. In addition, cholesterol is sure to affect liposome size, heighten permeability, and modulate the releasing of encased drug [56]. Finally, to overcome the disadvantages of liposomal carriers, various functional agents are applied to decorate on the surface of liposomes, such as antibodies, polyethylene glycols (PEGs), aptamers, ligands, proteins, peptides, and some other small molecules (Fig. 4.1).

Liposomal drug carriers can efficiently transport drugs to targeting tumor and heighten the accumulation of drugs, so they have great application potential and clinical value [58]. Liposomal preparations are usually administered by intravenous injection. When circulating in the vascular system, liposomes get nonselective adsorption with serum proteins and tend to be eliminated by macrophages, resulting in low targeting. Although lots of liposomes have shown positive results in vitro experiments, they hardly survive in the complex in vivo environment. To make liposomes more effective in vivo, the function of liposomes undergoes further optimization in various ways.

4.4.1 Overcoming the Quick Elimination by MPS

The conventional liposomes were found to get rapid elimination from circulation. As liposomes interact with negatively charged serum proteins, the mononuclear phagocyte system (MPS) works [59]. As a result, liposomes might accumulate in organs, mainly including spleens or livers, leading to the rapid decrease in the blood concentration of entrapped drugs [60, 61]. Except for the treatment of MPS diseases, the phenomenon significantly weakens the targeting of liposomes to their targeted tumors and produces toxicity to the MPS organs [62, 63].

Considering these problems, a large number of liposomes without entrapped drugs were injected into bodies ahead of time. This method was to shield the MPS and prolong the circulation time of the liposomes with drugs [64]. Juliano et al. [65] did some research on the blood circulation of the liposomes labeled with Tc. The results indicated that the radiation intensity of tumors in the group with MPS shielded was 1.5 times higher than that in the unshielded group; at the same time, tumors reflected two-time radioactivity of other tissues. Nevertheless, it was impossible to conduct this shielding method in clinical practice. Hence, modification of liposomal properties to increase the circulation time in vivo had become the focus of researches. Some early studies showed that reducing vesicle size, to a certain extent, could prolong the circulation half-lives in vivo [66]. A possible mechanism for the rapid elimination of liposomes was that those serum proteins had an influence on them and the surficial modifications of liposomes caused the increase in the circulation half-lives. In early studies, the focal point was on the differences between the unmodified phospholipid bilayers and the biological bilayers whose facial membranes were abundant in carbohydrates. The monosialoglyprotein GM1 was first added in the liposomes imparted by egg phosphatidylcholine (egg PC), leading to realizing the increased circulation time without MPS shielding [67]. It suggested less MPS intake and longer circulation half-lives of liposomes by substituting sphingomyelin for egg PC. People speculated that it might be for an increase in the facial hydrophilicity of these long-circulating liposomes composed of carbohydrates.

Some scholars found that the circulation half-lives of liposomes were increased when they added polyethylene glycol (PEG) as composition [68]. It was milestone progression for Maruyama et al. [69] and Cevc et al. [70] to modify PEG molecules on the surface of liposomes. The PEG could envelop liposomes and separate liposomes for serum protein. In this way, the PEGylated liposomes extremely weakened the quick elimination by MPS and made improvements in the circulation half-lives in vivo [71, 72]. Additionally, Ji et al. [73] indicated that the PEGylated neutral liposomes (NL) were more instable than cationic liposomes (CL) and anionic liposomes (AL) and NL loaded with DOX was inferior to CL and AL in antitumor activity.

However, the PEGylated liposomes are also faced with problems. First, if the PEGylated shell is not opportunely removed from tumor tissues, the uptake of the liposomes into the cancer cells may be inhibited, or it is hard to achieve lysosomal

escape [74]. In addition, Dams et al. [75] found that the PEGylated liposomes were observed to arouse accelerated blood clearance (the ABC phenomenon), which appeared at the first conduct of the PEGylated liposomes by intravenous injection. When injecting the PEGylated liposomes repeatedly at intervals, their pharmacokinetics should abnormally change. The occurrence of this phenomenon not only weakened the long-circulating advantages of the PEGylated liposomes but also caused serious damage to healthy organs or tissues.

4.4.2 Constructing Active Targeting Liposomes

The active targeting liposomes are constructed based on the interaction between ligands and receptors to realize specific active targeting. However, specific receptors have saturation effect, and these liposomes modified with ligands are weak in active targeting. In addition, complex microenvironment in tumor area tends to hinder them from approaching the targeting receptors [76, 77]. To solve these problems and promote the targeting efficacy, three methods are taken into consideration. First is to construct liposomes with dual-targeting molecules. Second is to utilize physical factors to realize active targeting. Third is to apply cell-penetrating peptide technology in liposomes.

To improve the targeting efficiency and accuracy, liposomes can be modified with two kinds of ligands [78]. More and more attentions come to dual-targeting liposomes. Li et al. [79] designed targeting liposomes modified with folate and transferrin for DOX delivery. Transferrin guided liposomes to penetrate through blood-brain barrier and then approached brain tumor. On the other hand, folate could also target the glioma cells and release the active pharmaceutical ingredients that made DOX effective. Furthermore, by inhibiting the ATP-binding cassette transporter, transferrin could decrease drug efflux and restrict drug resistance. The results indicated that compared with single-modified liposomes, dual-modified liposomes did better in active targeting without obvious DOX toxicity to heart.

Physical chemistry targeting liposomes mean application of some physicochemical methods to enable targeting agents effective in specific regions, such as pH-sensitive liposomes, photoactive liposomes [80]. By application of physical targeting technology in liposomes, liposomes could remain stable in complex microenvironment and accumulate in targeting tumor. Yu et al. [81] designed a novel liposome by modification with folate and the near-infrared imaging agent, naphthalocyanine green (IR780). The liposome featured with photoactivity and loaded DOX to kill cancer cells. The results indicated that the liposome significantly enhanced the targeting to liver tumor and the release of the entrapped drug could be under control. By diffusion to tumor tissues, the entrapped drug could extremely restrict the microcirculation of liver tumor.

Cell-penetrating peptides (CPPs) are segments of short positively charged peptides. By electrostatic interaction, CPPs can approach cell membrane and assist drugs enter cells without toxic effects. CPPs are lack of cell selectivity, and blood

enzymes tend to degrade CPPs in blood circulation. The application of CPPs in liposomes is expected to enhance the permeability of liposomes and promote the drug accumulation in tumor area [82].

Plenty of experiments have been conducted to make out what advantages the active targeting liposomes possess compared with the passive targeting ones and which would have the practical applying value. Several articles reported the improvements of active targeting liposomes in survival periods compared to passive targeting ones [83, 84], while no improvements in other cases [85, 86]. The passive and active targeting liposomes approached the target tissues in the same distribution method. Therefore, if they have similar circulation half-lives in vivo, active targeting liposomes will have no advantages in distributing to tumor tissues [87, 88]. More liposomes absorbed by target cells rather than target tissues seem to achieve improvements in the survival period. If entrapped agent releases ahead of intake, the anticancer effect will be hard to improve.

4.4.3 Realizing Triggered Release of Drug

Conventional liposomes release entrapped drugs by passive diffusion. Although the modification of PEG on the surface of liposomes contributes to more circulation time and higher targeting efficiency, more circulation time means liposomes have more opportunities to gradually release the active pharmaceutical ingredients ahead of time. Moreover, drug release is also influenced by serum proteins [89, 90]. Cholesterol is benefit to improve the stability of bilayers and reduced drug leakage from liposomes [91, 92]. It was also helpful to reduce the release of drugs in advance when the liposome membrane switched from the liquid phase to the solid phase [93]. The more stable bilayer exactly decreased the drug release ahead of time, but this might reduce the efficiency of passive diffusion in targeting area, which would lead to drug resistance.

To solve these problems, scholars are trying to construct novel liposomes to realize the triggered release of drug. Two triggers are mostly utilized—local triggers (such as enzymes and pH changes) and remote triggers (such as light, ultrasound, and heat).

Mangy attentions come to tumor hyperthermia in recent years. Hyperthermia is a feasible treatment for terminal cancers or tiny tumors. Compared with operation, hyperthermia takes less expenditure and seldom damages adjacent tissues. The heating source mainly includes ultrasound, laser, microwave, and radio. The heating source could raise temperature up to 50 °C. The active enzymes could be denatured, and transient cytotoxicity could be produced at such temperature [94]. At the same time, when thermosensitive materials applied, heat is expected to trigger the drug release. Chen et al. [95, 96] constructed a thermosensitive liposome by addition of ammonium bicarbonate. When the temperature raised to 42 °C, ammonium bicarbonate tended to degrade and produce bubbles, leading to the crash of bilayer structure and the release of entrapped drug.

Under light irradiation, some photosensitizer can release singlet oxygen and damage cancer cells, which is termed as photodynamic therapy. Skin cancer, oral cancer, and cervical cancer are expected to be cured under photodynamic therapy [97]. Moreover, light irradiation is able to trigger the drug release. There are some unsaturated bonds in the structure of liposomal bilayer, and singlet oxygen can break these unsaturated bonds, leading to the destruction of hydrophobic chains and the triggered release of drug [98].

Another method for triggered release is using enzyme-responsive liposomes. Some enzymes are abundant in tumor tissues, such as secreted phospholipase A2 (sPLA2), matrix metalloproteinases (MMPs), prostate-specific antigen (PSA), urokinase plasminogen activator (uPA), and elastase. These enzymes are expected to be triggers for drug release [99]. Li et al. [100] reported a MMP-2 reactive liposome with β-cyclodextrin modified. When approaching tumor area, under the function of MMP-2, the liposome tended to separate into two parts. One part was β-cyclodextrin, an anti-fibrotic drug; the other part was the liposome with RGD modified. The liposome showed stronger lethal effect to pancreatic cancer.

The release rate of drug plays a role in liposome function. If entrapped drug cannot release from carriers, carriers have no value. In addition, the release mode and rate should be taken into consideration, too.

4.4.4 Constructing Multifunctional Liposomes

In order to kill cancer cells, different chemotherapeutics tend to inhibit different signaling pathways. Nevertheless, one chemotherapeutic conducted repeatedly might cause drug resistance [101]. In addition, therapeutic effect of one drug is hard to achieve expected results. The common method to make up for it is increasing the dose, but the following toxicity is also a problem. Hence, to solve the problems of one-drug treatment, doctors tend to conduct combination therapy. Combination therapy is uniting two or more chemotherapeutics with complementary effects, which is expected to minimize side effects and inhibit drug resistance [102].

The uniting of several chemotherapeutics does benefit to therapeutic effect. Liposomes can load chemotherapeutics with similar function, resulting in less drug dose and improved anticancer effect. DOX is an anthracycline antibiotic and able to treat multiple cancers, such as lymphoma, lymphoma, and breast cancer [103]. DOX mainly takes function by destroying chromosome [104]. First, utilizing electrostatic action, DOX is able to intervene into DNA double helix. Next, DOX can inhibit the DNA-topoisomerase II and stop DNA double helix to rewind, leading to the end of the cell replication process. Third, DOX leads cells to apoptosis. Cisplatin is a conventional alkylating agent. Cisplatin leads to DNA break and cell apoptosis by terminating DNA synthesis or transcription [105]. Ramasamy et al. [106] reported a transferrin-modified liposome to deliver cisplatin and DOX. The results indicated that the liposome entrapping cisplatin and DOX got better anticancer effect than complex of cisplatin and DOX, let alone other one-drug formulations. Salinomycin

(SAL) is a carboxylic acid polyether antibiotic that can effectively inhibit the growth of tumor stem cells, but its poor water solubility limits its application. The combination of SAL and other chemotherapeutics has dramatically improved the therapeutic effect on tumors. Gong et al. [107] prepared three liposomes respectively loaded with SAL, DOX, SAL, and DOX (SLN, DLN, SDLN). The results indicated that SDLN could more effectively inhibit the growth of lung cancer than SLN and DLN in vivo and the number of CSCs in the tumor site was significantly reduced. They also found that SDLN had the best synergistic effect when the drug ratio SAL/DOX was 1:1. Therefore, exploring the optimal ratio of two drugs is a key issue in the preparation of dual drug-loaded nanoparticles.

Liposomes can deliver chemotherapeutics with different functions to take synergistic effect. Paclitaxel (PTX) is a natural extract and widely applied to treat multiple cancers, such as breast cancer, ovarian cancer, gastric cancer [108], non-small cell lung cancer and Kaposi's sarcoma [109]. This agent functions uniquely. At cell mitotic phase, PTX can lead tubulin proteins to polymerize and make the microtubules dysfunctional, eventually resulting in cell death. However, the anticancer effect of PTX is weakened for its low water solubility. Scientists tried to deliver PTX with liposomes, which significantly increased the water solubility of PTX and reduced side effects, including vomiting, nausea, and hypersensitivity reactions, compared to free PTX [108]. What's more, Fang et al. [110] designed a multilamellar liposome to deliver PTX and DOX. These two drugs function based on different mechanisms. The results suggested that after the treatment with dual agent liposomes, the survival period of tumor-bearing mice was much longer than that only treated with single drugs.

Gene transfection technology is introducing normal genes or genes with therapeutic effects into relative cells to cure diseases aroused by gene disorder. This type of treatment is called gene therapy. Scholars found that genetic changes played a crucial role in the development of tumors. The expression of miRNA and siRNA in tumor cells, especially in CSCs, is often abnormal, which affects the self-renewal and reproduction of cells. Therefore, miRNA or siRNA can be used as a therapeutic to inhibit cancer cells [111]. In addition, tumor suppressor genes can suppress tumor cells and CSCs by regulating the expression of specific enzymes. However, nucleic acid drugs have many disadvantages, such as low cell uptake, poor stability, and bad tissue specificity. Liposomes can effectively deliver nucleic acid drugs for their specific properties. MiRNA200c is significantly downregulated in breast CSCs, and increasing its content can restore the sensitivity of PTX. Liu et al. [112] selected CL to transport miRNA200c by charge attraction. After breast CSCs ingested the therapeutic for 12 h, miRNA200c could still be effectively released from the liposome, and breast CSCs became more sensitive to PTX. Kim et al. [113] designed a liposome to deliver small-molecule drug and modified a single-chain antibody against transferrin receptor on its surface. Using this nanocarrier to load wtp53, it was able to pass through the blood-brain barrier, downregulate methylguanine methyltransferase (MGMT) by wtp53, and weaken drug resistance of malignant glioma cells and CSCs to temozolomide (TMZ).

Nowadays, multidrug resistance has become the main obstacle to the chemotherapy of tumors. Nowadays, varieties of anticancer agents are combined to overcome drug resistance by nano delivery systems. The agents should be chemotherapy sensitizers or inhibit tumor cells from agent efflux. Cyclosporine A and verapamil are included [114]. However, these inhibitors of efflux pump have toxicity on healthy organs or tissues. Calcium channel inhibitors, such as verapamil, might induce hypertension, dizziness, and arrhythmia, while cyclosporine A often leads to immunosuppression, nephrotoxicity, and leukopenia [115–117]. These problems extremely restricted the application of efflux pump inhibitors. Resveratrol, a natural extract, attracts more focus on its functions in recent years. Some studies indicated that resveratrol took effects on anti-inflammatory, anti-aging, anti-oxidation, and reducing blood sugar [118]. Resveratrol was also reported to suppress tumor proliferation and induce cell apoptosis [119]. Guo et al. [120] constructed a PEGylated liposome to deliver resveratrol and PTX. The results were positive; the nano delivery system could effectively kill breast cancer cells and significantly restrict the development of tumors in mouse model without obvious toxicity.

4.5 Polymeric Micelles

In 1992, Yokoyama et al. [121] first reported polymeric micelles as nano drug delivery systems after the research of DOX-conjugated block copolymer micelles. Polymeric micelles have special core-shell structures that are self-assembled by amphiphilic polymers in aqueous phase. The particle size of polymeric micelles is generally less than 200 nm, and they have different shapes, such as balls, bars, and tubes, among which the balls are the most common ones. The hydrophilic shell of polymeric micelles can maintain their spatial stability and long circulation. The hydrophobic core can encapsulate hydrophobic drugs to increase water solubility of drugs. Like other nano drug delivery systems, polymeric micelles can approach tumors through the EPR. In addition, modification of ligands or antigens on the surface of polymeric micelles can achieve active targeting.

The chemical properties and molecular weight of the hydrophilic shell significantly affect the stability, pharmacokinetic, and tissue accumulation of polymeric micelles. The most common hydrophilic shell polymers are polyethylene glycol (PEG), polyethylene oxide (PEO), polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), and chitosan. The hydrophilic shell sometimes is made up of a mixture of various polymers. These hydrophilic polymers give stealth capabilities to polymeric micelles, allowing them to avoid absorption by the reticuloendothelial system (RES), which is significant to achieve long circulation time in vivo. PEG is nontoxic, non-immunogenic polymer and has good water solubility. PEG can effectively avoid the interaction with immunoglobulin, prevent polymeric micelles from uptake by phagocytes, and increase the circulation time in vivo [122]. N-succinyl chitosan is a derivative of chitosan. Compared with chitosan, N-succinyl chitosan is more

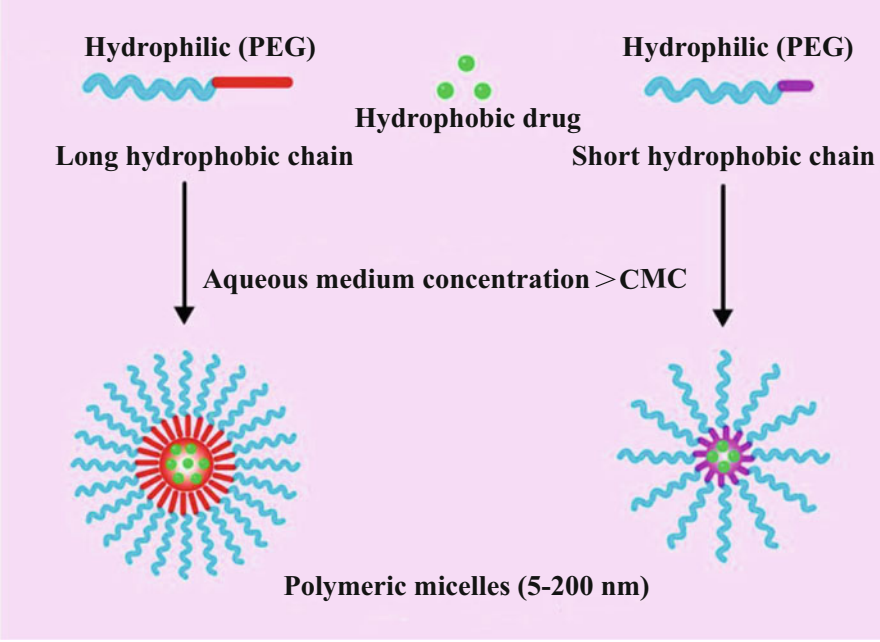

Fig. 4.2 General structure of polymeric micelles loaded with hydrophobic drug. Copied with permission [127]. Copyright 2019, Frontiers in Pharmacology

biocompatible and less toxic, has a longer half-life period, and can be effectively enriched in tumor sites. Thus, it is widely used in antitumor carries [123].

The hydrophobic core of polymeric micelles can be loaded with poorly soluble drugs to improve drug stability and bioavailability. The properties of hydrophobic core directly affect the stability, drug loading capacity, and drug release performance of polymeric micelles. Commonly used hydrophobic core polymers are polyesters and polyamino acids. Polyesters are easy to hydrolyze and have low toxicity and good biocompatibility. Polyesters mainly include polylactic acid (PLA), polycaprolactone (PCL), polyglycolic acid (PGA), and polylactide-co-glycolic acid (PLGA). Polyamino acids can be used as core fragments. They are easy to modify chemically and encapsulate drugs in physical or chemical methods. Polyamino acids mainly include poly-L-aspartic acid (PAsp), poly-L-histidine (PHis), poly-L-glutamic acid, and their derivatives. In addition, poly-β-amino ester (PbAE) and some short-chain hydrophobic lipids, such as distearoylphosphatidylethanolamine (DSPE), have also been widely used as hydrophobic core polymers in recent years [124, 125]. Moreover, if the hydrophobic core is more similar to the chemical structure of drugs, the solubilization effect of the polymeric micelles on drugs will be better, and the solubility of drugs will be higher. Yokoyama et al. [126] substituted the benzyloxy group in the hydrophobic core of PEG-PBLA micelles with hexadecyl esters. As a result, the solubility of the aliphatic anticancer drug KRN-5500 was significantly increased (Fig. 4.2).

Polymeric micelles have good thermodynamic and kinetic stability. The thermodynamic stability of polymeric micelles is affected by a variety of factors, such as the interaction between the shell polymers, the interaction between the hydrophobic drugs and the core, the length of the hydrophobic block copolymers, the hydrophobicity of the core, the length and density of the hydrophilic block copolymers, and the liquid environment [128]. Therefore, in order to ensure the good thermodynamic stability of polymeric micelles, the length of the hydrophobic and hydrophilic blocks and the surface density of the hydrophilic blocks need to be balanced. The CAC value is an essential indicator for measuring the thermodynamic stability of polymeric micelles. When the copolymer concentration is above CAC, the micelles can be stable at the thermodynamic level. The CAC value of polymeric micelles is usually in the range of 10^{-7} to 10^{-6} mol L^{-1}, which is lower than that of micelles formed with low-molecular-weight surfactants (10^{-4} to 10^{-3} mol L^{-1}) [125]. Therefore, polymeric micelles can maintain structural integrity after a series of dilutions and have good thermodynamic stability. When the concentration of the copolymers drops below the CAC value, the kinetic stability of polymeric micelles becomes more important. The kinetic stability of micelles is mainly related to the structure of the micelle core, the size of the hydrophobic blocks, and the ratio of the hydrophobic blocks to the hydrophilic blocks. Unlike micelles formed by low-molecular-weight surfactants, polymeric micelles have a more stable hydrophobic core structure and better kinetic stability. Therefore, when below the CAC value, the decomposition rate of polymeric micelles will slow down, which can maintain the structural integrity of polymeric micelles before reaching targeting site, prevent drug leakage, and effectively improve drug bioavailability.

Compared with traditional drug delivery carriers, polymeric micelles have many potential advantages, such as broader drug delivery range, stronger delivery capacity, longer circulation time, fewer side effects, and better antitumor efficacy, so they have been widely used in anticancer therapy.

4.5.1 Passive Targeting and Active Targeting Polymeric Micelles

Polymeric micelles can deliver drugs to tumor tissues through the EPR, enabling passive targeting of nanocarriers. Theoretically, the circulation time of polymeric micelles in vivo is one of the most critical factors of the distribution of micelles in tumor tissues. Therefore, the micelles with long circulation time can take more substantial EPR effect, and the accumulation of micelles in tumor tissue can be more. However, because the EPR of different tumors is significantly different, the EPR alone to deliver polymeric micelles to tumor tissue is not ideal [129]. The tumor-specific active targeting nanocarriers mainly utilize specific molecules expressed on the surface of tumor cells or rely on the tumor microenvironment to load the modification of target molecules to achieve active targeting.

One method is the application of monoclonal antibodies that specifically bind to tumor antigens. As an ideal tumor antigen, monoclonal antibodies are expressed on the surface of tumor cells and necessary for tumor cells to survive. In addition, they are not prone to mutation [130]. Monoclonal antibodies can be independently used as targeting carriers for antitumor drugs or can be coupled to drug delivery systems or form complexes with drugs. Torchilin et al. [131] bound anti-myosin antibodies to polyethylene glycol-phosphatidylethanolamine (PEG-PE) polymeric micelles in order to target lung cancer cells. Comparing the PEG-PE micelles, the drug-loaded micelles, and the antibody-modified micelles, it was found that all three micelles were stable in vivo. However, the active targeting of the monoclonal antibody-modified micelles was significantly higher than others. The antibody-modified micelles could effectively deliver drugs to early tumors, mature vascular tumors, and metastatic lung tumors.

Another method is receptor-mediated targeting. Receptors on the surface of tumor tissues or cells are closely related to the growth and proliferation of tumors, and some are specifically overexpressed. The receptors can induce internalization of tumor cells after binding to the corresponding ligand, which helps to kill tumor cells. These receptors can be used as specific targets for tumor targeting therapies. At present, the common receptor types are cytokine receptors, transferrin receptors, low-density lipoprotein receptors, hormone receptors, and folate receptors [132]. It is worth noting that folate is a small class of nonimmune molecules, which is nonirritating to body and binds explicitly to folate receptors. Many studies have confirmed that folate receptors are overexpressed on the surface of various cancer cells, including breast cancer, ovarian cancer, brain cancer, kidney cancer, lung cancer, and bone cancer cells, which is not expressed on normal cells [133]. Yoo et al. [134] first designed a PLGA-b-PEG copolymer and then connected DOX to the PLGA end to form a DOX-PLGA-PEG complex; on the other hand, they bound folate to the PEG end. The results showed that folate-modified polymer micelles were more toxic than free DOX and in vivo experiments also confirmed that folate-modified polymer micelles could deliver more micelles to tumor tissues. Abou-ElNaga et al. [135] connected folate to the surface of PTX-loaded PLGA micelle, which could greatly increase the sensitivity of PTX to ovarian CSCs in vivo and reduce the expression of drug resistance genes ABCG2 and MDR1.

The third method is tumor-activated drug. These drug delivery systems rely on inactive complexes to interact with tumor microenvironment or specific molecules on the cell surface, thereby activating the complexes and releasing drugs. These drug carriers can increase drug concentration in tumor tissues and kill tumor cells more effectively.

4.5.2 Drug Co-delivery Systems

The combination of two or more different therapies may produce synergistic effects, which is a promising strategy. The combination therapy can improve therapeutic

effect, reduce side effect, and even reach multiple targets at the same time. Physical encapsulation, chemical linking, or both of them are usually applied in polymeric micelles serving as drug co-delivery systems.

Ke et al. [136] utilized a PEG-PUC/PEG-PAC mixed micelle system to physically encapsulate thioridazine (THZ) and DOX for inhibiting tumor cells and CSCs. The results indicated that the system has a higher loading capacity for THZ and DOX. Compared with mixture of free DOX and THZ, mixed polymeric micelles have stronger antitumor activity in vivo. Li et al. [137] used PLG-PLGA polymeric micelles to deliver SAL and docetaxel (DTX). The micelle could effectively kill gastric tumor cells and gastric CSCs, and its tumor suppressive effect in vivo was stronger than single nanocarrier and dual drugs.

The microenvironment of tumor is different from normal tissues. Because cancer cells need to synthesize fatty acids, nucleic acids, and amino acids continuously, the energy requirements are much higher. Therefore, inhibiting the energy metabolism pathway can also effectively inhibit tumor growth, making cancer cells more prone to apoptosis [138]. Krishannamurthy et al. [139] selected PEG-PUC and PEG-PAC copolymers to self-assemble into polymeric micelles. The inhibitors of energy metabolism pathways, phenformin (Phen) and gemcitabine (Gem), were loaded in the micelle through hydrogen bonds and ionic interactions. The results showed that in in vitro experiments, the combination of two drugs was more toxic to lung cancer cells and lung CSCs than a single one, which significantly inhibited tumor growth. At the same time, the micelle did not cause liver and kidney toxicity and had good biological safety.

Nowadays, the combination of drugs and genes has become a promising antitumor therapy, which has the advantages of overcoming drug resistance and improving gene transfection efficiency [140]. Polymeric micelles can form PIC micelles through electrostatic interaction with negatively charged genes. The surface charge on PIC micelles enables it to be modified by molecules with opposite charges, which provides a new method to construct multifunctional carriers. Zheng et al. [141] prepared PEG-PLL-PLLeu copolymers for co-delivery of DTX and siRNA. DTX is physically embedded in the hydrophobic core of PLLeu, and siRNA is electrostatically adsorbed to the carrier by PLL. Compared with single DTX or siRNA micelle system, it had better tumor suppressive effects.

4.5.3 Environmentally Responsive Polymeric Micelles

Environmentally responsive polymeric micelles have become research hotspots, which mainly include pH-responsive polymeric micelles and thermoresponsive polymeric micelles.

There is a pH gradient in human body. The pH of physiological environment in vivo is 7.4, while the pH of endosomes is 5.5–6.0 and that of lysosome is 4.5–5.0. The pH of normal tissues and organs is higher than that of tumor tissues. Polymeric micelles can be got uptake by target cells through receptor-mediated endocytosis.

After entering cells, polymeric micelles are enriched in endosomes and then enter lysosomes. Therefore, the pH gradient between normal tissues and tumor cells can be utilized to synthesize drug carriers, which greatly increase the bioavailability of drugs and achieve targeting delivery. PH-responsive polymeric micelles usually have pH-sensitive bonds such as amidine bonds, amino groups, acetals, or ketals. Osada et al. [140] reported a pH-responsive polycarbonate micelle for the controlled release of PTX. The pH-responsive micelle consisted of a PEG shell and a polycarbonate core containing acetal. The research indicated that the micelle was very stable at pH 7.4, but the acetal in the hydrophobic core could rapidly hydrolyze at pH 5.0, making the micellar swell, resulting in releasing PTX rapidly. Staurosporine (STS) is a common protein kinase inhibitor that can effectively kill CSCs. The combination of STS and other chemotherapeutic drugs can synergistically inhibit the growth of tumors. PEG-β-PAsp and epirubicin (Epi) are connected by a hydrazone bond to form a drug delivery polymeric micelle (Epi/m), and the hydrazone bond can be broken in acidic environment, leading to drug release. Kinoh et al. [142] encapsulated STS in Epi/m to form dual-drug delivery micelles (STS/Epi/m). After the micelle entered tumor cells, they were triggered by the acidic environment and simultaneously released two drugs, STS and Epi, which effectively inhibited the growth of CSCs. At the same time, by inhibiting the ABC transporter, the drug resistance of CSCs was weakened, and CSCs resistant to Epi were effectively eliminated.

Generally, the temperature of normal tissues is lower than that of tumor tissues. According to this characteristic, thermoresponsive polymeric micelles can be used as drug targeting deliver. When temperature changes, thermoresponsive micelles will undergo a phase transition from dissolution to insolubility. Typical thermoresponsive polymers include poly-N-isopropylacrylamide (PNIPAM), polypropylene oxide (PPO), etc. Peng et al. [143] successfully prepared thermoresponsive polymeric micelles, poly-NIPA-co-DMAEMA, through the radical polymerization. Under local heating, the micelles could slowly release drugs and significantly inhibit the growth of C26-derived colon cancer cells.

4.6 Conclusion

As a new type of anticancer drugs, nano drug delivery systems have excellent advantages in increasing drug targeting, improving drug bioavailability, and enhancing drug stability. CSC theory believes that CSCs are the root cause of tumorigenesis, drug resistance, and postoperative recurrence. Therefore, the eradication of CSCs is of great significance for cancer treatment. The nano drug carriers which target CSCs have broad prospects in tumor treatment, but their clinical application still faces many problems.

First of all, CSCs and normal stem cells share many signal pathways and surface markers. Therefore, the process of targeting CSCs may cause damage to normal stem cells. Second, CSCs are heterogeneous too. CSCs with different sources have

different surface markers. The heterogeneity of CSCs limits the efficiency of targeting CSCs in tumor treatment. Third, the binding efficiency of nano drug delivery systems and targeting molecules needs to be studied further, so as their stability after entering the systemic circulation. Last but not the least, the toxicity of nano drug carriers which target CSCs needs to be observed and explored for a long time. With the development of CSCs research and nano drug delivery systems, the combination of the two will be closer in the future and will provide a strong guarantee for cancer treatment.

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