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RESEARCH HIGHLIGHT

Multifunctional polymer vesicles for cancer stem cells-targeted drug/siRNA therapy

Qiuming Liu[1,2], Jianzhong Du[1,2]

[1]*Shanghai Tenth People's Hospital, Tongji University School of Medicine, 301 Middle Yanchang Road, Shanghai 200072, China*
[2]*Department of Polymeric Materials, School of Materials Science and Engineering, Tongji University, 4800 Caoan Road, Shanghai 201804, China*

Correspondence: Jianzhong Du
E-mail: jzdu@tongji.edu.cn
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Cancer stem cells (CSCs) are responsible for origin, growth, recurrence, and metastasis of tumor, and are closely linked to the failure of chemotherapy due to their self-renewal and multilineage differentiation capability with an innate resistance to cytotoxic agents. We have recently reported a novel EpCAM (epithelial cell adhesion molecule)-monoclonal-antibody-labeled CSCs-targeting, noncytotoxic and pH-sensitive block copolymer vesicle as a nanocarrier of anticancer drug and siRNA (to overcome CSCs drug resistance by silencing the expression of oncogenes). This vesicle shows high delivery efficacy of both doxorubicin hydrochloride (DOX·HCl) and siRNA to the CSCs. Furthermore, the DOX or siRNA loaded CSCs-targeting vesicles exhibited much better CSCs killing and tumor growth inhibition capabilities with much lower toxicity to normal cells ($IC_{50,DOX}$ decreased by 80%) compared with non-CSCs-targeting vesicles. Overall, these vesicles have significant implications for overcoming CSCs chemo-resistance in cancer chemotherapy.

Keywords: Cancer stem cell; polymer vesicle; EpCAM; drug/siRNA delivery

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Cancer is one of the leading causes of morbidity and mortality worldwide. Cancer arised from the uncontrolled growth and division of cells is caused by the cooperation of mutations in DNA that activate genes that push cell division, and suppress natural anticancer mechanisms [1]. Current anticancer therapies are primarily based on the inhibition of cancer cell growth, killing of cancer cells, or a combination of both. Usually, initial treatments appear to be successful, but the disease re-occurs at a later date [2-4].

Many cancers, including hematopoietic and solid tumors, may be driven by a small subpopulation of cancer stem cells (CSCs). CSCs have the potential for self-renewal and multi-lineage differentiation, and are subsequently identified in various solid tumors and confirmed to play a critical role in the tumor occurrence, deterioration, metastasis, and recurrence [5-10]. This potential is the primary reason for us to deal with CSCs by chemotherapy to cure cancer, and to inhibit the tumor growth and metastasis.

The surface antigen epithelial cell adhesion molecule (EpCAM, CD326) is a glycoprotein of ~40 kd. It is one of the most important surface specific markers of CSCs for a variety of tumors, attributable to its high expression on

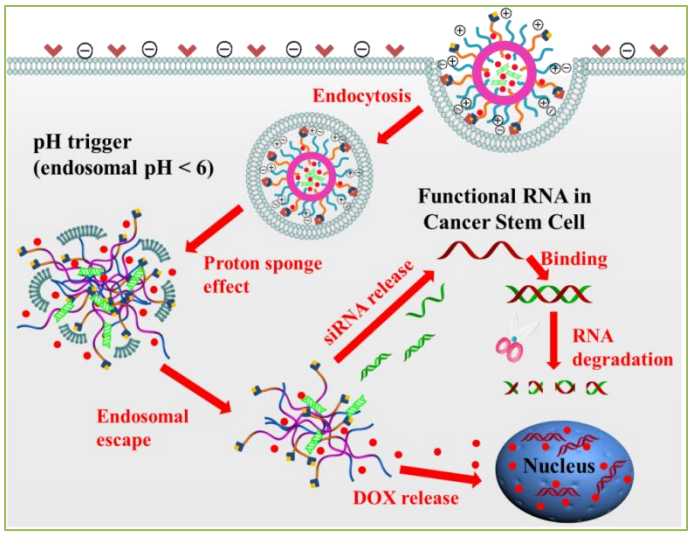

Figure 1. Schematic illustration for the synthesis of DOX·HCl and siRNA loaded polymer vesicles for efficient cancer stem cells-targeted intracellular drug and RNA delivery. Blue: biocompatible and hydrophilic PEO. Purple: pH-sensitive PDPA (hydrophilic when protonated at lower pH while hydrophobic when deprotonated at higher pH). Orange: pH-sensitive PAA (hydrophilic when deprotonated at higher pH). Red: hydrophilic anticancer drug DOX in its HCl salt form. Green: Fluorescein isothiocyanate (FITC)-siRNA-targeting functional RNA in cancer stem cells. Mazarine blue: streptavidin. Yellow: biotin-labeled anti-EpCAM monoclonal antibodies. Brown: The surface specific antigen of cancer stem cells, epithelial cell adhesion molecule (EpCAM). Black: positive charges on the surface of the vesicle and negative charges on the cell surface. Reprinted with permission [12].

rapidly proliferating tumors [11]. In early studies, EpCAM was proposed to be a cell-cell adhesion molecule. However, our and other research revealed that the EpCAM is not limited to cell adhesion but includes diverse processes such as signaling, cell migration, proliferation, differentiation and cell targeting [12]. Thus, EpCAM is a candidate protein for tumor diagnosis and therapy. It has been used for tumor therapy, involving monoclonal and bi-/tri-specific antibodies, vaccination strategies, toxin-conjugated antibody fragments, and an antibody fragment-targeted sTRAIL fusion protein [13-15].

EpCAM positive (EpCAM+) cells can be isolated from cancer cell lines, which have the ability to self-renew, to initiate tumor formation, and are intrinsically resistant to therapy. These specific characteristics of the EpCAM+ cells increased the difficulties of screening sensitive drugs to kill CSCs due to the multiple cell signal transduction pathways [16, 17]. Fortunately, small interfering RNA (siRNA) has been explored to overcome the drug resistance of cancer cells by silencing the expression of genes in signal transduction pathways [18, 19]. Nevertheless, the successful siRNA therapy needs efficient, stable, easy making, and low cytotoxic siRNA delivery carriers.

Nanoscale carriers with targeting units are promising for drug/siRNA delivery in modern pharmaceutics. The nanocarriers, such as polymer vesicles reported in our work [12, 20-23], will help the drug/siRNA to prevent non-specific distributing. Furthermore, they are inactivated and cleared shortly after administration before reaching action sites, leading to less toxicity to healthy organs. Recent advances in nanomedicine for cancer therapy support the idea that, instead of merely targeting the bulk non-CSCs, successful cancer curing requires efficient elimination of both undifferentiated CSCs and differentiated non-CSCs. Fortunately, CSCs targeting drug/siRNA delivery systems such as polymer vesicles present a promising strategy in cancer treatment.

We recently reported a novel noncytotoxic and pH-sensitive polymer vesicle for targeting EpCAM positive CSCs and for efficient intracellular drug/siRNA delivery (**Figure 1**) [12]. The vesicle is self-assembled from a pH-responsive triblock copolymer, poly(ethylene oxide)-*block*-poly[2-(diisopropylamino)ethyl methacrylate]-*block*-poly(acrylic acid) (PEO_{43}-*b*-$PDPA_{76}$-*b*-PAA_{17}). The biocompatible PEO is designed as the mixed coronas with hydrophilic PAA chains, while the pH-sensitive PDPA chains form the membrane of the vesicle. The PAA chains in the outer coronas were decorated with Streptavidin (SA), and then be bound with biotin-labeled anti-EpCAM monoclonal antibodies, leading to a specific recognition between vesicles and CSCs. This kind of EpCAM-Ab-labeled pH-sensitive polymer vesicles with positive charges could lead to a significant improvement in the loading and delivery of DOX·HCl and siRNA. Furthermore, the polymer vesicle can be internalized via endocytosis, thereby initiating the protonation of PDPA membrane to trigger the efficient and rapid release of the encapsulated DOX·HCl and siRNA in CSCs. Finally, the siRNA bound functional RNA will be degraded by exonuclease, whereas the DNA in the nucleus of the CSCs will be damaged by DOX·HCl, leading to better treatment of tumors.

DOX is a water-soluble anticancer drug in its hydrochloride salt form [21, 24, 25]. The drug encapsulation and intracellular release behaviors of DOX-loaded PEO_{43}-*b*-$PDPA_{76}$-*b*-PAA_{17} vesicles showed high delivery efficacy, high endosomal escape ability and excellent CSCs killing capability. Therefore, pH-regulated drug release, intracellular efficiency and cell killing efficiency of DOX-loaded vesicles with and without EpCAM labeling have been identified in normal liver L02 cells and EpCAM+ cancer stem cells.

Micro-RNAs (miRNAs), an abundant class of naturally occurring and small noncoding RNAs (about 21–25 nucleotides in length), have been identified as tumor

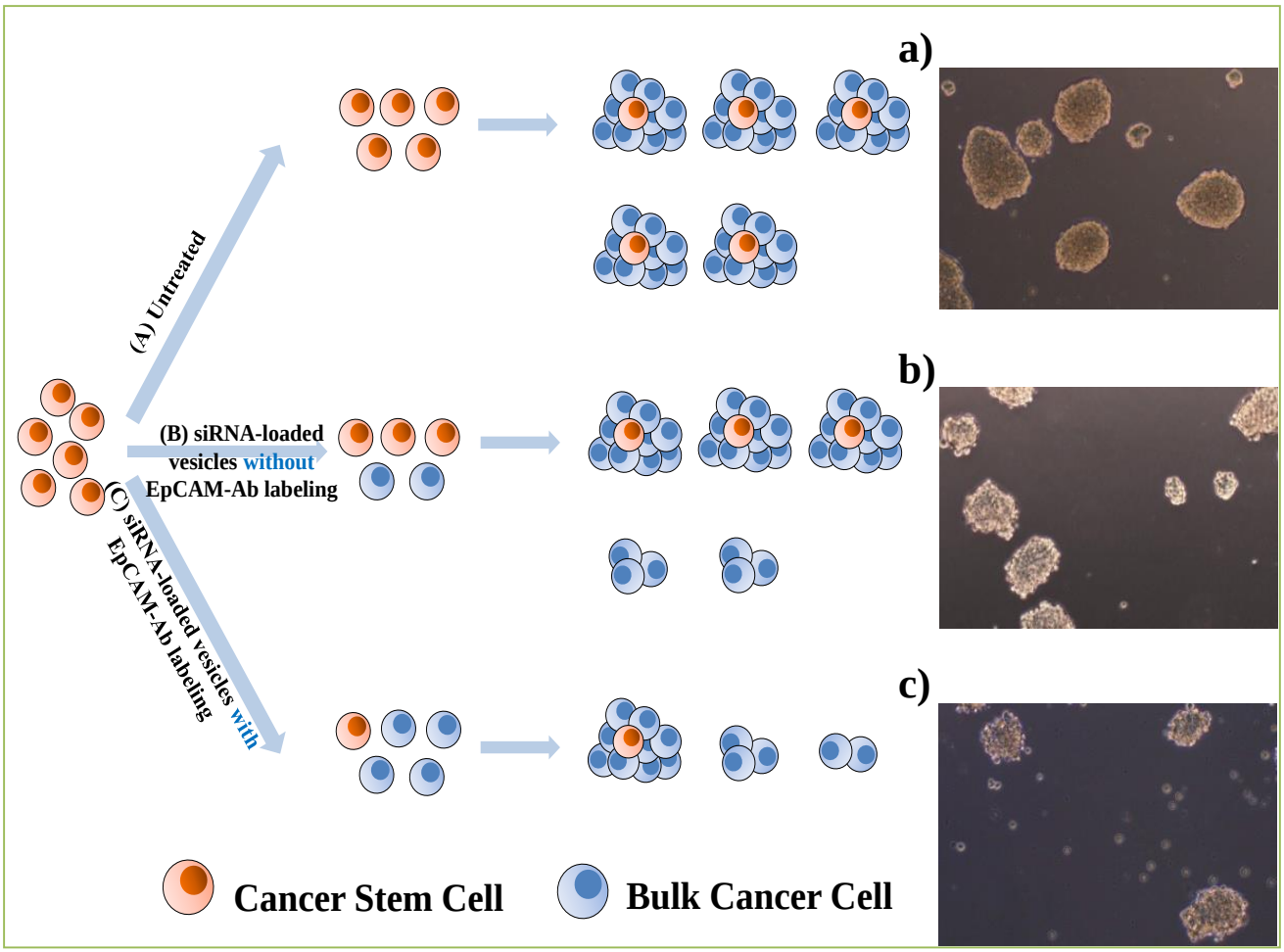

Figure 2. Formation of tumor spheroids by EpCAM positive cells pretreated by siRNA-loaded vesicles without and with EpCAM-Ab labeling. a) Cancer stem cells possess the spheroids forming features on the low-adhesion plates based on their reported capacity to self-renewal and differentiation at the single-cell level *in vitro*. b) and c) To overcome the drug resistance of cancer stem cells, the siRNA with the capability to silence the oncogene miRNA-429 expression was delivered by the polymer vesicles. Reprinted with permission [12].

promoters or suppressors, regulating the progression of cancers and CSCs. For example, miR-429 plays important roles in a wide range of common epithelial cancers, for example, gastric carcinoma, breast cancer, metastatic ovarian cancer, hepatocellular carcinoma, and so on [26, 27]. In hepatocellular carcinoma (HCC), miR-429 plays as a significant prognosis factor, which is up-regulated in HCC tissues and also in primary liver CSCs isolated from clinical samples. The enrichment of miR-429 in EpCAM positive CSCs contributed to hepatocyte self-renewal, malignant proliferation, chemoresistance and tumorigenicity.

Recently, we used the FITC-labeled siRNA (siRNA-FITC), a synthesized antisense RNA as the inhibitor of miR-429, to bind to miR-429 and to promote its degradation, which resulted in the down-regulation of miR-429 and turnover the stem-cell features of the EpCAM positive CSCs. To investigate the siRNA delivery efficiency by the polymer vesicles against the EpCAM positive CSCs, the siRNA-FITC-loaded EpCAM-Ab-labeled vesicles were co-incubated with magnetically sorted EpCAM positive CSCs for 24 h, and were observed under the fluorescence microscopy. The EpCAM-labeled vesicles can efficiently deliver the siRNA-FITC into the cytoplasma of EpCAM positive CSCs, and the siRNA delivery positive ratio is nearly 100%, indicating the high siRNA delivery efficiency by the polymer vesicles to the EpCAM positive CSCs.

Furthermore, we used RT-qPCR to quantitatively analyze the miR-429 level with or without siRNA treatment. The intracellular miR-429 relative level of EpCAM positive CSCs was decreased to only 22% of the non-treatment controls, which is better than the siRNA-loaded vesicles without EpCAM labeling.

Spheroids forming assay was performed to evaluate the suppression level of self-renewable and tumor occurring capabilities of EpCAM positive CSCs after the siRNA

treatment. As shown in **Figure 2**, after treatment with the siRNA-loaded EpCAM-Ab labeled vesicles, the EpCAM positive CSCs formed smaller, looser, and the least spheroids (**Figure 2c**) compared with untreated control cells (**Figure 2a**), or with cells after treatment with siRNA-loaded vesicles without EpCAM-Ab labeling (**Figure 2b**). These results demonstrated that the EpCAM-Ab labeled vesicles can specifically and efficiently deliver the siRNAs into EpCAM positive CSCs, which led to a significantly decreased expression of target cancer stem cell promoting miRNA (miR-429), and dramatically inhibited their self-renewable and tumor occurring capabilities.

The above results provide us with a new insight on multifunctional polymer vesicles for cancer stem cells-targeted drug/siRNA therapy. Moreover, these vesicles may have many other promising applications in nanomedicine.

Conflicting interests

The authors have declared that no competing interests exist.

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Abbreviations

CSCs: cancer stem cells; EpCAM: epithelial cell adhesion molecule; siRNA: small interfering RNA; DOX·HC: doxorubicin hydrochloride; IC_{50}: 50% inhibitory concentration; DNA: deoxyribonucleic acid; PEO: poly(ethylene oxide); PDPA: poly(2-(diisopropylamino)ethyl methacrylate); PAA: poly(acrylic acid); SA: streptavidin; Ab: antibody; FITC: fluorescein isothiocyanate; miRNA: micro-RNA; HCC: hepatocellular carcinoma.

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