The Application of Nano-drug Delivery System With Sequential Drug Release Strategies in Cancer Therapy

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Abstract: Currently, multidrug combinations are often used clinically to improve the efficacy of oncology chemotherapy, but multidrug combinations often lead to multidrug resistance and decreased performance, resulting in more severe side effects than monotherapy. Therefore, sequential drug release strategies in time and space as well as nano-carriers that respond to the tumor microenvironment have been developed. First, the advantage of the sequential release strategy is that they can load multiple drugs simultaneously to meet their spatio-temporal requirements and stability, thus exerting synergistic effects of two or more drugs. Second, in some cases, sequential drug delivery of different molecular targets can improve the sensitivity of cancer cells to drugs. Control the metabolism of cancer cells, and remodel tumor vasculature. Finally, some drug combinations with built-in release control are used for sequential administration. This paper focuses on the use of nanotechnology and built-in control device to construct drug delivery carriers with different stimulation responses, thus achieving the sequential release of drugs. Therefore, the nano-sequential delivery carrier provides a new idea and platform for the therapeutic effect of various drugs and the synergistic effect among drugs.

Key Words: sequential drug release, synergistic combinations, stimulus-responsive nanoparticles, anti-cancer therapy, space-time sequence

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INTRODUCTION

At present, tumors remain the world's leading health problem, and it is a difficult and cutting-edge area of medical research. Surgical resection, radiotherapy, and chemotherapy are commonly used in clinical treatment. Despite surgical resection is mainly used to remove tumor masses, due to the destruction of tumor tissue matrix, residual cancer cells will still lead to tumor recurrence[1,2] and even tumor cell metastasis. Radiotherapy is mainly used to treat tumors limited to a certain part of the body, and limited by radiation risk, it will cause some damage to normal tissues.[3,4] However, compared with the mentioned local tumor treatment methods limited to the anatomic area, chemotherapy, as one of the systemic treatments, plays a role in treating any part of the tumor in the body, especially for patients with metastatic tumors. However, because of the low specificity and side effects of its chemotherapeutic drugs, the curative effect of chemotherapy is far from satisfactory.[5,6] Multidrug combinations offer a resistance advantage over monotherapy, although the combination of two or more drugs often produces additional or synergistic therapeutic effects. Multidrug delivery at the same time can not ensure that each drug can play its maximum therapeutic role, and can not meet the delivery of each drug in time and space.[7–9] In addition, the combination of multiple drugs does not mediate the mutual constraints between drugs with different physicochemical properties.[10–13]

Therefore, based on the limitations of the above treatments, sequential administration therapy began to be discovered and developed. Sequential drug delivery therapy refers to a drug delivery system that can release multiple therapeutic drugs in a controlled and programmed time sequence or space. The system features the ability to release one or more drugs in a controlled sequence at different times or locations. Therefore, sequential therapy may be more advantageous than combination therapy. First, sequential therapy can avoid or reduce the mutual restriction between drugs and reduce the serious toxic and side effects caused by combined administration. Secondly, sequential administration is also an important way to solve the multidrug resistance (MDR) pump caused by chemotherapy. MDR pump is a form of drug pumping mediated by P-glycoprotein (P-gp). When tumor cells come in contact with chemotherapy drugs, the drugs enter the cells according to a concentration gradient. In the cells, the drugs combine with P-gp, and at the same time, adenosine triphosphate is hydrolyzed to obtain energy, which pumps the drugs out of the cells. The drug concentration in the cells keeps decreasing, which weakens the damage of drugs to the cells until it disappears, and finally, drug resistance appears. Therefore, it is an effective way to apply a sequential administration strategy in this case. Sequential release strategy firstly releases specific inhibitors to inactivate the P-gp pump and lose the ability to pump drugs out of cells and then releases chemotherapy drugs to kill cancer cells effectively, thus cleverly avoiding the risk of being pumped out of cells by P-gp by direct delivery of chemotherapy drugs.[14]

In contrast, the nano-drug delivery system (NDDS) with one or more small interfering RNA (siRNA) genes can also be applied to the sequential drug delivery strategy. It can control the release of its genes in advance and play the role of silencing all its drug-resistant genes to improve the sensitivity of tumor cells to drugs and exert stronger drug efficacy. Moreover, this strategy has long been reported as one of the strategies used in the NDDSs to enhance the antitumor effect. Not only that but with the development of science and technology and the appearance of personalized medicine, sequential administration strategy precision medicine has gradually become a hot topic for medical workers.[15,16]

The sequential delivery strategy is mainly used in the nano-delivery system of chemotherapeutic drugs (Table 1). The nano-drug delivery technology combined with sequential delivery strategy judges and responds to the changes of signals emitted from the focus of the disease. Give full play to the function of self-regulation and self-feedback to achieve selective drug delivery in timing, quantification, and positioning,

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TABLE 1. A Typical Delivery System Used by Sequential Release Strategies in Different Ways

Sequential release manner	Solved problem	Nano carries	References
pH	Reverse MDR cells	NDDS—CS/PAA@TPGS/PLGA NPs	17
	Mediated protein recombination, block angiogenesis, tumor dormant	Embedding, PTX/CA4 (mPEG) self-assembly	18
	OSCC	Hyperbranched polydactyl hydrazone (HPAH)	19
Redox	Colon cancer and thrombus	Polyglutamic acid dendrimer/NK—DOX-(NK-G2)n	20
Enzymes	Reverse MDR cells	D/N-PDA/Hb@HA	21
Magnetic	Sequential drug, bioimaging	([$NaYF_4$:Eu^{3+}/{DOX@CS}@PLGA]//[benzimidazole {Bim}/Fe_3O_4@PLGA])	22
NIR	Enhanced therapeutic effect	rGO-AuNRVe-DOX	23
GSH/pH	Reverse MDR cells	PDS-PDA(DOX/Ver)PEG HMSNs	24
NIR/pH	Enhance PTXL and GEM synergistic effect	PTXL-ss-PMAGP-GEM/NAG NLCs	25
	Reverse MDR cells	PCL-AuNC/Fe (OH) $_3$-PAA) JNP	26
3D printing	Reverse MDR cells	3D printing alginate tubes	27
TNT nanotube array	Reverse MDR cells	TNT combination PMS	28–30
Polymer coating	Primary and advanced liver cancer	Poly (ethtlene glycol)-block polylactic acid–co-ethanolide (mPEG-b-PLGA)	31

3D indicates 3-dimensional; AuNC, gold nanocage; DOX, doxorubicin; GEM, gemcitabine; GSH, glutathione; HMSN, hollow mesoporous silica nanoparticle; JNP, Janus nanoparticles; MDR, multidrug resistance; mPEG, methoxy polyethylene glycol; NAG, n-acetylglucosamine; NDDS, nano-drug delivery system; NIR, near-infrared radiation; NK, nattokinase; NLC, nanostructures; OSCC, oral squamous cell carcinoma; PAA, poly acrylic acid; PCL, poly (3-caprolactone); PDS, pyridine disulfide; PLGA, polylactic-glycolic acid; PMAGP, poly (6-Omethacryloyl-d-galactopyranose); PMS, polymer micelles; PTX, paclitaxel; PTXL, paclitaxel; rGO, reduced graphene oxide; TNT, titanium nanotube.

improve treatment efficiency and reduce side effects at the same time.[32] However, tumor microenvironment (TME) is a very complex internal microenvironment system. Compared with normal tissue, tumor tissue is characterized by its unique pathophysiological indexes, such as low pH value, intracellular high glutathione (GSH) concentration, enzyme-specific overexpression, and high reactive oxygen species (ROS) level.[33] These are the factors that affect the survival and development of tumor cells. These factors promote the occurrence and development of tumors, greatly reduce the effect of drugs, and bring great obstacles to the treatment of tumors. But in contrast, these unique pathophysiological indicators also provide researchers with a new idea for tumor therapy, thus the specific microenvironment responsive drug delivery system designed according to the characteristics of TME began to develop. Good results have been achieved. For example, The pH-sensitive drug delivery system releases specific drugs in a targeted sequence based on the pH in the different environments of the tumor. In this way, not only the loss of drugs in blood circulation is greatly reduced but also the bioavailability of drugs is improved. Targeted therapy can be carried out in different positions in tumors, which eliminates the restriction between drugs caused by the simultaneous release of multiple drugs and improves the therapeutic effect to some extent. In addition to the inherent environmental conditions of tumor cells or tissues, there are 2 parts of blessing applications. Part of it comes from the wise application of physical triggers for external applications, such as stimulus-specific responses to near-infrared, magnetic fields.[34,35] The other part comes from a completely different from the mentioned one. On this basis, 2 or more stimulus-response nano-carriers (such as GSH/pH and near-infrared radiation/pH) were designed. They are used in more complex tumor environments and are widely used.[36,37]

Sequential administration strategy is not only mainly used for the administration of chemotherapy drugs but also more and more widely used in cellular pathways. Cell signaling pathways plays a very important role in regulating apoptosis.[38,39] Chemicals or biological substances that affect cell signaling pathways and gene drugs that affect the apoptosis of sensitized tumor cells are used to sensitize tumor cells and induce apoptosis. The antitumor chemotherapy drugs are then released sequentially, so as to increase the accumulation of chemotherapeutic drugs near tumor cells. In addition, it also treats tumors from the perspective of blood vessel normalization. Specifically, the nano-coating technology is used to coat the vascular endothelial factor layer-by-layer, so that it can be continuously released at the minimum dose level, which can avoid the serious vascular leakage and hypotension caused by excessive vascular endothelial growth factor, and affect the therapeutic effect.[40] As shown in the figure (Fig. 1), in addition, there are many cutting-edge technologies that enable sequential delivery strategies to be adopted. For example, nanotube array technology has been able to continuously release 2 or more different hydrophilic chemotherapeutic drugs.[41] The combination of 3-dimensional (3D) printing technology and the sequential release strategy of lay a foundation for the manufacture of implantable devices.[42] In conclusion, through the above, it is not difficult to find that nanotechnology combined with sequential delivery will become a promising antitumor therapy strategy, which can effectively improve the drug resistance of a single drug, and the serious adverse reactions caused by the simultaneous use of drugs. This paper reviews recent research advances in several sequential drug delivery strategies with the aim of combining nanotechnology with sequential drug delivery strategies to improve the efficacy of NDDSs in tumor therapy.

SEQUENTIAL RELEASE NANO-CARRIERS

Polymer Nano-carriers

Since Yokoyama[43] proposed that polymer micelles (PMS) can be used as a new type of NDDS, micelles are expected to deliver anti-cancer drugs in cancer therapy. PMS are block copolymers that embed insoluble drugs in water by self-assembly through hydrophobic and electrostatic forces, forming a nanostructured micelle solution with a size of 10~100 nm.[44] PMS have good self-assembly ability and the

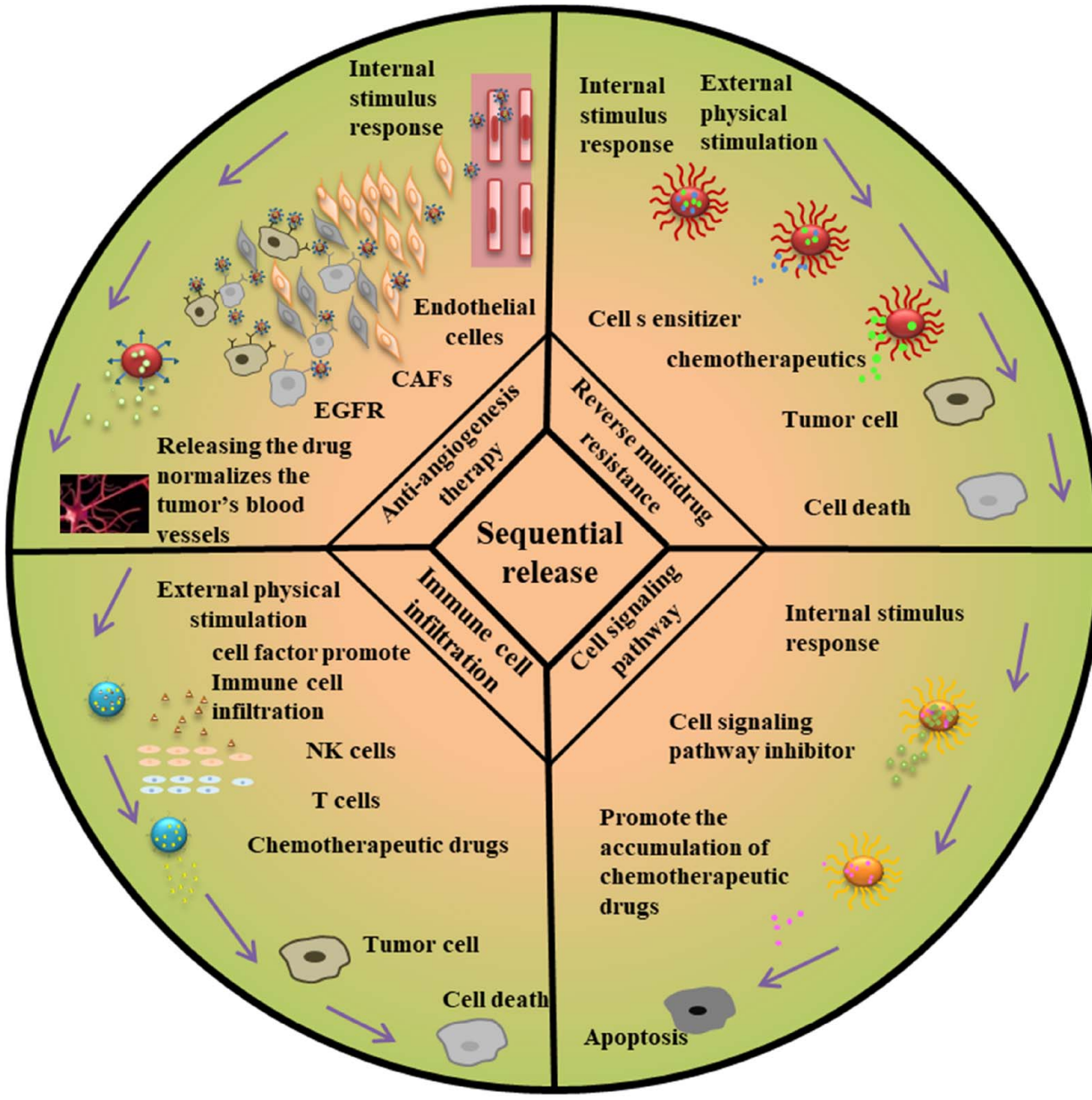

FIGURE 1. Sequential drug delivery strategies combined for the treatment of tumors and from different angles, such as reversing MDR, inhibiting cell signal pathway, antiangiogenesis, and immune cell infiltration. CAF indicates cancer-associated fibroblasts; EGFR, epidermal growth factor receptor; MDR, multidrug resistance; NK, natural killer. full color online

ability to dissolve hydrophobic anti-cancer drugs. With its unique shell-core structure, the appropriate micelle carrier can be freely selected according to the drug properties[45] Targeted sequential release of anti-cancer drugs can be achieved if specific antibodies, ligan, or some stimulation-sensitive systems are attached to hydrophilic segments of micelles. Therefore, it is called one of the most potential nano-drug carrier systems.[46,47]

Liposome Nano-carriers

Thanks to a series of characteristics, such as special hollow spherical structure, diverse particle sizes, modifiable phospholipid bilayer, excellent biocompatibility, etc, liposomes are widely used in the biomedical field as excellent delivery systems.[48] Until today, liposomes are still the most commonly used nano-carriers in clinical applications.[49] British researcher, Bang Ham, first discovered that liposomes are that disperse phospholipids in water. Phospholipids are amphiphilic, so in an aqueous environment, they can form a very stable bilayer, with hydrophilic head groups inserted into the water and hydrophobic tails extending into the air. The hydrophobic chains are repelled by water molecules and the liposomes self-assemble into a closed bilayer. The 3 forces that form liposomes are the hydrophilicity between polar head groups, the van der Waals force between hydrocarbon chains (which keeps long hydrocarbon tails together), and the hydrogen bond with water molecules. The amphiphilic nature of phospholipids is similar to that of natural cell

membranes. Its biocompatibility, biodegradability, nontoxicity, and nonimmunogenicity make liposomes interact well with human cell membranes, thus promoting effective cell absorption. Due to the structural diversity of liposomes, they can be modified by a large number of surface ligands, which are used to functionalize different types of diseases. One of the most important advantages of liposomes is that it has the ability to deliver a large number of drug payloads, which can ensure the controlled release of a variety of drug delivery, such as the release of small molecule interference RNA sensitized cells, followed by the release of chemotherapeutic drugs,[50] to achieve the best therapy effect. Based on these advantages, liposomes are considered to be a capable drug delivery system.[51,52]

Janus Nanoparticles

Janus is a double-faced door god in Roman mythology. Later, the word "Janus" was adopted by the field of scientific materials, and was expressed as a material showing different characteristics in two opposite.[53] Compared with other single nanomaterials, Janus nanoparticles (NPs) have the following advantages: (1) their own anisotropic characteristics can realize relatively independent diagnostic/therapeutic functions, (2) the asymmetric structure is used to realize the independent loading and release of a variety of drugs, and (3) it can effectively reduce the signal interference generated in NPs and effectively inhibit the proliferation of cancer cells in combination with a variety of treatment methods (such as photothermal therapy, photodynamic therapy, chemotherapy, sonodynamic therapy, radiotherapy, magnetothermal therapy, and imaging). Compared with conventional NPs,[54] typical Janus NPs can not only accommodate drugs with different solubility in different domains, reduce the interaction between the two drugs, and promote the independent release of individual drugs, but also control the release sequence of drugs.[55] In addition, the controllable size and morphology, unique surface characteristics, and response to a variety of stimuli of Janus NPs can realize the synergistic treatment of tumors.

RESPONSE OF ENDOGENOUS STIMULUS SIGNAL TO NANO-SEQUENTIAL DELIVERY SYSTEM

pH Response Nano-sequential Delivery System

As can be seen (Fig. 2), the TME is an extremely complex internal microenvironment system. Most tumor cells get energy for growth through an inefficient glycolysis pathway. In this process, lactic acid and protons are pumped out of the cells as the main products of glycolysis, which leads to the acidification of the tumor's extracellular environment to its pH of 6.2 to 6.9.[56] Meanwhile, during this period, the pH value of endosomes in tumor cells is about 5.0 to 6.0, and the pH value of zymosomes is 4.0 to 5.0.[57] Because of the unique pH microenvironment in tumor cells, a series of pH-responsive NDDSs have been designed and studied in some research work.

MDR refers to a state produced by cancer cells after exposure to anti-cancer drugs.[58] The main reason for MDR is the increase in efflux, the activation of metabolic enzymes, and the decrease of drug retention in the nucleus, which eventually leads to a decrease in drug intake, which leads to a decrease in drug accumulation. In the cell and the inability to obtain the effective concentration of antitumor therapy.[17] Therefore, to improve the therapeutic effect of anti-cancer drugs, anti-cancer drugs need to be delivered to the nucleus more effectively.

Wang et al[18] skillfully utilized the difference in pH in different parts of TME to develop NPs with intracellular pH sensitivity and sequential drug release. In this study, S@LNPs is composed of the vitamin E D-α-tocopherol polyethylene glycol (PEG) 1000 succinate-modified poly(lactic-co-glycolic acid) NPs (toxic polyethylene glycol 1000 succinate/polylactic-glycolic acid [PLGA] NPs) with large particle size was used as the shell, and of chitosan (CS)-poly (acrylic acid) NPs wrapped and loaded with small particles inside the drug etoposide. As shown in Figure 3, after the system enters MDR cells, it is triggered to degrade in the acidic environment of cytoplasm, endosome, or lysosome, and then the drug-carrying particles encapsulated in it are released. Because of its smaller particle size, the small particles then enter the nucleus through the nuclear pores and finally depolymerize in the alkaline

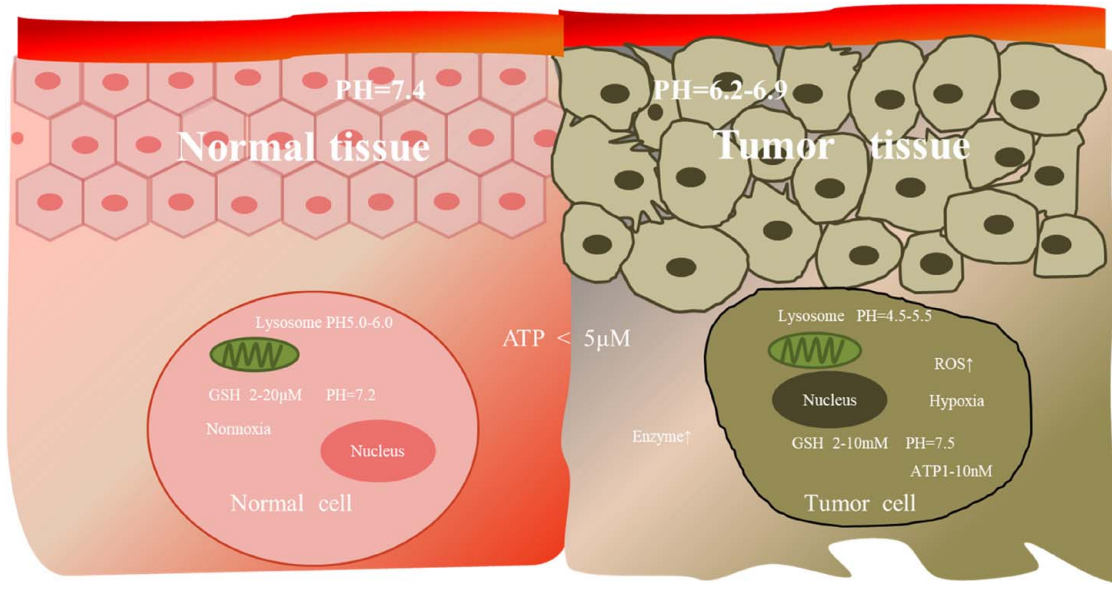

FIGURE 2. Schematic illustration of TME. ATP indicates adenosine triphosphate; GSH; glutathione; ROS, reactive oxygen species; TME, tumor microenvironment. full color online

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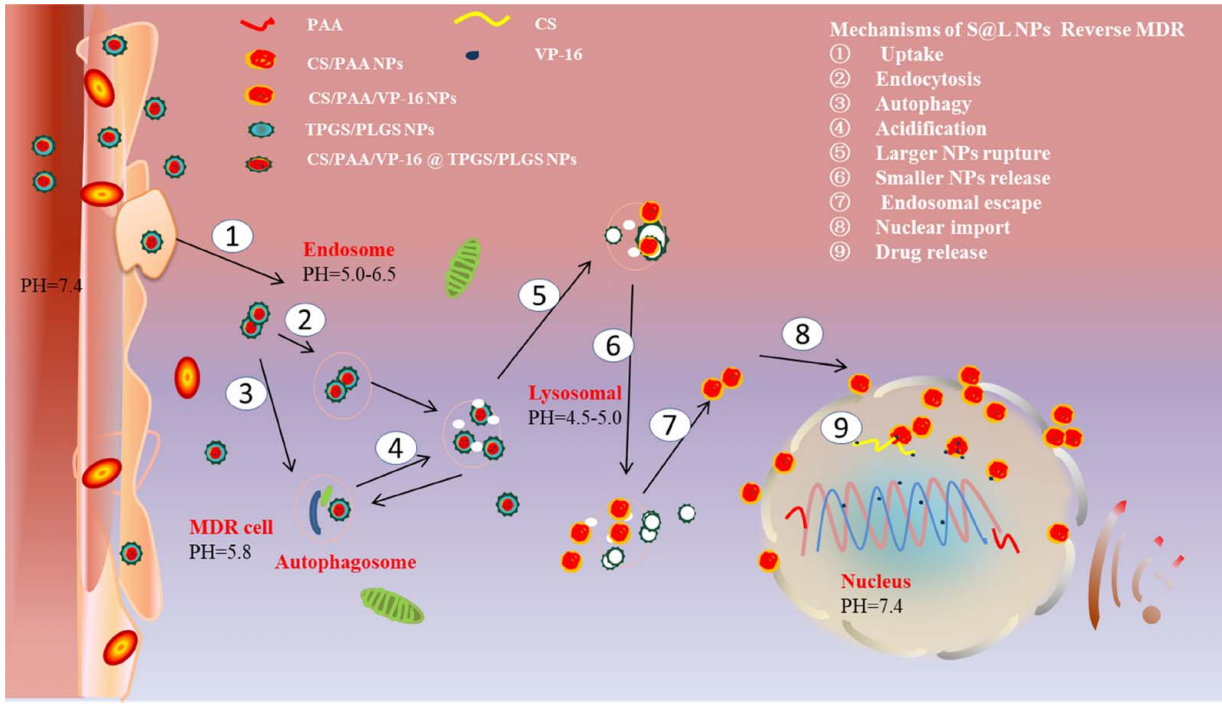

FIGURE 3. Illustration of the mechanism by which CS/PAA/VP-16@TPGS/PLGA NPs reverse MDR. CS indicates chitosan; MDR, multidrug resistance; NP, nanoparticle; PAA, poly acrylic acid; PLGA, polylactic-glycolic acid; TPGS, toxic polyethylene glycol 1000 succinate; VP-16, etoposide. full color online

environment of the nucleus, thus releasing the anti-cancer drug etoposide. Importantly, due to the external protection of large particles, drug-loaded small particles not only avoid being detained and degraded by lysosomes, but also successfully avoid the risk of being excreted by P-gp, finally achieving the goal of more and more drugs accumulating near the nucleus, and at the same time, playing a more effective antitumor role to a certain extent.

Combination therapy has advantages in realizing synergistic effects, overcoming MDR, and reducing nonspecific toxicity,[59] and has a broad prospect in the efficient treatment of cancer. In this study,[19] an NDDS was designed, which can deliver two drugs into lysosomes and mitochondria of cancer cells in turn. At first, doxorubicin (DOX) was inserted into the DNA double-strand of cytochrome c aptamer (Apt) hybridized with its complementary single-strand DNA through noncovalent interactions, and then condensed by dense poly (l-lysine) (DGL) to self-assemble NPs (DOX/DGL). To achieve mitochondrial targeting, mitochondria-penetrating peptide (MPP) as the targeting ligand and was linked to DOX/DGL, secondly, set MPP-modify ide DOX/DGL (DOX/MPP-DGL) was encapsulated into the aqueous core and deoxybouvardin (RA-V) was doped into the shell of pH-sensitive liposomes. To further improve the cancer selectivity, a cyclic pentapeptide was coalesced on the surface of liposomes, and DGL-liposome NPs were obtained. The system enters cellular lysosomes rich in αvβ3 integrin through receptor-mediated endocytosis. In the presence of acidic pH, the liposomes degrade and release RA-V and DOX/MPP-DGL, in which DGL acts as a proton sponge, causing the lysosomes to swell and rupture, which will be taken up by mitochondria after successful escape. Further, the dissociation of DNA duplex was induced by the high level of cytochrome c in mitochondria, resulting in rapid release. Because the fluorescence and cytotoxicity of DOX are quenched after insertion into the complex, DOX release can kill cancer cells synergistically with RA-V.

Saiyin et al[60] provided a new strategy for the effective treatment of MDR in oral squamous cell carcinoma by using the mechanism of autophagy inhibition. Hydrophobic DOX was coupled with hydrophilic, pH-sensitive hyperbranched polyacylhydrazone (HPAH). In contrast, autophagy inhibitor LY294002 (LY) can self-assemble into nanometer micelles in an aqueous solution because of its amphiphilic property and can be loaded into HPAH-DOX micelles, thus successfully synthesizing a pH-responsive framework degradable HPAH.[61] The system relies on pH to release drugs in sequence. In this system, LY is released first to inhibit autophagy of tumor cells, increase their drug sensitivity, and make DOX released later play a more effective antitumor effect.

Redox Response Nano-sequential Delivery System

Due to hypoxia and the production of a large number of reducing molecules (such as reductase and GSH), there is a large redox potential difference between tumor cells and the extracellular environment due to hypoxia and the production of a large number of reducing molecules (such as reductase and GSH).[62] GSH has also become one of the main redox pathways in mammalian cells, which are used to avoid cell damage caused by ROS and maintain the stability of thiolase and the integrity of cell membrane, that is, GSH is a g-glutamyl cysteine tripeptide.[63] In blood or extracellular matrix, GSH exists at a very low concentration of 2 to 20 μM. The rapid proliferation of tumor cells causes the content of GSH in tumor tissue to be at least four times higher than that in normal tissue.[64] This significant difference in GSH levels has been successfully applied to specifically trigger the controlled release of redox-sensitive NDDSs in tumor cells.[20]

In this study,[65] researchers obtained nattokinase-G2 (NK-G2) by amidation between the carboxyl group on the second-generation polyglutamic acid dendrimer (G2) and the amino group on NK. After that, the polymer conjugate $(NK\text{-}G2)_n$ was cross-linked by cysteamine-containing disulfide bonds, and it was encapsulated in adriamycin because of its hydrophobicity.[66] When the nano-delivery system reaches the TME, in the presence of a high concentration of GSH, after the disulfide bond is broken, the conjugate is internalized by the cancer cell and dissociates and releases DOX to kill the cancer cell.[67,68] Then the amide bond breaks to release NK to treat thrombotic complications, thus realizing the sequential release of complications while treating colon cancer.

Enzyme Response Nano-sequential Delivery System

Enzymes play an important role in most physiological reactions of the human body. Studies have shown that the pathology of tumor diseases is related to enzyme dysfunction or its expression imbalance. Among them, the expression and activity of protease, phospholipase, and other enzymes in tumor tissues are significantly higher than those in normal tissues, which have an important impact on the occurrence and development of tumors.[69] Based on this characteristic, it is of great significance to design and develop an enzyme-responsive antitumor drug delivery system, which is helpful to realize the controlled release of drugs in the tumor site.[70]

Enzyme-responsive nano-ions are linked to substrates of specific enzymes[71,72] at the response sites, and trigger responsiveness[73] with the help of enzymes with high specificity and expression in tumor cells, and change their functions or characteristics, such as exposure of functional ends, morphologic changes in designated directions, etc, so as to improve targeting, permeability and drug accumulation time, and achieve better antitumor effects.[21,74] In the course of exploring how to overcome drug resistance and solve the difficult problem of solid tumor treatment.[34,35,75] In this research, the author used dopamine and hemoglobin to polymerize to form intelligent nanocarrier (polydopamine [PDA]/Hb), then connected adriamycin and nitric oxide donor to the surface (D/N- PDA/Hb), and finally modified its surface with hyaluronic acid (HA) (D/N-PDA/Hb@HA) PDA/Hb@HA can degrade HA in TME by HAase, and enter tumor cells after adding HA and receptor to tumor cells.[22] And then the charge of PDA/Hb changed from a negative charge to a positive charge due to the acidity of the lysosome. Due to electrostatic repulsion, DOX and NO were released, which resulted in nitrosation of DNA in tumor cells and death of tumor cells. Subsequent NPs returned to the cytoplasm, and due to the influence of their neutral environment, the charge changed from positive to negative, and the release was suspended until they entered the next cell, and the charge turned over and drug release began. Until the drug is completely released (Fig. 4), the penetration efficiency of the sequential intracellular drug administration is higher than that of the solid tumor treated with enhanced permeability and retention effect. In summary, this study provides an opportunity for nanotechnology-based drug delivery and drug resistance reversal in the next generation of solid tumor therapy.

RESPONSE OF EXOGENOUS STIMULUS SIGNAL TO NANO-SEQUENTIAL DELIVERY SYSTEM

Magnetic Response Nano-drug Delivery System

A magnetic targeted drug delivery system is a targeted magnetic preparation made of magnetic NPs and drugs coated

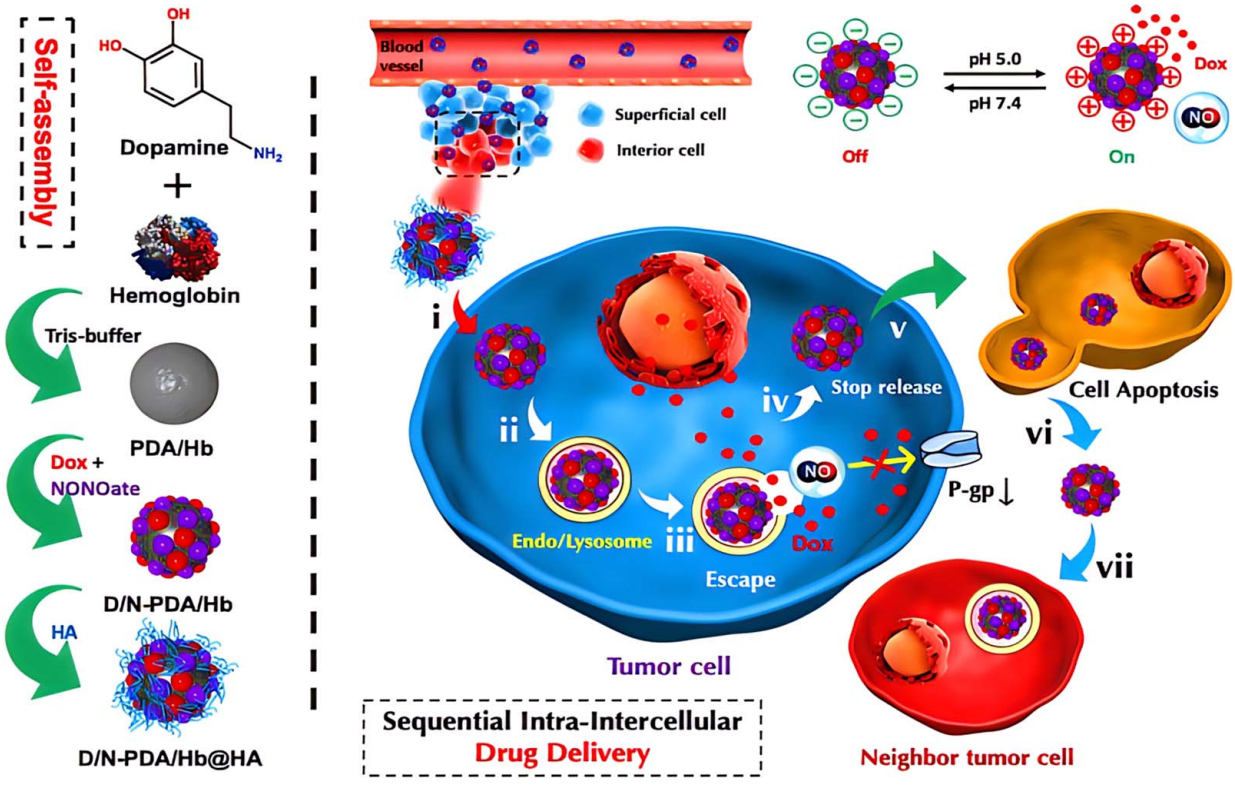

FIGURE 4. Schematic design of the sequential initiative drug delivery for deep drug-resistant solid tumor penetration.[21] P-gp indicates P-glycoprotein. full color online

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with polymer materials. It can enter the body through intravenous and arterial injection or oral administration and can be guided by an external magnetic field or an internal magnetic field to directionally enrich and target specific tumor cells or biomolecules and release drugs in the body. In this study,[76] inspired by the structure of rapeseed, the author designed a magnetic targeted drugs delivery system with a dual-cavity structure, which consists of Fe_3O_4 NPs, $NaYF_4$:Eu^{3+}, and NPs; composition of CS (CS NPs) NPs. In which poly (lactic-co-glycolide) (PLGA), CS NPS, and NaYF4:Eu^{3+} NPs are dispersed in tetrahydrofuran as solution A, and PLGA and Fe_3O_4 NPs are dispersed in tetrahydrofuran as solution B. Then, the AB solutions are respectively filled into syringes, connected by a double-needle system, and finally, the final drug delivery systems [(NaYF4:Eu^{3+}@CS)@PLGA]/Fe_3O_4@PLGA is prepared by electric spraying. In this system, different chemical drugs can be loaded, and they can be released separately according to the different components of their chambers, thus achieving the purpose of sequential release of the two drugs, and achieving a good therapeutic effect.

Near-infrared Radiation Response Nano-drug Delivery System

Cutaneous-mesenchymal transition (EMT) is a key factor that leads to tumor metastasis and the characteristics of tumor stem cells based on the remodeling of cytoskeleton and the increase of drug efflux so that the drug resistance of tumor cells affects the antitumor efficacy.[77–79] Previous studies have shown that the overexpression of β-tubulin III promotes the EMT of prostate cancer. In addition, chemotherapy also up-regulated stromal cell-derived factor 1-α, and the combination of this factor with CXCR4 not only affected the activation of EMT-related pathways but also inhibited paclitaxel (PTX)-induced microtubule stability.[80] Based on this, the authors of this paper found that targeting these genes to silence these genes can make cells re-sensitive to PTX and inhibit EMT in prostate cancer.[81] siRNA-mediated RNA interference is an effective method for specific silencing of target genes. Coating siRNA with nanotechnology can effectively improve the shortcomings of siRNA, such as short half-life and easy degradation in vivo.[82] As a result, the authors constructed a shell-core nano-targeted delivery system, which is composed of prostate-specific membrane antigen-Apt-modified DSPE/PEG_{2K} polymer, PTX wrapped in the core, 3 siRNAs (β-tubulin III, AR, and CXCR4) and calcium phosphate shell (PTX/siRNA NPs-Apt). When reaching the tumor tissue, the siRNAs on the shell of the NPs are first released into the cell, showing the effect of gene silencing, reversing EMT, overcoming the PTX resistance of prostate cancer, and then the core PTX is released to exert the killing effect of chemotherapy (Fig. 5). The results showed that the sequential release of siRNAs and PTX significantly reduced the number of tumor cells, promoted tumor cell apoptosis and down-regulated Bcl-2 and Ki67 proteins in prostate tumors. The results of western blot detection showed that the sequential release of siRNAs and PTX significantly increased the expression of E-cadherin and down-regulated the expression of AR, β-tubulin III, and CXCR4, which effectively reversed EMT and enhanced the efficacy of antitumor therapy. In this paper[83], the author designed a sequential co-delivery system for miR-21i/DOX co-delivery of near-infrared radiation responsive hollow gold NPs (HGNPs), which is called D-P-HGNPs/21i. After entering the tumor cells, the cancer cells sensitized by miRNA-21i were first released, and then the explosive release of DOX was realized by near-infrared triggering the collapse of HGNPs. The continuous transmission of miRNA-21i and DOX produced a synergistic apoptotic response, which increased the anti-cancer efficacy.

Metal plasma nanocrystals with local surface plasmon resonance have been widely used in the fields of biosensors,

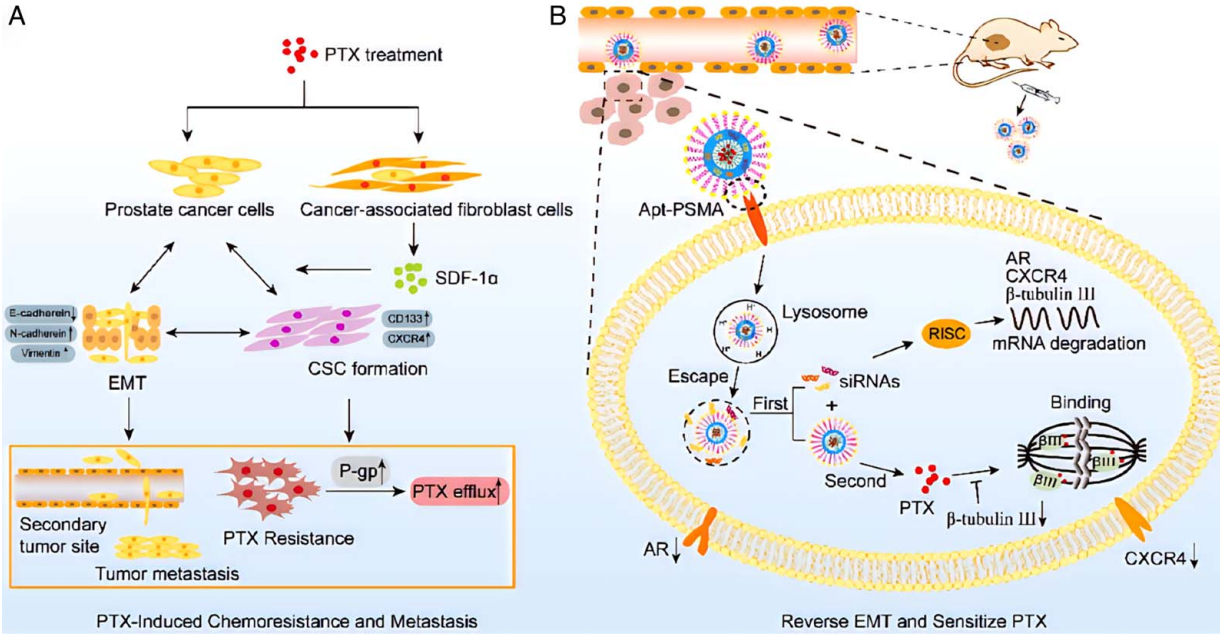

FIGURE 5. Sequential release of pooled siRNAs and PTX by Apt-functionalized shell-core NPs to overcome PTX resistance and inhibit metastasis of PCa. A, PTX treatment is found to promote PTX resistance and metastasis through inducing EMT. B, Reversing EMT by PTX/siRNAs NPs-Apt in PCa cells can sensitize PTX and inhibit metastasis[81]. Apt indicates aptamer; CSC, cancer stem cell; EMT, cutaneousmesenchymal transition; NP, nanoparticle; PCa, Prostate cancer cells; PSMA; prostate-specific membrane antigen; PTX, paclitaxel; SDF, stromal cell-derived factor; siRNA, small interfering RNA. full color online

biological imaging, photothermal therapy, and drug delivery.[83] Reduced graphene oxide (rGO) NPs have been widely used in the field of therapy because of their large drug-loaded surface area and photothermal effect.

It has been reported that the photothermal properties of rGO can be improved by coating or fixing plasma NPs on the surface of rGO. This enhanced photothermal effect is due to the fact that plasma NPs, not only as a photothermal source but also as a local nano-antenna, improve the optical absorption efficiency of rGO at the plasma frequency of NPs.[84] Song et al[85] developed a novel ultra-small plasma gold nanorod vesicle (rGO-AuNRVe-DOX) loaded with carbon-metal hybrid rGO-DOX (rGO-DOX) for photothermal therapy of comprehensive chemotherapy. The hybrid vesicle has a high loading capacity of DOX. Both the cavity of the vesicle and the large surface area of encapsulated rGO can be used to load DOX. Near-infrared light heating induces the release of DOX from the bubble, and the intracellular acidic environment induces the release of DOX from the surface of rGO. Using this construction scheme, the direct interaction between rGO-DOX and the physiological environment in the process of circulation and cellular internalization was avoided. Compared with single AuNR and the mixture of rGO and AuNRVe, the photothermal effect and PA signal of rGO-AuNRVe were significantly enhanced. In vivo experiments showed that mixed rGO-AuNRVe-DOX showed a higher therapeutic effect on cancer due to the synergistic effect of photothermal therapy and controlled release of chemotherapeutic drugs.

MULTISTIMULUS SIGNAL RESPONSE NANO-SEQUENTIAL DELIVERY SYSTEM

Glutathione/pH Nano-sequential Delivery System

The hollow core of hollow mesoporous silica NPs (HMSNs) can be used as an additional drug repository.[85] Hydrophilic drugs can be loaded into the internal space of hollow silica NPs with high loading efficiency, and hydrophobic drugs can be loaded onto the mesoporous surface of HMSNs by physical adsorption. Therefore, hollow silica NPs can be used as an effective solution for combined multidrug therapy.

In heterogeneous tumor environments, different drug uptake and drug concentrations limit the synergistic effect of drug administration.[86] In addition, combination therapy usually requires multiple injections, which may impair patient compliance and complicate the treatment process. Therefore, to improve the efficacy of combined chemotherapy, multidrug sequential administration to the target site is needed. Palanikumar et al,[24] to improve the efficacy of combined chemotherapy, hollow silica NPs were selected as its NDDS.

To produce the specific response of pH and GSH, pyridine disulfide, 2-(diisopropylamino (DPA)) ethyl methacrylate and PEG were used as cross-linked copolymers in the NDDS. At pH 7.4, the copolymer maintains the negative charge on the surface of its NDDS and reduces the nonspecific interaction with blood. When the pH value is pH 5.5 to 6.5, the amino groups on DPA have a protonation effect, which makes the surface of HMSN positive and increases the targeting for tumor site accumulation. After being internalized, the acidity and positive charge density increased, which caused the hydrophilic drugs contained in the polymer to burst and be released, and blocked the drug efflux of P-gp. In addition, the positive charge can also damage the cell membrane and promote the escape of HMSN into the cytoplasm.[87] In cytoplasm, pyridine disulfide, a substance containing disulfide bonds, is reduced and degraded by high concentration of GSH, so that hydrophobic drugs are released, further killing drug-resistant cancer cells. The results showed that when HMSN, with the double response of pH and GSH reached the TME, hydrophilic Ver and hydrophobic DOX were released in turn under the double response of pH and high concentration of GSH, and the drug concentration of DOX was significantly increased under the action of Ver (Fig. 6). Thus, a sequential drug delivery system is an effective strategy to enhance the antitumor effect.

Gemcitabine (GEM) and PTX paclitaxel (PTXL), two commonly used chemotherapeutic drugs for non-small cell lung cancer, have independent mechanisms of action and do not overlap in toxicity.[88,89] In this paper, the author studied a 2-drugs polymer conjugate for the treatment of non-small cell lung cancer. Firstly, the nano-structure amphiphilic block copolymer n-acetylglucosamine-poly (styrene-alt- maleic anhydride)$_{58}$mur-b-polystyrene$_{130}$ (PTXL-ss-PMAGP-GEM/n-acetylglucosamine nanostructured lipid carriers) was formed as the targeting part and emulsifier.[25] To accelerate the release of PTXL by the redox potential of cancer cells, DPS was introduced as a shear linker to form PTXL-ss-DPA. GEM is linked to the nano-structure by an ester bond. Under the condition of pH 6.0 and 10 mM, the release rate of GEM is slower than that of PTXL. Compared with the combination of PTXL and GEM as a free drug or in a nonreducing GEM-sensitive drug delivery system.

Near-infrared Radiation/pH Nano-sequential Delivery System

The heterostructure,[90] surface properties, and various functions of inorganic Janus NP can carry drugs with different solubility in different regions without affecting each other, and they can promote the independent release of individual drugs. Therefore, a novel amphiphilic poly (3-caprolactone) gold nanocage/ferric hydroxide-poly (acrylic acid) (defined as poly (3-caprolactone)-argentum nanocage/Fe(OH)$_3$-PAA) Janus NP was designed for the first time in this paper. In this system, Fe(OH)$_3$-PAA has the ability to respond to pH stimulation, magnetic resonance imaging, and hydrophilic drug loading. anocage was etched with haucl4 to form argentum nanocage and then modified with poly (3-caprolactone)-SH, so that this part has the ability of near-infrared stimulation response, computed tomography imaging, hydrophobic drug storage, and photothermal therapy.[26] In this paper, the nano-delivery system constructed by the author realizes the sequential release of multiple drugs by controlling the irradiation time of near-infrared laser and has a very shallow ability to improve the chemotherapeutic drugs that have been resistant to tumor cells. This carrier can be used in the combined application of sensitizing drugs and chemotherapeutic drugs, and the sensitized drugs can be released in advance by controlling the laser irradiation time so that drug-resistant cells can be sensitized temporarily. And then create opportunities for the treatment of chemotherapeutic drugs.

The authors[91] developed thalidomide combined with epidermal growth factor receptor targeting super-pH-sensitive nano-photosensitizer. Due to the fluorescence resonance energy transfer effect between Ce6 and Cy7.5, the photosensitivity of Ce6 is quenched at physiological pH. However, when $pH < pH_t$, the micelles rapidly dissociated into monomers, accompanied by fluorescence and singlet oxygen sensor green activation (under 660 nm irradiation). The normal effect of thalidomide on tumor vessels will increase the

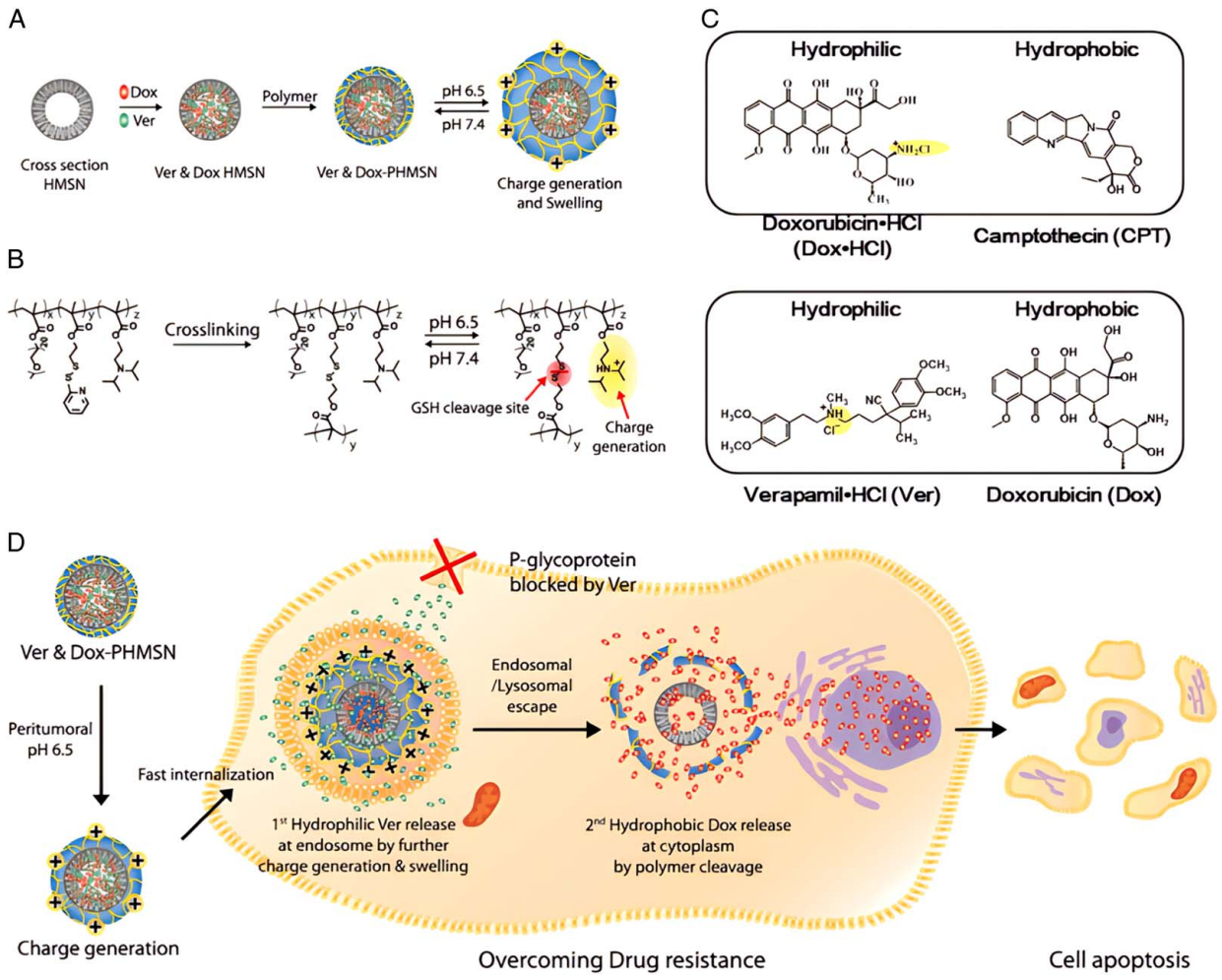

FIGURE 6. A and B Synthetic scheme for the preparation of a dual drug-loaded PHMSN using the polymer gatekeeper technique. B, Disulfide cross-linking and pH-dependent cationic charge reversal by the protonation of the diisopropylamine group. C, Two combination sets of hydrophilic and hydrophobic drugs. D, Schematic illustration of the cellular uptake, endosomal escape, and GSH-mediated stimulus for sequential drug release.[24] GSH indicates glutathione; PHMSN, hollow mesoporous silica nanoparticles. full color online

accumulation of passive and active targeted NPs in the tumor. Photoablation of stromal cells located in the perivascular area significantly improved the accessibility of antibody-modified nano-photosensitizers to receptor-overexpressed cancer cells (Fig. 7).

SEQUENTIAL RELEASE SYSTEM OF EXTERNAL CONTROL DEVICE

Three-dimensional Printing Nano-sequential Delivery System

3D printing technology is a brand-new chapter in pharmaceutical manufacturing, which has been widely explored in recent years. Compared with the traditional pharmaceutical industry, this technology has very significant advantages. For example, personalized dosage, sequential drug release, personalized local treatment equipment, etc. This technology can be said to be a revolutionary tool, which greatly increases the flexibility in production.[92] Generally speaking, 3D printing is a layer-by-layer process, and each layer can achieve the purpose of controlling the release of drugs successively through its sufficiently different composition. Moreover, this technology fully combines the current medical hotspot "personalized scheme," and it can perform personalized printing according to different backgrounds, habits, and metabolic patterns of patients. In short, 3D printing technology is likely to be a turning point in the revolution of the drug delivery system.[93,94]

In this paper, we use a 2-layer system to print a drug delivery device that can deliver drugs continuously.[27] The delivery system includes a 3D printing (through a coaxial extrusion system) of alginate tubes, containing PLGA cores to enhance the structural integrity of the alginate tubes.[60,95,96] In the study presented in this paper, the "proof of concept" 3D printing PLGA-filled alginate tube was demonstrated by studying the fluorescein released from alginate PLGA tube by fluorescein (in alginate sheath) and rhodamine (in PLGA core). The results show that the initial release of fluorescein and the delayed release of rhodamine are consistent with the properties of a layered system. Before the release of rhodamine from the core of PLGA, fluorescein diffused through the alginate sheath. This setting can enhance the sensitivity of multidrug-resistant antineoplastic drugs. First of all, release sensitizing drugs to attack tumor cells, so that drug-resistant tumor cells

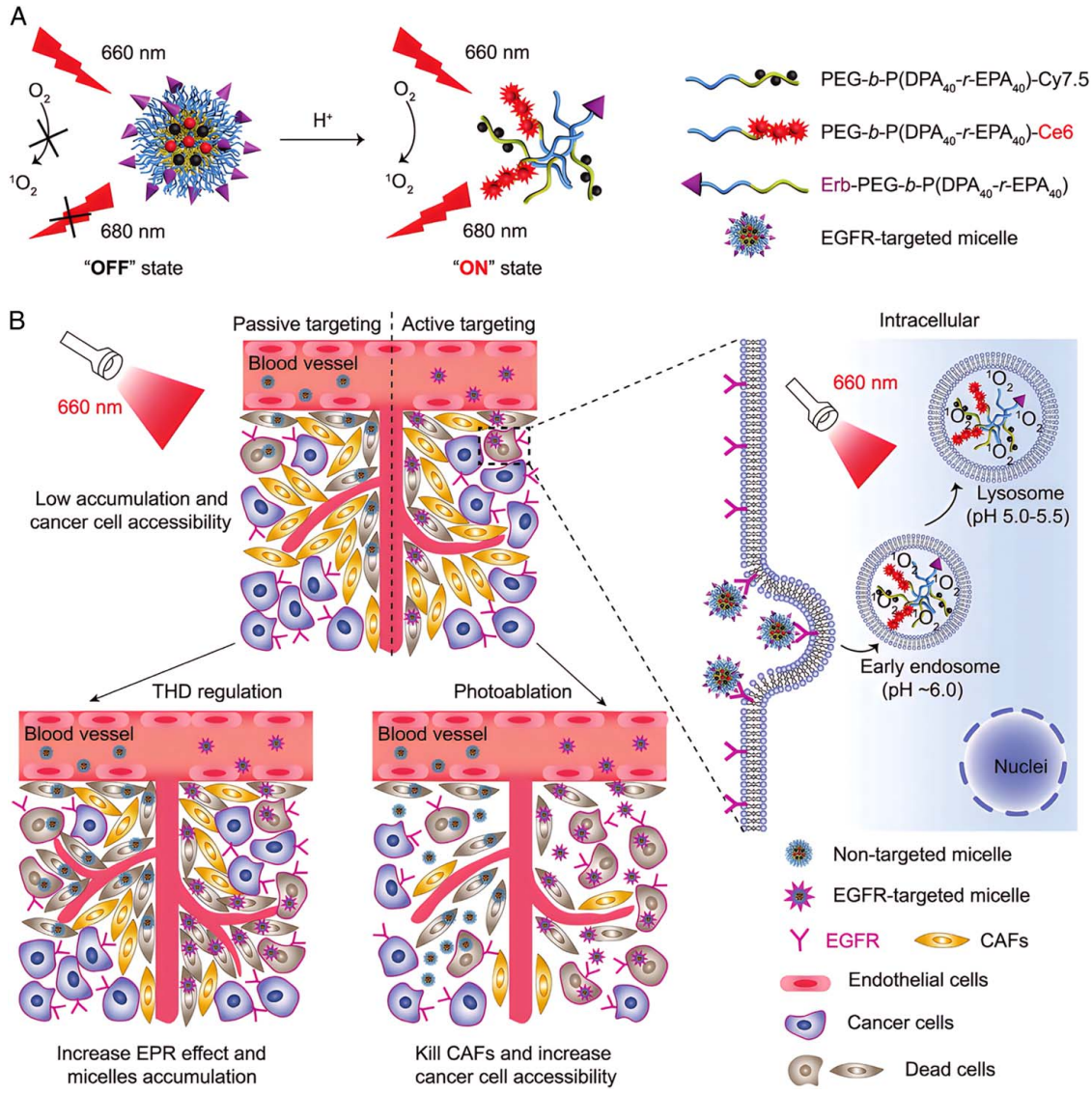

FIGURE 7. Schematic diagram of delivery of an acid-activated EGFR-targeted nano-photosensitizer regulated by the TME. A, Ce6 fluorescence activation and singlet oxygen generation of pH-modulated photodynamic micelles. B, Left panel: Thalidomide enhances tumor accumulation and stromal cell photoablation to improve in vivo PDT treatment with EGFR-targeted nanocapsular photosensitizer. Right panel: Photodynamic micelle specific internalization, dissociation in acidic endocytosis organelles, resulting in singlet oxygen killing tumor cells under 660 nm irradiation.[91] EGFR indicates epidermal growth factor receptor; PDT, photodynamic therapy; TME, tumor microenvironment. full color online

become sensitive, and then release chemotherapeutic drugs to maximize the therapeutic effect as much as possible. Reduce the waste of drugs and unnecessary side effects.

As a new tissue/organ manufacturing technology, 3D biological printing provides high precision in controlling the internal structure of the scaffold, with better mechanical strength and printing complex microstructure, which is comparable to the local tissue. However, the current biological printing technology still has difficulties in realizing biomimetic nano-resolution and synergistic bioactive spatial-temporal signals. The current 3D bio-printing technology (including fused deposition modelling) has difficulties in achieving biomimetic nano-resolution regulation of cell events.[97–99] However, surface modification or other postfabrication techniques are undoubtedly promising options for improving the biocompatibility and functionality of 3D bio-printing scaffolds.

In this study, a set of integrated manufacturing processes are realized for the first time, which combines bionic 3D structure design with postfabrication functionalization.[42] Research activities include: (1) Bio-printing of 3D fluid perfusion microstructural vascularized bone scaffolds by computer-aided design. (2) The bio-inspired intelligent release nano-coating was fabricated on the surface of the bio-printing scaffold (Fig. 8). The nano-coating was formed by adsorbing double growth factors (recombinant human bone morphogenetic protein and recombinant human vascular endothelial growth factor) by gelatin (Gel) and polylysine, respectively, and then assembled layer-by-layer on the 3D scaffold by electrostatic interaction (Gel/polylysine). Subsequently, under the action of matrix metalloproteinase-2 (MMP2) expression in human umbilical vein endothelial cells, gelatin was degraded,

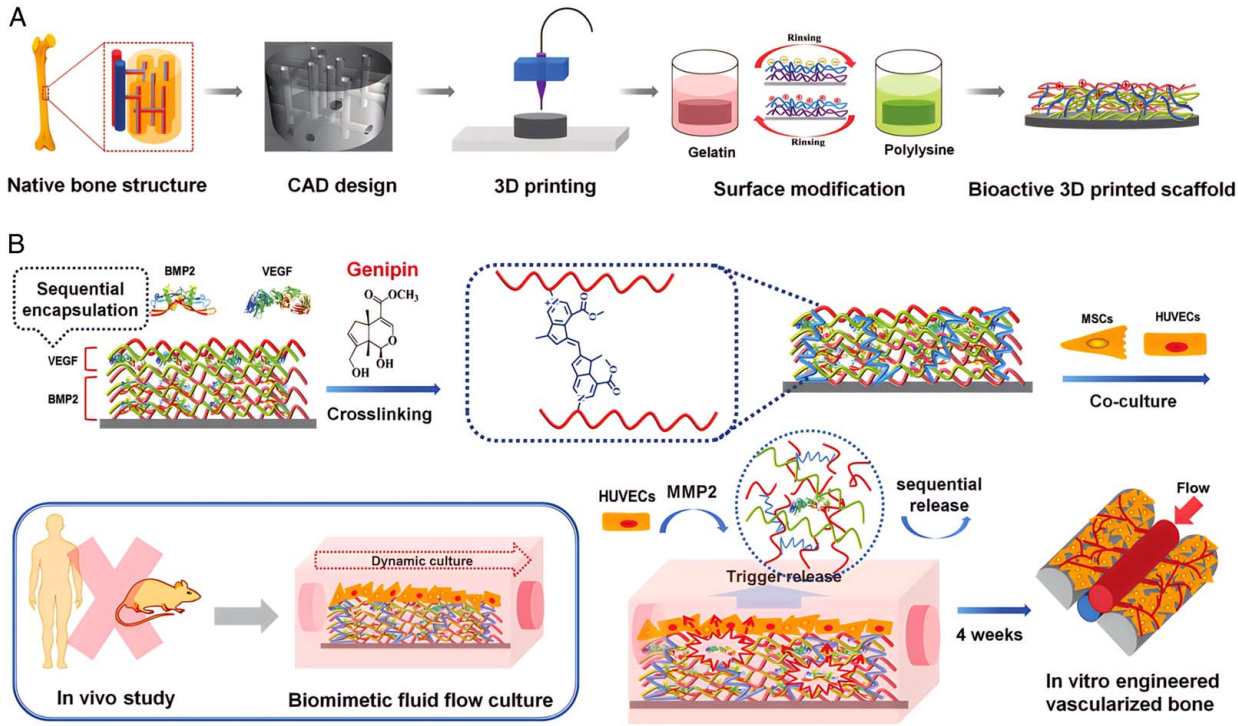

FIGURE 8. A and B, Schematic illustration of the fabrication process of nano-coating modified 3D bio-printed scaffolds.[42] 3D indicates 3-dimensional. full color online

and with the accumulation of MMP2, the nano-coating was split to coordinate spatial-temporal angiogenesis and osteogenic growth factor transmission.

Titanium Nanotube Nanotube Array Nano-drug Delivery System

Titanium nanotube (TNT) arrays were formed by self-ordered electrochemical anodization on titanium surface, and their excellent biocompatibility, controllable size, chemical stability, and bone bonding performance have become one of the most attractive implantable drug delivery solutions in recent years[100] Based on this, this paper proposes a new design strategy.[28–30] PMS are used as drug carriers and TNT arrays to prolong the release of low water-soluble drugs. The system provides 2 functions: multidrug delivery and sequential drug release (Fig. 9). Its basic principles are based on the unique

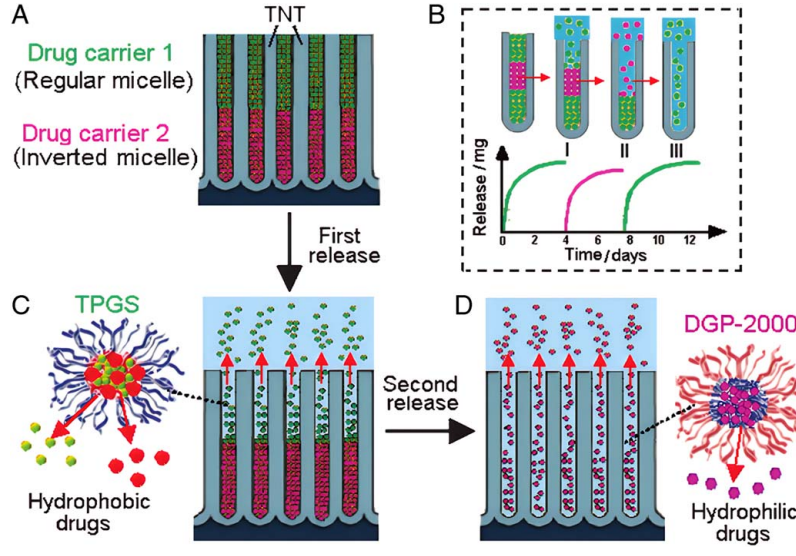

FIGURE 9. Scheme of multidrug release using TNT arrays and PMS as drug carriers. A, TNT loaded with two types of PMS, a regular micelle (TPGS) encapsulated with hydrophobic and inverted micelle (DGP 2000) encapsulated with hydrophilic drug. B, Scheme of sequential drug release with layered drug carriers with details of 2-step drug release (C and D).[28–30] DGP indicates 1,2- dimethyl myristate-rac-glycerol-3-methoxy polyethylene GlycolPMS; TNT, titanium nanotube; TPGS, toxic polyethylene glycol 1000 succinate. full color online

geometric characteristics of TNT and PMS. Specifically, conventional micelles are selected to cover hydrophobic micelles and inverted micelles of hydrophilic drugs with opposite interfacial properties (hydrophilic and hydrophobic) so that these layers do not mix with each other. Therefore, a method of publishing a series of sequential time controls is created. The immiscible layer of the proposed drug carrier will produce a unique nanotube release mode in continuous and independent steps, in which the number, time, and sequence of drug release can be controlled by loading conditions, TNT length, pore size, and surface properties of micelles.[22] Therefore, local drug delivery is considered to be an attractive solution, which overcomes the challenges of traditional drug delivery, such as low efficacy, poor bioavailability, uneven biological distribution, drug overdose, toxicity, and so on.

Polymer Path Nano-drug Delivery System

To overcome the side effects caused by damage to vascular endothelium after long-term systemic chemotherapy for cancer, the use of electrospun nanofibers loaded with antineoplastic drugs for local chemotherapy has attracted more and more attention.[41,101,102]

In this paper, a methoxy poly (ethylene glycol)-block polylactic acid-co-ethanolide (mPEG-b-PLGA) and dextran emulsion electrospun polymer patch with 10-hydroxycamptothecin (HCPT) in oil phase tea polyphenols (TPs) in water phase were prepared.[31] The electrospun polymer patch composed of core-sheath nanofibers assembles HCPT and TP on the same platform for the treatment of primary and advanced liver cancer in situ. After the membrane was applied to the orthotopic liver cancer, the 2 drugs were continuously released into the tumor. HCPT is a commonly used topoisomerase-1 inhibitor with a wide range of antitumor activities.[103,104] In general, HCPT can inhibit DNA replication and effectively prevent the proliferation of cancer cells. Phenolic derivatives, especially TP, can significantly inhibit the formation of ROS. TP extracted from green tea has been studied in many cancer prevention studies and showed significant ROS inhibition properties. The combination of the two drugs can induce mitochondrial-related apoptosis of cancer cells by increasing the expression of caspase-3.[21,105]

CONCLUSIONS AND PROSPECTS

The strategy of using nano-carriers combined with sequential release shows great potential in cancer therapy, mainly because this strategy improves the delivery efficiency of drugs targeting tumor tissues, and greatly improves the curative effect of anti-tumor therapy. In contrast, it can effectively solve the constraints caused by the combined application of two or more drugs. Each drug has its own unique pharmacodynamic and pharmacokinetic characteristics, and multiple chemotherapeutic drugs can be treated at the same time. From the perspective of pharmacodynamics, it may cause a certain loss to the curative effect of each drug. For example, the simultaneous release of multiple drugs may result in mutual restriction between drugs, and the maximum therapeutic effect of each drug cannot be achieved. In addition, it may produce more severe side effects than chemotherapy alone. Based on this, the emergence of a sequential drug delivery strategy has addressed the limitations of simultaneous treatment with multiple chemotherapeutic agents to some extent. In addition, this strategy can effectively combine more diverse drug release sequences in time and space, and avoid the differences in their pharmacokinetic characteristics. With the development of nanotechnology, today's nano-carriers involve the design and development of materials ranging from ~1 nanometer to even several hundred nanometers in size, making it possible to design and manufacture materials with certain molecular structures. Without the help of nano-carriers, the sequential release strategy can not be applied. In this review, we summarized the specific responses to internal stimuli (pH, redox, ROS, enzymes, etc) and external stimuli (near-infrared, magnetic response, etc). In addition, to obtain excellent antitumor effects through the sequential release of 2 drugs, there are also rapidly developing tumor sensitizers and chemotherapy drugs, drugs targeting cell signaling pathways, and gene drugs. In addition to the mentioned joint strategies, in recent years, advanced technologies, such as 3D printers, have also opened the door for the manufacture of specially designed drug/gene delivery vectors and arrays. These technologies simplify the preparation of carriers, which can load hydrophobic and hydrophilic drugs, and release them under different time and space parameters, thus reducing their toxicity. In fact, the sequential administration strategy of nano-carriers or external carriers prepared by advanced technology can also realize the sequential release of chemotherapeutic drugs, chemical substances, or biological substances in timely and controllable conditions, and to some extent, the antitumor efficacy. Although sequential drug delivery systems based on the stimulatory response of the internal and external tumor environment have been reported successively, the application of this therapeutic strategy in oncology treatment still faces many challenges. The tumors of different individuals, different tumors, and the internal and external microenvironments of the growth process are very different. What is more, during the internal circulation of the sequential drug delivery system, some normal cells also have some characteristics of the tumor cell microenvironment, which interferes with antitumor therapy. In addition, the complex interaction between tumor extracellular matrix and tumor cells also restricts antitumor therapy. In contrast, in the material research of sequential drug delivery systems, the in vivo solubility and biocompatibility of materials are the difficulties that need to be considered and paid attention to. There is a challenge to make progress, so it may bring some encouraging results to researchers in this field.

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