Advanced Drug Delivery Reviews 176 (2021) 113891

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/adr

Nanotechnology-based delivery of CRISPR/Cas9 for cancer treatment ☆

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ARTICLE INFO

Article history:
Received 18 March 2021
Revised 15 July 2021
Accepted 19 July 2021
Available online 26 July 2021

Keywords:
Cancer treatment
CRISPR/Cas9
Gene editing
Nanotechnology
Delivery vectors
Clinic application

ABSTRACT

CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats-associated protein 9) is a potent technology for gene-editing. Owing to its high specificity and efficiency, CRISPR/Cas9 is extensity used for human diseases treatment, especially for cancer, which involves multiple genetic alterations. Different concepts of cancer treatment by CRISPR/Cas9 are established. However, significant challenges remain for its clinical applications. The greatest challenge for CRISPR/Cas9 therapy is how to safely and efficiently deliver it to target sites in vivo. Nanotechnology has greatly contributed to cancer drug delivery. Here, we present the action mechanisms of CRISPR/Cas9, its application in cancer therapy and especially focus on the nanotechnology-based delivery of CRISPR/Cas9 for cancer gene editing and immunotherapy to pave the way for its clinical translation. We detail the difficult barriers for CRISIR/Cas9 delivery in vivo and discuss the relative solutions for encapsulation, target delivery, controlled release, cellular internalization, and endosomal escape.

Contents

☆ This review is part of the Advanced Drug Delivery Reviews theme issue on "Transl Drug Delivery".

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https://doi.org/10.1016/j.addr.2021.113891
0169-409X/

1. Introduction

As one of the deadliest diseases, cancer poses great threaten to human life [1]. Until now, many therapeutic methods, including radiation therapy, chemotherapy, or surgery, have been applied for cancer treatment, but the high recurrence frequency and chemo/radio resistance usually lead to poor treatment effect. It is well established that genetics play a significant role in cancer pathogenesis and growth by the accumulation of multiple genetic and epigenetic mutation [2]. The database of Catalogue of Somatic Mutations in Cancer highlights the direct impact of over 600 mutations in human tumors [3]. Therefore, to correct or disable the gene mutation in cancer cell should provide an alternative method for cancer treatment and the tool which can specifically induce genetic editing with high accuracy is required [4].

Currently, a new gene-editing technology named clustered regularly interspace short palindromic repeat associated protein 9 (CRISPR/Cas9) is discovered and developed [5]. In this system, the Cas9 endonuclease is accurately guided to target sites by a single guide RNA (sgRNA) and then produce the DNA double strand breaks (DSBs), resulting to site-specific genomic modification. Compared with conventional gene-editing methods, including ZFNs (zinc finger nucleases) and TALENs (transcription activator-like effector nucleases), CRISPR/Cas9-mediated gene editing is more flexible, effective and precise [6]. Since its first application in mammalian cells in 2013 [7,8], CRISPR/Cas9 technology has been studied extensively and its application has been expanded from the modification of the gene in cells to organisms [9]. The potential role of CRISPR/Cas9 in gene therapy has made it to become one of the hottest pots in cancer treatment. Different concepts of CRISPR/Cas9-mediated cancer therapy, including tumor-related genes manipulating, tumor immunotherapy, tumor research modelling and anti-cancer drug resistance overcoming are established in various cancer types [10].

To achieve effective and precise cancer treatment, the CRISPR/Cas9 components must directly go into the target cells by passing through different physical barriers. Furthermore, gene editing process needs the functionated Cas9 protein and sgRNA transporting to the nucleus at the same time [11]. Thus, the delivery method is especially important for CRISPR/Cas9-mediated editing therapeutics. Previously, both physical and viral vectors have been explored and utilized for CRISPR/Cas9 components delivery. The physical carrying approach, including microinjection, electroporation and hydrodynamic delivery show high efficiency for the application in vitro, but they are not satisfy requirement for in vivo applying [12]. Viral vectors are also facing some limitations in their transition into clinical grade therapeutics, which suffer with immunogenic response, limited packaging capacity, off-target effect and high cost in production [13–15]. As an alternative, non-viral vectors based on nanotechnology and material science have received more attention recently. Many different types of nanocarriers, including polymers, lipids, porous silicon, mesoporous silica nanoparticles, metal–organic frameworks etc., have been exploited in cancer therapy due to their low immunogenicity, high biocompatibility, and ideal cargo delivery capability [16]. Until now, studies on nanotechnology-based delivery of CRISPR/Cas9 mainly focus on the explanation and classification of materials. Herein, we will introduce the promising design of the nanocarrier and how they can enable the CRISPR/Cas9-based cancer treatment in vivo, even the future clinic applications. We first discuss the major characteristics of CRISPR/Cas9 gene-engineering technology and summarize its applications in cancer therapy. Then, we highlight different kinds of nanocarriers for anticancer cargo delivery. Finally, we focus on how to reasonably design the nanocarriers for CRISPR/Cas9 delivery in vivo, for improved oncogene editing and cancer immunotherapy. We consider the advances in nanotechnology-based delivery systems will broaden CRISPR/Cas9 therapy and give a promising method to meet the challenging clinical needs.

2. CRISPR/Cas9-mediated gene-editing system

2.1. Structure and feature of CRISPR/Cas9 system

CRISPR was first discovered in 1987, when a strange DNA pattern in the IAP gene of Escherichia coli was noticed [17]. This gene is composed of short ~30 base pairs of palindromic (relapsing/recurring) DNA repeats. The repeating pattern of direct repeats and similar size non-repetitive sequences are found commonly in many microbes in both domains of prokaryotes. Therefore, the family name, CRISPR is introduced [18]. Researchers found that CRISPR

had a specific role in bacteria's defense mechanism against foreign DNA (bacteriophages and plasmid conjugations) by intervening with them [19,20]. CRISPR with Cas genes associated with the prokaryotic immune system is shown later, where bacterial strains can adapt phage resistance by incorporating new spacer sequences. The sequences are homologous to the invasive nucleotides that come from the phage gene of the CRISPR and act through a gene disruption pathway [21–23]. This short 2–5 bp sequences from the region are identified as protospacer adjacent motifs (PAMs) [24].

CRISPR/Cas are mainly categorized into two classes (1 and 2) [3]. In class I CRISPR/Cas system, several Cas proteins are needed to achieve its application. But for the class 2 system, it only needs one protein [25]. Therefore, the class 2 system is more popular for gene-editing. Among different kinds of class 2 systems, CRISPR/Cas9 is the most extensively exploited due to its simplicity.

It is formed by three key components: the Cas9 protein, CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA) [26]. Specifically, Cas9 endonuclease contains six main domains, recognition (REC)1, REC2, Bridge Helix (BH), PAM Interacting (PI), HNH and RuvC (Fig. 1). The crRNA usually has a 20-nt protospacer sequence and an extra part used for tracrRNA complementary pairing. For tracrRNA, its structure has two functional parts used for crRNA and Cas9 protein binding, respectively. In addition, the crRNA-tracrRNA complex can be easily engineered as a single guide RNA (sgRNA) (Fig. 1) [27]. Briefly, the Cas9 endonuclease can be activated by binding with sgRNA using REC1 domain. Then it will search for the targeting DNA sequence which matches its PAM site. After the matching happening, the nuclease domains (HNH and RuvC) will cleave the target DNA like a scissor. During the whole process, PI domain in Cas9 protein is the key to initiate its endonuclease activity to bind the target DNA. In general, the coordination of several functional areas in CRISPR/Cas9 endows it with strong ability for gene editing.

2.2. Mechanism and advantage of CRISPR/Cas9 system

CRISPR/Cas9 technology can be served as a system for performing cutting of all tested DNA strands with great precision [27]. In this process, the sgRNA first binds to the target sequence after PAM reorganization and then leads Cas9 endonuclease to cleave the DNA strands up-stream of the PAM site, forming double-stranded breaks (DSBs) (Fig. 1) [28]. After DSBs are formed, the genome repair can be initiated in two pathways (Fig. 1), including the Non-Homologous End Joining (NHEJ) and Homology Directed Repair (HDR). As the most likely pathway to occur, NHEJ often results in the insertion/deletion (InDel) of edited DNA strands. The generation of InDel finally induces the frameshifts and/or the premature of stop codons [12]. Under the help of donor DNA template, HDR is enabled the correct DNA to be inserted into the target site accurately, whereas the NHEJ can join the break sequences directly. This can be exploited to insert the gene of interest by providing an adjacent exogenous DNA template to generate specially designed and modified DNA [29,30].

CRISPR/Cas9 system was first used in mammalian cells (both human and mouse cells) in 2013 [7]. Since then, it has been extensively applied to various research areas, such as biological study, biotechnology, medicine, agriculture etc. [31,32]. Compared with conventional genome engineering tools, CRISPR/Cas9 technology has shown its great advantages (Table 1). Briefly, the CRISPR/Cas9 system only requires a simple sgRNA for RNA-DNA reorganization, which is more specific, efficient, and less expensive than the protein-DNA-binding mechanism [33]. It is convenient and easy to personalize the CRISPR/Cas9 complex by only changing the sgRNA sequence. This allows it for multiple independent sites editing, simultaneously [2]. Furthermore, CRISPR/Cas9 is capable of chromosomal targets modification with high fidelity and low cytotoxicity.

2.3. Application of CRISPR/Cas9 in cancer treatment

2.3.1. Gene engineering in cancer

The main cause of tumors is the dysregulation of cell growth, including the activating of proto-oncogenes and the inactivating of tumor-suppressive genes [34,35]. Therefore, genome engineering technique offers a new hope for cancer treatment and the ability to edit multiple genes endows the CRISPR/Cas9 with great potential in cancer treatment.

According, the knock-out of oncogenes by CRISPR/Cas9 technology is a very helpful method to inhibit the tumor growth. On another hand, repairing tumor suppressor genes and restore their function can also inhibit the development of tumors [36]. Currently, CRISPR/Cas9 based gene therapy have been actively researched in lung cancer [37], breast cancer [38], head cancer [39], colorectal cancer [40], hepatocellular carcinoma [41], etc. Different kinds of target genes are involved, including EGFR (epidermal growth factor receptor), p53 (tumor protein P53), FAK (focal adhesion kinase), Nestin, BRCA (breast cancer gene), HER2/Neu (human epidermal growth factor receptor 2), TERT (telomerase reverse transcriptase), ALK (anaplastic lymphoma kinase), KRAS (Kirsten rat sarcoma viral oncogene homolog), BRAF (v-raf murine sarcoma viral oncogene homolog B), NOTCH1 (notch homolog 1), PTEN (phosphatase and tensin homolog), etc. CRISPR/Cas9 technology can also target specific disease-causing gene and construct cell- or animal-models of corresponding gene mutations. This allows for better understanding of the disease development mechanisms and treatment strategies. For example, Cheng-Chi Chao et al. used CRISPR/Cas9 to develop two KRAS-driven cancer systems: genome editing CRISPR/Cas9 for eliminating KRAS G12S mutant alleles and transcription regulation dCas9-KRAB for inhibiting KRAS transcription [42]. Experimental results shown that using CRISPR/Cas9 technology to specifically target oncogenic KRAS mutant alleles could significantly inhibit tumor growth [43]. Furthermore, CRISPR/Cas9 technology is also a good tool to study the pathogenesis of the diseases and identify the role of new oncogenes or tumor suppressor genes in the disease process. For instance, small cell lung cancer (SCLC) which is prone to recurrence is a high-grade neuroendocrine tumor, accounting for about 15% to 20% of lung cancer [44]. Studies have shown that p107 and p130 (members of the retinoblastoma protein family) have repeated mutations in approximately 6% of human SCLC tumors [45]. The results were verified by Tyler Jacks and coworkers who simulated the deletion of p107 and p130 in Trp53/Rb1 via an SCLC mouse model constructed using CRISPR/Cas9 system [46]. The research above proves the potential of the functions of other candidate genes frequently mutated in SCLC and will also help to verify future SCLC therapeutic targets. To date, the main research findings of cancer-related mutation by CRISPR/Cas9 in cancer treatment are shown in Table 2.

The CRISPR/Cas9 systems not only directly target oncogenes to inhibit tumor growth, but also carry out large-scale cancer gene screening to improve the efficiency of anti-cancer drug development. Recently, Wellcome Sanger Research Institute, GlaxoSmithKline (GSK), EMBL-EBI and OpenTarget used genome-scale CRISPR screening technology to construct a new cancer-dependent gene resource library and developed a gene score calculation framework for discovering new targets. The research team conducted CRISPR/Cas9 screening on>300 human cancer cell lines from 30 cancer types. They combined cellular adaptive effects, genomic biomarkers, and drug development targets to systematically prioritize new targets for specific tissues and genotypes. Using this method, the researchers finally verified that Werner syndrome ATP-

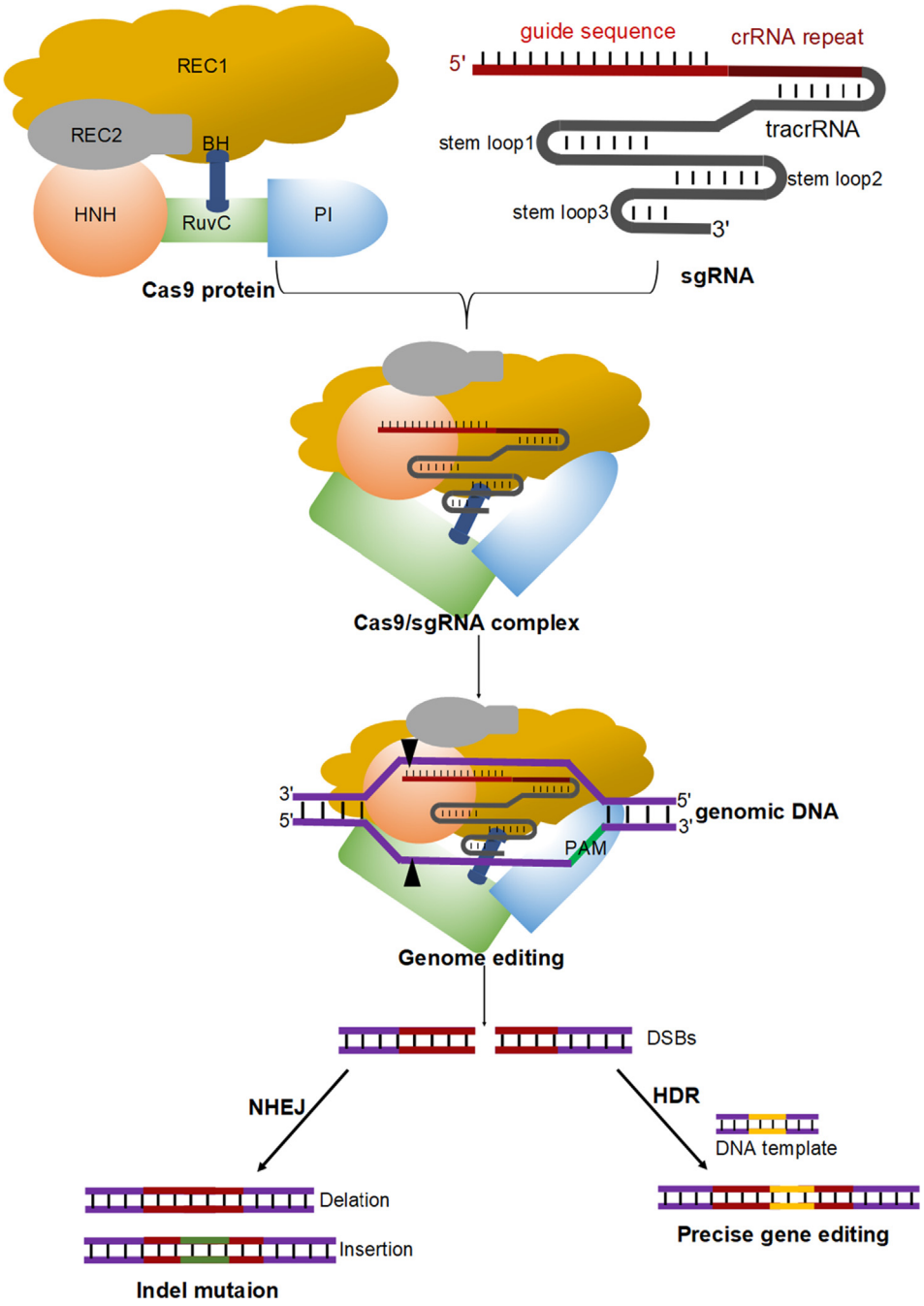

Fig. 1. Schematic structures of CRISPR/Cas9 (REC1, REC2, BH, PI, HNH and RuvC) and sgRNA (crRNA and tracrRNA) and molecular mechanism of its gene-editing process.

Table 1
Comparation of the different gene-editing technologies.

Technology	ZFNs	TALENs	CRISPR/Cas9
Endonuclease	FokI	FokI	Cas9
DNA targeting	ZF protein	TALE protein	SgRNA (crRAN + trancrRNA)
Targeting mechanism	Protein-DNA-binding	Protein-DNA-binding	RNA-DNA reaction
PAM	NA	NA	5′-NGG-3′
Targeting efficiency	Low	Low	High
Off-targeting	High	High	Variable
Operation cost	High	High	Low
Operation feasibility	Difficult	Moderate	Easy
Cytotoxicity	High	Low	Low

dependent helicase (WRN) could be used as a synthetic lethal target for a variety of cancer types with characteristics of microsatellite instability [93].

2.3.2. Clinical potential in cancer

According to the good performance of pre-clinical research, CRISPR/Cas9 system has been transformed into clinical applications. Since 2016, 18 clinical trials have been launched and 8 of them are immunotherapies for programmed cell death-1 (PD-1) protein. The detail information is shown in Table 3. Accordingly, PD-1 and programmed cell death ligands (PD-L1) play an important role in immune therapy [94]. PD-1 is a membrane protein found on many immune cells (T cells, B cells and myeloid cells), while PD-L1 is expressed on many tumor cells. The engagement

Table 2
CRISPR/Cas9 mediated gene editing for cancer-related mutations.

Cancer types	Gene targets	Cells or tissues	Function	Gene-related Function	Ref.
Lung cancer	*FGFR*	PC9, HCC827 and HCC4006 cell lines	Drug-resistant	overcome EMT-associated acquire drug resistance	[47]
	IGF1R	HCC827 cells	Drug-resistant	amplify the MET gene and acquire erlotinib resistance	[48]
	NOP10	H1975 and H358 cell lines	OG	inhibit cell growth, colony, migration, and invasion	[49]
	STK11	A549 cells	TS	promote tumor growth, resistance to therapies and cause early death	[50]
	KEAP1				
Breast cancer	*P53*	human organoids from breast epithelial subsets	TS	gained long-term culturing capacity	[51]
	PTEN				
	RB1				
	MIEN1	MDA-MB-231cells	OG	reduce disease progression and metastatic	[52]
	NAT1	MDA-MB-231 cells	Metabolism-related	reduce pyrimidine biosynthesis and β-oxidation of fatty acids	[53]
	HSD17B11	MDA-MB-231 cells	OG	mediate the selective cytotoxic effects	[54]
	PARP1	MDA-MB-231 and MDA-MB-436 cell lines	OG	enhance the effeteness of chemotherapy	[55]
	FASN	MCF-7 cells	OG	reduce proliferation, migration, and viability	[56]
HCC	*ADAMTSL3*	human SMMC7721 HCC cells	TS	increase cell proliferation and metastasis	[57]
	PTEN				
	EGFR	HepG2 cells	OG	inhibit cell proliferation	[58]
	SGOL1	Huh7 and SMMC-7721 cell lines	Drug-resistant	suppress the active of sorafenib	[59]
	NCAPG	HCCLM3 and HepG2 cell lines	OG	impair cell viability and increased cell death	[60]
Colorectal cancer	*RASGAP*	patient-derived CRC organoids	TS	enhance RAS-ERK signal and promote resistance to EGFR inhibition	[61]
	EPHA1	HRT18 cells	TS	increase cell spread and adhesion	[62]
	LGALS2	HEK293 cells	TS	increase cell growth	[63]
	ATF3	HCT116 and RKO cell lines	OG	inhibit cell invasion and proliferation	[64]
Ovarian cancer	*BIRC5*	SKOV3 and OVCAR3 cell lines	OG	inhibit epithelial to mesenchymal transition	[65]
	ZNF587B	SKOV3 and A2780 cell lines	Drug-resistant	overcome cisplatin resistance	[66]
	SULF1				
	BMI1	EOC cells	OG	reduce cell growth and metastasis, promote cell apoptosis	[67]
	OC-2	SKOV3 and CAOV3 cell lines	OG	inhibit cell proliferation, migration, and invasion	[68]
	EGFL6	SKOV3 cells	OG	inhibit cell proliferation, migration, and invasion	[69]
Melanoma	*PTGS2*	B16F10 cells	OG	inhibit cell proliferation, migration, and invasion	[70]
	SIRT6	A375 cells	TS	decrease cell growth and viability	[71]
	CD133	BAK-R cells	OG	reduce cell invasion and levels of MMP2/MMP9	[72]
	FBXO42	SK-MEL-147 cells	Drug-resistant	overcome trametinib resistance	[73]
	AHR	HCmel12 cells	OG	impaired tumor growth and lung metastatic	[74]
Glioma	*PLEKHG5*	U251-MG cells	OG	Inhibit NF-κB activation and autophagy	[75]
	EGFRvIII	H9-hESCs cells	OG	induced astrogenesis and cell proliferation	[76]
	MAP4K4	U138 cells	OG	reduced cell migration and invasion	[77]
	IGF2BP1	A172 cells	OG	inhibited cell survival and proliferation	[78]
	Lnc-THOR				
	Ninj2	A172 and U251MG cell lines	OG	inhibited cell survival, growth, proliferation, migration	[79]
Gastric cancer	*PDEF*	AGS cells	OG	inhibits migration and invasion	[80]
	SST	BGC823 cells	TS	enhances cells migration and invasion	[81]
	TFF1	mice	TS	promote tumorigenesis	[82]
Pancreatic cancer	*HIF-1α*	BxPC-3 cells	OG	suppress cells metastasis	[83]
	PSMA6	HPNE, HPAF-II, AsPC-1, and Mia PaCa-2 cell lines	OG	induce cells apoptosis	[84]
	KDM6A	BxPC3 and PANC1 cell lines	TS	enhance cells aggressive	[85]
Osteosarcoma	*CD44*	KHOSR2 and U-2OSR2 cell lines	Drug-resistant	inhibit migration and invasion; enhance drug sensitive	[86]
	ABCB1	KHOSR2 and U-2OSR2 cell lines	Drug-resistant	reverse drug resistance	[87]
	TP53	KHOS and KHOSR2 cell lines	OG	decrease cells proliferation, migration, and tumor formation	[88]
	Sox2	RB and p53 knockout mouse and mOS-202 cells	OG	loss cells viability and proliferation	[89]
Cervical cancer	*E6*	SiHa and C33-A cells and xenograft mouse	OG	inhibit tumor growth	[90]
	E7				
	Trio	Caski and HeLa cells	OG	decrease cells migration and invasion	[91]
	c-REL	HeLa cells	OG	decreased cell proliferation	[92]

FGFR: fibroblast growth factor receptors; *BCAR1*: Breast cancer anti-estrogen resistance protein 1; *IGF1R*: insulin-like growth factor; *NOP10*: H/ACA box protein; *STK11*: serine/threonine kinase 11; *KEAP1*: Kelch-like ECH-associated protein 1; *RB1*: retinoblastoma protein; *MIEN1*: Migration and Invasion Enhancer; *NAT1*: Human arylamine N-acetyltransferase 1; *HSD17B11*: HSD17B11; *PARP1*: Poly [ADP-ribose] polymerase 1; *FASN*: encodes Fatty acid synthase; *ADAMTSL3*: ADAMTS-like protein 1; *SGOL1*: Shugoshin-like 1; *NCAPG*: non-SMC condensin I complex subunit G; *RASGAP*: RAS p21 protein activator 1; *EPHA1*: ephrin type-A receptor 1; *LGALS2*: encoded Galectin-2 protein; *BIRC5*: baculoviral inhibitor of apoptosis repeat-containing 5; *ZNF587B*: cisplatin-related resistance gene; *SULF1*: Sulfatase 1; *BMI1*: Polycomb complex protein; *OC-2*: ONECUT Homeobox 2 gene; *EGFL6*: Epidermal growth factor-like domain multiple 6; *PTGS2*: Prostaglandin-endoperoxide synthase 2; *SIRT6*: Sirtuin 6; *CD133*: prominin-1; *FBXO42*: Kelch domain-containing F-Box protein 42; *AHR*: aryl hydrocarbon receptor; *PLEKHG5*: Pleckstrin homology containing family member 5; *EGFRvIII*: epidermal growth factor receptor variant type III; *MAP4K4*: mitogen-activated protein 4 kinase 4; *IGF2BP1*: insulin-like growth factor 2 (IGF2) mRNA-binding protein 1; *Lnc-THOR*: Long non-coding RNA THOR; *Ninjurin2*: a homolog of ninjurin1; *PDEF*: Prostate-derived Ets factor; *SST*: Somatostatin; *TFF1*: Trefoil factor 1; *HIF-1α*: Hypoxia-inducible factor 1-alpha; *PSMA6*: proteasome subunit alpha type-6; *KDM6A*: Lysine-specific demethylase 6A; *CD44*: Cluster of differentiation 44; *ABCB1*: P-glycoprotein 1; *TP53*: Tumor protein P53; *Sox2*: SRY (sex determining region Y)-box 2; *E6/E7*: human papillomavirus oncogenes; Trio: Triple functional domain protein; *c-REL*: NF-κB subunit

of PD-1 and PD-L1 usually suppresses antitumor T-cells activities, contributing to the escape of tumor cells from immune system. In 2016, Chinese scientists took the first human phase I clinical trial based on CRISPR/Cas9, and they also firstly used CRISPR/Cas9 technology to knock out PD-1 for Metastatic Non-small Cell Lung Cancer (NCT02793856) treatment. Except lung cancer, the clinical trials of CRISPR/Cas9 mediated PD-1 knock-out have also been applied for other types of cancers, including liver cancer

Table 3
List of Clinical Trials based on CRISPR/Cas9 system in cancer treatment.

Condition or disease	Target site	Phase	NCT No.	First Posted Time
B Acute Lymphoblastic Leukemia	TRAC/CD52	I	NCT04557436	2020.08.12
Renal Cell Carcinoma	CD70	I	NCT04438083	2020.06.18
Gastro-Intestinal (GI) Cancer	CISH	I/II	NCT04426669	2020.06.11
Advanced Hepatocellular Carcinoma	PD-1	I	NCT04417764	2020.06.05
Multiple Myeloma	BCMA	I	NCT04244656	2020.01.28
Relapsed or refractory B-cell malignancies	CD19	I	NCT04035434	2019.07.29
Solid Tumor, Adult	PD-1	I	NCT03747965	2018.11.20
High Risk T-Cell Malignancies	CD7	I	NCT03690011	2018.10.01
Solid Tumor, Adult	PD-1 and TCR	I	NCT03545815	2018.06.04
Prostate Cancer	PD-1	I/II	NCT03525652	2018.05.16
Relapsed or refractory leukemia and lymphoma	CD19 and CD20 or CD22	I/II	NCT03398967	2018.01.16
Multiple Myeloma; Melanoma; Synovial Sarcoma; Myxoid/Round Cell Liposarcoma	PD-1 and TCR	I	NCT03399448	2018.01.16
Tumors of the Central Nervous System	NF1	Suspended	NCT03332030	2017.11.06
$CD19^+$ leukemia and lymphoma	TCR and B2M	I/II	NCT03166878	2017.05.25
Esophageal Cancer	PD-1	II	NCT03081715	2017.03.16
Human papillomavirus-related malignant neoplasm	HPV16-E6/E7 HPV18 E6/E7	I	NCT03057912	2017.02.20
EBV positive advanced stage malignancies	PD-1	I/II	NCT03044743	2017.02.07
Metastatic Non-small Cell Lung Cancer	PD-1	I	NCT02793856	2016.06.08

NCT No. National Clinical Trial Number. TARC: T cell receptor (TCR) alpha constant; CISH: Cytokine-inducible SRC homology 2 (SH2) domain protein; PD-1: Programmed cell death protein 1; BCMA: Targeting B Cell Maturation Antigen; TCR: T-cell receptor; NF1: Neurofibromatosis type I; B2M: Beta 2 Microglobulin; HPV16-E6/E7: Human papillomavirus type 16 E6 and E 7 proteins; HPV18 E6/E7: Human papillomavirus type 18 E6 and E 7 proteins.

(NCT04417764), prostate cancer (NCT03525652), esophageal cancer (NCT0308171) and EBV (Epstein-Barr virus) positive advanced malignant tumors (NCT03044743).

Beyond that, T cells from cancer patients are also edited by CRISPR/Cas9 technology and then transferred back to patients after editing. Treatment methods using CRIPR/Cas9 technology include engineering editing of T cells, modifying TP53 and NF-1 (neurofibromatosis type 1) genes, inhibiting RIPK1 (receptor-interacting serine/threonine-protein kinase 1) and CISH (Cytokine-inducible SH2), inactivating TIL (temperature-induced lipocalin-1) or silencing TRC signals in $CD8^+$ cells. Researchers at the University of Pennsylvania first conducted the clinical trials. They used CRISPR/Cas9 to eliminate tumor immune escape. They investigated the importance of NY-ESO-1 redirected engineered T cells in a variety of cancer types (NCT03399448). Other clinical trials for $CD19^+$ leukemia and lymphoma (NCT03166878) were developed that used CRISPR/Cas9 system to destroy endogenous TCR and B2M (Beta-2-Microglobulin) genes to generate the allogeneic chimeric antigen receptor (CAR) T cells. This method helped avoid host-mediated immunity, thereby reducing the development of graft-versus-host disease. However, some patients are lacking the CD19 target. To tackle this, the clinical trial focused on CRISPR/Cas9-edited bispecific CD19 and CD20 or CD22 CAR-T cells was developed, in which the CD19-negative cells can be killed by CD20 or CD22 recognizing (NCT03398967).

Human papillomavirus (HPV) infection is positively correlated with the incidence rate of cervical cancer, in which HPV E6 and E7 genes are important for initiating and maintaining the growth of tumor cells [95]. When knocking out the E7 in cervical cancer, the tumor will be inhibited efficiently, and tumor cell apoptosis will be promoted. In a clinical trial group (NCT03057912), CRISPR/Cas9 was successfully used to destroy E6 and/or E7 to interfere with the proliferation and survival of cervical cancer cells.

Although CRISPR/Cas9-mediated gene editing shows its promising results in clinical studies, deeper study is still required to ensure that CRISPR/Cas9 can be applied for human cancer treatment safety and efficiency. Moreover, the off-target effect and delivery approaches of CRSIPR/Cas9 still need to be considered. Very recently, a lipid nanoparticle based CRISPR/Cas9 gene-editing system (NTLA-2001) had been used for transthyretin amyloidosis (ATTR) treatment and created the first-ever clinical data of therapeutic gene editing within patients' bodies (NCT04601051) [96]. The NTLA-20021 findings demonstrated our capability to clinical-scale nanotechnology-based CRISPR/Cas9 delivery platform for genetic disorders therapy.

3. Nanotechnology-based delivery system for cancer treatment

Owning to the achievements in cancer treatment, different types of anti-cancer components have been designed and shown satisfactory results in inhibiting the growth of cancer cells, including chemotherapeutic drugs (doxorubicin (DOX), 5-fluorouracil (5-Fu)), anti-cancer peptides (ACPs) and nucleic acid therapeutic agents [97–100]. However, direct injection of these drugs to the bloodstream can also damage healthy cells and lead to serious adverse effects. Besides, problems with rapid metabolism also affect the efficiency of the drugs. Due to that, many researchers begin to search solutions for the precise delivery of anti-cancer drugs to targeted lesion locations. The ideal advanced delivery system should simultaneously satisfy the needs of biocompatibility, high-dose cargo loading and controlled release functions. In addition, the carrier materials themselves should be chemically and biologically inert and must not react with anti-cancer substances. Recently, a lot of efforts made by researchers in nanotechnology area have clarified the possibility of multifunctional nanocarriers [101,102]. According to that, we summarize the recent research trends and present the general working mechanisms of various types of nanocarriers, including polymers nanoparticles (micelle, dendrimer, vesicle, and hydrogel), lipids nanoparticles (SLNs, NLCs, liposome and noisome), porous nanoparticles (porous silicon, mesoporous silica nanoparticles, metal–organic frameworks) and other kinds of inorganic nanoparticles, such as gold nanoparticles and quantum dots. The rough structure illustrations of these materials are given in Fig. 2.

3.1. Polymer nanoparticles

Polymer materials are regarded as a potential and powerful delivery platform because of their main characteristics, including long blood circulation, high drug encapsulation, good biocompatibility, and relatively simple functionalization [103]. Polymer complexes such as micelles, dendrimers, vesicles, and hydrogels have been used as nanocarriers for anti-cancer drugs delivery.

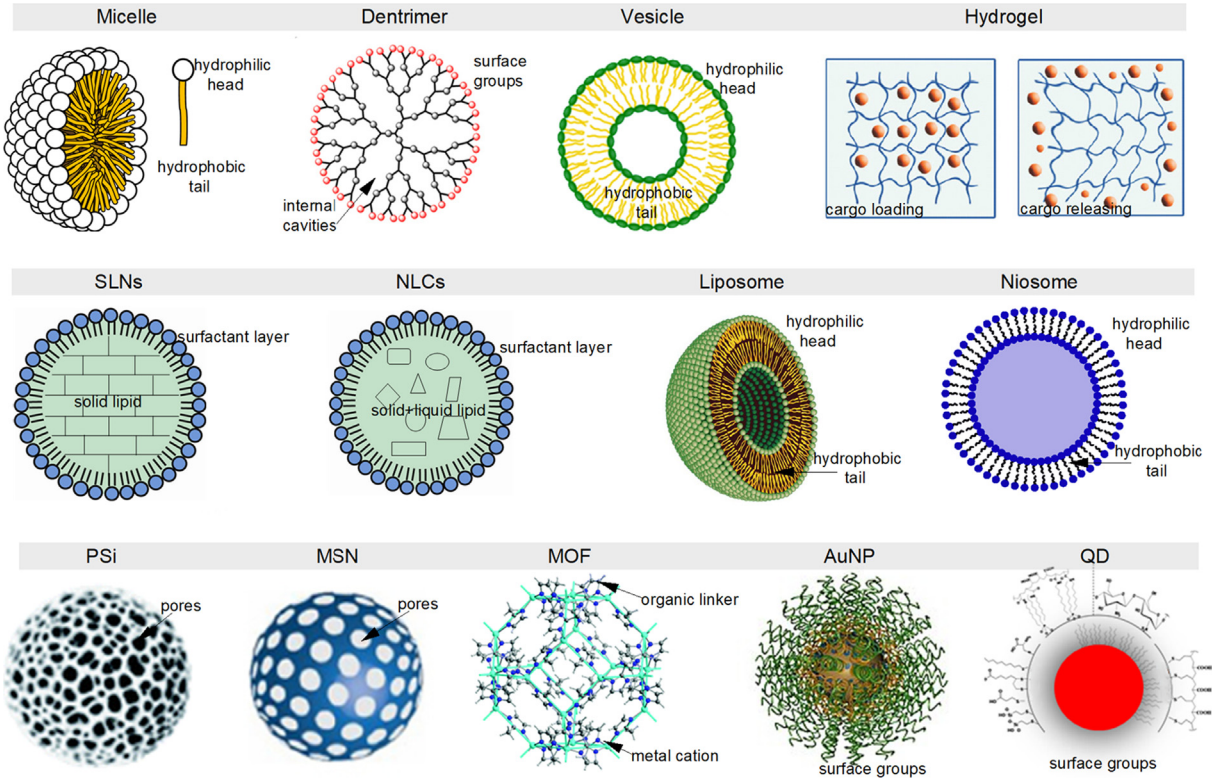

Fig. 2. Structural illustration of different nanocarriers for anti-cancer drugs delivery.

3.1.1. Micelles

Micelles composed of hydrophobic and hydrophilic components can be assembled into nano-scale spherical, elliptical, cylindrical or monolayer structures [104]. In aqueous medium, micelle core is hydrophobic, and micelle shell is hydrophilic, which can protect delivered cargoes from the immune clearance. Hence, micelles are especially suitable for the delivery and stabilization of hydrophobic anti-cancer drugs, like calprotectin and paclitaxel (PTX). The amount of the encapsulated drug is much more than its saturated concentration in aqueous solution. These structural characteristics make the micelles available for hydrophobic anti-cancer drugs delivery. They can improve drug stability, thereby improving the anti-cancer effect [105]. For example, Shi et al. prepared a stable micelle for PTX delivery (Fig. 3a) [106]. The micelle was assembled by methoxy poly(ethylene glycol)-b-(N-(2-benzoy loxypropyl)methacrylamide) (mPEG-b-p(HPMAm-Bz)) block copolymers. These micelles had attractive delivery capacity and drugs retention ability because of the π-π stacking interactions between aromatic groups in polymer chains and PTX. For anti-tumor study, the drug loaded micelles shown significant increase of PTX in tumor and improvement of PTX pharmacokinetics, inducing complete tumor suppression in different tumor models. The results implied that stable polymeric micelles based on π-π interaction are an effective nanocarrier for the improved delivery of hydrophobic drugs into tumors. Mi et al. successfully synthesized a new micelle (Vitamin E $TPGS_{2k}$) and found its critical micelle concentration (CMC) was much lower than that of conventional TPGS, which greatly enhanced its micelle stability (Fig. 3b) [107]v Therefore, $TPGS_{2k}$ micelles were developed for Docetaxel delivery and the encapsulation efficiency was as high as 80%. Compared with other drug delivery systems, TPGS2k shown more improved ability for drug delivering. $TPGS_{2k}$ was also coupled to ligands (e.g., folic acid) for targeted delivery of anticancer drugs, thus further enhancing cellular uptake and consequently in vitro therapeutic effects.

3.1.2. Dendrimers

Dendrimers are artificial, hyperbranched nano-polymer structures with many branches irradiating from the center to form the ideal three-dimensional geometry [108]. Dendritic polymers have three main parts: a centralized core, a middle or interior section made up of repeating branches which makes up each generation, and an outer shell defined by terminal functional groups. According to studies based on dendrimers, they are nanoscale molecules with the diameter ranging from a few nanometers to tens of nanometers. As one of its important applications, dendrimers can be used as carriers of various anti-cancer drugs [109]. Many studies of dendrimer-mediated targeted drug delivery have been developed. Sun et al. constructed a dendrimer/lipid nanocomplex using hydrophobic and hydrophilic dendrimers and phospholipids (Fig. 3c) [110]. They packed the anti-cancer drug DOX to the dendrimer of poly(amino ester) (PAE). And the anti-cancer drug methotrexate (MTX) was packed into the hydrophilic dendrimer, poly(amidoamine) (PAMAM) dendrimer, the loaded PAMAM dendrimer was also covered inside the lipid layer with the help of the anionic surfactant sodium dodecyl sulfate via both electrostatic and hydrophobic interactions. In addition, they investigated the release pathways of different nanocarriers in tumors. For DOX-PAE/lipid complex, it could fuse with cell membrane and then induce the intracellular or extracellular release of the dendrimer. But for MTX-PAMAM/lipid, it was internalized into the cancer cells by endocytosis. The change in the microenvironment generated the breakdown of MTX-PAMAM/lipid. In this process, two drugs were released in the tumor site, improving the synergistic effect of anti-cancer.

3.1.3. Polymersomes

Polymersomes are one kind of polymeric vesicles self-assembled from amphiphilic block-copolymers [111]. Unlike polymers such as micelles, polymersomes are completely enclosed bilayer structures with a certain amount of water inside. This

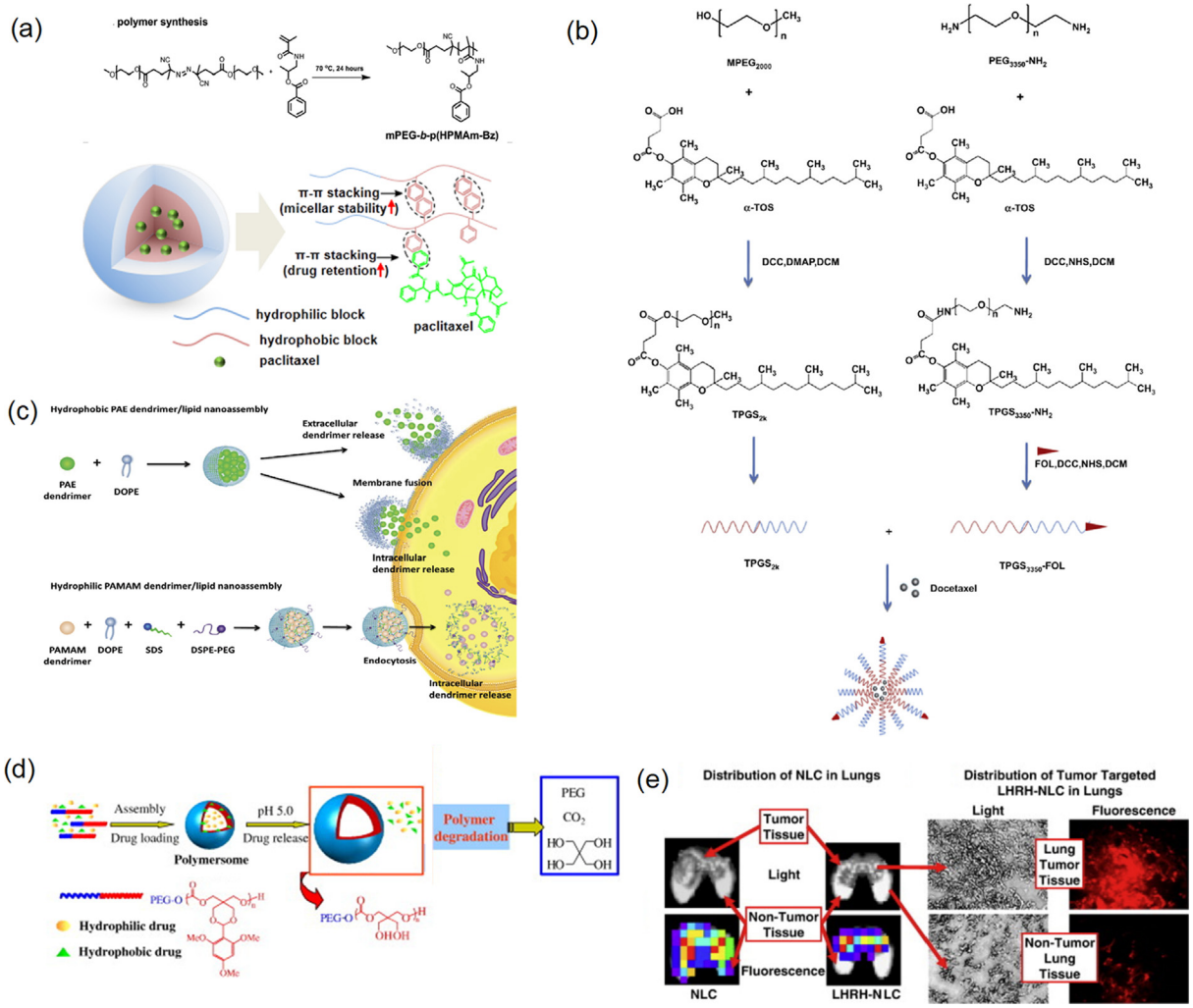

Fig. 3. (a) Preparation of mPEG-b-p(HPMAm-Bz) and drug loading process via π-π stacking. Adapted with permission from [106]. Copyright (2015), American Chemical Society. (b) Preparation scheme for $TPGS_{2K}$ and folic acid conjugated $TPGS_{2k}$ ($TPGS_{2K}$-FOL) micelles and Docetaxel loading process. Adapted with permission from [107]. Copyright (2011), Elsevier. (c) Illustration of lipidic nanocomplex from self-assembly of hydrophobic or hydrophilic dendrimers with phospholipids and their different pathways of dendrimer release. Adapted with permission from [110]. Copyright (2016), Royal Society Chemistry. (d) Illustration of pH-sensitive degradable polymersomes based on PEG–PTMBPEC diblock copolymer for triggered release of both hydrophilic (DOX) and hydrophobic (PTX) anticancer drugs. Adapted with permission from [113]. Copyright (2010), Elsevier. (e) Distribution of fluorescently labeled (Cy5.5) non-targeted and LHRH-tumor targeted NLC in mouse lungs bearing human lung cancer. Adapted with permission from [124]. Copyright (2013), Elsevier.

allows polymersomes to load with different kinds of drugs with hydrophilic and hydrophobic properties. Furthermore, their ideal biocompatibility, robustness, good colloidal stability, and simple functionalization approaches make polymersomes a powerful material for anti-cancer drug delivery [112]. To improve their therapeutic efficiency, the polymersome with stimuli-responsive ability has also been developed to obtain the controlled release of anti-cancer drugs at the tumor sites. Chen and coworkers constructed a pH-sensitive degradable polymersome for drug delivery. The polymersome was composed by diblock copolymer of poly (ethylene glycol) (PEG) and an acid-labile polycarbonate, poly(2,4 ,6-trimethoxybenzylidenepentaerythritol carbonate) (PTMBPEC) (Fig. 3d) [113]. They found the designed polymersome had the ability to load PTX and DOX simultaneously. The releasing profile of the delivered drugs was strongly pH dependent. Compared with the release process in physiological pH, more drug was released at mildly acidic pH (4.0 or 5.0) due to acetal hydrolyzing. Except for acetal, various pH-sensitive groups, including hydrazine, orthoester and vinyl ester have been applied to synthesize pH-responsive polymersomes [112]. In recent years, the other types of stimuli-responsive polymersomes like redox-, temperature-, photo have also been widely developed [114]. Importantly, the design of novel polymers should be a useful way to widen the fabrication of polymersomes in the application of anti-cancer drugs delivery.

3.1.4. Hydrogels

Hydrogels were one kind of soft materials with a three-dimensional network structure, which contain a lot of water molecules [115]. In the early days, due to the poor mechanical properties of hydrogels, drug stability and release could not be controlled, and its application in drug delivery was limited. Recently, the improvement of hydrogel materials by researchers has greatly increased its potential for drug delivery [116]. Existing studies have shown that hydrogel-based nanocarrier improves the efficacy of anti-cancer drugs and gene therapy by extending the life of drugs in vivo, promoting controlled drug release and reducing non-targeted exposure. The biggest limitation of current cancer treatments using siRNA technology is delivery, which includes localizing siRNA at specific target cell populations and maintaining

the ability of siRNA at a certain timepoint. Nguyen et al. designed a functional, biodegradable siRNA delivery system in which cationic linear polyethyleneimine (LPEI) was covalently incorporated into a photo-crosslinked dextran hydrogel through a biodegradable ester bond [117]. The covalent binding of LPEI was not affect the property and structure of the hydrogel, while the addition of siRNA and LPEI had little effect on its mechanical properties. The negatively charged siRNA was retained in the hydrogel by electrostatic interaction with positively charged LPEI, and the release profile could be controlled by adjusting the degradation rate of the hydrogel. The hydrogel delivery system that allowed the adjustable and controlled release of biologically active siRNA for a long time could be widely applied in various cancer gene therapy and regenerative medicine applications.

3.2. Lipid nanoparticles

For several years, lipid-based delivery systems have got serious attention mainly due to their capability of permeating challenging physiological barriers, simple synthesis, low cost and easy of large-scale production [118]. Depending on the preparation method and physicochemical characteristics, lipid nanoparticles can be categorized into different types, including solid lipid nanoparticles, nanostructure lipid carriers, liposomes and niosomes.

3.2.1. Solid lipid nanoparticles (SLNs)

Among different lipid-based delivery systems, SLNs which are made from solid lipids is the materials that have been studied earlier [119]. SLNs are spherical particle with the size between 50 and 1000 nm. Anti-cancer drugs encapsulation has been deeply studied in SLNs. There are three different approaches for drug encapsulation in the lipid matrix, including solid solution, drug-enriched shell, and lipid shell model [120]. In the solid solution model, the drugs are dispersed on a molecular basis in the lipid matrix. The drug-enriched shell is formed during the cooling process of SLNs preparation due to phase separation between drugs and lipids. The lipid shell model, or drug-enriched core, is obtained when the drugs is precipitated before the lipid is recrystallized. Hence, SLNs can incorporate the drugs hydrophilic or hydrophobic and be constructed as a controlled and targeted delivery system. For instance, Rehman et al. reported a thermo-responsive SLN for 5-Fu delivery [121]. The nanocarrier was fabricated by a mixture of solid and liquid lipids (lauric acid, oleic acid, and linoleic acid). Drug releasing study indicated > 90% loaded 5-Fu was released at 39 ℃ after 5 h due to the solid–liquid phase transition at melting point, whereas just 22%-34% of drug releasing was observed at 37 ℃. Apart from stimulative drug releasing, SLNs-based nanocarrier can also achieve targeting drug delivery through surface engineering. Wang and coworkers developed Pluronic 85 (P85) coated SLN and applied it for PTX delivery [122]. The nanocarrier was synthesized using the hot homogenization approach with a PTX loading content of 4.9%. Different with free PTX releasing, he studies suggested PTX loaded SLN could release drug sustainedly. After administration, the HA modification endowed the PXT loaded SLN with high accumulation in tumor site and significant tumor suppression.

3.2.2. Nanostructure lipid carriers (NLCs)

NLCs are another lipid-based nanoparticle formed from the mixture of solid and liquid lipids, which have different structures according to the production method and lipid blend composition [120]. In the imperfect structure, the crystallization of NLCs is altered by few oils. The amorphous structure can obtain by special lipids mixture, like hydroxyoctacosanyl hydroxystearate with isopropyl myristate. The multiple structure is one solid lipid matrix with little oil ingredients, producing by mixing a solid lipid with an additional quantity of oil. Compared with SLNs, NLCs have higher drug loading efficiency because of their special crystal structure [119]. In addition, the presence of liquid lipids in NLCs can reduce the expulsion of loaded drugs after formulation and storage period. Therefore, NLCs have been widely explores as potential topical delivery vectors. For instance, Wang et al. constructed one kind of NLCs using melt-emulsification method and applied it for anti-cancer drugs (PTX and DOX) delivery for lung cancer treatment [123]. The nanocarriers shown encapsulation efficiency of 82% and loading efficiency of 10% for PTX and 84% and 10% for DOX, respectively. The cancer treatment ability was investigated and shown good results both in NCL-H460 cells and NCL-H460 infected mice. Taratula et al. synthesized a versatile NLC-based nanocarrier and used to co-deliver of anti-cancer drugs and siRNA (Fig. 3e) [124]. The NLCs was prepared by mixing the oil phase containing precirol ATO5, squalene and lipophilic emulsifier and aqueous phase containing tween-80, DOTAP and DSPE-PEG-COOH. After modified with luteinizing hormone-releasing hormone (LHRH), the NLC-based nanocarriers achieved the goals, including (1) co-delivery of DOX and PTX along with siRNAs effectively; (2) targeting to lung cancer cells without healthy lung tissues damage; (3) effective tumor growth control by the synthetic effect of anti-cancer drugs and drug resistance suppression.

3.2.3. Liposomes

Liposomes refer to small lipid vesicles with a particle size between 50 and 1000 nm [125]. Liposome are mainly made of a hydrophilic head part and a hydrophobic tail. As liposomes have unique advantages over traditional drug therapies, including protecting drugs from degradation, delivering drugs to targeted sites, and having the ability to reduce drug toxicity, therefore, liposomes were widely studied and applied for anti-cancer drugs delivery [126]. The liposome-based nanoparticle nucleic acid delivery system is currently potential and effective gene therapy methods, and a variety of lipid-based vectors already approved for clinical trials. Liposomes were first used by Felgner et al. to encapsulate DNA and deliver it to fuse with the plasma membrane of cultured mammalian cells, leading DNA internalization and expression [127]. Gene therapy based on CRISPR/Cas9 technology focuses on the formation of lipid nanoparticles by combining negatively charged CRISPR plasmid, mRNA, and gRNA with cationic liposomes in the presence of electrostatic interaction. The lipid-based nanocarrier not only helps CRISPR/Cas9 components to go through the cell membrane, but also protects them from degradation and immune response. Commercialized lipids are a potent and effective delivery vehicle. For example, Lipofectamines and RNAiMAX are both designed to deliver different CRISPR/Cas9 components and used for gene therapy of cancer. Zuris et al. reported a lipid-based nanoparticle that had achieved efficient delivery of a modified Cas9/sgRNA RNPs, which reached 80% gene modification in human cells [128]. In addition, RNAiMAX has also been used to deliver different kinds of proteins, like the Cre tyrosine recombinase, TALE repeat array and transcription activators into targeted cells, stem cell colonies or the hair cells in the inner ear of live mice. This study proved that protein delivery by liposomes was an effective way for gene editing.

3.2.4. Niosomes

Niosomes are other kind of lipid vesicles consisted of non-ionic surfactants and cholesterol [129]. The first noisome formulation was constructed by L'Oreal in 1975 and then developed as an alternative controlled drug delivery system to liposomes. Niosomes can overcome some problems appearing in liposome-based nanocarriers, liking sterilization, large-scale production, and stability [130]. For drug delivery, niosomes can encapsulate the hydrophobic drugs in bilayer membranes and hydrophilic drugs in aqueous

chambers. Generally, loading drugs into niosomes contain two types of methods. Directly entrapping or passive loading is the most common and easiest method that just needs dissolving hydrophobic drugs in organic regent and hydrophilic drugs in water among the noisome preparation process. For example, Seleci et al. encapsulated an anti-cancer drug, topotecan (TPT), into the designed noisome (PEGNIO) by dispersing TPT in aqueous phase and span 60, cholesterol, DSPE-PEG (2000) in chloroform [131]. After modifying with a cancer targeting and penetrating peptide (tLyp-1), the obtained formulations were performed efficient anti-glioma treatment effect. In remote loading, drugs can pass the membrane barrier of noisome by aid of pH or ions. Usually, pH-assisted method was achieved by hydration of surfactant and cholesterol in the presence of citric acid (pH 4.0). Then the alkali drug became ion form and precipitated in the environment of lower pH, leading to the encapsulation into the vesicle [148]. In another study, the ion gradient of trans membrane had also been developed for DOX remote loading and delivery [132].

3.3. Porous nanoparticles

Porous nanoparticles are widely applied for anti-cancer drug carrier's construction with optimistic properties, including uniform pore sizes, well-organized morphology, modifiable architectures, and large surface areas [133]. In addition, controlled drug releasing can be engineered in porous nanoparticle-based nanocarriers by conjugating stimuli-responsive pore-blockers or sensitive hybrid coats.

3.3.1. Porous silicon nanoparticles (PSi)

PSi is composed by crystalline silicon that has introduced nanopores in its microstructure [16]. The most used approach to synthesize PSi is a top-down approach by electrochemical anodization and etching [134]. PSi has many particularly attractive properties that can facilize the delivery of anti-cancer drugs. Firstly, PSi has high porosity (50–80%) and big pore volume (up to 2.0 cm^3/g) to achieve high drug loading degree. Drugs can be loaded by immersion or by chemical conjugation. For example, Landgraf et al. prepared one PSi with the pore size of 12 ± 6 nm and surface area around 500 m^2/g used for camptothecin (CPT) loading [135]. The loading capacity was calculated to be 150 ± 10 μg of CPT per mg of PSi, which shown significant therapeutic effect on breast cancer in vivo. In another study, daunorubicin (DNR) and dexamethasone (DEX) were sequentially loaded into oxidized Psi [136]. The loading process was achieved by first conjugating DNR and then conjugating DEX to the surface of PSi. Secondly, the versatile surface functional moieties and high surface area (580 m^2/g) of PSi can do surface modification for responsive drug release and achieving other biological function. Recently, stimulative-responsive moieties, such as heptakis(6-amino-6-deoxy)-β-cyclodextrin, glycol-block-poly(L-histidine), polymer (N-isopropylacrylamine) based polymer and ruthenium complex, have been modified on PSi to achieve controlled drug releasing [137]. In addition, various targeting ligands have been used for surface functionalization of PSi. For instance, carbonic anhydrase IX modified PSi shown high targeting ability toward cancer cells in the condition of hypoxia [138]. The PSi that conjugated with Ly6C antibody had high accumulation ability in pancreatic tumors (9.8 ± 2.3%) after 4 h administration as compared to 0.5 ± 1.8% with nontargeting. Finally, PSi has good biocompatibility that can degrade completely in human body. Generally, silicon oxide is a nontoxic food additive and has been approved by the U.S. Food and Drug Administration (FDA) as meeting basic health care requirements.

3.3.2. Mesoporous silica nanoparticles (MSNs)

MSNs are porous particles with pore sizes in the range of 2 to 50 nm, where the pore channels are used to accommodate drug molecules when are used in drug delivery systems [139]. It has some attractive advantages such as rich and homogeneous mesopores, adjustable particle size (10–1000 nm) and large pore volume range (2–20 nm), flexible morphology, big specific surface area, easy surface functionalization, attractive biocompatibility, and biodegradability. MSNs have a high porosity and is therefore able to encapsulate high doses of anticancer substances [140]. During transmembrane transport, MSNs are stable enough for resisting various biological erosions during intracellular transport. After internalization, MSNs can escape from endosome and go into the cytoplasm, leading to the protection for the loaded drugs. In addition, targeting and cryptic coating functionalization to MSNs can also be applied to improve their active targeting, improve membrane transport, and avoid the leakage of delivered cargoes before endocytosis of MSNs [141]. An effective MSN-PEM aptamer-based cancer cell targeting, and drug controlled-releasing nanocarrier was constructed by Zhu et al (Fig. 4a) [142]. By sequentially depositing polyvinylpyrrolidone (PVPON) and thiolated polymethacrylic acid (PMASH) on the MSNs, the obtained multilayer electrolyte (PEM) was used to prevent advanced drug-leakage and to achieve controlled drug-release under the condition of reducing. Finally, the DNA aptamer sgc8 against cancer cells was selected as a target recognition molecule to achieve a high capability of cancer cell recognition. As a result, the MSN-PEM-aptamer-based nanocarrier integrated the advantages of high drug delivery efficacy, stimuli-response release and high cell targeting ability. The anti-cancer capability of small interfering RNAs (siRNAs) has several limitations, for example, how to develop usable delivery tools for protecting siRNAs from degradation, delivering siRNAs to target cells with high specificity and facilitating their endosomal escape and cytoplasmic distribution. Ashley et al. shown that MSN-supported lipid bilayers (or "primary cells") have many properties that could overcome the limitations of existing delivery platforms (Fig. 4b) [143]. The loading capacity of delivered siRNA was 10 to 100 times larger than the corresponding lipid-based nanoparticles and shown significant stability in the physiological conditions. Protocells loaded with siRNA mixtures bonded to cells relied on the existence of appropriate targeted peptide molecular. This facilitated the delivering of silencing nucleotides into cytoplasm by successful endosomal escape. The expression levels of siRNA recognized gene were inhibited, which effectively induced growth suppression. Control cells without antigen expression could be recognized by the siRNA-loaded protocells, resulting no cell apoptosis. Their study highly demonstrated the exact specificity of the target protocells. Therefore, protocells offered unique properties for the therapeutic oligonucleotide's delivery considering their loading capacity, targeting capacity and potency.

3.3.3. Metal-organic frameworks (MOFs)

MOFs are novel type of organic–inorganic hybrid material. It is synthesized by coordination-oriented self-assembly of metal cations and organic ligands, presenting a one-dimensional, two-dimensional or three-dimensional coordination network structures [144,145]. As a vehicle for anti-cancer drugs, MOFs exhibits many desirable characteristics, which are embodied in the very high surface area used for drug adsorption, the large pore size used to encapsulate drugs, and the in vivo biodegradation caused by unstable metal–ligand bonds, and the versatility of grafting drugs or targeting molecules by post-synthesis modification [146]. The development and application of nano-scale MOFs (NMOFs) in drug delivery not only maintains the excellent performances of the bulk, but also improves the pharmacokinetics of the packaged drugs. Different kinds of anticancer drugs, including DOX, 5-Fu, Topote-

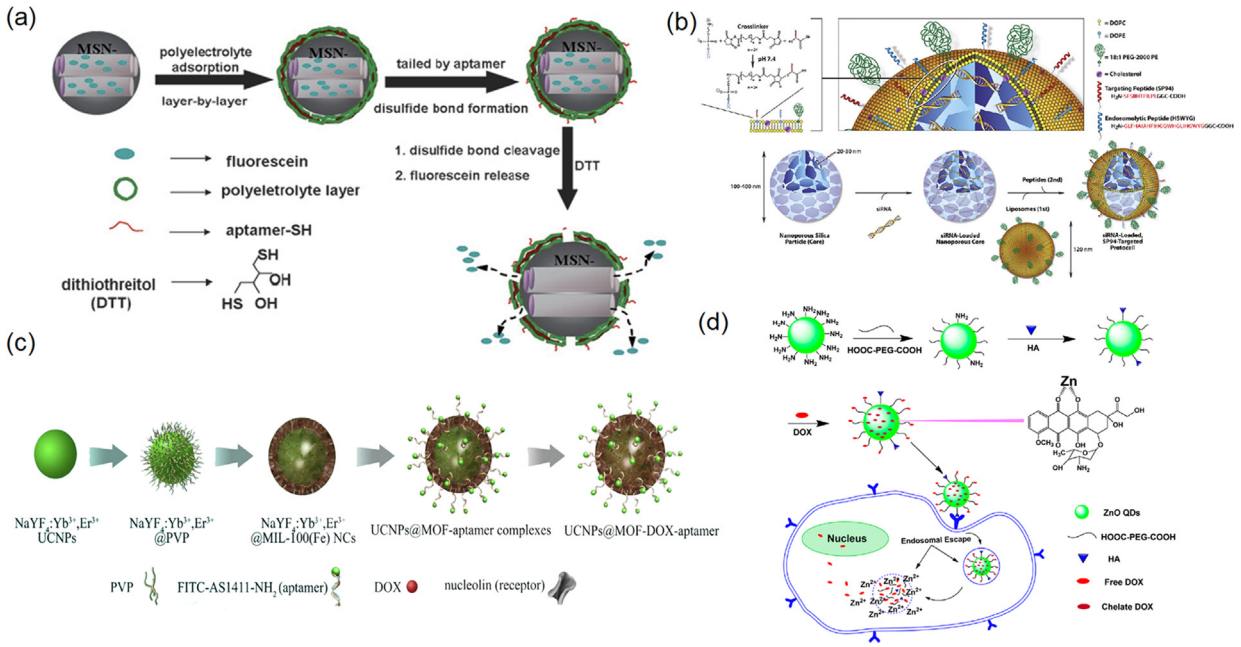

Fig. 4. (a) Schematic illustration of drug loading and controlled releasing of MSN-PEM-aptamer conjugates. Adapted with permission from [142]. Copyright (2009), Royal Society Chemistry. (b) Synthesis of siRNA-loaded MSN-supported lipid bilayers. Adapted with permission from [143]. Copyright (2012), American Chemical Society. (c) Synthetic process of the targeted UCNPs@MOF core–shell NCs. Adapted with permission from [148]. Copyright (2015), Springer Nature. (d) Synthesis, drug loading, releasing and cancer therapy process of HA-ZnO-PEG system. Adapted with permission from [159]. Copyright (2016), American Chemical Society.

can (TPT) and therapeutic RNA have been delivered by MOFs [147]. In addition, multifunctional NMOFs for targeted bioimaging and drug delivery have been extensively applied in early diagnosis and cancer treatment. Deng et al. prepared the UCNPs@MOFs nanocomposite core–shell materials with up-conversion luminescent nanoparticles (UCNPs) as the core and MOFs as the shell under room temperature (Fig. 4c) [148]. After loading the anticancer drug DOX, the aptamer AS1411 was grafted onto the surface of UCNPs@-MOFs by covalent bonding to target cancer cells and enhance intracellular uptake. The hybrid nano-platform can up-convert fluorescence luminescence under 980 nm excitation for cell imaging and carry drugs into selected cancer cells. It has shown great potential in simultaneously targeting markers, monitoring cancer progression and cancer therapy. Except for one drug carrying, MOFs had also been used for dual-drug delivery for cancer therapy. Liu et al. developed a UiO-66-NH_2-based nanocarrier which could load the first anti-cancer drug (dichloroacetic acid, DCA) in site and load the second anti-cancer drug (5-Fu) after synthesis [149]. To achieve the responsive and targeting delivery, 3,3′-dithiodipropionic acid anhydride (DTDPA) and folic acid (FA) were modified onto the nanocarriers surface. After endocytosis by cancer cells via FA targeting, the GSH-responsive disulfide bonds were cut by GSH in cancer cells. As a result, the contained drugs were releasing with the MOFs disassembly and the enhancing anti-cancer effects by DCA, and 5-Fu was achieved.

3.4. Others

Recently, many kinds of inorganic nanoparticles are showing their great potential in biomedical application because of their unique properties. In this session, we will discuss the most popular inorganic nanoparticles, including gold nanoparticles and quantum dots and their application in anti-cancer drug delivery.

3.4.1. Gold nanoparticles (AuNPs)

AuNPs have very large specific surface area and can be used to attach various biological macromolecules [150,151]. AuNPs treated with cationic polymer surface can be connected to anionic drugs (such as siRNA and plasmid DNA) through electrostatic interaction or covalent bonding [152]. Furthermore, the modification of cell specific targeting components to AuNPs can promote active cancer-specific drug delivery and passive enhanced penetration and retention (EPR) effects. AuNPs also have unique photoelectric properties, which are caused by the collective coherent oscillation of the free electrons in AuNPs-surface plasmon resonance (SPR) [153]. Under a specific wavelength of radiation, the dispersed AuNPs exhibit strong absorption and scattering ability. Besides, the SPR effect can be adjusted by changing the size, shape, and composition of AuNP. This makes AuNPs successfully applied as nanoprobes to biomedical diagnosis and analysis [154]. Rizk et al. synthesized AuNPs of various shapes and sizes, and then used tumor cells uptake to measure the uptake of AuNPs and selected the AuNPs with the highest uptake [155]. Then, two anticancer drugs (TGF-β1 antibody and methotrexate) and the targeting component folate were conjugated to AuNPs. As results, the multifunctional AuNPs used TGF-β1 antibodies to reduce extracellular TGF-β1 levels used folic acid to target cancer cells and used methotrexate to enhance the cellular cytotoxicity. The optimization of AuNPs will improve the efficient of the drug delivery capability, and due to the tunability of multifunctionalities, AuNP can have numerous clinical applications in cancer therapy.

3.4.2. Quantum dots (QDs)

QDs are very small nanoparticles composed of hundreds or thousands of atoms and have particles size between 1 and 10 nm [156]. Because of their great light stability, tailored optical properties and multimodality, QDs show more advantages in fluorescence imaging for the purpose of biomedical diagnosis. A variety of surface modifications can further expand the application range of QDs [157]. With its small size, this inorganic–organic composite nanomaterial contributes to unobstructed whole body blood circulation. The attached targeting molecules can not only perform specific reorganization at the tumor area and can inactivate the toxicological and pharmacological effects of QDs materials in vivo. Like other nanoparticle-based drug delivery, QDs have a large enough specific surface area to attach drugs [158]. By binding various tumor-targeting molecules

on the QDs' surface, drug delivery can be achieved at the same time. For instance, Cai et al. designed a DOX delivery platform based on a ZnO quantum dots (Fig. 4d) [159]. To enhance its stable under physiological fluid, they modified ZnO with dicarboxyl-terminated poly (ethylene glycol) (PEG). Moreover, for specifically binding, hyaluronic acid (HA) was also introduced to ZnO. By the formation of metal-DOX conjugation and covalent interactions, DOX molecules were loaded into the ZnO systems successfully and controlled released into cancer cells included by pH-sensitive dissociation. Significantly, cytotoxic Zn^{2+} was releasing as the ZnO QDs degradation. Due to the Zn^{2+}-induced cytotoxicity, a synergistic therapy with DOX was accomplished.

4. Nanotechnology-based delivery of CRISPR/Cas9 for cancer treatment

4.1. Barriers for CRISPR/Cas9 delivery systems

According to the previous discussion, CRISPR/Cas9-based therapeutic methods possess great potential for developing the new cancer therapy approaches. The delivery of CRISPR/Cas9 agents to cancer site is preconditioned to achieve its application for precise treatment. Today, the nanotechnology-based delivery systems are becoming more and more attractive in therapeutic gene editing, such as gold/lipid-CRISPR system for PLK-1 gene editing in melanoma, lipid-CRISPR system for human hepatitis B virus PCSK9 editing in human hepatitis B virus and polymer-CRISPR system for MTH1 gene editing in ovarian cancer [11,160]. However, to develop the nanocarrier for CRISPR/Cas9 with safety and efficiency still remains challenges due to the restriction of many different barriers in vivo (Fig. 5).

The first challenge is efficient encapsulating the CRISPR/Cas9 components. Usually, there are three mainly formats to do gene-editing using CRISPR/Cas9, including the plasmid encoding the Cas9 protein and sgRNA, the Cas9 mRNA and sgRNA mixture and the Cas9 protein and sgRNA ribonucleoprotein (RNP) [13]. The primary CRISPR/Cas9 plasmid from Zhang Group (pX330, Addgene plasmid #42230) is a single plasmid encoding the Cas9 nuclease and sgRNA, which possess a molecular weight of 5.55×10^3 kDa and negative charges of 1.74×10^4 [7]. Using SpCas9 as an example, the mRNA is approximately 4300 nt and contains 4300 negative charges [161]. With a big Cas9 protein (160 kD), the RNP's size is about 10 nm, while contains the negatively charged sgRNA. Therefore, it's difficult to condense the CRISPR/Cas9 system into a single delivery carrier considering their big size and charged surface. After administration, the delivery systems need to overcome multiple physiological and pathological barriers in vivo and each barrier will directly affect the final tumor treatment effect. For system delivery, CRISPR/Cas9 loaded nanoparticles first face the blood barrier, including degradation of CRISPR/Cas9 components by various enzymes in plasma[95], clearance of nanocarriers by the mononuclear phagocyte system or macrophages[162], aggregation of nanoparticles caused by opsonization[163] and filtration of nanocarriers by the glomerulus[164]. After blood circulation, the nanocarriers must overcome the barrier of tumor tissue to accumulate in tumor tissue before they meet target cells. This process will inevitably encounter vascular endothelial cells and interstitial areas. In particular, leaky blood vessels (usually in tumors and inflammatory sites) promote the extravasation of delivery systems from blood vessels[165]. The blood–brain barrier formed by the tightly connected cerebral vascular endothelial cells and astrocytes greatly hinders the CRISPR/Cas9 components delivering to brain

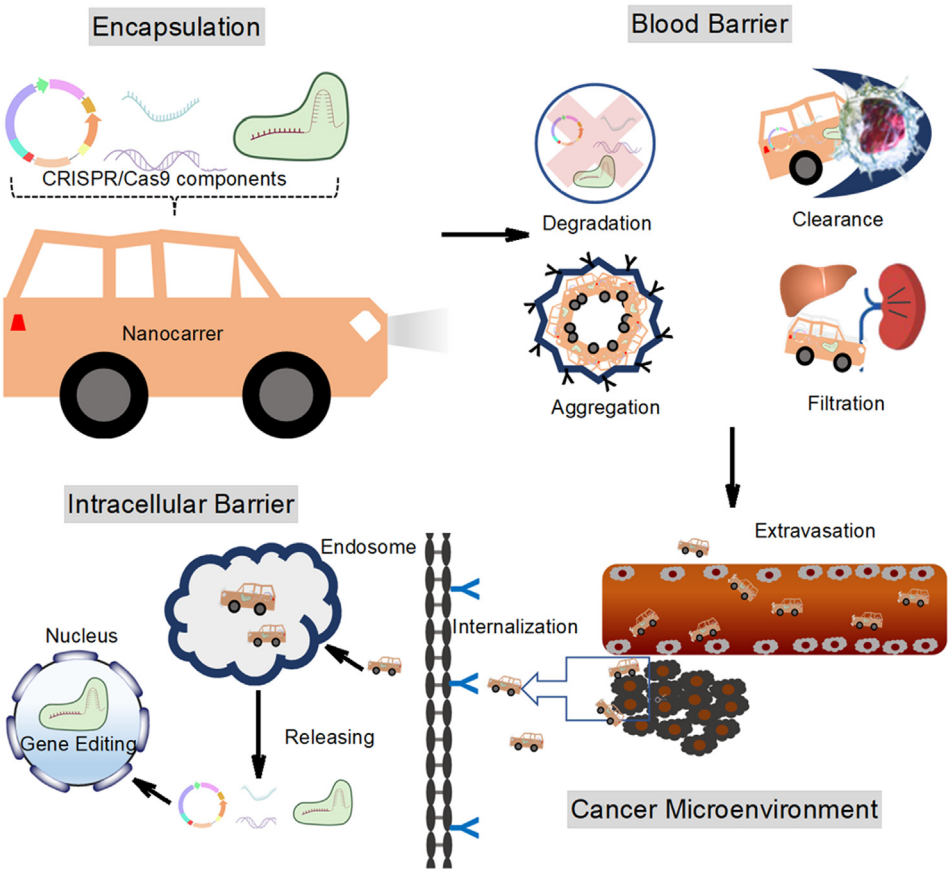

Fig. 5. Schematic illustration of different barriers in the process of CRISPR/Cas9 delivering for cancer therapy.

[166]. Due to the negatively charge of extracellular matrix, the nanocarriers with positive charge are easy trapped in the interstitial area [167]. In addition, the lack of lymphatic drainage, dense extracellular stroma, high tumor interstitial pressure, and the acidic and hypoxic tumor microenvironment increase the difficulty of nanocarriers entering the tumor. There is also a transcellular membrane barrier in the CRISPR/Cas9 delivery process from tumor tissue into cell. After they are released from endosomes or lysosomes, they also need enter nucleus for initiate gene-editing. Intracellular barriers mainly include cell membrane, endosome, and nuclear membrane. Before CRISPR can play a therapeutic role, it is necessary to overcome all the above-mentioned barriers. Therefore, the design and optimization of delivery vectors for efficient CRISPR/Cas9 delivery have become extremely important.

4.2. Delivery of CRISPR/Cas9 for cancer treatment

In recent years, wide interest in nanotechnology-based vectors for CRISPR/Cas9 delivery has evoked much research, indicating its potential prospect. Nanocarriers can be produced and optimized by rationally designing desired nanoparticles. In addition, the optimization of nanocarriers can be achieved by proper chemical modifications [165]. Here, we will introduce the critical consideration in the design of CRISPR/Cas9 nanocarriers to overcome different delivery barriers for cancer therapy.

4.2.1. Enhanced delivery for cancer gene editing

4.2.1.1. Efficient encapsulation. Compared with the gene-editing efficiency in vitro, the editing efficiency of CRISPR/Cas9 in vivo is significantly lower, which is partly determined by the lower delivery efficiency of the nanocarriers. The lower editing percentage may be enough for alleviating certain disease (e.g., liver tyrosinemia, muscular dystrophy), but not working for other diseases, such as cancer which needs essentially high editing. Usually, electrostatic interaction and effective bonding with specific component in CRISPR/Cas9 system are major methods for its efficient packing into nanocarriers.

Comparing to some not-in-one delivery platforms, incorporating CRISPR/Cas9 components into a single nanocarrier could be a more efficient way to simultaneously deliver CRISPR/Cas9 to the same cancer cell [168]. Because of the stronger negative charge on the surface of CRISPR/Cas9 components, the most popular method to encapsulate the big CRISPR/Cas9 cargo is interacting with the nanoparticles with positive charge by electrostatic attraction. Many positively charged materials, such as cationic lipid-based nanoparticles, polymers and polypeptides, have been designed for CRISPR/Cas9 packaging [169–171]. For example, Zhu et al. synthesized one poly (β-amino ester) (PBAE) based nanoparticles using 1,4-butanediol diacrylate, 4-amino-1-butanol and 1-(3-aminoprpyl)4methylpiperazine, which condensed CRISPR/short hairpin RNA (shRNA) plasmid by electrostatic forces [172]. By controlling the expression level of HPV16 E7, the nanocarrier suppressed the cancer cells proliferation and cervical tumor growth efficiently in vivo. The treatment was much better than the methods that just treated the tumors with control and plasmid only. Sametime, the researchers found the nanoparticles decreased the expression of the HPV16 E7 protein level and deregulated the related cell signaling in HPV16 transgenic mice. Usually, hyperbranched polymers (HBPs) has improved delivery ability for different cargoes compared with their corresponding linear structures [173]. In the past few years, delivery of many kinds of guest molecules using HBPs has been widely investigated [174,175]. Haag et al. studied the encapsulation efficiency of hyperbranched PEI modified with different fatty acids, such as C18, C16, C11 and C6. The designed hyperbranched structure allowed about 150 guest molecules to be encapsulated and transported [176]. Importantly, the functionalized hyperbranched PEI exhibited a higher loading ability than the linear one. Therefore, Gao and coworkers reported a nanocarrier based on hyperbranched copolymers (hPPCs) that used PBAE as the condensing part and poly(amide-amine) (PAMAM) as the branching unit for CRISPR/Cas9 plasmid delivery (Fig. 6a) [177]. When the mass ration of the polymers and plasmids came up to 30:1, the plasmids were totally incorporated by the copolymer. In addition, the copolymer with the highest degree of branching shown the largest plasmids encapsulation ability and highest transfection efficiency. When delivering the HPV16 E7 targeted CRISPR/Cas9 plasmid, the designed platform shown ideal anti-cancer ability both in vitro and in vivo due to the knock-out of HPV16 E7 oncogene.

As alternative vectors, inorganic nanoparticles are also designed for CRISPR/Cas9 agents delivering by electrostatic force. Zhou et al. reported a biodegradable 2D black phosphorus nanosheets (BPs) CRISPR/Cas9 RNP (Fig. 6b) [178]. BPs with the puckered honeycomb structure had high surface-to-volume ratios. Due to the negative charge of both BPs and RNP, they first conjugated Cas9 protein with three repeats of nuclear localization signals (NLSs) at C terminus to obtain thee positively charged Cas9N3. After loaded the RNP onto the BPs, the remarkable loading capacity was achieved up to 98.7%. After intratumorally injection, the designed Cas9N3-BPs with the coding region of EGFP induced seriously reducing of EGFP signal in the tumor sites at a relatively low dose. Recently, gold nanoclusters (GNs) based delivery platform (named as LGCP) which used GNs as core and lipids as shell had been synthesized for CRISPR/Cas9 plasmids delivery (Fig. 6c) [179]. By conjugating HIV-1 transactivated of transcription peptide to GNs, plasmids targeted to Polo-like kinase-1 (Plk1) could be loaded through electrostatic interactions. Finally, the strategy had been utilized to treat melanoma, leading to high reduction (>70%) of Plk1 protein expression in cancer cells and great suppression (75%) of the melanoma progression in vivo.

Apart from electrostatic interaction, other encapsulation methods according to the unique properties of sgRNA or Cas9 protein also developed for CRISPR/Cas9 components condensing. Enlightened by its property of single-stranded DNA that could pair with sgRNA, Gu and coworkers designed DNA nanoclew (NC) with ability to partially base-pair with the sgRNA for RNP delivery (Fig. 6d) [180]. The DNA NCs were prepared using rolling circle amplification with palindromic sequences encoded twelve nucleotides complementary to the 5′-end of the sgRNA (NC-12) that derived self-assembly. The delivery nanoparticles were formed by incubating Cas9 and sgRNA first, then adding NC-12 and PEI, which transferred the RNP into U_2O_S cells efficiently and achieved the GFP knock-out in vivo. Liu et al. reported one boronic acid-rich cationic polymer to bind RNP into stable nanoparticles [181]. Boronic acid could coordinate with cationic groups on proteins through nitrogen-boronated interaction. Sometimes, the aromatic ring in it could bind with proteins via cation-πinteractions. After self-assembly into nanoparticles, the RNP loaded polymer induced 40% EGFP knockout, 23.1% adeno-associated virus integration site 1 (AAVS1) indel rate, 21.1% hemoglobin subunit beta (HBB) indel rate in HEK-293 cells and efficient catenin beta-1 knockout in HTC-116 and HT-29 cells, which was greater over the commercially available RNP delivery agent CRISPRMAX (CMAX).

4.2.1.2. Long circulation. Besides the efficient packaging, prolonged blood circulation is another important need for systemic administration of CRISPR/Cas9 based cancer therapy in vivo [182]. As mentioned above, the CRISPR/Cas9 components in nanocarriers required to overcome three blood barriers at least, including opsonization, clearance and physical filtration. Generally, the longer the nanoparticles circulate in the bloodstream the more they can reach the cancer sites.

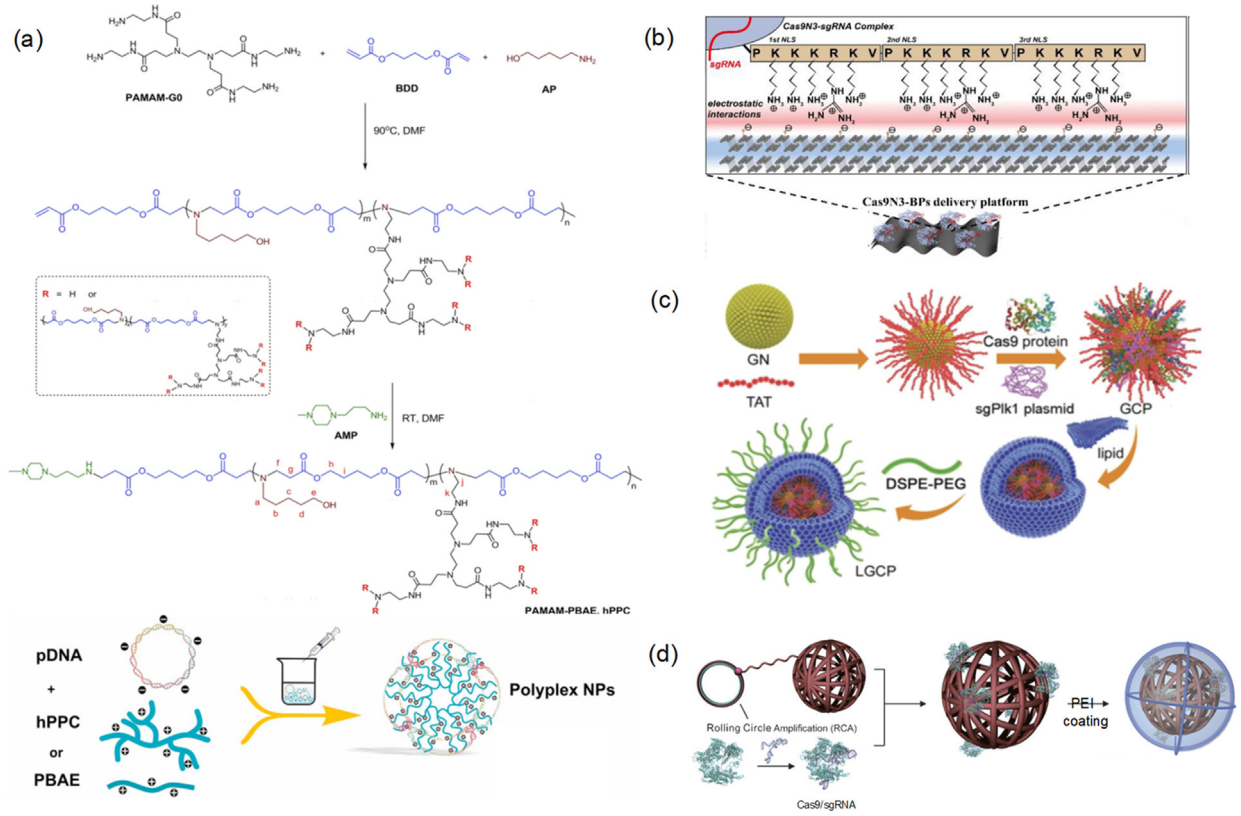

Fig. 6. (a) Synthesis scheme and CRISPR/Cas9 encapsulation process of hPPCs. Adapted with permission from [177]. Copyright 2020, Elsevier. (b) Design of the Cas9N3-BPs delivery platform. Adapted with permission from [178]. Copyright 2018, John Wiley and Sons. (c) Schematic diagram of the synthesis process of the LGCP. Adapted with permission from [179]. Copyright 2017, John Wiley and Sons. (d) Design of the DNA NC based CRISPR/Cas9 delivery system. Adapted with permission from [180]. Copyright 2015, John Wiley and Sons.

A simple and effective solution to protect the CRISRP/Cas9 from denature and enlarge the circulation time of nanocarrier is surface modification, such as PEGylation. Polyethylene glycol (PEG) is a non-toxic polymer and has been widely used as a vehicle or base in foods, cosmetics, and pharmaceuticals. The PEGylation of proteins was first described in 1977 and had been proved as an efficient method to increase blood circulation [183]. Similar with that, PEG coating on nanoparticles have been investigated for systemic application [184–186]. The first PEGylated product (Doxil®) approved by FDA was came in 1995. Compared with free drug, Doxil shown a longer drug half-time (72 h) and blood circulation half-life (36 h), which increased the bioavailability of DOX significantly [187]. The protective mechanism of PEGylation is that coated PEG chains can produce a hydrated cloud due to its hydrophilic nature [188,189]. Therefor, modifying the nanoparticles with PEG can cover them from aggregation, opsonization and phagocytosis, hence extending nanoparticle's circulation in blood. Jubair and co-workers reported a CRISPR/Cas9 delivery platform using PEGylated liposomes which prepared by hydration of Freeze-Dried Matrix (HFDM) Method [190]. After optimizing the packaging efficiency, they also studied the protecting and delivering ability for CRISPR/Cas9 plasmid out of the bloodstream, which shown that the PEGylation liposomes protected plasmid against degradation in serum up to 6 h. In addition, no DNA was detectable within 4 h after injecting into the bloodstream. For cancer treatment, the CRISPR/Cas9 with the sgRNA targeted to HPV E6 and E7 oncogenes was loaded into PEGylated liposomes during HFDM process, leading to effective tumor growth inhibition and treatment in animals. Similarly, Zhang et al. constructed a PEGylation modified cationic lipids with core–shell structure and applied for CRISPR/Cas9 plasmid delivering (Fig. 7a) [191]. The core of designed nanocarrier was applied to condense CRISPR/Cas9 plasmid yield lipid/chondroitin/protamine/plasmid DNA (LNP/DNA) and the DSPE-PEG shell was used to increase circulation half-life. The designed nanocarrier was observed with more stability in different culture media compared with commercial regent Lipofectamine 2000 and the stability was

maintained along with the incubation time increasing. Then the LNP/DNA targeted to PLK-1 exhibited high transfection efficiency (47.4%) of Cas9/sgPLK-1 in A374 cells. After intratumor injection, Cas9/sgPLK-1 plasmid was successfully delivered into melanoma tumor-bearing mice and deregulated PLK-1 level significantly, thereby obtaining great tumor growth suppression (>67%) in vivo. Apart from PEGylated lipid/liposome, PEGylated polymer is another effective vector for CRISR/Cas9 delivery. For example, Liu and coworkers constructed a multistage delivery nanoparticle (MDNP) and achieved targeted delivering of CRISPR/Cas9 to tumor [192]. MDNP was constructed to be a core–shell structure, in which the shell was consisted of 2,3-dimethylmaleic anhydride (DMMA)-modified poly(ethylene glycol)-*b*-polylysine (mPEG113-*b*-PLys100/DMMA). When circulation in bloodstream, the shell endowed MDNP with negative charge and reduced the immune clearance. As a result, remarkable tumor growth inhibition was observed after systemic administrating of MDNP/dCas9-miR-524 to tumor-bearing mice. In another study, the researchers added the FDA-approved F127 to their nanocarrier to boost transfection efficiency of CRISPR/Cas9 (Fig. 7b) [193]. The mechanism of F127 was similar with PEGylation that improved colloidal stability and reduced nonspecific adsorption of some proteins due to PEG corona [194]. The nanocarrier was constructed by the mixing of quaternary ammonium-terminated PPO (PPO-NMe3), F127 and plasmid to produce micelles formulation via the hydrophobic and

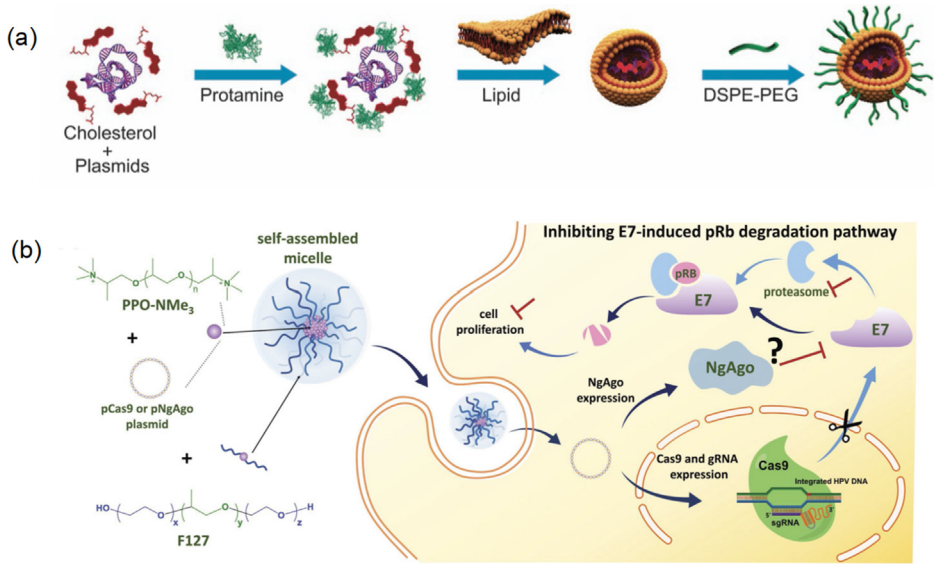

Fig. 7. (a) The packaging and encapsulation processes of the Cas9/sgRNA plasmid by PEGylation modified cationic lipids. Adapted with permission from [191]. Copyright 2017, Spring Nature. (b) Design and HPV oncogene manipulation of the self-assembled micelle system. Adapted with permission from [193]. Copyright 2018, John Wiley and Sons.

electrostatic interaction. In their study, the researchers optimized and adjusted the final micellar formulation ratio to 40/40/1 (w/w/w) of F127/PPO-NMe3/pCas9. Then, they use this strategy to do the HPV18-E7 oncogene editing. Compared with commercial transfection regent Lipofectamine 200, the designed delivery platform shown a higher gene disruption rate in vitro and an obvious tumor growth without health tissues damage in mice.

4.2.2. Targeted approaches for cancer gene editing

To improve gene editing specificity and decrease undesired off-target effects in cancer therapy, targeting delivery of CRISPR/Cas9 components to cancer cells is the simplest but necessary approach [195,196]. For the nanotechnology based gene editing system, the ability of nanocarriers concentrate in areas of tumor site is divided into two means of targeting: passive and active. Passively targeted nanocarriers, which utilized the EPR effect and newly suggested endothelial transcytosis [197], are the most widely studied strategy for targeting cancer treatment [198]. However, due to multiple physiological barriers and a high degree of nanoparticles extravasation through the tumor vasculature, only a small percentage of nanoparticles can accumulate and penetrate into tumors [199]. According to a recent meta-analysis study, the accumulation rate is less than 1% even in high-EPR xenografted tumors [199]. As a complementary strategy to passive targeting, active targeting was widely developed for improving tumor localization of nanocarrier by increasing their targeting efficiency and increasing retention at the target tumor site [200]. Active cancer targeting strategy is usually modifying the nanocarrier with specific components that have strong affinity with the receptor in target cancer cells or tumor vascular endothelium, which results to cellular uptake increasing due to receptor-mediated endocytosis [201]. Accordingly, cancer cells can be set apart from normal cells through the surface receptor and over-expressed antigen. Therefore, in this part, we want to discuss the active targeting strategy for nanotechnology based CRISPR/Cas9 delivery systems and summarize several different kinds of typical targeting moieties in cancer therapy.

4.2.2.1. Antibodies. Antibodies, belonging to the superfamily of immunoglobulin, has been extensively explored as targeting ligands. Many different kinds of antibodies have higher affinity to several receptors of antigens over-expressed on the cancer membrane [202,203]. In cancer therapy, the application of monoclonal antibodies have been going on 20 years and the patient responsive have been well-understood [204]. Therefor, antibodies should be a natural choice for nanoparticles-based targeting as therapeutics. Among various receptors on cancer cells, human epidermal growth factor receptor (EGFR), shows great promising in cancer targeting due to its vital function in many cells activities, including growth, migration and metabolism [205]. Until now, many EGFR targeting ligands are discovered and utilized to many different delivery platforms, including lipids, polymers, Fe_3O_4 nanoparticles, up-conversion nanoparticles and MOFs [206]. As proven, these systems can be uptake through receptor-mediated endocytosis and reduce the side effects and cytotoxicity of the loaded therapeutic cargoes. Many nanoformulations (>24), including Doxil® and Abraxane® have been utilized in clinic approved by the FDA and various stages of clinical trials are undergoing.

However, taking full-length antibodies as a targeting molecule can increase the size of conjugated nanoparticles and immunogenicity, thereby reducing the therapy efficiency due to fast clearance. This can be overcome by the next-generation antibodies, such as single-chain fragments, antigen-binding fragment, and so on [207,208]. Single-chain antibody fragments which can retain the ability to bind with antigens are just one fifth of full antibodies' size, leading to much safer active targeting [208]. For instance, Rosenblum et al. described a EGFP-targeted amino-ionizable lipid (cLNP) used for CRISPR/Cas9 delivery. After loading Ca9 mRNA and sgRNA, the cLNP was coated with anti-EGFP (lgG2A, Anchored Secondary scFv Enabling Targeting (ASSET)) which could bind to an over-expressed receptor on ovarian cancer cells [169]. In this study, anti-EGFR-modified cLNPs shown higher accumulation in disseminated tumors than IgG isotype controlled cLNPs, giving a powerful prove of the targeting approach for tumors. In addition, a single administration of this designed nanocarriers achieved up to ~80% PLK1 gene knock-out in vivo. The tumor growth was greatly reduced and overall survival of mice with serious ovarian cancer malignant ascites was increased ~80% after injection of the targeting sgPLK1-cLNPs. As one of the most important promising nanocarrier, cell-derived extracellular vehicles (EVs) were

widely developed for biomolecules delivery, particularly therapeutic components, due to their less immunogenicity and good encapsulation efficiency [209,210]. He and coworkers reported one epithelial cell-derived EVs and used it to delivery CRISPR/Cas9 targeted to sgIQ1.1, which shown efficient liver cancer treatment [211]. Similarly, Jang's group reported a cancer-derived exosomes and applied it as an attractive delivery platform for CRISPR/Cas9 (Fig. 8a) [212]. After selective accumulating in ovarian cancer tumors in vivo, CRISPR/Cas9 loaded EVs induced significant suppression of poly (ADP-ribose) polymerase-1 (PARP-1) expression and apoptosis of ovarian cancer. However, EVs based nanocarriers can also be adsorbed by normal cells and organs somehow, generating undesired cellular internalization of non-targeted cells. To promote their targeting capability, Xu et al. modified the epithelial cell derived EVs with a chimeric-antigen receptor (CAR) (Fig. 8b) [213]. The CAR included a single-chain variable fragment for CD19 targeting, leading to anti-CD19-CAR-EV. Compared with normal EVs, CAR modified EVs could accumulate rapidly around cancer cells and effective release the CRISPR/Cas9 components in vivo, achieving targeting gene-editing of MYC. This study supported that CAR modification could facilitate the targeting delivery of EVs-based nanocarriers.

4.2.2.2. Aptamers. Similar with the properties of antibodies, aptamers (single-stranded oligonucleotides) possess specific target recognition and binding affinities. Aptamers can be engineered to conjugate to a variety of targeting sites ranging from small molecules (DNA, RNA, or oligonucleotide) to proteins and carbohydrates [214]. Depending on systematic evolution of ligands by exponential enrichment (SELEX), certain targeted aptamers are able to be sort out from a huge library of random sequences [215]. Because of generated for a range of molecules expressed in tumors. Recently, aptamers have been extensively developed and utilized for cancer treatment via binding to the molecules expressed in tumors [216,217]. Therefore, aptamers are very potential targeting units in the nanoparticle-based delivery systems.

AS1411 is one of DNA oligonucleotide being rich in G. It can bind specifically to nucleoli, a protein which is generally over expressed on the membrane of cancer cells and has a low content on the surface of normal cells [218]. The nanocarriers decorated with AS1411 has been developed for CRISPR/Cas9 delivery. For example, Cheng et al. fabricated a tumor targeting delivery system with core–shell structure based on a natural polymer (Fig. 9a) [219]. CRISPR/Cas9 plasmid was binding to protamine sulfate (PS) via electrostatic interactions to generate the core (plasmid@PS). Then, the carboxymethyl chitosan (CMC) and KALA peptide complexes with negative charge were coated onto the positive core. To give the nanocarrier the capacity of tumor cell and nuclear targeting, AS1411 incorporated CMC was introduced. The genome editing platform decreased the expression of CDK11 IN MCF-7 cells significantly. In addition, CDK11 knockout can also up-regulate cancer suppression and immune-related protein, resulting good potential for cell behaviors regulation in tumor treatment. Except CDK11 knockout, they also prepared a delivery vector modified with AS1411 for knockout of cancer related gene CTNNB1 (Fig. 9b) [220]. In this study, negatively charged CMC was modified with AS1411-functionlized CMC (ACMC) and cell penetrating peptide (TAT) firstly and then introduced it to positively charged core made by PS/CaCO3/plasmid co-precipitation to generate the final delivery nanoparticles (ACMC/TCMC/PS/CaCO3/plasmid, ATNP). The delivery of CRISPR/Cas9 plasmids targeting to CTNNB1 gene shown high efficiency, resulting in a significant knockout of CTNNB1 gene. At same time, the expression level of tumor preventing protein, such as vimentin, snail, MMP-2, MMP-9, CD44 had

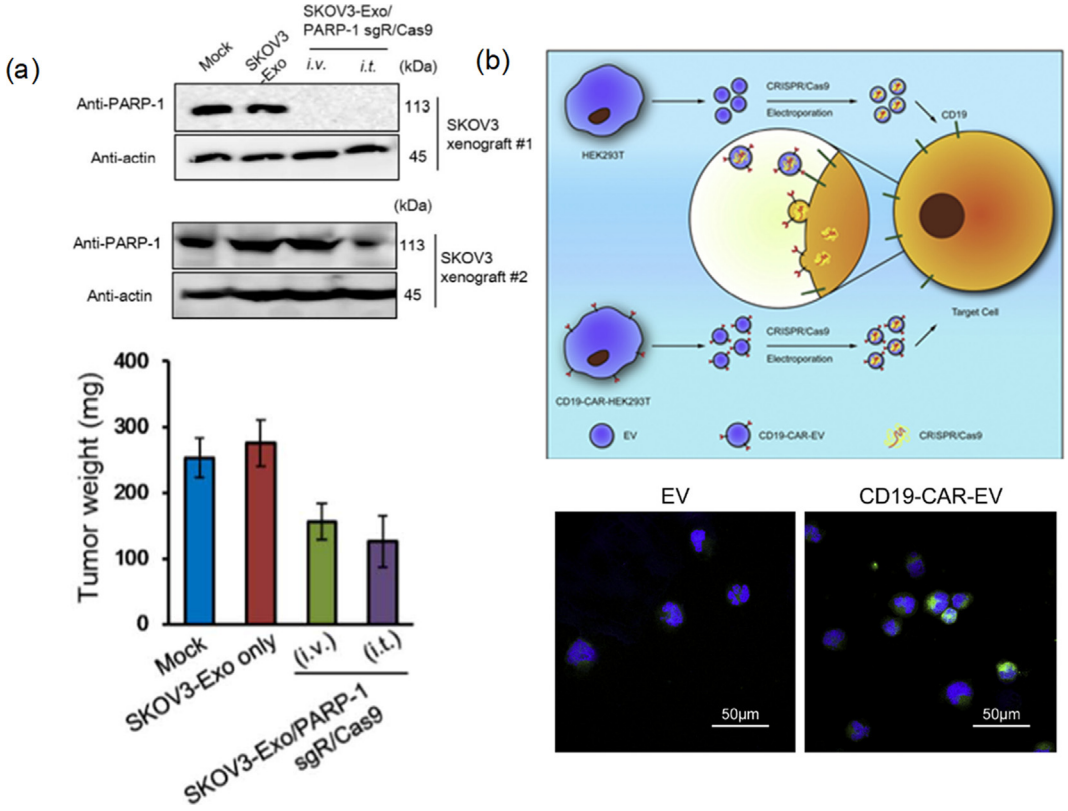

Fig. 8. (a) Anti-cancer effects of cancer exosomes loaded with CRISPR/Cas9 in SKOV3 xenografts. Adapted with permission from [212]. Copyright 2013, Elsevier. (b) Schematic illustration of the design and cellular internalization of anti-CD19-CAR-EV. Adapted with permission from [213]. Copyright 2020, Elsevier.

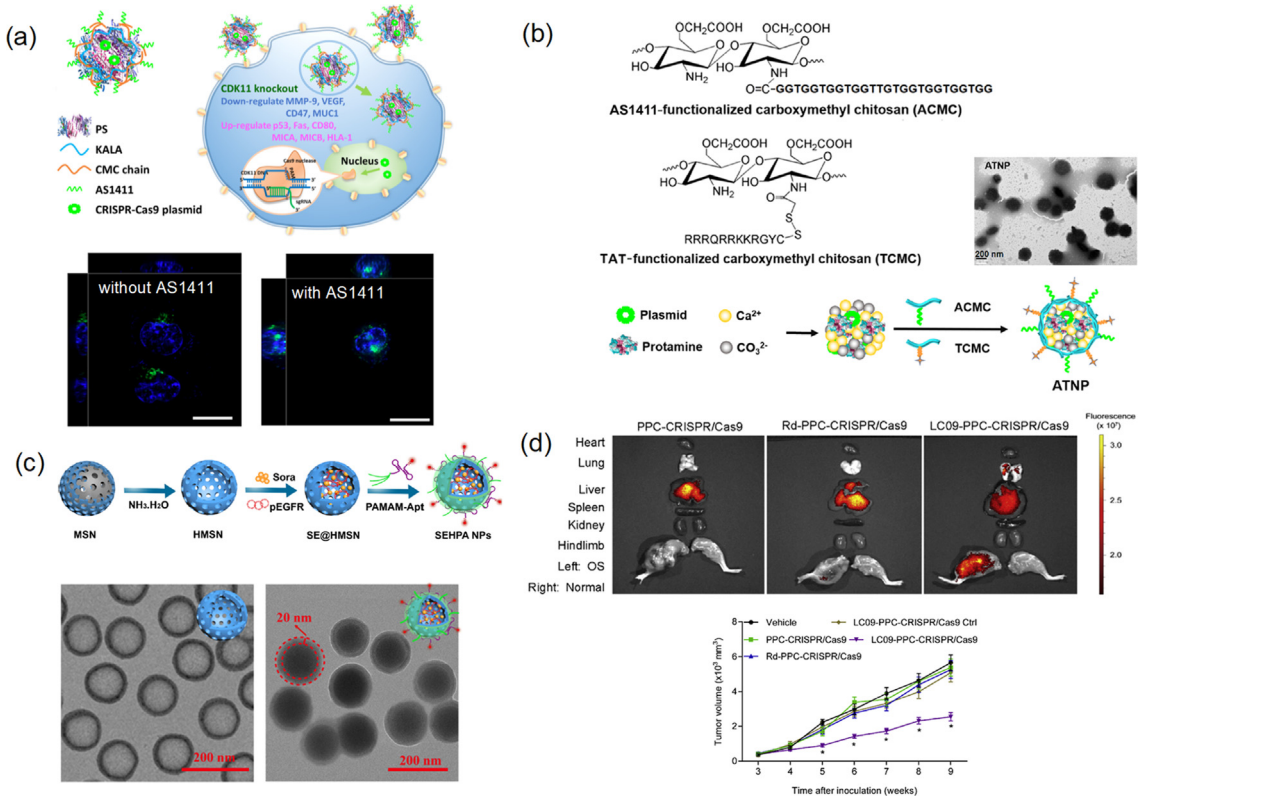

Fig. 9. (a) The cellular delivery results of CRISPR/Cas9 plasmid by the multi-functional delivery system. Adapted with permission from [219]. Copyright 2017, Elsevier. (b) Structure of ACMC and TCMC and the preparation of CRISPR/Cas9 plasmid delivery system. Adapted with permission from [220]. Copyright 2019, American Chemical Society. (c) Preparation of MSN-based nanocarrier and TEM images of the nanocarrier without and with P-Apt shell. Adapted with permission from [59]. Copyright 2020, American Chemical Society. (d) Distribution and tumor therapy results of LC09-PPC-CRISPR/Cas9 from syngeneic orthotopic OS mice. Adapted with permission from [227]. Copyright 2017, Elsevier.

been downregulated. Except for AS1411, EpCAM (epithelial cell adhesion molecule) aptamer is another efficient targeting ligand for cancer therapy [221,222]. EpCAM is generally over-expressed on cancer cells (up to 1000-fold), but expression level in normal cells is quiet low. Zhang and coworkers have developed one EpCAM aptamer modified sorafenib and CRISPR/Cas9 co-delivery system for hepatocellular carcinoma (HCC) treatment (Fig. 9c) [58]. The delivery platform utilized hollow MSNs for CRISPR/Cas9 plasmid loading and modified the loaded MSNs with anti-EpCAM aptamer and polyamidoamine (PAMAM) conjugates onto the surface. As a result, the final nanocarrier demonstrated high EGFR knock out efficiency (>60%) with low off-target effect in vitro and caused 85% tumor growth inhibition in vivo. In addition, the tumor accumulatio results shown that the presence of EpCAM aptamer prompted the nanocarrier with high accumulation at the tumor site and good safety without side-effect to health organs.

Apart from the aptamers used for several tumor cells targeting, a few aptamers for specific receptor binding were successfully applied for the targeting delivery platforms. Among them, prostate-specific membrane antigen (PSMA) aptamers are the best studied and most common specific aptamers [223]. It is reported that PSMA-targeted delivery of docetaxel using PSMA ligand nanoparticles has been evaluated in clinical trials [224]. In addition to PSMA aptamers, the A10 RNA aptamer, a 57-bp nuclease-stabilized 2′-fluoropyrimidine RNA molecule can also be recognized as the extracellular domain of PSMA [225]. The A10 aptamer has been linked to cationic liposomes by post-insertion method to form a delivery platform for CRISPR/Cas9 that target PLK1

in prostate cancer cell [226]. The complexes of aptamer/liposome/CRISPR/Cas9 were proved possessed a high specificity to cell types binding. Sametime, it shown a high-performance gene editing in vitro and a prominent tumor suppression in vivo with a lower immune response. In another study, the osteosarcoma cell-specific aptamer (LC09) was chosen and applied to modify PEG-PEI-Cholesterol (PPC) lipopolymer that encapsulated CRISPR/Cas9 plasmids encoding VEGFA (Fig. 9d) [227]. As results, LC09 endowed the delivery platform with selective distribution of plasmids in orthotopic OS. As a result, effective VEGFA gene-editing and orthotopic OS malignancy inhibition were achieved in tumor. In order to improve the targeting capacity of EVs, DNA aptamers have also been used for surface modification of EVs and guided them to tumor sites by receptor-ligand binding [228,229]. Furthermore, DNA aptamers can reduce the nonspecific binding of EVs to non-target cells by adding negative charge to EVs [230]. For example, Zhuang et al. designed a TLS11a that specific targeted to liver cancer cells anchored EVs from HEK293T for CRISPR/Cas9 delivery [231]. The DNA aptamer was conjugated with a 3D tetrahedral DNA nanostructure (TDN) which could provide specific trend of DNA without steric hindrances and better control of the aptamer binding simultaneously [232]. After binding the TDNs with aptamer on the surface of EV through cholesterol anchoring, this delivery system shown efficient tumor-specific accumulation both in vitro and in vivo, inducing the downregulation of WNT10B and remarkable tumor growth inhibition.

4.2.2.3. Peptides. Targeting peptides which are naturally occurring or artificially synthesizing are one kind of low weight molecular. They are generally composing of less than 50 amino acids [233]. Compared with antibodies, they have superior cell/tissue permeating capacity due to the low molecular weight while maintain the

function of specific receptors binding on cancer cells. In the past few years, several cell surface binding peptides have been discover successfully according to combinatorial library methods, which is much useful for cancer targeting [234]. Therefore, some peptides, such as chlorotoxin, RGD peptide and EPPT peptide, have been used for the construction of nanocarriers to provide cell- or tissue-specific targeting [235,236]. Peptide-conjugated nanoparticles have demonstrated popularity for CRISPR/Cas9 targeting delivery.

The Arg-Gly-Asp (RGD) peptide, which has high affinity to αvβ3 integrin overexpressed in many cancers, has been applied in experimental targeting strategy [237]. Accordingly, RGD targeted nanoparticles shows enhanced selective endocytosis into cancer cells and angiogenic endothelial cells [238]. For example, Li and coworkers constructed a RGD-R8 modified core–shell artificial virus (RRPHC) and utilized it for CRISPR/Cas9 targeting delivery (Fig. 10a) [239]. As reported, R8-RGD was prepared by conjugating RGD to a cell penetrating peptide R8, which had much increased cellular uptake efficiency [240]. RRPHC was made of a core of fluorinated polymer (PF33) that bonded to CRISPR/Cas9 and a versatile shell of RGD-R8-PEG-HA. When loaded with Cas9-hMTHI that targeted to MTH1 (the MutT Homolog1) gene, RRPHC exhibited effective targeting of ovarian cancer and disruption of MTH1 in vivo. Except for RGD, R8 have been also used to conjugate to reverse sequence of RGD (dGR) to form R8-dGR, which can recognize two different kinds of receptors on tumor cells (αvβ3 integrin and neuropilin-1) [241]. In addition, the capabilities of tumor targeting, and tissue penetrating were obviously enhanced. This approach has been used for CRISPR/Cas9 plasmids delivering to pancreatic cancer reported by He's group [83]. In their study, plasmids encoding Cas9 and hypoxia-inducible factor-1α (HIF-1α)-targeting sgRNA were encapsulated into R8-dGR modified cationic liposome, along with PTX. The formed nanocarriers (R8-dGR-Lip) shown enhanced targeting ability to BxPC-3 cells and deep penetrating to tumor sites, successfully leading downregulation of HIF-1α and inhibition of tumor growth, but no severe toxicity in vivo. By inhibiting its downstream molecules, the antiproliferation and antimetastatic effect of PTX were significantly increased. Therefore, this system shown good combination therapy effect in pancreatic tumor model. Similarly with R8-dGR, another RGD variant iRGD (internalizing RGD) have been described as an attractive approach to delivery different components deep into tumors by peptide mediation [242]. Recently, Chen et al. designed and synthesized hydrogel nanoparticles (LHNPs) with core–shell structure for CRISPR/Cas9 delivery (Fig. 10b) [243]. The core of LHNPs was formed by PEI hydrogel used for Cas9 protein encapsulating and the shell was constructed from cationic DOTAP lipids conjugated with cell penetration peptide mHph3 and cell targeting peptide iRGD. Due to the multiple-targeting ability of iRGD to αvβ3/αvβ5 integrins and neuropilin-1, the concentration of iRGD-modified LHNPs in tumors was 2.6 times higher than that treated with non-modified LHNPs. When encapsulating the CRISPR/Cas9 targeting PLK1 gene, LHNPs based delivery platform inhibited tumor growth both in body and in brain of U87 cell xenografted mice. In addition, the targeted manner by LHNPs delivered CRISPR/Cas9 to residual tumor cells and as a result, the residual tumors were eliminated, and their recurrence was prevented.

4.2.2.4. Small molecules. As another targeting ligand, small molecules also possess specificity to overexpressed receptors in tumors and have been widely explored in the application of cancer-targeted delivery [244]. Among them, folic acid stands out as most promising targeting molecule due to its high affinity of the folate receptor. Accordingly, folate receptor (FR) is overexpressed in mand different kinds of human malignancies (almost 40%), including breast cancer, ovary cancer, kidney cancer and bladder cancer, but low expression in normal tissue [245]. The density of folate receptor increase with the development of cancer. Folate-anchored therapeutics can be internalized into the endosome through receptor-mediated endocytosis. This endows many folate-conjugated drugs or imaging agents going to clinical trials [246]. Consequently, folate was extensively employed as a targeting ligand for nanoparticle-based cancer therapy and recent investigations have further increased its targeting function. For example, Zhao et al. synthesized a folic acid conjugated cationic γ-cyclodextrin-oligoethylenimine star polymer and used it as an effective targeted gene delivery system [247]. Compared with the polymer without folic acid, folic acid modified one had 2–4-fold higher efficiency of gene transfection in FR-positive KB cells. The recycling of FR back to the cell membrane was observed after polymer internalization, which promoted continuous cellular uptake mediating by FR and gene delivery. The folic acid targeting method has also been used for CRISPR/Cas9 based cancer therapy [248]. The present study reported a folate receptor-targeted liposome

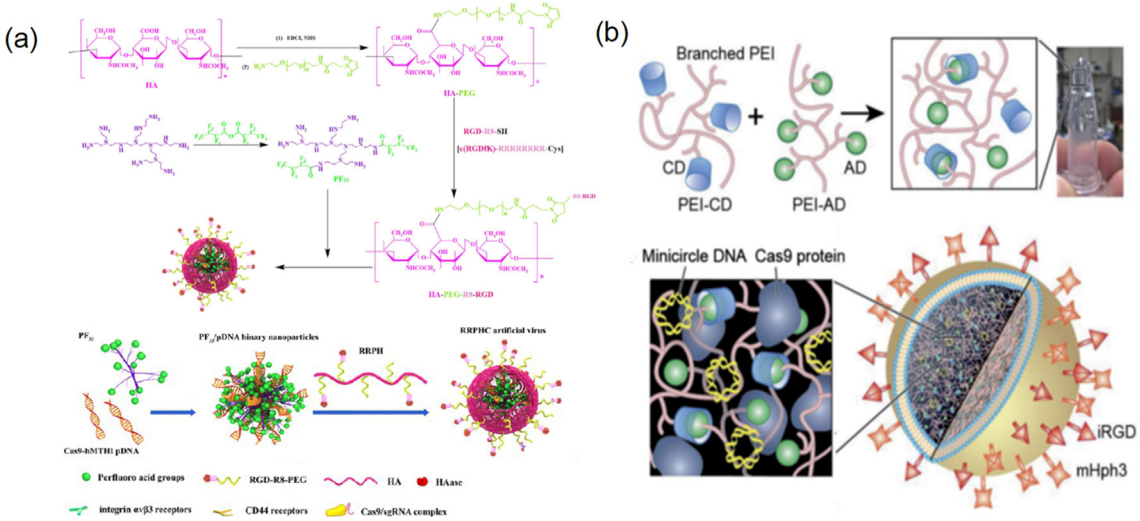

Fig. 10. (a) synthesis scheme of PF33 and RRPH. Adapted with permission from [239]. Copyright 2017, American Chemical Society. (b) Formation of PEI hydrogel through CD-AD mediated host–guest interaction and core–shell structure of LHNPs formed by DOTAP liposome and PEI hydrogel. Adapted with permission from [243]. Copyright 2017, John Wiley and Sons.

(F-LP) for CRISPR/Cas9 plasmid delivering. The plasmid encoded with the sequence targeting the ovarian cancer-related DNA methyltransferase 1 (DNMT1) gene (gDNMT1). The nanocarriers (F-LP/gDNMT1) which were stable for injection introduced effectively mutated endogenous DNMT1 and decreased methylation of genomic DNA of ovarian cancer in vivo. Consequently, the designed nanocarrier inhibited growth of PTX-sensitive and -resistant ovarian tumors and showed few adverse effects compared to high-dose PTX injection. Except for FR, Laminin receptor (Lam 67R) which shows high expression level on membrane of human prostate cancer cell has also been used for small molecule targeting, such as epigallocatechin-gallate (EGCG) [249]. EGCG is a major phytochemical component of green tea and has been known to bind to Lam67R with excellent specificity and selectivity. When injecting of EGCG-modified AuNPs to the prostate tumor-bearing mice, 72% retention of the radioactive ^{198}AuNPs was observed in tumors and 80% reduction of tumor volumes was obtained [250]. In a similar study, EGCG and gelatin-DOX (GLT-DOX) conjugated AuNPs shown markable proliferation suppression of PC-3 cancer cells owning to the Laminin67R receptor-mediated endocytosis [251]. Chung et al. found that the Herceptin® loaded micellar nanocomplexes with EGCG demonstrated significant tumor specificity and growth inhibition than free Herceptin [252]. By coating EGCG on ZIF type MOFs (ZIF-C), Poddar and coworkers achieved targeted delivery of CRISPR/Cas9 and efficient treatment of prostate cancer [253]. Accordingly, EGCG coated nanocarriers exhibited a significant increase (23 ± 8%) of cell internalization and cleavage of the ribosomal protein SA gene (PRSA). Effect of PRSA knockdown by CRISRP/Cas9 caused higher killing effect to PC-3 cells compared with normal prostate cells.

4.2.3. Improved cellular internalization for cancer gene editing

After recognizing by targeted cancer cells, most synthetic nanoparticle-based vectors with therapeutic biomolecules enter the cells via endocytosis [254,255]. In this process, nanocarriers from extracellular fluid are engulfed by cell via its membrane invagination and go inside the cell, generating a membrane-bounded vesicle-endosome [256]. Usually, the inside environment of endosomes are acidic (pH, 5.0) and have multiple digestive enzymes that induce biomolecules inactive easily. Therefore, it makes most sense to overcome these barriers to achieve CRISPR/Cas9 based cancer therapy. The solutions of introducing loaded CIRPSR/Cas9 into cytoplasm are discussed in the following section.

4.2.3.1. Endosomal membrane disruption:. pH-buffering effect: Accordingly, pH-buffering (or proton sponge effect) is the most common method to facilitate nanoparticle-based delivery systems escaping from endosome by the disruption of membrane [257]. This phenomenon has observed in some cationic polymers containing protonatable secondary and/or tertiary amine groups, such as PEI and PAMAM [258,259]. When trapping in endosomes, these amine groups with "proton sponge" property become protonated and resist the acidification of endosomes, which can induce high concentration of H^+ and Cl^- pumping and water flowing into endosomes. Eventually, the osmotic pressure causes swelling and disruption of endosomes, releasing the polymers into cytosol. This effect provides direct support for the design of polymer based CRISPR/Cas9 delivery vectors. Recently, Liu et al. reported a multi-stage nanocarriers (MDNP) which could improve tumor-treatment delivery of CRISPR/Cas9 (Fig. 11a) [192]. The delivery system was designed as a core–shell structure. In this structure, the core was a cationic polyplex came from CRIPR/Cas9 plasmid and phenyboronic acid (PBA)-modified PEI (PEI-PBA) and the shell was constructed by 2,3-dimethylmaleic anhydride (DMMA)-modified poly(ethylene glycol)-b-polylysine (mPEG113-b-Plys100/DMMA). After internalized by cancer cells, the delivery system effectively escaped from endosome after 4 h incubation and released plasmids into cytosol owning to the protonating of PEI-PBA. Intravenous administration of pDNA loaded MDNP that targeting to tumor suppressor gene miR-524 achieved effective miR-524 upregulation and remarkable tumor growth retardation in tumor-bearing mice. Similarly, another PEI modified nanocarriers for CRISPR/Cas9 delivery were prepared and employed efficient releasing of Cas9 RNP into colorectal cancer cells, resulting in high gene-editing actively in vitro [170].

Apart from protonable polymers, zeolitic imidazolate framework-8 (ZIF-8) has also used for CRISPR/Cas delivery due to its attractive endosomal escape ability. This mainly comes from the excellent pH-buffering capacity of 2-methylimidazole linkers in ZIF-8. Alsaiari and coworkers have reported one nanoscale ZIF-8 that could encapsulate CRISPR/Cas9 RNP with high loading efficiency of 17% (Fig. 11b) [260]. The endosomal escape of RNP encapsulated ZIF-8 (CC-ZIFs) was clearly observed after 3 and 6 h transfection, after which CRISPR/Cas9 RNP was released to the cytoplasm successfully. Compared with the results in other liposome systems with the same cells in the same period, CC-ZIF-8 had distinct advantage to escape from endosome. Taking EGFP as model gene, the delivery platform resulted in the decrease of EGFP fluorescence up to 37% and EGFP gene expression by 3-folds after 4 days incubation. In another example, ZIF-8 coated by biomimetic cancer cell membrane also constructed for CRISPR/Cas9 delivery, resulting in 3-fold inhibition in EGFP expression and 24% downregulation in EGFP fluorescence [261].

Cationic lipid induced hexagonal H_{II} conformation: For the cationic lipid-based nanocarriers, the endosomal escape is mainly induced by the inverted non-bilayer conformation termed hexagonal HII. Indeed, strong affinity between cationic lipid and the endosomal membrane enable them to mix together [255,262]. This process leads to inverted nonplayer conformations and thereby destroys the integrity of endosomal membrane. After these steps, genetic cargos are released into the cytoplasm with no sealing by the closed endosomal membrane. Therefore, the interaction between the cationic lipid and the anionic membrane is essential to induce the efficient escape and release of genetic cargos. In addition, the existence of "helper" lipids like dioleoyphosphatidyle-thanolamine or cholesterol can boost cationic lipid-mediated cell transfection and promote the HII phase formation. For in vivo application, the ionizable cationic lipid with week surface charge is more attractive than the cationic lipid with permanently charged quaternary amine groups. This is owing to ionizable cationic lipid has less non-specific adsorption with the protein in serum under physiological pH [263]. Thus PEG-lipid analogues were an efficient method to strengthen the cationic lipid induced HII conformation, which was accomplished by including either pH-sensitive PEG analogues or exchangeable PEG-lipid analogues [264].

4.2.3.2. Endosome bypass. Another method to reduce the trap of nanocarriers into endosome is bypassing the endosome, which means the nanocarriers entering cytoplasm through other pathways, such as some cases of caveolae-mediated endocytosis and receptor-mediated endocytosis [265]. For example, Wang et al. reported a PEGylated nanoparticles (named P-HNPs) based on the cationic α-helical polypeptide poly(γ-4-((2-(piperidin-1-yl)ethyl)aminonethyl)benzyl-^{L}-glutamate) and unutilized it for the delivery platform of CRISPR/Cas9 plasmid and sgRNA [266]. The cell-penetrating α-helical polypeptide was proved that could increase cell-uptake and cargoes release via the caveolae-mediated formation. This bypass pathway more directly translated the CRISPR/Cas9 components to the Golgi or endoplasmic reticulum, which facilitated their nuclear transport. As results, single or multiplex gene-editing was achieved and editing efficiency was calculated up to 47.3% in vitro. When delivering the sgRNA tar-

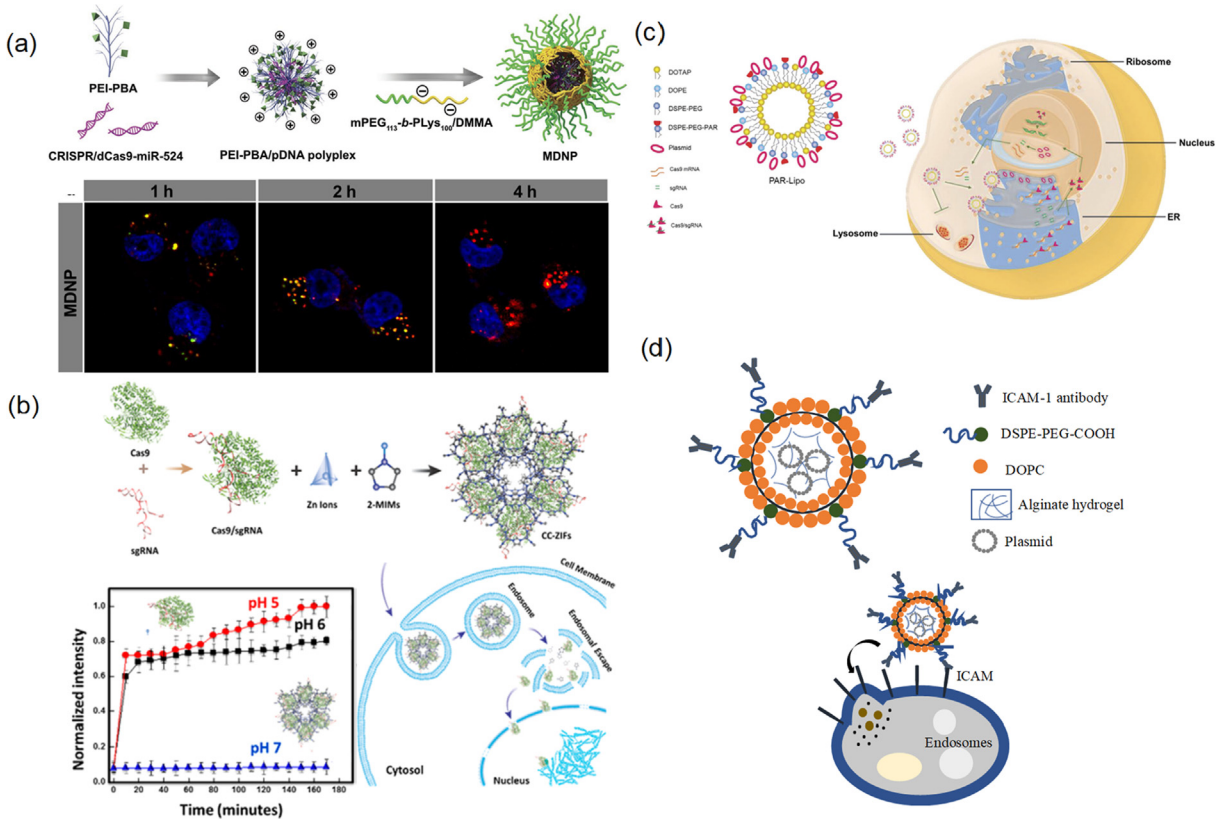

Fig. 11. (a) Preparation illustration and endosomal escape of MDNP containing TOTO-3 (red) labeled pDNA. Adapted with permission from [192]. Copyright 2019, John Wiley and Sons. (b) pH dependent release of Cas9/sgRNA from CC-ZIFs and endosomal escape of CC-ZIFs. Adapted with permission from [260]. Copyright 2018, American Chemical Society. (c) The possible mechanism for enhanced gene editing efficiency by PAR-Lipo to skip the lysosomes capture and induce an increased entrance of the system into nucleus via an ER-nucleus rout. Adapted with permission from [267]. Copyright 2020, John Wiley and Sons. (d) The design of tNLGs and CRISPR plasmids releasing into the cytosol of targeted TNBC cells via ICAM1-mediated membrane fusion pathway. Redrawn from [268].

geted to PLK1 gene, P-HNPs accomplished high PLK1 knock-out (35%) in Hela tumor tissue and tumor growth inhibition (71%), achieving remarkable animal survival (60%) within 60 days. In another study, Yin and coworkers developed a cationic liposome containing DOTAP for CRISPR/Cas9 components delivery (Fig. 11c) [267]. In this study, cationic peptide-pardaxin (PAR) was modified to the liposome to keep the liposome away from lysosomal pathway and guide the liposome to the ER after cellular uptake. Accumulation of PAR-Lipos in the ER improved the binding of Cas9 and sgRNA, eventually enhanced gene editing efficiency in vitro. After intravenously injection of PAR-Lipo/plasmid targeting to oncogene CDC6, the mice shown significant tumor growth suppression compared with Lipo2000/plasmid complexes. In addition to peptide, antibody has also been utilized to improve CRISPR/Cas9 plasmids release into the cytosol directly (Fig. 11d) [268]. A nanolipogel system (tNLG) for tumor-specific CRISPR gene editing was constructed by covalently conjugated ICAM1 antibody on its surface. The study shown the tNLGs selectively recognized and entered triple-negative breast cancer (TNBC) cells via an ICAM1 receptor-mediated membrane fusion pathway, allowing no endosome entrapment of CRISPR/Cas9 plasmid. When delivering the plasmid targeting to breast cancer oncogene, Lipocalin 2 (Lcn2), tNLGs induced > 81% Lcn2 gene knockout in TNBC tumor tissues and > 77% tumor growth suppression in an orthotopic TNBC model.

4.2.4. Controlled release for cancer gene editing

Considering the specific tumor microenvironment and its pathological difference with normal tissues, the stimuli-responsive nanocarriers are rationally developed to control the release of CRISPR/Cas9 components. Generally, the nanocarriers can be responsive to external stimuli, including photo, thermal and ultrasound, etc. and internal stimuli, including pH, redox-regents, ATP and hypoxia etc. [269]. This would decrease the off-targeting risk of CRISPR/Cas9 based cancer therapy and achieve high therapeutic performance. Here, we summary several reported controlled release nanocarriers for CRISPR/Cas9-based oncogene editing.

4.2.4.1. pH-responsive. Recently, nanocarriers with pH-responsive property have been widely applied for controlled release of CRISPR/Cas9. This can be explained by the nature of low pH (pH 5–6.5) inside the organelles of tumor cells and extracellular environment and the pH value in cytosol, blood and normal tissues is almost close to 7.4 [270]. Different kinds of pH-responsive materials, including lipid/liposome, copolymers and inorganic nanoparticles or crystals, have been developed for controlled CRISPR/Cas9 release in tumor, which possess the pH-triggered disassemble property.

Using a thin film method, Zhen et al. constructed nano-liposomes with cationic phospholipid, pH-responsive phospholipid, DSPE-PET2000 and cholesterol for efficient CRISPR/Cas9 encapsulation (95%) [271]. The surface charge of the nano-liposomes increased with decreasing pH and the pH-sensitive induced release was proved in which shown above 80% cargo releasing within 20 h. Intratumoral injection of nano-liposomes targeted to splicing HPV 16 E6/E7 in nude mice could obtain efficient knock-out of HPV 16 E6/E7 and significantly inhibit tumor growth. In addition, injection of the nano-liposome-CRISPR/Cas9

did not cause changes in the tissue structure of main organs, indicating the less biotoxin effects. In another study, Qi et al. prepared a pH-responsive polycation (ARP) through a simple one-pot ring-opening polymerization. The designed ARP was with abundant ortho ester linkages and hydroxyl groups and decorated with fluorinated alkyl chains to form final structure, ARP-F (Fig. 12a) [272]. ARP-F could condense CRISPR/Cas9 plasmid through electrostatic interaction to form a stable nanoparticle. Due to the ortho ester linkages, ARP-F shown obvious structure degradation in acid solution and the plasmid release promotion by acidic degradation had been proved by DNA electrophoresis assays. The subsequent delivery of pCas9-surv (one typical all-in-one Cas9 plasmid that target and knockout survivin gene) mediated by ARP-F exhibited impressive tumor repression performance both in vitro and in vivo. Besides, the knockout of surviving gene increased the sensitivity of tumor cells to anti-cancer drug (temozolomide), providing an efficient combination therapy of cancer.

The pH-responsive disassembly can also happen in some metal-containing self-assembled materials. For example, gold nanoclusters (AuNCs) with carboxylic groups can self-assemble with SpCas9 protein through electrostatic interaction (Fig. 12b) [273]. This self-assembly behavior had strong pH dependence. SpCas9-AuNCs could stay stable at higher pH but could disassemble at lower pH owning to the protonating of carboxylic groups in AuNCs, leading to the release of SpCas9. When used together with the HPV 18 E6 sgRNA, the assembled SpCas9-AuNCs induced remarkable oncogenic E6 gene knock-out (34%) in cervical cancer cells. Therefore, the function of tumor-suppressive protein p53 was restored. Sametime, there were no obvious knockout effect by SpCas9-AuNCs happening on normal cells. In another study, Alsaiari et al. encapsulated Cas9 RNP to ZIF-8 by the directly self-assembling of Zn^{2+}, 2-methylimidazoles likers and Cas9 RNPs [260]. The self-assembly process was pH-dependent due to the protonation capacity of imidazole linkers. The results shown that less than 3% of Cas9 was release under physiological conditions while 60% and 70% were release in 10 min at pH 6 and 5, respectively. The delivery of Cas9 RNP targeted the EGFP coding region led to 37% decrease of the EGFP fluorescence and 3-folds reduction of the EGFP gene expression level. Wang and coworkers reported a pH-responsive silica-metal–organic framework hybrid nanoparticle (SMOF) through a simple water-in-oil emulsion method (Fig. 12c) [274]. SMOF nanoparticles with both silica and ZIF-8 structures could incorporate hydrophilic payloads. The obtained encapsulating content was calculated to 9.5% for CRISPR/Cas9 RNP and encapsulating efficiency was>90%. Because of the same pH-induced degradation of ZIF-8 component, SMOF shown a fast release of encapsulated RNP. As a result, RNP-encapsulated SMOF nanoparticles exhibited high gene-editing efficiency in murine retinal pigment epithelium (RPE) tissue by subretinal injection.

4.2.4.2. GSH-responsive. Owing to their sensitive reduction capacity in tumor sites, the redox-responsive delivery system has been studied extensively in CRISPR/Cas9 delivery [275]. Among them, the nanocarriers with glutathione (GSH)-triggered release property are one of the most potential platforms in recent years. The intracellular concentration of GSH (2–10 mM) is much higher than the concentration in normal regions or extracellular, which ranges from 2 to 10 μM [276]. In recent years, more than a few GSH-responsive delivery platforms have been developed for gene editing delivery, including bioreducible lipid nanoparticles [277], phenylboronic acid-derived lipid nanoparticles [278], and copolymers [279]. The disulfide bond which can be easily cleaved by intracellular GSH is the main functional group in GSH-responsive nanocarriers. In addition, it can also be used to conjugate with some bioactive compounds [276,280].

Wan and coworkers described a supramolecular polymer which could perform the controlled release of Cas9 RNP (Fig. 13a) [170]. The supramolecular polymer system was synthesized by

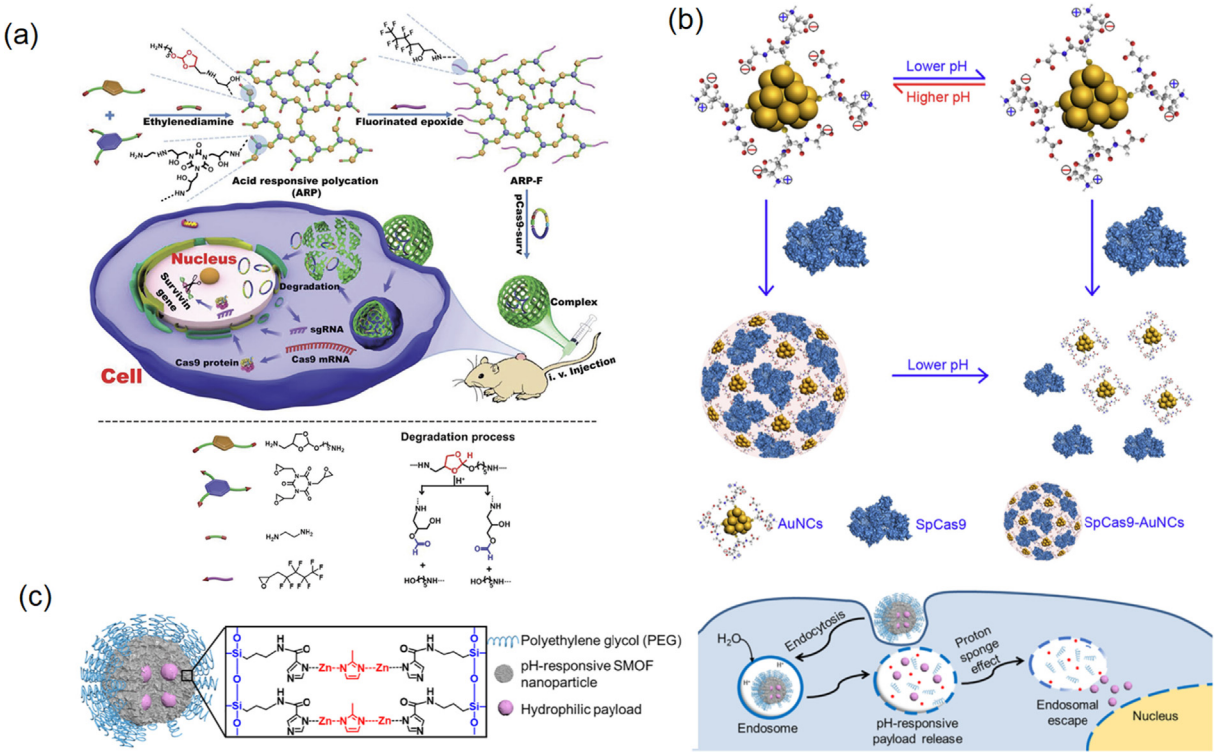

Fig. 12. (a) Preparation illustration of ARP-F and its intracellular delivery process of CRISPR/Cas9 induced by pH responsive. Adapted with permission from [272]. Copyright 2018, John Wiley and Sons. (b) Schematic illustration of pH-induced assembly disassembly of SpCas9-AuNCs. Adapted with permission from [273]. Copyright 2019, American Chemical Society. (c) Schematic illustration of SMOF nanoparticles and its intracellular trafficking pathways. Adapted with permission from [274]. Copyright 2020, Elsevier.

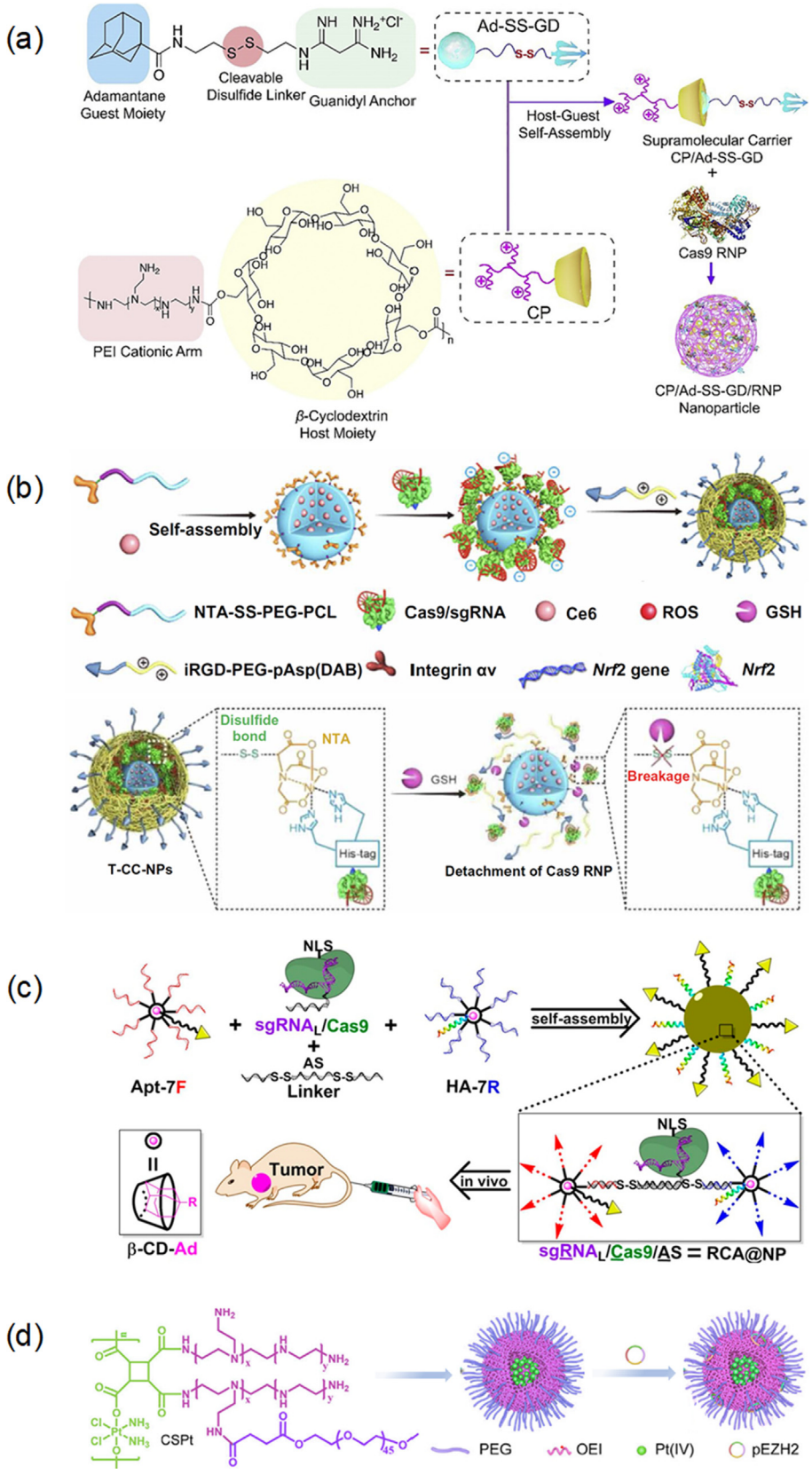

Fig. 13. (a) Preparation illustration of CP/Ad-SS-GD/RNP nonensemble and intracellular RNP delivery induced by GSH. Adapted with permission from [170]. Copyright 2020, Elsevier. (b) Preparation illustration of NIR-sensitive and reducing agent-sensitive nanoparticles containing Nrf2-targeting Cas9/sgRNA. Under the license of CC BY-NC. (c) Preparation illustration of the branched DNA-based nanoplatform for sgRNA/Cas9/antisense delivery and GSH-responsive releasing. Adapted with permission from [282]. Copyright 2019, American Chemical Society. (d) Structure and preparation of the chain-shattering Pt(IV)-backboned polymeric nanoplatform for EZH2-targeted CRISPR/Cas9 delivery. Redrawn from [283]

supramolecular assembly between disulfide-bridge biguanide adamantine (Ad-SS-GD) and β-cyclodextrin-conjugated low-molecular-weight polyethyleneimine (CP) to generated CP/Ad-SS-GD. In the presence of GSH, the RNP releasing from CP/Ad-SS-GD could reach 90% in 72, whereas only 21.9% of RNP had been released in the absence of GSH. When delivering the RNP targeted to mutant KRAS, the nanocomplexes generated remarkable tumor proliferation inhibition in both colorectal cancer cells and colorectal cancer-xenografted models. The approach offered a therapeutic gene-editing modality for the colorectal cancer treatment. For combined cancer therapy, Deng et al. demonstrated a responsive micelle for CRISPR/Cas9 RNP and antitumor photosensitizer chlorin e6 (Ce6) codelivery (Fig. 13b) [281]. The micelle, referred as iRGD-PD was synthesized by nitrilotriacetic acid-disulfanediyldipropionate-polyethyleneglycol-b-polycaprolactone (NTA-SS-PEG-PCL) and iRGD-PEG-b-polyasparte-g-1,4-butanediamine [internalizing RGD-PEG-pAsp(DAB)]. The disruption of the disulfide bond between NTA and PEG induced the RNP detachment responded to GSH, resulting in the disruption of antioxidant regulator Nrf2 both in vitro and in vivo. Sametime, the gene-editing of Nrf2 improved the tumor sensitivity to the NIR/Ce9-genetrated ROS in CNE-2 xenograft mice. This study indicated the efficient synergy of CRISPR/Cas9-based gene-editing and photodynamic therapy. Apart from the polymer-based GSH-responsive nanocarrier, Ding et al. prepared a branched DNA with disulfide bond for CRISPR/Cas9 system delivery (Fig. 13c) [282]. In their study, the β-cyclodextrin (β-CD-7 N3) conjugated with azide was covalently cross-linked with DBCO-modified DNA 7F or 7R and then branched DNA (7F and 7R), linker (antisense with two disulfide linkages) and RNP assembled to generate RCA@NP. The antisense and RNP could be successfully released for the co-assembled nanoparticles when incubated with GSH. By targeting PLK1, the administration of DNA nanocarriers significantly inhibited cell proliferation of MCF7 human breast cancer cells and suppressed tumor growth in MCF7 tumor-bearing mice.

As another GSH-responsive mechanism, a Pt(IV)-backboned polymeric nanoparticles (NP_{CSPt}) has been developed for the delivering of EZH2-targetd CRISPR/Cas9 plasmid ($NP_{CSPt/pEZH2}$) and as the platform of tumor synergistic treatment (Fig. 13d) [283]. NP_{CSPt} was constructed by taking Pt(IV) prodrug as monomer of the particle bone and then grafting low toxic oligomethylenimine ($OEI1_{.8K}$) to endow plasmid binding. After internalization, the CSPt in $NP_{CSPt/pEZH2}$ was breakdown induced by the high concentration of GSH due to the transformation of Pt(IV) to Pt(II). The plasmid DNA were separated and dispersed in the cytoplasm effectively, leading to commendable EZH2 knock-out efficiency in vitro (32.2%) and in vivo (21.3%). The downregulation of H3K27me3 due to EZH2 suppression could increase the accessible possibility of released Pt(II) to nuclear DNA and enhance cell apoptosis. As a result, significant growth inhibition against subcutaneous xenograft tumor was accomplished, showing the great feasibility of gene editing-chemo combination for cancer treatment.

4.2.4.3. Photo responsive. Photo-responsive nanocarriers mediated by light irradiation has been actively explored and realized remote-control of CRISPR/Cas9 releasing with high accuracy and resolution. Although there are many photosensitive materials which respond to ultraviolet (UV) and visible light, their application for CRISPR/Cas9 delivery systems are restricted. UV light has high phototoxicity and the tissue penetration for both UV and visible lights is shallow (less than1 mm) [284]. In comparation, near-infrared (NIR) light shows much lower phototoxicity and deeper tissue penetration up to 5 mm [285]. The NIR-sensitivity of nanocarriers is often produced by functional groups that can change their conformations or suitable optical materials. Usually, this process can convert NIR light to ROS or heat efficiently.

Pan et al. designed a NIR-light sensitive nanocarrier and used it to deliver CRISPR/Cas9 for cancer treatment (Fig. 14a) [286]. The nanocarrier was constructed by coating lanthanide-doped up-conversion nanoparticles (UCNPs) with silica shells and subsequently, surface functionalizing with carboxylic group to form UCNPs@SiO_2-COOH. To achieve remote NIR-controlled releasing, the UV-photocleavable ONA molecules was modifying on UCNPs@SiO_2-COOH via ester bond (UCNPs@SiO_2-ONA), followed by Cas9 protein conjugation (cross-liner chemistry) and sgRNA attachment. The UCNPs acted as "nanotransduces" which could convert NIR light (980 nm) into local UV light to cleavage ONA. As a result, CRISPR/Cas9 released from nanocarriers and entered the nuclei at 6 h, while the signal only could be observed in the cytoplasm without the irradiation of NIR light. By targeting a cancer therapeutic gene PLK1, this strategy successfully suppressed cancer cell proliferation and tumor growth, providing potential lung cancer treatment method by the remote control of NIR light. Without specific Cas9 conjugation or modification, Yan and coworkers synthesized a NIR photolabile semiconducting polymer nanotransducer (pSPN) and demonstrated its controlled-regulating of CRISPR/Cas9-based gene engineering [287]. pSPN was self-assembled from semiconducting polymer which was conjugated with PEG2000 and PEI600 through thioketal moiety. CRISPR/Cas9 plasmids were encapsulated via electrostatic interaction. Under NIR light (680 nm) irradiation, the backbone generated singlet oxygen ($^1O_2$) to cleave thioketal linker and liberate PEI600, resulting in CRISPR/Cas9 plasmid releasing and gene editing. Taking GFP gene as model, the editing efficiency was 15- and 1.8-fold more than the results without NIR irradiation in living cells and mice.

The NIR-responsive releasing of CRISPR/Cas9 can also achieved in some nanocarriers with photothermal conversion ability. For instance, a lipid/AuNPs complex (LACP) was constructed by TAT peptide modifying and lipids (DOTAP, DOPE, cholesterol, PEG2000-DSPE) coating and used for Cas9-sgPlk-1 plasmids responsive delivery [288]. Because of localized surface plasmon resonance effect of AuNPs, which generated heat to induce LACP bond broken, the intracellular release of Cas9-sgPlk-1 plasmids was triggered under laser irradiation. Cellular delivery studies shown that 20 min irradiation yielded the maximum plasmids releasing signal in A375 melanoma cells. After Cas9-sgPlk-1 entering the nucleus, this system realized knock of Plk-1 gene and efficient cancer treatment in vivo. Similarly, Peng and coworkers constructed a NIR laser sensitized CRISPR/Cas9 nanomachine (LACM) that was capable to deliver the sgRNA into cancer cells (Fig. 14b) [289]. The LACM was designed using gold nanorods (AuNR) to absorb NIR and decorating them with plenty of protector DNAs. The targeting sgRNA could hybridize to the protector DNA, thereby realizing conjugating of the sgRNA on AuNR and liberate from the protector DNA due to the heat induced denatures upon NIR irradiation (808 nm). It was found that LACM performed NIR-activated editing of EGFP and EMX1 genes in A549 and HEK293T cells. Sametime, the knock-down of PLK1 gene induced significant apoptosis of cancer cells. Alternatively, Chen et al. used AuNR-based nanocarrier to deliver Cas9 plasmid that encoded with a heat-inducible promotor, HSP70 (Fig. 14c) [290]. The nanocarrier was coated with biocompatible polystyrene sulfonate (PSS) and cationic β-cyclodextrin-polyethyleneimine. Then Cas9 plasmid was encapsulated to form APC. Instead of controlling plasmid releasing, the photothermal effect of AuNR was used to regulate Cas9 expression and activity. Due to the NIR-II-absorbing feature of APC, the optogenetic regulation was validated in deep tissue with a high resolution. By adjusting NIR laser exposure time and irradiation time, the editing degree could be finely programed. For in vitro transfection, the APC induced a remarkable knock-out level in different genomic loci (EGFP, AAVS1 and RHBDF1) upon optogenetic activation. In addition, therapy of deep cancer

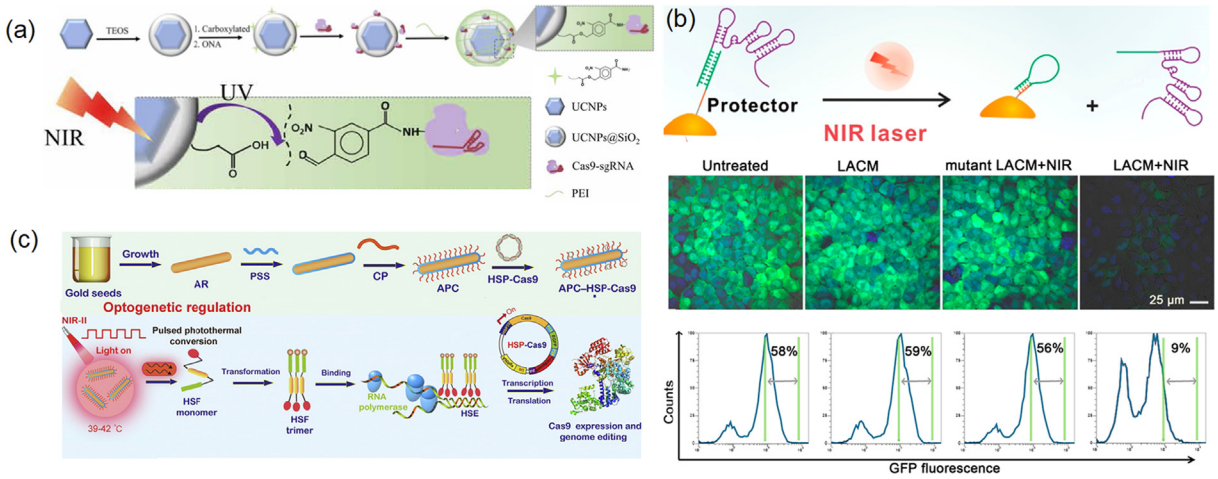

Fig. 14. (a) Preparation of the UCNP-based CRISPR/Cas9 delivery system and mechanism of NIR light-controlled gene editing. Under the license of CC BY-NC. (b) The release of the sgRNA from protector induced by NIR irradiation and EGFP editing results of LACM. Adapted with permission from [289]. Copyright 2020, American Chemical Society. (c) Preparation illustration of the APC with Cas9 plasmid that encoded HSP70 and mechanism of inducible optogenetic regulation of Cas9 expression and gene editing. Under the license of CC BY-NC-ND.

and cue of fulminant hepatic failure with minimized off-target effect were achieved by the NIR-responsive programmable editing strategy.

4.2.5. Delivery strategies for cancer immunotherapy

Recently, immunotherapy is increasingly becoming an efficient strategy for cancer therapy [291]. Instead of attacking tumor directly, cancer immunotherapy is to stimulate natural immune system of the human body to fight against cancers. Even though the progress made until now in cancer treatment is promising, there are still some limitations, including the lack of proper treatment choice for certain cancer types, noneffective for some patients, potential immune toxicities and resistance of the tumor microenvironment [292]. Considering that nanotechnology has the potential to transport, enhance and modulate cancer immunotherapeutic components, the combining of nanotechnology and immunotherapy is supposed to conquer some of the limitations [293], especially CRISPR/Cas9-mediated nanoplatforms. The immune checkpoint blockade therapy with CRISPR/Cas9 systems has been developed and used for cancer treatment. Among that, PD-1/PD-L1 pathway is the most studied one [294]. Nowadays, several studies have been developed to explore how the convergence of nanotechnology and CRISPR/Cas9 can be used to improve cancer immunotherapy.

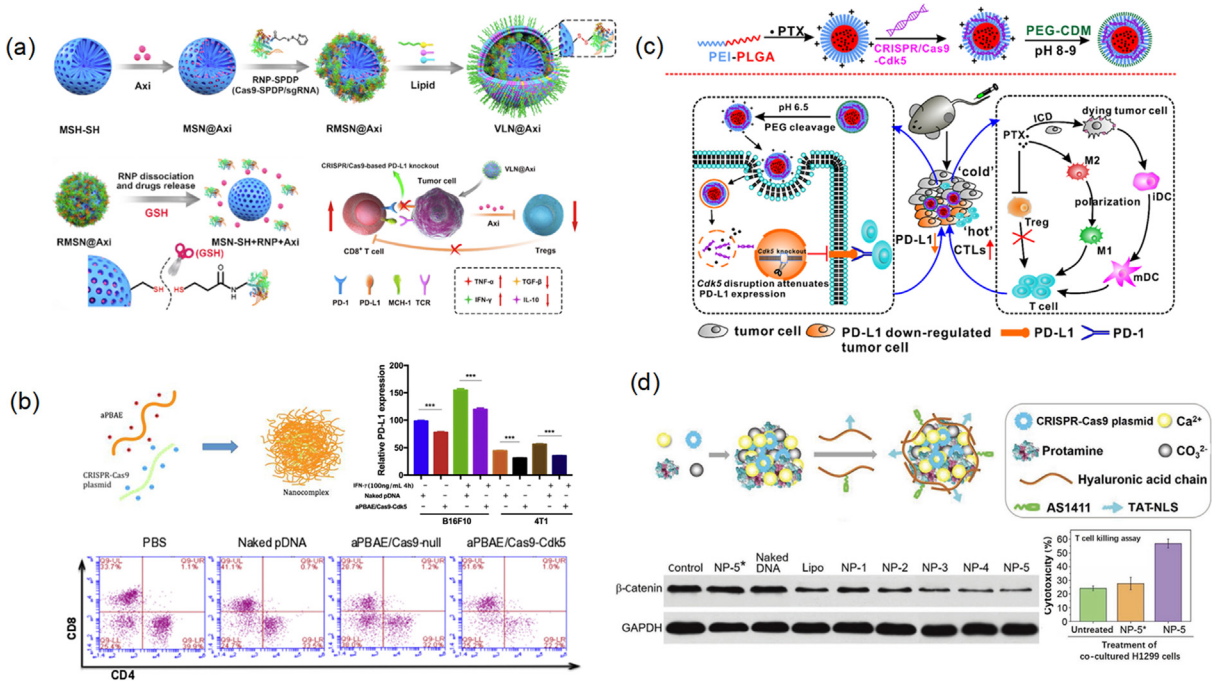

Fig. 15. (a) Preparation illustration of VLN codelivery system and GSH induced RNP releasing for gene-editing. Adapted with permission from [295]. Copyright 2020, Elsevier. (b) Preparation of PBAE/CRISPR-Cas9 nanoparticle and its application in PD-L1 attenuation therapy. Under the license of CC BY-NC-ND. (c) Illustration of pH-responsive nanoparticles for PTX and CRISPR/Cas9-Cdk5 delivery and pH-induced gene disruption and immunochemotherapy. Adapted with permission from [298]. Copyright 2020, American Chemical Society. (d) Preparation of the multi-functionalized delivery system of CRISPR/Cas9 plasmid and β-catenin editing results in tumorous H1299 cells by the delivery system. Adapted with permission from [171]. Copyright 2020, John Wiley and Sons.

By targeting to PD-L1 encoding gene in cancer cells, Liu et al. reported a virus-like nanoparticle (VLN) to co-delivery CRISPR/Cas9 system and small molecule drugs (axitinib) (Fig. 15a) [295]. VLN had a core–shell structure, in which CRISPR/Cas9 and axitinib were loaded in MSNs-based core and further coated with a lipid shell came from DOTAP, DOPE, PEG2000-DSPE. The structure allowed VLN maintaining stable during blood circulation and releasing the cargoes by GSH-induced bond cleaving. In vitro and in vivo studies demonstrated the VLN shown effective PD-L1 knockout based on CRISPR/Cas9 and disruption of PD-1/PD-L1 checkpoint pathway, resulting in the suppress of melanoma. Furthermore, the co-delivery of axitinib promoted the tumor growth inhibition by reducing the immunosuppressive Tregs. Apart from knockout PD-L1 directly, to attenuate PD-L1 expression via other kinds of gene editing provides an alternative approach for immunotherapy. Deng and colleagues successfully designed a CRISPR/Cas9 gene-editing platform delivered by cationic copolymer and applied it to reduce PD-L1 expression on tumor cells (Fig. 15b) [296]. They first constructed CRISPR/Cas9 plasmid that targeted cyclin-dependent kinase 5 (Cdk5). The knockout of Cdk5 could decrease PD-L1 expression owning to the interference with interferon regulatory factor-2 (IRF2) and interferon regulatory factor 2-binding protein 2 (IRF2BP2) [297]. By condensing the plasmid with poly(β-amino esters) (PBAEs) via electrostatic

interaction, the nanocomplex exhibited transfection efficiency of 95% in B16F10 cells and 70% in 4 T1 cells, which were much higher than that mediated by PEI and commercial liposome HP. By knocking out Cdk5, the expression level of PD-1 on tumor cells was remarkable decreased, resulting in significant tumor growth repression in murine melanoma and lung metastasis suppression in triple-negative breast cancer. In addition, the designed nanocomplex induced powerful immune responsive by T cell. The population of CD8 + T cells highly increased, while the population of Tregs highly decreased. In another study of Cdk5 knockout, researchers fabricated a pH-sensitive nanocarrier to accomplish the co-delivery of CRISPR/Cas9-Cdk5 plasmid (Cas9-Cdk5) and chemotherapeutic agent (PTX) (Fig. 15c) [298]. The designed platform was core–shell structure. The inner core composed by cationic copolymer poly-(ethyleneimine)-poly(lactic-co-glycolic acid) (PEI-PLGA) used for Cas9-Cdk5 and PTX encapsulation. The shell was CDM modified PEG, which could be broken under weakly acidic condition. Within the first 24 h, the results shown 70% PTX got released in the pH 6.5 and 5.5, while only 35% PTX was released in the pH 7.4. Because of the existence of PEI, the nanocarriers exhibited high transfection efficiency in both B16F10 and CT26 cells. Significant PD-L1 expression had been attenuated via the knockout of Cdk5, generating the restore of cytotoxic T lymphocytes (CTLs) mediating antitumor immunity in melanoma and colorectal tumor models. Moreover, PTX loaded in the nanocarriers induced the immune checkpoint blockade effect, reduced Tregs and reprogramed tumor microenvironment. Recently, a multifunction nanocarrier which could deliver CRISPR/Cas9 plasmid for β-catenin knockout was constructed and explored it application in immunosuppression reversing (Fig. 15d) [171]. The silence of β-catenin could impair PD-L1 expression on tumor cells. The plasmid was encapsulated in the core consisted of $CaCO_3$ and protamine. Then aptamer (AS1411) conjugated hyaluronic acid (AHA) and peptide (TAT-NLS) conjugated hyaluronic acid (PHA) were assembled onto the core. With the targeting and cell penetrating components, the delivery system resulted in high β-catenin editing efficiency (up to 40.2%). After β-catenin inhibition, the expression of PD-L1 was significantly downregulated in both H1299 and Hela cells, leading to the efficiently recognizing and eliminating of edited cancer cells by CD8 + T cells.

5. Conclusion and perspectives

As a powerful gene-editing tool, CRISPR/Cas9 produces dramatic impact to the field of molecular biology and gene therapy. It has been widely adopted worldwide and utilized to target gene engineering in various cells and organisms. At that point, CRISPR/Cas9 systems bring new hope to cancer treatment. It not only possesses great application potential for cancer genes manipulation, but are also used to regulate gene expression, construct tumor models, achieve tumor immunotherapy and are used to explore anti-cancer drugs. They have been utilized to treat many kinds of cancers, such as brain, liver, lung, bladder, colorectal, et al., and has accomplished remarkable results in vitro and in vivo. There are high demands for the clinical translation of CRISPR/Cas9 system in cancer treatment. So far, most of the CRISPR/Cas9-based clinal trials are happening in vitro, which needs the isolation of cells form patients and the transportation back into the patients after gene correcting with CRISPR/Cas9 system. And the delivery of CRISPR/Cas9 agents for this purpose depends heavily on viral vectors and physical approaches.

Particularly, the lack of safe and efficiency delivery systems is the biggest obstacle for the clinical application of CRISPR/Cas9. Apart from the high encapsulation ability and biocompatibility, the delivery system needs to overcome many physical barriers and direct carry CRISPR/Cas9 components to the target sites, realizing the accurate and effective tumor treatment. With the rapid development of nanotechnology, the vectors designed from different nanoparticles are widely studied, showing enormous potential. To date, nanotechnology-based vectors, such as polymers, lipids, PSi, MSNs, and MOFs have been reported for anticancer cargoes delivery. Furthermore, nanotechnology and nanotechnology-based delivery systems provide improved therapy effect and reduced adverse side effects.

Hence, the development of nanocarriers to delivery CRISPR/Cas9 into targeted cancer is the general direction of clinic application. As evident trough the many examples presented in this review, the cancer therapeutic potential of these delivery systems is great. First, personalized nanoparticles are ideal for efficiently packaging and protecting of different forms of CRISPR/Cas9 components through self-assembly. Second, the nanocarriers have abilities to accommodate many different, very reactive chemical groups which can be used to do surface modification for more effective blood circulation, intracellular uptake, and better targeting. Third, the nanocarriers integrated chemical moieties possess responsive CRISPR/Cas9 release profile under the trigger of specific intracellular environment or extracellular signals for spatiotemporal gene editing. These smart delivery systems based on nanotechnology have significantly improved the cancer therapy ability and decreased undesirable off-target effect of CRISPR/Cas9 treatment.

Nanotechnology-delivered gene-editing with CRISPR/Cas9 is a new dawn in the field of cancer treatment. To study the underlying working mechanism of CRISPR/Cas9, nanoparticles, cancer cells or tissues and translation of finding from laboratory to clinic are the ultimate target of nanocarrier development. Although most of the present CRISPR/Cas9 nanocarriers cannot fulfill all the requirements of clinical trials, the perspective is certainly positive. Any limitations will eventually be addressed and solve with the sustained attention of researchers from different fields. We believe that future advances in nanotechnology-based vectors will scale-up production and broaden its application sphere of CRISPR/Cas9-based therapeutic genome editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work is supported by Academy of Finland (Grant No. 328933) and Sigrid Jusélius Foundation (decision no. 28002247 K1) and the program of China Scholarships Council (No. 201803170073).

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