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Overcoming challenges in the delivery of STING agonists for cancer immunotherapy: A comprehensive review of strategies and future perspectives

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ABSTRACT

STING (Stimulator of Interferon Genes) agonists have emerged as promising agents in the field of cancer immunotherapy, owing to their excellent capacity to activate the innate immune response and combat tumor-induced immunosuppression. This review provides a comprehensive exploration of the strategies employed to develop effective formulations for STING agonists, with particular emphasis on versatile nano-delivery systems. The recent advancements in delivery systems based on lipids, natural/synthetic polymers, and proteins for STING agonists are summarized. The preparation methodologies of nanoprecipitation, self-assembly, and hydrogel, along with their advantages and disadvantages, are also discussed. Furthermore, the challenges and opportunities in developing next-generation STING agonist delivery systems are elaborated. This review aims to serve as a reference for researchers in designing novel and effective STING agonist delivery systems for cancer immunotherapy.

1. Background

STING agonists are recently being widely investigated and applied in cancer immunotherapy due to their capacity of triggering innate immunity in the local tissues and efficiently alleviate the immunosuppressive environment by triggering the cGAS (cyclic GMP-AMP synthase) -STING signaling pathway [1–4]. For now, several STING agonists such as ADU-S100, E7766, and GSK3745417 have been approved for clinical trials of cancer immunotherapy [5–7]. Besides, STING agonists were also developed as adjuvating subunit vaccines of pathogens like influenza, SARS-CoV-2, HIV, *etc* [8–10]. However, low cytosol delivery derived from its physiochemical properties such as electronegativity, hydrophilicity, short half-life, *etc.* significantly confined its further clinical translation and application [11].

The cGAS-STING signaling pathway is activated by a specific ligand binding to the STING protein located in the endoplasmic reticulum [12,13]. The endogenous dinucleotide, 2′3′-cyclic GMP-AMP (cGAMP), is generated by cGAS when it recognizes DNA in the cell, and binds to the endoplasmic reticulum (ER)-localized adaptor protein STING to activate the cGAS/STING signaling pathway [14,15]. After a series of reactions, the binding of cGAMP to STING recruits TANK-binding kinase 1 (TAK1) [16] and interferon regulatory factor 3 (IRF3) [17] and ultimately upregulates type I interferons (IFNs) expression [18–20], which results in the maturation, migration and activation of dendritic cells (DCs), T

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cells, and natural killer (NK) cells [21,22]. However, some negatively charged and hydrophilic STING agonists, such as the cyclic dinucleotide monophosphates (CDN) are hard to permeate the cell membrane and internalize into the cytoplasm [23–25]. Consequently, the low intracellular uptake efficiency of STING agonist result in insufficient presentation efficiency of STING agonists to antigen-presenting cells (APCs) Besides that, the enzymatic degradation of STING agonists in blood circulation can lead to a fast clearance rate and short half-life of STING agonists [26]. Natural CDNs, for instance, can be subject to degradation by phosphodiesterases, particularly ectonucleotide pyrophosphatase/-phosphodiesterase 1 (ENPP1), which is present in the blood, thus restricting their administration method to intratumoral injection in most clinical trial scenarios [27–30]. Recently, the development of synthetic non-phosphate STING agonists like 5,6-dimethylxanthenone-4-acetic acid (DMXAA) partially improves the resistance to enzyme degradation and enhances its half-life [31–33]. However, its affinity for STING is not comparable to that of naturally derived CDNs like cGAMP. For example, in a clinical trial for advanced non-small cell lung cancer, DMXAA administration failed to improve frontline antitumor efficacy because it could not bind to human STING [34,35]. Interdisciplinary collaborations encompassing materials science, nanotechnology, and pharmaceutics have emerged as promising strategies to tackle the aforementioned challenges.

In fact, delivery systems for STING agonists, such as liposomal formulations and PLGA micro/nanoparticles, have already been developed and utilized [36–38]. By utilizing the nanoprecipitation method, STING agonists were encapsulated into the hydrophobic core of micro/-nanoparticles and thus effectively prevent its direct contact or interaction with the physiological environment, and consequently, the bioactivity and half-life of STING agonists were highly improved. Besides, micro/nanoscale delivery systems change the cellular uptake of STING agonists from direct diffusion to endocytosis, which enhances intracellular and presentation efficiency to antigen-presenting cells. Although significant research has been conducted to develop delivery systems for STING agonists in materials science and nanomedicine, few reviews have comprehensively discussed the principles of designing and preparing a delivery system based on the physicochemical properties of STING agonists and the chosen preparation methodology.

Therefore, in this review, the advancement of delivery systems based on lipids, natural/synthetic polymers, protein, *etc.* for STING agonists was collectively summarized in terms of preparation methodology namely nanoprecipitation method, self-assembly, and hydrogel. The advantages and disadvantages of each preparation methodology were also included in this manuscript. Besides that, the underlying issues of developing the next-generation STING agonists delivery systems were also included and discussed. In this regard, we envision the review may provide a reference for researchers to design new and novel STING agonist delivery systems.

2. cGAS-STING pathway and STING agonists

Innate immunity not only controls the infection and transmission of pathogens in the early stages of infection but can also play an important role in generating an enhanced response during secondary challenges with various stimuli [39,40]. cGAS-STING signaling pathway is an elemental part of innate immunity that participates in evacuating intracellular pathogens through the detection of double-stranded nucleic acid (dsDNA) [41–43]. Together with the other pathogen recognition receptor (PRR) agonists like Toll-like receptors (TLRs), retinoic acid-inducible gene (RIG-I)-like receptors, and nucleotide-binding oligomerization domain (NOD)-like receptors, STING agonists are widely investigated as an adjuvant for developing subunit vaccine formulations to defense virus infection [44–48].

Current cancer immunotherapy strategies focus on enhancing antitumor adaptive immune responses, but their therapeutic effects have been limited to certain tumor types [49,50]. However, the innate immune system's role in tumor immunosurveillance and generating antitumor immune responses has long been recognized [51], and new strategies targeting innate immunity in cancer treatment have emerged. Recently, research has shown that the interaction between STING activation and the tumor microenvironment (TME) is also important [52–54].

The activation of STING leads to the downstream regulation of cytokines that bridge innate and adaptive immunity [55–57]. In particular, STING activation promotes the secretion of cytokines such as Type I IFNs, which enhance the levels of cytotoxic T cell responses and type 1 T helper cell (Th1)-based responses [58]. Additionally, the elevated levels of Type I IFNs promote the activation and maturation of DCs [59,60] facilitating antigen (cross) presentation to $CD4^+$ T cells [61] or $CD8^+$ T cells [62–66]. The cGAS-STING pathway has the capacity to facilitate the transformation of a "cold" tumor immune environment into a "hot" tumor-immune microenvironment. The distinction between hot and cold tumors is based on the cytotoxic T cell landscape within a tumor [67]. Cold tumors are characterized by limited immune cell infiltration and a weak immune response within the tumor microenvironment [68], while hot tumors have abundant immune cell infiltration and an active immune response [69,70]. Therefore, converting cold tumors into hot tumors can max the effectiveness of antitumor and immunotherapy. And the cGAS-STING pathway is also recognized as a key mechanism that initiates the anti-tumor innate immune response and can modulate the TME [71–73].

STING agonists can be divided into natural form and synthetic form (Fig. 1 and Table 1). Natural forms are referred to as cyclic dinucleotide monophosphate including cyclic dimeric guanosine monophosphate (c-di-GMP), cyclic dimeric adenosine monophosphate (c-di-AMP), and cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) from bacterial or human sources [74]. Besides, Rp, Rp-20, 30-*c*-di-AMPSS (ADU-S100), the synthetic phosphorothioate analog, was also widely investigated and has been approved in several clinical trials for cancer immunotherapy [75–77]. On the other hand, the synthetic forms, which do not contain phosphate group, are mainly analogs based on the structures of xanthenone-4-acetic acid (XAA), (*e.g.* DMXXA), acridanone (*e.g.* 10-carboxymethyl-9-acridanone), 2-phenyl-2-thio-acetamide (*e.g.,* C11), xanthone (*e.g.* α-Mangostin), amidobenzimidazole (*e.g.,* diABZIs), 3-oxo-3,4-dihydro-2H-benzo[b] [1,4] thiazine-6-carboxamide skeleton (*e.g.,* G10), picolinamide skeleton (*e.g.* SINCRO), tetrahydro-dispiro-pyrazine-indene-hexaone (*e.g.,* DSDP) and N-naphthalen-benzo dioxole carboxamide (*e.g.,* BNBC) [78–80]. In contrast with the natural forms, these synthetic small molecules are more resistant to enzyme degradation and have a longer half-life in blood circulation. However, their affinity to the cell membrane is unsatisfactory. For example, DMXAA was reported to not bind to human STING in Phase III clinical trials for advanced non-small cell lung cancer treatments [81,82]. As of date, there have 12 clinical trials have been either terminated or ongoing in exploring the feasibility of STING agonists like ADU-S100, E7766, and GSK3745417 in the treatment of solid tumors or lymphoma (Table 2) [83,84]. Due to the low cytosolic delivery of STING agonists, particularly the natural forms because of their negative charge and hydrophilic properties, most of the clinical trials administering STING agonists such as ADU-S100, E7766, and GSK3745417, use intratumoral injection. This approach enables direct targeting of the tumor site with relatively well-defined primary concentrations while limiting systemic exposure and associated toxicities [85–88]. In some cases, they are also administered intravenously or subcutaneously.

Moreover, due to their physiological function on innate immunity, STING agonists are often combined with immune checkpoint inhibitors (ICIs), such as anti-programmed death 1 and cytotoxic T lymphocyte-associated antigen 4 antibodies [91–94]. On one hand, the effectiveness of cancer immunotherapy with ICIs relies on the presence of a pre-existing antitumor T cell response within the tumors, often referred to as "hot tumors" [95]. On the other hand, the activation of the STING

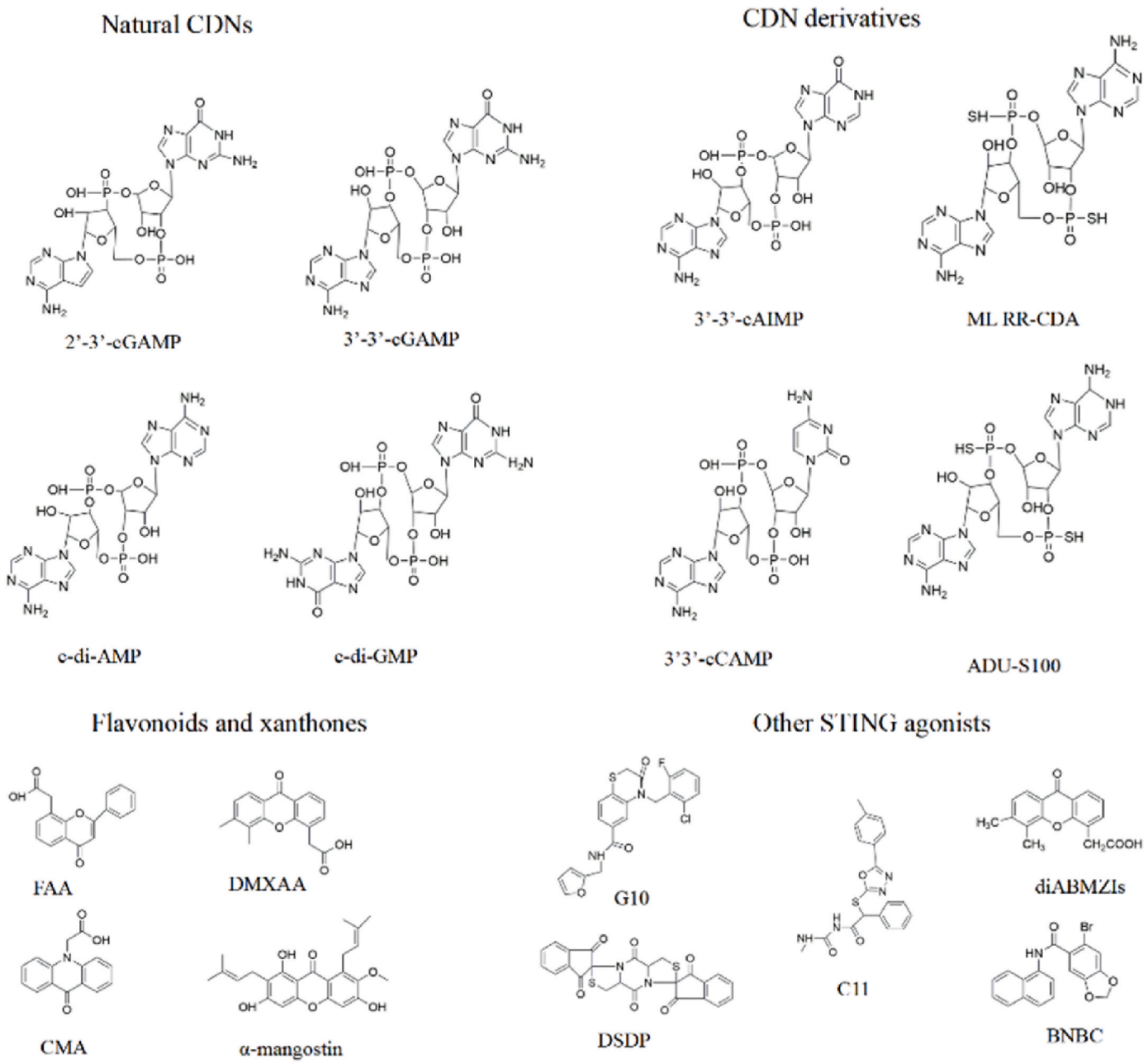

Fig. 1. Chemical structures of various STING agonists.

pathway can enhance innate immunity, promote antigen presentation and T cells activation, thereby facilitating the transformation of the tumor microenvironment from a "cold" state to a "hot" state. Additionally, the combination of STING agonists with immune checkpoint inhibitors has the potential to mitigate immune suppression, which is often triggered by the upregulation of the immune checkpoint programmed death-ligand 1 (PD-L1) expression due to the elevation of type I interferon (IFN) levels [96–98]. Therefore, accumulating clinical evidence has confirmed that the combination of immune checkpoint inhibitors and STING agonists is a key factor in enhancing immune responses and improving the effectiveness of cancer immunotherapy. Clinical trials combining STING agonists and ICIs are currently in progress [99,100].

Overall, STING agonists hold great clinical potential in the treatment of pathogen infection and various solid tumors. However, their physiological and chemical properties, such as enzyme degradation, short residence time, immunocellular toxicity, and low cytosolic delivery, significantly limit their clinical translation and application [101]. Therefore, delivery strategies utilizing nanotechnology, hydrogel, and other platforms are emerging and developing in recent years [102,103]. Nano-complexation or physical encapsulation in materials offers a way to preserve STING agonists from enzyme degradation or other harsh physiological environments [104,105]. In addition, cationic surface modifications have been found to promote cytosolic delivery efficiency, and further chemical modifications of materials can be used to fine-tune the release kinetics of the loaded drugs, minimizing potential side effect [106,107] The following section will discuss strategies utilizing various novel biomaterials for the delivery of STING agonists, based on their preparation methods or techniques.

Table 1
Listing of the features of both natural and synthetic STING agonists.

STING agonists	Characteristics	Classification	References
Natural STING agonists	Hydrophilic small molecules with negative charges; They are susceptible to enzymatic degradation; Low bioavailability in target tissues, unwanted toxicities and narrow therapeutic windows.	2′3′-cGAMP, 3′3′-cGAMP, c-di-GMP, c-di-AMP	[5,89,90]
Synthetic STING agonists	The metabolic stability has been significantly improved; A longer half-life in blood circulation; The affinity to the cell membrane is unsatisfactory.	CDN derivatives, Flavonoids and xanthones, Other STING agonists	[1,5,78]

3. Nano delivery strategy

3.1. Nanoprecipitation

The nanoprecipitation technique is the most conventional and widely used method for the preparation of various nano-drug delivery systems in the past decades owing to its facile, reproducible, scalable, and low-cost properties [108,109]. The principle of nanoprecipitation is compatible with both hydrophobic and hydrophilic solutes used for nanoparticle generation. It involves a spontaneous emulsification process that only requires complete miscibility of the polymer's solvent and non-solvent [110]. For instance, when a solute, such as a hydrophobic polymer, is dissolved in a solvent (*e.g.*, ethanol, tetrahydrofuran or THF, acetone …), the mixture leads to supersaturation and phase separation, ultimately resulting in the controlled and reproducible formation of nanoparticles [111]. The nanoprecipitation of materials is achieved by removing the solvent in which the materials are dissolved, which can be done by adjusting the pH, salt concentration, solubility conditions, or the addition of a non-solvent phase [112]. This alteration in solvent quality allows for the formation of nanoparticles. In the delivery of STING agonists, lipids and commercial biodegradable polymers such as polylactide (PLA) and polylactide-*co*-glycolide (PLGA) have received the most attention from researchers.

The emulsion/double emulsion method is currently the primary technique used in the preparation of liposomes loaded with STING agonists. However, with the advancement of nanotechnology, alternative fabrication techniques like thin-film hydration and extrusion are emerging as options for better control of the monodispersity of liposomes. These newer techniques offer improved precision in the production of nanoparticles and can provide more uniform sizes, which is essential for consistent drug delivery. Among all these lipid formulations, dipalmitoylphosphatidylcholine (DPPC) is the most abundant disaturated neutral phospholipid that is being used in the development of liposomes loaded with STING agonists [113,114]. DPPC-based liposome formulations can be efficiently formulated with cholesterol, DPPE-PEG2000, or DSPE-PEG2000 in an optimal ratio to encapsulate STING agonists such as cGAMP or cdGMP into the hydrophobic core through solvent/non-solvent alternation. For instance, in the work of E. Karathanasis et al., an immunostimulatory liposome (immuno-LP) formulated with DPPC, cholesterol, and DSPE-PEG_{2000} at a molar ratio of 77:20:3 were prepared through thin-film hydration method for co-delivery of cdGMP and monophosphoryl lipid A (MPLA) to treat pancreatic ductal adenocarcinoma (Fig. 2A). The as-prepared immuno-LP was ~60 nm in diameter with a loading efficiency of cdGMP and MPLA at about 60 % and 50 % respectively. Study on an orthotopic murine Panc02 model has demonstrated the systemic administration of immuno-LP loaded with STING pathway agonist (cdGMP) and Toll-like receptor 4 agonists (MPLA) could effectively promote the expansion of APCs and infiltration of lymphocytes, leading to an 11-fold increase in the level of Interferon β (IFN-β). This treatment effectively alleviated the immunosuppressive tumor microenvironment in pancreatic tumors (cold tumor). Consequently, the median survival time of mice treated with immuno-LP was prolonged from 24 to 56 days compared to the untreated group.

The team of E. Karathanasis investigated the feasibility of using the dual-agonist lipid formulation in combination with PD1 blockade for the treatment of aggressive cancers [116]. In this work, a similar formulation was used except that the cholesterol was replaced with 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). They found that this nanoformulation was able to deliver the encapsulated immune agonists

Table 2
Listing of STING agonists approved for clinical trials.

Agonists	Treatment modality	Company	Administration method	Indications	Current status	NCT code
ADU-100	Combined with anti-CTLA4 mAb	Aduro Biotech; Novartis	Intratumoral injection (*i.t.*)	Advanced/metastatic solid tumors; lymphoma	Phase I (terminated)	NCT02675439
ADU-100	Combined with anti-PD-L1 mAb	Novartis	*i.t.*	Advanced solid tumors; lymphoma	Phase Ib (terminated)	NCT03172936
ADU-CL-20	Combined with anti-PD-L1 mAb	Aduro Biotech	*i.t.*	Metastatic/recurrent head and neck squamous cell carcinomas	Phase II (ongoing)	NCT03937141
BMS-986301	Monotherapy/combined with anti-PD-L1 mAb or anti-CTLA4 mAb	Bristol-Myers Squibb	*i.t.*	Advanced solid tumors	Phase I (ongoing)	NCT03956680
DMXAA	Combined with carboplatin and Paclitaxel	Antisoma; Novartis	Intravenous injection (*i.v.*)	Non-small cell lung cancer	Phase III (terminated)	NCT00662597
E7766	Monotherapy	Eisai Inc.	*i.t.*	Advanced solid tumors; lymphoma	Phase Ia/Ib (ongoing)	NCT04144140
E7766	Monotherapy	Eisai Inc.	*i.v.*	Bladder cancer	Phase I (ongoing)	NCT04109092
GSK3745417	Monotherapy/combined with anti-PD-L1 mAb	GSK	*i.t.*	Advanced solid tumors	Phase I (ongoing)	NCT03843359
IMSA-101	Monotherapy/combined with anti-PD-L1 mAb	ImmuneSensor Therapeutics	*i.t.*	Advanced solid tumors	Phase I/IIa (ongoing)	NCT04020185
MK-1454	Monotherapy/combined with anti-PD-L1 mAb	Merck & Co	*i.v.*	Advanced/metastatic solid tumors; lymphoma	Phase I (ongoing)	NCT03010176
MK-2118	Monotherapy/combined with anti-PD-L1 mAb	Merck & Co	*i.v.*	Advanced/metastatic solid tumors; lymphoma	Phase I (ongoing)	NCT03249792
SB-11285	Monotherapy/combined with anti-PD-L1 mAb	Spring Bank Pharmeceuticals	*i.v.*	Advanced solid tumors	Phase Ia/Ib (ongoing)	NCT04096638

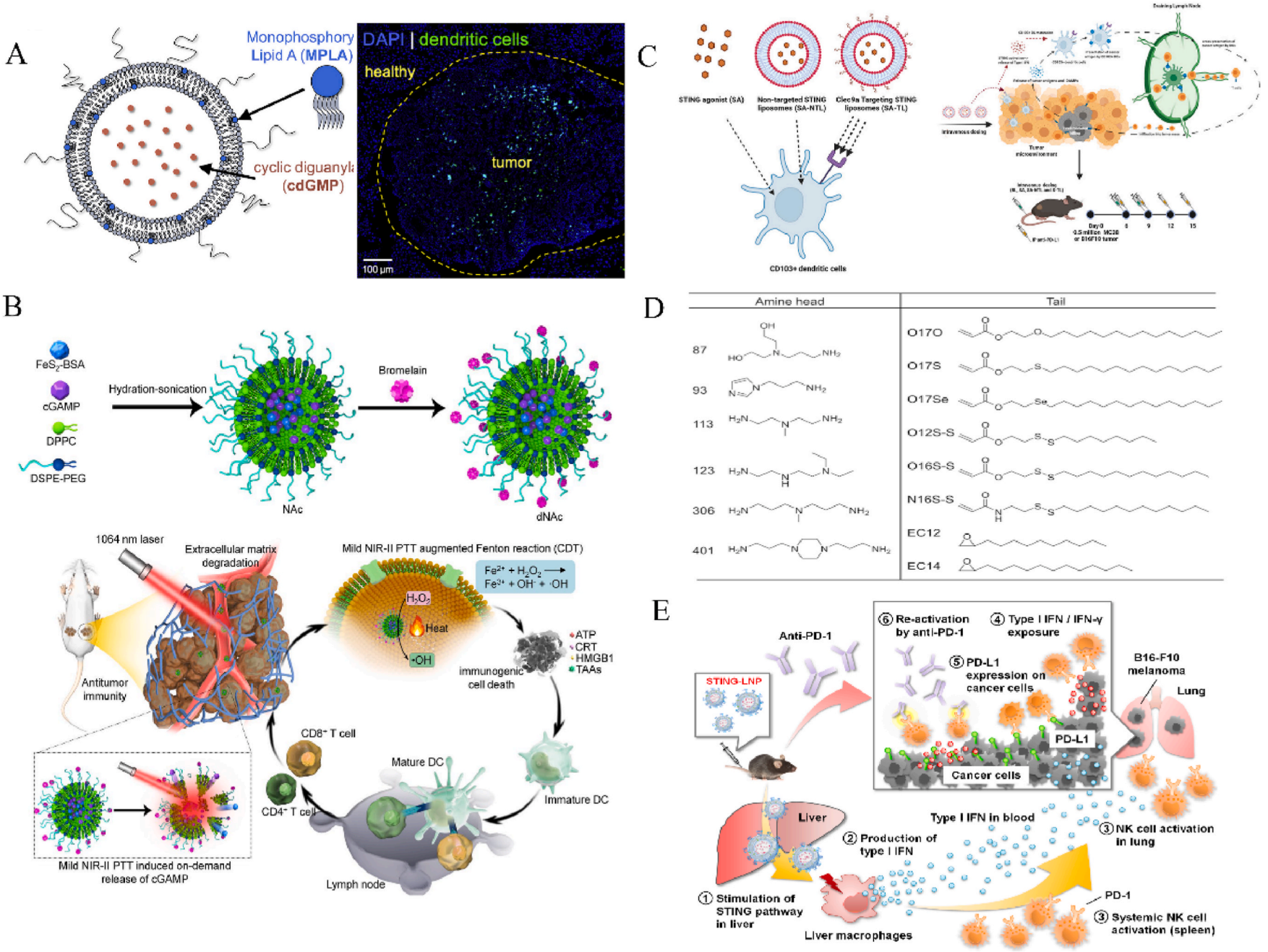

Fig. 2. (A) Schematical illustration of DPPC-based liposome co-loaded with cdGMP and MPLA [115]. Adapted with permission. Copyright © 2020 Elsevier B.V. All rights reserved. (B) The preparation procedures of BSA-FeS_2 and cGAMP co-loaded liposomes (NAc) and their underlying antitumor mechanism [118]. Adapted with permission. Copyright © 2023 BioMed Central Ltd unless otherwise stated. (C) WH peptide grafted DOTAP-based liposome for effective targeting of Batf3 dendritic cells to improve cancer immunotherapy [119]. Adapted with permission. Copyright © 2022 Elsevier B.V. All rights reserved. (D) The library of amine heads and tails of lipidoids used for screening the delivery of STING agonists [120]. Adapted with permission. Copyright © 2021 The Authors, some rights reserved. (E) Summary for reducing anti-PD-1 resistant by STING-LNP [121]. Adapted with permission. Copyright © 2021, BMJ Publishing Group Ltd & Society for Immunotherapy of Cancer. All rights reserved.

directly to the tumor site, producing a higher level of type I IFN β. In a 4T1 mouse model of triple-negative breast cancer (TNBC), the use of immune agonist-loaded nanoparticles promoted a significant amplification in antigen-presenting cells (APCs) and natural killer cells in blood and tumor tissue, resulting in enhanced systemic anti-tumor immunity [117]. Moreover, a study across different tumor models indicated that a combination therapy involving anti-PD1 could stimulate the "exhausted" $CD8^+$ T cells that infiltrate the tumor, leading to improved tumor clearance via the immuno-LP mechanism. The response rate of anti-tumor immunological memory in mice in these tumor models was reported to be as high as 71 %, with the generation of both B and T cell *de novo* epitopes, resulting in a comprehensive memory response and developing effective "cold"-to- "hot" strategies.

In addition, Lu et al. further broadened the application of these DPPC-based liposome formulations in chemodynamic-immunotherapy of cancer (Fig. 2B) [118]. BSA-FeS2, cGAMP, DPPC, DSPE-PEG, and DSPE-PEG-NHS were formulated in a mass ratio of 0.5:0.5:25:4:1 using the hydration-sonication method to prepare BSA-FeS2 and cGAMP co-loaded liposomes (NAc). To enhance tissue penetration of NAc and promote enzymatic degradation on the extracellular matrix (ECM), bromelain was chemically modified on the surface of NAc. The dynamic light scattering (DLS) measurement revealed an average size of ~200 nm for NAc with a negatively charged surface. Upon NIR-II photoactivation, both primary tumors and liver and lung metastases were significantly inhibited in a 4T1 xenograft murine model following intravenous injection of NAc. This suggests a potential synergistic effect of STING agonists in multi-modality cancer therapy.

STING agonists like cGAMP have hydrophilic and negatively charged properties, which hinder their internalization into the cytosol. To achieve more effective cytosol delivery, cationic lipids such as 1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP) have been widely employed for the delivery of STING agonists. Besides, cholesterol and DSPE-PEG2000 are also indispensable components in the formulation of cationic lipid-based delivery systems for STING agonists. To date, DOTAP-based liposomes loaded with STING agonists have demonstrated great antitumor efficacy in various tumor-bearing models such as triple breast cancer, melanoma, and colorectal cancer [121–123]. It should be mentioned in the work of Mansoor Amiji et al. [119], DOTAP-based liposomes were modified with WH peptide (WPRFHSSVFHTHGGGC; a peptide could target to *Batf3* dendritic cells for improvement of vaccination efficiency) through maleimide-thiol reaction with $DSPE\text{-}PEG_{2000}$ (Fig. 2C). In addition, the WH peptide can bind to Clec9a, which is an attractive target for STING agonist-loaded formulations and can contribute to the antitumor effects. As a result, the prepared nanoparticles had a positive surface charge with a zeta potential of 22.9 mV and a size of approximately 121 nm. The encapsulation efficiency and loading efficiency of ADU-S100 were around 55 % and 2.1 %, respectively. The lipid formulation with a low dose of ADU-S100 (0.1 mg/kg) efficiently provoked the antitumor immune response in both MC38 and B16F10 tumor-bearing mice. The plasma levels of cytokines, including IFN-α, CXCL10, and CCL5, were significantly increased 16 h after administration, demonstrating the advantages of targeting Clec9a through modification of the WH-peptide. Besides that, the combination therapy with anti-PD-L1 further demonstrated this Clec9a targeting lipid formulation could alleviate the immunosuppressive tumor microenvironment and effectively induce the "cold" tumor to a "hot" tumor. Except for these classic cationic DOTAP-based liposomes, novel cationic lipids like 93-O17S–F, and YSK12–C4 are also developed and applied in the delivery of STING agonists. For instance, in the work of Xu et al., cGAMP-loaded 93-O17S–F lipidoid nanoparticles (LNPs/cGAMP) were also prepared through the thin-film hydration method [120]. The lipid was formulated with cholesterol, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and C16-PEG2000-ceramide at a wt/wt ratio of 16:4:1:1. Previous reports have shown that the structure of 93-O17S–F, which contains cyclic imidazole heads, can target T cells *in vivo* (as depicted in Fig. 2D) and therefore has the potential to enhance cross-presentation. Building on this, Xu et al. investigated the potential of combinative therapy using 93-O17S–F and doxorubicin (DOX) for the treatment of melanoma. The intratumor injection of LNPs/cGAMP effectively enhance the survival ratio of the B16F10 tumor-bearing mice, as 35 % of mice recovered completely from the primary tumor and 71 % of mice recovered completely from a subsequent tumor challenge. Low-dose pretreatment of DOX-induced local apoptosis and released tumor-associated antigens (TAAs). The subsequent local injection of positively charged LNPs/cGAMP captured TAAs through electrostatic interactions and facilitated their delivery to the draining lymph nodes (DLNs). Taking advantage of T-cell targeting property derived from 93-O17S–F, LNPs/cGAMP provoked STING activation and enhanced cross-presentation *in vivo* simultaneously. Likewise, pH-sensitive cationic lipid (YSK12–C4) with high fusogenic activity and endosomal escape capability was also used for the loading of cdGMP through the t-BuOH dilution procedure. This work also demonstrated the feasibility of STING agonist-loaded liposome in the combinative anti-PD-L1 treatment of melanoma (Fig. 2E) [121].

The nanoprecipitation technique also outweighs the preparation of polymer nanoparticles loaded with STING agonists [124,125]. Synthetic polyesters like PLGA have vastly been investigated in the intracellular delivery of STING agonists and testified its potential on improving the antitumor efficacy of immunotherapy [126]. For instance, Gao et al. prepared PLGA-based nanovaccine loaded with cGAMP (PLGA/STING) through the double emulsion solvent evaporation method. The PLGA/STING were further coated with a cancer cell membrane containing 12-mer Clec9a + binding peptide (PLGA/STING@EPBM), to enhance the antigen cross-presentation (Fig. 3A) [127]. The average size of as-prepared PLGA/STING@EPBM was ~157 nm with ~80 % encapsulation efficiency. The zeta potential of PLGA/STING@EPBM was about −21.1 mV considering the surface modification of the cell membrane. The antitumor effect of PLGA/STING@EPBM was confirmed on several tumor models including the 4T1 breast orthotopic tumor model and B16-OVA model. The combination with radiotherapy was also investigated on B16-OVA and TC1 tumor models in this work. The results suggested that, on the basis of increasing Clec9a + DCs in tumor tissues triggered by radiotherapy, subcutaneous injection of PLGA/STING@EPBM could inhibit tumor growth and effectively prolong the survival ratio. Together with the aforementioned chemo-immunotherapy or mono-immunotherapy, this work proved and broadened the suitability of STING agonists in cancer treatment modalities. Furthermore, the potential of PLGA nanoparticles loaded with adjuvants cocktail including STING agonists, retinoic acid-inducible gene 1 (RIG-I) agonists, and toll-like receptor (TLR) agonists on cancer immunotherapy was also investigated by Desai et al. [128]. In this work, CpG ODN 1826 (TLR9 agonist), 5′ppp-dsRNA (RIG-I agonist), cGAMP (STING agonist), and a melanoma peptide (TRP-2) were all encapsulated into PLGA nanoparticles (iaNP) via double emulsion method (Fig. 3B). According to the analysis of dynamic light scattering (DLS), iaNP with a negatively charged surface (−21.7 mV) was about 258 nm in diameter. The loading capacity of each laden drug was 69.1 % (CpG ODN1826), 29.8 % (5′ppp-dsRNA), 56.5 % (cGAMP), and 80 % (TRP-2) respectively. The *in vivo* investigation verified systemic administration of such iaNP loaded with adjuvants cocktail could significantly induce a broader adaptive response through stimulating various lymphocytes like $CD4^+$ T cells, $CD8^+$ T cells, and NK cells. As a result, the tumor growth of the B16F10 tumor-bearing model was effectively inhibited with increased levels of TNF-α and IFN-γ.

Polyethylene glycol (PEG) copolymer is a widely used reagent for drug delivery due to its steric stability, which prevents further uptake by the reticuloendothelial system (RES) and prolongs the half-life of drugs, increasing their circulation time in the body [129,130]. As a type of polyether, PEG offers low immunogenicity, high hydrophilicity, and biocompatibility, making it a popular choice for delivering STING agonists [131,132]. In addition, PEG can also be involved in the synthesis of the biomaterial carriers, which themselves can stimulate the STING

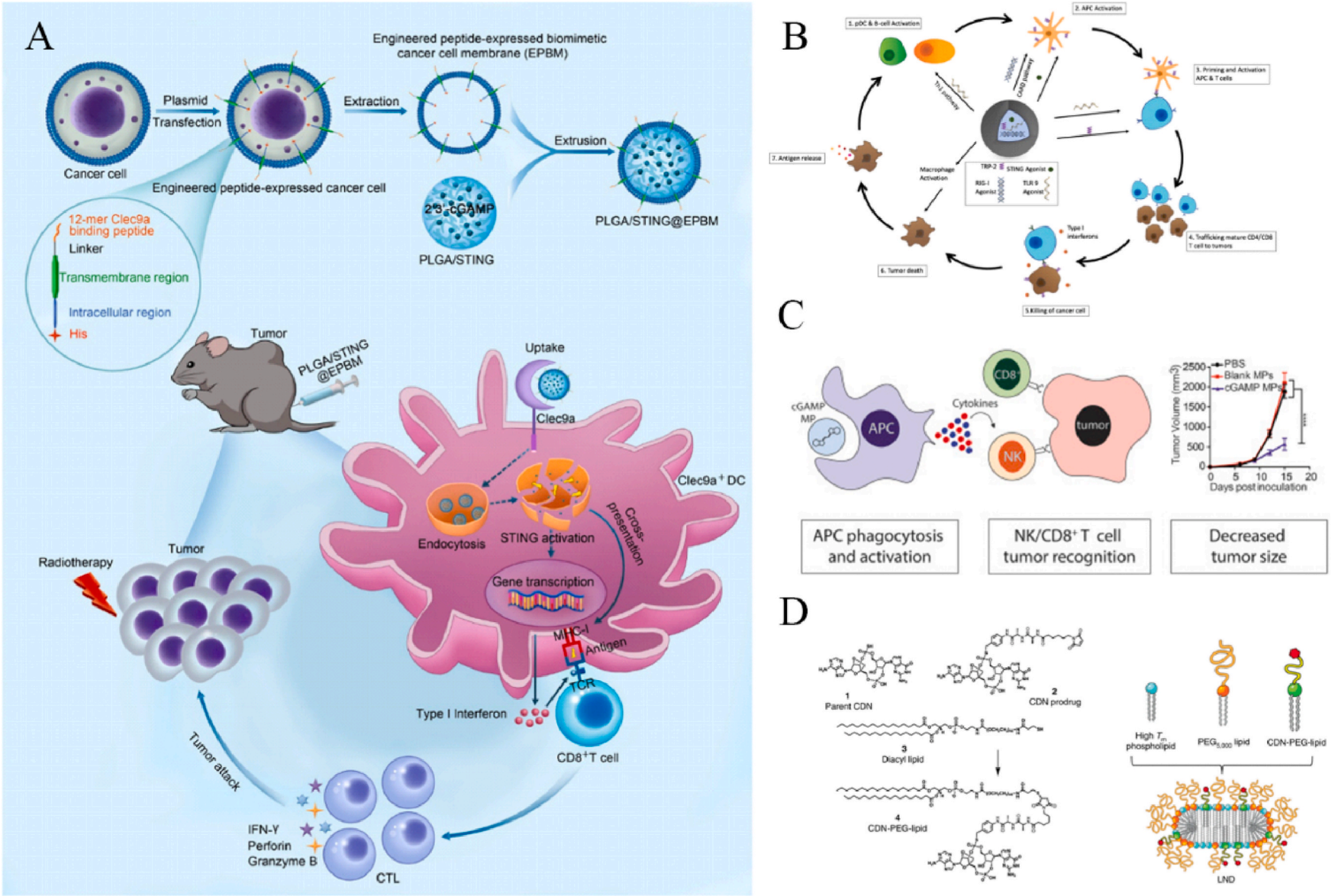

Fig. 3. (A) Preparation of PLGA/STING@EPBM and its mechanism to improve cancer immunotherapy [127]. Adapted with permission. Copyright © 2021 American Chemical Society. (B) Potential antitumor pathways activated by iaNPs loaded with CpG ODN 1826 (TLR9 agonist), 5′ppp-dsRNA (RIG-I agonist), cGAMP (STING agonist), and a melanoma peptide (TRP-2) [128]. Adapted with permission. Copyright © 2021American Chemical Society. (C) Preparation of polymeric acetalated dextran microparticles loaded with cGAMP through electrospray method, and its application in cancer immunotherapy [134]. Adapted with permission. Copyright © 2019 Elsevier Ltd. All rights reserved. (D) Chemical structures of the CDN prodrug and its schematic illustration of liposome-CDN [135]. Adapted with permission. Copyright © 2022, The Author(s).

pathway, thus eliminating the need to add separate STING agonists. For instance, in the work of Gao et al., PEG-b-PR, a pH-sensitive polymer, was designed and synthesized through atom transfer radical polymerization for cancer immunotherapy [133]. PEG-b-PR polymer nanoparticles loaded with Ovalbumin (OVA) used as a model antigen were then prepared using the solvent evaporation method. cGAS-dependent and STING and cGAS-independent STING activation were verified by in vitro culture of bone-marrow-derived macrophages (BMDMs) and human monocyte THP-1 cells. A specific interrelationship between PC7A NPs and STING was demonstrated. Subsequently, after local subcutaneous injection of PC7A NPs, the expression of indoleamine 2, 3-dioxygenase 1 (IDO-1), IFN-stimulated genes (ISGs) (*e.g.*, IRF7, CXCL10, *etc*.) increased significantly over time, which directly activated the STING pathway and promoted antigen cross-presentation to enhance anti-cancer immunotherapy.

Natural polymer like dextran accounts for a large part in the delivery of STING agonists due to their plenty of advantages such as biocompatibility, degradability, and the ease of surface modifications [136]. The research group led by Kristy M. Ainslie and Jenny P–Y Ting made significant contributions to the development of an acetalated dextran-based vaccine/adjuvant for the treatment of tumors and seasonal influenza [137–139]. Jenny P–Y Ting and colleagues introduced electrospray as a novel method to achieve monodisperse micro/-nanoparticles and enhance encapsulation efficiency, as opposed to the classic double emulsion method commonly used in polymer-based formulations. In addition, their previous report revealed that compared to the acidic metabolites of polymers, which may have a negative impact on vaccine efficacy, the hydrolysis byproducts of acetalated dextran were primarily biocompatible dextran, acetone, and ethanol. Moreover, the degradation of acetalated dextran was pH-dependent as the degradation rate of acetalated dextran was faster in the acidic endosomal environment (pH 5.0) than in the neutral environment (pH 7.4). This pH-responsive degradation property enables better cytosol delivery of laden STING agonists and more effective antigen presentation. In the latest research (Fig. 3C) [134], acetalated dextran microparticles loaded with cGAMP (Ace-DEX cGAMP MPs) were prepared through monoaxial electrospray for the study of antitumor mechanisms. The hydrodynamic diameter of Ace-DEX cGAMP MPs ranged between 0.687 and 1.12 μm. Evaluation of mice inoculated with B16F10 melanoma cells showed that cGAMP MPs could effectively improve the delivery of STING agonists and promote the activation of the STING pathway. Further evaluation showed that the ACE-DEX CGAMP MPS formulation stimulated a significant increase of tumor-infiltrating leukocytes, generated robust immune responses against tumors and tremendously inhibited the tumor sizes in mice.

In addition to nanoformulations that physically encapsulate STING agonists through the nanoprecipitation method, some STING agonists, such as DMXAA, were also conjugated to hydrophobic segments (such as lipids) to synthesize amphiphiles. These amphiphiles were then used to prepare nanoparticles or other nanoformulations through the nanoprecipitation method, which improved the encapsulation efficiency of the STING agonists [140–144]. For example, Irvine et al. [135], used thiol-maleimide coupling to conjugate cyclic dinucleotides containing dialanine peptide linker to a thiol-terminated PEG-phospholipid (Fig. 3D). They then prepared liposomes loaded with cyclic dinucleotides (liposome-CDN) through ethanol precipitation, resulting in a size of approximately 60 nm. By adjusting the formulation of liposome-CDN with PEGylated lipids and phospholipids, its parameters such as size, shape, surface charge, and rigidity can be tuned to better target tumor tissues. Moreover, liposome-CDN demonstrated enzyme-responsive release kinetics to peptidase. In mouse models bearing MC38 tumors, intravenous injection of liposome-CDN was found to effectively reduce tumor growth and maintain a survival ratio of approximately 75 % for 50 days after tumor inoculation. Mechanistic investigation revealed that liposome-CDN enhanced the secretion of cytokines such as IFN-β, TNF-α, and provoked innate and adaptive immunity in tumor tissues by promoting the infiltration of specific cytotoxic T lymphocytes and activating DCs. Liposome-CDN has shown great promise as a formulation for facilitating the conversion of cold tumors into hot tumors.

Based on all the formulations developed for the delivery of STING agonists, it can be confirmed that the nanoprecipitation technique is a convenient, rapid, relatively low-cost, and well-established method for fabricating micro/nanoparticles for drug encapsulation, not limited to STING agonists. However, the use of organic solvents in the nanoprecipitation technique is an inevitable issue that needs to be addressed for further applications. Additionally, as hydrophilic small molecules, the encapsulation efficiency and loading capacity of STING agonists through the nanoprecipitation technique were found to be unsatisfactory. Furthermore, the structure of lipids or polymers should be tailored further to improve the release kinetics of STING agonists and prevent undesirable global inflammation reactions resulting from unfavorable release in off-target tissues/organs. In the case of synthetic polymer carriers, the detrimental effect on the STING agonists due to the acidic byproducts of polymers is another critical issue that needs to be resolved (Table 3).

3.2. Self-assembly

Self-assembly, which is characterized by the spontaneous organization of several components under direct specific molecular interactions or indirect adjustments of the environment, is another widely used strategy for preparing micro/nanoparticles [154]. The molecular interactions that are involved in various biological processes can be modulated by adjusting the environment or the concentrations of certain components. These interactions can be broadly classified into several categories, including hydrophobic interactions, electrostatic interactions, hydrogen bonding, molecular dipole interactions, metal coordination bonding, and π−π stacking [155–158].

As mentioned in the previous section, STING agonists are primarily hydrophilic and anionic dinucleotides. Therefore, the most commonly used strategy for their delivery involves creating nanoparticles that can electrostatically interact with hydrophilic cationic materials. One popular choice for loading STING agonists is polyethylenimine (PEI), a classic and conventional vector for delivering nucleic acids [159,160]. Zhong et al. developed a copolymer called Poly (ethylene glycol)-b-poly (dithiolane trimethylene carbonate-*co*-trimethylene carbonate)-*b*-polyethyleneimine (PEG-P(TMC-DTC)-PEI) for efficient delivery of ADU-S100 (Fig. 4A) [160]. Through electrostatic interaction between ADU-S100 and PEI containing in the structure of PEG-P(TMC-DTC)-PEI, the encapsulation efficiency and loading content of this self-assembled polymersome could be high up to 86.0 % and 17.2 wt% with an average size at ~ 47 nm. The antitumor evaluation on B16F10 melanoma-bearing mice demonstrated that the intratumoral injection of this nanoformulation (1 mg/kg ADU-S100) could effectively inhibit tumor growth and elicit a long-lasting specific immune response. The combinative treatment with radiotherapy further indicated this nanoformulation could evoke pro-inflammatory TME as cytotoxic T lymphocytes ($CD8^+$ and $CD4^+$ T cells) and pro-inflammatory factors including IFN-β and TNF-α were both significantly elevated. Likewise, Wang et al. introduced another conventional cationic polypeptide, protamine, to form nanocomplexes with cGAMP through electrostatic interaction. For more effective cytosol internalization, the nanocomplexes were further coated with nd1, 2-dioleoyl-3-trimethylammonium-propanechloridesalt (DOTAP) [161]. The final LP-cGAMP formulation was spherical in shape with a neutral surface charge and an average size of 80 nm. Investigation on both B16F10 and BRAF-mutated murine melanoma models proved the LP-cGAMP formulation exhibited a great antitumor effect on both models by promoting the infiltration of cytotoxic T lymphocytes. In addition, the authors demonstrated the potential of combination therapy with anti-PD-L1. The co-treatment of LP-cGAMP and anti-PD-L1

Table 3
Listing of the advantages and disadvantages of the formulations.

Preparation methodologies	Advantages	Disadvantages	Reference
Nanoprecipitation	Don't require external energy input (*e.g.* no required high sheer homogenization techniques, ultracentrifugation or surfactants); The reaction conditions are facile and mild; A fast and low-cost process.	Non-uniform sizes, low concentration preparation and the arguably inability to freeze dry the particles; The use of organic solvents; The acidic byproducts of polymers carriers can have adverse effects on the STING agonists.	[109,111, 145–147]
Self-assembly	Wide applicable substrate, good stability, strong drug delivery capability and long circulation time; Simpler and more facile preparation process; Significantly improved STING agonist encapsulation efficiency and loading content.	Potential toxicity and side effects of cationic materials; Inadequate production of proteins requiring glycosylation and proper folding within the bacterial cytoplasm.	[147,148]
Surface absorption	Drug release can be readily achieved and controlled.	The surface charge and colloidal stability of nanocarriers are influenced; Forming and breaking the chemical bonds are affected by the steric hindrance of the drug molecules on a surface; The bonds in the linkage vary in their degree of reversibility; Mostly limited to solid tumors.	[147,149]
Hydrogel	Mild preparation conditions, with rare need for organic solvents; High porosity, injectability, low toxicity, good biocompatibility, and biodegradability; Porous structure, high capacity for drug loading; High water absorbing affinity.	The burst initial release and in-situ cross-linking.	[150–153]

resulted in more durable inhibition of tumor growth compared to the single treatment groups. The survival ratio of mice was maintained at approximately 50 % for at least one month after the final intratumoral injection, indicating the potential of this combinative therapy in the treatment of melanoma.

Except for the aforementioned classic polymers, novel synthetic polymers such as poly (β-amino esters) (PBAEs), and dendrimers were also designed for the delivery of STING agonists [165–167]. For instance, Kim et al. synthesized a kind of PBAEs (PBAE 447 polymer) through a two-step Michael addition reaction and applied it to encapsulate cdGMP through electrostatic interaction [166]. The as-prepared PBAE 447/cdGMP nanoparticles with positive surface charge were ~100 nm in diameter. The author also investigated the antitumor effect of PBAE 447/cdGMP nanoparticles on B16F10 melanoma-bearing mice and confirmed its potential on improving the efficacy of immune checkpoint inhibitors and enhancing immunotherapy. According to the results, the intratumoral injection of PBAE 447/cdGMP nanoparticles (at a dose of 2 μg/mouse) for four times on Days 3, 6, 9, and 12 after tumor inoculation significantly reduced the growth of melanoma. In another work by Sun et al., triblock copolymers poly (ethylene glycol)-*b*-poly-(DTMASN38)-*b*-poly[2-(diethylamino)-ethyl methacrylate] (PEG-*b*-PSN38-*b*-PDEA) were designed and successfully synthesized [162]. Cleavable chemotherapeutics, SN38 (the active metabolite of the cytotoxic drug irinotecan), was engrafted in the backbone of this copolymer through reversible addition-fragmentation chain transfer polymerization. The engraftment of SN38 served as a hydrophobic core during the self-assembly of this amphiphilic copolymer, with PDEA containing tertiary amines in the copolymer responsible for molecular interaction with DMXAA (5,6-dimethylxanthenone-4-acetic acid) through electrostatic interactions (Fig. 4B). With adjusting the ratios of the PSN38 block and the PDEA block, the encapsulation efficiency of DMXAA changed from 13 to 86 %. PEG_{5k}-*b*-$PSN38_{4.5k}$-*b*-$PDEA_{1.5k}$ was finally chosen for the following investigation to load DMXAA considering higher encapsulation efficiency and lower polydispersity index. As purified by dialysis, DMXAA-loaded PEG_{5k}-*b*-$PSN38_{4.5k}$-*b*-$PDEA_{1.5k}$ nanoparticles (PS3D1@DMXAA) were spherical in shape with an average diameter of 25 nm, which enable its fast penetration and accumulation in tumor tissues. The antitumor effect of PS3D1@DMXAA was then evaluated on three tumor models (B16-melanoma, primary colon cancer, and lung metastasis of 4T1 breast tumor). Relevant data proved that the intravenous injection, instead of intratumoral injection, of PS3D1@DMXAA (8 mg/kg DMXAA) could significantly inhibit tumor growth, indicating the synergistic effect between SN38 and DMXAA. Specifically, the PS3D1@DMXAA nanoparticles were designed to respond to the tumor microenvironment by dissociating the SN38 hydrophobic core, inducing apoptosis of tumor cells and triggering the release of chemokine CCL4. This chemokine promoted the infiltration of CD103+ dendritic cells (DCs) into the tumor, leading to the activation of $CD8^+$ T cells and ultimately the inhibition of tumor growth. Meanwhile, efficient cytosolic delivery of DMXAA through PS3D1@DMXAA provoked STING activation in CD103+ DCs and promoted the migration of mature CD103+ DCs into the tdLN. PS3D1@DMXAA thus alleviated the immunosuppressive TME and facilitated the infiltration of TAA-specific effector $CD8^+$ cytotoxic T cell through CXCL9/CXCL10, which consequently amplified the antitumor efficacy.

Considering the phosphate groups present in the structure of most STING agonists, metal coordination bonding is also emerging as a strategy for drug delivery. Moreover, researchers have demonstrated metal ions are crucial for immune regulation like Ca^{2+} (T cell activation), K^+, Na^+ (activation of inflammasome), Zn^{2+}, and Mn^{2+} (activation of cGAS-STING signaling). In the work of James J. Moon et al., Mn^{2+} self-assembled into the coordination polymers after mixing with cyclic di-AMP (CDA), cyclic di-GMP (CDG), and cGAMP in water with an average size ranging from nanometers to micrometers [168]. However, the CDA–Mn^{2+} coordination polymers were unstable under physiological conditions and were dissociated rapidly in phosphate-buffered saline (PBS) solution. Therefore, to improve the stability of CDA-Mn2+ coordination polymers, a conventional and classic liposomal formulation (dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(histidine)11, DOPE-H11) was introduced to coat onto the surface of CMPs. As described in this work, the lipids served as an additional coordination ligand for chelating CDA and a hydrophobic core to stabilize the CDA–Mn^{2+} coordination polymer. The final CMP with neutral surface charge was about 118 nm in size and the loading efficiency of CDA and Mn^{2+} was 39.6 and 25.3 %, respectively. *In vitro* investigation on THP-1 cells indicated, in the presence of Mn^{2+}, the treatment of CMP could activate the immune function by inducing the phosphorylation of TBK1 and p65, and consequently amplify the STING signaling cascade and the secretion of type 1 IFNs. The evaluation of three tumor models, namely CT26 tumor-bearing BALB/c mice, B16F10 tumor-bearing C57BL/6 mice, and a novel tobacco carcinogen-associated syngeneic squamous cell carcinoma model, all proved the local injection of CMP could

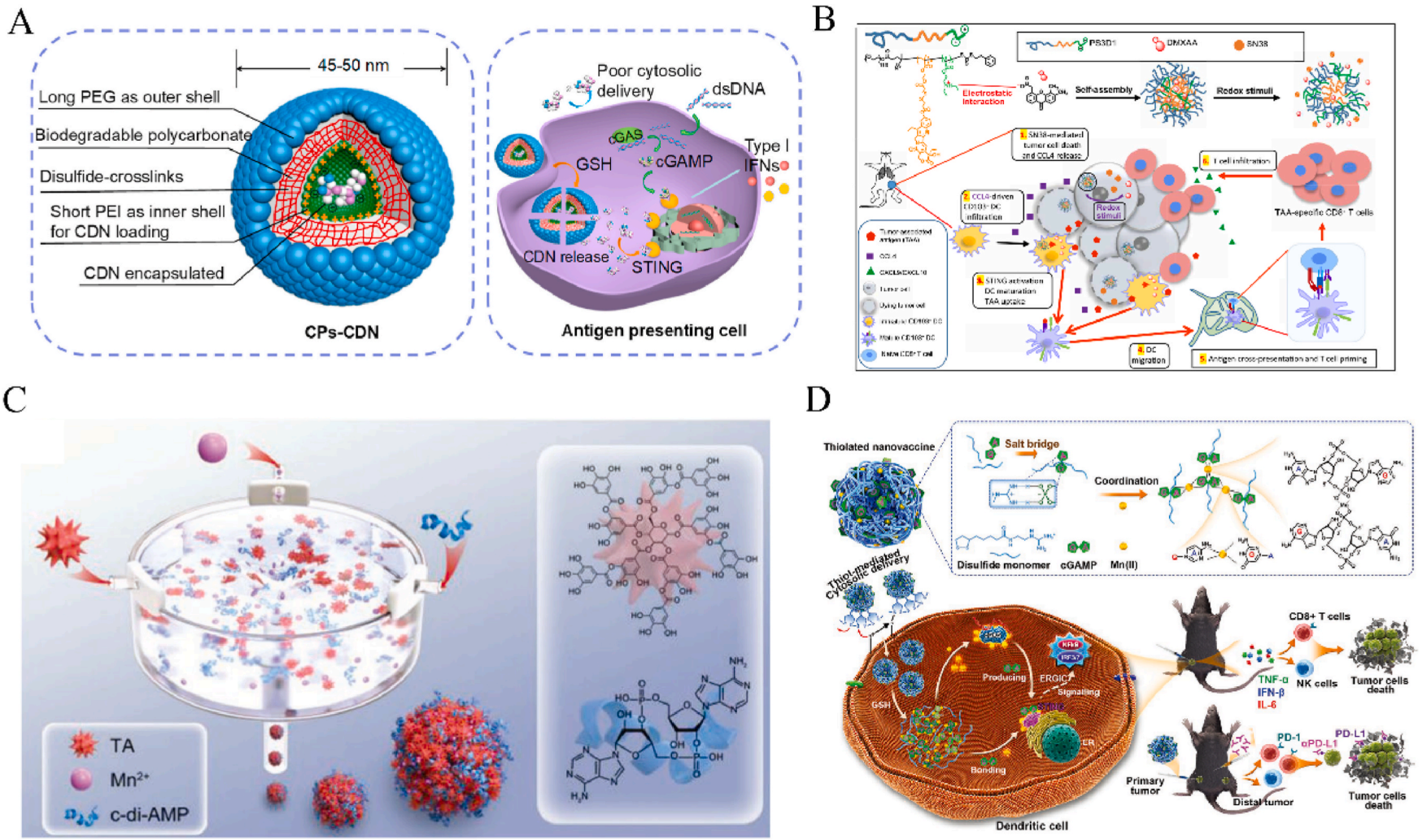

Fig. 4. (A) Schematic illustration of CPs-CDN and the cytoplasmic delivery of ADU-S100 mediated by them [160]. Adapted with permission. Copyright © 2022 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. (B) Self-assembly of PS3D1@DMXAA and its underlying mechanism on activating innate and adaptive immunity in tumor tissues [162]. Adapted with permission. Copyright © 2020 The Authors, some rights reserved. (C) Preparation of TMA-NPs by FNC technique through non-covalent interaction [163]. Adapted with permission. Copyright© 2022 Wiley-VCH GmbH. (D) Preparation of Mn-cGAMP nanoparticles through metal coordination bonding between guanidine-containing disulfide monomer and cGAMP and its application for combinative treatment with anti-PD-L1 [164]. Adapted with permission. Copyright © 2021 Wiley-VCH GmbH.

promote the secretion of cytokines including IFNβ, TNFα, CXCL9, and CXCL10, and thus induce robust antitumor immune responses. This includes the maturation of DC in lymph nodes and the repolarization of intratumoral macrophages from M2 to M1, consequently facilitatiing the transformation of "cold" tumors into "hot" tumors. Inspired by this work, Xiao et al. further claimed that the instability of the CDA–Mn^{2+} coordinate could be ascribed to the unsaturated phosphate groups or Mn^{2+} and the uncontrollable interaction of these two components [163]. On the basis of this speculation, in our recent research, an FDA-approved polyphenol called tannic acid was utilized to stabilize the CDA–Mn^{2+} coordination polymer and create TMA-NPs for the first time. The synthesis and release of TMA-NPs are regulated by several interactions, including the interaction of the phenolic group with unsaturated Mn^{2+}, the electrostatic interaction of the phenolic group with amine groups of c-di-AMP, and the protonation of TA in an acidic environment. In this study, a flash nanocomplexation (FNC) technique was used to fabricate TMA-NPs through the non-covalent interaction of these components in a homogeneous, continuous, and reproducible manner (Fig. 4C). The as-prepared TMA-NPs were spherical in shape with a size of 25.6 nm. The loading efficiency of Mn^{2+} and CDA was high up to 81.3 % and 85.4 %, respectively. The dual tumor model established by our team showed that TMA-NPs could cause a significant increase in the proportion of NK cells, $CD8^+$ T cells, and $CD45^+$ lymphocytes. Apart from the great antitumor efficacy of the 4T1-bearing tumor xenograft model and metastasis model, we confirmed the feasibility of TMA-NPs in the radioimmunotherapy of large tumor. The ELISA study on the tumor tissues after administration demonstrated that c-di-AMP levels were significantly higher in the TMA-NPs + X-ray treatment group than in other control groups. The significant increase of chemokines such as CXCL10, CCL5, IFN-β, and TNF-α also proved that TMA-NPs + X-ray could activate the STING-mediated anti-tumor effects in 4T1 tumor-bearing mice and significantly increased the survival rate of mice. Overall, this work provided a new strategy to deliver STING agonists and represented the combinatorial potency of TMA-NPs and radiotherapy on large tumor regression by amplifying the STING signaling cascade. Likewise, Yang et al. designed a guanidine-containing disulfide monomer and applied it to assembly with cGAMP through the salt bridge and electrostatic interaction [164]. The thiolated and Mn^{2+} coordinated cyclic dinucleotide nanovaccine (Mn-cGAMP NVs) were finally prepared after Mn^{2+} ions were coordinated into this assembly through metal coordination bonding (Fig. 4D). Through this preparation method, the loading content of Mn^{2+} and cGAMP was about 22 wt% and 20 wt% respectively. Mn-cGAMP NVs can directly co-deliver cGAMP and Mn^{2+}. Meanwhile, polysulfides on the NVs surface not only avoid lys-endosomal degradation but also enhance STING activation, thus improving the anti-tumor immune response. In addition to their application in cancer immunotherapy, it is worth noting that metal-STING agonist coordinates have also been investigated for use as biosensors. In the work of Tseng et al., cdGMP, TB^{3+}, and Ag^+ were mixed in an aqueous environment and self-assembled into nanoparticles (CPNPs) with an average size of 30 nm through electrostatic interaction and metal coordination bonding [169]. The addition of Ag^+ was reported to improve absolute quantum yield (QY) and emission lifetimes to different extents. Combining with Fe_3O_4 NPs, the CPNPs-based sensing system was applied for luminescence turn-on sensing of c-di-GMP in bacterial lysates.

Apart from metal coordination bonding, hydrophobic interaction is an effective strategy for the delivery of STING agonists [171]. In a study done by the group of John T. Wilson, a novel poly (ethylene glycol)-block-[(2-(diethylamino)ethyl methacrylate)-*co*-(butyl methacrylate)-*co*-(pyridyl disulfide ethyl methacrylate)] (PEGDBP) copolymers was fabricated by loading with cGAMP (STING-NP) through a direct hydration method [172–174]. pH-sensitive, cationic 2-(diethylamino)ethyl methacrylate (DEAEMA) groups and hydrophobic butyl methacrylate (BMA) moieties were integrated through a direct hydration method and further cross-linked via reduction of dithiothreitol (DTT) for higher encapsulation efficiency. The as-prepared STING-NP was 80 nm in size with a neutral surface owing to the PEGylation. The authors demonstrated both intratumoral and intravenous injections of such STING-NP could elicit strong and robust immune responses in B16F10 tumor-bearing mice. Recently, they also broaden their application in the immunotherapy of neuroblastoma. On the basis of these results, in another work by John T. Wilson et al., several neoantigens including OVA, Reps1 (RVLELFRAAQLANDDVVLQIMELC), TRP2 (SVYDFFVWL), Adpgk (GIPVHLELASMTNMELMSSIVHQQVF), M27 (REGVELCPGNKYEMRRHGTTHSLVIHD), and M30 (PSKPSFQEFVDWENVSPELNSTDQPFL) were co-encapsulated respectively into STING-NP. And they further verified the adjuvant property of STING-NP to be applied in the development of nanovaccine in the treatment of melanoma.

Moreover, taking advantage of bioengineering, Zhang et al., fused human heavy-chain ferritin subunits (HFn) with functional peptides, including RGE, Pep-1, and CGKRK peptides, at the N-terminus through biosynthesis of *E.coli* BL21(DE3) to endow HFn with glioma-targeting and penetration properties (Fig. 5) [170]. According to the description, the self-assembly/dissemble of HFn was pH-dependent as HFn could dissemble at pH 2.0 and could assemble into nanoparticles at pH 8.0. Therefore, the STING agonist, SR717, was encapsulated within HFn by adjusting the pH value. The hydrodynamic diameter of such SR717-loaded HFn nanoparticles (SR717@RGE-HFn) was 15 nm and the surface charge was about −6.8 mV. The investigation on an orthotopic glioma model demonstrated that intravenous injection of SR717@RGE-HFn (5 mg/kg SR717) could effectively induced a strong innate immune response within the tumor microenvironment, thereby postponing the progression of glioma tumors and prolonging the survival of mice models.

Overall, in contrast with the nanoprecipitation method, the preparation process of self-assembly is more facile and simpler. Remarkably, the whole aqueous preparation (or small volume organic solvent in the cases of hydrophobic interaction) methodology prevents the usage of organic solvent that is widely applied in the nanoprecipitation technique, which efficiently enhanced the biosafety of the nanoformulation and accelerates its clinical translation. Owing to the specific intermolecular interaction, the encapsulation efficiency and loading content of STING agonists were also significantly improved. Moreover, the emergence of homogeneous mixture techniques such as FNC enables a continuous, scalable, and reproducible preparation manner, which can accelerate clinical translation. However, while electrostatic interactions are a prominent strategy, they can also pose risks. One major concern is the potential toxicity and side effects associated with cationic materials, both natural and synthetic. When cationic surfaces come into contact with physiological fluids after systemic administration, they have the propensity to form large complexes. This can give rise to the formation of thrombi in macro/micro-vessels, thereby posing a significant health risk. Additionally, fusion protein delivery systems based on bacterial expression often fall short in producing proteins that necessitate glycosylation and proper folding within the bacterial cytoplasm. Consequently, the development of a mammalian expression system that facilitates posttranslational modification and proper folding is urgently needed to address these challenges (Table 3).

3.3. Surface absorption

Surface absorption is a widely used strategy in inorganic materials like mesoporous silica nanoparticles (MSNs) for the delivery of STING agonists. The underlying mechanism is also based on the intermolecular interaction *per se* electrostatic interaction [175–177]. However, instead of forming polyplexes to prevent the outer environmental interference to the laden STING agonists, STING agonists in this respect was loaded onto the cationic surface of solid nanoparticles through electrostatic interaction. For instance, in the work of Wu et al. [176], TA-silane (*N*-trimethoxysilylpropyl-*N,N,N*-trimethylammonium chloride) containing quaternary ammonium group was coated onto MSNs to endow a cationic

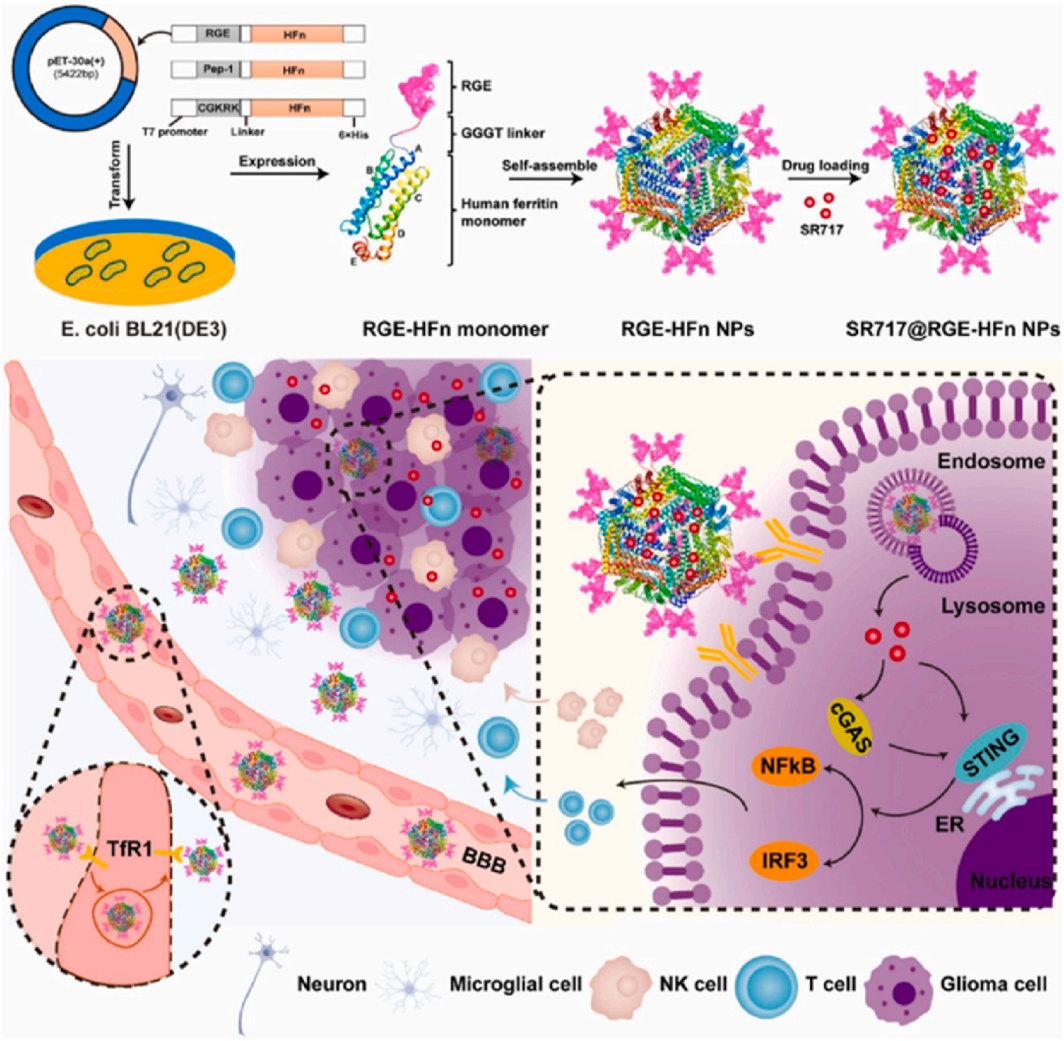

Fig. 5. Biosynthesis of HFn with functional peptides, including RGE, Pep-1 and CGKRK peptides through *E.coli* BL21(DE3) and its cytosol delivery in glioma tumor tissues [170]. Adapted with permission. Copyright © 2022 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd.

surface to load negative charged cyclic diguanylate monophosphate (cdG) onto the surface of the nanoparticles (cdG@RMSN-PEG-TA). The as-prepared cdG@RMSN-PEG-TA was about 26 nm in diameter with 28 mV cationic surface charge at pH 7.4, enabling its accumulation and penetration in tumor tissues. Intratumoral injection of such cdG@RMSN-PEG-TA demonstrated to be able to effectively inhibit the tumor growth of 4T1-bearing tumor xenograft model through promoting the infiltration of lymphocytes including CD11c + dendritic cells, F4/80+ macrophages, cytotoxic T lymphocytes.

Besides that, Zhu et al. reported a cytosine-rich i-Motif DNA-coated polymeric nanoparticles to efficient loading of cGAMP (STING-NVs) [178]. As described in their work, i-Motifs with multiple domains of consecutive cytosine underwent conformational reconfiguration with adjustment of pH (pH 5–7) and could pair with cGAMP to form four-stranded C-quadruplexes through protonated C: C^+ base intermolecular or intramolecular pairing. The as-prepared STING-NVs exhibited vaccination efficiency both in vitro and *in vivo* as its co-culture with RAW 264.7 cells could induce the repolarization of macrophages from M1 to M2. Moreover, on B16F10 tumor-bearing mice, the intralesional administration of STING-NVs effectively ameliorated the immunosuppressive tumor microenvironment through downregulating M2 gene markers (Ym1, Arg1, and Mrc1) and upregulating M1 gene markers (TNF, IL-6, and NOS-2). Consequently, the tumor growth and survival rate of B16F10 tumor-bearing mice were both improved.

Overall, surface absorption is a facile and feasible strategy for the delivery of STING agonists similar to the self-assembly strategy. The intermolecular or intramolecular electrostatic interaction enables a whole aqueous preparation methodology without the usage of organic solvent and a higher encapsulation efficiency. Moreover, taking advantage of the inherent physiological function of inorganic materials, the whole STING agonists delivery system could better integrate multiple treatment modalities into one single formulation [149]. Nevertheless, its drawback is obvious as the STING agonists were all coated on the surface of the nanoparticle instead of the core or matrix, which poses a great risk and challenge to preserve the bioactivity of the laden nucleic acid drugs. Moreover, the use of such nanoformulations may be limited to solid tumors because most of them can only be administered intratumorally (Table 3).

3.4. Other strategies

New techniques are rapidly emerging in the past few years. For instance, Jaklenec et al. introduced a soft lithography technique to fabricate cubic PLGA microparticles. The team prepared the master molds of the microparticle base and cap on the silicon wafers by SU-8 lithography. A thin PDMS mold made of PDMS base and the curing agent was used as the negative molds. Next, PLGA films with a thickness of about 1650–1750 μm were placed between the PDMS map mold and a

Teflon film for compression, heating, and cooling to yield the PLGA caps. Finally, the above steps are repeated to obtain the base of microparticles without using a Teflon film. cGAMP was then filled into the cubic PLGA microparticles through a BioJet Ultra picoliter dispensing instrument (BioDot) [179]. The as-prepared cubic PLGA microparticles exhibited an external dimension of 400 μm: 400 μm: 300 μm (length: width: height) and the wall thickness of each dimension was 100 μm. The loading capacity of cGAMP is 8.4 % by volume (Fig. 6A 1). The PLGA microparticles were subjected to characterization by the author, utilizing scanning electron microscopy (SEM) (Fig. 6A 2–4), high-resolution X-ray computed tomography (CT) (Fig. 6A 5), and optical microscopy (Fig. 6A 6). The results unveiled the ability to create large arrays (over 300 per array) of these microparticles with exceptional precision. Remarkably, according to the author's description, the release kinetics of cGAMP in these cubic PLGA microparticles exhibited a programmable sequence of pulses for days to weeks, which could release the laden cGAMP at predetermined time intervals. Specifically, these cubic PLGA microparticles released the laden drugs in pulses at about 1 ± 0, 4 ± 0, 8 ± 0, 11 ± 1, 15 ± 1, 18 ± 1, and 97 ± 2 days without premature drug release. Subsequent antitumor investigations conducted on B16F10 and 4T1 tumor-bearing mice revealed that a single intratumoral injection of cubic PLGA microparticles effectively suppressed tumor proliferation and improved the survival rate of the mice. This approach significantly reduced the frequency of injections required while still achieving a notable antitumor effect across different types of cancers.

Exosome, a kind of biological nanoscale (40–100 nm) spherical lipid bilayer vesicles secreted by cells [180–182], is a novel technique that is being widely studied in applications like nucleic acid (siRNA) delivery owing to its effect on mediating signaling between cancer cells and tumor resident APCs [183–185]. Compared to synthetic drug delivery systems like liposomes, micro/nanoparticles, and micro/-nanoemulsions, the endogeneity of exosomes is an inherent and unique advantage for its clinical translation. Therefore, for the negatively charged and hydrophilic STING agonists with low cell membrane penetration efficiency, the exosome is an ideal alternative carrier for the delivery of STING agonists. In the work of Kalluri et al., cGAMP was loaded into exosome (iExo^{STINGa}) isolated from HEK293T cells through the co-incubation method [186]. The antitumor efficacy of iExo^{STINGa} was also evaluated on B16F10 tumor-bearing mice and the data indicated that intratumoral injection of iExo^{STINGa} could promote the influx of proliferating CD8^{+} T cells and evoke the specific antitumor response through activating the STING signaling pathway. In another work by Sathyanarayanan et al., instead of a co-incubation method to load STING agonists, DNA cassettes encoding PTGFRN were cloned to a plasmid and transfected into the HEK293T cells via electroporation [187]. Overall, exosome-mediated delivery of STING agonists effectively improved the issues like low cell membrane penetration, short residence time, and lack of specific uptake into APCs after intratumoral injection.

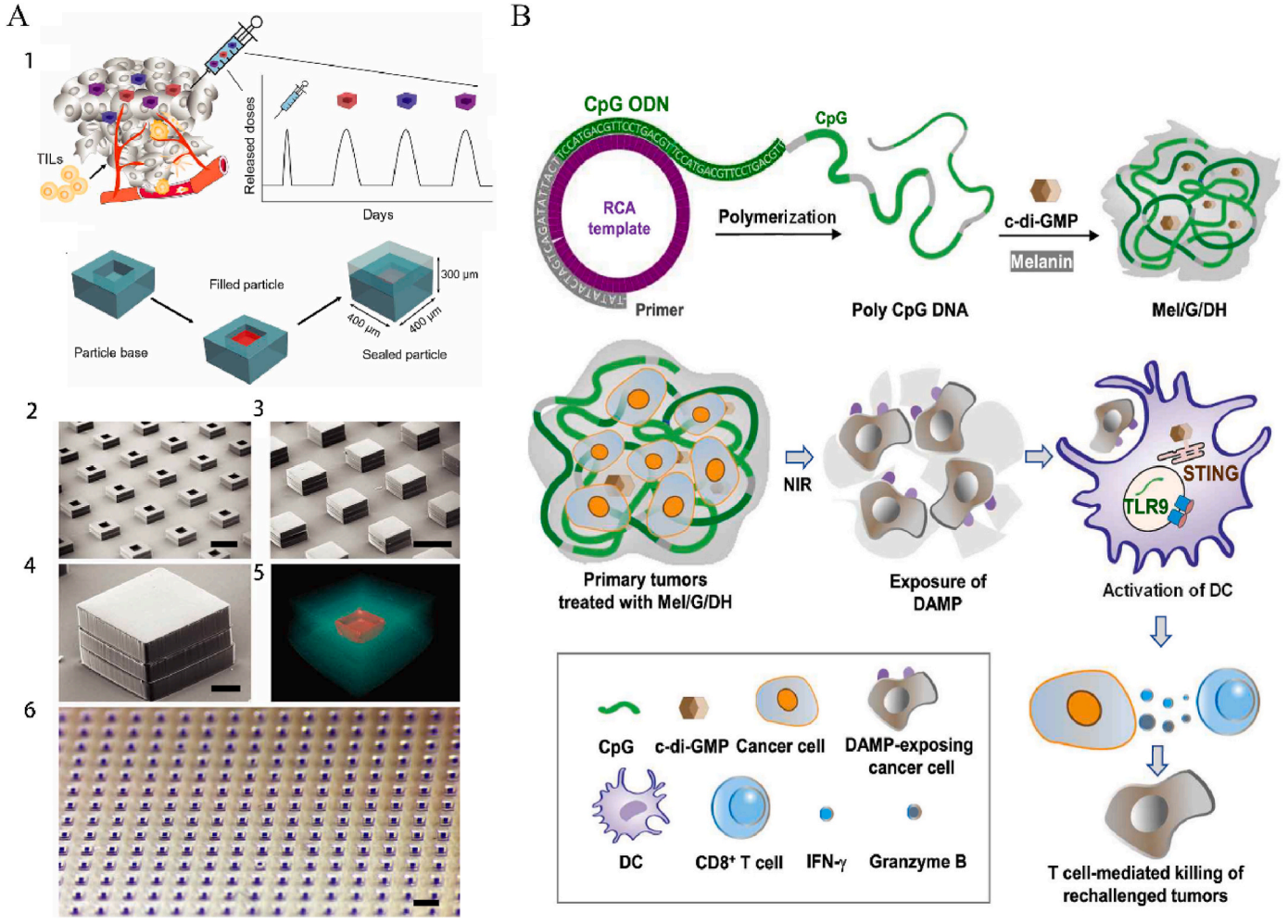

Fig. 6. (A) Preparation of cubic PLGA microparticles loading with cGAMP through a BioJet Ultra picoliter dispensing instrument [179]. Adapted with permission. Copyright © 2020 The Authors, some rights reserved. (B) Preparation of DNA CpG hydrogel loading with melanin and cdGMP by rolling-circle amplification and its application for the combinative treatment of cancer immunotherapy and photothermal therapy [195]. Adapted with permission. Copyright © 2020 The Authors. Published by Elsevier B.V.

Nevertheless, there are still several issues that need to be resolved before exosome-mediated delivery of STING agonists can be translated into clinical applications and further utilized. (1) The yield and purity of exosomes are still the first bottleneck confining its clinical application [188–190]. The combination of the current preparation methods such as ultracentrifugation, size-based isolation techniques, polymer precipitation, and immunoaffinity capture techniques partly improve the above issue [191]. (2) The specific mechanism of the secretion and fusion mechanism of exosomes is still under-discovered [192]. (3) The encapsulation efficiency and loading capacity of exosome are need to be optimized and improved [193,194]. (4) The comprehensive investigations on its own biological functions, pharmacokinetics, and toxicology are urgent to be performed, which is helpful to accelerate its clinical translation.

4. Hydrogel

Hydrogels are three-dimensional cross-linking networks made of hydrophilic polymers [196,197], which hold great potential in clinical application owing to their advantages of biocompatibility, low cost, and injectability, *etc* [198–201]. Hydrogels establish a mesh-like structure through physical or chemical crosslinking to preserve their three-dimensional integrity while in a swollen state [202,203]. Physically crosslinked hydrogels encompass various common interactions, such as hydrogen bonding, electrostatic attraction, hydrophobic interactions, and non-covalent interactions, among others. On the other hand, chemical crosslinking involves the formation of covalent bonds within the hydrogel, resulting in a more enduring and stable network [196]. As to date, more than 30 injectable hydrogel-based formulations have been approved by the FDA such as Fibrel ® (Serono Laboratories), Radiesse ® (Bioform Medical), and UFLEXXA® (Ferring Pharmaceuticals Inc.), *etc* [204]. and the most well-known may be commercial successes like INFUSE® (Medtronic, lnc.) and Vantas® (Endo Pharmaceuticals). Unlike the wide usage of organic solvent in the nanoprecipitation method, the preparation procedures of hydrogel formulations were in mild condition, which is helpful to preserve the bioactivity of biomacromolecules like nucleic acid (including STING agonists). Besides that, by adjusting the cross-linking density, the release kinetics of laden drugs could be tuned to meet the various demands of different treatment modalities. Given that most STING agonists in clinical trials are administered intratumorally, injectable hydrogel holds great potential to become an ideal platform to deliver STING agonists and accelerate their clinical application.

In 2018, Goldberg et al. first introduced traditional hyaluronic acid (HA) to prepare a 3D scaffold loading with CDA to investigate its potential in local immunotherapy of tumors [205]. According to the results of *in vivo* release kinetic, HA hydrogel loaded with CDA exhibited a slower release rate especially in the first 4 h in contrast with the fast release rate of free CDA. Further investigation on 4T1-tumor-bearing mice suggested the local administration of this hydrogel could distinctively prevent lung metastasis and tumor recurrence. This work provided a new strategy to prevent local tumor recurrence and distal metastasis after surgical resection by evoking local innate immunity by mediating the STING signaling pathway.

With the advancement of bioactive materials, novel materials like polypeptides or DNA were also reported to be exploited as a hydrogel for the delivery of STING agonists [206,207]. For example, Cui et al. conjugated hydrophobic chemotherapeutic camptothecin (CPT) to a tumor-penetrating peptide (iRGD) to form an amphiphile that could self-assembly into supramolecular nanotubes [208]. After intratumoral injection, the electrostatic interaction between the negatively charged CDA and its surrounding environment facilitates the formation of a supramolecular hydrogel called CDA-NT. This hydrogel is capable of condensing CDA within the physiological conditions of the tumor microenvironment. The *in vivo* release kinetics indicated CDA-NT could extend the release phase of CDA for one more week, which enabled the sustained action of provoking the tumor immune response. Moreover, the local injection of CDA-NT was demonstrated to significantly reduce tumor growth and thus dramatically enhance the survival ratio in the GL-261 brain tumor mice model. Mechanistically, the local administration of CDA-NT provoked both innate and adaptive immunity by promoting the infiltration of various lymphocytes, macrophages, or natural killer cells. Intriguingly, in the recent work of Oh et al., a DNA CpG hydrogel was designed and prepared by rolling-circle amplification. Melanin (photothermal agent) and cdGMP were co-loaded into the hydrogel (Mel/G/DH) to achieve the combinative treatment of immunotherapy and photothermal therapy (Fig. 6B) [195]. To enhance the immunosuppressive tumor microenvironment, the authors employed hyperthermia to trigger the release of danger-associated molecular pattern signals (DAMPs) within tumor tissues. This approach effectively activated dendritic cells (DCs) and elicited an immune response. Together with the inherent effect of cdGMP on the STING signaling pathway, local administration of Mel/G/DH could effectively inhibit the tumor growth of CT26-tumor-bearing mice combined with the irradiation of near-infrared laser. Moreover, the immunosuppressive tumor microenvironment was greatly improved, transitioning from a cold tumor to a hot tumor, as the elevated infiltration of cytotoxic T lymphocytes and the decrease of Treg cells could be detected in tumor tissue or draining lymph nodes.

Taken together, hydrogels loaded with STING agonists hold great potential in solid tumor treatment especially the post-treatment after surgical resection owing to their biodegradability, injectability, and high affinity to tissues [151,152,209]. Nevertheless, it is important to address the issue of burst release that often occurs in hydrogel formulations. This rapid initial release of STING agonists can have unintended consequences, such as excessive activation of local innate immunity and the potential for global inflammation. Therefore, careful consideration must be given to achieving desirable release kinetics to mitigate these concerns. Furthermore, the in-situ cross-linking property of hydrogels may pose a limitation in their application to hematological malignancies, as these malignancies typically lack a fixed tumor bed. This property hinders the precise localization of the hydrogel, which can impede its effectiveness in specifically targeting these types of cancers. Overall, hydrogel formulations have demonstrated promise for STING agonist delivery. Nevertheless, addressing the challenges of burst release and in-situ cross-linking is crucial to optimize their therapeutic potential (Table 3). Moreover, it is essential to explore alternative delivery strategies in order to effectively target hematological malignancies.

5. Conclusion

On the basis of different novel materials, various delivery systems of STING agonists were successfully designed and prepared through methodologies such as nanoprecipitation technique, and self-assembly. Through nanocomplexation or physical encapsulation in materials, the bioactivity of STING agonists was well preserved from enzyme degradation and the other harsh physiological environment. Moreover, the cytosol delivery of STING agonists to APCs was significantly improved after further facile surface modification or targeting conjugation (*e.g.,* Clec9a), which thus have been reported can be administered intravenously in some cases. However, there are still several issues to be addressed before the clinical translation of these delivery systems can be fully realized. Firstly, preparation techniques need to be improved for continuous, scalable, and reproducible production. Homogeneous mixture techniques such as FNC and microfluidics could be promising solutions. Secondly, delivery materials should be designed and tailored to improve intracellular delivery and antigen presentation efficiency, considering size, surface charge, and specific targeting ligands. Specifically, the lymphatic drainage of STING agonists delivery systems with smaller sizes (<50 nm) would be more prone to accumulate in lymph nodes. Besides, for the intravenous injection, a smaller size (<50 nm) has higher tissue penetration, which can enhance the accumulation of

STING agonists delivery systems in target tissues or organs. Finally, the release kinetics of these delivery systems should be tuned for spatio-temporal control, especially for cancer therapy. The premature release of STING agonists may lead to the over-activation of the STING signaling pathway or an undesirable global inflammation, which would enhance the incidence of auto-immune diseases such as psoriasis. Rational designing stimuli-responsive delivery systems in response to tumor microenvironments such as pH, redox, or exogenous stimuli like hyperthermia and light would be beneficial to control the release of STING agonists spatiotemporally and simultaneously. With the advancement of the research on cGAS-signaling pathways and materials science, we believe that these issues will be explicitly addressed and a new generation of STING agonists delivery systems will be developed and applied in the clinical treatments of various diseases in a safer, more controllable, and effective manner.

Data statement

No data was used for the research described in the article.

Authors' contributions

Yanyu Huang, Hong Zhang, Liangping Luo and Zeyu Xiao: Writing - Review & Editing, Supervision; Cuiqing Huang and Ni Shao: Conceptualization, Writing- Original draft preparation; Jifeng Chen, Duo Wang and Genwen Hu: Formal analysis, Resources; all authors discussed, reviewed and edited the final manuscript. All authors have read and agreed to the published version of the manuscript.

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.

Data availability

No data was used for the research described in the article.

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