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# Polyvalent design in the cGAS-STING pathway

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ABSTRACT

Polyvalent interactions mediate the formation of higher-order macromolecular assemblies to improve the sensitivity, specificity, and temporal response of biological signals. In host defense, innate immune pathways recognize danger signals to alert host of insult or foreign invasion, while limiting aberrant activation from autoimmunity and cellular senescence. Of recent attention are the unique higher-order assemblies in the cGAS-STING pathway. Natural stimulation of cGAS enzymes by dsDNA induces phase separation and enzymatic activation for switchlike production of cGAMP. Subsequent binding of cGAMP to STING induces oligomerization of STING molecules, offering a scaffold for kinase assembly and signaling transduction. Additionally, the discovery of PC7A, a synthetic polymer which activates STING through a non-canonical biomolecular condensation, illustrates the engineering design of agonists by polyvalency principles. Herein, we discuss a mechanistic and functional comparison of natural and synthetic agonists to advance our understanding in STING signaling and highlight the principles of polyvalency in innate immune activation. The combination of exogenous cGAMP along with synthetic PC7A stimulation of STING offers a synergistic strategy in spatiotemporal orchestration of the immune milieu for a safe and effective immunotherapy against cancer.

## 1. Introduction

Innate immunity is an ancient form of host defense primarily characterized by recognition of evolutionarily conserved pathogen structures [1]. Molecular pattern recognition receptors (PRRs) are invariant, germline-encoded protein receptors of the host, which recognize both non-self pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) from self, amplifying the signal of PAMP or DAMP recognition through signaling cascades [2–4]. The resulting amplification generally converges in secretion of cytokines by host cells, particularly leukocytes and antigen-presenting cells, activating adaptive immunity to generate specific effector responses towards PAMPs or DAMPs [5]. The signaling amplification and antigen presentation of non-self is highly contextual, depending on the anatomical and subcellular localization of antigen within the host [6,7].

The presence of double-stranded deoxyribonucleic acid (dsDNA) in the cytosol is generally an aberrant phenomena in eukaryotic cells, indicative of infection or host disease [8]. The recognition of dsDNA by cyclic guanosine monophosphate-adenosine monophosphate (cyclic GMP-AMP, cGAMP) synthase (cGAS) amplifies an innate response with production of cGAMP as a secondary messenger [9,10]. Evolutionary adaptations within this pathway utilize polyvalent interactions to limit recognition of self dsDNA while preserving sensitivity towards abnormal-self or non-self dsDNA [11–14]. Downstream, the stimulator

*Abbreviations:* AMP, adenosine monophosphate; CDN, cyclic dinucleotide; cGAMP, cyclic guanosine monophosphate-adenosine monophosphate; cGAS, cyclic guanosine monophosphate-adenosine monophosphate synthase; CTD, C-terminal domain; CTL, cytotoxic T lymphocyte; CTT, C-terminal tail; DAMP, danger-associated molecular pattern; dsDNA, double-stranded deoxyribonucleic acid; ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; F(ab), antigen binding fragment; GMP, guanosine monophosphate; IFN, interferon; IgG, immunoglobulin G; IgM, immunoglobulin M; IRF3, interferon regulator factor 3; ISG, interferon stimulated gene; KSR, kinase suppressor of Ras; LBD, ligand binding domain; LLPS, liquid-liquid phase separation; MAPK, mitogen activated protein kinase; NTD, nucleotidyltransferase; PAMP, pattern-associated molecular pattern; PC7A, polymer with a cyclic seven-membered amine ring; PEG, polyethylene glycol; PMMA, poly(methyl methacrylate); PRR, pattern recognition receptor; STING, stimulator of interferon genes; TBK1, TANK-binding kinase 1; UPS, ultra-pH-sensitive.

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of interferon genes (STING) is the adaptor protein of cGAMP. STING undergoes a substantial conformational change with subsequent oligomeric assembly upon binding with cGAMP [15,16]. This facilitates the phosphorylation and recruitment of TANK-binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3) molecules, leading to enhanced innate activation [17,18]. Herein, we discuss how nature harnesses polyvalency in the regulation of cGAS-STING signaling and highlight the development of synthetic polymers, which induce biomolecular condensation, as an emerging therapeutic principle for cancer immunotherapy.

## 2. Higher-order assemblies in cell biology

Polyvalency is a characteristic of macromolecules which have the capacity to form multiple simultaneous interactions. Representative polyvalent interactions include receptor-ligand pairs each with multiple binding-units (Fig. 1A). The quantity of interactions is the molecular valence state, defined as *i*, and influences system binding behavior. The binding strength of a typical monovalent interaction ($i = 1$) is quantifiable by the kinetic rate constants ($k_{on}$ and $k_{off}$) and binding *affinity* ($K_B$). For example, an immunoglobulin antigen-binding fragment (F(ab)) is a minimal structural component displaying affinity for a specific antigen [19]. The F(ab) molecule binds to antigen in a monovalent stoichiometry and is subjected to equilibrium association and dissociation. However, the incorporation of two F(ab) components into an immunoglobulin G (IgG) antibody shows bivalent binding ($i = 2$) to antigen (Fig. 1A). Effectively, bivalency causes stronger total binding *avidity* between IgG and antigen through a decrease in the kinetic $k_{off}$ rate as well as an increased probabilistic binding geometry [20,21].

In a polyvalent system ($i \geq 3$) avid binding describes the holistic kinetics and energetics through summation of individual affinities [22], each contributing energetically towards a higher-order assembly [23]. For example, immunoglobulin M (IgM) is a polymeric immunoglobulin molecule with decavalent binding to and high avidity for antigen due to its high valency and structural configuration [24]. Quantitatively, the *enhancement* factor, $\beta$, is the ratio between two binding affinities: $K_B^{poly}$ and $K_B^{mono}$, empirically comparing the change in system binding [22]. For a canonical IgM molecule which has specificity towards particular 2, 4-dinitrophenyl ligands on the bacteriophage ϕX174, the association affinity is in the $10^{10-12}$ $M^{-1}$ range as compared to $10^{4-5}$ $M^{-1}$ range for its respective monovalent component, defining a $\beta$ factor of $10^{6-7}$-fold [25]. Functionally, IgM shows utility in early activation of immune complement during infection, displaying the influence of high avidity binding on biological response [26]. In addition, avid IgM macromolecules may form a supramolecular assembly with its complementary antigen, inducing subsequent aggregation or assembly [27]. This higher-order assembly of polyvalent molecules is a general organizing principle, endowing additional function beyond stoichiometric assembly, including modulating enzymatic kinetics [28–30], enabling cytoskeletal arrangement and assembly [31–33], and regulation of gene transcription [34–36].

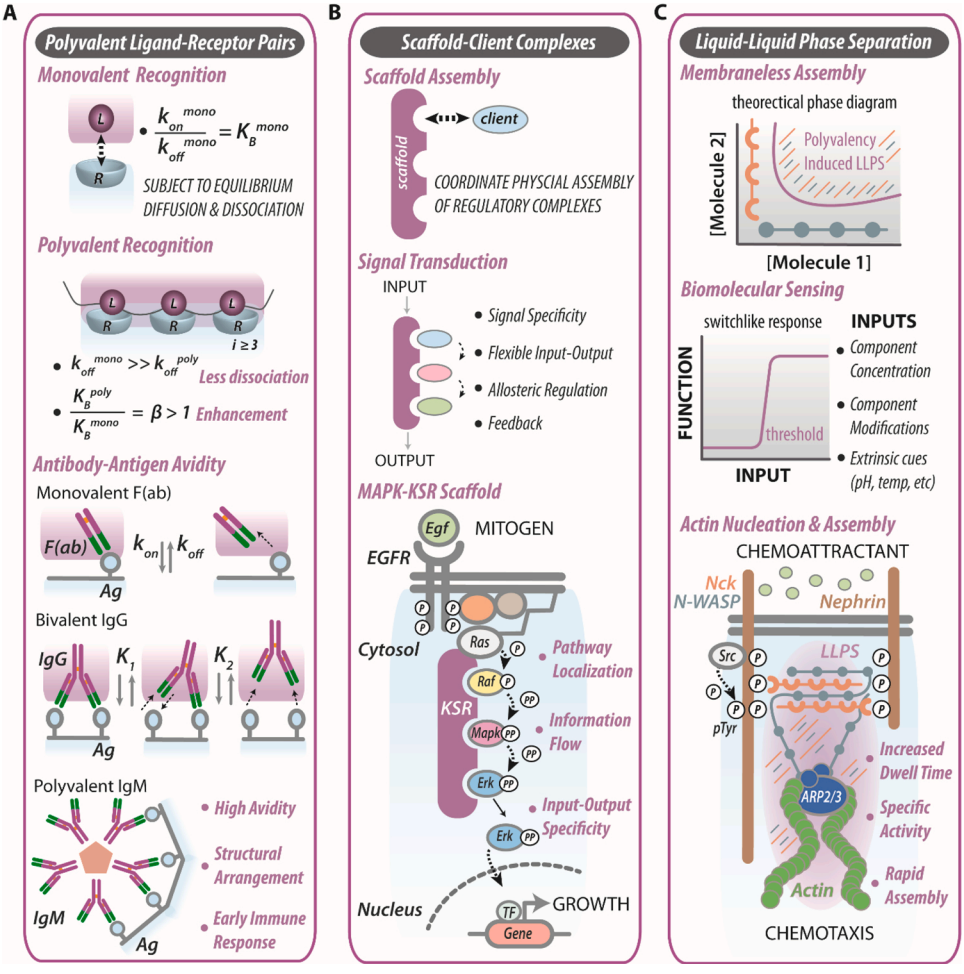


**Fig. 1.** Polyvalency enables formation of higher-order structures with a switchlike response to input stimulus signal. (A) The affinity between a monovalent ligand and receptor pair ($K_B^{mono}$) is determined by the ratio of the association and dissociation rate constants ($k_{on}^{mono}$ and $k_{off}^{mono}$, respectively). When the same ligand receptor pair binds in a polyvalent fashion, the dissociation constant is decreased significantly ($k_{off}^{poly} << k_{off}^{mono}$). This change in molecular association is quantified by molecular avidity ($K_B^{poly}$) betweem polyvalent pairs. The ratio between binding avidity and affinity constants is known as enhancement factor (β). The F(ab) portion of an antibody shows monovalent binding to antigen (Ag). IgG displays bivalent binding to antigen, which reduces kinetic dissociation and enhances avidity. IgM is a polyvalent molecule which has high affinity for antigen. (B) Scaffold proteins are multivalent molecules which physically assemble and recruit monovalent client molecules. These complexes have many diverse functions in signal transduction pathways, including complex signal processing function. The MAPK-KSR signaling scaffold recruits several kinases after EGF binding and EGFR phosphorylation, enabling specific phosphorylation and transcription of growth programs. (C) Interactions between polyvalent molecules causes phase separation known as membraneless assemblies. The spontaneous nature of liquid droplets reduces the time and energy required to form higher-order structures as compared to static structures. A functional consideration of LLPS assemblies is the switchlike amplfication of output signals in response to an input stimulus. Interactions between Nephrin, Nck, and N-WASP on the cell membrane increase condensate dwell time through LLPS for rapid actin assembly and chemotaxis.

### 2.1. Protein assembly in signal transduction pathways

Signal transduction describes a sequential process of protein activation in response to ligand binding of a protein receptor, converging in a specific biological response. Intermediate proteins in the signaling cascade influences threshold behavior, signal amplification, and spatial regulation of the signaling response [37]. Particularly, high valency *scaffold* proteins improve specificity in signal transduction through higher-order assembly (Fig. 1B). Scaffolds interact with other modular proteins in a polyvalent configuration or induce stoichiometric recruitment and binding of low valency *client* molecules [38]. Specifically, the mitogen activated protein kinase (MAPK) signaling pathway canonically utilizes scaffold assemblies to link multiple kinases in a single cascade [39]. In mammalian cells, the kinase suppressor of Ras (KSR) is a scaffold adaptor which localizes Ras-GTPase with other signaling kinases including Raf, MAPK, and extracellular signal-regulated kinase (ERK) [40,41]. Along this pathway, diverse regulatory elements and behavior facilitate emergent behavior such as pathway branching, allostery, and feedback for specific convergence upon cellular growth and proliferation programs [42,43]. These amplification events can display ultrasensitive or *switchlike* behavior, a hallmark of positive cooperativity [44].

### 2.2. Liquid-liquid phase separation of proteins

Molecular self-organization and assembly occurs through out-of-equilibrium thermodynamic processes, which require significant free energy investment for formation [45,46]. To minimize the cost of self-assembly and optimize self-organization, biological systems spatiotemporally enrich molecules within transient, dynamic structures, through a process known as liquid-liquid phase separation (LLPS) [47]. These multi-component assemblies form by polyvalent weak, non-covalent interactions [48,49] and undergo liquid-liquid demixing through spontaneous immiscibility events characteristic of polymer-polymer separation [50,51]. In addition to conserving free energy, intracellular LLPS entities conserve essential and limited cellular components, such as lipid bilayers, by separating in a membraneless capacity [52].

Functionally, condensates optimize enzymatic reaction conditions by influencing kinetics, specificity, or inhibition [53]. Specifically, the process of actin nucleation and polymerization in podocyte cells of the kidney utilizes high-avidity, low-affinity polyvalent interactions between several components: phosphorylated tyrosine (pTyr) residues in the transmembrane receptor Nephrin, the Src homology 2 (SH2) domains on the adaptor protein Nck, and the proline-rich motifs (PRMs) on its ligand N-WASP (Fig. 1C) [54]. The system undergoes membrane-tethered LLPS after chemoattractant molecules bind to Nephrin, increasing the dwell time of the Nephrin-Nck-N-WASP protein assembly and rapidly polymerizing actin via ARP-2/3 complex recruitment for a sensitive chemotactic response [54]. Significantly, additional biological systems exploit LLPS to enhance the sensitivity of a particular input signal to achieve a *switchlike* or positively cooperative response [48,55,56]. Comparatively, while both LLPS and scaffolding serve as organizing assemblies for amplifying molecular signals within the cell, a key distinction is the interaction between polyvalent scaffolds and monovalent clients as compared to the heterotypic interaction between two or more scaffold proteins. While polyvalent-monovalent assemblies regulate sensitivity through mass transport of client proteins and availability of free scaffold binding sites [38], polyvalent-polyvalent assemblies enhance sensitivity dependent on the local concentrations of its constitutive polyvalent components [57]. Thus, both assemblies depend on the relative concentration of polyvalent components, with heterotypic polyvalent pairs having increasing propensity to phase separate, enhancing transduction sensitivity.

## 3. Natural polyvalency in the cGAS-STING pathway

The canonical cGAS-STING signaling cascade is an innate immune pathway for recognition of cytosolic dsDNA as a danger signal. Upon sequence-independent binding of dsDNA, cGAS enzymatically synthesizes the secondary messenger, cGAMP, for downstream binding and activation of STING [9,10,58]. STING is a endoplasmic reticulum (ER) transmembrane receptor protein which undergoes a conformational change and translocates from the ER to the Golgi apparatus after activation [59]. Activated STING recruits the adaptor protein TBK1 [17] for phosphorylation of the transcription factor interferon regulatory factor 3 (IRF3), resulting in the expression and secretion of type I interferon (IFN) [60]. In general, the cGAS-STING pathway is an essential immune defense mechanism against DNA-containing pathogens as well as an alert system for cancer or environmental insult. The fundamental nature of the cGAS-STING pathway necessitates tight regulation to prevent stimulation by host genomic and mitochondrial DNA, while allowing rapid signal amplification in response to aberrant cytosolic dsDNA [12,61,62]. Evolutionary adaptions in the cGAS-STING pathway utilize polyvalent interactions for formation of higher-order assemblies, achieving a specific and rapid response.

### 3.1. dsDNA-induced phase condensation of cGAS enzyme

The recognition step in the cGAS-STING pathway involves cytosolic dsDNA binding to cGAS in a sequence-independent manner. Due to the lack of sequence-specificity for dsDNA and the abundant presence of dsDNA within cells, cGAS activation obtains specificity, in part, through dsDNA-length dependent activation [63,64]. Structurally, cGAS contains an intrinsically disordered and positively charged N-terminal domain as well as an ordered C-terminal nucleotidyltransferase (NTD) domain [65,66]. The dsDNA molecules bind to both domains of cGAS in a 2:2 dsDNA to cGAS stoichiometry. Subsequent conformational changes to cGAS enable enzymatic production of the secondary messenger cGAMP [67,68]. Critically, dsDNA binding to cGAS induces foci formation, which facilitate cGAMP production [9,69]. Recent studies reveal these foci are liquid-like droplets formed through LLPS and are mediated by polyvalent ionic interactions between the negatively-charged phosphate backbone of dsDNA and the positively-charged N- or C-terminus of cGAS (Fig. 2A) [11]. While dsDNA activates cGAS independently of genetic sequence, the length of dsDNA segments impacts the size and frequency of LLPS as well as cGAMP production [11]. This understanding suggests the valence state of the dsDNA polymer is critical for activation of the cGAS-STING pathway, wherein a minimal threshold requires 45 base pairs in the dsDNA for substantial enzymatic production of cGAMP by cGAS [11,63,64]. Functionally, the cGAS-DNA biomolecular condensate concentrates cGAS enzyme and AMP/GMP substrates in droplets for enhanced enzymatic production of cGAMP [11,70]. Furthermore, cellular sequestering of cGAS in a monomeric configuration within the nucleosome prevents aberrant cGAS activation by inhibiting polyvalent condensation, suggesting the higher order assembly is regulated by the polyvalent interactions between dsDNA and cGAMP [61,71–73]. Overall, this phenomenon enables a switchlike response in cGAMP production, amplifying the initial dsDNA stimuli for further activation of downstream STING.

### 3.2. Oligomerization of STING dimers for downstream immune activation

STING is an ER membrane-bound protein consisting of four transmembrane helices, a dimeric cytosolic ligand-binding domain (LBD), and a C-terminal tail (CTT) [74]. Functionally, STING (also known as TMEM173, MITA, ERIS, and MPYS) is essential in production of IFN-β in response to pathogen infection [74–77]. The IFN response is mediated by cyclic dinucleotide (CDN) ligand binding to STING [78] wherein the eukaryotic natural ligand of STING is 2'3'-cGAMP [58,79]. CDN binding

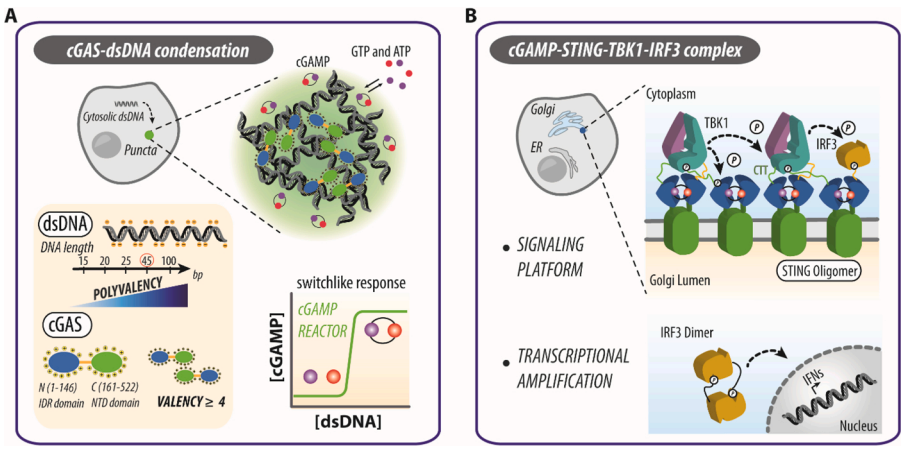


**Fig. 2.** Higher-order assemblies in the natural cGAS-STING pathway. (A) Cytosolic dsDNA enters the cytoplasm upon infection by DNA-containing pathogen or by damage to host. This dsDNA binds with cGAS to form a biomolecular condensate through LLPS. The function of this assembly is to concentrate cGAS with the substrates (GMP, AMP) to catalytically synthesize cGAMP. (B) cGAMP binds to STING on the ER membrane, which causes lid-closing of the STING dimer and oligomerization to form a higher-order scaffold. The whole complex translocates to the Golgi apparatus through ERGIC where TBK1 binds and phoshophrylates STING residues as well as other TBK1 molecules. This platform recruits IRF3 which is also phosphorylated. Activated IRF3 dimerizes and translocates to the nucleus, initiating transcription of type I IFNs.

in a V-shaped pocket at the dimer interface induces STING activation by promoting 'lid-closing' conformational changes to the cytosolic LBD [15,80,81]. The conformational change causes release of the CTT, exposing an oligomerization interface on the LBD [15]. STING molecules begin side-by-side packing on the ER membrane and assemble into a higher-order oligomeric structure [16], which migrates to the ER-Golgi intermediate compartment (ERGIC) and is observable as puncta in the perinuclear region (Fig. 2B) [82]. The oligomeric cGAMP-STING assembly further recruits TBK1 to a conserved motif in the CTT of STING [18]. Although there is constitutive interaction between STING and TBK1 in the absence of cGAMP, STING-TBK1 binding is enhanced by cGAMP-activation through STING oligomerization [17]. TBK1 molecules cluster on oligomeric STING and phosphorylate the activation loop of an adjacent TBK1 molecule in a trans-autophosphorylation mechanism, which is normally inaccessible in a cis configuration [83,84]. Further, phosphorylation of STING by TBK1 must occur on a neighboring STING dimer in the assembly due to geometric constraints [17]. TBK1 phosphorylation of adjacent TBK1 molecules *in trans* as well as neighboring STING dimers can only occur in the context of an oligomeric cGAMP-STING-TBK1 higher order assembly, endowing additional functional specificity on the pathway.

The STING-TBK1 complex recruits the transcription factor IRF3, acting as a signaling platform through mediating STING-TBK1-IRF3 interactions concurrently [85]. TBK1 phosphorylates STING at an evolutionarily conserved, IRF3-binding motif, *pLxIS* (p is a hydrophilic residue, x is any residue, and S is the phosphorylation residue), which is also present on other adaptor molecules: mitochondrial antiviral-signaling protein (MAVS), TIR-domain-containing adapter--inducing interferon-β (TRIF), and TLR adaptor interacting with SLC15A4 on the lysosome (TASL) [60,86]. IRF3 traffics and binds to the phosphorylated STING-TBK1 assembly and is subsequently phosphorylated by TBK1 at an identical *pLxIS* motif on IRF3 itself [87]. Phosphorylated IRF3 homodimerizes, translocates to the nucleus, and engages with an interferon-stimulated response element (ISRE) on DNA for transcription of IFN-β and CXCL10 [88,89]. Overall, the higher-order assembly of STING oligomers, induced by cGAMP, enables a sensitive and specific mechanism for activation of TBK1 and IRF3 through polyvalent structural avidity. Further, regulation and inhibitory structures of cGAMP-STING-TBK1 occur through similar, but excessive, polyvalent interactions including an LLPS transition to a gel-like state [90] or complete inhibition of the oligomerization interface [16].

## 4. Synthetic polyvalent STING agonists with prolonged innate activation

### 4.1. An amphiphilic block copolymer, PC7A, directly activates STING

Therapeutic stimulation of STING by natural or synthetic agonists represents an attractive target in cancer immunotherapy, due to the link between innate immunity stimulation and a cell-mediated adaptive response [91]. Nanoparticles represent alluring therapeutic or vaccination platforms because the ability to encapsulate small molecular cargo as well as size-dependent accumulation within myeloid cells, which have high capacity to secrete soluble cytokines [92]. Therefore, we synthesized a library of ultra-pH-sensitive (UPS) polymers with characteristic linear or cyclic tertiary amine sidechains to screen for a candidate STING agonist delivery platform (Fig. 3A) [93]. UPS polymers self-assemble into nanoparticles in aqueous environments and respond to tunable pH-thresholds with remarkably strong positive cooperativity [94]. The nanoparticles have a poly(ethylene glycol) (PEG) shell and a uniform (diameter ≈ 30 nm) micelle structure, showing suitable biocompatibility properties. Upon exposure to pH below a particular threshold, the micelles spontaneously disassemble into a positively-charged unimer state. Previously work demonstrates the applicability of this platform in delineating acidotic tumor margins for image-guided surgical resection [95] or for PET imaging [96]. Furthermore, UPS responses to endo-lysosomal pH enables digitization [97] and perturbation [98] of the endosomal maturation process. Screening of the UPS library revealed polymers with specific cyclic sidechains disrupt endosomal membranes and allows cytosolic delivery of cargo such as adjuvants and antigens [99].

Encapsulation of tumor-associated antigens or tumor neoantigens in UPS nanoparticles enabled formation of a micellar nanoparticle vaccine [100]. Screening of the UPS library by a T cell killing assay identified direct immune activation by a polymer with a cyclic, seven-membered amine ring (PC7A). The immune response was characterized by cytotoxic T lymphocyte (CTL) antigen-specific killing and a high titer of antigen-specific IgG1 and IgG2c. Interestingly, other linear and cyclic amine polymers did not initiate strong immune activity or T cell killing. Mechanistic investigation revealed strong STING-IFNα/β-dependence but independence of myeloid differentiation primary response protein (MyD88)/TRIF and MAVS pathways. Subcutaneous administration of the nanoparticle vaccine in mice targeted draining lymph nodes and initiated an immune response against multiple tumor models. The resulting tumor growth suppression outperformed treatment by established adjuvants such as cytosine and guanine with phosphodiester backbone oligonucleotides (CpG ODNs) and polyinosinic: polycytidylic acid (poly(I:C)). These results show PC7A has intrinsic immune adjuvant effects, a unique property of a polymeric nanoparticle.

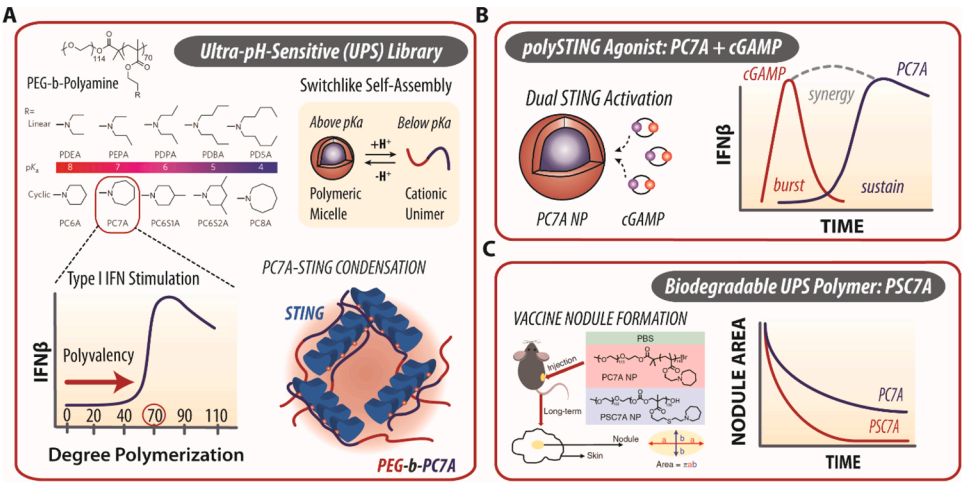


**Fig. 3.** Non-canonical activation of STING by a synethic PC7A polymer. (A) A library of UPS polymers with distinct sidechains and $pK_a$ show positive cooperativity in response to pH as an input signal. PC7A shows polyvalency-dependent binding of and condensation with STING dimers. Adapted from [100]. (B) cGAMP is formulated within the core of PC7A nanoparticles as a dual STING agonist. Treatment of monocytes shows distinct spatiotemporal IFNβ in response to cGAMP or PC7A NP alone. (C) A hydrolyzable polycarbonate backbone is substituted for the PMMA backbone to establish a biodegradable, polyvalent STING agonist. After subcutaneous injection, a nodule forms as a result of local inflammation and wound healing response. This nodule remains persistent in PC7A treated mice but vanishes in PSC7A-treated mice over time. Adapted from [106].

STING activation by PC7A was compared to CDNs such as 2′3′-cGAMP [101]. While both PC7A and cGAMP demonstrated the intracellular re-distribution of STING molecules and phosphorylation of downstream proteins (TBK1 and IRF3) in mouse embryonic fibroblasts, the temporal activation of STING by PC7A was slower than cGAMP (Fig. 3B). The kinetic differences in STING activation suggested a differing mechanism in STING activation by PC7A. Mechanistically, PC7A binds to a non-competitive surface site on the α5 helix of STING that is distinct from the cGAMP pocket. Mutation of two acidic residues (E296A/D297A) sufficiently abolishes polymer binding, while cGAMP-resistant STING variants (e.g., R232 H) retains PC7A activation. As a result, PC7A polymer serves as a supramolecular scaffold and directly engages polyvalent interactions to multimerize STING molecules for immune activation (Fig. 3A) [101].

The formation and size of STING-PC7A puncta was dependent on polymer repeating unit, suggesting a critical role in the valency of PC7A sidechains [101]. Downstream transcription of interferon stimulated genes (ISGs) such as CXCL10, was also dependent on the number of repeating units. STING activity reached a maximum when the repeating unit was at 70 (Fig. 3A). Interestingly, longer polymers (e.g., >70 repeating units) induced higher phase condensation levels, but STING activity was reduced. We attribute the attenuated STING activity to the weaker signaling capacity of oversized condensates with excessive cross-linking and poor molecular dynamics. This hypothesis was supported by the lower reversibility and slower fluorescence recovery rate of STING-GFP after photobleaching. A recent study also observed a cGAMP dose-dependent STING condensation, wherein a high dose of agonist treatment induced a gel-like transition and suppressed innate immune signaling [90]. Together, these results emphasize the critical role of polymeric binding valence and suggests precise control of phase separation achieves optimal immunological outcome.

The seven-member tertiary-amine ring of PC7A (azepanyl moiety) activates STING even in a monomeric configuration. Lipids containing a heterocyclic amino head group, formulated within mRNA-containing lipid nanoparticles, demonstrate dose-dependent STING-activation [102]. The resulting mRNA vaccination platform shows potent DC maturation and activation for presentation of translated tumor associated antigen to CTLs, enabling an effective antitumor immune response against established sold tumors in mice. While the cyclic amino lipids activate STING, it is unclear if the lipids share the same binding site as PC7A or bind within the CDN binding pocket. Molecular dynamics of the heterocyclic lipid and the STNG ligand binding domain suggest association within the CDN binding pocket [102], which is not indicated in the mutagenic study of STING binding to PC7A as discussed earlier [101].

### *4.2. cGAMP-loaded PC7A nanoparticles synergize for enhanced STING activation*

Since PC7A and cGAMP activate STING through distinct binding moieties and spatiotemporal profiles, encapsulation of cGAMP within PC7A nanoparticles (polySTING) synergizes dual activity in STING-mediated immune activation. Intratumoral treatment of tumor-bearing mice with polySTING showed robust tumor growth inhibition and prolonged survival as compared to cGAMP and PC7A alone [101]. In polySTING treatment, cGAMP initiates an initial *burst* in IFNβ secretion whereas PC7A achieves a *sustained* activity (Fig. 3B). This dual-activation strategy prolongs the innate immune activation. Moreover, incubation of fresh surgically-resected human tissue with polySTING shows elevated STING-activation and type I IFN response in sentinel lymph node specimens as well as in several types of resected human tumor tissues. In particular, $CD45^+$ myeloid cells are primarily responsible for the type I IFN response after polySTING treatment in patient samples, indicating the STING-mediated immunomodulation of leukocytes in the tumor microenvironment by nanoparticles.

### *4.3. A biodegradable block copolymer improves the therapeutic window of STING activity*

Therapeutic administration of STING agonists for cancer immunotherapy generally requires repeat dosing [103]. A concern with PC7A is increased dose accumulation after repeated administration. While the poly(methyl methacrylate) (PMMA) backbone of the PC7A polymer is relatively inert and non-toxic, accumulation of polymeric PC7A sidechain agonists could cause adverse effects and dose-limiting toxicity [104,105]. Therefore, we rationally designed and synthesized a biodegradable polymer with similar sidechains as PC7A, known as PSC7A (Fig. 3C) [106]. This degradable polymer is composed of a polycarbonate backbone, which spontaneously hydrolyzes over time, causing degradation into small molecule monomer sidechains or oligomeric material. An *in vitro* hydrolysis assay showed a degradation half-time of the polymer to be 12 or 27 days at pH 6.5 or pH 7.4, respectively. Similar to PC7A, PSC7A also directly binds to the STING CTD. Moreover, encapsulation of tumor antigen in the PSC7A nanoparticle formed a stable biodegradable nanoparticle vaccine. The acute response of PSC7A vaccine towards STING activation and CTL response in mice is of equal magnitude compared to PC7A. However, PSC7A shows significant reduction in systemic inflammatory cytokines after acute treatment. Over time, PC7A induces a persistent inflammatory nodule on the skin at the injection site. This nodule was initially observed with PSC7A, but the nodule reduced in size and was completely diminished after 40 days, demonstrating a favorable safety

profile and increasing the therapeutic dosing window (Fig. 3C).

## 5. Comparing STING agonists

### 5.1. Small molecule STING agonists

Traditional STING agonists are small molecule CDN analogs [107]. In addition to 2′3′-cGAMP, several naturally occurring CDNs include cyclic di-adenosine monophosphate (c-di-AMP) [108], cyclic di-guanine monophosphate (c-di-GMP) [78], and 3′3′-cGAMP [109]. However, degradation by phosphodiesterases, anionic hydrophilicity prohibiting passive membrane transport, and poor pharmacokinetics, limit the therapeutic application of natural CDNs [104,110–112]. Moreover, agonists initially considered as promising therapeutic candidates, such as DMXAA [113] and CMA [114], only activate murine but not human STING. Synthetic CDN analogs, such as thiophosphate c-di-GMP analogs [115], ADU-S100 [104], IAC-8779 and IACS-8803 [116], CL656 [117], and E7766 [118] were developed to overcome these limitations (Table 1). Several clinical trials are ongoing with intra-tumoral administration of CDNs for cancer immunotherapy, which are the focus of other reviews [119,120]. Generally, CDNs show low biological activity and limited therapeutic efficacy, necessitating the further development of STING-activating therapies.

Non-nucleotide CDNs exist as an alternative to CDNs (Table 1). Representative examples include the small molecules SR-717 [121] and MSA-2 [122], which activate STING with a similar 'lid-closing' conformation as cGAMP [123]. In contrast, the class of amidobenzimidazole (ABZI) non-CDN agonists activates STING in a 'lid-open' manner but within the CDN-binding pocket [124,125]. Further molecular and structural biology investigations are necessary to delineate the biological consequences of activating STING in its open or closed conformations. Critically, the administration route of these compounds is not limited to intratumoral injection, enabling systemic administration either intravenously or orally. However, concerns still exist regarding the precise delivery of these compounds for potent activation of STING in relevant dendritic cell populations within tumor or secondary immune organs.

### 5.2. Engineered STING agonist delivery platforms

Nano-engineering offers unique advantages by simultaneously enabling potent and safe on-demand delivery of small molecule STING agonists (Table 2). As discussed previously, several polymeric micelles with heterocyclic amines show inherent STING activity through direct binding to a non-competitive surface on the ligand binding domain of STING [100–102,106]. The polymer agonists display polyvalent condensation of STING molecules, inducing prolonged innate immune responses [101], but it is still unknown if this mechanism is specific to PC7A polymer or to all heterocyclic amine molecules with the azepanyl moiety. Further, several classes of nanoparticles with hydrophilic cores such as polymersomes [126–129], liposomes [130–134], extracellular vesicles [135,136], and mesoporous silica [137,138] show stable loading of anionic CDNs. Both polymersomes and liposomes show efficient cytosolic delivery of CDN agonists, typically through stimuli-responsive moieties, and have potential for high loading capacity of anionic CDN payload. In particular, unique synergy and convergence on IFNβ secretion between $Mn^{2+}$ ions and CDN-induced STING activation shows potential for dual-delivery of non-redundant payloads within an engineered liposome formulation [134]. Extracellular vesicles, such as exosomes or viral vectors, show intrigue in regards to feasibility for development but uniformity and yield of production remain concerns.

The sustained delivery of STING agonists involving microparticles [139–141] or hydrogels [142,143] are developed to avoid repeated administration of the agonists, while enabling an optimal time window for immune activities to overcome tumor recurrence or metastasis. The inherent size of these biomaterials is less appealing in regards to acute biological clearance and clinical utility but remain selections for investigation because of favorable long-term biodegradable properties as well as tractability and feasibility in formulation. Additionally, the delivery of STING protein lacking the transmembrane domain and in complexation with cGAMP (cGAMP-STINGΔTM) is a novel approach for STING activation by triggering the signaling pathway independently from endogenous STING [144,145]. It is unclear if these therapeutics induce the natural polyvalent assembly of cGAMP-STING-TBK1 on the ER membrane or, conversely, assemble in the cytosol for TBK1/IRF3 recruitment. Moreover, antibody-drug conjugates represent a strategy for systemic targeting of STING agonists to cell types of interest and prolonging the drug half-life as compared to small molecule circulation [146]. A major challenge herein is the development of a covalent, yet labile, linkage between antibody and agonist for cytosolic delivery and STING activation.

A key distinction between the classes of STING activators is the polyvalent condensation induced by PC7A and PSC7A, which enables sustained STING activity over 6−48 h [101]. In comparison, cGAMP activates STING with downstream functions peaking at 6 h after treatment. The distinctions in temporal kinetics of STING activation need to be investigated further in the contexts of immunological activation and priming of adaptive responses. However, the synergy between lid-closing activation and polymer-induced condensation of STING enables dual 'burst' and 'sustain" temporal profiles, which proves effective in the immunotherapy against solid tumors [101].

**Table 1**
Small Molecule STING Agonists.

| STING Agonist | Type | Mechanism | Taxonomy | Reference |
|---|---|---|---|---|
| 2'3'-cGAMP | CDN | Cell Intrinsic | Metezoan | [15,58] |
| 3'3'-cGAMP | CDN | Cell Intrinsic | Bacterial | [109] |
| c-di-AMP | CDN | Cell Intrinsic | Bacterial | [108] |
| c-di-GMP | CDN | Cell Intrinsic | Bacterial | [78] |
| DMXAA | Non-CDN | Lid-Closed | Mouse | [113] |
| CMA | Non-CDN | Lid-Closed | Mouse | [114] |
| Dithio c-di-GMP | CDN | Lid-Closed | Human | [115] |
| ADU-S100 | CDN | Lid-Closed | Human | [104] |
| IACS-8779 | CDN | Lid-Closed | Human | [116] |
| IACS-8803 | CDN | Lid-Closed | Human | [116] |
| CL656 | CDN | Lid-Closed | Human | [117] |
| E7766 | CDN | Lid-Closed | Human | [118] |
| SR-717 | Non-CDN | Lid-Closed | Human | [121] |
| MSA-2 | Non-CDN | Lid-Closed | Human | [122] |
| di-ABZI | Non-CDN | Lid-Open | Human | [124,125] |

A list of synthetic small molecules which direcetly bind and activate STING. 'Type' indicates the class of drugs: cyclic dinucleotides (CDN) or CDN mimetics (non-CDN). 'Mechanism' details the inherent occurance of CDN's (cell intrinsic) or the structural configuration of STING (lid-closed/lid-open) after synthetic agonist binding. 'Taxonomy' specifies either the biological kingdom for cell-intrinsic agonists or the biological species which are activated by synthetic agonists.

## 6. Conclusion and future outlook

Polyvalent interactions drive many of biology's organizing principles and functions. Polyvalency strengthens higher-order assemblies by reducing the dissociation rate and enhancing the overall association avidity of molecular recognition groups. The functional requirements imparted on the innate immune system utilize polyvalent condensation for enhanced signal processing and amplification. The interactions in the cGAS-STING pathway amplify presence of cytosolic dsDNA through cGAMP secondary messenger signaling, terminating in the transcription and secretion of type I IFN. How this pathway prevents aberrant activation is appreciated by the understanding of its regulatory polyvalent structural assemblies. Further, a synthetic polymer, PC7A, activates STING in a novel manner, inducing polyvalent condensation and prolonging type I IFN response. This therapeutic strategy synergizes with

**Table 2**
Engineered STING Agonists for Immunotherapy.

| Class | Unique Component | Agonist Cargo | Route | Key Finding | Reference |
|---|---|---|---|---|---|
| Polymeric Micelle | PEG-*b*-PC7A | N/A (Intrinsic) | *s.c.* | Polyvalent Condensation | [100,170] |
| | PEG-*b*-PC7A | cGAMP | *i.t.* | Dual Kinetic STING Activity | [101] |
| | PEG-*b*-PSC7A | N/A (Intrinsic) | *s.c.* | Biodegradable Polymeric Agonist | [106] |
| Polymersome | PEG-*b*-(DEAEMA-*co*-BMA) | cGAMP | *i.t./i.v.* | Endosome Disruption | [126,127,128] |
| | PEG-*b*-P(FcMA -*co*-PEMA) | diABZI | *i.v.* | Hydroxyl Radical Sensitization | [129] |
| Liposome | Heterocyclic Amine Lipid | N/A (Intrinsic) | *s.c.* | Direct STING Binding | [102] |
| | Cationic Lipid | cGAMP/ c-di-GMP | *i.t./s.c.* | Traditional Delivery Systems | [130,131] |
| | Pulmonary Surfactants | cGAMP | *i.h.* | Synergy with Influenza Vaccine | [133] |
| | $Mn^{2+}$ Chelated Lipid | c-di-AMP | *s.c./i.v.* | Metal-Ion Modulation | [134] |
| Extracellular Vesicle | Virus-like Particles | cGAMP | N/A | Endogenous cGAMP Packaging | [135] |
| | HEK293 T Exosome | cGAMP | *i.t.* | Passive Diffusion into Cytosol | [136] |
| Mesoporous Silica | PEGylated Silica | di-GMP | *i.t.* | *In situ* Vaccination | [137] |
| | Labile Silica Matrix | c-di-AMP | *i.t.* | Biodegradable Low Density Silica | [138] |
| Microparticle | Acetylated- Dextran | cGAMP | *i.t./i.m.* | Traditional Delivery Systems | [139,140] |
| | PLGA | cGAMP | *i.t.* | Pulsatile cGAMP Release | [141] |
| Hydrogel | Hyaluronic Acid | c-di-AM(PS)2 | *Local* | Gradual Release Post-Surgery | [142] |
| | Peptide-Drug Nanotube Complex | c-di-AMP | *i.t.* | Gradual Release of Agonist | [143] |
| STING Protein | Exogenous STINGΔTM Protein | cGAMP-STINGΔTM | *s.c.* | Self-Assembly of cGAMP-STING ΔTM Dimer into Tetramers | [144] |
| | Omomyc Protein | cGAMP-STINGΔTM | *i.t.* | Cell-Penetrating Fusion Protein for Cytosolic Delivery | [145] |
| Antibody Drug Conjugate | Immunosynthen | Unknown | *i.v.* | Targeted Delivery of Agonist via Antibody | [146] |

A list of engineered systems which activate STING either through intrinsic mechanisms or delivery of STING-activating CDN. 'Class' refers to the overarching type of STING agonist delivery system. 'Unique Component' refers to the distinguishing feature of the system. 'Cargo' refers to the specific STING-activating molecule loaded within the system. 'Route' refers to the administration route for therapeutic utility. s.c. is subcutaneous; i.t. is intratumoral; i.v. is intravenous; i.h. is inhalation; i.m. is intramuscular. 'Key Finding' defines how the system activates STING or offers utility as a delivery platform.

cGAMP for a dual-activating STING agonist platform wherein cGAMP provides a 'burst' and PC7A enables a 'sustain' in type I IFN secretion. Additionally, a biodegradable backbone of a similar polymer, PSC7A, directly activates STING but improves the therapeutic window of repeat dosing as it reduces vaccine-induced nodule formation over time.

Discrimination between self and non-self is highly dependent on the contextual presence of molecular patterns [147]. Generally, the balance in recognition of *microbial non-self*, *missing self*, and *altered self* against *normal self* requires continual exposure to self-molecules and robust inhibitory pathways to prevent aberrant auto-immunity [148]. Immune surveillance mechanisms must have robust recognition of microbial non-self and altered self-patterns (Fig. 4A). A proposed model for non-self antigenic discrimination in the presence of abundant self-antigen is the 'discontinuity theory', which proposes the temporal aspect of antigen exposure determines the resulting immune response [149,150]. An enduring effector response against a structurally abnormal stimulus (such as in the case of cancer immunotherapy) requires intermittent exposure with clear removal of stimulus to prevent overexposure [151]. This is contrasted to the persistent presence of innate stimulus, which causes immune tolerance [152], hypersensitivity [153], or exhaustion [154]. Moreover, the 'trained innate immunity' model may induce amplified adaptive response upon repeat innate stimulus triggers [155]. Through both immunogenic discontinuity as well as trained innate immunity stimulation, we theorize a novel agonist design principle herein.

We propose a polyvalent agonist design to introduce intermittent immune activation in the context of cancer immunotherapy (Fig. 4B). This principle relies on immunostimulatory molecules which have relatively low monovalent affinity for intended immune receptors [156]. Methods to measure binding affinity include isothermal titration calorimetry [157], fluorescence anisotropy [158], and surface plasmon resonance [159]. We suggest these immunostimulatory molecules serve as monomeric building blocks with functional groups for polymerization. Examples of polymerization include reversible addition-fragmentation chain transfer (RAFT) [160], atom transfer radical polymerization (ATRP) [161], or ring opening polymerization (ROP) [162]. Considerations for additional functions include amphiphilic block copolymer segments for stability and controlled self-assembly [163], pH-responsive or functional moieties for controlled release of cargo [94,164], and membrane destabilizing groups for

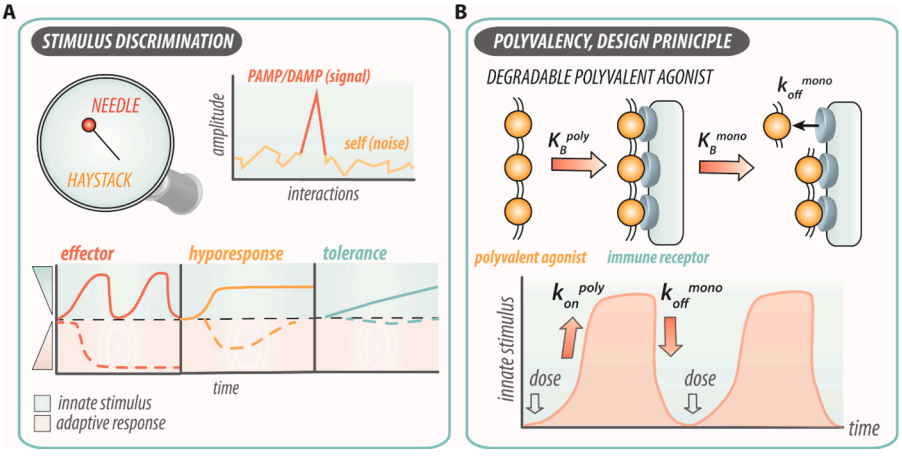


**Fig. 4.** A design principle for exploiting polyvalency in innate immune activation. (A) Immune receptors are constantly engaged in many interactions, particularly from low-affinitiy self molecules. However, PAMPs or DAMPs bind specific PRRs with high affinity, amplifying signals downstream. With a specific time-dependent pattern of innate activation (intermittent, sustained, or persistent; solid line, light green background), an adaptive response (dotted line, salmon color background) initiates a durable effector response or behaves in a hyporesponsive or tolerogenic manner. (B) A polyvalent macromolecule displays binding avidity ($K_B^{avidity}$) to innate immune receptors (light blue). We propose a biodegradable design which disintegrates into monomeric components over time. The affinity between monomeric ligand and the immune receptor is greatly reduced, allowing for ligand-receptor dissociation. This is reflected by an ON/OFF stimulus response curve as a function of time, which fits with an 'intermittment dosing' regimen for optimal effector immune response.

cytosolic delivery of nanomaterial [165].

The binding avidity of polymeric material to the immune receptors shows enhancement as compared to monomeric ligands. To prevent hyperactivation or tolerance, we propose biodegradable polymeric backbones, such as polycarbonate (degradation rate constant $\approx 10^{-10}$ $s^{-1}$) [106], polyester ($10^{-8}$ -$10^{-9}$ $s^{-1}$) [141,166], or polyphosphoester ($10^{-6}$ $s^{-1}$) [167] as well as many other backbones [168,169]. Intermittent dosing of biodegradable material is possible without persistent hyperactivation and resulting immune-related toxicity due to a reduction in biological activity upon degradation [106]. Ultimately, combining the natural and engineering means of activating the cGAS-STING pathway presents a potent design strategy for rational development of novel innate immunostimulatory agonists. Further work will refine this model to understand the specific molecular interactions between polyvalent ligand and innate immune receptors for safe and efficacious cancer immunotherapy.

## Data availability

Data will be made available on request.

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