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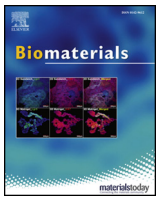

Review

Bioinspired nucleic acid structures for immune modulation

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ABSTRACT

Nucleic acids have both extensive physiological function and structural potential, rendering them quintessential engineering biomaterials. As carriers of precisely-tunable genetic information, both DNA and RNA can be synthetically generated to form a myriad of structures and to transmit specific genetic code. Importantly, recent studies have shown that DNA and RNA, both in their native and engineered forms, can function as potent regulators of innate immunity, capable of initiating and modulating immune responses. In this review, we highlight recent advances in biomaterials inspired by the various interactions of nucleic acids and the immune system. We discuss key advances in self-assembled structures based on exogenous nucleic acids and engineering approaches to apply endogenous nucleic acids as found in immunogenic cell death and extracellular traps. In addition, we discuss new strategies to control dinucleotide signaling and provide recent examples of biomaterials designed for cancer immunotherapy with STING agonists.

1. Introduction

A substantial effort has recently been placed on the development of natural and synthetic materials to engineer immunity. This research thrust has been particularly prevalent in the field of oncology, where the “breakthrough” status of cancer immunotherapy has given way to a wide array of clinically-driven innovations. This wave of innovation has, along with its great successes, also revealed limitations of initial immune-targeted therapies. Namely, the complexity of the immune system requires a multi-pronged approach in order for the next-generation vaccines and immunotherapies to reach their full potential [1]. The rapid development and commercialization of combinatorial therapeutic elements, such as immune checkpoint blockers and vaccines, are testament to the promise and urgency of this call [2].

The innate immune system and its role in priming the host for therapeutic efficacy are among the targets of this broadened view of immunotherapy. As the biological first responders to bodily insult, innate immune cells act as master regulators of the responses that follow. While this fact has been exploited therapeutically in the form of vaccine adjuvants [3], the recent emphasis on combinatorial therapies has returned innate immunity to the focus of scientists and clinicians alike [4].

Innate immune cells are equipped with a wide array of sensors termed pattern-recognition receptors (PRRs), intra- and extracellular proteins which recognize both pathogen-associated molecular patterns (PAMPs), conserved elements of bacterial and viral lineages, and danger-associated molecular patterns (DAMPs), classical indicators of host damage. The presence of either PAMPs or DAMPs initiates tightly-regulated immune activation cascades, which inform the roles of adaptive immunity. A significant number of PRRs respond to nucleic acids (NAs), molecules that can be considered both PAMPs and DAMPs [5]. The discovery of these receptors and their ligands has spurred substantial clinical advances towards the development of NAs as standalone therapeutics and combinatorial adjuvants [6]. This immunological facet of NA biology has been reflected in recent progress in new NA-based materials. In some configurations, these approaches are purely mechanical and structural, incorporating immunogenic sequences into already-established NA structures. In other configurations, the biomaterial itself is inspired by physiological responses to NAs.

In this review article, we summarize the recent developments in NA-based biomaterials and their applications in immunotherapies and vaccines. We first address the relevant immunobiological and clinical foundations of each NA-immune phenomenon, followed by a discussion of various engineering approaches for constructing and utilizing these immune-modulating materials for therapeutic applications. In particular, we highlight key advances in the areas of self-assembling

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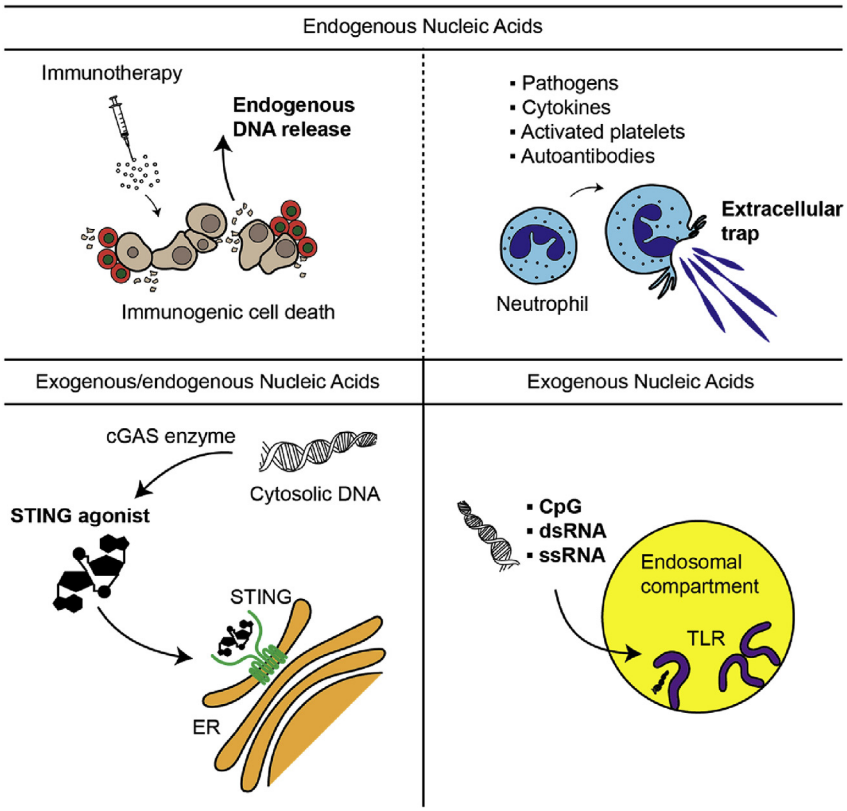

Fig. 1. Immunobiological foundations of bioinspired nucleic acid (NA) structures. Endogenous NAs (top) from immunogenic cell death and extracellular traps can trigger immune responses. Exogenous NAs (bottom right) released by bacteria and viruses are recognized by innate immune cells. Both exogenous and endogenous NAs can be processed into cyclic dinucleotide STING agonists (bottom left) to drive immune activation.

structures based on exogenous NA patterns [7–9]; novel approaches to mimic endogenous immune-NA phenomena, such as immunogenic cell death [10] and phagocyte extracellular traps [11]; and new strategies to precisely control the second messenger (dinucleotide) signaling via the use of STING agonists [12] (Fig. 1). These immunoengineering strategies focused on bioinspired nucleic acid structures and biomaterials may improve the potency and safety of immunotherapies and vaccines in the near future.

2. Danger from the outside: Exogenous DNA and RNA

2.1. Immunobiology of exogenous nucleic acids

Perhaps the most classical role of NAs in immunity is their recognition by immune cells during microbial or viral infection. The immunogenicity of foreign DNA was shown to be dependent on the heightened quantity and hypomethylated status of CpG sequences in non-mammalian genomes [13,14] and mediated by the PRR Toll-like receptor 9 (TLR9) [15]. From this foundation, a wide array of intra- and extracellular sensors has been characterized for various configurations of NAs. The structure and detailed functions of these various receptors have been extensively reviewed elsewhere [5,16–18] and will only be briefly discussed here.

Broadly, nucleic acid sensing falls into two compartments: external and internal sensing. External sensing is achieved by Toll-like receptors 3 (double-stranded RNA), 7 and 8 (single-stranded RNA), and 9 (DNA, particularly enriched with unmethylated CpG sequences). These receptors are found in the endosomes of innate immune cells, thus ensuring activation only by external entities taken up by the cell. On the other hand, internal sensing is critical for the recognition of infected cells when bacterial or viral pathogens have entered the cytosol and potentially begun the process of replication. Cytoplasmic RNA is generally detected by RIG-I and its family of RIG-I-like receptors (RLRs), while DNA is detected by an expanding list of receptors, including cyclic GMP-AMP synthase (cGAS), Absent in Melanoma 2 (AIM2), and several members of the DEAD/DEAH-box helicase family [18]. Additionally, the inflammasome has been reported to sense exogenous DNA, both from bacterial and viral sources [19].

The predominant response to foreign DNA is the production of type I interferons [18] although CpG sequences are also known to induce other pro-inflammatory cytokines, such as IL-6, IL-12, TNF-α, and IFN-γ [20,21], and inflammasome-activating NAs have been shown to induce IL-1β in macrophages via NF-κB [19]. The downstream effect of these pathways is the generation of a pro-inflammatory milieu in which type I interferons cause a T helper 1 (T_H1) skewing and enhanced antigen cross-presentation [22]. Such an environment encourages local and systemic immune involvement, which can be exploited via the therapeutic use of NAs, either as adjuvants to a co-administered vaccine or as standalone treatments.

2.2. Clinical leveraging of exogenous nucleic acids

In the clinic, it has long been known that killed pathogens are far less efficient as vaccines, compared with their live counterparts [23]. Subsequent studies found that the superiority of live vaccines stems from their strong capacity to activate and proliferate $CD8^+$ effector T cells [24]. A recent, further exploration into this phenomenon revealed viability-associated PAMPs, or “vita-PAMPs”, that elicit distinct immune responses to live bacteria as opposed to killed bacteria [25]. These PAMPs are mRNA species unique to viable bacteria and disappear after killing. As the authors showed, these PAMPs are vital to the induction of both pro-inflammatory cytokines and type I interferons, thus elucidating a critical role for native exogenous NAs in the establishment of an immune response.

In addition, exogenous NAs found in NA-based vaccines have immunostimulatory properties. This vaccination strategy, designed to deliver the genetic coding for a target immunogen, involves exogenous NAs with a self-adjuvanting propery, as seen with plasmids containing unmethylated CpG regions. These regions are critical to the efficacy of a DNA vaccine, as enzymatic methylation drastically decreases potency

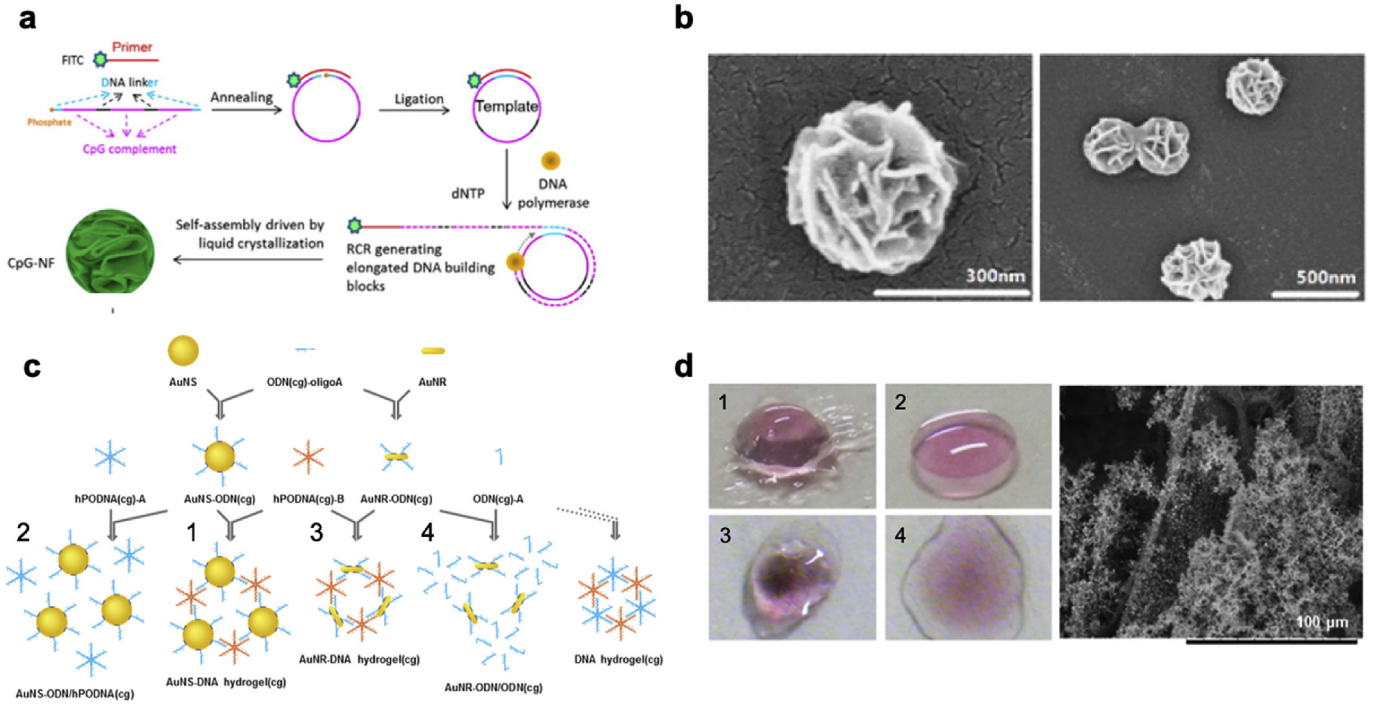

Fig. 2. Exogenous NA-inspired structures. (**a**) Rolling-circle design template for CpG DNA immunonanoflowers, shown in SEM in panel (**b**). Scale bars in (**b**) are 500 nm (left) and 300 nm (right). (**a, b**) reproduced with permission [7]. (**c**) Schematic for DNA hydrogel formed by various combinations of gold nanospheres (AuNS), gold nanorods (AuNR), and CpG DNA. (**d**, left) Brightfield images of hydrogels corresponding to the numbers in (c). (**d**, right) SEM of gold nanosphere-DNA hydrogel. (**c,d**) reproduced with permission [9].

[26]. DNA vaccines approved for use in veterinary applications [27] have utilized this advantage and are under clinical development for vaccination against human papillomavirus, Ebola virus, and Marburg virus [28,29].

2.3. Engineering approaches based on exogenous nucleic acids

A wide variety of structures have been designed to exploit the immunostimulatory properties of exogenous NAs. Recent innovations in this field are highlighted below.

2.3.1. Oligonucleotides and encapsulation

Oligonucleotides (ONs) are short synthetic NA constructs endowed with a particular sequence for either a biological or structural function. A subset of these entities, oligodeoxynucleotides (ODNs), which were critical in the initial discovery of microbial nucleic acid immunogenicity [13,15], were then the subject of rapid research such that a sizable library of ODNs, matched for TLR agonism or antagonism along with stimulation strength, has been generated [30]. ONs have collectively proved to be highly useful in the clinic as both standalone therapies [6,31] and as adjuvants [32], including immunostimulatory agents designed to target TLR9 (various classes of CpG ODNs), TLR3 (polyinosinic:polycytidylic acid, poly(I:C)), and TLR7/8 (imidazoquinolines).

Recent research has expanded beyond TLR-targeting of ONs towards targeting other types of PRRs, including RIG-I [33] and MDA-5 [34]. In addition, researchers have sought to engineer ONs with heightened functionality. In one such example, enantiomeric substitution was used to protect TLR9-directed ODNs from nuclease degradation [35]. After a thorough *in vitro* screen, the authors put forward two candidate agonists that elicited strong cytokine production and inhibited tumor growth in six distinct murine tumor models. In a separate demonstration, a RIG-I agonist 5′-triphosphorylated double-stranded RNA ON was modified with a reduction-sensitive poly(ethylene glycol) (PEG) tail designed to mute the immunogenicity of the RIG-I ON until it reached a reducing environment within cells [36]. In this study, optimization of the conjugated PEG tail resulted in a wealth of information regarding the size, positioning, and chemical requirements necessary to achieve stimulti-responsive immunological silencing.

In addition to being administered as standalone entities, ONs are often also encapsulated in nano- and microparticles to enhance delivery, prolong retention, and increase immunostimulatory potential. In many such applications, ONs are co-encapsulated in vaccine formulations to increase immune receptivity. The depth and history of this particular application necessitates a standalone treatment; thus, readers are directed to an excellent review of ON-adjuvanted particulate systems [37]. There have also been recent innovations in the delivery of standalone ONs, such as the encapsulation of the TLR7/8 agonist R848 (resiquimod) in β-cyclodextrin nanoparticles for the repolarization of the tumor microenvironment [38]. In this study, R848 was identified among a screen of 38 potential therapeutics to reverse pro-tumorigenic M2-polarized macrophages to their M1-polarized counterparts. Encapsulation of R848 into nanoparticles significantly improved its capacity to reeducate macrophages *in vitro* and *in vivo* relative to soluble R848 and blank nanoparticle controls, and administration of this formulation led to improved survival in an MC38 tumor model. Another study explored the pharmacokinetic and pharmacodynamic advantages of nanoparticulate ONs by conjugating an imidazoquinoline-based TLR7/8 agonist (IMDQ) to a block copolymer nanoparticulate system [39]. The authors showed that conjugation to nanoparticles led to the retention of intratumorally-injected IMDQ, as compared to the rapidly systemic distribution of soluble ON, and that this retention significantly altered the immune phenotype of sentinel lymph nodes and spleen. Similar to the previous study, the authors translated this phenomenon into improved therapeutic outcomes in a B16 melanoma tumor model. In both reports, the therapeutic efficacy of the encapsulated ONs was enhanced in combination with checkpoint blockade therapy. Furthermore, TLR3 was engaged by the electrostatic association of poly(I:C) to a polyethyleneimine-coated calcium phosphate nanoparticle [40]. After identifying high uptake by macrophages *in vitro* and the liver *in vivo*, the

authors noted high pro-inflammatory cytokine and type I IFN production by primary hepatocytes, liver sinusoidal endothelial cells, and Kuppfer cells. The breadth and strength of this response emphasizes the therapeutic potential of such an approach.

2.3.2. Self-assembling nucleic acid particulates

NAs have been used to promote self-assembly into particulates with immunostimulatory properties. In one example, structures termed "immunonanoflowers" were generated using a rolling circle templating, an NA replication schema in which a small circular oligonucleotide is used to drive repetitive synthesis [7] (Fig. 2a and b). These structures induced potent cytokine production in macrophages *in vitro*. In another example, DNA dendrimer sequences were self-assembled into particulates with external CpG hairpin loops [8]. Assembly of these hairpin loops into dendrimer structures greatly increased immunostimulation over the CpG loop control as assessed by RAW264.7 macrophage cocultures. Furthermore, the addition of a TAT cell-penetrating peptide added an additional boost to cytokine production.

2.3.3. Nucleic acid hydrogels

Nucleic acid hydrogels have been constructed via various methologidies, including self-assembly [41,42], aligning of linkers with NAs [43], or DNA-mediated crosslinking [44]. Incorporation of immunostimulatory motifs, particularly the exogenous unmethylated CpG motif, is an attractive strategy to engage innate immunity with these macrostructures, and is often employed in combination with other therapeutic interventions. In a classic example of this approach, doxorubicin was incorporated into a CpG hydrogel, a combination that exhibited superior anti-tumor efficacy *in vivo*, compared with doxorubicin delivered either in free form or in a non-CpG-containing hydrogel [45]. Interestingly, the CpG DNA hydrogel alone also exhibited a moderate anti-tumor effect, indicating the power of these structures from which combination therapies can be built.

In a more recent example of immunostimulatory NA hydrogels, a cationized model antigen, ovalbumin, was used to complex CpG DNA into macrostructures, which induced potent immunoactivation [46]. These structures were able to delay the tumor growth and increase animal survival in a murine tumor model of EG7-OVA, whereas an equivalent non-stimulatory inverted sequence, known as GpC, gel used as a control was not able to mediate such a response. Another recent innovation in hydrogel design is the use of polypod-like NA structures, sequence-matched structures that align multiple strands of NAs into asterisk-shaped configurations [47]. Such structures, in a hexapod arrangement, were employed alongside gold nanoparticles, enabling a combination photothermal-immunotherapy triggered by laser irradiation [9] (Fig. 2c and d). The hexapod-like DNA outperformed the crosslinked ODN structure in both *in vivo* cytokine production and antitumor efficacy in an EG7-OVA tumor model. The phenomenon of using small, precisely engineered NA building blocks to incorporate into larger structures has also been applied to other immunological functions. For instance, TLR9 antagonist ODNs were built into structures resembling the Kanji character *Takumi*, which were then incorporated into higher-order hydrogels. Uptake of these structures by macrophages and dendritic cells dampened pro-inflammatory cytokine production after secondary exposure to immunostimulatory CpG ODN [48], showing their potential for treatment of autoimmune diseases with overt immune activation.

3. Damage from the Inside: Immunogenicity of Host Nucleic Acids

3.1. Immunobiology of Endogenous Nucleic Acids

While exogenous NAs and their patterns are indicative of danger to the host, endogenous NAs, particularly DNA, are indicative of damage. DNA released during acute forms of cell death can activate similar pathways as exogenous DNA, including TLR9 and AIM2 [49]. In addition, DNA released in this manner is often found to be complexed with other DAMPs, such as HMGB1 [50]. Even more potent than standard genomic DNA is mitochondrial DNA (mtDNA), which is released during injury [51]. mtDNA, inherited from the endosymbiotic bacterial origins of mitochondria, retains its original parent's unmethylated state and therefore activates the immune system with similar potency as bacterial DNA [52,53].

Further building upon the immunogenicity of mtDNA are extracellular traps (ETs), unique structures released predominantly by neutrophils [54] in which NAs, both genomic and/or mitochondrial [54–56], are externalized in a fibrous mesh. While these structures have been postulated to play a critical role in the prevention of bacterial dissemination, they also localize many immunostimulatory proteins, including HMGB1 and the cathelicidin-derived peptide LL37, on their DNA scaffold, resulting in a highly potent and immunostimulatory entity [57–59]. The remarkable immunogenicity of these structures, as well as their deleterious effects in an array of pathological states as diverse as cancer [60–62], autoimmunity [57,59,63–65], and thrombosis [66], have engendered debate in the field about the evolutionary advantages of ET production [67–69]. These arguments are a testament to the immunological potency of these structures, and while ETs have been shown to be critical for bacterial clearance, the balance still seems to favor host damage rather than repair and/or homeostasis.

Interestingly, neutrophils and other ET-producing cells, including eosinophils [70,71], mast cells [72], basophils [71,73], and macrophages [74,75], leverage not only the traditional immunostimulatory role of the ET DNA backbone but also the fibrous nature of its strands, thereby introducing a novel immunological function of NAs. In a structural characterization of ETs, one group described a wide range of fiber openings between tens of nanometers to several microns [76], which can therefore ensnare a wide variety of pathogens, cells, and debris. Another group noted the similarity between ETs and fibrin meshes under scanning electron microscopy [77]. This phenomenon plays out biologically in the trapping of not only circulating bacteria [78,79] but also other entities as large as circulating cancer cells [60] and as small as circulating cytokines [80]. Thus, in these structures, not only is the immunological potential of NAs elevated to new heights, but novel functions are also gained.

3.2. Clinical Leveraging of Endogenous Nucleic Acids

The immunostimulatory properties of endogenous NAs have been exploited in two main applications in the clinic. The first and most established of the two is the induction of immunogenic cell death (ICD), a general classification encompassing any form of cell death that exhibits antigenicity and adjuvanticity [81]. Such adjuvanticity can come in a vast number of forms, but among them is the release of various NA species from dying cells [82], which can also be found packaged in exosomes [83,84], and the release of other intracellular and nuclear DAMPs such as high mobility group box 1 protein (HMGB1) [85]. HMGB1 is particularly noteworthy with regard to NAs, as it has been shown to increase the kinetics and potency of DNA activation via TLR9 [86], leading to elevated cytokine production [50]. HMGB1 interacts with the receptor for advanced glycation endproducts (RAGE) and triggers AIM2 inflammasome and the autophagy pathway [87]. ICD also promotes the release of calreticulin and heat shock proteins from cancer cells, leading to uptake of cellular materials and debris. In addition, ICD induces the release of CXCL10, ATP, and Annexin A1, which promote the long- and short-distance chemotaxis of immune cells towards the site of cancer cell death [81]. ICD is most commonly encountered in cancer therapy, where chemotherapeutic drugs such as anthracyclines, oxaliplatin, cyclophosphamide, and bortezomib, in addition to radiotherapy and photodynamic therapy increase both the antigenicity and adjuvanticity of dying tumor cells [81,88]. These observations have led to clinical trials exploring various ICD inducers for cancer treatment [85]; however, this disease context also makes ICD

exploitation particularly succeptible to evasion via tumor mutation. Indeed, TLR3 is established as a prognostic indicator for cancer survival, as its higher expression and availability to ICD-induced NA release is associated with improved clinical outcomes [89].

The second pathophysiological arena in which the immunogenicity of endogenous NAs is addressed in the clinic is in the area of ET production. While the dangers associated with ETs are well-enumerated, there are pathologies in which the lack of ETs is problematic. One notable example of such a condition is chronic granulomatous disease, in which patients lack a functional NADPH oxidase that affects free radical production, pathogen killing, and ET production. In the clinic, gene therapy has been tested to rectify this disease, restoring the ability to produce ETs and leading to the resolution of a persistent, therapy-refractory *Aspergillus* infection in a human patient [90,91].

3.3. Engineering Approaches Based on Endogenous Nucleic Acids

The diverse modalities by which the immune system interfaces with endogenous NAs provides a number of distinct tools for engineers in their design of novel materials. Researchers have found inspiration in the complexation of NAs with other co-stimulatory DAMPs, a potent immunogenic combination which mirrors the conditions of ICD. In one such example, synthetic nuclear DAMP complexes (nDCs) consisting of DNA, histones, and HMGB1 were constructed [92]. These complexes were shown to be potent in cytokine production and induction of both apoptosis and necrosis in macrophages. A similar approach utilized a base PAMAM dendrimer structure with electrostatically associated HMGB1 and DNA, which was then coated in a folic acid-PEG-chitosan layer [10]. The resulting nanocomplexes promoted efficient gene transfection and protein expression in folate receptor-expressing cells *in vitro*. Interestingly, however, the authors' motivation in employing HMGB1 was its capacity for nuclear localization rather than its immunogenicity, highlighting its multi-faceted role. In addition, HMGB1 family member HMGN1, which is also classified as a DAMP [93], has been combined with R848 and cyclophosphamide, a mixture termed "TheraVac" [94]. Intratumoral administration of "TheraVac" produced significant immunostimulation, leading to tumor regression in murine models of CT26 colon carcinoma and Renca renal carcinoma. The authors also described the flexibility of the ICD-mimicking combination by exchanging cyclophosphamide for anti-PD-L1 in the treatment of EG7 thymoma tumors. Heat shock proteins have also been employed in ICD-inspired NA engineering approaches. In one such example, a mimic of a cancer cell undergoing ICD was constructed using a phospholipid bilayer encircling a CpG-loaded calcium phosphate core and decorated with B16OVA melanoma cell surface antigens as well as the active peptide of heat shock protein 70 (αHSP70p) [95]. This artificial cell system primed T cells *in vivo* and also activated natural killer (NK) cells. This response led to a regression of B16OVA tumors as well as a decline in lung metastatic nodules.

Another design cue taken from immune-associated endogenous NAs is the trapping and bactericidal behavior of DNA in ETs. In a directly bioinspired application of this phenomenon, DNA-histone microwebs were synthesized which morphologically and functionally resembled ETs [11] (Fig. 3). These microwebs could trap and kill microbes while enhancing the efficacy of antibiotic regimens, therefore uniquely positioning these biomimetic NA structures as an antimicrobial biomaterial platform. In addition, this platform leveraged partially unmethylated DNA in its structure, mimicking not only the structure and trapping ability of ETs but also their inclusion of the immunologically potent mtDNA. Notably, a recent paper suggested that extracellular release of oxidized mtDNA stimulates robust type I IFN responses through a pathway dependent on the DNA sensor STING [55]. As there have been recent reports of NA-architected nanocarriers for targeted drug delivery to mitochondria [96–98], it would be interesting to apply these engineering strategies to target and modulate mtDNA for immunotherapies.

4. Second messenger immunogenicity and STING

4.1. Immunobiology of dinucleotides and STING

While exogenous and endogenous NAs have potent immunostimulatory functions as discussed above, they can also serve a more subtle role in immunity: that of a PAMP-associated second messenger. Exogenous NAs that have successfully bypassed external sensing and appear in the cytosol are processed by cGAS into cyclic dinucleotides (CDNs), which engage stimulator of interferon genes (STING) molecules positioned along the endoplasmic reticulum. This triggers the interferon regulatory factor 3 (IRF-3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling pathways and leads to type I interferon and pro-inflammatory cytokine production. In addition to protection against viral and bacterial infections, recent findings have also revealed a critical role of STING in initiating immune responses against cancer [99]. While much remains to be investigated on how spontaneously arising malignancies initiate the immune response, one recent study suggests that cGAS in tumor cells but not of the normal cells is essential to the generation of endogenous CDN which acts as the source for STING activation and subsequent immune responses [100]. These tumor-derived CDNs induce local release of type I IFN that activates NK cells. Intratumoral NK cells produce CCL5 and XCL1, which attract conventional type I DCs that in turn carry tumor-antigens to draining LN for priming T cells [101]. This provides an insight into how the cascade of immune responses stimulated by STING agonists leads to adaptive immune responses.

4.2. Clinical exploitation of dinulceotides and STING

Importantly, STING agonist treatment induces significant anti-tumor immune responses, leading to complete tumor regression in multiple murine tumor models [102]. Compared with other PRR-binding adjuvants, STING agonists showed a distinct potency in that their sole treatment led to complete regression of established tumors in preclinical settings. The role of STING agonists in triggering type I interferon response leading to activation of various immune cell types suggests many potential applications for cancer treatment. Based on many promising preclinical studies, two types of STING agonists are currently under clinical evaluation as a single treatment or in combination with immune checkpoint blockers [103,104]. Nevertheless, there are concerns surrounding the use of STING agonists. The small molecular size of STING agonists results in fast systemic dissemination after administration, potentially causing severe off-target side-effects. Therefore, most preclinical and clinical studies with STING agonists have been limited to direct intratumoral injection, which limits their use to treating local tumors. A recent report on STING activation in T cells leading to cell death exposes a negative effect of STING activation on cell types other than innate immune cells [105]. In addition, since natural STING agonists are based on inherently unstable CDN structures, they exhibit a fast clearance from blood circulation.

4.3. Engineering approaches to modify or enhance cyclic dinucleotides

One of the ways to address these issues and accelerate clinical translation of STING agonists is to improve the pharmacokinetics of CDNs. It is important to control the dose and regimen for each treatment since these parameters could significantly alter the resulting immune responses. It was recently suggested that a high dose intratumoral injection of STING agonist ablates the primary tumor but compromises systemic immune response, thereby failing to regress distant tumors [106]. Interestingly, the opposite was observed when the same compound was given at a lower dose. Therefore, biomaterial-based platforms for controlled release of STING agonists may offer a solution to these issues.

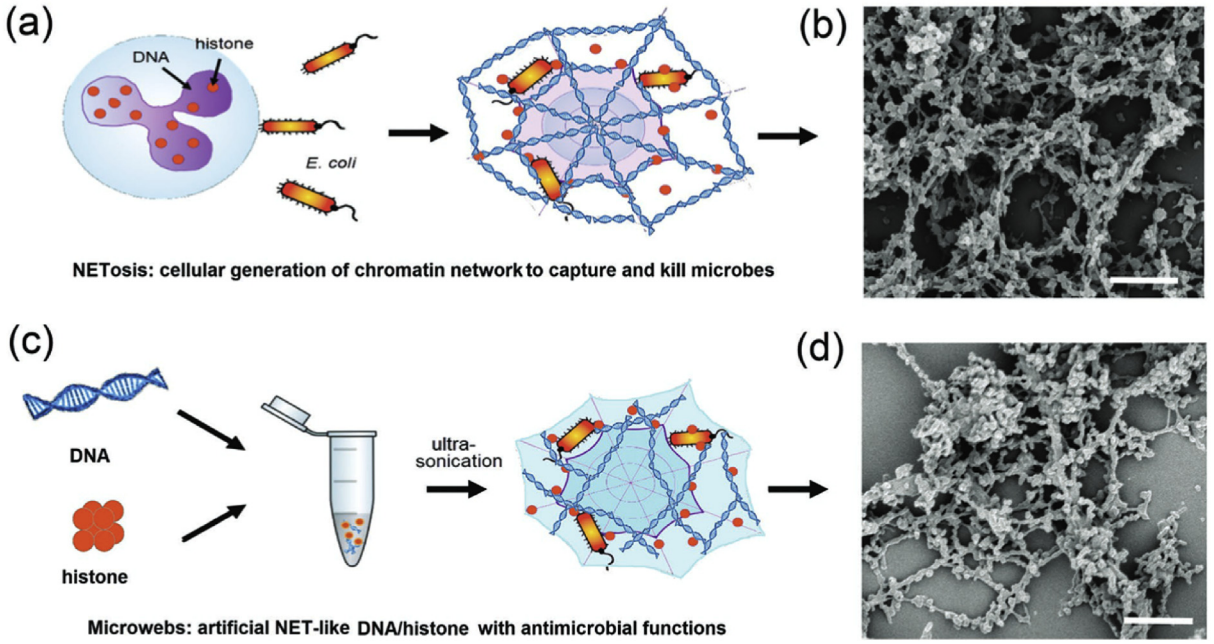

Fig. 3. Particulate system inspired by endogenous extracellular traps. (**a,c**) Schematics of formation of ETs (**a**) and artificial microwebs (**c**), leading to similar fibrous ultrastructures visible under SEM (**b,d**) (scale bar = 1 μm). Reproduced with permission [11].

4.3.1. Liposomal encapsulation

One approach to engineer STING agonist delivery is via the use of liposomes. PEGylated liposomes loaded with cyclic di-GMP (CDG) significantly increased the amount of CDG delivered to draining lymph nodes (dLN) upon subcutaneous administration, whereas free CDG rapidly diffused into the blood stream [107]. CDG-liposomes combined with a liposomal peptide vaccine elicited a superior vaccine-specific $CD4^+$ T cell response and B cell differentiation in dLNs, compared with soluble formulations. Notably, the combination regimen kept the systemic toxicity to a minimal level while eliciting stronger antibody titers, compared with that induced by a TLR-4 agonist, monophosphoryl lipid A (MPLA). In another study, liposomes were used to load cyclic GAMP (CGA) [108]. Due to its size and slightly cationic charge, the CGA-loaded liposomes were able to induce higher type I IFN response *in vitro,* compared with the soluble control. In a mouse model of triple-negative breast cancer, multiple intravenous injections of CGA-liposomes induced repolarization of pro-tumoral M2-like macrophages to a tumor-suppressive M1-like phenotype. This treatment also increased infiltration of $CD4^+$ and $CD8^+$ T cells in the tumor microenvironment and suppressed the tumor growth. In another study, CGA-liposomes allowed for delivery of CGA to lung metastases of melanoma in a mouse model and exerted more potent anti-tumor efficacy, compared with a soluble formulation [109]. In addition, intratumoral injection of CGA-liposomes in orthotopic melanoma resulted in retention of STING agonist within in the tumor microenvironment, leading to ablation of the primary tumor.

4.3.2. Polymer encapsulation

Other than liposomes, several studies have attempted to use polymers to load STING agonists. Use of poly(beta-amino ester) (PBAE) nanoparticles to load two different STING agonists, ML-RR-CDA and RR-CDG, was one elegant example to enhance cellular uptake of the formulation and induce stronger STING activation [110]. This approach provided a cationic charge that promoted cellular uptake *in vitro* as confirmed by flow cytometry using various cell types. Due to the enhanced cellular uptake, stronger IRF-3 activation was observed, compared with soluble control samples. In a mouse model of B16F1 melanoma, nanoparticle delivery of STING agonists combined with an immune checkpoint inhibitor suppressed tumor growth, with the efficacy comparable to 10-fold higher dose of soluble STING agonist treated alone. Acetalated dextran microparticles also have been used to deliver CGA. These MPs were used to simultaneously load multiple adjuvants that would otherwise have different pharmacokinetics [111]. Dual loading of CGA and a TLR7/8 agonist, resiquimod (R848), into the microparticles induced robust pro-inflammatory cytokine release from mouse bone marrow derived dendritic cells (BMDC) *in vitro* and generated superior antigen-specific T cell activation *in vivo*.

Polymersomes have been also employed for delivery of STING agonists. With functional moieties embedded within the polymeric composition, one can endow polymersomes with additional functionalities that may increase the efficacy of the loaded drug. In a recent study, endosomolytic block-copolymers were used to create a polymersome that released CGA in response to the acidic endosomal condition (Fig. 4) [12]. This strategy allowed for facile delivery of CGA into the cytosol, thereby increasing immunostimulation in various cell lines *in vitro* and in a mouse B16F10 melanoma model. Importantly, intratumoral injection of the nanoformulation led to an increased level of activated neutrophils and T cell infiltration and decreased M2-like macrophages within the tumor microenvironment, compared with soluble controls. Moreover, DCs in tumor dLN expressed higher level of CD86, a costimulatory ligand. Owing to these effects, CGA delivery with polymersomes achieved survival benefits in tumor-bearing mice with effective inhibition of tumor growth as observed in therapeutic studies using a mouse B16F10 melanoma model.

4.3.3. STING activation by hydrogels

In addition to nanoparticle formulations, STING agonists have been delivered via hydrogels. Current clinical studies rely on intratumoral injection of STING agonists, which suffer from a fast dissemination from the injection site into the systemic circulation. Therefore, using hydrogels as depots for slow, controlled release of STING agonists can be an effective strategy to achieve better therapeutic outcomes while limiting systemic toxicity. For example, cationic multidomain peptides (MDPs) have been used for mixing and crosslinking with anionic molecules, including the CDA ML RR-S2, leading to self-assembled hydrogels [112]. The unique shear-thinning property of the MDP hydrogel allowed for intratumoral injections and retention. Compared with the well-known collagen hydrogel, the MDP hydrogel significantly

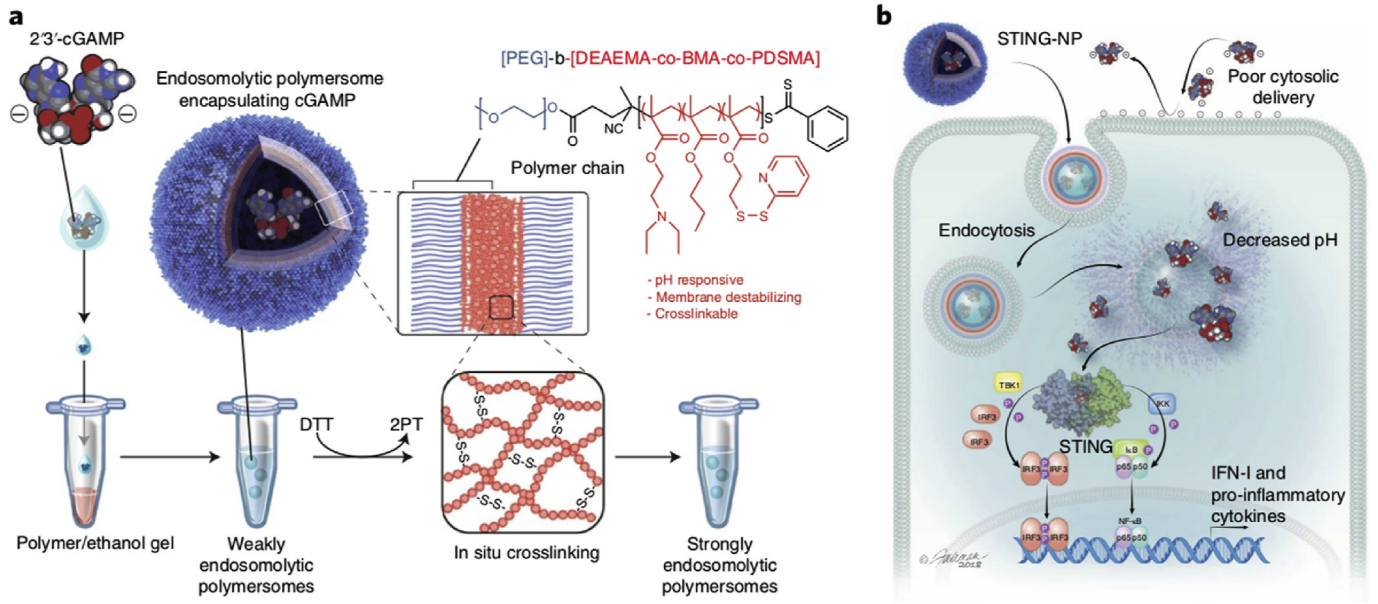

Fig. 4. Encapsulation of CDN for improved delivery to STING agonist. (**a**) Schematic of STING agonist-loaded polymersome composed of pH-responsive di-block copolymers. (**b**) STING agonist-loaded polymersomes are able to enter through cellular membranes and release STING agonist into the cytosol by disrupting the endosomal layers. Reproduced with permission [12].

extended the release profile of ML RR-S2 CDA. In a mouse model of head and neck squamous cell carcinoma, intratumoral injection of hydrogels carrying ML RR-S2 CDA significantly inhibited tumor growth and improved animal survival, compared with soluble or collagen gel control groups.

One of the benefits of using hydrogels for local delivery of STING agonists is that the platform can be placed at a desired location with controlled release of the cargo. This can be especially advantageous when used at post-surgical resection sites. When a tumor is removed by surgical resection, it transiently induces an immunosuppressive environment via the normal wound healing process. Therefore, administering immunomodulatory drug-loaded hydrogels at the surgical site may prevent post-surgical side effects, such as metastasis and relapse, by slowly releasing the drug. For example, hyaluronic acid (HA)-based hydrogel scaffolds were used to deliver R848 and 2′3′-c-di-AM(PS)2 (Rp,Rp), a STING agonist [113]. In a mouse orthotopic model of breast cancer, the hydrogel system was better able to suppress tumor regrowth after tumor resection, compared with soluble controls, thus demonstrating the importance of localized and prolonged release of STING agonist. In a similar study, Matrigel, which is at a liquid phase in low temperatures and transforms to a gel-like solid at body temperature, was used to deliver CDA [114]. Placement of CDA-containing gels at the resection site outperformed soluble CDA in preventing tumor relapse in mouse model of head and neck squamous cell carcinoma.

4.3.4. Modification of cyclic dinucleotides

There have been many attempts to increase the structural stability of nucleotide-based STING agonists. One example is the synthesis of nonhydrolyzable analogs of CGA that are resistant to hydrolysis by ENPP1, one of the hydrolases present in intracellular compartment of cells [115]. A number of analogs were synthesized by introducing phosphothioate diester linkages between the two nucleic acids. The resultant analogs exhibited hydrolysis-resistance to ENPP1 while maintaining strong affinity to human STING molecules. In a recent study, a STING agonist consisting of amidobenzimidazole (ABZI) was synthesized [116]. The synthesized STING agonist consisted of two ABZIs linked to form a diABZI structure, which had enhanced binding affinity to human STING, compared with an NA-based STING agonist, CGA. This compound effectively triggered the release of type I IFN and pro-inflammatory cytokines from human peripheral blood mononuclear cells. Notably, intravenous injection of the compound exerted potent anti-tumor efficacy in a mouse model of colon carcinoma. Systemic administration of STING agonists would broaden the general applicability of STING agonist-based cancer immunotherapy and may benefit more patients.

5. Conclusions and future outlook

Here we discussed a variety of NA-based structures designed to mimic or enhance their interactions with the immune system, representative examples of which are summarized in Table 1. In these arenas – foreign exogenous NAs indicating danger, endogenous NAs indicating damage, and second messenger NAs – the innate immune system utilizes different sets and combinations of PRRs to detect and respond to NAs. Thus, engineering strategies highlighted in this review allow for fine-tuning of NA-immune interactions for achieving their therapeutic potential.

Despite the diversity of bioinspired structures presented herein, this work also highlights several facets of NA immunobiology which are not being exploited towards the generation of bioinspired NA structures. In the context of exogenous NAs, the potent ONs generated to target TLR3, 7, and 8 have often been incorporated into nanoparticulate vaccine systems but, to our knowledge, have not been built up into engineered structures in a similar manner as TLR9-directed ONs. This discrepancy represents a profound engineering opportunity, not only to generate standalone RNA-based structures, but to assemble entities which combine the PRR agonism of RNA, DNA, and other danger signals to induce an even more potent immune response. Likewise, the limited examples of endogenous NAs offer an opportunity for innovation. In this case, the recency of both ICD and ET immunobiology explains the sparsity of current bioinspired structures and also provides two burgeoning classifications upon which engineering can build.

Furthermore, despite many promising platforms studied for STING agonist encapsulation and delivery, there still remains potential for further innovations. Potential immune-modulatory effects by the carrier materials and rapid release profiles of STING agonists from drug delivery platforms covered here are among the aspects that should be improved. Also, while codelivery with other immunomodulants may

Table 1
Representative studies of bioinspired NA-based structures.

NA Inspiration	Defining Characteristics	Nucleic Acid & Analogs	Engineered Structure	Biological Application	Ref.
Exogenous	ss or dsRNA DNA with unmethylated CpG	R848	β-cyclodextrin nanoparticles	MC38 colon cancer	38
		CpG hairpin loops	DNA dendrimer	Macrophage uptake and activation	8
		poly(I:C)	Calcium phosphate nanoparticles	Macrophage uptake and activation	40
		CpG ODN	Electrostatic hydrogel	EG7OVA T-cell lymphoma	46
Endogenous	Complexation with other immunostimulants Unmethylated CpG (mtDNA)	Mouse genomic DNA	DNA-histone-HMGB1 complexes	Macrophage activation	92
		Plasmid DNA	HMGB1-DNA complexed PAMAM dendrimer	Genetic engineering	10
		Bacteriophage DNA	ET-mimicking microwebs	Bacterial trapping and killing	11
Second Messenger (STING)	Cyclic dinucleotide	CDG	PEGylated lipid nanoparticle	HIV gp41 vaccination	107
		CGA	PEGylated lipid nanoparticle	B16F10 melanoma	109
		CDA	Matrigel-based hydrogel	SCCVII HNSCC	114
		ML-RR-S2-CDA	Multi-domain peptide-based hydrogel	MOC2-E6E7 oral cancer	112
		2′3′-c-di-AM(PS)2 (Rp,Rp)	Hyaluronic acid-based hydrogel	4T1-Luc2 breast cancer	113

provide ways to improve the therapeutic efficacy, most of the combination studies with STING agonists have been limited to the use of immune checkpoint blockers. Since diverse cell types are involved in STING-dependent immune responses, it would be interesting to study combinatorial effects of other immunomodulators with STING agonists. STING activation initiates a cascade of immune responses from type I IFN to $CD8^+$ T cell activity; therefore, codelivery with vaccines or other PRR-binding adjuvants may synergize to achieve potent anti-tumor efficacy. For example, there have been a number of attempts to achieve synergistic effects by combining STING activation with CAR-T therapy [117] or formulating with vaccines [118,119], producing promising results in preclinical studies. Also, utilizing multiple PRR-mediated innate immune pathways by combining STING agonists with different types of adjuvants showed enhanced immune responses [111,120], all of which suggest a great potential for STING agonists as an immunotherapeutic agent.

Lastly, when summarizing these innovations, it is important to heed the caution of Campbell and colleagues [121], who noted that translation of immunological phenomena from preclinical models to clinical application can face roadblocks due to the differential expression profiles of PRRs in mouse versus human. Nevertheless, there is a great need to further understand the immunological roles of exogenous and endogenous NAs and to develop new engineering strategies to maximize their therapeutic potential for applications in vaccines and immunotherapies [122].

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