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DNA-based Biomaterials for ImmunoEngineering

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Abstract

The biointerface between man-made DNA biomaterials and the host modulates immune responses toward immunoactivating or immunosuppressive cascades. DNA-based biomaterials introduce DNA into the extracellular environment during implantation or delivery, and subsequently intracellularly upon phagocytosis or degradation of the material. Therefore, the immunogenic functionality of biological and synthetic extracellular DNA should be considered to achieve desired immune responses. In vivo, extracellular DNA from both endogenous and exogenous sources holds immunoactivating immune functions which can be traced back to the molecular features of DNA such as sequence and length. Extracellular DNA is recognized as a damage-associated molecular pattern (DAMP) or a pattern-associated molecular pattern (PAMP) by immune cell receptors, activating either pro-inflammatory signaling pathways or immunosuppressive cell functions. Although extracellular DNA promotes protective immune

Conflict of Interest

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responses during early inflammation such as bacterial killing, recent advances demonstrate that unresolved and elevated DNA concentrations may contribute to the pathogenesis of autoimmune diseases, cancer, and fibrosis. Therefore, addressing the immunogenicity of DNA enables immune responses to be engineered by optimizing their activating and suppressive performance per application. To this end, we review emerging biology relevant to the generation of extracellular DNA, DNA sensors, and its role concerning existing and future man-made DNA biomaterials.

Graphical Abstract

Recent advances in extracellular DNA biology present new opportunities for DNA-based biomaterial design with tuned immune responses. This review describes the manner in which DNA interacts with immune cells, the sources of extracellular DNA, and finally, the emerging biotechnologies that incorporate DNA molecules. Leveraging the immunomodulatory capacity of DNA enables tailored materials that prime specific immunoactivating or immunosuppressive outcomes.

Keywords

DNA; immunoregulation; biomaterials; eDNA; NETs

1. Introduction

An emerging class of biomaterials incorporates DNA molecules as structural or bioactive components including vaccine adjuvants, hydrogels, and synthetic DNA complexes.^{[1]–[3]} In addition to DNA's genetic and structural functions, a paradigm shift has elucidated the immunogenic properties of DNA, now recognized to stimulate immune responses.^[4] Numerous physiological events lead to the externalization of DNA including cell death, disease, and injury, resulting in disruptive concentrations of extracellular DNA in tissues and in circulation.^[5] Extracellular DNA is capable of engaging with the immune system as depicted in (Figure 1), therefore as synthetic DNA-based biomaterials emerge, their potential immune responses must be considered. DNA sensors are mediators of extracellular DNA

immune responses observed *in vivo*, including cyclic GMP-AMP synthase (cGAS) and toll-like receptors (TLRs). Defining novel DNA sensors and their mechanisms is an active area of investigation.^[6] Likewise, the structural and chemical properties of DNA that trigger DNA sensors continue to be uncovered.^[6] This review summarizes known DNA interaction modalities with the immune system, the sources of extracellular DNA, and finally, the relevant and emerging biotechnologies that incorporate DNA molecules. A focus is geared towards innate immune responses and double-stranded DNA (dsDNA). Leveraging the immunomodulatory capacity of DNA enables tailoring materials to initiate specific immune outcomes depending on the application. For instance, priming aggressive immune responses for localized tumor-interfacing biomaterials, or immunosuppressive functions to prevent chronic inflammation on implant surfaces. Achieving this requires an improved understanding of the DNA properties, such as backbone chemistry, base pair sequence, charge, and length, that can function as design levers for the immune response.

2. The role of extracellular DNA in the immune response

The immune system is designed to protect the host against infections by distinguishing between self- and non-self motifs, and by identifying pathogen and damage signals. Upon recognition of a “non-self” or foreign molecule, an array of effector functions are triggered and orchestrated by immune cells to achieve pathogen clearance and restore homeostasis.^[7] These immunoactivating defense mechanisms include the release of pro-inflammatory cytokines, immune cell recruitment, programmed cell death, and phagocytosis. Ideally, immunoactivating responses are supplanted by immunosuppressive functions once the pathogen or agonist is resolved. This immune resolution is necessary to coordinate healing and anti-inflammatory functions such as diminished chemokine and cytokine secretion, replacement of damaged tissues with re-established matrix, tissue remodeling, and restored homeostasis.^[8]

2.1 DNA sensors and their effector functions

Extracellular DNA is sensed as a danger signal capable of eliciting an immune response.^[7] Immune cells detect extracellular DNA as a pathogen-associated molecular pattern (PAMP), or a damage-associated molecular pattern (DAMP), depending on its origin, for example, bacterial versus nuclear DNA origin. DNA binds to cell surface, cytosolic, or endosomal receptors known as pattern-recognition receptors (PRRs) which serve as DNA sensors that activate an initial innate immune response.^[6] PRRs have evolved to recognize both 1) conserved patterns of pathogen contents, and 2) host cellular damage indicated by aberrant or extracellular DNA, and they are critical to a host's ability to fight infections. DNA sensors can be categorized based on location within the cell and their targets. One class of cell-surface receptors are the toll-like receptors (TLRs), which when activated can induce inflammatory type-1 interferon or NF- κ B responses.^[9] TLRs are also found in the endosomal compartment of cells, which trigger anti-viral or anti-microbial responses when pathogens are phagocytosed and trafficked into lysosomes. Cytoplasmic nucleic acid receptors include retinoic acid-inducible gene I (RIG-I) like receptors (RLR), and cyclic GMP-AMP synthase (cGAS).^[10] Upon activation, these signaling pathways culminate in the nuclear transcription of interferon-stimulating genes (ISGs),^[11] type-1 interferons, and NF-

kB responses. Additionally, AIM2 is a cytosolic sensor that once activated by DNA assembles ASC and caspase1 to generate IL-1B, a cytokine considered to mediate the inflammatory response by promoting apoptosis.^[12]

2.2 Immunostimulatory features of DNA

While the identity and mechanisms of DNA sensors *in vivo* continue to be unraveled, the nucleic acid sensor TLR-9 carries particular relevance to extracellular DNA detection.^[6]^[13]^[14] TLR-9 is a transmembrane protein that primarily resides within the intracellular endosomal membranes of dendritic cells, B cells, granulocytes, and monocytes.^[13] This sensor detects cleaved fragments of pathogenic nucleic acids following phagocytosis. Professional phagocytes internalize pathogens into endosomal compartments which then fuse with lysosomes to destroy the pathogen in a toxic environment composed of enzymes, reactive oxygen species, and a low pH.^[10] The phagocyte recognizes the presence of the pathogen upon TLR-9 binding to fragments of the bacterial double-stranded DNA via TLR-9's leucine-rich-receptor (LRR) domain.^[15] The nucleic acid binding induces a conformational change which activates TLR-9 signaling pathways that result in nuclear NF- κ B transcription of pro-inflammatory cytokines, or type-1 interferon production.^[16] IFN- α , IFN- β and other pro-inflammatory cytokines are then secreted to signal the pathogen's presence, and serve to amplify the immune response. The magnitude of TLR-9 responses, and that of other DNA sensors, is dependent on the properties of the immunoactivating DNA. While nuclear DNA can trigger activation, foreign DNA motifs mediate the strongest immune responses.^[17] One such motif is cytosine-guanosine (CpG) sequence, comprised of nucleotides linked by a phosphate group in the 5'-to-3' direction.^[18] Unmethylated CpG sequences along the DNA backbone are one recognition signature of viral and bacterial DNA. These sequences induce a stronger TLR-9 response than methylated DNA, which is more characteristic of mammalian genomes.^[19] However, Yasuda et al. observed that mammalian DNA still induces TLR-9 activation when the mammalian DNA was rich in CpG islands.^[19] Minimal activation was detected in the absence of CpG islands in mammalian DNA, suggesting that DNA fragments containing this feature are pro-inflammatory. Interestingly, mitochondrial DNA (mtDNA) stimulates elevated TLR-9 responses by being sensed as a foreign pattern despite its endogenous origin.^[20]^[21] This effect is attributed to the high concentration of unmethylated CpG sequences found on mtDNA therefore resembling viral DNA over mammalian DNA.^[22]^[23] Mounting evidence suggests that mtDNA adopts inflammatory functions as a DAMP molecule when it is released into the extracellular space during cellular stress and viral infections.^[24]–^[26]

TLR-9 illustrates the manner in which the structural qualities of DNA, such as methylation and base sequence, can dictate the magnitude of DNA sensor activation. Other properties that modulate DNA's immunogenic potential include: length, oxidation, and post-translational modifications. Extracellular DNA varies widely across species, cell populations, and individuals. This diversity contributes to a wide range of immune responses when cells are confronted with extracellular DNA, ranging from immunoactivating to immunosuppressive outcomes. A selection of the DNA structural features that trigger immune responses are outlined below in (Table 1). The DNA origin and its physico-

chemical characteristics influence which DNA sensors are activated, therefore modulating immune outcomes.

Bacterial and viral extracellular DNA is an immunostimulatory PAMP that activates TLR-9, evoking antimicrobial and antiviral responses.^{[17][27][28]} One immunostimulatory property, shared by both forms of exogenous DNA, is attributed to their unmethylated CpG motifs. These responses have been characterized for several viruses including MCMV, HSV-1, and HSV-2.^{[29]–[31]} Bacterial DNA has been shown to induce antigen-presenting cells to express pro-inflammatory cytokines, including IL-12 and TNF- α , and to induce Th1 immune responses.^[32] Although the immunomodulatory properties of DNA have been widely delineated for TLR-9, the immunoactivating triggers of DNA remain to be characterized for several DNA sensors. For instance, the contributions of individual DNA sensors and their activation during sterile inflammation are currently unknown.^[6] The structural features such as length, protein complexes, U-turns that promote DNA recognition with other sensors such as the cGAS-STING and inflammasome pathways continue to be elucidated.^{[33]–[35]} cGAS is activated independent of backbone sequence of cytosolic DNA strands, PAMPs, and DAMPs.^[36] cGAS activation has been demonstrated by length- and structure-dependent features. DNA fragments of 15–20 base pairs induced sensor activity *in vitro*, while longer DNA fragments were necessary to activate cGAS in cells.^{[37]–[39]} Finally, U-bend structural features along the DNA strand was also found to amplify cGAS signaling.^[40]

2.3 Immune Evasion via Extracellular DNA

When considering biomaterial-based immunosuppressive strategies, it is interesting to consider the viral and bacterial approaches that have evolved to mask pathogenic DNA from host PRR detection as potential biomimetic strategies to attenuate immune responses.. These immune evasion strategies, identified *in vitro* or used by pathogens, block various components of DNA sensing pathways. In general, the strategies hamper type-1 IFN and pro-inflammatory cytokine production which proves beneficial for an invading pathogen, but is detrimental to the host.^[41] For instance, CpG-driven signaling was inhibited by blocking endosomal acidification with the molecular compounds bafilomycin and chloroquine.^[42] In another example, certain adenoviruses adapted to contain low CpG-DNA sequences, thus avoiding TLR-9 surveillance.

2.4 Emerging immune functions of endogenous extracellular DNA

Emerging immunobiology reveals that in addition to pathogen-derived DNA, self-derived DNA, particularly neutrophil extracellular traps (NETs) and mtDNA, also function as agonists of the immune system.^{[24][43]} Although a robust inflammatory response is desired for bacterial and viral infections, dysregulated clearance and aberrant responses to host-derived extracellular DNA contribute to the pathogenesis of autoimmune diseases, cancer, and other malignancies.^{[44][45]} Accumulations of extracellular DNA in tissues or in circulation are hypothesized to overwhelm the immune response by engaging DNA sensors persistently, thereby prolonging immunoactivation and inflammation.^{[44][46]} For example, in systemic lupus erythematosus (SLE), patients have defective DNase or DNase inhibitors resulting in unresolved extracellular DNA and NETs.^[47] This lack of DNA clearance is thought to initiate immune hyperactivation by stabilizing TLR-9-DNA complexes, and the

development of auto-antibodies against “self” DNA, leading to inflammatory flares in which the immune system targets destructive effector functions against its own tissues.^{[44][48]}

Altogether, an improved understanding of the underlying features of extracellular DNA in various disease contexts, and their resulting immunomodulatory mechanisms presents opportunities to tailor immune responses with man-made biomaterials. Establishing design principles to address the immunomodulatory nature of DNA subtypes benefits the technologies and biomaterials which incorporate extracellular DNA. In general, base sequence, post-translational modifications, methylation, oxidation, and species origin, should be considered in order to avoid auto-immunity or pro-inflammatory responses, and instead, to promote immunoregulatory and healing cascades.

3. Sources of Extracellular DNA

3.1 Types of Cell Death

The human body embodies numerous types of cells whose turnover is extremely rapid: approximately one million cells per second.^[49] Those dying and dead cells can promote both immunostimulatory and -suppressive responses. Particularly white blood cells (WBCs) are mainly responsible for immune responses. WBCs are composed of neutrophils, lymphocytes, monocytes, eosinophils, and basophils, among others.^[10] WBCs recognize and clear dying and dead cells in response to released signaling cues including DNA.

According to an annual review of regulated cell death, cell death can be categorized into twelve types.^[50] Among them, apoptosis, necrosis, and ETosis are the keystone types of cell death that release fragmented DNA to the extracellular matrix (ECM).^[44] Apoptosis is a programmed death process where cell contents, including condensed chromatin, are enzymatically cleaved and compartmentalized into lipid vesicles.^[51] Those vesicles can rupture and release the inflammatory contents including cleaved chromatin, also known as oligonucleosomes, as a source of extracellular DNA. However, most of them are recognized and engulfed by macrophages. As such, it does not immediately provoke an inflammatory response and rather suppresses inflammation by releasing mediators such as IL-10 and TGF- β .^[52] In contrast, necrosis is a non-programmed death process where cell swelling and loss of cell membranes occur.^[53] As a result, intracellular contents including DNA are directly released to the ECM and exposed to WBCs, which stimulates rapid inflammation.

In 2004, Brinkmann *et al.* discovered a new cell death process termed NETosis that is caused by neutrophils.^[54] Neutrophils release decondensed chromatin combined with granule proteins such as myeloperoxidase and neutrophil elastase when they encounter pathogens such as bacteria, fungi, parasites in order to entrap them by a DNA-protein mesh, so called neutrophil extracellular traps.^{[55]–[57]} Recent studies reveal that this defense mechanism is not limited to neutrophils, but prevalent in other immune cells such as eosinophils, mast cells, monocytes, and macrophages.^[58] Extracellular traps (ETs) provide a large surface area of exposed DNA and warrant effective clearance of pathogens. Moreover, ETs function as a hub for recruiting other biomolecules and cells and participate in the pathogenesis of various diseases such as lupus, atherosclerosis, thrombosis, fibrosis, sepsis, and cancer.^{[59]–[64]} In

summary, the three types of cell death described generate a distinct composition, morphology and size of DNA and play different roles in inflammation.

3.2 Types of Released DNA

DNA goes through distinct processing during apoptosis, necrosis, and ETosis, (Figure 2). Those distinct DNA types can either stimulate or suppress inflammatory responses.^[52] Apoptosis generates relatively short DNA by-products through nuclease-induced degradation and encapsulates the degraded DNA in microvesicles with a regulated distribution.^[65] Conversely, necrosis produces intact and long DNA with a random distribution and directly releases the exposed DNA. Similarly, ETosis releases a DNA-granule protein mesh in an exposed format. Exposed DNA enhances interactions and inflammatory responses as opposed to enclosed, vesicle-sequestered DNA. Notably, ETs comprise either nuclear and mitochondrial DNA through suicidal and viable processes, respectively.^{[66]–[68]} Those distinct DNA types can either stimulate or suppress inflammatory responses.

3.3 Clearance of Released DNA

Extracellular DNA is cleared by phagocytes and cleaved by nucleases as part of the cellular clearance and repair process. In addition to phagocytosis, nucleases serve as another extracellular DNA regulatory mechanism. These are comprised of DNase I, DNase II, TREX-1, and DNaseIL3 and can be found both in the blood and intracellularly.^{[69]–[71]} A balance, or threshold, is required between DNA production and degradation during early inflammation. While an inability to generate or respond to extracellular DNA makes the host susceptible to bacterial and viral infections, elevated DNA concentrations and ineffective DNA clearance may induce long-term effects.^[4] Therefore, effective DNA clearance is necessary to maintain cellular and tissue homeostasis. The dysregulated DNA clearance resulting from impaired nucleases, impaired DNA sensors, or overwhelming extracellular DNA release during infection and disease may lead to prolonged pro-inflammatory responses and autoimmunity, (Figure 3). The development and progression of autoimmune diseases have been linked to the persistent exposure to extracellular DNA including: rheumatoid arthritis, inflammatory myocarditis, Aicardi-Goutieres syndrome, and SLE.^{[5][72]} Attenuating DNA cleavage leads to DNA aggregation and the formation of immune complexes in the extracellular environment. These complexes prolong immune cell recruitment and persistently trigger intracellular DNA receptors to produce pro-inflammatory cytokines, as described in section 2.4. While apoptotic bodies promote DNA clearance, other DNA release methods including ETosis and necrosis are believed to pose difficulty in DNA clearance by phagocytes, thereby contributing to inflammation.^[73] Therefore, the design of novel DNA-based biomaterials must consider DNA clearance mechanisms, DNA sensor interactions, and DNA nuclease confrontation in order to achieve the desired “threshold” response to extracellular DNA.

4. Man-made DNA materials for ImmunoEngineering

DNA is a genetically encoded biomaterial with a huge capacity to store genetic information, and it also leads the transcription of mRNA and the primary sequence of proteins. Emerging

DNA-based biomaterials were classified broadly into six categories. Their functions and applications are summarized in (Table 2), and illustrated in (Figure 4).

4.1 Synthetic oligodeoxynucleotides (ODN)

To examine the immune response of a specific DNA sequence and amplify its immune functionality, short strands of DNA-based materials, oligodeoxynucleotides (ODN) were synthesized for gene silencing, treatment of tumors, autoimmune diseases, and infection.^{[1][3][18][74]–[77]} Compared to a long DNA strand, shorter ODN allow efficient cellular uptake. Among them, CpG motifs were the most frequently investigated ODN. Unmethylated CpG motifs, DNA segments prevalent in bacteria can be recognized as pathogen-associated molecular patterns (PAMPs) by human plasmacytoid dendritic cells (pDCs) and B cells through intracellular signaling pathway TLR-9.^{[18][77][78]} As a result, these immune cells acquire enhanced ability to present antigen to T cells and release cytokines, including tumor necrosis factor (TNF), interleukins (IL-1, IL-6, IL-12, IL-18), and interferon gamma (IFN- γ). These cytokines can further stimulate T cells and natural killer cells, upregulating their antitumoral activity.^[79] Therefore, during the treatment of tumors and cancer, a CpG moiety is often used as an adjuvant of chemotherapy or radiotherapy.^[80]

While non-methylated CpG motifs from bacterial DNA are an immunostimulatory material, the poly-G motif represents a class of immunosuppressive ODN materials derived from the mammalian genome. Contrary to the CpG motif, poly-G motifs deactivate B cells, macrophages and pDCs by blocking TLR-9 receptors in pDCs and B cells, or by inhibiting the activation of cGAS pathways in human monocytes.^{[81]–[84]} Secretion of tumor necrosis factor- α (TNF- α) and IL-12 are also inhibited, and the immune responses are downregulated by these DNA-based materials. The immunosuppressive ODNs have been applied in the treatment of autoimmune diseases.^{[85]–[87]} For example, in patients with systemic lupus erythematosus (SLE), the anti-DNA antibodies are produced in excessive amounts, which forms complexes with extracellular DNA and slows down the DNA's degradation.^[85] The slow degradation of eDNA and their complexes triggers the overactivation of immune system. Poly-G ODN, such as the TTAGGG motif, reduces the production of anti-DNA antibodies and suppresses the secretion of proinflammatory cytokines. Immunosuppressive ODNs have been shown to delay the onset and progression of glomerulonephritis, and improve the clinical outcome in the treatment of arthritis.^[87] Additionally, some ODNs, such as IMT504 can be used as immuno-regenerative materials. For patients with chronic inflammation and cancer, the diseased tissues have reduced capacities for self-repair, and pro-inflammatory cytokine signaling continues over a prolonged time. This leads to desensitized cell receptors and immune dysregulation. Transplantation of mesenchymal stem cells presents a promising therapeutic strategy to regenerate the immune system. Immuno-regenerative ODN can induce the proliferation of mesenchymal stem cells and therefore it is a promising therapy for degenerative immune diseases.^{[88][89]}

4.2 Chemically modified long DNA

Chemical modifications on DNA can change its physicochemical properties, regulate gene expression, and tune their immune responses. Two common biochemical modifications of DNA include methylation and oxidation. In mammalian genomes, DNA methylation typically occurs on the cytosines of the CpG dinucleotides, but they are also observed on N6-adenine in mammalian embryonic stem cells. DNA methylation is associated with genomic imprinting, embryonic development, aging, and cancer.^{[90]–[92]} In the context of immunology, DNA methylation is generally related to transcriptional silencing.^[93] Dynamic methylation and demethylation of DNA modulates gene expression, yet their correlation with disease, particularly with malignancies, is not well understood. A recent genomic study reveals that the DNA sequence largely determines the local pattern of DNA methylation, suggesting methylation is a useful biomarker for diagnosis of cancer at the cellular level.^[93] In clinical applications, DNA methylation has been identified as an effective biomarker with improved accuracy in early cancer diagnosis of breast, lung, brain, and the digestive tract.^{[94]–[97]} DNA oxidation results from oxidative damage mainly on guanine, 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-OH-dG). The damage is caused by accumulated reactive oxygen species, and correlates with cardiovascular disease, neurodegenerative disease, ischemia-reperfusion injury, cancer, and aging.^[98] Oxidized extracellular DNA is often released into the extracellular space by apoptotic dead cells, where it is recognized as a damage-associated molecular pattern (DAMPs) through the TLR-9 pathway as described above. Oxidized DNA conveys a stress signal to cells and induces the release of cytokines including TNF- α from macrophages.^[99] Further studies need to address its impact on immune cells and their effectiveness as biomarkers of human diseases, particularly in stress-related disorders.^[100]

4.3 DNA hydrogels and nanostructures

Pure DNA hydrogels can be formed by crosslinking different DNA monomers into 3D networks.^[101] By varying the species and concentration of DNA monomers, DNA was designed to fulfill various biomedical applications including drug delivery, cell encapsulation and immunoregulation.^{[2][102][103]} Tailoring the sequence of DNA can be used to adjust the responsiveness of DNA to different stimuli, such as enzymes and heat.^[104] For instance, cargo embedded in a DNA hydrogel can be released in a controlled manner in the presence of endonucleases such as DNase. Like simple DNA strands, DNA hydrogels trigger secretion of proinflammatory cytokines from immune cells. Sequence-based immunostimulatory or immunosuppressive effects have been identified in DNA hydrogels.^{[2][105]}

Compared to their DNA strand components, cross-linked DNA gels are physicochemically more stable, and their degradation typically takes a much longer time. The prolonged retention time of DNA hydrogels *in vivo* may be beneficial for boosting the immune responses over prolonged time frames in cancer therapy, as well as the development of adaptive immunization. However, the slower degradation of DNA hydrogels could also be sensed as a danger signal that triggers overactive immune responses *in vivo*. The application of DNA hydrogels in immunoengineering thus requires careful considerations on the degradation-related gel properties, such as degree of cross-linking, length of DNA moiety, as

well as the concentration of DNA mediators, such as DNase around the specific site of implantation. When DNA forms a hybrid hydrogels with other macromolecules, the combined degradation time can be significantly altered, subject to the accessibility of DNase to the DNA in the gel structure. [2]

DNA origami nanostructures comprise an additional form of immunomodulatory DNA biomaterials. [106][107] DNA origami uses various short ‘staple’ strands that hybridize with domains on a scaffold, folding into precise super-architectures. [108] In one example, DNA origami structures demonstrated immunomodulatory characteristics through structural or chemical modifications that tuned their susceptibility to nuclease degradation. Specific structural changes included minimizing free termini and single strands, while chemical modifications included incorporation of nuclease-sensitive sequences to modulate their susceptibility to nuclease degradation. [108]–[110] By manipulating the *in vivo* stability, these materials were able to acquire immunoactivating or immunosuppressive characteristics. In another example, a hollow DNA origami tube with a length of 80 nm and a diameter of 20 nm was decorated with up to 62 CpG anchor sequences. This structure had pronounced immunostimulation through the TLR-9 pathway and endosomal targeting. [111] DNA origami-based nanostructures offer programmable and flexible designs, making them promising drug-delivery vehicles for various biomedical and immunoengineering applications. [112]

4.4 DNA-nanoparticle conjugates

While extracellular DNA typically induces active immune responses, polyvalent oligonucleotide-coated metallic nanoparticles have demonstrated unusual insensitivity to the immune system. [113] The immune stealth property of this material originates from a high packing density of charged ODN on the nanoparticles, yielding a hydrophilic, brush-like barrier that may prevent the enzymatic recognition of ODN. In applications such as biosensors and bioimaging, the non-specific adsorption of protein frequently causes biofouling of the sensor and imaging materials; also, cellular uptake of nanoparticles, such as macrophage phagocytosis, can be an alternative way to eliminate the biomaterials, causing failure in sensing and imaging. The immune stealth property of ODN-nanoparticle conjugates hence may provide an avenue to elongate the retention time of man-made DNA particles *in vivo*. Interestingly, when ODN was chemically modified with a spacer on nanoparticles, the resulting conjugates showed enhanced cellular uptake and tumor growth inhibition. [114] This phenomenon is attributed to the free rotation of ODN on nanoparticles following the addition of a molecular spacer, which allows free access of immune cells to the conjugates. The seemingly controversial observations highlight the importance of structural design and its immune functions in DNA biomaterials.

The duplex structure of DNA also provides a chiral template for the assembly and stabilization of metallic nanoclusters. [115][116] When thiolated DNA (DNA-SH) conjugates with silver to form nanoclusters, the resulting conjugates show strong fluorescence emission and extended shelf-time, which can be used for nuclear imaging. [117][118] Compared with CpG motifs alone, the CpG-Ag nanoclusters increase IL-6 levels due to the enhanced efficiency of phagocytosis and higher resistance to DNase degradation. [118] Recently, metal-

organic frameworks (MOF) nanoconjugated with therapeutic CpG ODNs were also shown to increase the immunostimulatory properties of the ODN when compared to unconjugated CpG ODNs. MOF's are highly crystalline inorganic–organic hybrids that are constructed by bridging metal ions or clusters with organic ligands.^[119] In addition to enhanced cellular uptake, MOF's induced higher production of TNF- α and IL-6 cytokines by antigen-presenting cells.^[120] The use of metallic nanoparticles as conjugates of DNA therefore can achieve simultaneously desired imaging and immunostimulatory properties for applications that include vaccines and imaging.

In addition to imaging and sensing applications, DNA nanoparticle conjugates have been used in the photothermal treatment of tumors. When photothermal nanoparticles, such as metallic nanoparticles, clusters or graphene are conjugated with ODN and delivered into targeted tumor cells, their absorption of near-infrared light (650–900nm) converts vibration energy into heat. The localized heating from this process can kill cancers and tumors without damaging surrounding healthy tissues.^[121] Since ODNs are immunostimulatory materials, conjugation potentially allows combination of photothermal treatment with immunotherapy, with better therapeutic efficacy and alleviated pain for cancer patients. Further studies in this area are needed to improve the specificity of ODN-mediated recognition and targeting to cancer cells.

4.5 DNA-biomolecule complexes (proteins, antibodies, and RNA)

Chromatin DNA, which is packed around histones into nucleosomes through electrostatic forces, can be released into extracellular environment through ETosis, necrosis and apoptosis. During ETosis, histone is citrullinated via protein arginine deiminases (PAD4), resulting in the disassembly of the chromatin structure and release of ETs.^[122] Cytoplasmic proteins, such as neutrophil elastase, cathepsin G, LL-37 have shown a high affinity to the DNA component of ETs. While ETs are believed to exert the primary immune function of bacteria trapping and inhibition to bacterial infections, the accumulation of NETs *in vivo* also correlates with many autoimmune diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), psoriasis and vasculitis.^[123] In these autoimmune diseases, antibodies are often colocalized with extracellular DNA, causing slow degradation of ETs and long-term organ damage. Nevertheless, due to the complex compositions of ETs, identifying the immune function of each ET component, as well as identifying the therapeutic targets in ETs for autoimmune diseases remains a challenging matter. Synthetic immune biomaterials provide a bottom-up approach to test and construct the function of DNA-protein complexes. For instance, using synthetic DNA-LL37 complexes, Stephan et al. found that DNA-LL37 complexes are an antimicrobial component which attack mycobacteria in macrophage phagolysosomes.^[124] The synthetic approach can also potentially be used to identify the pathological roles of specified NET components in autoimmune disease.^[125] As an example, complexes of mitochondrial DNA-LL37 have been found to evade autophagy and hence trigger atherosclerosis. These DNA-protein complexes therefore are potential therapeutic targets for autoimmune diseases.^{[126]–[128]} Furthermore, some synthetic DNA-antibody, DNA-cytokine complexes offer new possibilities for the repair of damaged DNA or as new therapeutic targets for deficient DNA-repair malignancies. For instance, an anti-DNA antibody found in lupus patients, 3E10, can

efficiently inhibit the DNA repair in tumor cells and decrease their tolerance to anti-cancer therapy.^[129] The use of synthetic DNA-protein complexes and coatings would accelerate the screening of anti-cancer antibodies and facilitate a better understanding and design of novel medicines.

With respect to DNA origami nanostructures, protein-coatings may serve to modulate their conferred immune response. DNA origami nanostructures have observed improved structural integrity and function through electrostatically-driven protein coatings.^[130] For example, when coated with proteins such as bovine serum albumin (BSA), the nanostructures displayed less degradation by DNase1, increased cellular transfection, and attenuated immune responses of splenocytes.^[131]

Finally, and additional DNA complex structure are cytosolic immunostimulatory complexes that form when DNA combines with the protein-encoding nucleic acids, RNA. Synthetic versions of these DNA:RNA complexes have been generated to represent various endogenous, viral, and bacterial compositions. Interestingly, different signaling pathways have been activated depending on the hybrid composition. For instance, enzymatically generated homopolymers, and were shown to activate macrophages through the cGAS-STING signaling pathway and generated antiviral type 1 interferon responses.^[132] The complexes were thought to activate the DNA sensor through a conformational manner, resembling dsDNA. In another example, the duplexes were synthesized to contain viral sequence motifs and activated antiviral responses in dendritic cells through TLR-9 sensing.^[133] Finally, synthetic bacterial-like DNA:RNA hybrids were found to activate NLRP3 inflammasome-dependent responses.^[134] Their exact mechanisms of DNA sensor activation remain to be fully described. However, synthetic RNA:DNA complexes illustrate the potential for biomaterials to characterize immune responses that may advance the understanding of host defenses and autoimmunity.

5. Conclusion and Perspectives

5.1 Targeting new DNA sensors and signaling pathways

Synthetic DNA-based materials have been applied in regulating immune responses for two decades, most of these materials stimulate our immune system through TLR-9 pathways. With the advancement of current knowledge in cellular biology, a variety of new cytosolic DNA sensors and signaling pathways have been identified, such as cGAS-STING, NF-KB, AIM2-inflammasomes, and the RIG-I-like signaling pathway. Synthetic DNA materials targeting these sensors and signaling pathways remain largely unexplored, yet their potential impacts to fundamental science and clinical treatment of diseases is well-recognized. For instance, recent findings indicate a strong connection between damaged DNA and the activation of cGAS, which further amplifies inflammation, cellular senescence, and cancer.^[135] Small molecular inhibitors, such as RU.521 can selectively inhibit the activation of cGAS and suppress the expression of interferons in macrophages, thus potentially useful in the treatment of autoimmune diseases.^[136]

5.2 Biomimetic DNA materials

Our immune system has evolved advanced functional extracellular DNA materials, examples include extracellular traps with web-like structures to trap and kill pathogens. This natural biomaterial constitutes an important part of innate immune system, and it also interacts with immune cells for the development of adaptive immune responses. Biomimetic DNA materials, which may include the composition-structure-function relationship of these complexed eDNA structures, offer new opportunities in advancing the knowledge of natural immune structures, as well as the interactions between biomaterials and the immune system. The knowledge could inspire new approaches towards advanced therapeutic treatment, such as DNA vaccinations.^[136] Moreover, these eDNA structures also correlate with infection of bacterial/viral diseases (such as biofilm formation), pathology of autoimmune diseases and cancers under certain physiological conditions.^[137] Biomimetic studies on the performance of these diseased DNA structures using synthetic materials or mimicking the pathological environment would help to reveal clinical targets of immune diseases and the clues of cancer progression, which would open new doors for the clinical treatment of these diseases.

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Figure 1. The role of DNA in the immune system.

Beyond its role as an information-encoding molecule (left), DNA has immune functions when introduced into the extracellular milieu. DNA engages with immune cells to initiate either immunoactivating and immunosuppressive responses. Depicted on the right is extracellular DNA activating the DNA sensor, TLR-9, upon internalization by phagocytosis. TLR activates NF- κ B signaling leading to the release of pro-inflammatory cytokines, amplifying the immune response. Created with BioRender.

Figure 2. Types of cell death and resulting DNA release.

The top row depicts types of cell death and morphology and the bottom row illustrates DNA size and morphology upon cell death. The columns from left to right correspond to apoptosis, necrosis, and ETosis, respectively. Created with BioRender.

Figure 3. The Production-Degradation Balance of Extracellular DNA.

The immune system requires a balance between extracellular DNA production and degradation. The mechanisms of release and their pathological implications are outlined. Over-production may lead to cell death by-products, defective nucleases, and cellular stress. [5][44][53] These have been found to have implications in lupus, cancer, and arthritis. [45]–[47] On the other hand, insufficient extracellular DNA by phagocytosis or enzymatic clearance, such as DNase 1, can lead to host susceptibility of infections. [6][23][43][44] Created with BioRender.

Figure 4. Engineered DNA biomaterials.

Emerging DNA-based biomaterials were classified broadly into categories: ODN, chemically-modified DNA, DNA hybridization, DNA-nanoparticle conjugates, and DNA-protein complexes.

Table 1.
Immunostimulatory Features of Extracellular DNA.

The structural features of extracellular DNA induce responses in the immune system.

Classification	Features
Exogenous	
Bacterial DNA	• Unmethylated CpG motifs induce TLR-9 response ^[17]
Viral DNA	• CpG-rich regions induce TLR-9 response ^[7]
Endogenous	
Nuclear DNA	<ul style="list-style-type: none"> • dsDNA, U-bend ^[7, 36] • Modifications: oxidation, methylation, decondensation ^[54,68,93] • Immune DNA-protein complexes: HMGB1, auto-antibodies, LL-37 ^[35,125] • Length: Minimal 50–80 bp for AIM2, Minimal 20–40 bp for cGAS ^[12, 39]
Mitochondrial DNA	<ul style="list-style-type: none"> • Unmethylated CpG motifs (TLR-9) ^[24] • Oxidation^[26]

Table 2.
DNA-based biomaterials for ImmunoEngineering.

Emerging DNA-based biomaterials and their interactions with the immune system. Their applications range widely including cancer and autoimmune disease therapeutics, imaging, and diagnostics.

Oligodeoxynucleotides	Interaction with immune system	Application
a) Immunostimulatory ODN (E.g. unmethylated CpG motif from bacterial DNA)	Activate pDCs and B cells through TLR-9 pathway; Increased proinflammatory cytokine release (TNF, IL-1, IL-6, IL-12, IL-18, IFN- γ)	Activate the immune system to respond to cancer, allergy and infection. ^[1,3,75,76]
b) Immunosuppressive ODN (E.g. Poly-G ODN)	Deactivate B cells, macrophage and pDCs by blocking receptors of TLR-7 and TLR-9 pathways	Depression of autoimmune disease and septic shock ^[81–87]
c) Immuno-regenerative ODN (E.g. IMT 504; PyNTTTTGT)	Stimulate proliferation and differentiation of mesenchymal stem cell ^[88,89]	Repair immune systems for treatment of neuropathic pain, osteoporosis, diabetes and sepsis
Chemically modified DNA		
a) Methylation/demethylation	Affect gene expression and differentiation of B cells, enhanced activity	Biomarker for diagnosis of cancers ^[93–97]
b) Oxidation	Convey a damage associated signals and induce release of TNF- α	Correlated with stress disorders ^[98–100]
DNA hydrogels and nanostructures		
a) Pure DNA gels	Prolonged circulation/degradation time	Drug delivery ^[101]
b) Hybrid DNA gels	DNA hybrid materials can also be sensed through TLR-9	Imaging, biosensing, and drug delivery ^[2,102–105]
c) DNA-origami nanostructures	Tuned TLR9 sensing and nuclease degradation	Drug delivery ^[108]
DNA-anoparticle conjugates		
a) DNA-nanoparticle (E.g. Au, Ag, Pd, Carbon nanotubes, MOF, Graphene)	Spacer/assembly of ODN strongly affects the recognition and cellular uptake by immune cells. Localized heating by electromagnetic radiation	Biosensors, imaging, photothermal treatment of tumors ^[113–118]
b) DNA- nanocluster (E.g. cages, sheets)	Enhanced immune response and cell imaging	Biosensors, imaging, photothermal treatment of tumors ^[117,118]
DNA-biomolecule complexes		
a) DNA-histone	Extracellular traps correlate with native immune defense, autoimmune diseases and cancer	Potential therapeutic targets for lupus patient
b) DNA-cytoplasm proteins (E.g. neutrophil elastase, LL-37)	Attack bacteria in macrophage phagolysosomes ^[124] ; MtDNA-LL37 can cause atherosclerosis ^[125]	Potential therapeutic targets for atherosclerosis
c) DNA-cytokines (E.g. HMGB-1, CCL-7,8, CXCL-10,11)	Stimulate cytokine production through TLR-9-MyD88 pathway. ^[119] It induces production of DNA-antibody via TLR2/microRNA-155 pathway ^[127]	Potential therapeutic targets for autoimmune pathogenesis of systemic sups erythematosus (SLE) ^[128]
d) DNA-antibody	Cause autoimmune diseases, such as lupus nephritis	Potential therapeutic targets for autoimmune disease and cancer ^[129]
e) DNA:RNA hybrids	Engage various innate signaling pathways depending on nucleic acid origin	Potential therapeutic targets for infections and autoimmune diseases ^[132–134]