

Engineering of DNA Micro- and Nanoparticles: Towards Vaccine Delivery

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Abstract

Vaccines are an effective tool for preventing and controlling various diseases by inducing adaptive immunity. Nanomaterials play an important role in vaccine development. Micro- and nanocarriers can be engineered to improve the therapeutic efficacy of vaccines by (i) preventing the degradation and systemic clearance of vaccine antigens and (ii) facilitating the uptake of vaccines in antigen-presenting cells (iii) co-delivering adjuvants and antigens at desired intracellular compartments for optimal immunotherapy. However, it is important to engineer a carrier that is both effective and safe. Micro- and nanoparticles based on DNA have shown great potential for biological applications, owing to the programmable sequences, predictable interactions, versatile modification sites, and high biocompatibility of DNA strands.

This thesis aims to develop facile strategies to synthesize DNA particles for vaccine delivery by self-assembly approaches. First, a simple strategy to synthesize DNA microcapsules is reported. The cytosine-phosphate-guanosine oligodeoxynucleotides (CpG) motif is an efficient vaccine adjuvant that can effectively stimulate the immune system to secrete cytokines. By loading and crosslinking Y-shaped DNA building blocks (containing CpG motifs) into sacrificial calcium carbonate templates, monodisperse and spherical DNA capsules were obtained. These DNA microcapsules were internalized into cells efficiently, accumulated in endosomes, and induced immune cells to secrete high-level of cytokines.

Next, we developed a template-assisted and versatile approach for synthesizing a new set of multifunctional particles through the supramolecular assembly of tannic acid (TA) and DNA molecules. Uniform and stable DNA-TA particles with different morphologies could be easily synthesized by using different types of DNA strands. Intriguingly, different DNA sequences can be encoded into this DNA-TA particle for applications in immunotherapy or gene delivery. The incorporation of CpG motifs and ovalbumin into the particles allows the intracellular antigen/adjuvant co-delivery to amplify cytokines production in macrophages, through synergistic effects. In addition, green fluorescent protein (GFP)-expressing plasmid DNA could be transfected by using the DNA-TA particles in HEK293T cells.

Finally, nanometer-sized particles were engineered by exploiting the one-pot supramolecular assembly of TA, DNA, and PEG for intracellular delivery of CpG motifs. TA-DNA-PEG nanoparticles with different sizes could be fabricated by adding different molecular weight PEG chains. TA-DNA nanoparticles with tunable size were also synthesized by varying the molar ratio of TA and DNA. The obtained nanoparticles can enhance the cellular uptake of

CpG oligonucleotides and consequently the production of cytokines in macrophages. Overall, the engineered DNA-based particles have potential for co-delivering nucleic acids and protein antigens in immune cells to enhance the immunological response against infectious diseases and cancer.

Declaration

This is to certify that:

- i. The thesis comprises only my original work towards the degree of Doctor of Philosophy (PhD) except where indicated in the Preface;
- ii. Due acknowledgement has been made in the text to all other material used;
- iii. The thesis is less than 100,000 words in length, exclusive of tables, bibliographies, appendices, and footnotes.

Yijiao Qu

Preface

Chapter 3 of this thesis has been previously published in the following article:

Qu, Y., Ju, Y., Cortez-Jugo, C., Lin, Z., Li, S., Zhou, J., Ma, Y., Glab, A., Kent, S.J., Cavalieri, F. and Caruso, F. Template-Mediated Assembly of DNA into Microcapsules for Immunological Modulation. *Small* **2020**, 16, 2002750.

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Publications

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Chapter 1

Literature Review

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1.1 Introduction

To date, vaccination is considered as the most efficient way for prophylactic protection against infectious diseases.¹ Since the first vaccine created by Edward Jenner against smallpox, vaccines have successfully saved millions of lives from preventable diseases.² Vaccines containing an attenuated/subunit of pathogens elicit immunological memory without inducing symptoms of diseases, which prime the immune system to fight a real infection.³ Various types of vaccines have been developed, including virus vaccine, viral vector vaccine, subunit vaccine, and nucleic acid vaccine.⁴ Despite the tremendous progress made in vaccine development for disease control and prevention, there are still some limitations related to vaccine formulations. The immunization induced by vaccines is sometimes not effective against infections, hence a high dose or multiple low doses are required to produce a sufficient initial immune response.⁵ Moreover, most of the vaccines are sensitive to temperature, hence strict storage and shipment conditions are required.⁶ Considerable efforts have been devoted to improving the stability and effectiveness of vaccines.⁷

Nanotechnology is a valid approach to solve the existing key technical challenges in vaccine delivery.⁸ Nanomaterials are broadly classified into organic, inorganic, and bio-derived subtypes.⁹ Due to the diversity in composition, nanomaterials provide versatile platforms that can be tailored for specific applications.¹⁰ Nanoengineered vaccine delivery systems have shown numerous advantageous properties including; 1) enhanced stability of bioactive cargos against hydrolytic enzymes during administration; 2) enhanced penetration of antigens through interstitial and mucosal barrier in specific tissues; 3) surface modification of carriers by ligands for targeting specific cells; 4) controlled release of cargos in response to specific environmental stimuli; 5) delivery of multiple bioactive substances including antigens and adjuvant to antigen-presenting cells (APCs); 6) various routes of vaccine administration, such as subcutaneous and intravenous injection.^{10,11} To date, a wide range of nanomaterials with different structures and biological functions have been developed to provoke immune responses against infectious diseases.¹² Collectively, nanoplateforms have shown a great immunomodulatory impact on vaccination efficiency.

This chapter aims to provide an overview of vaccine development, including the chemical composition, principles of biological activity, subtypes, and challenges. The application of nanotechnology and nanomaterials for the delivery of vaccines is described. Furthermore, an

overview of recent trends in vaccine design and delivery, with special attention to the application of DNA nanotechnology, is provided.

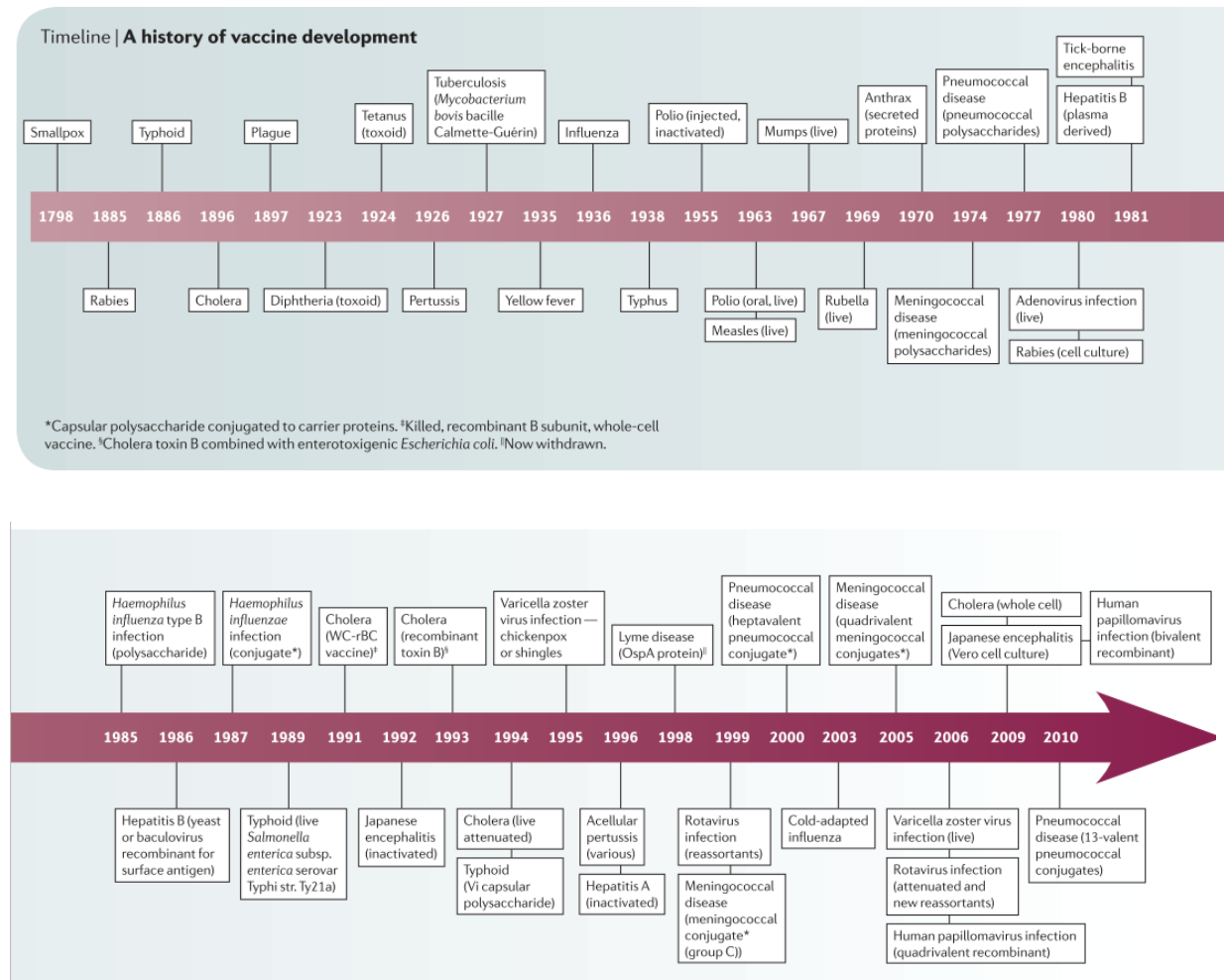
1.2 Vaccines

Vaccines are the most efficient way to prevent infectious diseases and one of the greatest achievements of medicine. Vaccines can be generally defined as a biological preparation, which aims to activate immune responses against infectious diseases by the presentation of an antigen to the immune system. When the vaccinated individual is exposed to the specific pathogen, a rapid and strong immune response occurs to protect the body from infection.¹³ The optimal properties of vaccine include long-lasting immunity, lack of autoimmunity, ease of administration, and storage.² Normally, adjuvant is combined with antigen in vaccine to achieve stronger immune responses. Importantly, all ingredients of vaccines should be safe, and particularly the vaccine itself should not cause a disease state.

1.2.1 History of Vaccine Development

Vaccines have been developed to protect against a range of infectious diseases, since Edward Jenner pioneered the concept of vaccines and created the smallpox vaccine in 1798 (**Scheme 1.1**).¹⁴ He observed that milkmaids who had caught cowpox did not catch and suspected that cowpox protected them against smallpox. The word vaccine is derived from the Latin *vacca*, meaning “cow”, since the first vaccine was derived from a virus affecting cows. In the 1880s, Louis Pasteur discovered that he could attenuate living organisms (e.g., bacteria and viruses) by heating—now this process is known as pasteurization. Then, Barbara Hopkins and Alexander Glenny reported that the toxicity of diphtheria toxin could be reduced by the action of formalin in 1923. There was a methodological breakthrough in vaccinology from 1950 to 1985: the culture of viruses in cell culture, which accelerated the development of vaccines. In the 1960s, three classical attenuated virus vaccines, which had less reactogenic effects but retain their immunogenicity, were developed against measles virus, mumps virus, and rubella virus. In the 1980s, two important strategies for vaccine developments were introduced: the conjugation of bacterial capsular polysaccharides to proteins, and protein genetic engineering. In the late 20th century, advances such as recombinant protein expression and genetic engineering led to a range of new vaccines being created and licensed for use in humans and animals. Over the past decade, based on the continuous development of molecular genetics, various new vaccine delivery systems (e.g., topical formulations, viral vectors and, DNA vaccines) have been developed. In particular, the recent introduction and emergency use

authorization of DNA and mRNA vaccines against COVID-19, is a significant milestone in vaccine development. Despite the success of vaccines to date, efficient and safe vaccines are still lacking for various infectious diseases, such as tuberculosis and human immunodeficiency virus (HIV).



Scheme 1.1. A history of vaccine development. Reproduced from reference [14].

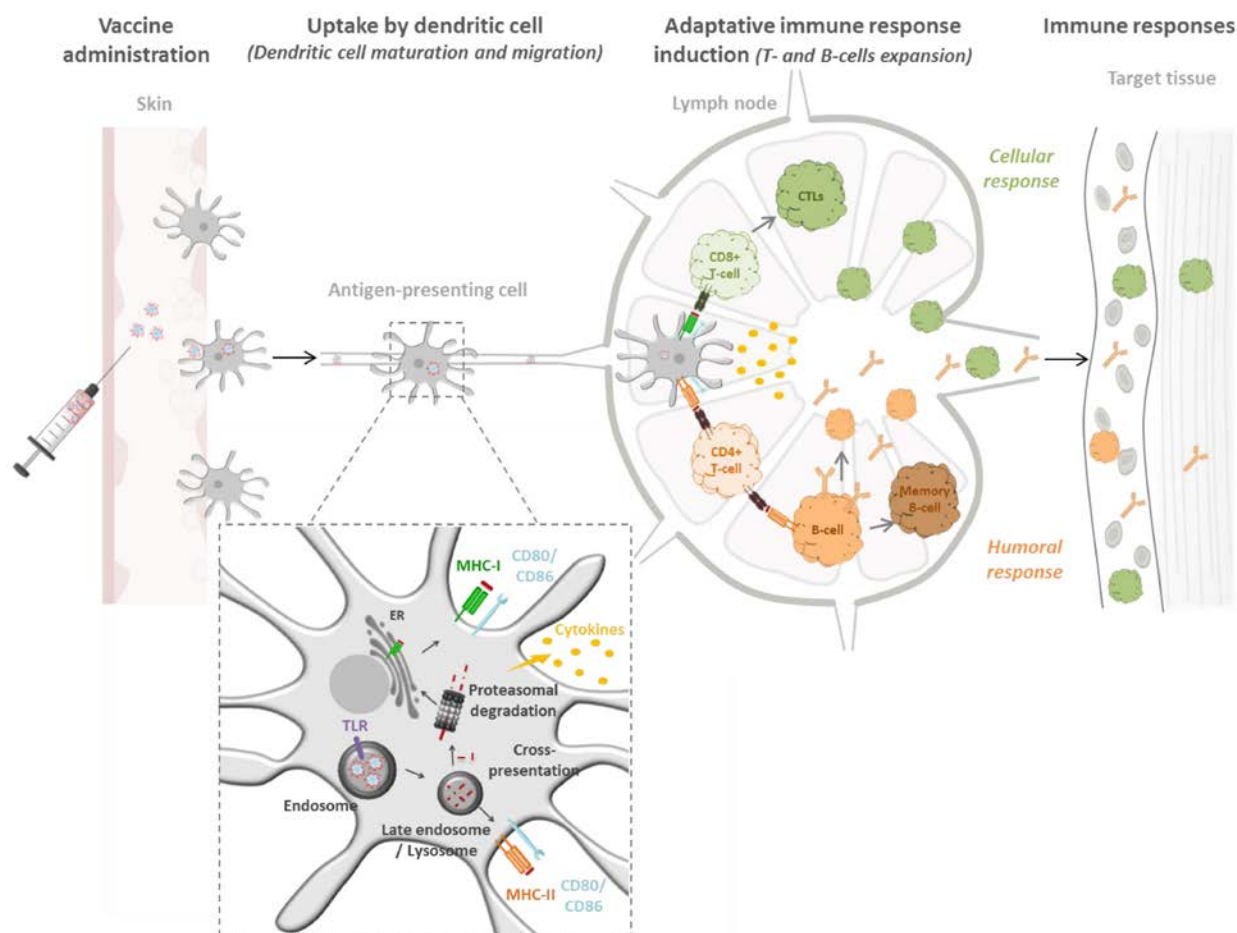
1.2.2 Vaccine Immunology

To comprehend the rationale of vaccination, it is important to understand how the immune system works. The immune system is composed of both innate and adaptive immune responses.¹⁵ Innate immunity comprises physical, chemical, and cellular defences to fight the pathogens, which is quick, non-specific, and without immunologic memory. Contrary, adaptive immunity comprises cellular immunity and humoral immunity, which is slow, specific, and with immunological memory. Normally, it takes days to weeks to develop adaptive immunity. Cellular immunity deals with antigens presented on antigen-presenting cells (APCs) or

expressed on the cell surface and carried out by T cells. While humoral immunity (antibody-mediated immunity) deals with the free-circulating antigens and carried out by B cells and antibodies.

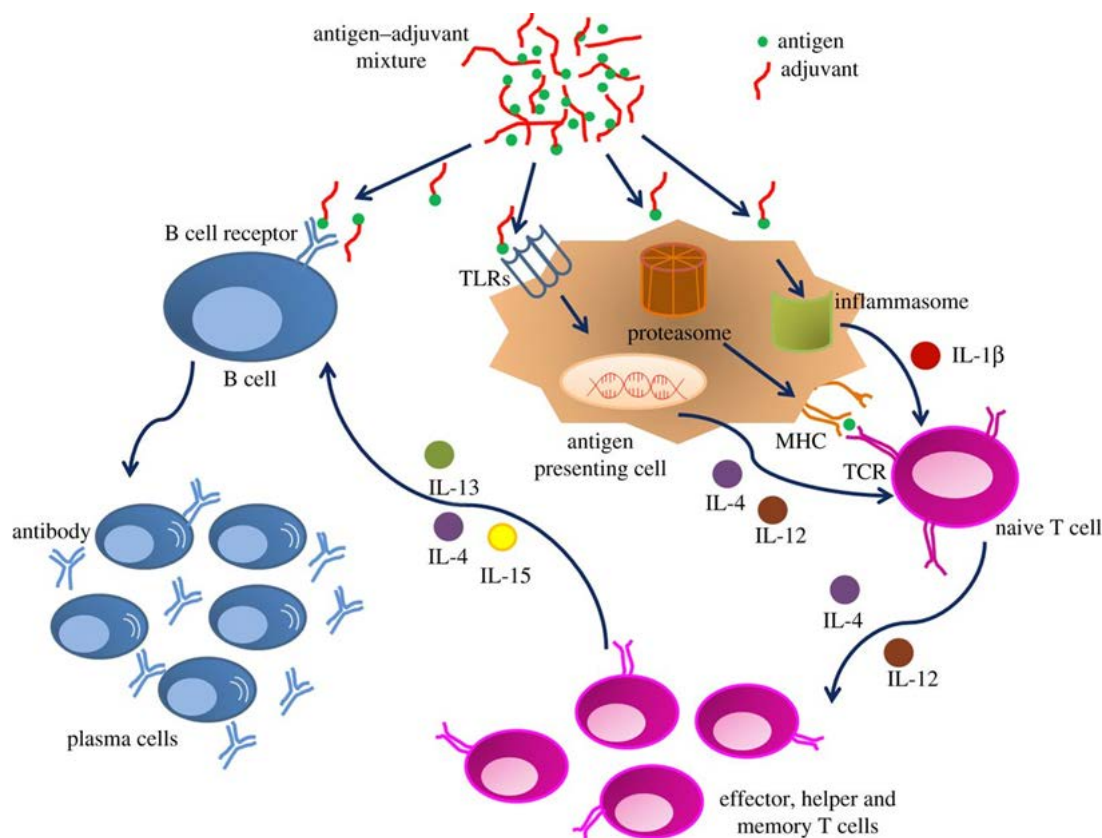
APCs, including neutrophils, monocytes, macrophages, and dendritic cells (DCs), play an important role in both innate and adaptive immunity. When an infection overtakes the innate immunity, APCs call help from the adaptive immunity.¹⁶ The immature APCs involves in the capture, uptake, processing of antigen as phagocytes in innate immunity. After the antigens are captured and phagocytosed by APCs, they are digested into many fragments with endo/lysosomes and then presented on the cell surface by the major histocompatibility complex (MHC) molecules. Internalization of antigen also induces APCs maturation and migration out of the muscle to lymphoid organs. While moving to secondary lymphoid tissue, APCs cells enable to activate naïve T cells by the antigens on their surface binding to T cells. T cells are a type of white cells and are precursors for effector and memory T cell subsets. CD8⁺ and CD4⁺ T cells are generated based on antigen presentation by MHC-class I molecules and MHC-class II molecules respectively. Normally, intracellular antigens are presented by MHC-class I molecules to activate CD8⁺ T cells, known as cytotoxic T cells. Extracellular antigens are presented by MHC-class II molecules to activate CD4⁺ T cells, known as T helper cells. B cells are a type of white blood cell that can differentiate into plasma B cells to produce antibodies by the assistant with CD4⁺ cells.⁹

When a vaccine is administered via intramuscular, intradermal, oral, or mucosal route, cells in the surrounding tissues interact with antigens and are activated to produce proteins (e.g. cytokines and chemokines) (**Scheme 1.2**).¹¹ In most cases, APCs will be attracted to the injection site to process the antigen locally.¹⁷ After processing the antigen, the attracted APCs migrate to the draining lymph node (dLN), where most of the immune cells accumulate. The further presentation of antigen on APCs activate T cells occurs via MHC-class I or MHC-class II molecules. The activated CD4⁺ T cells further stimulate B cells to generate antibodies. The activated CD8⁺ T cells, B cells, and produced antibodies then enter the circulation to form part of the adaptive immune system. After being vaccinated, some T and B cells are converted into long-lasting memory cells, which grant the immune system immunologic memory. This enables a rapid immune response upon subsequent exposure to the specific pathogen, which protects body from infection.



Scheme 1.2. Immune responses stimulated by vaccine. Administered NPs are firstly taken up by APCs, and the processed antigen is presented by the proper MHC molecules to invoke cellular response and humoral response. Reproduced from reference [11].

A substance called adjuvant, is normally used to help to enhance the immune response when given with an antigen in vaccine. Adjuvant is derived from the Latin word (*adiuvare*), meaning to help or aid. In particular, Toll-like receptor (TLR) ligands are increasingly used in vaccine design. The antigen-adjuvant mixture can directly interact with B cells to produce antibodies against antigens (**Scheme 1.3**).¹⁸ Alternatively, the mixture can be phagocytosed and processed through TLRs, proteasomes, or inflammasome pathway. Adjuvant will activate the TLR pathway to produce cytokines and antigen will be presented on the surface of APCs via MHC molecules to naïve T cells.^{19,20} In turn, the activation of T cell and cytokine production will enhance the interaction between T cells and B cells.



Scheme 1.3. Schematic diagram of a possible mode of action of vaccine (combination of antigen and adjuvant). Reproduced from reference [18].

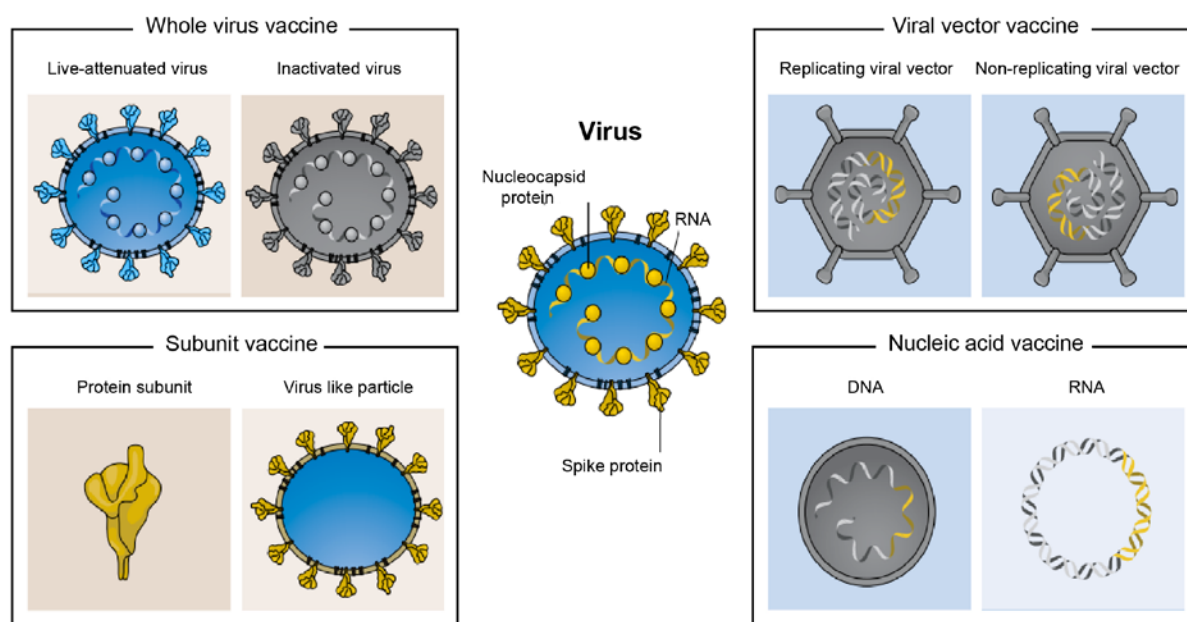
1.2.3 Vaccine Subtypes

Several types of vaccine have been developed, based on composition and process, they can be classified into live attenuated/inactivated virus vaccine, viral vector vaccine, subunit vaccine, nucleic acid vaccine (**Scheme 1.4**).⁶

Live attenuated/inactivated virus vaccines are traditional and well-established vaccines, which are similar to the native pathogens, but have no or less virulence.²¹ A typical virus comprises nucleic acid (RNA or DNA) surrounded by a protein coat known as a capsid and spike proteins outside that help the virus to attach to specific cell surfaces. A disease-causing micro-organism could be attenuated and killed by heat or chemical treatment. Usually, the DNA or RNA of viruses are destroyed during the treatment, so the virus can not cause diseases. Live attenuated or inactivated whole organism-based vaccines have been employed to control and successfully eradicate many severe infectious diseases, such as smallpox, rubella, and measles. Compared to inactivated virus vaccines, live attenuated virus vaccines are still alive to induce a robust immune response with reduced virulence. These vaccines are easy to prepare, cheap, and provide good immunity against infection. However, there are serious concerns about this

vaccine, such as the potential to reverse to a virulent condition by the back-mutation and cause diseases in immuno-compromised individuals. Generally, inactivated virus vaccines provide a weaker immune response than live attenuated vaccines, so multiple doses may be needed to get enough immune response. These vaccines activate an antibody-mediated immune response, not a cell-mediated one, while live attenuated viruses could elicit both these two immune responses. In addition, inactivated virus vaccines could be used in immunocompromised individuals who are not able to use live attenuated virus vaccines, due to the low virulence.

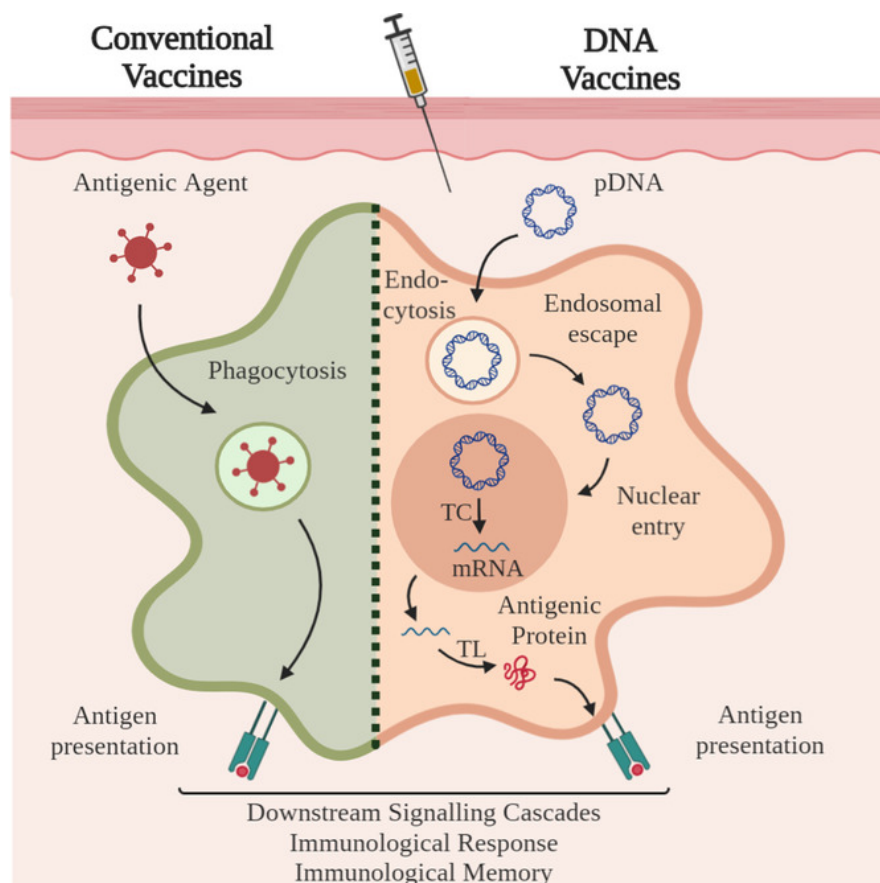
Viral vector vaccines are recombinant viruses, in which viral antigen genes were added using recombinant DNA techniques.⁵ Normally, the viral vector is the virus that will not cause disease. Viral vector vaccines can either be replicating or non-replicating. The replicating viral vector can not only produce viral protein in the infected cells, but also by replicating can infect new cells and then produce copies of viral protein. In contrast, the non-replicating viral vector is designed not to replicate but could still produce the viral antigen in the infected cells to trigger an immune response. Viral vector vaccines could lead to endogenous viral antigen production, so both cellular and humoral immune responses are elicited. Generally, this kind of vaccine can provide sufficient protection by one single dose, for example, the vesicular-stomatitis virus-based Ervebo vaccine against Ebola virus.²²



Scheme 1.4. An overview of the different vaccines, including whole virus vaccine, viral vector vaccine, subunit vaccine, and nucleic acid vaccine. Reproduced from reference [6].

Unlike virus vaccine and viral vector vaccine, subunit vaccines only introduce a fragment of a pathogen to immune system and stimulate immunity. These fragments of the pathogen are normally proteins/polysaccharides in the absence of genetic material, so they could not replicate and cause disease, therefore subunit vaccines are considered very safe. However, subunit vaccines could not elicit a strong immune response, normally multiple doses may be needed, as in the cases of subunit vaccine against shingle and hepatitis B. Subunit vaccines typically are composed of an antigen, a fragment of the pathogen to provide protective immunity, and the adjuvant, a substance to enhance immune response to the antigen.²³ Virus-like particles (VLPs) which have similar morphology to natural viruses, represent a specific type of subunit vaccine.²⁴ VLPs are composed of synthetic proteins to form shells that are similar to natural viruses, including their antigenic components. They are not infectious due to a lack of genetic material, which makes VLPs a safe substitute for attenuated vaccines. As VLPs still preserve the antigen pattern, they can elicit strong immune responses.

Nucleic acid vaccines contain genetic material, which can express the antigen of a pathogen within the host to stimulate the immune system.^{25,26} Depending on the material, the nucleic acid vaccine could be either DNA or RNA based, both contain the genetic information to produce an antigen. In the case of DNA vaccines, a piece of DNA encoding the antigen of a pathogen is inserted into small DNA molecules (called plasmids).⁷ Plasmids can express the antigen to trigger the immune response and replicate within the host independently of the chromosomal DNA.²⁷ The synthesis of plasmid DNA is very fast and cheap by using bacteria. Thus, the DNA vaccine has great potential to treat many diseases, including cancers, diabetes, and atherosclerosis.²⁸ However, the main safety concern associated to the DNA vaccine is the possibility that the injected DNA may be integrated into the host's chromosomal DNA, which may lead to side effects.²⁹ Similar to DNA vaccine, a piece of RNA encoding the antigen of the pathogen is inserted into self-amplifying RNA messenger RNA (mRNA) to provide immunity. RNA vaccines are based on the molecular templated use by cellular factories to produce proteins, which can direct their self-replication within the cytoplasm.^{30,31} When RNA vaccines are introduced into the cells, multiple copies of the antigen-encoding mRNA will be replicated and express high levels of antigen. In contrast, the plasmid of DNA vaccines needs to be translocated into the nucleus, transcribed, and exported back to the cytoplasm to get expressed (**Scheme 1.5**).²⁷ Thus, RNA vaccines can trigger the immune system more quickly and efficiently than DNA vaccines. However, RNA vaccines are temperature and enzyme-sensitive and require strict storage and shipment condition.



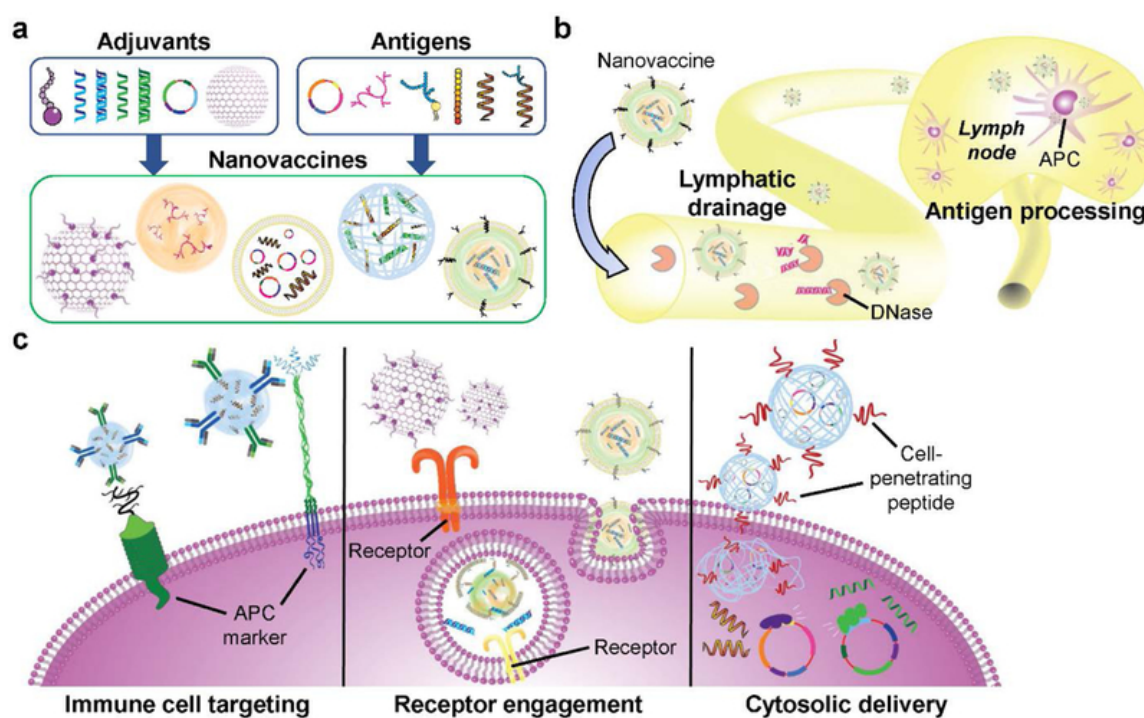
Scheme 1.5. A comparison between conventional and DNA vaccines. Conventional vaccines (left): the antigenic agent is phagocytosed and then presented by APCs. DNA vaccines (right): Plasmid DNA is endocytosed, escape from the endosomes into the cytosol, and internalized by the nucleus, followed by the expression of the antigenic protein. The antigen presentation leads to downstream signaling cascades provoking an immunological response and the establishment of immunological memory. Reproduced from reference [27].

1.2.4 Vaccine Challenges

As traditional vaccines based on the whole virus (live-attenuated/inactivated) became less popular due to difficulties with mass production and potential risk of side effects, nucleic acids/based vaccine and viral vector vaccine are considered as the next generation vaccines, to tackle emerging epidemic outbreaks.²⁹ The main disadvantage of these vaccines is the instability of the vaccine material, which require extremely strict storage and shipment conditions.⁶ Subunit vaccine composed of a part of a pathogen are also becoming very popular nowadays, because of large scale production, high stability compared to other vaccine materials.³² Although this vaccine is safer and easier to prepare, subunit components are usually not enough to stimulate desired immunity and long-term protection. Thus, the incorporation of

adjuvants in the formulation, as additional immune-stimulating signals, is highly needed to enhance the immunogenicity of antigens.²⁸

Nanovaccines are emerging as novel systems to improve efficacy and reduce side effects of vaccines.³³ Nanocarriers can be designed and formulated with custom size, shape, and surface and loaded with antigens and adjuvants.¹⁷ There are several advantages of nanoengineered vaccine, (a) multiple immune agents, antigens, and adjuvants, can be integrated into one system using nanoparticle; (b) nanovaccines can protect cargoes against environmental degradation and drain to the lymphatic drainage and (c) various chemical and structural properties of nanovaccines can be tuned to achieve the maximum immune activation (**Scheme 1.6**).³⁴



Scheme 1.6. Advantages of nanoparticles for vaccine design. Reproduced from reference [34].

1.3 Nanoengineered Vaccine Delivery Systems

Nanomaterials offer many potential benefits for the development of vaccines including increased antigens stability, improved biodistribution, and enhanced vaccine immune response.³⁵ Various nanoengineered particles have been employed to deliver both immunomodulators and other therapeutic agents.³⁶⁻³⁹ The cargos could be loaded into the delivery system through encapsulation, adsorption, covalent conjugation and so on.^{9,40} Based

on the materials utilized to prepare the systems, nanocarriers can be categorized into three types, including inorganic, polymer- and lipid-based particles, and bio-derived particles.⁴¹

1.3.1 Lipid-Based and Polymer-Based Particles

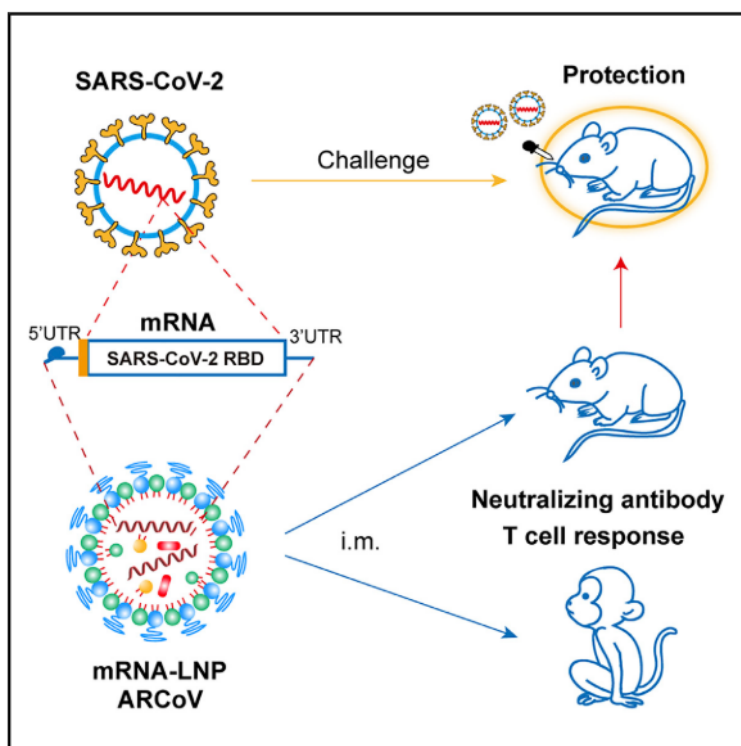
Lipid-based and polymer-based nanocarriers have been extensively investigated in vaccine development.⁴² It has been clinically proven that these systems have minimal toxicity and preserve the functionality of drugs effectively.¹ In particular, lipid-based and polymer-based carriers are promising systems for DNA, RNA, and protein delivery.⁴³

Lipid-based nanoparticles have been well studied for vaccine delivery owing to their ease of scale-up, proven clinical safety, and biocompatibility.⁴⁴ Liposomes are the most studied lipid-based delivery systems. They are vesicles composed of one or more lipid bilayers which can be formed by self-assembly of lipids in water leading to a hydrophilic core and hydrophobic bilayer. Liposomes retain the cargo in the hydrophilic core and protect the cargos inside the core from enzymatic degradation in the surrounding biological environment and increase the circulation time.⁴⁵

Liposomes modified with the cell-penetrating peptide octa-arginine were used to co-deliver antigen and adjuvant for enhanced immune responses. Polyinosinic:polycytidylic acid (polyI:C), a synthetic dsRNA which recognized by TLR3 to activate immune cells, incorporated into the liposomes could induce significantly higher cytotoxic T lymphocytes (CTL) responses and inhibit tumor growth, compared to soluble antigen/polyI:C.⁴⁶ Post-modification of liposomes with polymers is an effective method to grant liposome specific function and desired characteristics. For example, liposomes have been modified with the positively charged polymer polyethyleneimine (PEI), to induce electrostatic adsorption of proteins on liposomes.⁴⁷ The PEI-modified liposome nanoparticles were loaded with large amounts of antigens, enhance antigen cellular uptake and presentation by APCs, which resulted in enhanced antigen cellular uptake and presentation by APCs, production of proinflammatory cytokines and upregulation of co-stimulatory markers.

Lipid-based nanoparticles (LNP) are currently used to prepare a vaccine against coronavirus disease 2019 (COVID-19) due to the ongoing SARS-CoV-2 pandemic. Qin' lab reported a vaccine candidate, mRNA (mRNA-LNP) encoding the receptor-binding (RBD) domain of SARS-CoV-2 was encapsulated by a lipid nanoparticle (**Scheme 1.7**).³⁰ Robust production of antibodies was stimulated by the lipid nanoparticle-encapsulated SARS-CoV-2 and cellular response was elicited by intramuscular immunization of mRNA-LNP in both mice and non-

human primates. The candidate vaccine is still being tested in the clinical trial. Furthermore, this vaccine is easy to manufacture and can be stored at room temperature for at least 1 week. The most promising vaccine against COVID-19 developed by Pfizer/BioNTech is using LNP as a delivery platform.⁴ The vaccine consists of mRNA encoding coronavirus's spike protein encapsulated in a lipid nanoparticle. After injection, the cells internalize the mRNA vaccine, expressed the spike protein, and activate the host's immune system to recognize the virus. The efficacy of Pfizer/BioNTech's vaccine was found 95% according to the phase III trial studies.

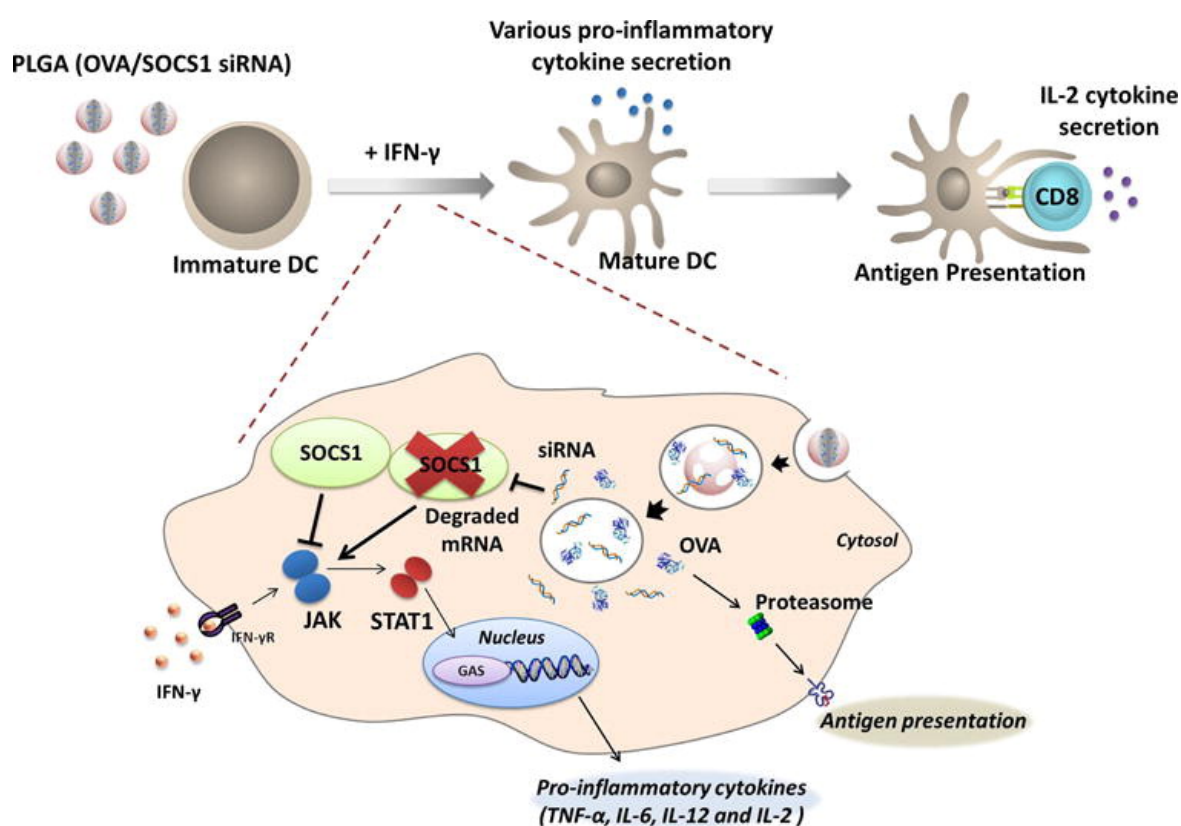


Scheme 1.8. Schematic illustration of the development of mRNA-LNP vaccine targeting the RBD of SARS-CoV-2. Reproduced from reference [30].

Polymer-based particles have become popular nanocarriers for controlled and targeted drug delivery, owing to the versatility of both synthetic and natural polymers.^{48,49} Several polymer-based therapeutic nanoparticles have been proven to have minimal toxicity to patients clinically and approved for clinic use.⁵⁰ Many polymer-based nanoparticles are biodegradable, which makes them suitable for the engineering of drug delivery formulations.⁵¹ However, in some cases, the degradation products may cause toxic effects and the production of proinflammatory molecules.

A great range of synthetic polymers has been used to fabricate particles for vaccine delivery, including polyethylene glycol (PEG), polylactic-co-glycolic acid (PLGA), and

polyethyleneimine (PEI).⁵² PLGA has been extensively investigated and approved by FDA for therapeutic purposes, owing to its excellent biocompatibility and biodegradability.⁵³ Various antigens derived from pathogens have been delivered by PLGA particles, such as *Bacillus anthracis*, hepatitis B virus (HBV), HIV antigens, protein, and tumor-associated antigens.⁵⁴⁻⁵⁸ Heo and his co-workers developed PLGA nanoparticles to co-deliver siRNA targeting the immunosuppressive gene with other immune agents (**Scheme 1.8**).⁵⁹ The combination of siRNA, albumin (OVA), and TLR7 agonist significantly enhanced APCs maturation and immune response in vivo. These results showed that polymeric materials are versatile delivery carriers for multiple cargo co-delivery.



Scheme 1.9. Schematic representation of the application of PLGA (OVA/SOCS1 siRNA) NPs for the immunomodulation of DCs. Reproduced from reference [59].

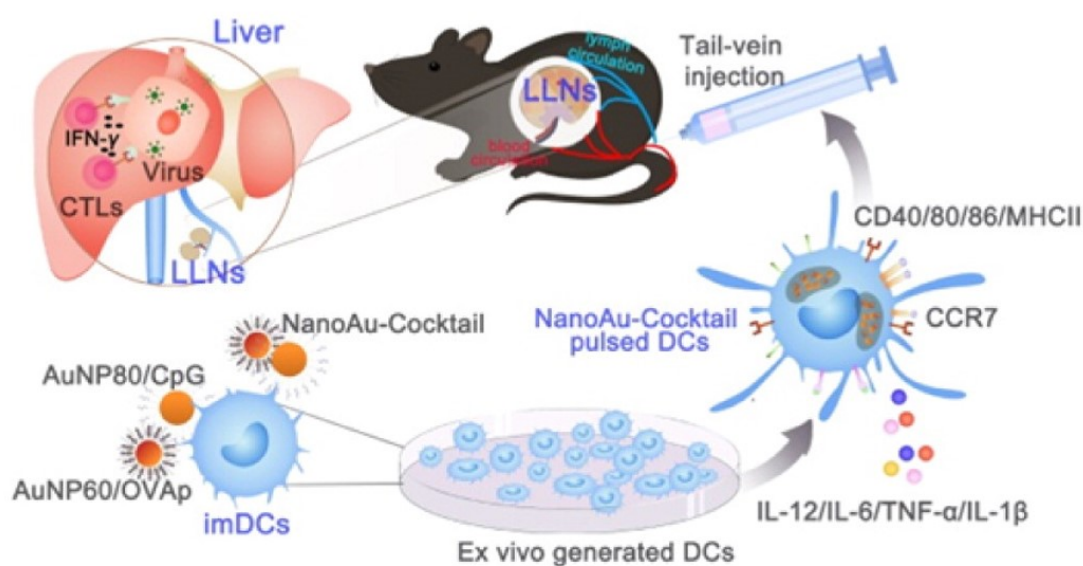
Natural polymers are promising materials for vaccine delivery owing to high biocompatibility, biodegradability, and abundance in nature.⁶⁰⁻⁶² Chitosan is a cationic polysaccharide that is usually obtained from the exoskeletons of crustaceans and other sources such as fungi.⁶² Chitosan was used as a platform for Newcastle disease, DNA, and HBV vaccines.⁶² One study showed that compared to soluble antigen, chitosan nanoparticles loading antigen could significantly enhance the upregulation of co-stimulatory markers on APCs and secretion of

pro-inflammatory cytokines (e.g. $\text{TNF-}\alpha$, IL-6, and IL-1 β). OVA loaded chitosan particles elicited the presentation antigen via MHC-I and MHC-II molecules in DCs and promoted higher proliferation of CD4 $^{+}$ T cells and CD8 $^{+}$ T cells than soluble OVA.⁶³

1.3.2 Inorganic Particles

Inorganic nanoparticles such as carbon nanomaterials, gold nanoparticles, and silica-based nanomaterial have been investigated as nanocarriers for vaccine delivery.⁶⁴ Though most of these particles are not degradable, they are relatively stable and can be easy to generate on a large scale with desired size and shape.⁶⁵ Inorganic particles provided delivery platforms for a large dose of antigens and adjuvants to enhance the effect of vaccines, due to their high surface to volume ratio.⁶⁶ The cargos could be loaded into the delivery system through electrostatic adsorption or covalent conjugation.

Gold nanoparticles (AuNPs) are normally used to deliver small molecules, peptides, nucleic acid, and antibodies, as they are bio-inert, non-toxic, and easily fabricated into different shapes and sizes.⁶⁷ It was found that both shape and size can affect the immune effect of AuNPs, which leads to different levels of immune responses. For example, Zhan's group reported spherical gold nanoparticles for CpG and OVA co-delivery (**Scheme 1.9**).⁶⁸ These nanoparticles could enhance antigen presentation, upregulation of co-stimulatory markers, and increased cytokine production. This study showed the ability of AuNPs to increase DCs homing and antigen response.



Scheme 1.10. Mechanism of NanoAu-Cocktail pulsed DC induction of antiviral T cell responses. Reproduced from reference [68].

Iron oxide nanoparticles (IONPs) are the typical contrast agents of magnetic resonance imaging (MRI), which were approved by FDA for clinical use in 1996.⁶⁹ Recently, IONPs have attracted great interest in vaccine development, owing to their superparamagnetic properties and high biocompatibility.⁷⁰ The superparamagnetic property makes IONPs a promising tool for oriented drug delivery.⁷¹ IONPs could be guided and concentrated in desired regions using an external magnetic field. For instance, silver decorated IONPs have been used to deliver various antibiotics for targeted antimicrobial therapy.⁷² Antibiotics can bind to silver loaded IONPs by physical adsorption and silver can bind to IONPs via electrostatic interaction. Due to the electron transfer between silver and IONPs in this system, a silver coating positively charged surface was created, which allowed the adsorption of different antibiotics. The fabricated nanocomposites revealed antimicrobial activity towards *B. pumilus*.

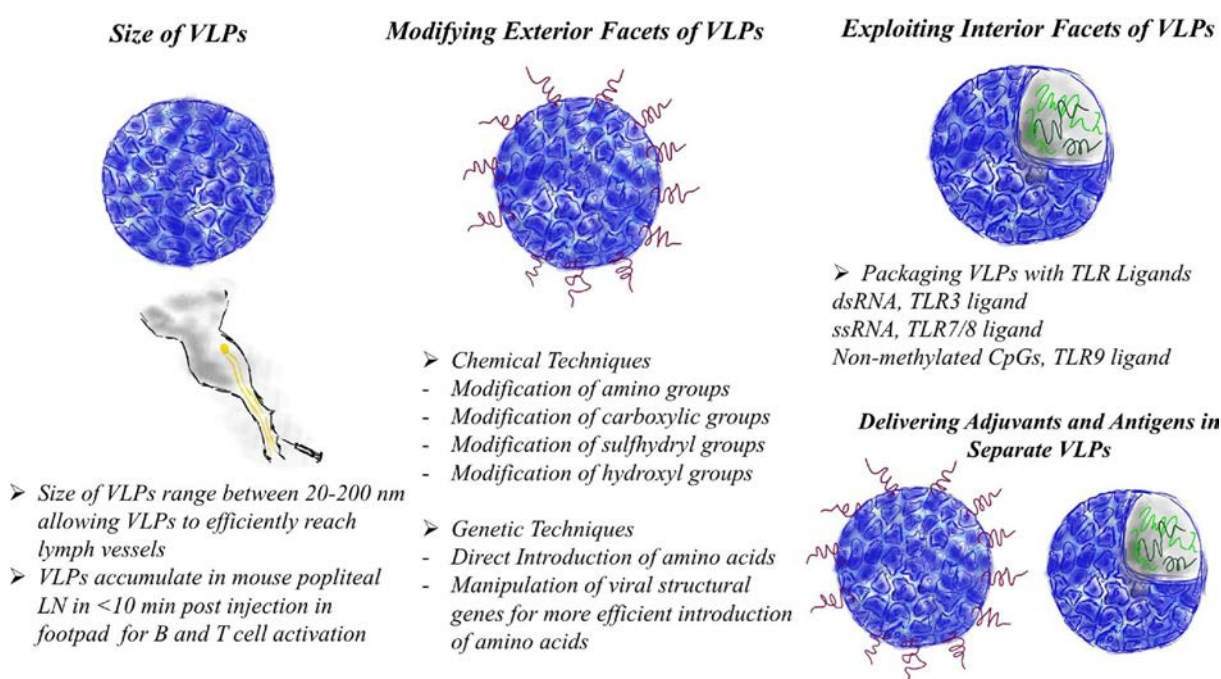
Carbon nanoparticles (CNPs) are another commonly investigated nanoparticle for drug and vaccine delivery, due to their high biocompatibility and various shapes (nanotubes, graphene, fullerene, nanodiamonds, etc).^{73,74} Large surface areas provide the possibility of multivalent conjugation of cargos and modification sites. Normally, carbon nanotubes (CNTs) used as carriers have a diameter of 0.8–2.0 nm and a length of 100–1000 nm, while mesoporous carbon spheres have a diameter of 500 nm.⁴² For example, a two-dimensional nanosheet, graphene oxide (GO) was reported to deliver cytosine-phosphate-guanosine oligodeoxynucleotides (CpG) motifs.⁷⁵ Uniform small GO nanosheets can be efficiently delivered into murine RAW264.7 macrophages and exhibit immunostimulatory activity.

Silica nanoparticles (SiNPs) have been extensively studied for biomedical applications including drug delivery and bioimaging, owing to their specific properties, such as uniform pore size, large surface area, and tuneable pore and particle sizes.^{76,77} SiNPs are highly biocompatible materials for cancer diagnosis and treatment and have been approved by the FDA for cancer diagnosis and treatment. Because of the abundant surface silanol groups, further modification on SiNPs could be achieved, which results in additional functionalities for enhancement of cellular uptake, cell recognition, and absorption of specific biomolecules. The nanocarriers based on silica with a size of 50-200 nm could provide controlled-release and high cargo loading efficiency to co-deliver adjuvant and antigen.⁷⁸ Gu's lab used luminescent porous SiNPs as carriers to deliver an agonistic antibody of CD40, which is a co-stimulatory receptor expressed on the surface of APCs.⁷⁹ The inherent NIR photoluminescence of SiNPs could be exploited as a tracking agent to investigate the interaction of SiNPs with the immune system. These nanoengineered SiNPs significantly enhanced the activation of B cells by 30-40 folds.

1.3.3 Bio-Derived Particles

Bio-derived particles^{10,51,80}, such as virus-like particles, protein particles, and nucleic acid particles, have emerged as vaccine carriers, due to their high biocompatibility, biodegradability, and various natural sources.⁸

Virus-like particles (VLPs) are self-assembled by the viral capsid proteins without the genetic DNA/RNA.³² The size, shape, and immunogenicity of VLPs are similar to viruses but lack infectious ability. VLPs are the ideal vaccine delivery system due to the evolved viral structure, which is optimized to interact with immune cells naturally and induce potent immune responses (**Scheme 1.10**).⁸¹ In 1986, the first VLP vaccine for HBV was commercialized and this was the first nanoparticle class that reached the market.⁸² The successful multiple human papillomavirus (HPV) vaccines, such as Gardasil and Cervarix, employ VLPs as delivery systems.⁸³ Therefore, the safety and lack of toxicity of VLPs have been extensively proved by these commercialized vaccines. A VLP-based nanovaccine loaded with CpG motifs and a peptide antigen was developed by Speiser et al. and had been tested in Phase IIa in clinical trials.⁸⁴



Scheme 1.11. Major findings in VLP-based vaccine development and technologies. Reproduced from reference [81].

Naturally occurring proteins can self-assemble into stable nanoparticles, with organized structures and controlled sizes.⁸⁵ These particles have particularly attracted attention for

vaccine design, as they can be used as platforms for the delivery of well-organized antigens that can potentially mimic the repetitive surface architecture of a natural microbe.^{86,87} A hollow, vault nanoparticle was synthesized by self-assembly of vault proteins, which were composed of 96 units with a size of 40 nm wide and 70 nm long.⁸⁸ An immunogenic protein, the major outer membrane protein of *Chlamydia muridarum*, can be encapsulated inside the vault nanoparticles during the self-assembling process when mixed with the vault proteins. These self-assembled nanoparticles containing the antigen enhanced anti-chlamydia immunity and microbial eradication.

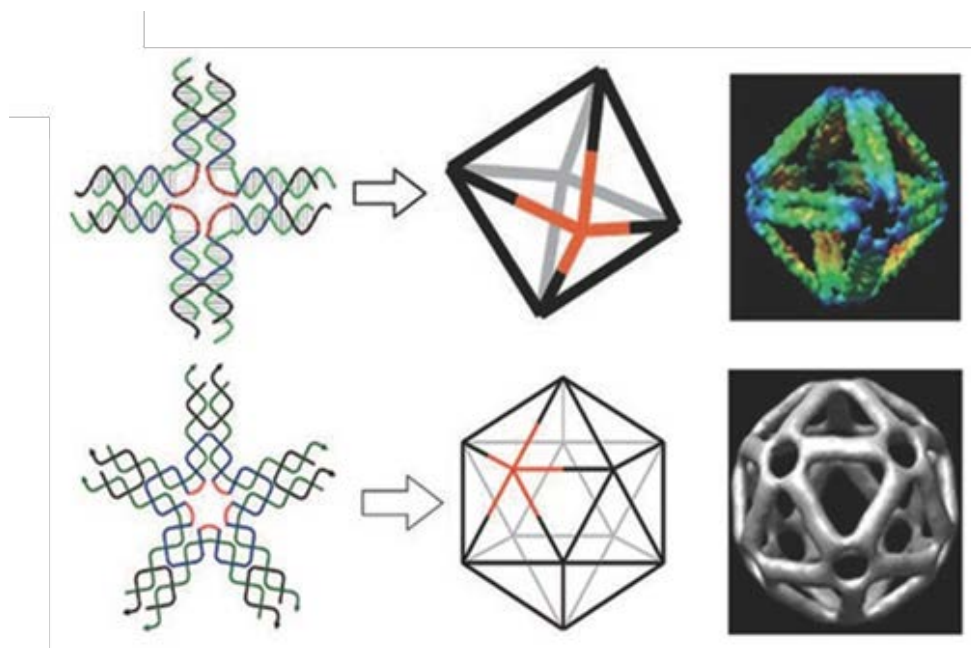
Self-assembled DNA nanostructures have attracted great interest in drug and vaccine delivery areas due to their desirable properties, including controlled size, shape, excellent biocompatibility, and biodegradability.⁸⁹ Different chemical moieties and biomolecules could be precisely conjugated to DNA carriers through complementary base pairs hybridization for targeting cells.⁹⁰ Various methods have been developed to build different DNA nanocarriers, such as tile-based structures and DNA origami.⁹¹ By designing special DNA sequences, DNA nanostructures can change conformation to release their cargos, responding to stimuli (pH, enzymes) in the specific microenvironment. DNA nanostructures have been designed for the targeted delivery of antigens and adjuvants. Co-delivery multiple immunomodulators by DNA nanostructures could achieve a synergistic enhancement of immunostimulatory activity.⁹² Takakura' group presented an injectable hydrogel system that was composed of water, salts, and CpG oligodeoxynucleotides (ODNs).⁹³ The complex gel significantly enhanced the immunostimulatory activity of CpG ODNs and also the immune response after ovalbumin (OVA) incorporated into the DNA hydrogel.

1.4 DNA Nanotechnology and DNA-Based Particles

DNA nanotechnology has been developed exponentially over the past decades, owing to the inherent properties of DNA, such as programmable sequences, predictable intermolecular interactions, versatile modifying sites, convenient chemistry synthesis, and high functional group density. Nanoengineered DNA-based particles can be assembled with desired size and shape at various dimensions and designed surface properties. These characteristics make DNA particles promising platforms to fabricate highly ordered and well-controlled systems with high biocompatibility, biodegradability, high cargo loading capacity, and easy modification of the surface.

1.4.1 DNA Nanotechnology

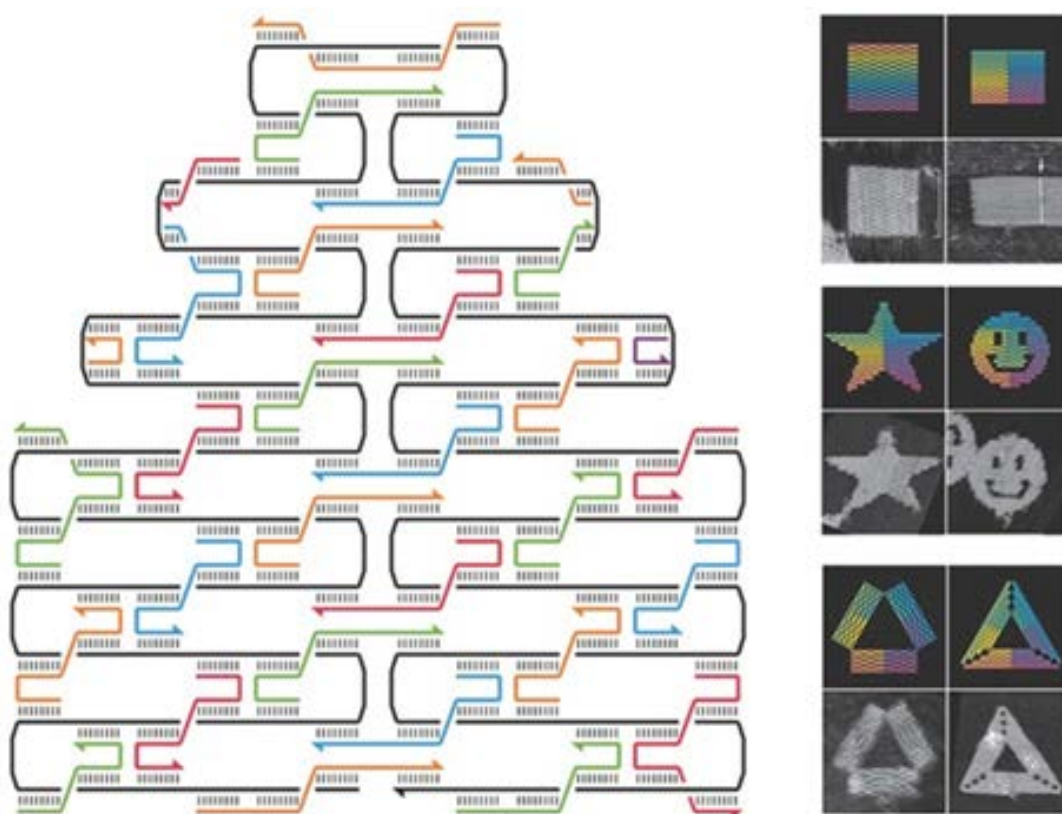
In 1982, Seeman, the pioneer of DNA nanotechnology, proposed that by breaking the homology using four DNA strands with designed sequences, an immobile DNA Holiday junction could be constructed. Subsequently, Seeman reported that the branched junction with cohesive ends could be used as tiles to form infinite 2D lattices by self-assembly.⁹⁴ Since the creation of 2D lattices, the field of structural DNA nanotechnology started to take off. Later, Seeman's group developed more fundamental DNA tiles, including double crossover (DX) composing of two DNA double helices linked together by two strand exchanges.⁹⁵ DNA tiles could self-assembled into 1D and 2D arrays through the interactions between tiles via sticky ends. Based on the groundwork, a triangular motif showed the ability to extend 3D to form 3D lattices.⁹⁶ Then, a range of dendritic DNA tiles have been designed to generate polyhedrons, such as octahedrons and icosahedrons (**Scheme 1.11**).^{97,98} Despite the success of forming various DNA nanostructures, the assembling strategy based on DNA tiles has some inherent drawbacks. The process of this method is time-consuming and error-prone and requires the exact stoichiometric.



Scheme 1.12. DNA octahedron and icosahedron self-assembled from DNA tiles. Reproduced from reference [98].

Rothemund developed the novel “DNA origami” strategy in 2006. To fabricate the desired shape, a long single-stranded genetic DNA of M13mp18 was utilized as a scaffold and then held the scaffold in place by the assistant with multiple specifically designed staple strands

(**Scheme 1.12**).⁹⁹ A series of shapes with a size of about 100 nm were created, such as smiley faces, triangles, squares, and five-pointed stars. Compared to the tile-based method which required high purity of DNA strand, DNA origami can be assembled using raw synthetic DNA strands. Moreover, strict stoichiometry is not required for DNA origami assembly. In 2009, Shih's group reported the 3D shape formed by packing multiple single-layer 2D origami.¹⁰⁰ Owing to the multiple packing of 2D DNA origami, 3D DNA nanostructures fabricated by this strategy are often highly rigid.¹⁰¹ Yan's lab then reported a range of 2D and 3D single-layered DNA nanoarchitecture through the twist-and-curvature approach.¹⁰² The origami strategy provided a simple approach to synthesize versatile 2D and 3D DNA nanostructures and computer-aided automation was also involved in the design process to make it handy. However, the size of origami nanostructures is limited by the long single-stranded DNA scaffold. Hundreds of staple strands are required to fold the long single-stranded DNA scaffold into the desired shape, which makes the technique cost-ineffective. Moreover, the process of purifying and accumulating intact origami nanostructures to certain concentration for biological applications is tedious and time-consuming.¹⁰³

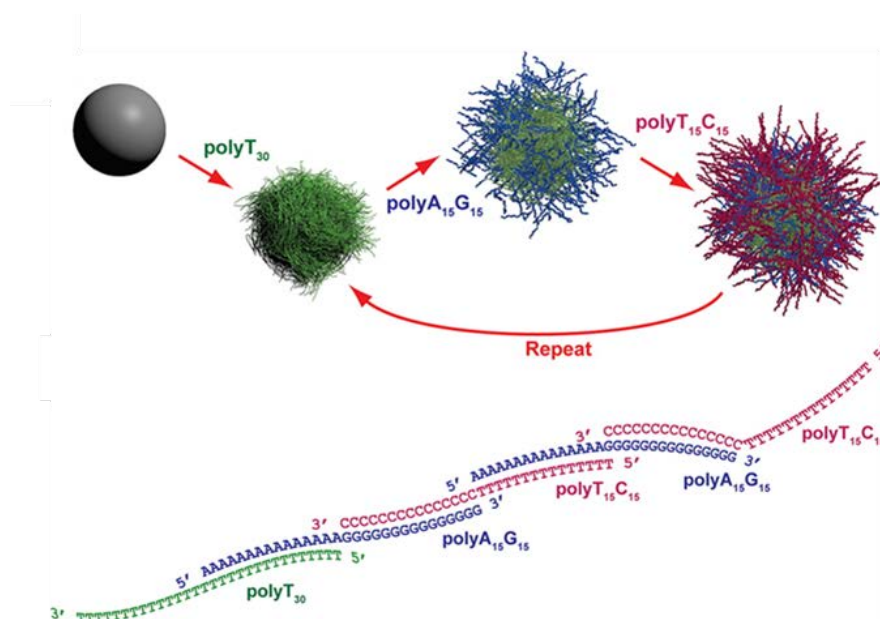


Scheme 1.13. Schematic illustration and characterization of 2D origami. Reproduced from reference [99].

1.4.2 Template-Mediated Assembly of DNA Capsules

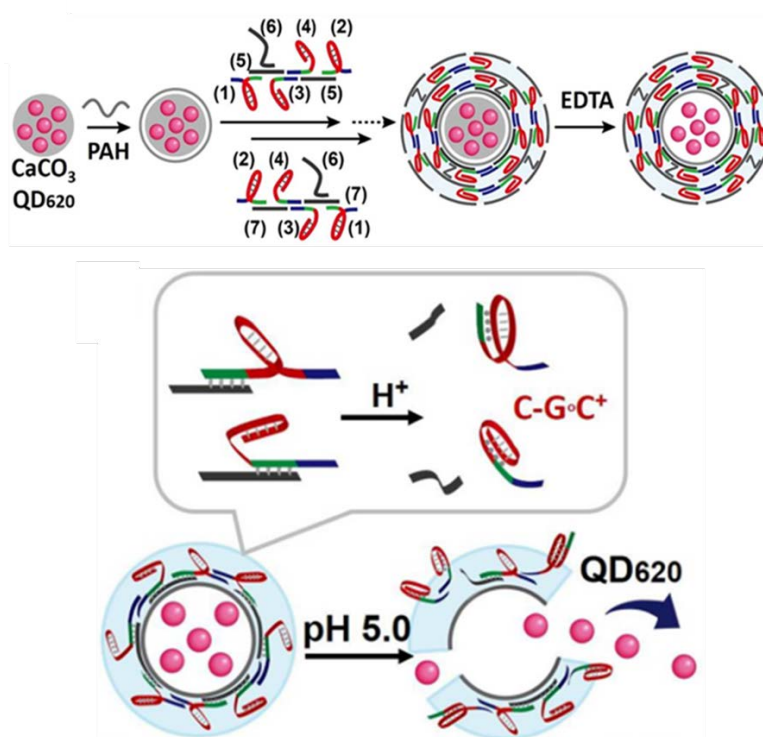
Template-mediated assembly of synthetic and natural polymers into multilayer capsules has shown great promise for engineering drug delivery vehicles. The most typical method to fabricate capsules with desired structure and function is the layer-by-layer (LbL) technique.¹⁰⁴ This method involves the sequential deposition of materials on inorganic particles used as templates, such as solid or mesoporous silica particles and porous calcium carbonate, which have an extremely large surface-to-volume ratio. Subsequently, these inorganic templates can be easily dissolved (e.g., SiO₂ by HF or CaCO₃ by EDTA) and hollow capsules can be formed.¹⁰⁵ Different substrates could be encapsulated in these capsules, which demonstrate the potential for drug delivery.¹⁰⁶

Caruso's lab used this method to assemble multi-layers capsules comprised entirely of DNA (**Scheme 1.13**).¹⁰⁷ Through a layer-by-layer deposition process, multiple DNA layers were assembled on the amine-modified core SiO₂ particle through the hybridization between the base pairs (G pairing with C and T pairing with A).^{108,109} Then the SiO₂ template was dissolved by HF to yield the pure DNA capsules. The physiochemical properties of the DNA capsules, including structure and permeability, were tuneable by changing, which the sequences and length of the specific oligonucleotides used in the assembly. They also developed the controlled degradable DNA capsules with specially designed sequences that could be recognized by a restriction enzyme.¹¹⁰



Scheme 1.14. Schematic illustration of the self-assembly of DNA microcapsules using silica template. Reproduced from reference [107].

Then, Willner's group developed a series of stimuli-responsive DNA-based capsules using CaCO_3 template. For example, the synthesis of the quantum dot (QD)s-loaded pH-responsive DNA capsules were reported (**Scheme 1.14**).¹¹¹ Porous CaCO_3 particles loaded with the QDs with modification of polyallylamine hydrochloride (PAH). Followed by a layer-by-layer process, DNA layers (1)/(2)/(3)/(4)/(5)/(6) and (1)/(2)/(3)/(4)/(6)/(7) were assembled on the core particles using hybridization between the DNA layer units. After dissolving the CaCO_3 templates, QDs-loaded DNA capsules composed of six layers were obtained. The layers (1–4) that acted as responsive units could have different formations under different pH conditions. When the pH of system was 7.2, these units existed as a duplex-stabilized tether to maintain the structure of DNA capsules. While pH changed to 5.0, these units reconfigured into the C–G·C⁺ triplex, leading to the disassembly of DNA capsules and then release of the QDs. The same group developed several types of DNA capsules responsive to enzymes, ATP, UV, and pH.¹⁰⁵



Scheme 1.15. Schematic diagram of the self-assembly of pH-responsive DNA microcapsules using CaCO_3 template. Reproduced from reference [111].

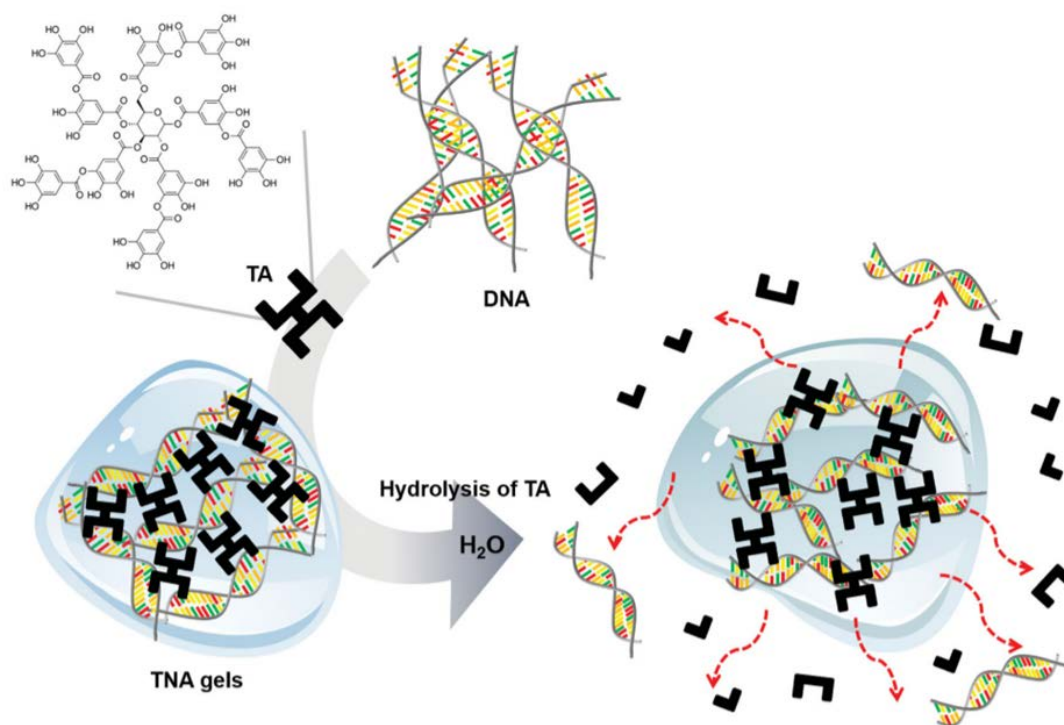
1.4.3 Other Strategies for DNA Nanostructure Construction

Apart from tile-based, origami-based, and template-mediated well-defined DNA structures, which have shown precisely programmed hybridization, size, and shape, the construction of

DNA nanoparticles through different strategies has also been developed, including DNA hydrogel, DNA nanoflowers, and other DNA hybrid nanostructures.

DNA hydrogels can be prepared by linking branched DNA monomers, such as Y-shape or an X-shape by hybridization or prepared by DNA and other polymers.¹¹² As the branched DNA monomers have sticky ends, which are designable, then different monomers could be linked through hybridization on sticky ends. For instance, Xing et al. reported a DNA hydrogel composed of a Y-shaped scaffold and a liner double-strand liner. The DNA hydrogels showed a sol-gel transition upon thermal and enzymatic treatment and these responsive properties could be tailored by adjusting the sequence of the building blocks.¹¹³

Zhang and his colleagues developed a single chain DNA-based nanoarchitecture, called immune-nanoflowers (NFs). The formation of NFs is based on self-assembly of a long DNA sequence via rolling circle replication, independently from Watson–Crick base pairing. By integrating CpG motifs into the DNA sequence, NFs with immunostimulatory effect could be generated. NFs with a size around 300 nm are nuclease resistant, can be efficiently uptaken by cells, and stimulate a high-level production of TNF- α and IL-6 in RAW264.7 cells.¹¹⁴



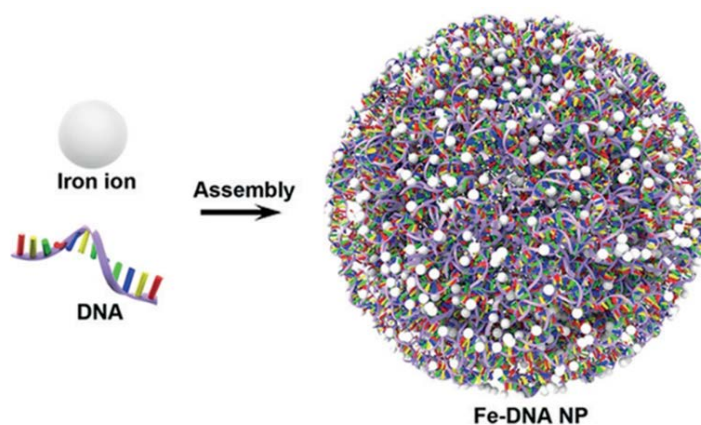
Scheme 1.16. Schematic depiction of the formation and degradation of TA-DNA gels. Reproduced from reference [116].

Tannic acid (TA), as a representative of polyphenol, has been explored for transporting various drugs, enzymes, and DNA.¹¹⁵ Owing to the unique chemical groups (e.g., catechol and galloyl

moieties), TA can interact with diverse materials or substrates through a variety of interactions, such as hydrogen bonds, metal coordination and electrostatic interactions. Lee and co-workers have demonstrated the use of TA and DNA to form DNA hydrogels named TNA hydrogels (**Scheme 1.15**).¹¹⁶ TA reversibly cross-linked with the phosphodiester bonds in DNA, which is different from widely used complementary base pairing in DNA assemblies. Moreover, the TNA hydrogels showed disassembly or degradation upon exposure to physiological buffers.

The interaction between TA and DNA also enables the development of self-assembly TA-DNA nanoparticles. For example, the co-encapsulation of an antigen and CpG adjuvant have been obtained by using TA and the synthetic non-degradable polymer pluronic F127 for in vivo vaccination.¹¹⁷ The nanovaccine targeting lymph nodes promotes robust immune responses and efficiently reduces tumor growth. Besides, combining programmed cell death protein 1 (PD-1) checkpoint blockade with nanovaccine leads to a significant reduction of tumor size and prolonged life of tumor-bearing mice. These results suggest that the nanovaccine could modulate the tumor microenvironment to achieve the reversal of immune checkpoint inhibitor resistance.

Interaction between DNA and metal ions has been investigated for several decades.¹¹⁸ Recently, a novel method to synthesize a new set of DNA-based nanostructures via coordination-driven self-assembly of Fe^{II} ions and DNA was reported by Li et al (**Scheme 1.16**).¹¹⁹ Fe-DNA nanoparticles with controlled sizes could be fabricated by controlling the assembly process. Furthermore, functional DNA was incorporated into the hybrid nanoparticles to demonstrate their drug delivery capability. The Fe-DNA nanospheres could efficiently deliver functional DNA into cells with enhanced biological functionality in vitro and in vivo.



Scheme 1.17. Schematic representation of the synthesis of Fe-DNA nanoparticles. Reproduced from reference [119].

1.5 Scope of the Thesis

In this thesis, DNA-based particles have been developed for vaccine delivery. The difficult hurdle to overcome is the development of simple and reproducible technologies to control the vaccine carrier size and physicochemical properties, which could affect the pharmacological effects.

Chapter 1 provided an overview of vaccines, nanoengineered particles, and DNA nanotechnology.

In Chapter 2, an overview of instruments and techniques utilized in this thesis was introduced. Numerous techniques were used to characterize the engineered particles and investigate their behaviour in biological environments.

In Chapter 3, a facile strategy to synthesize capsules made solely of DNA was developed. These DNA microcapsules made of CpG ODNs show stability in serum and immunostimulatory effects. These DNA capsules were internalized into cells and accumulated in endosomes efficiently and induce a high level of cytokine production. More than four million copies of CpG ODNs can be delivered to a cell with the uptake of a single DNA capsule. We envision that these DNA capsules can not only serve as gene delivery vehicles but also act as adjuvants to achieve synergistic immune effects for immunotherapy.

In Chapter 4, a facile and versatile approach for synthesizing multifunctional particles through the supramolecular assembly of tannic acid (TA) and DNA molecules was reported. Different morphologies of particles were fabricated by employing different types of DNA building blocks. The incorporation of CpG motifs into the particles resulted in high cytokine production in macrophages in vitro. Moreover, combining CpG motifs and ovalbumin in the microparticle, enhanced the cellular delivery of antigens and provoked a stronger cellular response. This work highlights the potential of TA as a universal tool for directing the supramolecular assembly of DNA into a potential platform for the intracellular delivery of genes, antigens, and adjuvants.

In Chapter 5, TA-DNA-PEG nanoparticles were formed by the supramolecular assembly of TA, DNA, and PEG. Different size TA-DNA-PEG nanoparticles were obtained by using different molecular weight PEG chains. These nanoparticles showed high cellular uptake efficiency and could effectively escape from endo/lysosome compartments. Moreover, the intracellular delivery of antigen/adjuvant by these systems stimulated macrophages to secrete an increased level of cytokines, compared to free antigen/adjuvant. Furthermore, nanoparticles

via a direct supramolecular assembly of TA and DNA were fabricated. The size of TA-DNA nanoparticles could be tailored by changing the molar ratio of TA to DNA. This work shows the potential of polyphenol-based nanoparticles for intracellular drug delivery.

1.6 References

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Chapter 2

Instrumentation and Techniques

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2.1 Aim

This chapter describes the key instrumentation and techniques used in this thesis briefly. The specific instruments, materials, and protocols used in this thesis will be presented in the respective chapters.

2.2 Instrumentation and Techniques

2.2.1 Ultraviolet-Visible Spectroscopy

Ultraviolet-Visible (UV-Vis) spectrophotometry is an analytical technique used to quantitatively characterize a sample in solution. It uses light in the ultraviolet and visible regions (wavelength 200-800 nm) to interact with the sample and measure the absorption of light by the sample. Molecules containing non-bonding electrons can absorb photons from ultraviolet or visible light, which promotes the electrons from the ground state to the excitation state. The wavelength of adsorbed light is dependent on the molecular structures of the sample, as the wavelength of adsorbed light is longer for those molecules containing conjugated systems of p electrons or more easily excited electrons. The longer the conjugated system, the longer the wavelength of the light absorbed.¹ When light passes through a sample, the sample will absorb light energy with different absorption intensities related to the wavelength of the light source. This generates an absorption spectrum that allows the detection of the compound in a solution.

The concentration of a compound in a solution can be quantified by using UV-Vis spectroscopy. Light from light source firstly passed through a diffraction grating monochromator, leading to light with a single wavelength (light intensity: I_0). The intensity of light that passes through a sample is reduced owing to the absorption of light by the sample. Then the intensity of the transmitted light (light intensity: I) is detected by a photomultiplier detector. A controller will transfer the detected signal to a computer that connects to the spectrophotometer. Using Beer-Lambert Law (Equation 2.1), the absorption of light at a specific wavelength ($A(\lambda)$) is determined by the concentration of the absorbing compound in the sample solution (c).

$$A(\lambda) = \log_{10} \left(\frac{I_0}{I} \right) = \varepsilon(\lambda)cL \quad (\text{Equation 2.1})$$

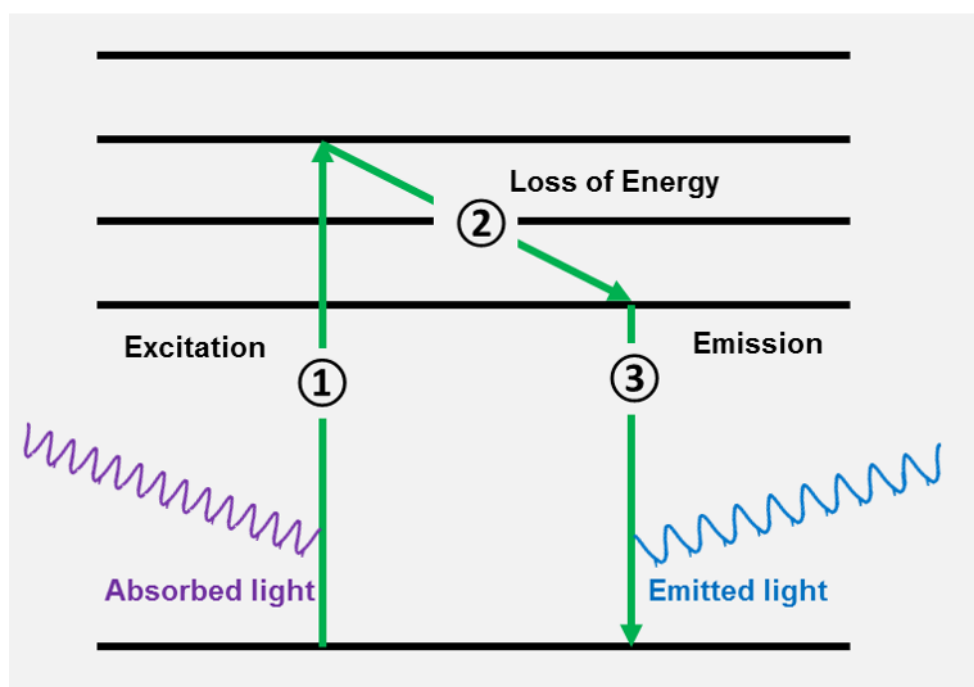
Where $A(\lambda)$ is the measured absorbance at wavelength λ (dimensionless); I_0 is the intensity of the incident light at wavelength λ (W m^{-2}); I is the intensity of the transmitted light at

wavelength λ (W m^{-2}); $\epsilon(\lambda)$ is the extinction coefficient at wavelength λ ($\text{M}^{-1} \text{cm}^{-1}$); L is the path length of the specimen (cm); c is the concentration of the specimen (M).

In this thesis, UV-Vis measurements were used to determine the concentration of DNA solutions using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA). Cell viability via MTT and cytokine release test (ELISA) were conducted by using Infinite M200 microplate reader.

2.2.2 Fluorescence Spectroscopy

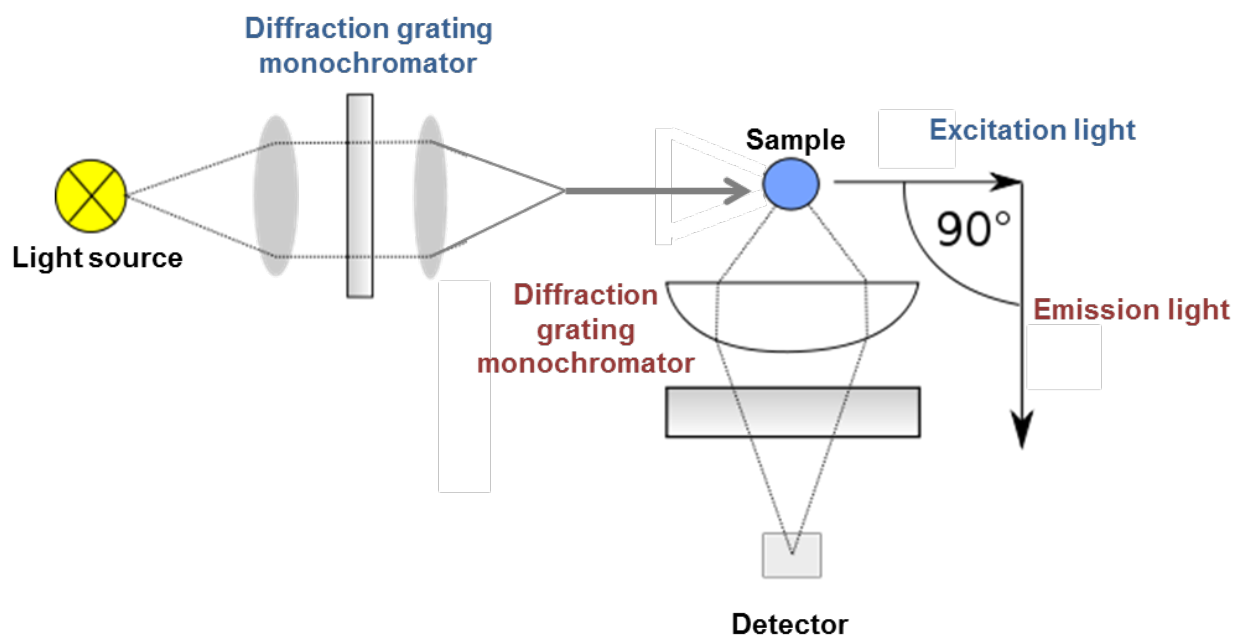
A fluorophore is a molecule that can absorb and emit light energy. When light passes through a sample, fluorophores absorb photons, the energy level of their electrons rising to a higher excited state. According to the wavelength of the light source, fluorophores can reach different energy levels of the excited state. However, the fluorophores at a high energy level are not stable. Subsequently, the molecule will drop down from the excited state to the ground state and the excess energy is released as fluorescent lights.^{2,3} The emitted light has lower energy than the absorbed light, because of the loss of energy during the short excited-state lifetime (**Scheme 2.1**).³ Therefore, emitted fluorescent light can be distinguished from the excited light which has a shorter wavelength.



Scheme 2.1. Schematic illustration of the cyclical fluorescence process. Reproduced from reference [3].

Fluorescence spectroscopy is a technique to measure the fluorescence emission and concentration of a sample with high sensitivity.

A fluorometer is the equipment used to measure fluorescence. A typical fluorometer consists of a light source, two monochromators, a sample holder, and a photon detector (**Scheme 2.2**).⁴ A mercury-vapor or xenon arc lamp is used as a light source with a broad spectrum. Light firstly passes through a diffraction grating monochromator for the selection of the desired excitation wavelength of a certain sample. A solution containing a fluorophore in a cuvette is strike by the excitation light. The emission light in all directions will reach a photon detector at 90° to the light source after they pass through the second diffraction grating monochromator. This can reduce the background noise, like transmitted or reflected incident light, detected by the detector. By acquiring the fluorescence intensity against the measured wavelength with a constant excitation wavelength, a fluorescence emission spectrum can be obtained. By plotting the fluorescence against measured excitation wavelengths with a constant emission wavelength, a fluorescence excitation spectrum can be generated.

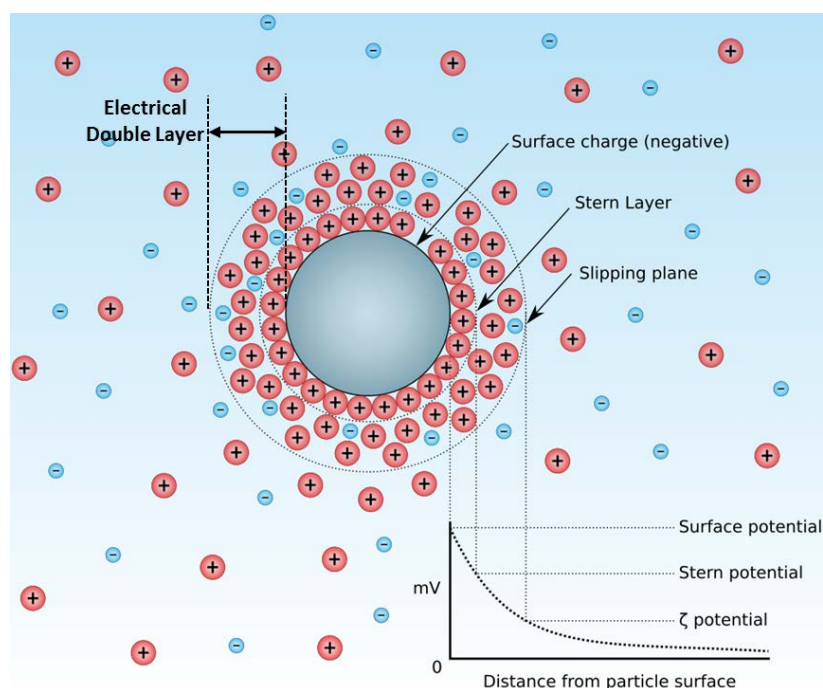


Scheme 2.2. Illustration of a typical fluorometer setup. Reproduced from reference [4].

In this thesis, a Fluorolog-3 Model FL3-22 spectrophotometer (Jobin Yvon Inc., NJ, USA) equipped with a HgXe lamp was used for all experiments.

2.2.3 Zeta-Potential Measurement

Zeta-potential (ζ -potential) is the electric potential of dispersed particles at a defined plane relative to a point in the suspension away from the particle surface (**Scheme 2.3**).⁵ Suspended particles in a liquid should maintain a net surface charge, but it is not easily determined directly. Because charged particles will attract counter ions, which will bind to the surface of particles relatively strongly to form the Stern Layer.⁶ Above the Stern Layer, there is a relatively diffused region consisting of an ion cloud (both ion and counterions), loosely associating with the particles. At the boundary of the ion cloud, defined as the slipping plane, ions no longer bind or associate with the particle surfaces. ζ -potential is the electrical potential at the slipping plane, while the surface charge of a particle is the electric charge on the particle surface. ζ -potential, which is largely affected by the ionic strength and pH of the dispersion medium, is used as a reference to the surface charge of a dispersed particle.



Scheme 2.3. Schematic illustration of the distribution of ions in an electrical double layer around a charged colloidal particle in solution. The ζ -potential is measured at the slipping plane. Reproduced from reference [7].

Microelectrophoresis is a technique to determine the ζ -potential of suspended particles. In electrophoresis, an electric field is applied across a particle suspension, and then the particles

with a ζ -potential drift toward the oppositely charged electrode. The viscous forces of the medium impede the movement of charged particles, resulting in a constant velocity related to the magnitude of the ζ -potential. This velocity can be determined using the dynamic light scattering (DLS) technique where the light scattering of the moving particles is detected and analyzed. The ζ -potential can then be measured by using Smoluchowski's equation (Equation 2.2):

$$v = \frac{\epsilon_R \epsilon_0 E}{\eta} \zeta \quad (\text{Equation 2.2})$$

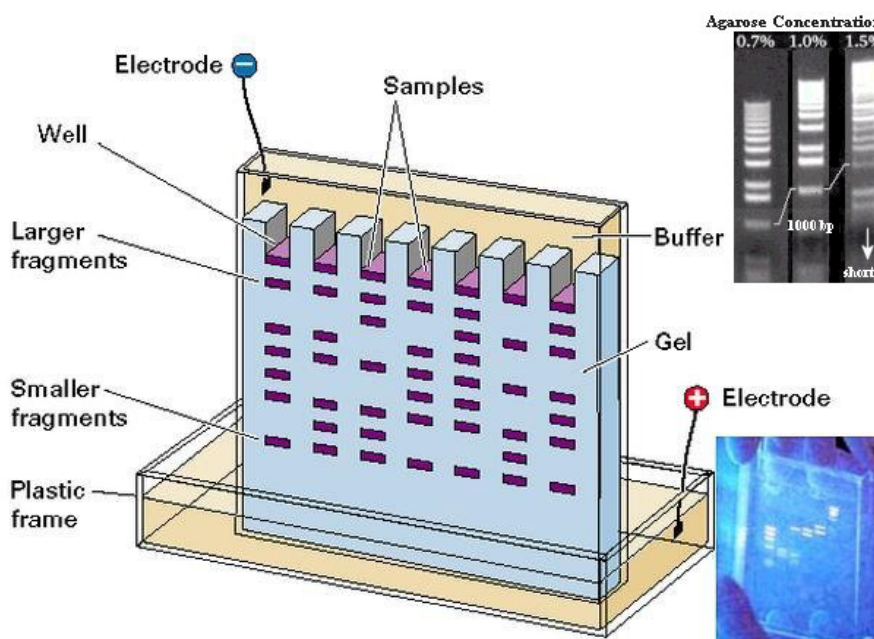
where v is the measured velocity of the particle (cm s^{-1}); ϵ_R is the relative permittivity of the solution (F m^{-1}); ϵ_0 is the permittivity of the vacuum (F m^{-1}); E is the strength of the electric field applied across the particle suspension; η is the dynamic viscosity (Poise); ζ is the ζ -potential of the moving particles (mV).

In this thesis, all ζ -potential measurements were operated using a Malvern Zetasizer 2000 (Malvern Instruments, Malvern, UK) to determine the overall surface charges of particles.

2.2.4 Gel Electrophoresis

Gel electrophoresis is a technique to separate biomolecules (e.g., nucleic acids and proteins) based on their molecular weights.⁸⁻¹⁰ Usually, a gel was made of agarose or polyacrylamide due to their porous structures. When an electric field is applied across the gel, the specimen placed in the gel will move through the gel from the negatively charged electrode to the positively charged electrode (**Scheme 2.4**). The smaller biomolecules move through the gel more quickly compared to the larger ones, because the smaller molecules can travel through the pores of gel more easily. Therefore, gel electrophoresis can separate proteins/nucleic acids with different weights. The molecules in the gel need to be stained for visualizing.

Agarose gel is usually used for nucleic acid and proteins with higher molecular weights owing to its larger pore size, while polyacrylamide gel is typically used to separate proteins due to its uniform pore size. Nucleic acids (DNA and RNA) are negatively charged, while proteins are both positively and negatively charged. Therefore, proteins need to be pre-incubated with an anionic surfactant, sodium dodecyl sulfate (SDS), to obtain evenly distributed negative charges around them before performing electrophoresis. The pH of the specimen solution can change because of the application of an electric field, so it is important to use buffers in gel electrophoresis to maintain a stable pH.



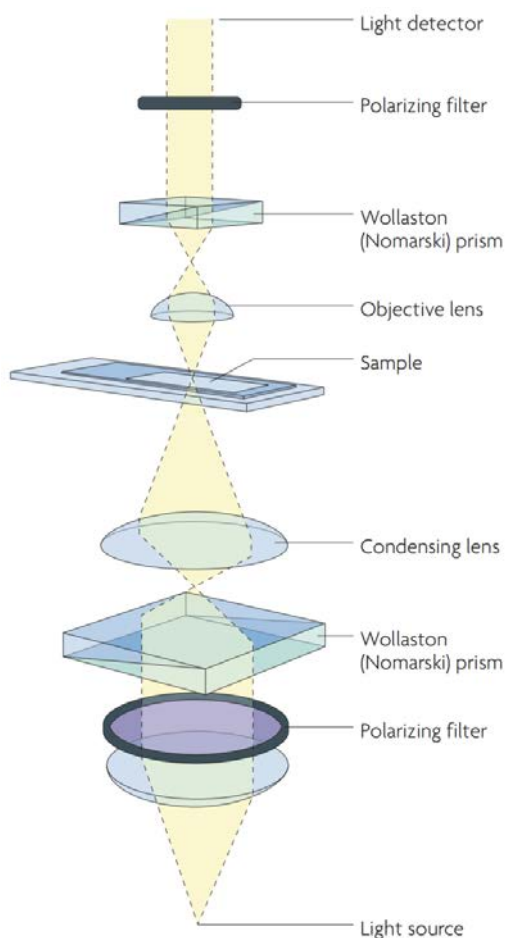
Scheme 2.4. Illustration of the use of gel electrophoresis to separate biomolecules. Specimens with negative charges pass through the gel from the cathode to the anode. Smaller molecules move faster than larger molecules due to the porous structure of the gel. The figure is adapted from reference [11].

2.2.5 Differential Interference Contrast Microscopy

Differential interference contrast (DIC) microscopy is a specially equipped bright field microscopy used to improve the contrast of transparent samples, which are hard to visualize under bright-field illumination.¹²⁻¹⁴ In conventional bright field microscopy, only a magnified image of the specimen can be obtained. However, for transparent specimens with lower optical density, such as living cells, bacteria, and polymer capsules, the magnified image is obtained with reduced contrast. Thus, in the 1950s, DIC microscopy was developed to enhance the contrast of specimen by using interferometry technique.¹⁴

A typical DIC microscopy consists of a light source (illuminator), two polarizing filters, two Wollaston (Nomarski) prisms, a condensing lens, an objective lens, and a light detector (**Scheme 2.5**).¹⁵ Light from the illuminator passes through a polarizing filter and is polarized at 45°. The polarized light then passes through a Wollaston (Nomarski) prism to be separated into two perpendicular beams. Subsequently, the two beams are focused by a condensing lens and pass through the specimen at two adjacent points (~0.2 μm apart). When traveling through adjacent areas of the specimen, the two beams will undergo slightly different optical paths depending on the refractive index or thickness of the passing points. Then, the two beams are

focused by an objective lens and pass through a second Wollaston (Nomarski) prism and a polarized filter, leading to recombination of the two beams into a polarized light at 135° . When the two beams are combined, the interference between them raises the contrast of the image with brighter or darker fields according to the optical path differences. Finally, the light is collected by a light detector, and the generated image can be both observed by eyes and captured by a digital camera.

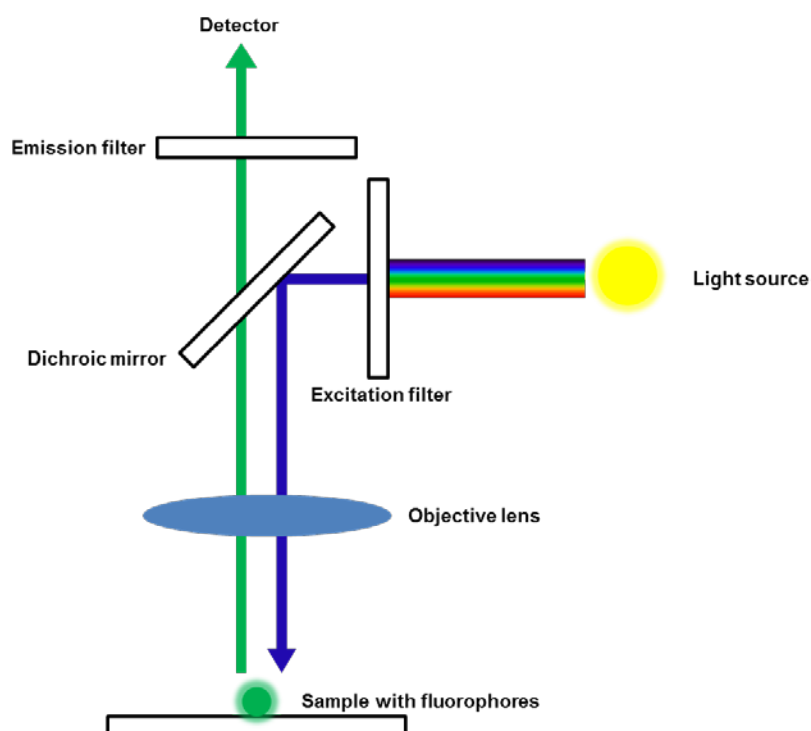


Scheme 2.5. Schematic illustration of the light path through the optical components of a DIC microscope. Reproduced from reference [15].

2.2.6 Fluorescence Microscopy

Fluorescence microscopy is an optical microscopy used to visualize fluorescent specimens. In a fluorescence microscopy, a high-intensity light with a specific wavelength is used to excite the fluorophores in the specimen, while visible light is used in a bright field microscopy to

illuminate the specimen. A typical fluorescence microscopy consists of a light source, an excitation filter, a dichroic mirror, and an emission filter (**Scheme 2.6**).¹⁶ A light from the light source (e.g., mercury-vapor or xenon arc lamps) passes through the excitation filter to select a specific wavelength that enables illuminate the specimen appropriately. When the filtered light with a specific wavelength illuminates the specimen with fluorophores, they will absorb the illumination light to be excited. Subsequently, the fluorophores in the specimen will emit light to return to the ground state. The emission light has a longer wavelength and lower energy than excitation light, which allows that the emission light can be distinguished from the excitation light. Then the emission filter blocks the excitation light and lets the emission light pass through to be detected by a detector. The dichroic mirror is used to accomplish the functions of excitation and emission filters. The chooses of both the filters and dichroic mirrors are depended on spectral excitation and emission properties of the fluorophores in the specimen. By using this technique, the distribution of a mono-color fluorophore can be imaged at a time and multi-color images of different fluorophores can be obtained by overlaying various mono-color images as well.



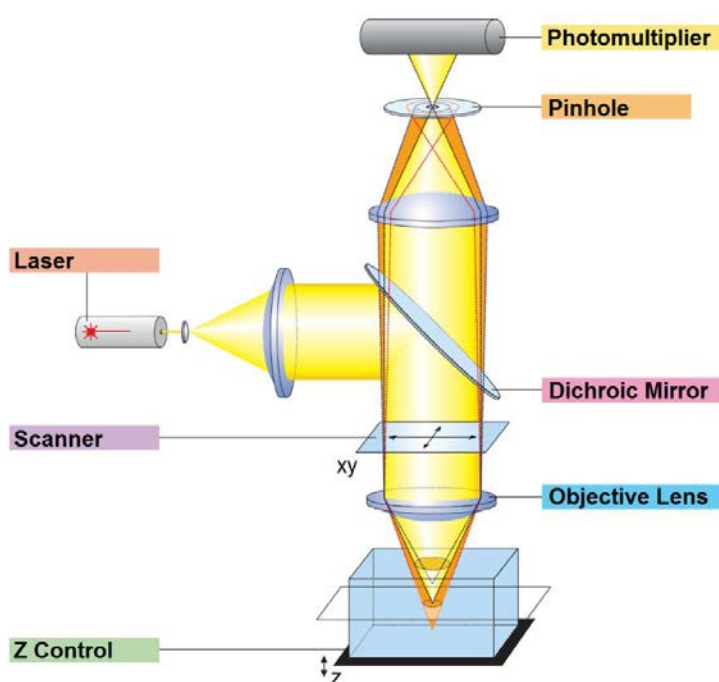
Scheme 2.6. Simplified schematic illustration of a fluorescence microscope setup.

Reproduced from reference [16].

In this thesis, all characterizations by CLSM were performed with a Nikon A1R confocal microscope fitted with a 60× 1.4 NA oil immersion objective.

2.2.7 Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) is an advanced fluorescence microscopy for imaging fluorescent specimen with high optical resolution in three dimensions.¹⁷ The operating principle of CLSM (**Scheme 2.7**) is similar to that of conventional fluorescence microscopy.¹⁸ The main difference is that CLSM has a confocal pinhole located between the photon detector and dichroic mirror to rejecting out of focus fluorescent light. As a result, the image only comes from a thin focused section of the specimen, and a clear three dimensions image of the specimen can be generated by scanning many thin sections through the specimen. Furthermore, more than one type of fluorescence can be imaged by changing the excitation light. In a CLSM, a laser is used as the light source in CLSM to permit spot illumination and a photomultiplier (PMT) is used to detect the emitted fluorescent light. CLSM is useful to detect the cell association and internalization with particles, as cells at different planes can be scanned.

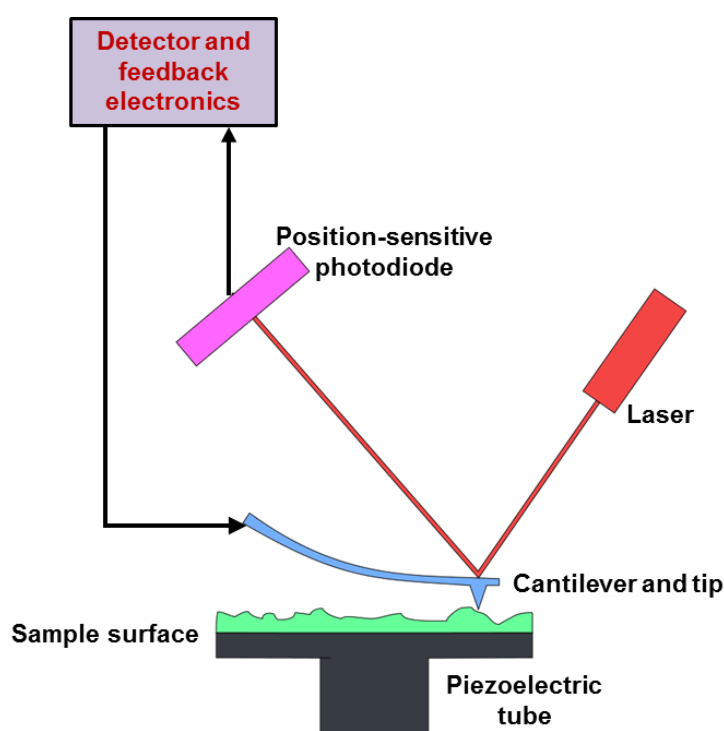


Scheme 2.7. Simplified schematic of CLSM. Reproduced from reference [18].

In this thesis, all characterizations by CLSM were performed with a Nikon A1R confocal microscope fitted with a 60× 1.4 NA oil immersion objective.

2.2.8 Atomic Force Microscopy

Atomic Force Microscopy (AFM) is a scanning probe microscopy, which is used for imaging surface morphology (on a nanometer scale) and measuring surface forces.^{19,20} Because AFM does not use an electron beam, it can be operated in the ambient environment, and wet condition. The operating principle of AFM is based on a series of interaction forces between a sharp tip (probe) and the surface of the specimen. The interaction forces include van der Waals forces, electrostatic forces, capillary forces, mechanical contact force, chemical bonding, and other forces.



Scheme 2.8. Illustration of the typical setup of an atomic force microscope. Reproduced from reference [20].

An atomic force microscope typically consists of a spring-like (Hookean) cantilever with a sharp tip at one end, a laser source, a series of mirrors and lenses, a piezoelectric positioner, and a position-sensitive photodetector (**Scheme 2.8**). The cantilevers have different spring constants used for different types of specimens (rigid or soft) and operating modes. As the specimen is scanned and the tip contacts with the surface of the specimen, the forces between the tip and the specimen will cause a deflection of the cantilever according to Hooke's Law.

The laser beam directed to the top surface of the cantilever is deflected and then detected by a position-sensitive photodetector, which measures the deflection of the special variations in the laser beam. To protect the tip avoiding the collision with the specimen, a feedback control loop is used to adjust the tip to specimen distance to maintain a constant force between the tip and the surface of the specimen, instead of a constant scanning height of the tip. The signal detected by the position-sensitive photodetector is converted to electrical signals, which are then transmitted to the piezoelectric positioner to control the position of the cantilever. During the scanning, x- and y-directional controls can be achieved by the operator via monitoring the output display, while z-directional control is achieved via the feedback loop – a function of the response to the signals collected by the piezoelectric positioner.

Usually, the AFM can be operated in three modes based on the nature of the type motion, including contact mode, non-contact mode, and tapping mode.²⁰ In contact mode, the tips contact with the surface of the specimen constantly by the piezoelectric positioner. While the tip does not contact the surface of the specimen in non-contact mode, which operated with the attractive regime. In tapping mode, the tip in the cantilever is oscillated at or near its resonance frequency and has less contact with the specimen. As the tapping getting closer to the specimen, the tip-specimen interaction forces acting on the cantilever decrease the amplitude of the oscillation. The tapping mode is used more frequently in AFM. Because the specimen is less damaged by the tip owing to the fewer contacts. The AFM, which provides a three-dimensional surface profile of the sample, is useful for imaging nanoscale features of polymer films and capsules.

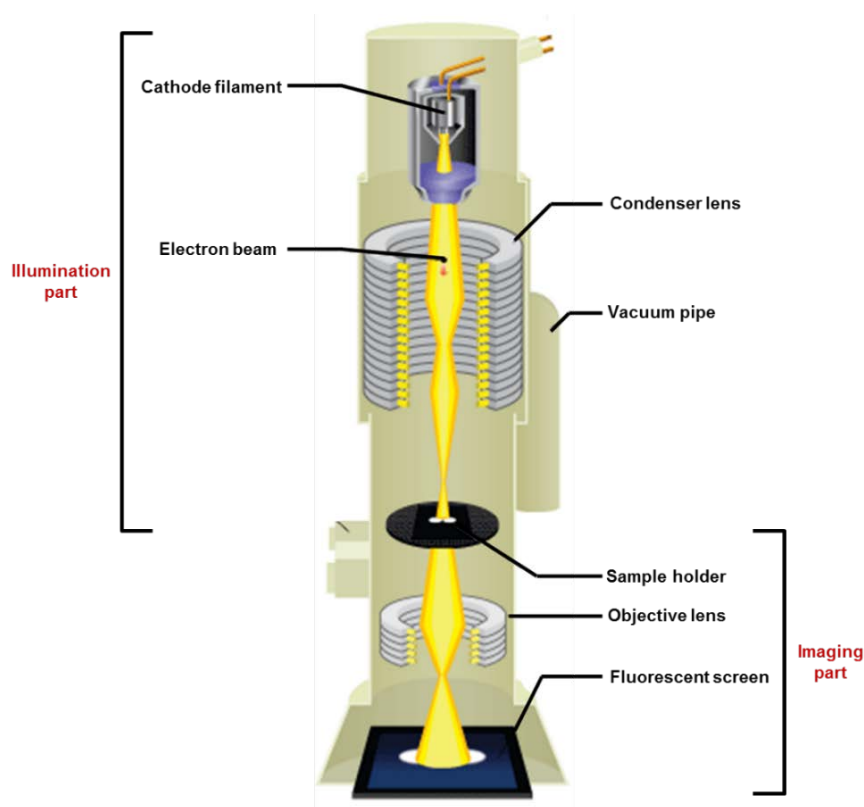
In this thesis, AFM images were acquired using an MFD-3D Asylum Research instrument (CA, USA). Typical scans were conducted in tapping mode using MikroMasch silicon cantilevers (NSC/CSC).

2.2.9 Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) is a microscopy with extremely high resolution, down to the nanoscale. Instead of using photons in light microscopy, TEM utilizes a beam of electrons that have de Broglie wavelength 100, 000 times smaller than that of the photons, leading to a largely enhanced resolution.²¹ When the electrons pass through the ultra-thin specimen (typically < 100 nm), only the electrons that have transmitted through the specimen will reach the electron detectors to generate the image. Depending on the thickness and the density of the specimen, fewer electrons pass through will generate a brighter field indicating

a thinner or less dense specimen, while more electrons pass through will generate a darker field indicating a thicker or denser specimen.

A typical transmission electron microscope consists of an electron emission source (electron gun), a set of electromagnetic lenses, and an electron detector (**Scheme 2.9**).²² High-energy electron beams are emitted from an electron gun at the top of the microscopy, which operates at high voltages (100-300 kV). The electrons travel through a vacuum in the column of the microscopy, to avoid the scattering of electron beams by molecules in the air. Then the electrons are accelerated towards the specimen by electromagnetic lenses, including condenser, objective, intermediate, and projector lenses. They can control the energy and wavelength of the electrons within a very thin beam, which allows the fine-tuning of the magnification and focuses of the image. Then the transmitted electrons hit a fluorescence screen at the bottom of the microscopy for visualization and the image can be captured by a charge-couple device (CCD) camera.



Scheme 2.9. Simplified schematic illustration of a transmission electron microscope setup. Reproduced from reference [22].

In this thesis, TEM imaging was performed by using the FEI TecnaiTM G2 Spirit instrument (OR, USA), operating at 120 kV.

2.2.10 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is usually used to obtain information about the surface topography of specimens. Scanning electron microscope consists of an electron gun, a set of electromagnetic lenses, and electron detectors, which is similar to TEM.^{23,24} In SEM, a high-energy beam of electrons is produced from the electron gun, accelerated down, and traveled through a set of lenses and apertures, leading to a focused beam of electrons that hits the surface of the specimen. As the electrons are scattered by the specimen, secondary electrons are produced and then detected by electron detectors to form images displayed on a computer screen. The intensity of detected electrons emission depends on the surface property of the specimen. Therefore, SEM is used for characterizing the surface topography of the specimen. In addition, the surface topography of the specimen needs to be electrically conductive, so the specimen usually is sputtered with gold and platinum. Normally, SEM is operated under a vacuum condition, both the column and chamber are evacuated by a set of pumps. Unless some specially designed SEM, they can be operated under low vacuum and wet conditions.

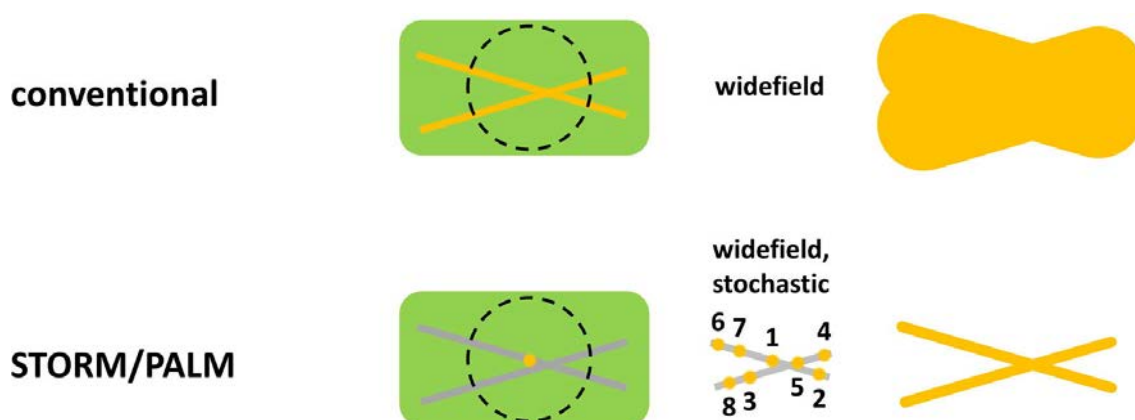
In this thesis, SEM images were captured on the FEI Quanta 200 field-emission scanning electron microscope, operating at an accelerating voltage of 120 kV.

2.2.11 Stochastic Optical Reconstruction Microscopy

Stochastic Optical Reconstruction Microscopy (STORM) is an advanced super-resolution microscopy that is used for single-molecule imaging that allows resolving structures beyond diffraction limited spatial resolution.²⁵

In conventional imaging, a vague image is generated by an object composed of two crossed line, due to the diffraction of light (**Scheme 2.10**).²⁶ A diffraction limited spot with a size $\sim 250\text{nm}$ is indicated by the dashed circle, which leads to the low resolution of conventional microscopy. While STORM imaging provides a high-resolution image of the fluorophores labeling the sample based on the stochastic activation of individual fluorophores with photoactivatable properties. During acquisition of STORM images, the individual fluorophore (numbered 1–8) molecules randomly switch between a fluorescent and dark state (blinking). By determining its position coordinates from the photons detected for each activation event (often as a Gaussian function), a single fluorophore can be precisely localized. This blink

process is randomly repeated several times until most of the fluorophores have blinked to be imaged. In addition, it is important to make individual fluorophores are sparse enough, so that at any time only one fluorophore is activated within a diffraction-limited region.



Scheme 2.10. Schematics showing a comparison between conventional and super-resolution fluorescence microscopy. Adapted from reference [26].

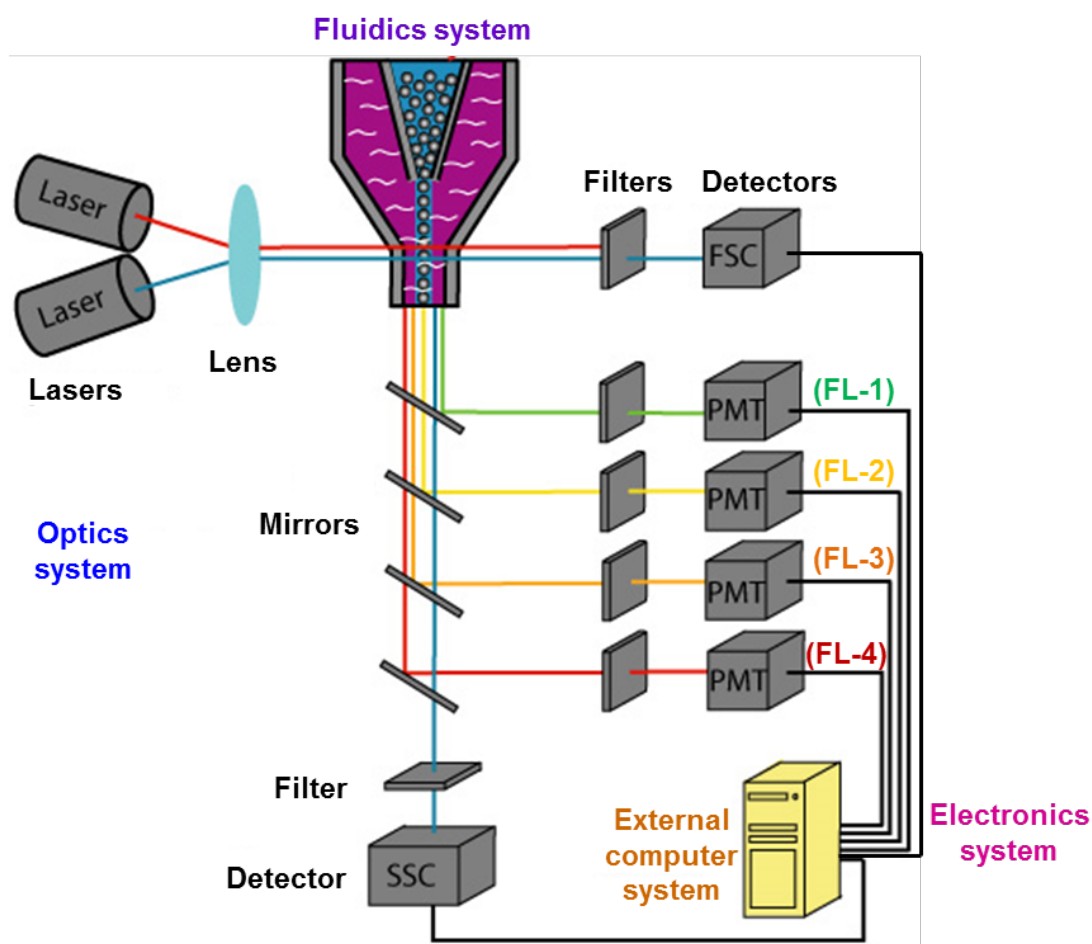
In this thesis, STORM images were acquired using a Nikon N-STORM system configured to achieve total internal reflection fluorescence imaging (TIRF).

2.2.12 Flow Cytometry

Flow cytometry is an analytical technique used for measuring multiple parameters of the particle (e.g., cells, bacteria, organic and inorganic particles) flowing in a fluid stream through a light beam.^{27,28} Typically, the size range of particles from 200 nm to 2 μ m is suitable for measurement. The physical properties of a particle, including the size, granularity, fluorescence intensity, and particle concentration, can be measured by using flow cytometry. In flow cytometry, particles pass through a flow system and are illuminated by a laser beam individually. The light scattering by a single particle will be detected and analyzed to provide the information about parameters of the particle.

A typical flow cytometry consists of a light source (light), a flow system with a coaxial stream, a series of dichroic mirrors and filters, photon detectors, and an electronics system for signal processing (**Scheme 2.11**).²⁹ The flow system with a coaxial stream is utilized to make particles in the center of the inner sample fluid pass through the interrogation point of a laser beam individually (one particle a time). The individual particle is illuminated by a laser beam and

particles passing through the laser beam will scatter light. Fluorophore-tagged particles will be excited by a laser within the corresponding excitation wavelength range and emit fluorescence light. A series of optical including filters, mirrors, and detectors will direct and collect scattered light and the emitted fluorescent signals. The scattered light can be collected at two angles, namely forward scatter (FSC) and side scatters (SSC). The FSC detector collects the scattered light in the forward direction of the laser path, which is the diffracted light by the particles and related to the size of particles. While SSC detector collects the scattered light in the perpendicular direction to the laser path, which is then refracted and reflected light by the particles and related to the granularity of particles. Then the electronics system converts the detected light signal to electronic signals, which can be further converted to digital signals for data analysis by an external computer connected to the flow cytometry.

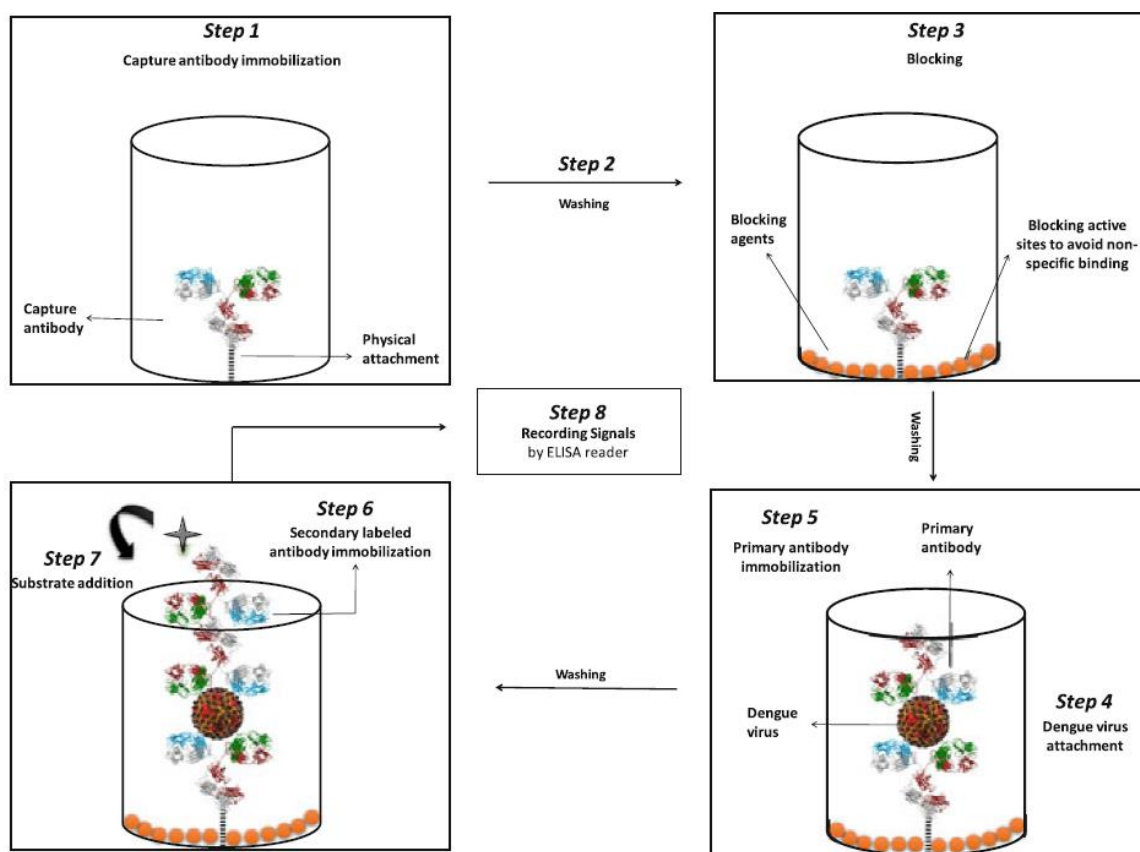


Scheme 2.11. Schematic illustration of a typical configuration and operation of flow cytometry. Reproduced from reference [29].

In this thesis, Flowjo was used for the analysis of all data collected by Apogee A50-Micro (Apogee Flow Systems Ltd, Hertfordshire, UK) flow cytometers.

2.2.13 Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) is an immunological plate-based assay commonly used to measure and quantify biological samples in liquid, such as antibodies, proteins, antigens, peptides, and hormones. ELISA assay is generally conducted in 96 well plates, which allows several samples to be measured in one experiment. The plate used in ELISA should be a special absorbent plate to allow the antibodies to stick on its surface.



Scheme 2.12. Diagram of a sandwich ELISA procedure. Reproduced from reference [30].

A sandwich ELISA is a typical ELISA, which used two types of antibodies to detect samples, e.g. cytokines. The ELISA plate is coated with a specific capture antibody and then the unbound capture antibody is washed out (**Scheme 2.12**).³⁰ The sample with antigens is added to the ELISA plate and the antigen will be immobilized by the capture antibody which is sticking on

the plate. Subsequently, the detection antibody labeled with an enzyme is applied, resulting in a complex with the immobilized antigen. The plate needs to be washed with a detergent solution (Tween 20) to remove any of the non-specifically bound antibodies, between each step. A substrate that can react with the enzyme is added to the plate, leading to a detectable colored product that could be measured by a plate reader. To determine the concentration of antigen in a sample, a standard sample needs to be used to provide a standard curve, so that the concentration of the antigen can be calculated based on the standard curve. The most typical group of enzymes and substrate are horseradish peroxidase and 3,5,3',5'-tetramethylbenzidine. In this thesis, ELISA kits (Invitrogen, USA) were used for all cytokine assays.

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Chapter 3

Template-Mediated Assembly of DNA into Microcapsules for Immunological Modulation

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3.1 Manuscript

FULL PAPER



Template-Mediated Assembly of DNA into Microcapsules for Immunological Modulation

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There is a need for effective vaccine delivery systems and vaccine adjuvants without extraneous excipients that can compromise or minimize their efficacy. Vaccine adjuvants cytosine-phosphate-guanosine oligodeoxynucleotides (CpG ODNs) can effectively activate immune responses to secrete cytokines. However, CpG ODNs are not stable in serum due to enzymatic cleavage and are difficult to transport through cell membranes. Herein, DNA microcapsules made of CpG ODNs arranged into 3D nanostructures are developed to improve the serum stability and immunostimulatory effect of CpG. The DNA microcapsules allow encapsulation and co-delivery of cargoes, including glycogen. The DNA capsules, with >4 million copies of CpG motifs per capsule, are internalized in cells and accumulate in endosomes, where the Toll-like receptor 9 is engaged by CpG. The capsules induce up to 10-fold and 20-fold increases in tumor necrosis factor (TNF)- α and interleukin (IL)-6 secretion, respectively, in RAW264.7 cells compared with CpG ODNs. Furthermore, the microcapsules stimulate TNF- α and IL-6 secretion in a concentration- and time-dependent manner. The immunostimulatory activity of the capsules correlates to their intracellular trafficking, endosomal confinement, and degradation, assessed by confocal and super-resolution microscopy. These DNA capsules can serve as both adjuvants to stimulate an immune reaction and vehicles to encapsulate vaccine peptides/genes to achieve synergistic immune effects.

Cytosine-phosphate-guanosine oligodeoxynucleotides (CpG ODNs) have been widely used as vaccine adjuvants owing to their immunostimulatory effects.^[2] CpG ODNs are recognized by Toll-like receptor 9 (TLR9), which is expressed and located in the endosomes of immune cells, including macrophages, dendritic cells, and B cells.^[3] The engagement of CpG ODNs with TLR9 leads to the activation of the transcription factor nuclear factor- κ B and mitogen-activated protein kinase pathways.^[4] These signaling pathways promote the secretion of proinflammatory cytokines, such as tumor necrosis factor (TNF)- α and interleukin (IL)-6, and initiate a cascade of innate and adaptive immune responses.^[5] However, different biological barriers can hinder the activity of CpG ODNs. For instance, CpG ODNs are not stable in serum due to their sensitivity to deoxyribonuclease (DNase) and are difficult to transport through cell membranes because of their negative charge.^[6] Therefore, significant efforts have been made to enhance the stability of CpG ODNs against degradation by DNase to improve their delivery efficacy and prolong the immune response. The

backbone modification of CpG ODNs with phosphorothioate (PT)^[7] is effective for inhibiting the degradation of CpG ODNs but potential side effects include reduced immune response, lymphoid follicle destruction, and organ enlargement. Alternatively, the delivery of CpG ODNs using nanoparticles has been

1. Introduction

Vaccines are an effective tool for controlling human diseases. The efficacy of vaccines can be greatly enhanced through improving innate immunity with the co-delivery of potent adjuvants.^[1]

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attempted to protect CpG ODNs from degradation, resulting in improved adjuvant activity.^[8–11] Nanomaterials have provided promising opportunities to deliver CpG ODNs through encapsulation, adsorption, and covalent conjugation.^[12] In particular, DNA nanostructures have been proposed for CpG delivery.^[13–15] Li et al. recently designed a functional DNA tetrahedral structure for noninvasive intracellular delivery of CpG ODNs.^[16] The DNA nanostructures were readily internalized by immune cells and stimulated secretion of proinflammatory cytokines. Schuller et al. reported the efficacy of DNA origami structures in delivering CpG ODNs to target cells.^[17] Although these DNA nanostructures could efficiently deliver CpG ODNs for immune stimulation, a number of limitations remain, including poor stability in serum, expensive and nonscalable preparation approaches, and limited drug loading capacity.

Recently, DNA microcapsules have emerged as a versatile platform for biological applications, including biosensing, bioimaging, and drug delivery, owing to their high cargo loading capacity, nontoxicity, and tunable size and thickness.^[18–21] A common method employed to fabricate DNA capsules is via the layer-by-layer (LbL) technique,^[22–24] which requires chemically modified SiO₂ and CaCO₃ templates, multiple deposition cycles of specific predefined DNA sequences, and numerous washing steps.^[25–28] An alternative and potentially scalable process for preparing DNA microcapsules made solely of CpG ODNs arranged into 3D nanostructures would be desirable not only to prolong the stability of the immunostimulant in serum, but also to increase the payload delivery to allow encapsulation and co-delivery of multiple cargoes. To our knowledge, nanostructured DNA capsules composed solely of CpG ODNs, which differ from DNA origami structures because of their size and loading capacity, have not been reported to date. Previous studies have shown that antigen-presenting cells (APCs), including macrophages and dendritic cells, can internalize micrometer-sized particles locally and carry particles to lymph nodes upon subcutaneous injection.^[29–31] Studies have also shown that micrometer-sized particles can induce higher immune stimulation than nanometer-sized particles owing to the higher uptake efficiency by APCs.^[32,33]

In the present study, we report a general method to fabricate DNA capsules using CaCO₃ particles as templates. Briefly, a Y-shaped DNA building block bearing CpG sequences (Y-CpG) was loaded into CaCO₃ particles with high loading efficiency, followed by cross-linking using a DNA duplex linker. After dissolving the CaCO₃ templates, nanostructured DNA capsules bearing CpG motifs and with uniform size were obtained. Unlike the single-stranded DNA (ssDNA) and the Y-CpG building block, the obtained capsules were stable in the extracellular milieu. They were efficiently taken up by macrophages and displayed high immunostimulatory activity, which correlated to their intracellular trafficking, endosomal confinement, and degradation, as evaluated by confocal and super-resolution microscopy. Furthermore, to demonstrate the versatility of our approach, a high-molecular-weight bio-macromolecule, i.e., glycogen, was readily co-loaded into the DNA capsules. The structural characteristics, i.e., hollowness, tunable size, and cross-linkable Y-shaped DNA units, make these DNA capsules unique with their simple design and synthesis, high stability, and high loading capacity and functionality. Overall, we

highlight the potential use of the present capsules to co-deliver a controlled dose of CpG adjuvant and high-molecular-weight antigens for future vaccine and immunotherapy applications.

2. Results and Discussion

2.1. Synthesis and Characterization of CpG_{cap}

DNA capsules, hereafter referred to as CpG_{cap}, were prepared from a Y-shaped building block containing CpG sequences using template-mediated assembly (Figure 1a). The DNA strands adsorbed onto the CaCO₃ templates via electrostatic interactions between the phosphate groups of DNA and positively charged calcium ions present on the surface of the CaCO₃ template.^[34] First, the Y-shaped building block (Y-CpG) was assembled by mixing three different single strands (Y-1, Y-2, and Y-3) bearing the same sticky ends (Figure 1a; Table S1, Supporting Information). Through complementary base pairing, the three single strands hybridized to form a Y shape with the sticky ends remaining single-stranded and available for further base pairing (Figure 1a). To endow CpG_{cap} with strong immunostimulatory effect, three CpG motifs were embedded in one Y-CpG building block. The prepared DNA building blocks without further purification were characterized by agarose gel electrophoresis (Figure S1, Supporting Information). The DNA building blocks showed sharp bands without any detectable smear, demonstrating the successful assembly of the desired products. The Y-CpG was then loaded onto porous CaCO₃ particles of 2 μm in diameter (Figure S2, Supporting Information) as shown in Figure 1a. The loading of Y-CpG was quantified by measuring the concentration of DNA in the supernatant—a loading efficiency of 85% was determined. After repeated washing to remove excess Y-CpG, the particles were mixed with the duplex DNA linker, which has sticky ends complementary to the sticky ends of Y-CpG, to cross-link the immobilized Y-CpG by hybridization. Finally, the CaCO₃ templates were dissolved using ethylenediaminetetraacetic acid (EDTA) solution, yielding microcapsules made solely of DNA. The number of particles before and after template removal was determined by flow cytometry, which showed that the yield of intact microcapsules was 90 ± 5%. By quantifying the amount of DNA loaded into the particles, we estimated that each CpG_{cap} was composed of ≈1.5 million Y-CpG units, embedding 4.5 million CpG motifs. Compared to DNA nanostructures that can deliver only a small number of copies (<100) of CpG,^[16,17] the presently engineered CpG microcapsules possess a considerably higher adjuvant loading capacity. The density of CpG motifs of CpG_{cap} was tenfold higher than the density of CpG motifs of DNA origami.^[17] More than 4 million copies of CpG can potentially be delivered to a cell with the uptake of a single CpG_{cap}.

To visualize and characterize the DNA capsules by fluorescence microscopy, Y-CpG labeled with Alexa Fluor 488 (AF488) was used. Confocal microscopy images showed that the CpG_{cap} were well dispersed in cell culture medium, with diameters of 2.5 ± 0.3 μm (Figure 1b). This indicated swelling of ≈25% relative to the original template diameter, which is typical of highly cross-linked hydrogel-like structures. Helium-ion microscopy (HIM) analysis revealed the homogeneity

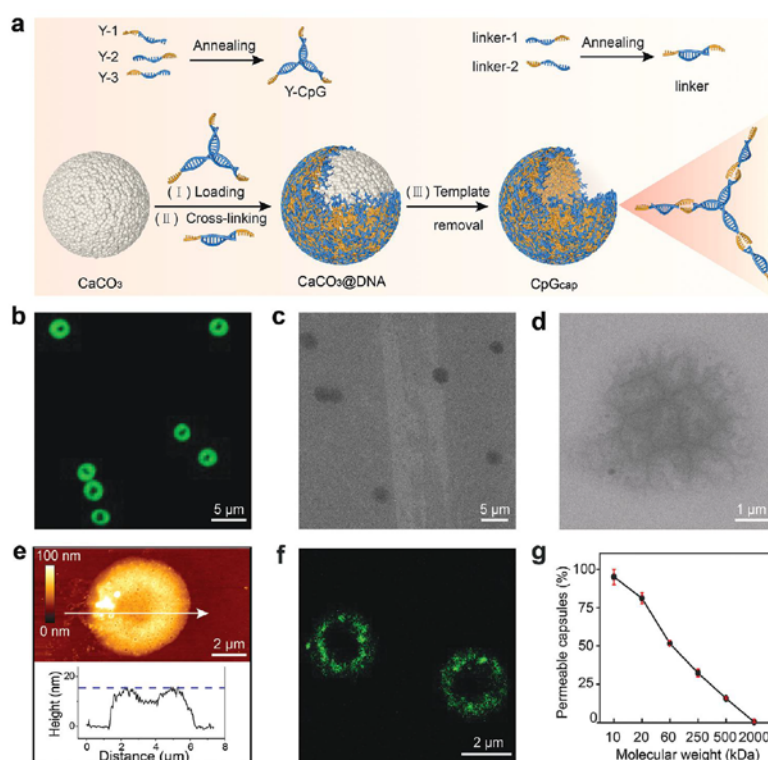


Figure 1. Preparation and characterization of CpG_{cap} . a) Schematic representation of the template-mediated assembly of CpG_{cap} : I) loading of CaCO_3 particles with Y-CpG; II) cross-linking by the duplex DNA linker; and III) removal of CaCO_3 particles to yield CpG_{cap} . The yellow strands on DNA represent sticky ends from the Y-CpG and linker, which are complementary to each other. b) CLSM, c) HIM, d) TEM, e) AFM, and f) STORM images of CpG_{cap} . g) Percentage of permeable CpG_{cap} plotted against different molecular weights of FITC-dextran (x-axis is not drawn to scale).

and size uniformity of the CpG_{cap} (Figure 1c). Transmission electron microscopy (TEM) images revealed the cross-linked and porous structure of CpG_{cap} (Figure 1d; Figure S3, Supporting Information) endowed with a brushed surface. The thickness of CpG_{cap} was around 10 ± 3 nm, as assessed by atomic force microscopy (AFM) (Figure 1e; Figure S4, Supporting Information). The TEM and AFM analyses both showed that CpG_{cap} collapsed upon air drying, suggesting that CpG_{cap} were soft and highly hydrated in aqueous solution. Because of the limited resolution (250 nm) of the confocal microscopy imaging of the microcapsules (Figure 1b), the nanostructured capsules' shell was analyzed by stochastic optical reconstruction microscopy (STORM). STORM is a super-resolution microscopy technique with single-molecule sensitivity and a high spatial resolution (typically 20 nm) based on the accurate localization of single photoswitchable fluorescent molecules such as Alexa Fluor 647 (AF647). DNA microcapsules were therefore assembled using AF647-labeled DNA linker and imaged by STORM in the original hydrated state. As observed from the STORM image in Figure 1f, the microcapsules featured a hollow structure and a thickness of 300 ± 50 nm. It is worth noting that

the single-molecule localization performed by STORM enables imaging of the shell of the microcapsules that includes the brushes of DNA chains protruding out of the capsules. Comparison between the thickness values determined by AFM and STORM suggests that the shell of the capsules is highly hydrated and swollen. The permeability of CpG_{cap} was investigated using dextran analytical standards at different molecular weights (M_w). The microcapsules were more permeable to macromolecules with low M_w ranging from 10 to 60 kDa, less permeable to macromolecules with high M_w (250–500 kDa), and impermeable to 2000 kDa dextran, (Figure 1g; Figure S5, Supporting Information).

To demonstrate the versatility of our CpG_{cap} in co-loading high-molecular-weight biomacromolecules, positively charged glycogen nanoparticles ($M_w = 350$ kDa, diameter = 20 nm) were loaded within the cavity of CpG_{cap} , as described in the "Experimental Section." HIM, TEM, and AFM images revealed the formation of spherical glycogen-bearing CpG_{cap} (Gly- CpG_{cap}) with a uniform size (2.5 μm) (Figure S6a–c, Supporting Information). The thickness of Gly- CpG_{cap} was ≈ 15 nm, as measured by AFM (Figure S6c, Supporting Information). The surface of

Gly-CpG_{cap} was not as smooth as that of CpG_{cap}, as noted from the presence of small “dots” on the capsule surface (black and bright dots in Figure S6a–c in the Supporting Information), indicating that the positively charged glycogen nanoparticles were embedded and immobilized within the porous CpG_{cap}. It is likely that there are electrostatic interactions between the negatively charged DNA and positively charged glycogen.^[35] This was confirmed by fluorescence microscopy imaging of Gly-CpG_{cap}, prepared using AF488-labeled DNA and Alexa Fluor 555-labeled glycogen, which showed green and red overlapping emission (Figure S6d, Supporting Information). Overall, these results reveal that high-molecular-weight biomacromolecules can be embedded into CpG_{cap}.

To study the stability of CpG_{cap} in fetal bovine serum (FBS) containing a low concentration of DNase,^[36] CpG_{cap} were incubated with cell culture medium containing 10% FBS at 37 °C and the mixture was analyzed by gel electrophoresis (Figure S7, Supporting Information). CpG_{cap} maintained structural integrity even after incubation in FBS for 24 h, demonstrating the remarkable stability of CpG_{cap} in cell culture medium. The stability in serum of CpG_{cap} can be ascribed to the entanglement and confinement of the DNA strands in the 3D nanostructure of the microcapsules, which reduces the enzyme binding affinity and prevents chain distortion that is required for the catalytic degradation.^[36,37] However, a DNA degradation study performed using a high concentration of DNase (1 U mL^{−1}) in phosphate-buffered saline (PBS) showed that CpG_{cap} degraded slowly but they were significantly more stable than a double-stranded DNA (linker) (Figure S8, Supporting Information). These results indicate that the engineered CpG_{cap} are stable in the extracellular environment (e.g., serum) but may degrade in environments with high concentrations of nucleases, potentially in the endo-lysosomal vesicles that contain hydrolytic enzymes.

2.2. Cytotoxicity and Cellular Internalization of CpG_{cap}

To investigate the interactions of CpG_{cap} with immune cells, the viability of RAW264.7 cells was examined after incubation with CpG_{cap}. CpG_{cap} showed no detectable cytotoxicity at the different capsule-to-cell ratios examined after incubation for 24 h (Figure 2a), as measured using a thiazolyl blue tetrazolium bromide (MTT) cell viability assay. To compare the association of CpG_{cap}, ssDNA, and Y-CpG with RAW264.7 cells, cells were incubated with the three systems for 2 h, using the same concentration of AF488-labeled oligonucleotides, and analyzed by flow cytometry after washing three times with PBS. CpG_{cap} showed high cellular association (Figure 2b) when compared with Y-CpG and ssDNA. The association of CpG_{cap} with RAW264.7 cells was time dependent, and the percentage of positive (CpG_{cap}-associated) cells reached 50% after 2 h of incubation and plateaued at 85% after 24 h of incubation (Figure 2c). The cellular association and internalization of Y-CpG and ssDNA are likely limited by their degradation in the cell culture medium or their negative charge. Conversely, the internalization of CpG_{cap} is likely mediated by endocytosis processes as observed for other 3D DNA nanostructures.^[38,39]

To gain further insight into the cellular internalization and trafficking of CpG_{cap}, confocal laser scanning microscopy (CLSM) was used. Figure 2d shows the CLSM images of RAW264.7 cells treated with AF488-labeled ssDNA, Y-CpG, or CpG_{cap} for 24 h. Weak fluorescence was observed in cells incubated with ssDNA and Y-CpG, whereas strong green fluorescence was observed in the CpG_{cap}-treated cells. The internalization of CpG_{cap} in cells was tracked using CLSM as a function of incubation time (0.5, 2, 4, 8, 24, and 36 h). After incubation for 0.5 h, a low fluorescence signal was detected in the cells, suggesting that uptake required a longer incubation time (Figure 2e; Figure S8, Supporting Information). A progressive increase in cellular uptake of CpG_{cap} was observed as the incubation was prolonged from 0.5 to 36 h, as suggested by the strong fluorescence signal arising from internalized CpG_{cap}. Close inspection of single cells (Figure S9, Supporting Information) suggested that the internalized CpG_{cap} were smaller than the original CpG_{cap} (before internalization). Overall, these results show that CpG_{cap} are non-cytotoxic to RAW264.7 cells *in vitro*, can be efficiently internalized by cells within 2 h of incubation, and experience a reduction in size following internalization.

2.3. Deformation and Degradation of CpG_{cap} During Internalization

The reduction in size of CpG_{cap} (green fluorescence in Figure S9 in the Supporting Information) is likely due to deformation upon internalization by cells and/or degradation by DNase in the endosomes.^[40,41] To gain more insight into the mechanism of internalization of CpG_{cap}, RAW264.7 cells were imaged at the early stage of incubation (2 h) and after staining the cell membrane. Figure 3a and Figure S10 (Supporting Information) show representative images of the endocytic process. CpG_{cap} first interacted with the cell membrane and induced the formation of ruffles (Figure 3a-I). Figure 3a-II,III shows that flexible CpG_{cap} can deform during the uptake process. Previous studies have shown that soft polymeric capsules inside cells are deformed upon ingestion because of the mechanical stress exerted by the cell membrane.^[42,43] The acquired images suggest that RAW264.7 cells can exert high shear forces on CpG_{cap} during their internalization. As CpG_{cap} are highly hydrated, hollow, and flexible, they are prone to deformation upon internalization. After internalization, the CpG_{cap} enveloped by the plasma membrane are localized in subcellular vesicles (Figure 3a-IV) and likely undergo endocytic trafficking. Numerous studies have reported that DNA nanostructures, such as DNA origami, remain confined in endosomes and lysosomes.^[44] Abundant enzymes exist in endosomes/lysosomes to facilitate the degradation of proteins and oligonucleotides. Hence, DNA nanostructures are likely degraded in the endo-lysosomes after cellular internalization.^[41]

We investigated the intracellular degradation of CpG_{cap} in cells using super-resolution microscopy techniques such as STORM and structured illumination microscopy (SIM). After incubation for 2 h with cells, CpG_{cap} were both internalized and surface bound (Figure 3b,c). Notably, CpG_{cap} attached to the outer surface of cell membrane were intact (Figure 3b),

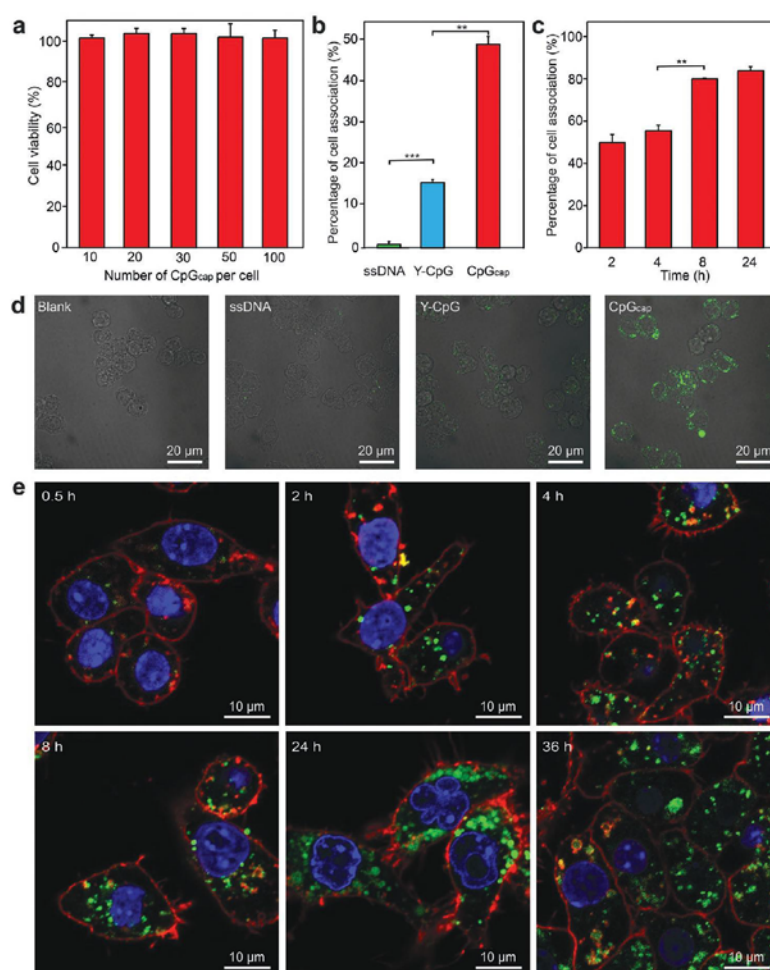


Figure 2. Cytotoxicity and cellular uptake of CpG_{cap} by RAW264.7 cells. a) MTT assay of cell viability. CpG_{cap} were incubated with cells for 24 h. b) Comparison of cell association of ssDNA, Y-CpG, and CpG_{cap} after 2 h of incubation with cells. c) Cell association kinetics of CpG_{cap} after incubation for 2, 4, 8, and 24 h. d) CLSM images of untreated cells (blank) and cells treated with ssDNA, Y-CpG, or CpG_{cap} for 2 h. e) CLSM images of cells after incubation with CpG_{cap} for 0.5, 2, 4, 8, 24, and 36 h at a capsule-to-cell ratio of 100:1. Green, AF488-labeled CpG_{cap}; blue, nuclei; and red, cell membrane. t-test: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

whereas those found inside the cells were present as small fragments with sizes ranging from 100 to 500 nm (Figure S11, Supporting Information). These results confirm that CpG_{cap} are readily degraded after internalization.

2.4. Intracellular Trafficking of CpG_{cap} in Macrophages

To further investigate the intracellular trafficking process of CpG_{cap}, the co-localization of CpG_{cap} with specific biomarkers of early endosome antigen 1 (EEA1), late endosome Ras-related protein 7 (Rab7), and lysosomal-associated membrane protein 1

(LAMP1) was analyzed. It is worth noting that TLR9 is located in the endosome of macrophages and serves as the receptor for CpG sequence recognition and immune cell activation. Hence, the endosomal accumulation of CpG_{cap} is an essential prerequisite for the efficient delivery of CpG ODNs.

After cells were incubated with AF488-labeled CpG_{cap} for 2 h, the unbound free CpG_{cap} were washed out and cells were kept under incubation in fresh medium for 2, 4, and 8 h. Cells were fixed at different time points (0–8 h), and different biomarkers, EEA1, Rab7, and LAMP1, in the cells were stained with the corresponding monoclonal antibodies. Strong co-localization of CpG_{cap} and EEA1 (protein localized in early endosome) was

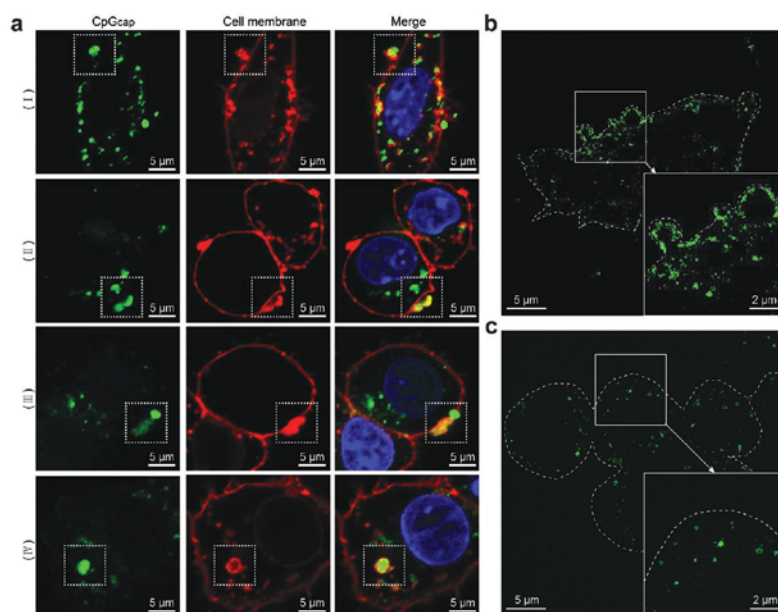


Figure 3. Microscopy images representing different stages of the macropinocytosis process. a) CLSM images of cells treated with CpG_{cap} for 2 h: I) formation of a ruffle at the cell membrane; II) association of CpG_{cap} with the cell membrane; III) macropinosome closure; and IV) internalization of CpG_{cap}. Green, AF488-labeled CpG_{cap}; blue, nuclei; and red, cell membrane. b) STORM and c) SIM images of cells treated with CpG_{cap}.

observed after incubation for 2 h and quantified using the Pearson's co-localization coefficient (PCC). The co-localization persisted 8 h postincubation (Figure 4; Figure S12, Supporting Information) as indicated by the high PCC values (0.800–0.900). CpG_{cap} showed partial co-localization with Rab7 (protein localized in late endosomes) at all of the time points (PCC $\approx$ 0.5). Interestingly, we did not observe appreciable co-localization between the fluorescence signals of CpG_{cap} and markers for lysosomes (LAMP1) over the entire incubation period of 10 h. Our data suggested that CpG_{cap} did not progress through the typical route of the endo-lysosomal pathway. Instead, over 10 h of observation, CpG_{cap} remained within the early and late endosomes, where degradation occurred to ultimately release their active cargo (CpG ODNs) at high local concentrations (4.5 million CpG motifs per capsule). This highlights the potential of these DNA capsules in engaging receptors that are localized within the endosome and subsequently trigger signaling pathways, as well as the potential for co-delivered vaccine antigens to be released for presentation by antigen-presenting cells.

2.5. Immunostimulatory Effect of CpG_{cap}

In response to CpG ODN activation, immune cells, such as dendritic cells and macrophages, secrete several cytokines, including TNF- α and IL-6, which in turn amplify immune responses to vaccines and immunotherapies.^[45] To investigate whether CpG ODN-containing CpG_{cap} efficiently induce cytokine release by macrophages, the DNA capsules (CpG concentration of

240×10^{-9} M) were incubated with RAW264.7 cells at 37 °C for 8 h. The immunostimulatory activities of CpG_{cap} and the control samples (ssDNA, Y-CpG, and GpC_{cap}) were assessed by measuring the triggered secretions of proinflammatory cytokines (TNF- α and IL-6) in the culture medium supernatant of stimulated RAW264.7 cells using an enzyme-linked immunosorbent assay (ELISA). Incubation with ssDNA- and Y-CpG-induced limited secretion of TNF- α (Figure 5a) and IL-6 (Figure 5d) in RAW264.7 cells. In contrast, CpG_{cap} induced high levels of TNF- α and IL-6 in RAW264.7 cells (Figure 5a,d). Furthermore, CpG_{cap} stimulated TNF- α and IL-6 secretion in a concentration- (Figure 5b,e) and time-dependent (Figure 5c,f) manner. To investigate the TLR9 targeting specificity of CpG_{cap} on immune activation, capsules were prepared using Y-building blocks that contained GpC dinucleotides instead of CpG (GpC_{cap}) as controls. After incubation of an equivalent number of GpC_{cap} with RAW264.7 cells, negligible secretion of TNF- α and IL-6 was observed (Figure 5a,d). This result confirms that the immunostimulatory effect of CpG_{cap} is mediated by the specific interaction between CpG ODNs and TLR9 receptor. The difference in the immunostimulatory effect exerted by CpG_{cap} could be ascribed to the more efficient cellular uptake of CpG_{cap} when compared to that of ssDNA and Y-CpG.

A single internalized CpG_{cap} can potentially deliver 4.5 million CpG motifs that are available to bind the endosomal TLR9 receptor upon degradation and disassembly. It was recently discovered that the quality and quantity of TLR9 signaling by CpG in murine dendritic cells are dependent on structural properties, especially ligand density and size of the

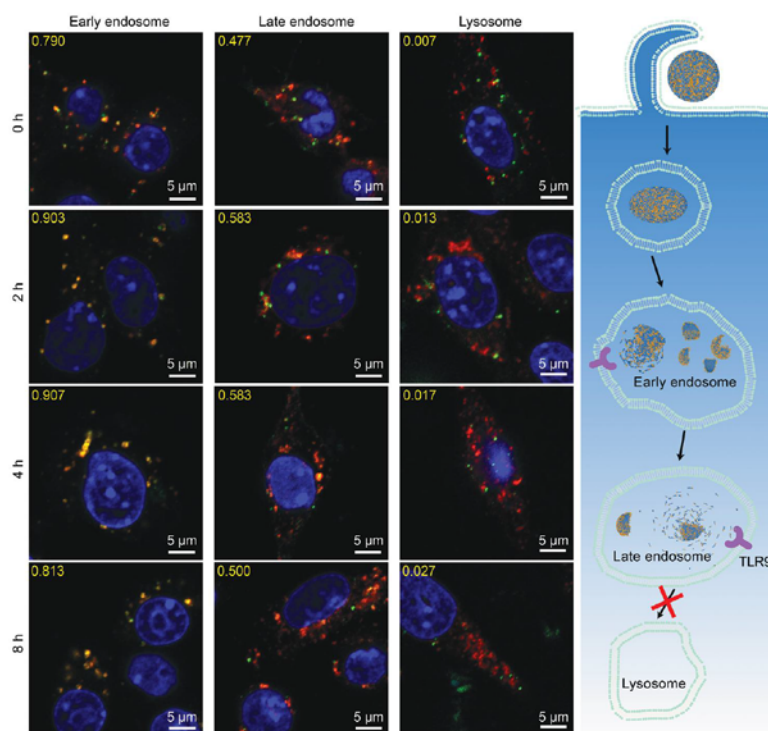


Figure 4. Intracellular trafficking of CpG_{cap} in RAW264.7 cells. CLSM images showing the intracellular trafficking of CpG_{cap} in RAW264.7 cells at varying incubation times of 0, 2, 4, and 8 h (following initial incubation for 2 h) and associated schematic. The capsule-to-cell ratio was 100:1. Compartments in cells (red) were stained with EEA1 monoclonal antibody (early endosome), anti-Rab7 monoclonal antibody (late endosome), or anti-LAMP1 monoclonal antibody (lysosome). Green, AF488-labeled CpG_{cap}; blue, nuclei; and red, early endosome/late endosome and lysosome. Pearson's co-localization coefficients are displayed in yellow.

CpG-presenting micro- and nanocarriers.^[31,39] The interaction of stimulatory CpG with TLR9 was investigated and the results indicated that activated TLR9 dimerizes prior to signaling.^[31] Therefore, we speculate that the effective dimerization and/or clustering of engaged TLR9 molecules require a high CpG density on the carrier surface or a threshold concentration of CpG must be reached for proper signaling. The dose and time dependency observed in our study are consistent with this hypothesis as a threshold of internalized capsules must be reached to observe the maximum stimulatory effect (Figure 5b,e). In addition, STORM imaging (Figure 3b; Figure S11, Supporting Information) indicated that the disassembly of CpG_{cap} into sub-micrometer debris occurred within 2 h of incubation, at which time a poor immunostimulatory effect was observed (Figure 5c,f). This indicates that the signaling process and the kinetics and magnitude of the stimulatory effect are controlled by the disassembly and degradation of CpG_{cap} to release a stimulatory CpG dose. Interestingly, the cytokine (TNF- α) release induced by the internalization of Gly-CpG_{cap} by RAW264.7 cells (Figure S13a, Supporting Information) was only slightly decreased when compared with CpG_{cap} (Figure S13b, Supporting Information). These results suggest that CpG_{cap}

should be capable of co-delivering both bioactive CpG ODNs and high-molecular-weight biomacromolecules into RAW264.7 cells and efficiently induce immune responses.

3. Conclusion

A facile method to fabricate DNA capsules comprised of immunostimulatory CpG oligodeoxynucleotides using a sacrificial template was demonstrated. The major advantage is that the microcapsules are composed entirely of the active oligonucleotides, with potential to co-load therapeutics or vaccine antigens for a synergistic effect. The DNA capsules were endowed with immunostimulatory properties by incorporating CpG ODNs into the DNA building blocks and were degradable. Macrophages internalized the CpG_{cap} effectively, which accumulated in the early and late endosomes where TLR9 was located. This led to TNF- α and IL-6 secretion caused by CpG-immune stimulation. Overall, our results demonstrate how the composition and structural properties of CpG_{cap} can directly modulate intracellular internalization, trafficking, interaction with TLR9, and ultimately the stimulatory response of macrophages. The

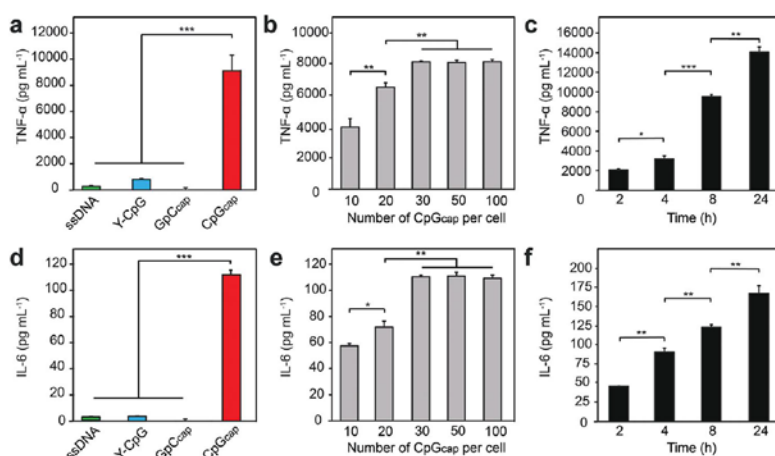


Figure 5. Cytokine release from RAW264.7 cells stimulated by CpG_{cap}. a,d) ELISA measurements of TNF-α and IL-6 release stimulated by different DNA structures after incubation for 8 h with RAW264.7 cells (concentrations of CpG motifs were equivalent, 240 × 10⁻⁹ M). b,e) TNF-α and IL-6 secretion from RAW264.7 cells incubated with CpG_{cap} at varying concentrations for 8 h. c,f) TNF-α and IL-6 secretion from RAW264.7 cells incubated with CpG_{cap} (CpG: 240 × 10⁻⁹ M, capsule-to-cell ratio of 70:1) for 2, 4, 8, and 24 h. The results are expressed as the mean of cytokine concentration ± standard deviation from three measurements. *t*-test: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

template approach of DNA capsule assembly could enable further particle engineering, including size modulation (Figure S14, Supporting Information). This is important for designing improved, tunable vaccines, and immunomodulatory therapies against cancer, infectious and autoimmune diseases. Although beyond the scope of the present study, we expect that in the subcutaneous *in vivo* environment our soft and deformable microcapsules may undergo passive drainage to lymph nodes and be internalized by peripheral APCs. DNA capsules composed entirely of DNA containing CpG ODNs can not only be employed as drug carriers, but also act as adjuvants. This highlights the potential of DNA capsules as both a vehicle for gene delivery and an immunomodulator.^[10,46,47]

4. Experimental Section

Materials: All oligonucleotides used in this study were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China). MTT, agarose, dimethyl sulfoxide (DMSO), PBS, calcium chloride dihydrate (CaCl₂·2H₂O), sodium carbonate (Na₂CO₃), poly(sodium 4-styrenesulfonate) (PSS, *M_w* = 70 kDa), Dulbecco's phosphate-buffered saline (DPBS), and EDTA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), trihydrochloride (Hoechst 33 342, 10 mg mL⁻¹), and Alexa Fluor 594 wheat germ agglutinin conjugate (AF594-WGA) were obtained from Life Technologies. PicoGreen DNA quantitation assay was from Thermo Fisher Scientific. Ultrapure water with a resistivity of >18 MΩ cm was used in all experiments and obtained from a three-stage Millipore Milli-Q Plus 185 purification system. All chemicals were used without further purification.

Minimum Information Reporting in Bio-Nano Experimental Literature (MIRIBEL): The studies conducted herein, including material characterization, biological characterization, and experimental details, conformed to the MIRIBEL reporting standard for bio-nano research, and a companion checklist of these components is included in the Supporting Information.^[48]

CaCO₃ Synthesis Protocol: CaCO₃ particles were synthesized using a previously described protocol.^[49] Briefly, PSS (5 g) was dissolved in Milli-Q water (500 mL) to form 10 mg mL⁻¹ PSS solution. Then, a precursor solution consisting of 1 M Na₂CO₃ (1 mL), 10 mg mL⁻¹ PSS (0.5 mL), and Milli-Q water (3.5 mL) was prepared. In a separate flask, 10 mg mL⁻¹ PSS solution (20 mL) was added to Milli-Q water (175 mL) with vigorous stirring. After stirring for 1 min, 1 M Ca(NO₃)₂·4H₂O solution (5 mL) was added with vigorous stirring for 2 min. The precursor solution (5 mL) was then added and the resulting mixture was stirred vigorously for 1 min. Then, the mixture was allowed to stand for 3 min, followed by further vigorous stirring for 1 min. The obtained CaCO₃ particles were dried overnight in an oven at 80 °C and subsequently ground using a mortar and pestle. The fine CaCO₃ powder was then calcined under air flow at 500 °C for 2 h to remove any organic materials.

Preparation of Y-CpG Building Block and Linker: The Y-shaped DNA building block was prepared using an enzyme-free self-assembly procedure. The building block was prepared by mixing the three oligonucleotide strands (Y-1, Y-2, and Y-3) in Tris-HCl buffer (10 × 10⁻³ M Tris-HCl, 1 × 10⁻³ M EDTA, pH 8.0, containing 150 × 10⁻³ M sodium chloride), and the final concentration of each strand was 20 × 10⁻⁶ M. The duplex linker was prepared using a similar procedure from two different oligonucleotide strands (see Table S1 in the Supporting Information). The DNA strands were annealed by heating at 95 °C for 5 min and cooled to 4 °C at a rate of 1 °C min⁻¹. Y-CpG and linker were then directly used without further purification. Y-GpC was also prepared as described above and was used to prepare the control capsules (GpC_{cap}).

Preparation of CpG_{cap}: The CpG_{cap} were synthesized using CaCO₃ particles of 2 μm in diameter as templates. The porous CaCO₃ particles (1 mg) were suspended in Milli-Q water (10 μL, 10% w/v). Then, Y (20 μL, 20 × 10⁻⁶ M) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (20 μL, 10 × 10⁻³ M, pH 7.2, 500 × 10⁻³ M sodium chloride, and 50 × 10⁻³ M magnesium chloride) were added to the CaCO₃ particle suspension with stirring at 2000 × g for 8 h. After 8 h of adsorption, the CaCO₃ particles were washed three times with HEPES buffer (100 μL) by centrifugation (2000 × g, 2 min) to remove excess Y. Then, the linker (30 μL, 20 × 10⁻⁶ M) was added to the pellet to cross-link the Y adsorbed onto the CaCO₃ particles, with stirring for 4 h at 2000 × g. The CaCO₃ particles were washed three times with HEPES buffer (100 μL) by centrifugation (2000 × g, 2 min) to remove excess linker.

To dissolve the CaCO_3 template, EDTA ($50 \mu\text{L}$, $100 \times 10^{-3} \text{ M}$, pH 7.4) was added to the pellet suspension ($20 \mu\text{L}$). After 5 min of incubation at room temperature, CpG_{cap} were obtained, which were then purified using a 40 kDa column (Zeba Spin Desalting Columns, molecular weight cutoff = 40 000, 0.5 mL). Drug (CpG) loading efficiency (85%) was measured by spectroscopic quantification of the concentration of DNA in the supernatant before and after incubation with CaCO_3 templates. The loading efficiency was calculated as follows: loading efficiency (%) = $100 \times (1 - \text{amount of Y-shaped DNA in the supernatant} / \text{total amount of Y-shaped DNA added})$.

Gel Electrophoresis Analysis: The DNA structures were analyzed by electrophoresis in a 1% agarose gel in $1 \times \text{Tris/borate/EDTA}$ buffer ($90 \times 10^{-3} \text{ M}$ Tris, $90 \times 10^{-3} \text{ M}$ boric acid, and $10 \times 10^{-3} \text{ M}$ EDTA, pH 8.0) at 80 V for 1 h at 4°C . Mobility retardation of DNA was evaluated by staining the gel with SYBR Gold reagent and imaged using a ChemiDoc XRS β Imaging System (BioRad, USA).

Characterization of CpG_{cap} : TEM analysis was conducted using an FEI Tecnai TF20 instrument at an operation voltage of 80 kV . The sample for TEM imaging was prepared by placing the test sample solution ($5 \mu\text{L}$) on formvar-carbon-coated copper grids, which were pre-cleaned using plasma. After deposition for 2 h , the grid was washed with Milli-Q water ($10 \mu\text{L}$) 15 times using a filter paper to absorb the water. Then, the grid was treated with a drop of 1% uranyl acetate for 15 s , and the excess solution was removed using a filter paper. The grids were kept at room temperature to evaporate the excess solution. A JPK NanoWizard II atomic force microscope was used to measure the thickness of CpG_{cap} . In a typical analysis, the test sample solution ($10 \mu\text{L}$) was deposited onto a clean glass for 2 h . The sample was washed by placing water ($10 \mu\text{L}$) onto the surface that was then removed using a filter paper. Washing was repeated 15 times. The sample was placed in a desiccator for 3 h and imaged in tapping mode with MikroMasch silicon cantilevers (NSC/CSG). For the thickness measurement, 20 CpG_{cap} were analyzed. The same sample preparation was used for the HIM (Orion Nanofab, Zeiss) studies. STORM images of CpG_{cap} labeled with AF633 in aqueous buffer (e.g., PBS buffer, pH 7.4, in the presence of $50 \times 10^{-3} \text{ M}$ 2-mercaptoethanol, and an oxygen-scavenging system based on glucose oxidase/catalase as per manufacturer protocol) were acquired. The images were acquired on a Nikon N-STORM system configured to achieve total internal reflection fluorescence (TIRF) imaging. The focus and TIRF angle were adjusted and tuned to maximize the signal-to-noise ratio. Fluorophores were excited by 647 nm laser lines. No activation by ultraviolet light was used. Fluorescence was collected through a Nikon 100×1.4 numerical aperture (NA) oil immersion objective and passed through a quad-band-pass dichroic filter. The dispersion of CpG_{cap} was mixed with fluorescein isothiocyanate (FITC)-dextran solutions (1 mg mL^{-1}) prepared with different molecular weights (10, 20, 60, 250, 500, and 2000 kDa) dextran. CLSM images of the capsules were taken within 10 min of mixing, and the capsules with dark interiors were considered to be impermeable, whereas capsules with interiors of similar fluorescence intensity to the outer environment were considered to be permeable. For the analysis, 100 capsules were examined. Solutions of CpG_{cap} were separately mixed with FBS (10%) and incubated at 37°C . After incubation for 0, 2, 4, 8, or 24 h , the mixtures were run on a 1% agarose gel and stained with SYBR Gold reagent. CpG_{cap} and double-stranded DNA were incubated with DNase (1 U mL^{-1}) for 1 h . Picogreen was used to fluorescently label DNA, and the fluorescence was measured using a fluorometer (Fluorolog, Horiba).

Synthesis of Positively Charged Glycogen Nanoparticles: The synthesis of positively charged glycogen nanoparticles was performed according to a previously reported protocol.^[35] Briefly, bovine liver glycogen (100 mg , Sigma-Aldrich) was dissolved in 0.6 M acetic buffer (6 mL , pH 5.0). Then, sodium periodate (0.12 mmol) was added and the reaction proceeded for 2 h with stirring while protected from light. Ethylenediamine (0.6 mmol) and sodium cyanoborohydride (1.2 mmol) were added. The reaction was incubated overnight, and the product was purified by dialysis against Milli-Q water and freeze-dried. For fluorescence labeling, the positively charged glycogen nanoparticles (4 mg mL^{-1}) were dissolved in 0.1 M sodium bicarbonate buffer (0.5 mL), pH 8.0, and mixed with 1 mg mL^{-1} Alexa Fluor 555-N-hydroxysuccinimide dye ($20 \mu\text{L}$). The reaction

proceeded for 4 h at room temperature (21°C), followed by purification on Illustra NAP-5 filter columns (GE Healthcare) according to the manufacturer's protocol.

Cell Cultures: RAW264.7 cells were purchased from the American Type Culture Collection. RAW264.7 cells with a passage number of 8–26 were cultured in complete DMEM supplied with 10% FBS at 37°C , 5% CO_2 , and 95% humidity.

MTT Assays: RAW264.7 cells were seeded into 96-well culture plates at a density of 1×10^4 cells per well overnight. After cells were incubated with CpG_{cap} at different capsule-to-cell ratios (10:1, 20:1, 30:1, 50:1, and 100:1) for 24 h , the culture medium was removed. Then, MTT solution ($150 \mu\text{L}$, 0.5 mg mL^{-1}) was added to each well, followed by 4 h of incubation at 37°C . The formazan crystals were dissolved by adding DMSO ($50 \mu\text{L}$) to each well. The absorbance of the supernatant was measured at 570 nm using a microplate reader (TECAN, InfiniteM200, Switzerland).

Flow Cytometry Analysis: RAW264.7 cells were seeded in 24-well culture plates at a density of 1×10^5 cells per well overnight. Then, cells were incubated with AF488-labeled ssDNA, Y, or CpG_{cap} (Y-2 equivalent $160 \times 10^{-3} \text{ M}$) in fresh DMEM supplemented with 10% FBS for 2 h at 37°C , harvested, and washed three times with PBS. The fluorescence intensity of the cells was then determined by flow cytometry (A50-Micro, Apogee Flow Systems Ltd.). For the time course experiments, cells were treated with AF488-labeled CpG_{cap} at 37°C for 2, 4, 8, or 24 h , harvested, and washed three times with PBS. Then, the fluorescence intensity of the cells was determined by flow cytometry.

CLSM Imaging: RAW264.7 cells were seeded at 3×10^4 cells per well in Labtek 8-well chamber slides and incubated overnight. The medium was replaced with fresh culture media. CpG_{cap} labeled with AF488 were added at a capsule-to-cell ratio of 100:1. After 0.5, 2, 4, 8, 24, or 36 h of incubation, the medium was removed. The cells were washed three times with PBS to remove unbound CpG_{cap} from the cells and to prevent nonspecific binding on the cell surface. Cells were fixed with 4% paraformaldehyde for 15 min and gently washed three times with DPBS. The membrane was stained with AF594-WGA ($5 \mu\text{g mL}^{-1}$) for 5 min , and the nucleus was stained with Hoechst 33 342 ($1 \mu\text{g mL}^{-1}$) for 10 min . The cells were imaged with a Nikon A1R confocal microscope with a 60×1.4 NA oil immersion objective. The same sample preparation was used for super-resolution imaging by SIM (OMX V4 Blaze).

Cell Imaging by STORM: For the STORM analysis, RAW264.7 cells were seeded at 3×10^4 cells per well in Labtek 8-well chamber slides and incubated overnight. The medium was replaced with fresh standard media. CpG_{cap} labeled with AF633 were added at a capsule-to-cell ratio of 100:1. After 2 h of incubation, the medium was removed. The cells were washed three times with PBS to remove unbound CpG_{cap} from the cells and to prevent nonspecific binding on the cell surface. Cells were fixed with 4% paraformaldehyde for 15 min and gently washed three times with DPBS. STORM images were acquired as per the description in "Characterization of CpG_{cap} ."

Intracellular Trafficking Evaluated by Confocal Microscopy: RAW264.7 cells were seeded at 3×10^4 cells per well in Labtek 8-well chamber slides and incubated overnight. The medium was replaced with fresh standard media. CpG_{cap} labeled with AF488 were added at a capsule-to-cell ratio of 100:1. After 2 h of incubation, the medium was removed. The cells were washed three times with PBS to remove unbound CpG_{cap} from the cells and to prevent nonspecific binding on the cell surface. Cells were incubated further in clean medium for 0, 2, 4, or 8 h . Cells were fixed with 4% paraformaldehyde for 15 min , washed, permeabilized with 0.1% Triton X-100 solution in PBS for 5 min , and washed again three times with 1% bovine serum albumin in PBS. Samples were then incubated overnight with mouse anti-EEA1 monoclonal antibody, rabbit anti-Rab7 monoclonal antibody, or rat anti-LAMP1 monoclonal antibody ($2.5 \mu\text{g mL}^{-1}$). The cells were washed three times and incubated for 1 h with goat antimouse, goat antirabbit, or goat antirat AF647 conjugate antibody ($2 \mu\text{g mL}^{-1}$). Cells were gently washed three times with DPBS and incubated with Hoechst 33 342 ($1 \mu\text{g mL}^{-1}$) for 10 min to stain the nucleus. The cells were imaged with a Nikon A1R confocal microscope with a 60×1.4 NA oil immersion objective.

Cytokine Assays: RAW264.7 cells were seeded into 96-well culture plates at a density of 1×10^4 cells per well overnight. Then, cells were incubated with ssDNA, Y-CpG, or CpG_{cap} (CpG motifs equivalent: 240×10^{-9} M) in fresh DMEM supplemented with 10% FBS. The concentration of CpG motifs was selected based on a previous study.^[37] Cells were incubated at 37 °C for 8 h (for TNF- α analysis) or 24 h (for IL-6 analysis), and the supernatants were collected and stored at 80 °C until use. The levels of TNF- α and IL-6 in the supernatants were determined by ELISA using antibody pairs specific to these cytokines, using protocols recommended by the manufacturer (TNF- α : Cat. No. 88–7324 and IL-6: Cat. No. 88–7064, Invitrogen). For the concentration-dependent experiment, cells were treated with CpG_{cap} at different capsule-to-cell ratios (10:1, 20:1, 30:1, 50:1, and 100:1) at 37 °C for 8 h (for TNF- α analysis) or 24 h (for IL-6 analysis), and the supernatants were collected and stored at –80 °C until use. For the time-course experiment, cells were treated with CpG_{cap} at a capsule-to-cell ratio of 100:1 at 37 °C for 2, 4, 8, and 24 h, and the supernatants were collected and stored at –80 °C.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

CpG motif, DNA capsules, immunostimulation, intracellular trafficking, self-assembly

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3.2 Supporting Information

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Supporting Information

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Template-Mediated Assembly of DNA into Microcapsules for
Immunological Modulation

*Yijiao Qu, Yi Ju, Christina Cortez-Jugo, Zhixing Lin, Shiyao Li,
Jiajing Zhou, Yutian Ma, Agata Glab, Stephen J. Kent, Francesca
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Supporting Information

Template-Mediated Assembly of DNA Microcapsules for Immunological Modulation

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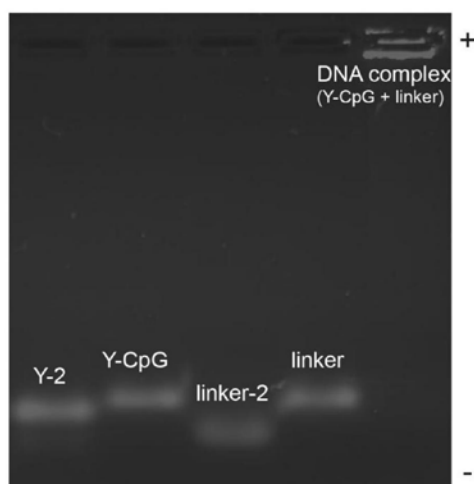
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Table S1. Sequence of oligonucleotides used in this work

Strand	Sequence ^{a)}
Y-1(CpG)	TGCAAGCTGTTGAGTC <u>GACGTT</u> AAGAGCCTGCTGAAGCGTCTGCGA
Y-2(CpG)	TGCAAGCTGTTGTCGCAG <u>ACGCTT</u> CACTGAGCACAG <u>ACGTT</u> GACGA
Y-3(CpG)	TGCAAGCTGTTGTCGTC AACGTCTGTGCTCAGGCAGGCTCTTAACGTCGACT
linker-1	CAA CAGCTT GCA TGC AGG CTT GAG CAG ACA GAG CCT TGA GCC TA
linker-2	CAA CAG CTT GCA TAG GCT CAA GGC TCT GTC TGC TCA AGC CTG CA
Y-1(GpC)	TGCAAGCTGTTGAGTGCAGCTTAAGAGCCTGCTGAAGGCTCTGGCA
Y-2(GpC)	TGCAAGCTGTTGTGCCAGAGCCTTCACTGAGCACAGAGCTTGAGCA
Y-3(GpC)	TGCAAGCTGTTGTGCTCAAGCTCTGTGCTCAGGCAGGCTCTTAAGCTGCACT
ssDNA	TGCAAGCTGTTGTCGCAG <u>ACGCTT</u> CACTGAGCACAG <u>ACGTT</u> GACGA

^{a)}The potent immunostimulatory CpG motif (GACGTT/GACGCT) is underlined.

**Figure S1.** Gel electrophoresis. Agarose gel electrophoresis of single strands and building blocks: Lane 1, Y-2; Lane 2, Y-CpG; Lane 3, linker-2; Lane 4, linker; and Lane 5, DNA complex (Y-CpG + linker).

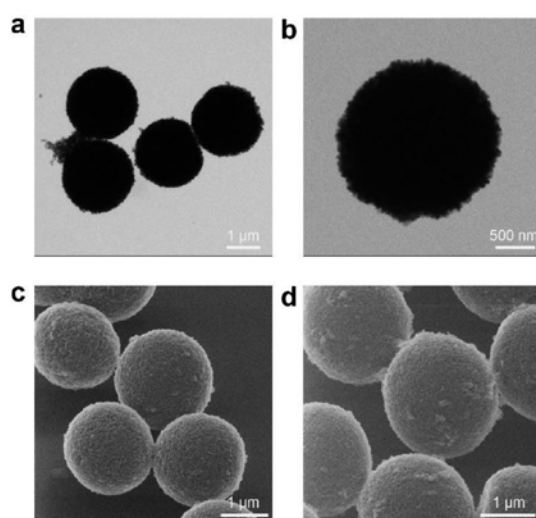


Figure S2. Characterization of CaCO_3 particles. (a) and (b) TEM images of porous CaCO_3 particles. HIM images of (c) porous CaCO_3 particles and (d) $\text{DNA}@\text{CaCO}_3$ particles.

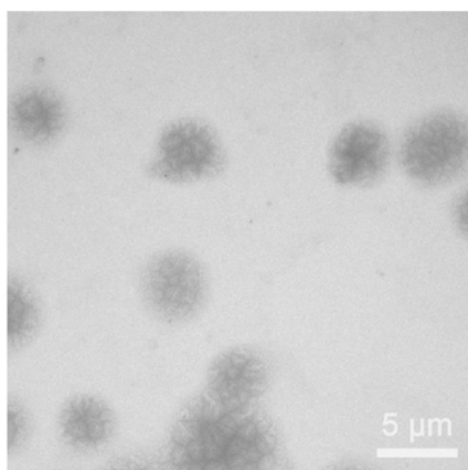


Figure S3. TEM image of CpG_{cap} .

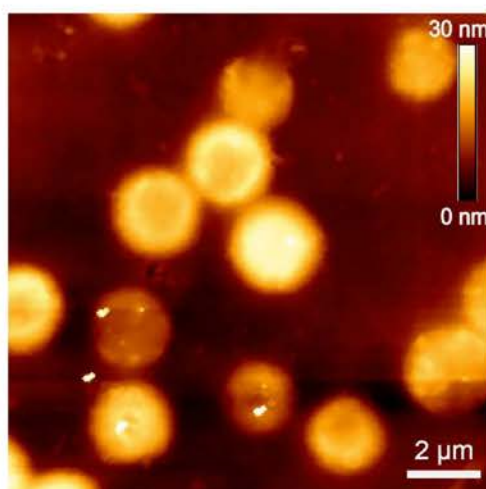


Figure S4. AFM image of CpG_{cap}.

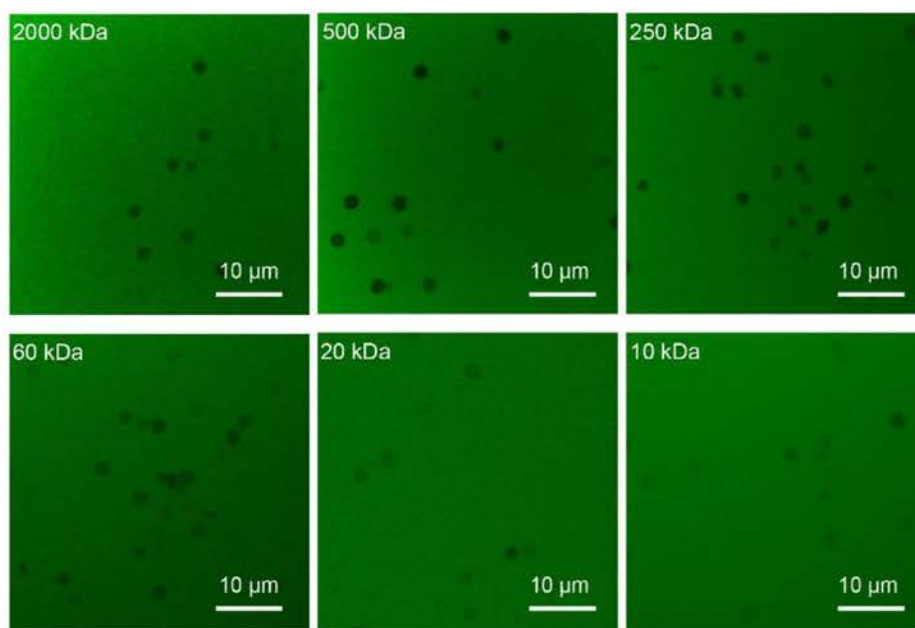


Figure S5. CLSM images of the permeation of FITC-dextran at varying molecular weights of dextran (kDa) into CpG_{cap}.

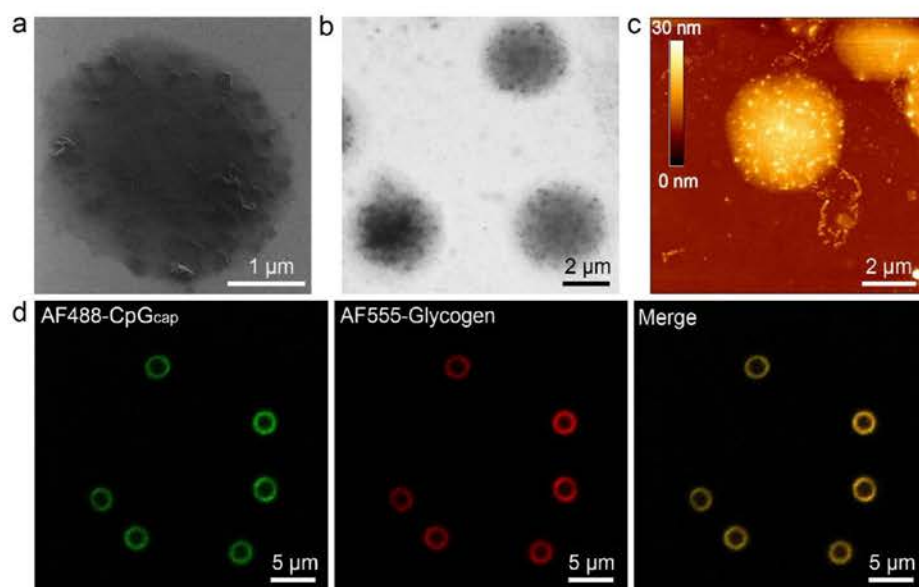


Figure S6. Characterization of Gly-CpG_{cap}. (a) HIM, (b) TEM, (c) AFM, and (d) CLSM images of Gly-CpG_{cap}.

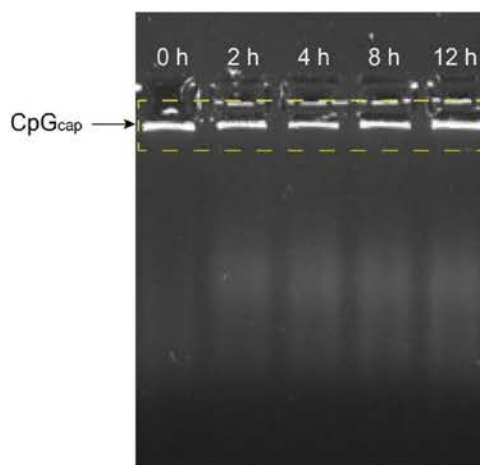


Figure S7. Electrophoretic analysis of the stability of CpG_{cap} incubated in 10% non-heat-inactivated FBS at 37 °C for 2, 4, 8, and 12 h.

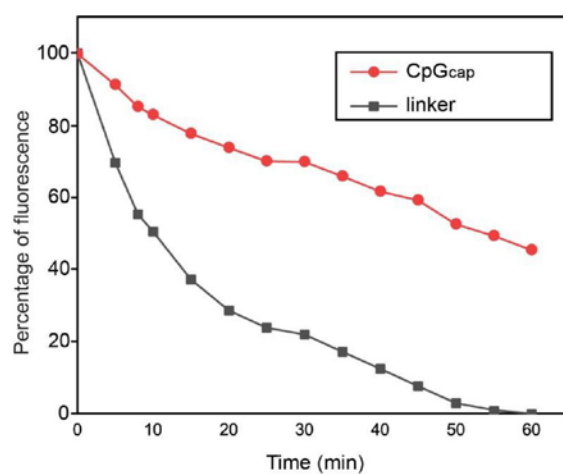


Figure S8. Degradation profiles of CpG_{cap} and double-stranded DNA (linker) by DNase (1 U mL^{-1}), as assessed by the fluorescence of the capsules. Picogreen was added to CpG_{cap} and linker to generate a fluorescence signal.

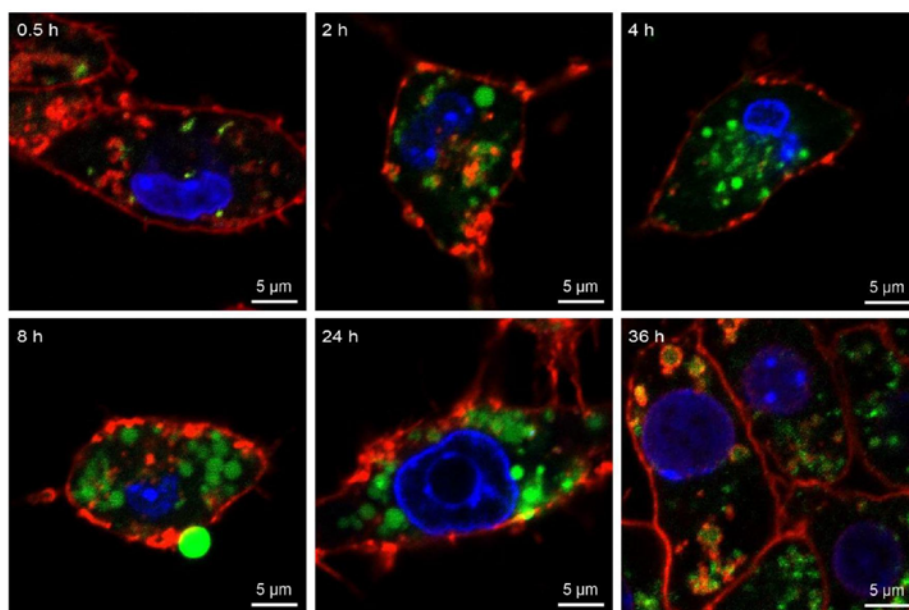


Figure S9. CLSM images of RAW264.7 cells after incubation with CpG_{cap} for 0.5, 2, 4, 8, 24, and 36 h at a capsule-to-cell ratio of 100:1. Green, AF488-labeled CpG_{cap}; blue, nuclei; and red, cell membrane.

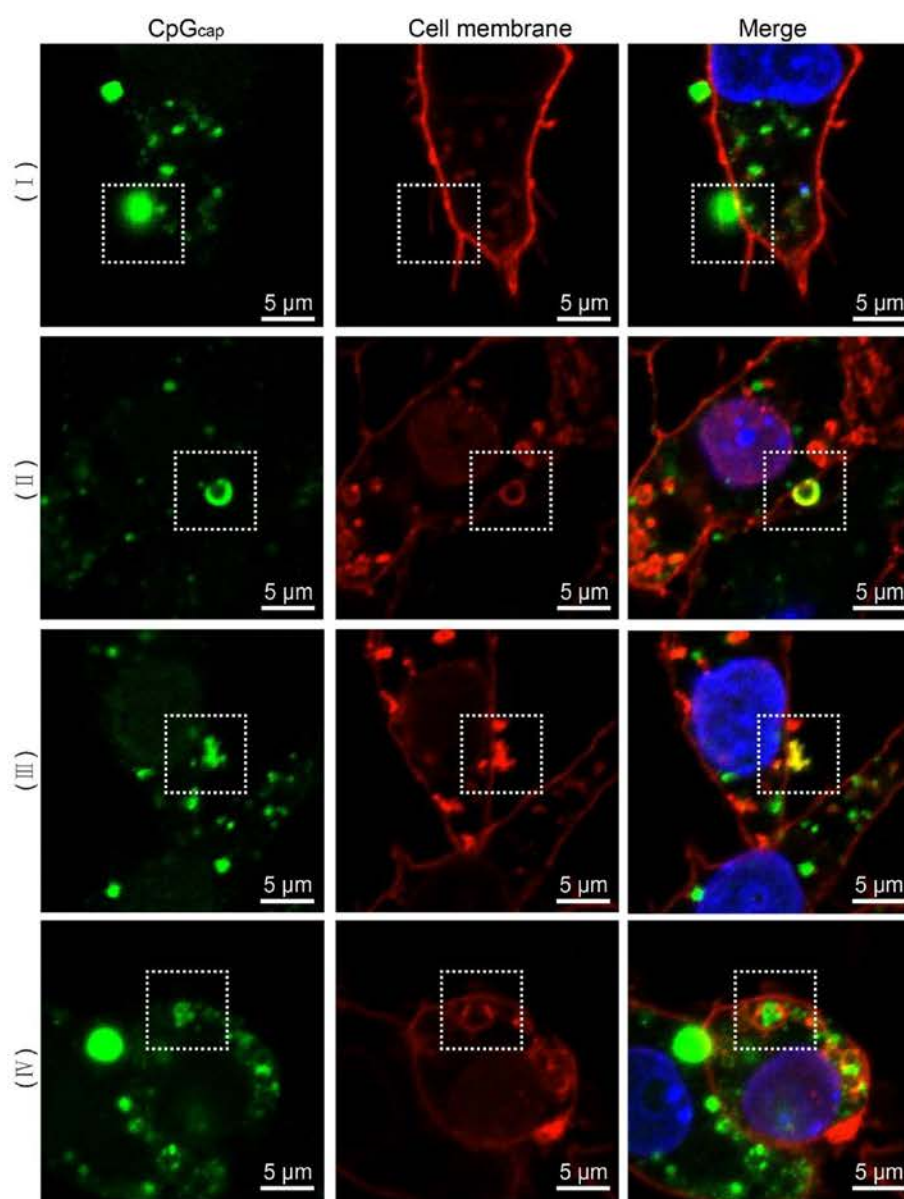


Figure S10. Microscopy images representing different stages of the micropinocytosis process. (a) CLSM images of cells treated with CpG_{cap} for 2 h: (I) formation of a ruffle at the cell membrane; (II) association of CpG_{cap} with the cell membrane; (III) macropinosome closure; and (IV) internalization of CpG_{cap}.

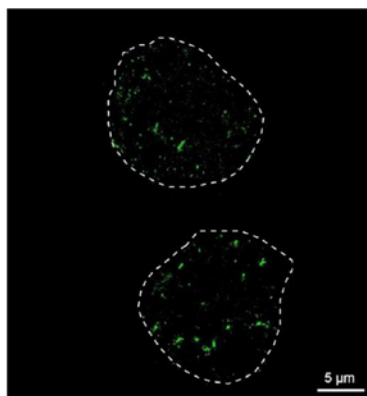


Figure S11. STORM image of RAW264.7 cells after incubation with CpG_{cap} for 2 h at a capsule-to-cell ratio of 100:1. Green, AF488-labeled CpG_{cap}.

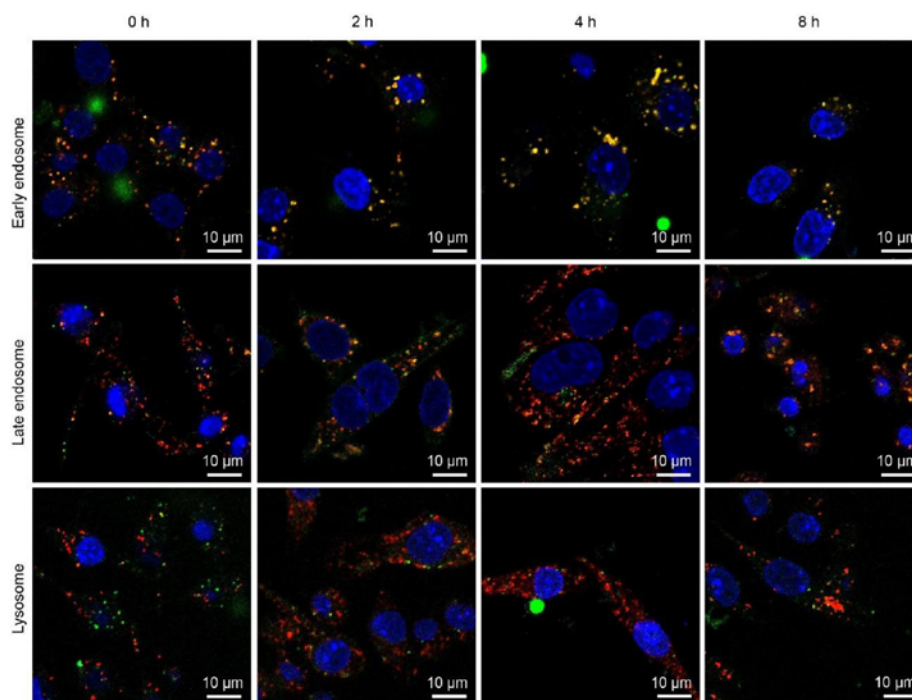


Figure S12. Intracellular trafficking of CpG_{cap} in RAW264.7 cells. CLSM images showing the intracellular trafficking of CpG_{cap} in RAW264.7 cells at varying incubation times of 0, 2, 4, and 8 h (following initial incubation for 2 h). The capsule-to-cell ratio was 100:1. Compartments in cells (red) were stained with EEA1 monoclonal antibody (early endosome), anti-Rab7 monoclonal antibody (late endosome), or anti-LAMP1 monoclonal antibody (lysosome). Green, AF488-labeled CpG_{cap}; blue, nuclei; and red, early endosome/late endosome and lysosome.

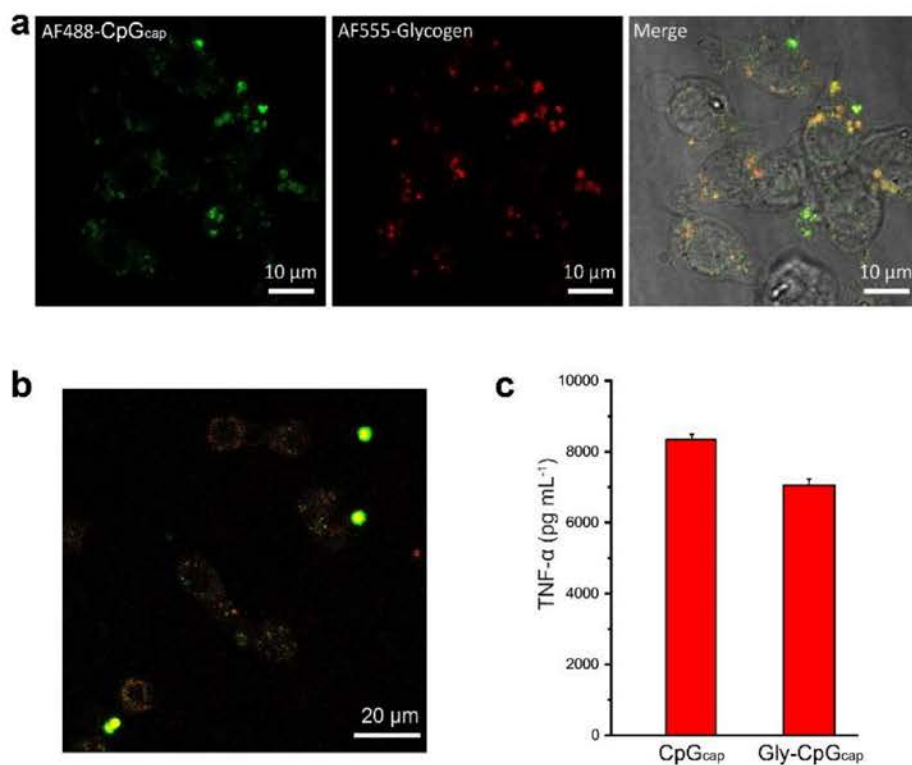


Figure S13. (a) CLSM images of Gly-CpG_{cap} incubated with cells for 24 h. Red, AF555-labeled glycogen; green, AF488-labeled CpG_{cap}; and yellow, merged images of glycogen and CpG_{cap}. (b) CLSM image of Gly-CpG_{cap} incubated with cells in cell culture medium for 2 h. (c) TNF-α secretion from RAW264.7 cells incubated with CpG_{cap} or Gly-CpG_{cap} for 8 h. The results are expressed as the mean of cytokine concentration $\pm$ standard deviation from three measurements.

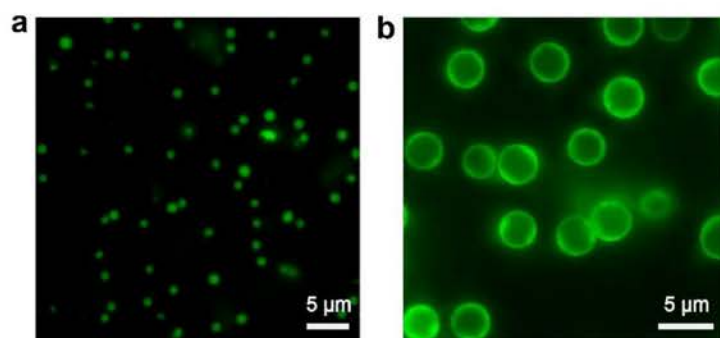


Figure S14. (a) DIC image of DNA particles using 1 μm porous silica particles as sacrificial templates. (b) DIC image of DNA microcapsules using 3.5 μm porous CaCO₃ particles as sacrificial templates.

Chapter 4

Supramolecular Polyphenol-DNA

Microparticles for Adjuvant and Antigen Co-Delivery

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4.1 Aim

DNA-based materials have attracted great interest due to the tunable structure design and encoded biological functionality of nucleic acids. Developing a simple and general approach to synthesize DNA-based materials with fine control over morphology and bioactivity is important to expand their applications.

4.2 Introduction

Nucleic acids are an important class of functional molecules with enormous potential for various biomedical applications, including diagnostics and as active agents for the modulation of cell behavior at the genetic level, e.g., gene silencing or expression, immune-modulation, and vaccines.¹⁻³ Currently, the most promising vaccines used worldwide are those against coronavirus disease 2019 (COVID-19), which use DNA and RNA in viral or non-viral assemblies to express SARS-CoV-2 viral antigen.⁴⁻⁶ Naked or free DNA and RNA molecules cannot reach intracellular targets easily due to their poor stability in the extracellular environment (e.g., sensitivity to nucleases) and low intracellular uptake (e.g., inhibited by their negative charge).⁷ Therefore, functional carriers have been developed to serve as efficient nucleic acid delivery vehicles to protect the payload from degradation and improve cellular uptake.⁷

Biomacromolecules are ideal building blocks for the construction of advanced gene and protein delivery vehicles because of their excellent biocompatibility and biodegradability.⁸⁻¹¹ In particular, polyphenols are abundant and cost-effective natural compounds that have been employed in a range of traditional industries, such as wine and tea making.¹² Due to the universal adherent property of polyphenols and their potential biomedical applications, many polyphenol-based materials have been reported recently.¹³⁻¹⁶ The chemical groups in polyphenols enable them to interact with diverse materials through a variety of interactions, such as hydrogen bonds, metal coordination, and electrostatic interactions.¹² Among them, tannic acid (TA), has been explored for transporting various drugs, enzymes, and DNA.¹⁷⁻¹⁹ For example, Lee et. al have demonstrated the use of TA and DNA to form DNA hydrogel.²⁰ However, to our best knowledge, the self-assembly of particle systems solely based on DNA and polyphenols has not been reported.

Herein, we report a template-mediated self-assembly strategy to generate DNA-TA particles stabilized by supramolecular interactions without the use of extraneous agents. We recently

used template-mediated assembly to form pure DNA capsules comprised of unmethylated cytosine-guanine (CpG) sequences as vaccine adjuvants.²¹ While these DNA capsules exhibited high immunostimulatory activity, they required specifically designed DNA sequences as crosslinkers to render the capsules stable and had limited capability of loading a wide range of nucleic acids and the co-delivery of various types of cargo, e.g., proteins, peptides, which remains challenging.^{22,23} Based on this premise, we hypothesized that supramolecular self-assembly beyond the conventional complementary base pairing could be leveraged for designing hybrid DNA particles to expand their applications.²⁴ In the current work, different morphologies (e.g., capsules and toroids) of DNA-TA particles can be achieved by using nucleic acids with different molecular weights (e.g., 46 nt to 3 kbp) and structure (e.g., ssDNA, dsDNA, and yDNA). The assembled DNA-TA particles were highly resistant to serum nucleases. The versatility of this approach for loading DNA was demonstrated by co-delivering CpG and a model antigen (ovalbumin) for immunological modulation of RAW264.7 cells, as well as plasmid DNA for gene transfection. This work shows the potential of DNA-TA particle assemblies as a platform for advanced vaccines, gene technologies, immunotherapies, and infectious diseases.

4.3 Experimental Section

4.3.1 Materials

All oligonucleotides used in this study were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China). Tannic acid (TA), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), agarose, dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, Na_2CO_3 , poly(sodium 4-styrenesulfonate) ($M_w \sim 70000$), Dulbecco's phosphate-buffered saline (DPBS), fetal bovine serum (FBS), bovine serum albumin (BSA), rat anti-lysosomal-associated membrane protein 1 (LAMP1) monoclonal antibody, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trihydrochloride (Hoechst 33342), Dulbecco's modified Eagle's medium (DMEM), Alexa Fluor 594-wheat germ agglutinin conjugate (AF594-WGA), lipofectamine RNAiMAX, and trypsin were obtained from Life Technologies. Rabbit anti-Rab7 monoclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-early endosome antigen 1 (EEA1) monoclonal antibody was purchased from BD Biosciences (San Diego, CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits of tumor necrosis factor (TNF)- α and interleukin (IL)-6 were purchased from Invitrogen. Plasmid DNA (plasmid

expressing enhanced green fluorescence protein (pEGFP, 3 kbp) was obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO, Australia). All chemicals were used as received without further purification. Ultrapure water with a resistivity of greater than 18 MΩ cm was used in all experiments and obtained from a three-stage Millipore Milli-Q Plus 185 purification system.

yDNA-1: TGCAAGCTGTTGAGTCGACGTTAAGAGCCTGCTGAAGCGTCTGCGA

yDNA-2: TGCAAGCTGTTGTCGCAGACGCTTCACTGAGCACAGACGTTGACGA

yDNA-3: TGCAAGCTGTTGTCGTC AACGTCTGTGCTCAGGCAGGCTCTTAACGTCGACT

ssDNA: TGCAAGCTGTTGTCGCAGACGCTTCACTGAGCACAGACGTTGACGA

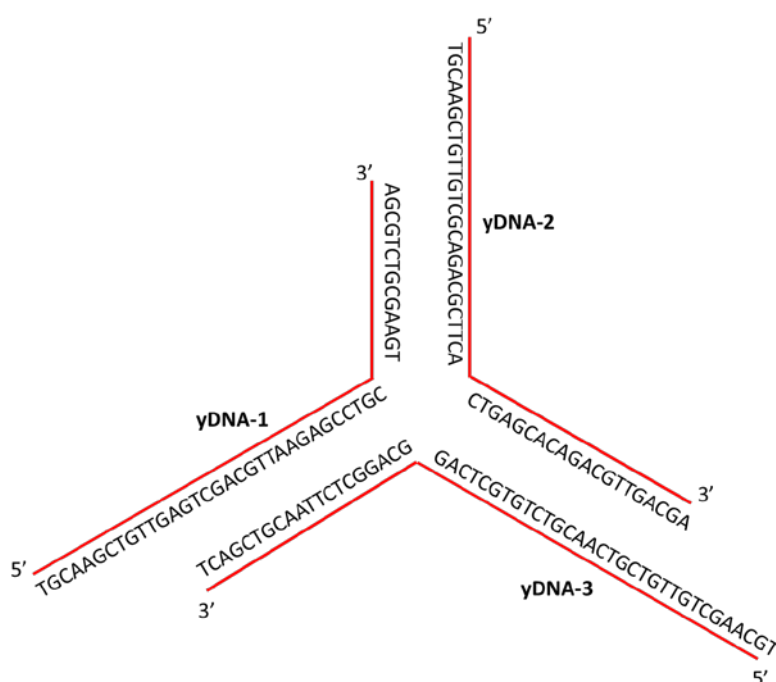
dsDNA-1: CAA CAGCTT GCA TGC AGG CTT GAG CAG ACA GAG CCT TGA GCC TA

dsDNA-2: CAA CAG CTT GCA TAG GCT CAA GGC TCT GTC TGC TCA AGC CTG CA

yGpC-1: TGCAAGCTGTTGAGTGCAGCTTAAGAGCCTGCTGAAGGCTCTGGCA

yGpC-2: TGCAAGCTGTTGTGCCAGAGCCTTCACTGAGCACAGAGCTTGAGCA

yGpC-3: TGCAAGCTGTTGTGCTCAAGCTCTGTGCTCAGGCAGGCTCTTAAGCTGCACT



Scheme 4.1. Putative structure of yDNA, which was prepared with three ODNs (yDNA-1, yDNA-2, yDNA-3) with the halves of each ODN being partially complementary to a half of the other two ODNs.

4.3.2 Preparation of yDNA

The Y-shaped DNA (yDNA) building block containing cytosine–guanine (CpG) sequences was prepared by mixing three oligonucleotide strands yDNA-1, yDNA-2 yDNA-3, in Tris-HCl buffer (10×10^{-3} M Tris-HCl, 1×10^{-3} M EDTA, pH 8.0, containing 150×10^{-3} M NaCl) and the final concentration of each strand was 20 μ M. The mixtures were heated at 95 °C for 5 min and slowly cooled to 4 °C at a rate of 1 °C min⁻¹. yDNA strands were then directly used without further purification. As a control, yDNA strands containing GpC sequences were prepared by mixing three oligonucleotide strands (yGpC-1, yGpC-2, and yGpC-3), as described above.

4.3.3 Preparation of DNA-TA Particles

The DNA–TA particles were synthesized using CaCO₃ particles (2 μ m) as templates (details of the synthesis in Chapter 3). The CaCO₃ particles (1 mg) were suspended in 10 μ L of Milli-Q water. Next, 20 μ L of yDNA (20 μ M) and 20 μ L of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM, pH 7.2, 500 mM NaCl, 50 mM MgCl₂) were added. The mixture was then incubated for 8 h under stirring. After 8 h of incubation, TA (10 μ L, 40 mg mL⁻¹) was added to the solution for 1 min. The coated CaCO₃ particles were washed with Milli-Q water three times to remove excess TA by centrifugation (2000 *g*, 2 min). The CaCO₃ template was dissolved by adding 100 μ L of EDTA (100 mM, pH 4) to the pellet suspension. Following dissolution for 5 min at room temperature, the resulting yDNA–TA particles were washed with Milli-Q water three times by centrifugation (2000 *g*, 2 min). Using different DNA building blocks that is single-stranded DNA (ssDNA; 10 μ L, 120 μ M), double-stranded DNA (dsDNA; 30 μ L, 20 μ M), and plasmid (20 μ L, 0.35 mg mL⁻¹), and 20 μ L HEPES buffer, ssDNA–TA particles, dsDNA–TA particles, and plasmid–TA particles were prepared following the same protocol. To prepare ovalbumin (OVA)–yDNA–TA particles, OVA (10 μ L, 1 mg mL⁻¹) was incubated with 200 μ L of yDNA–TA particles at room temperature for 0.5 h, then washed with Milli-Q water three times by centrifugation (2000 *g*, 2 min) to remove free OVA.

4.3.4 Characterization of TA-DNA Particles

An FEI Tecnai TF20 instrument at an operation voltage of 120 kV was used for transmission electron microscopy (TEM) analysis. Prior to TEM imaging, 5 μ L of sample solution was deposited on formvar carbon-coated copper grids that were precleaned using plasma. A JPK NanoWizard II atomic force microscope (AFM) was used to measure the thickness of the particles. Prior to AFM imaging, 10 μ L of sample solution was dropped onto a clean glass

substrate overnight and imaged with MikroMasch silicon cantilevers (NSC/CSC) in tapping mode. An FEI Quanta 200 field-emission scanning electron microscope (SEM) was used to capture the particles operating at an accelerating voltage of 10 kV. The dried samples were sputter-coated with gold prior to SEM imaging. ssDNA-TA particles, dsDNA-TA particles, and plasmid-TA particles were characterized by the same method. The ζ -potentials of the particles were measured in Milli-Q water on a Zetasizer Nano-ZS instrument (Malvern Instrument, UK).

4.3.5 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

For the analysis, a suspension (100 μ L) of yDNA-TA particles were incubated in 1 mL DMEM containing 10% FBS for 1 h at 37 °C. The particles were then washed with PBS solution three times to obtain protein corona-coated particles. NuPAGE LDS sample loading buffer was used to strip the adsorbed proteins from the particles at 70 °C for 10 min. After transferring the eluted proteins to a new tube, 50 mM dithiothreitol, a reducing agent, was added to cleave the disulfide bonds in the proteins at 70 °C for 10 min. The gel was run at 200 V for 50 min.

4.3.6 Agarose Gel Electrophoresis Analysis

The stability of the yDNA-TA particles were analyzed by electrophoresis in a 1% agarose gel in $1 \times$ Tris/borate/EDTA buffer (90×10^{-3} M Tris, 90×10^{-3} M boric acid, and 10×10^{-3} M EDTA, pH 8.0) at 80 V for 1 h. SYBR Gold was used to stain the gel to observe the mobility retardation of DNA. Then, the gel was imaged using a ChemiDoc XRS p Imaging System (BioRad, USA). Degradation of the yDNA-TA particles by DNase was conducted under the same conditions.

4.3.7 Cell Culture

RAW264.7 cells, HeLa cells, and HEK293T cells were purchased from the American Type Culture Collection. RAW264.7 cells with a passage number of 18–36, HeLa cells with a passage number of 5–10, and HEK293T cells with a passage number of 23–35 were cultured in complete DMEM supplied with 10% FBS at 37 °C, 5% CO₂, and 95% humidity.

4.3.8 MTT Assay

RAW264.7 or HeLa cells were plated into 96-well culture plates at a density of 1×10^4 cells per well for 24 h. The yDNA-TA particles at different particle-to-cell ratios (10:1, 20:1, 30:1, 50:1, and 100:1) were added to cells and incubated for 24 h. After the removal of the cell culture medium, MTT solution (150 μ L, 0.5 mg mL⁻¹) was added to each well and incubated at 37 °C for 4 h. Then, 50 μ L of DMSO was added to each well to dissolve the formazan crystals. A

microplate reader (TECAN, InfiniteM200, Switzerland) was used to measure the absorbance of the supernatant at 570 nm.

4.3.9 Flow Cytometry Analysis

RAW264.7 cells were plated in a 24-well culture plate at a density of 1×10^5 cells per well for 24 h. The AF488-labeled yDNA–TA particles were then added at a particle-to-cell ratio of 100:1 and incubated with the cells at 37 °C for 2, 4, 8, or 24 h. After harvesting and washing with PBS three times, the fluorescence intensity of the cells was determined by flow cytometry (A50-Micro, Apogee Flow Systems Ltd.). For the time-course experiment, RAW264.7 cells were plated in a 24-well culture plate at a density of 1×10^5 cells per well for 24 h. The AF488-labeled yDNA–TA particles were then added at a particle-to-cell ratio of 100:1 and incubated with the cells for 2 h. The medium was then discarded and any unbound yDNA–TA particles were removed by washing the cells with PBS three times. Following a further 8, 24, and 48 h period of incubation, the fluorescence intensity of the cells was determined by flow cytometry.

4.3.10 CLSM Imaging

RAW264.7 cells were plated in Labtek 8-well chamber slides at 3×10^4 cells per well and incubated for 24 h. The AF488-labeled yDNA–TA particles were added at a particle-to-cell ratio of 100: 1 for 0.5, 1, 2, 4, 8, 24, or 36 h of incubation. Then, cells were washed with PBS three times and fixed with 4% paraformaldehyde for 15 min. After gently washing with PBS three times, the cell membrane was stained with AF594–WGA ($5 \mu\text{g mL}^{-1}$) for 5 min, and the cell nucleus was stained with Hoechst 33342 ($1 \mu\text{g mL}^{-1}$) for 10 min. The cells were then imaged with a Nikon A1R confocal microscope fitted with a 60× 1.4 NA oil immersion objective.

4.3.11 Intracellular Trafficking Evaluated by CSLM

RAW264.7 cells were plated in Labtek 8-well chamber slides at 3×10^4 cells per well and incubated for 24 h. The AF488-labeled yDNA–TA particles were added at a particle-to-cell ratio of 100:1 for 2 h. Then the cells were washed with PBS three times to remove unbound yDNA–TA particles and incubated further in fresh medium for 2, 4, or 8 h. Then, cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 solution in PBS for 5 min, washing with 1% BSA in PBS three times during each step. Mouse anti-EEA1 monoclonal antibody, rabbit anti-Rab7 monoclonal antibody, or rat anti-LAMP1 monoclonal antibody ($2.5 \mu\text{g mL}^{-1}$) was added to the samples and incubated overnight. After

washing with 1% BSA three times, cells were incubated with goat anti-mouse, goat anti-rabbit, or goat anti-rat AF647 conjugated antibody ($2\ \mu\text{g mL}^{-1}$) for 1 h. Cells were gently washed with DPBS three times and incubated with Hoechst 33342 ($1\ \mu\text{g mL}^{-1}$) for 10 min to stain the nucleus. The cells were imaged with a Nikon A1R confocal microscope equipped with a 60×1.4 NA oil immersion objective.

4.3.12 Cytokine Assays

RAW264.7 cells were plated into 96-well culture plates at a density of 5×10^4 cells per well and incubated overnight. Then, cells were incubated with yDNA, yDNA–TA particles, OVA, or OVA–yDNA–TA particles (yDNA equivalent $120\ \text{nM}$, OVA equivalent $500\ \mu\text{g mL}^{-1}$) in fresh medium. Cells were incubated at $37\ ^\circ\text{C}$ for 8 h (for TNF- α analysis) or 24 h (for IL-6 analysis). The levels of TNF- α and IL-6 in the supernatants were determined by ELISA using antibody pairs specific to these cytokines and protocols recommended by the manufacturer. For the concentration-dependent experiment, the yDNA–TA particles at different particle-to-cell ratios were added to cells and incubated at $37\ ^\circ\text{C}$ for 8 h (for TNF- α analysis) or 24 h (for IL-6 analysis), and the cytokine level in the supernatants was measured. For the time-dependent experiment, the yDNA–TA particles at a particle-to-cell ratio of 100:1 were added to the cells and incubated at $37\ ^\circ\text{C}$ for 2, 4, 8, and 24 h, and the cytokine level in the supernatants was measured. For the time-course experiment, the yDNA–TA particles at a particle-to-cell ratio of 100:1 were added to the cells and incubated at $37\ ^\circ\text{C}$ for 2, 4, 8, 24, and 48 h (following initial incubation for 2 h), and the cytokine level in the supernatants was measured.

4.3.13 In vitro Transfection by Flow Cytometry and CSLM

HEK293T cells were plated in 24-well plates at a density of 4×10^4 per well overnight. pEGFP–TA particles were then added to the cells ($1\ \mu\text{g pEGFP}$ per well). The lipofectamine–pEGPF complex was used as the positive control, whereas naked pEGFP was used as the negative control. Following incubation for 4 h, the serum-free media was changed with fresh medium. After further incubation for 72 h, the transfected cells were harvested and washed with PBS once. The gene transfection efficiency was evaluated by flow cytometry by scoring the percentage of cells expressing EGFP. HEK293T cells were plated in Labtek 8-well chamber slides at 2×10^4 cells per well and incubated overnight. Then pEGFP–TA particles were added to the cells ($1\ \mu\text{g pEGFP}$ per well). The transfected cells were imaged with a Nikon A1R confocal microscope fitted with a 60×1.4 NA oil immersion objective.

4.4 Results and Discussion

4.4.1 Synthesis and Characterization of yDNA-TA Particles

Microparticles were prepared using template-mediated supramolecular assembly of tannic acid (TA) and Y-shaped double-stranded DNA units (yDNA). First, yDNA was prepared by hybridization of three complementary single strands (Table S1), which were adsorbed onto the CaCO_3 porous template (2 μm in diameter) via electrostatic interactions. Next, the particles were mixed with an aqueous solution of TA to cross-link the immobilized yDNA by forming a network as shown in **Figure 4.1a**. The CaCO_3 template was dissolved using ethylenediaminetetraacetic acid solution, yielding yDNA-TA particles stabilized by the interactions between DNA and TA. The yDNA-TA particles showed a negative zeta potential (-24.5 mV) in water (**Figure 4.1b**). We estimated that each yDNA-TA particle is composed of approximately 2.4 million yDNA units, by quantifying the yDNA left in the supernatant. Confocal laser scanning microscopy (CLSM) image of yDNA labeled with Alexa-488 (AF488) showed uniform well-dispersed yDNA-TA particles in an aqueous solution (**Figure 4.1c**), approximately $1.5 \pm 0.1 \mu\text{m}$ in size. Transmission electron microscope (TEM), scanning electron microscopy (SEM), and 3D images confirmed the well-defined toroid-like geometry of yDNA-TA particles (**Figure 4.1d and e** and **Figure S4.1**). The thickness of yDNA-TA particles was around 400 nm was measured by atomic force microscopy (AFM) (**Figure S4.2**). The interaction mechanism between DNA and TA was explored using a hydrogen bond breaking agent (e.g., urea), surfactants (e.g., Tween 20 and sodium dodecyl sulfate), and organic solvents (e.g., tetrahydrofuran and dimethyl sulfoxide). The particles were disassembled to a different extent in 100 mM urea, sodium dodecyl sulfate, Tween 20, dimethyl sulfoxide, and tetrahydrofuran (**Figure S4.3**) as confirmed by counting the intact particles by flow cytometry. Considering their distinct behaviors in these media, we infer that both hydrogen bonding and π - π stacking interactions are involved in stabilizing these particles.

We speculate that the efficient infiltration and TA-mediated crosslinking of yDNA into the porous CaCO_3 template results in the toroidal morphology observed for yDNA-TA particles, which have thick and compact shells likely not gas permeable. Consequently, when EDTA was added to remove templates, the pressure of the produced CO_2 inside may cause the shell breakage and form holes at the opposite sides of the particle. To confirm this hypothesis, CaCO_3 templates with different sizes were employed to synthesize yDNA-TA microparticles. Both SEM and TEM images displayed that these yDNA-TA particles had a toroidal shape and

shrank approximately 50% after templates dissolution (2.8 μm to 1.5 μm , 4 μm to 2 μm , and 7 μm to 3.2 μm) (**Figure S4.4**). Interestingly, thin-shelled microcapsules were obtained when the opposite deposition sequence (TA then yDNA, denoted as TA-yDNA) was used, indicating that morphological diversity can be achieved by simply switching the building block deposition order. SEM and TEM images showed the morphology of obtained thin shell capsules with a diameter around $1.5 \pm 0.2 \mu\text{m}$ (**Figure 4.1f and g**). The thickness of TA-yDNA capsules was approximately 70 nm as determined by AFM (**Figure S4.5**), which was significantly thinner than the 400 nm shell thickness of yDNA/TA particles. The adsorption of the highly adherent TA on the porous CaCO_3 template likely caused clogged pores limiting the deposition of the yDNA to the outermost layer and enabling the formation of a thin and gas-permeable yDNA-TA network. The ability of the CO_2 produced during the dissolution of the core to diffuse out likely results in a more uniform shell and capsules-like morphology.

To further display the versatility of the present method, a high molecular weight circular plasmid DNA (3 kbp) was used to synthesize plasmid-TA capsules (loading plasmid first followed by TA). The plasmid-TA capsules had thin shells as observed by SEM and TEM (**Figure 4.1h and i**). Single-stranded linear oligonucleotides (ssDNA) and double-stranded linear oligonucleotides (dsDNA) were also used to generate DNA-TA particles (**Figure S4.6**). Overall, these results indicate the general applicability of this method to fabricate supramolecular assemblies of DNA with TA-two natural and biodegradable biomolecules, without resorting to other polymers. Uniform DNA-TA particles with different morphology could be simply synthesized by using different types of DNA and molecular weights, including ssDNA, dsDNA, yDNA, and plasmid. The functionality of the various DNA combined with sequence specificity that can be encoded into the DNA-TA system make them attractive for different applications such as immunotherapy and gene delivery.

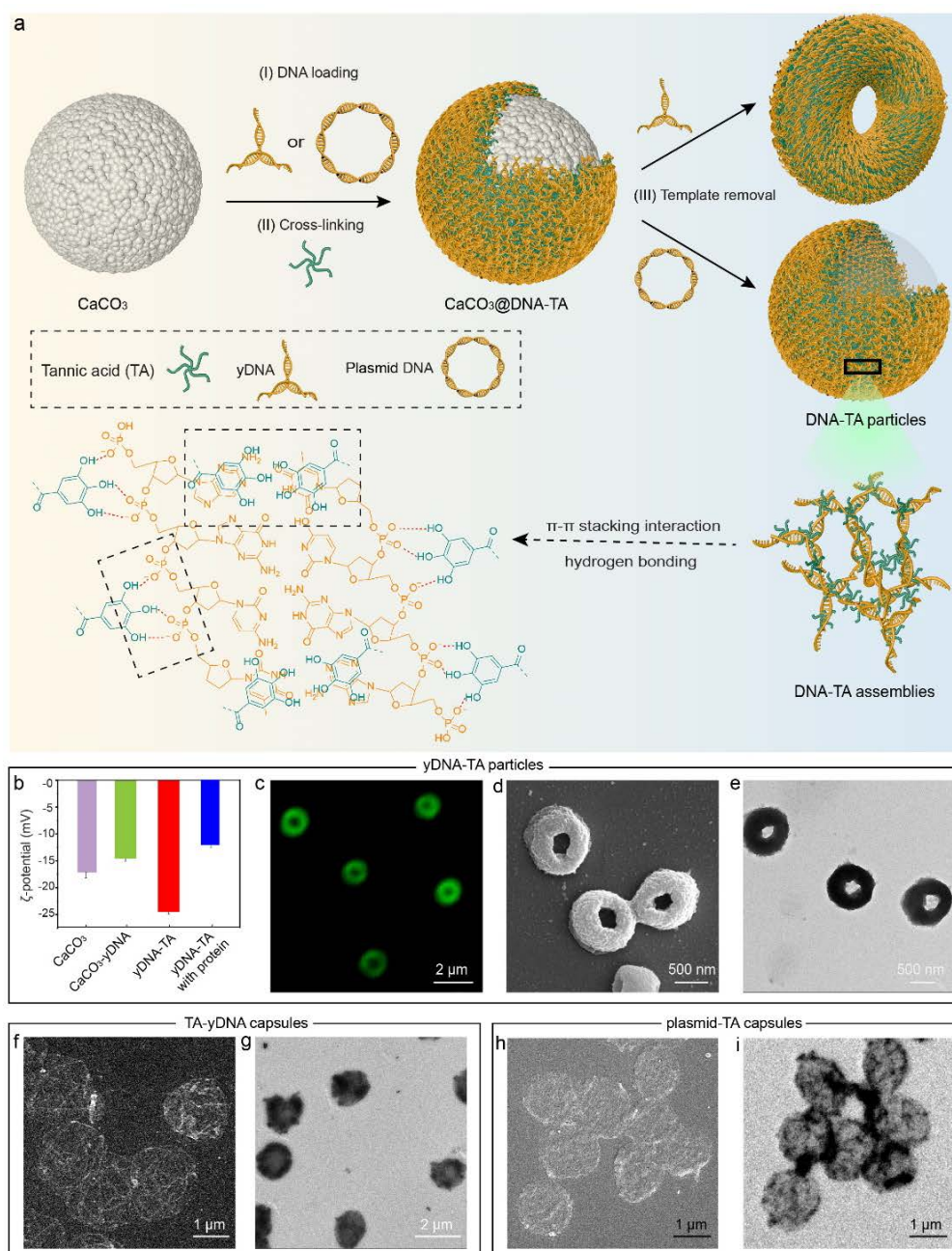


Figure 4.1. Preparation and characterization of DNA-TA and TA-DNA particles. (a) Schematic representation of the assembly of DNA-TA particles: (I) loading of CaCO_3 particles with DNA (yDNA or plasmid DNA); (II) cross-linking by TA; and (III) removal of CaCO_3 template core to yield DNA-TA particles. The possible interactions involved in the stabilization of the DNA-TA assemblies are shown. (b) ζ -Potentials of the templates, yDNA-loaded templates, yDNA-TA particles (after template removal), and protein corona-coated yDNA-TA particles. (c) CLSM, (d) SEM, and (e) TEM images of the yDNA-TA particles. (f) SEM and (g) TEM images of the TA-yDNA capsules. (h) SEM and (i) TEM images of the plasmid-TA capsules.

4.4.2 Serum Stability and Cell Interactions of yDNA-TA Particles

Stability in the extracellular environment and intracellular biodegradability are prerequisites for effective intracellular drug delivery. Hence, the stability and biodegradability of yDNA-TA particles were investigated. After incubation with cell culture medium containing 10% fetal bovine serum (FBS), the ζ -potential of yDNA-TA particles changed from -25 mV to -12.5 mV, owing to the adsorption of the proteins from the serum to form a protein corona on the particle (Figure 4.1b). Polyacrylamide gel electrophoresis further confirmed that serum proteins were absorbed (**Figure S4.7**).²⁵ yDNA-TA particles maintained the toroidal structure after 24 h incubation with 10% FBS medium as shown by CLSM and SEM images (**Figure S4.8** and **Figure S4.9**). Furthermore, agarose gel electrophoresis was used to show the stability of yDNA-TA particles incubated with 10% FBS medium at 37 °C (**Figure 4.2a** and **Figure S4.10**). After incubation in FBS for 24 h, yDNA-TA particles maintained intact structure which demonstrated the stability of yDNA-TA particles in medium. DNase (1 U/mL) was used to show the biodegradability of yDNA-TA particles; the yDNA embedded in the particles appeared to be slowly degraded in the presence of DNase (**Figure 4.2b** and **Figure S4.11**). These results confirm the stability yDNA-TA particles, which may be attributed to their compact structure that make yDNA less accessible to the enzymes present. In the case of endo/lysosomal compartments which are rich with nucleases, yDNA-TA particles could potentially be degraded. The high stability and biodegradability of yDNA-TA particles suggest their high potential use for intracellular drug delivery.

The cytotoxicity of yDNA-TA particles was examined by incubating them with RAW264.7 and HeLa cells for 24 h. yDNA-TA particles didn't show detectable cytotoxicity to RAW264.7 cells at the different particle-to-cell ratios (i.e., from 50 to 400) for 24 h incubation (**Figure S4.12** and **Figure S4.13**). To examine the internalization efficiency of yDNA-TA particles, cell association experiments were performed using AF488 labeled yDNA and flow cytometry analysis. The association of yDNA-TA particles with RAW264.7 cells was in a time-dependent manner and the percentage of positive (yDNA-TA particles associated) cells reached 50% after 1 h and 90.5% after 8 h of incubation (**Figure 4.2c**). The continuous internalization of yDNA-TA particles as a function of incubation time (0.5, 1, 2, 4, 8, 24 h, and 36 h) was monitored using CLSM (**Figure 4.2d** and **Figure S4.14**). After incubation for 0.5 h, negligible internalization of yDNA-TA particles was observed. An increase in cellular uptake of yDNA-TA particles with increasing incubation time from 0.5 to 36 h was observed (Figure S4.14). For example, 3.3 ± 0.4 yDNA-TA particles per cell were observed at 24 h. Collectively, these

results indicate that yDNA-TA particles are safe to RAW264.7 cells and can be internalized within 2 h of incubation with the internalization of particles increasing for longer incubation.

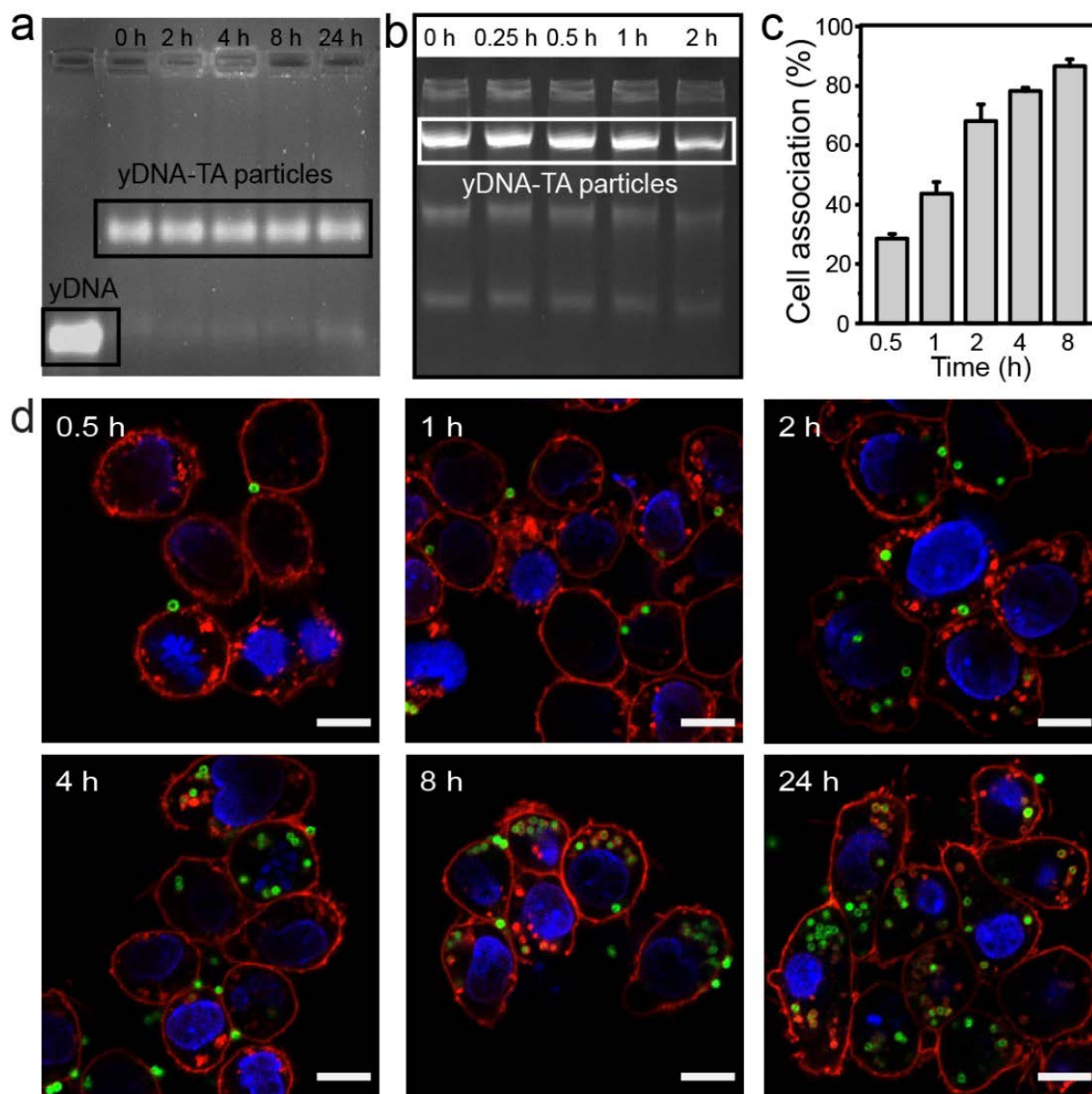


Figure 4.2. Stability and cellular interactions of the yDNA-TA particles with RAW264.7 cells. (a) PAGE analysis of the yDNA-TA particles incubated in 10% FBS at 37 °C for 2, 4, 8, and 24 h. (b) Agarose gel electrophoresis analysis of the yDNA-TA particles incubated in DNase (1 U mL⁻¹) at 37 °C for 0.25, 0.5, 1, and 2 h. (c) Time-dependent association of the yDNA-TA particles with RAW264.7 cells. (d) CLSM images of cells after incubation with the yDNA-TA particles for 0.5, 1, 2, 4, 8, and 24 h. Green, AF488-labeled yDNA-TA particles; blue, cell nuclei; red, cell membrane. Scale bars: 10 μm.

4.4.3 Degradation and Intracellular Trafficking of yDNA-TA Particles

The intracellular degradation of yDNA-TA particles was investigated by incubating RAW264.7 cells with yDNA-TA particles labeled by AF488. After 2 h incubation, washed out the non-associated yDNA-TA particles and kept incubating cells in fresh medium for 8, 24, and 48 h, respectively. Cells were collected at different time points and the mean fluorescence intensity (MFI) of cells was determined by flow cytometry. We observed that the MFI of cells decreased as a function of time, indicating that yDNA-TA particles were either degraded by the intracellular nuclease or eliminated through the process of exocytosis (**Figure 4.3a**). To determine the actual size of yDNA-TA particles deployed in the cytosol with a nanometric resolution we employed structured illumination microscopy (SIM). Compared to free yDNA-TA particles (**Figure 4.3b**), the diameter of intracellular yDNA-TA particles decreased from $1.5 \pm 0.1 \mu\text{m}$ to $1.3 \pm 0.1 \mu\text{m}$ (**Figure 4.3c and d**).

To directly visualize the colocalization of yDNA-TA particles with organelles, specific biomarkers of early endosome antigen 1 (EEA1), late endosome Ras-related protein 7 (Rab7), and lysosomal-associated membrane protein 1 (LAMP1) were used. After 2 h incubation with cells, the unassociated AF488-yDNA-TA particles were washed out and cells were incubated in fresh medium for further 2, 4, 8 h, and 24 h. At different time points, cells were fixed, and endo/lysosomes were stained with the corresponding antibodies. In the first 10 h incubation, yDNA-TA particles appear surrounded by late endosomes and/or lysosomes, whereas we did not observe appreciable colocalization with the early endosomes for the entire observation period (**Figure 4.3e** and **Figure S4.15**). The results suggest that after internalization yDNA-TA particles were rapidly translocated from early endosomes to late endosomes and lysosomes. Interestingly, at a prolonged incubation time (24 h) yDNA-TA particle did not show colocalization with the endocytic organelles, appeared smaller in size, and less fluorescent. Overall, this study suggested that the yDNA-TA particle may undergo partial degradation or disassembly by exposure to endo/lysosomal enzymes and subsequent translocation in the cytosol. A similar result was observed in HeLa cells, where yDNA-TA particles appear to escape endo/lysosome after 24 h (**Figure S4.16**). The limited pH buffering capacity of TA in the “pH change window” (pH 5-6.3) of the vesicles may facilitate the endosomal escape by the “proton sponge effect” mechanism.²⁶ The basis of endosome escape of yDNA-TA particle is not well understood and is still under investigation. Taken together, these results indicate that yDNA-TA particles undergo erosion during endo/lysosomal trafficking and can potentially allow the release of nucleic acids cargo intracellularly.

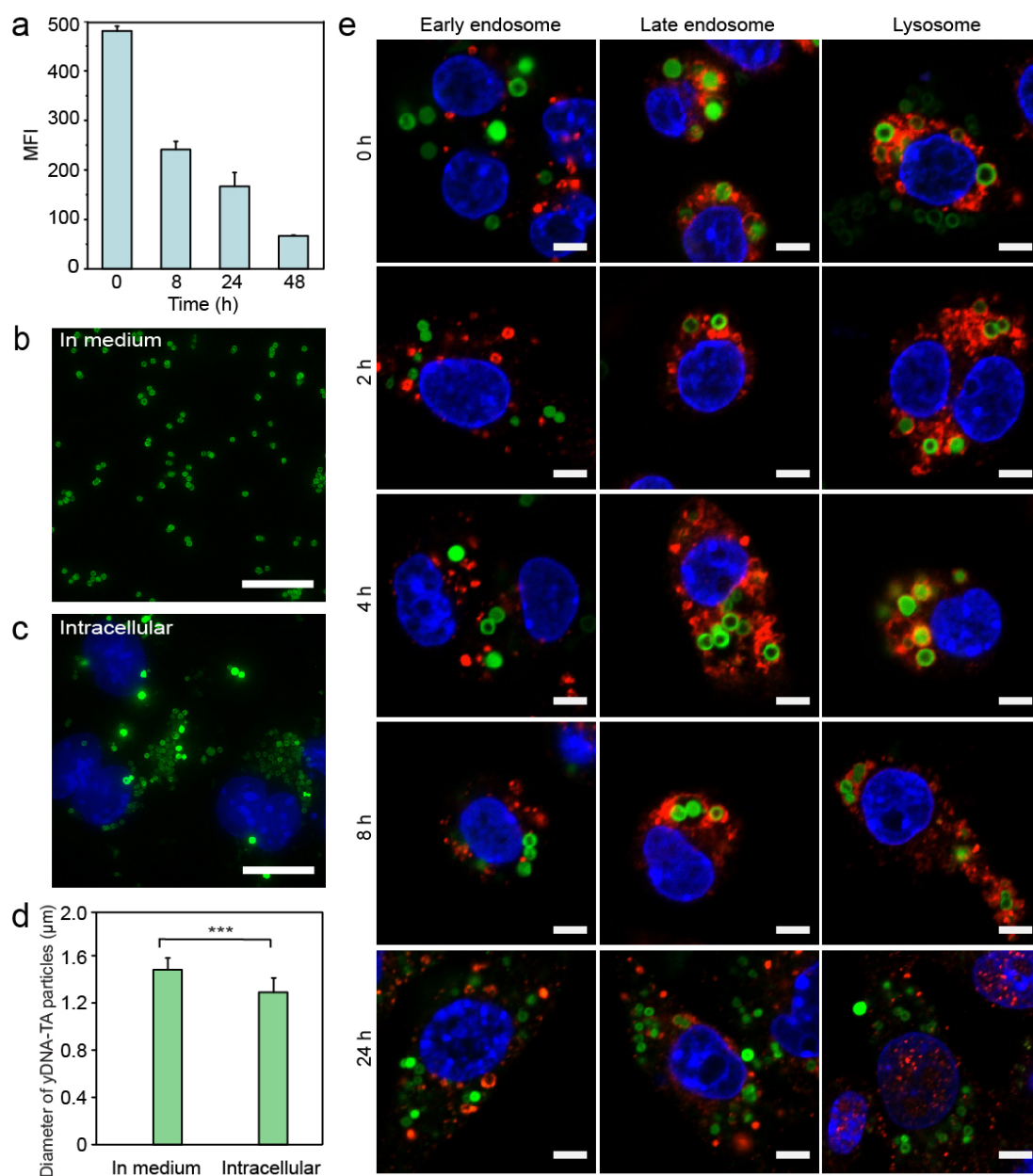


Figure 4.3. Intracellular degradation and trafficking of the yDNA–TA particles in RAW264.7 cells. (a) MFI of cells treated with the yDNA–TA particles at varying incubation times of 0, 8, 24, and 48 h (following initial incubation for 2 h). SIM images of the yDNA–TA particles (b) in the cell culture medium and (c) inside cells following incubation for 24 h. Scale bars: 20 μm. (d) Diameter profiles of the yDNA–TA particles in medium vs intracellular (data are shown as the mean ± standard deviation, $n > 50$). *** $P < 0.001$. (e) CLSM images showing the intracellular trafficking of the yDNA–TA particles in RAW264.7 cells at varying incubation times of 0, 2, 4, 8, and 24 h (following initial incubation for 2 h). Compartments in cells (red) were stained with EEA1 monoclonal antibody (early endosomes), anti-Rab7 monoclonal antibody (late endosomes), and anti-LAMP1 monoclonal antibody (lysosomes). Green,

AF488-labeled yDNA-TA particles; blue, nuclei; red, early endosomes or late endosomes and lysosomes. Scale bars: 5 μ m.

4.4.4 Immunostimulatory Effect of yDNA-TA Particles

We then investigated the potential of yDNA-TA particles to deliver immunostimulatory unmethylated cytokine guanine oligonucleotide (CpG motifs). Immune cells will produce a variety of cytokines, such as TNF- α and IL-6, by the activation of CpG motifs.^{27,28} CpG motifs are recognized by Toll-like receptor 9 (TLR9), which is expressed and located in the endosomes of immune cells.^{29,30} To impart immune stimulation to yDNA-TA particles, three CpG motifs were inserted into a yDNA building block. To evaluate the immunostimulatory activity of yDNA-TA particles, the secretion of cytokines (TNF- α and IL-6) in RAW264.7 cell culture medium was detected by ELISA. TNF- α and IL-6 secretion of RAW264.7 cells were very low after incubation with yDNA (**Figure 4.4a**). On contrary, yDNA-TA particles stimulated high levels of TNF- α and IL-6 in RAW264.7 cells. yDNA-TA particles showed significantly high immunostimulatory effects, which may be due to the effective internalization of yDNA-TA particles. Additionally, yDNA-TA particles stimulated the production of TNF- α and IL-6 in a concentration- and time-dependent manner (**Figure 4.4b and c**). To study the specificity targeting of yDNA-TA particles to TLR9 immune activation, the particles were prepared using yDNA containing GpC dinucleotides instead of CpG (yGpC-TA particles) as a negative control. After incubating yGpC-TA particles with RAW264.7 cells, negligible production of TNF- α and IL-6 was detected (**Figure 4a**). This result confirmed that the immunostimulatory effect of yDNA-TA particles was mediated by the specific interaction between CpG motifs and TLR9. We also investigate the ability of yDNA-TA particles to exert prolonged immune stimulation in RAW264.7 cells after internalization. After 2 h of incubating yDNA-TA particles with the cells, the unbounded yDNA-TA particles were washed off. The cells were further incubated in fresh medium for 2, 4, 8, and 24 h. Then TNF- α and IL-6 production from RAW264.7 cells at varying incubation times were measured. TNF- α and IL-6 were continuously produced and increased within 8 h (**Figure 4.4d**). Although the yDNA-TA particles remain intact during the intracellular trafficking, these findings indicated that the erosion of yDNA-TA enabled the negligible increase in cytokine production after 8 h incubation is consistent with the observed translocation of yDNA-TA particles from endo/lysosome to the cytosol. Collectively, these results suggested that yDNA-TA particles can deliver bioactive CpG into macrophages efficiently and invoke a strong and sustained immune response.

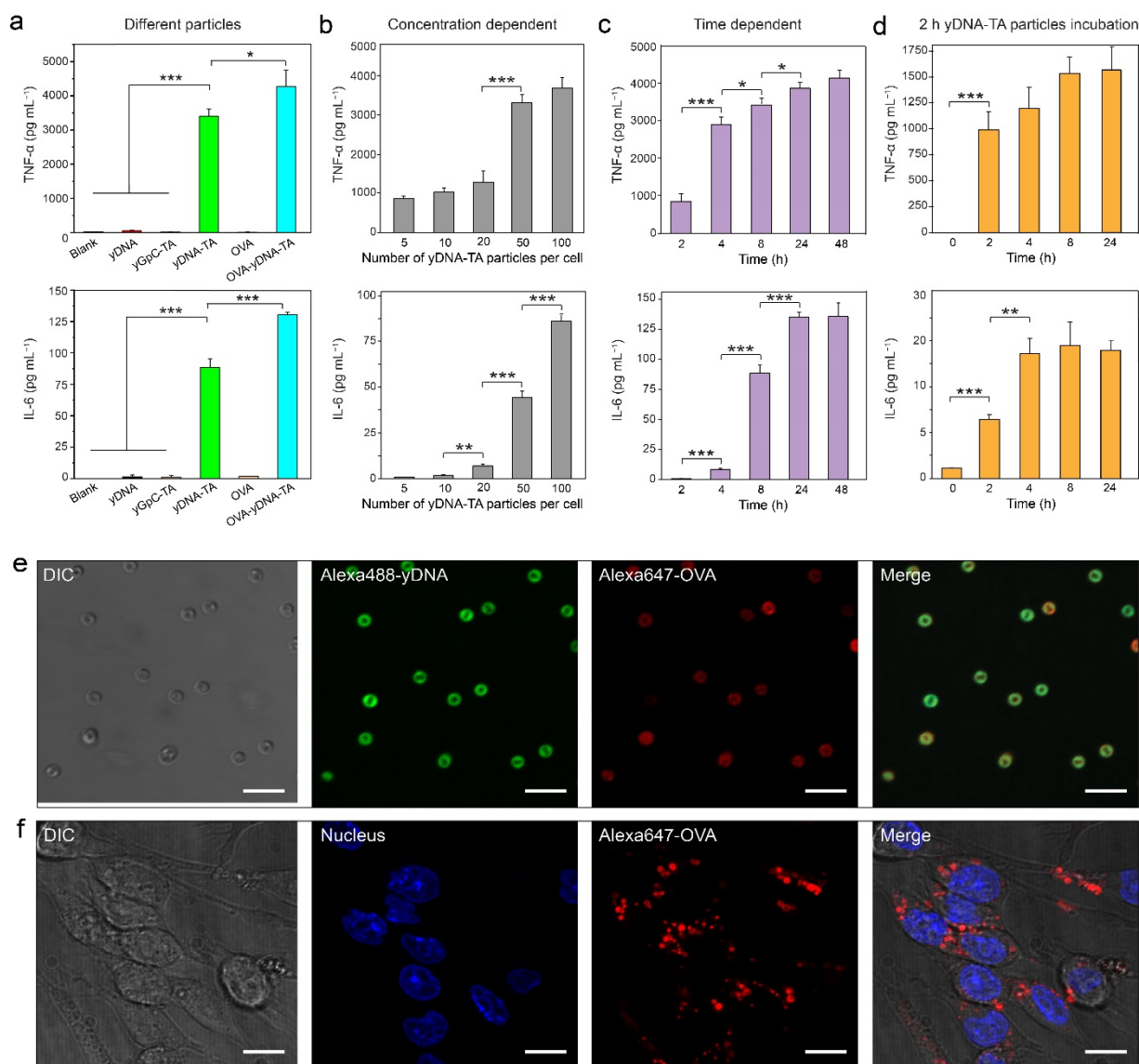


Figure 4.4. Cytokine release from RAW264.7 cells stimulated by the yDNA-TA particles. (a) ELISA measurements of TNF- α and IL-6 release stimulated by different DNA structures after incubation for 8 h (TNF- α) or 24 h (IL-6) with RAW264.7 cells (the concentration of the CpG motif in all samples was 120 nM; [OVA] = 500 $\mu\text{g mL}^{-1}$). (b) TNF- α and IL-6 secretion from RAW264.7 cells incubated with the yDNA-TA particles at varying particle-to-cell ratios for 8 h (TNF- α) or 24 h (IL-6). (c) TNF- α and IL-6 secretion from RAW264.7 cells incubated with the yDNA-TA particles ([CpG motifs] = 120 nM, particle-to-cell ratio = 100:1) for 2, 4, 8, 24 h and 48 h. (d) TNF- α and IL-6 secretion from RAW264.7 cells incubated with the yDNA-TA particles ([CpG motifs] = 120 nM, capsule-to-cell ratio = 100:1) at varying incubation times of 0, 2, 4, 8, and 24 h. (following initial incubation for 2 h). The results are expressed as the mean of cytokine concentration $\pm$ standard deviation from three measurements. t-test: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. (e) Differential interference contrast (DIC) and CLSM images of

OVA-yDNA-TA particles; scale bars: 5 μm . (f) DIC and CLSM images of cells after incubation with OVA-yDNA-TA particles for 24 h at a particle-to-cell ratio of 100:1; scale bars: 10 μm . Green, AF488-labeled yDNA; red, AF647-labeled OVA; yellow: merged yDNA and OVA; blue, nucleus.

For particulate vaccines, antigen and adjuvant are usually loaded in the same platform to invoke a stronger immune response.³¹ The combination of CpG motifs and an antigen within yDNA-TA particles may exert a synergetic effect in stimulating the immune system. Ovalbumin (OVA) was employed as, a model antigen, in combination with the immune stimulatory effect of CpG.³² Owing to the protein binding activity of TA, OVA was post-loaded onto yDNA-TA particles (OVA-yDNA-TA particles) by deposition and then washing with water (**Figure S4.17**). Alexa 647 (AF647)-labeled OVA was absorbed on yDNA-TA particles as indicated by the CLSM image (**Figure 4.4e**). After 24 h incubation with RAW264.7, a strong fluorescence arising from internalized OVA-yDNA-TA particles was observed (**Figure 4.4f**). This suggested that OVA-yDNA-TA particles can be internalized into cells efficiently after 24 h. The cytokine (TNF- α and IL-6) release induced by OVA-yDNA-TA particles in RAW264.7 cells increased compared with that of yDNA-TA particle and free OVA (Figure 4.4a). These results showed that yDNA-TA particles provide a platform for the efficient co-delivery of adjuvant and antigen.

4.4.5 DNA-TA Capsules for Efficient Gene Delivery

To further demonstrate the potential of DNA-TA particles for delivering nuclei acid in the cytosol, a plasmid expressing enhanced green fluorescence protein (pEGFP, 3 kbp) was used as the DNA building block. Particles composed of pEGFP and TA (pEGFP-TA particles, Figure 4.1h and i) were incubated with HEK293T cells for 4 h and then the medium was replaced by fresh medium. By determining the percentage of EGFP-expressing cells 72 h post-transfection, the transfection efficiency was quantitive. The transfection efficiency of pEGFP-TA particles was similar to that of the lipofectamine-pEGFP complexes (a gold standard for transfection of plasmids into HEK293T cells), demonstrating the successful and efficient plasmid DNA transfection in HEK293T cells (**Figure 4.5a**). Noticeably, pEGFP-TA particles showed a significantly higher transfection efficiency than free pEGFP as a negative control. As consistent with the flow cytometry, CLSM images of HEK293T cells transfected with pEGFP-TA particles showed a strong fluorescence signal arising from EGFP expression (**Figure 4.5b**).

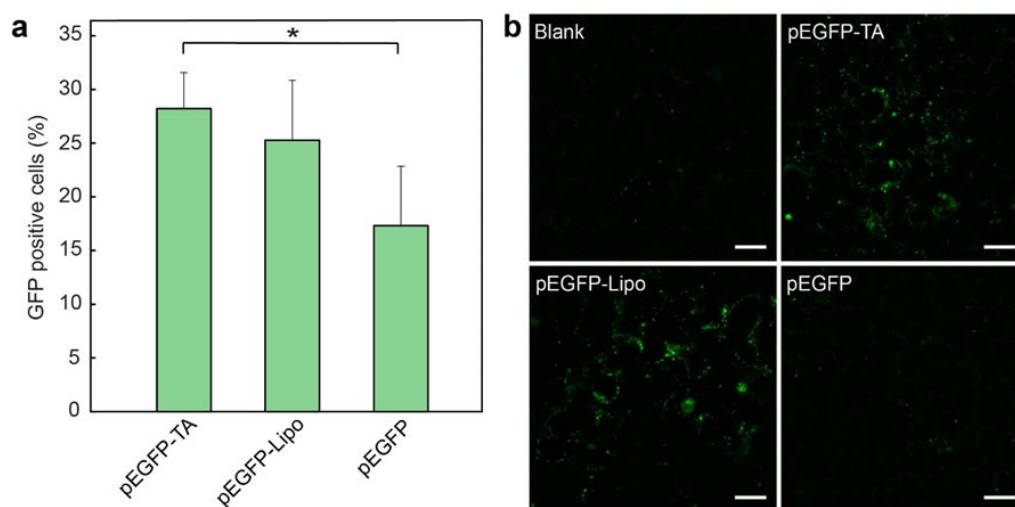


Figure 4.5. In vitro transfection efficiency of pEGFP-TA. (a) In vitro transfection efficiency of pEGFP-TA measured in HEK293T cells using flow cytometry. Data are shown as means $\pm$ standard deviations ($n = 3$), $*p < 0.05$. (b) CLSM images of HEK293T cells after transfection with pEGFP-TA particles (equivalent of 1 μ g plasmid DNA per well and incubation for 72 h at 37 $^{\circ}$ C). pEGFP-lipofectamine (pEGFP-Lipo) complex and naked pEGFP were used as positive and negative controls, respectively. Scale bars: 20 μ m.

4.5 Conclusion

We developed a facile and general method to synthesize various DNA-based microparticles by exploiting the non-covalent interaction of polyphenols and DNA, resulting in particles with diverse physicochemical properties and biological functions. The particles exhibited high stability in serum likely due to their compact structure that can protect DNA from enzymatic degradation. The effective internalization of DNA-TA particles by macrophages and HEK293T cells to induce cytokine production and expression of proteins, respectively, highlight their potential as a delivery platform. This platform applies to a wide range of functional DNA as demonstrated using CpG oligonucleotides, plasmid DNA, and a combination of oligonucleotide and protein cargoes, and offers possibilities for the loading of other nucleic acid-based therapeutics, including messenger RNA and small interfering RNA for a range of applications. The ease of preparation of DNA-TA particles combined with their tunable properties and biological function, stability in serum, and capacity for intracellular delivery provides a promising strategy to advance the field of DNA-based nanomedicine.

4.6 Supporting Information

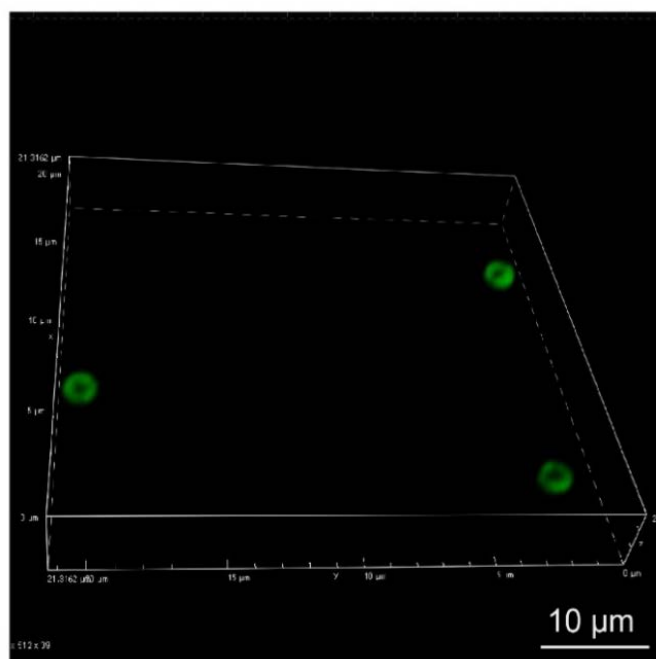


Figure S4.1. Three-dimensional CLSM image of the yDNA-TA particles.

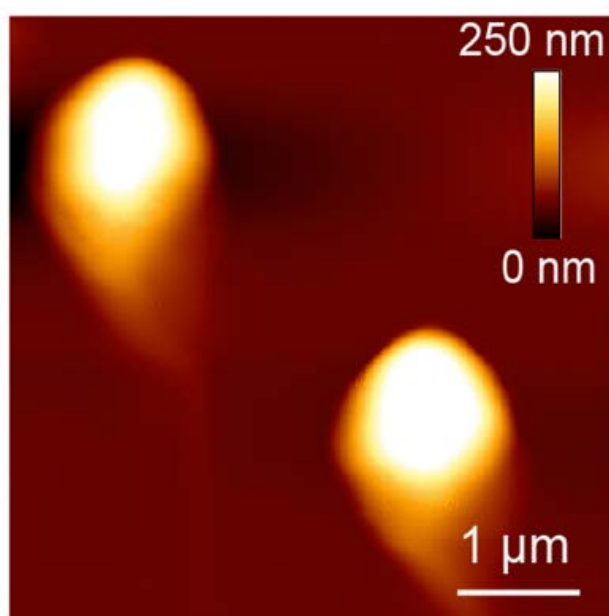


Figure S4.2. AFM image of yDNA-TA particles.

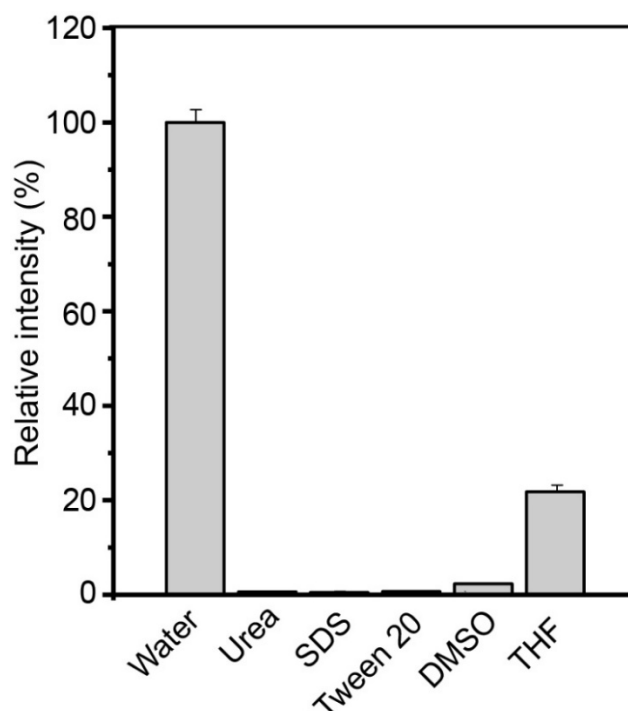


Figure S4.3. Stability of the yDNA–TA particles dispersed in various media, as measured by counting the number of particles by flow cytometry. The error bars represent standard deviations ($n = 3$).

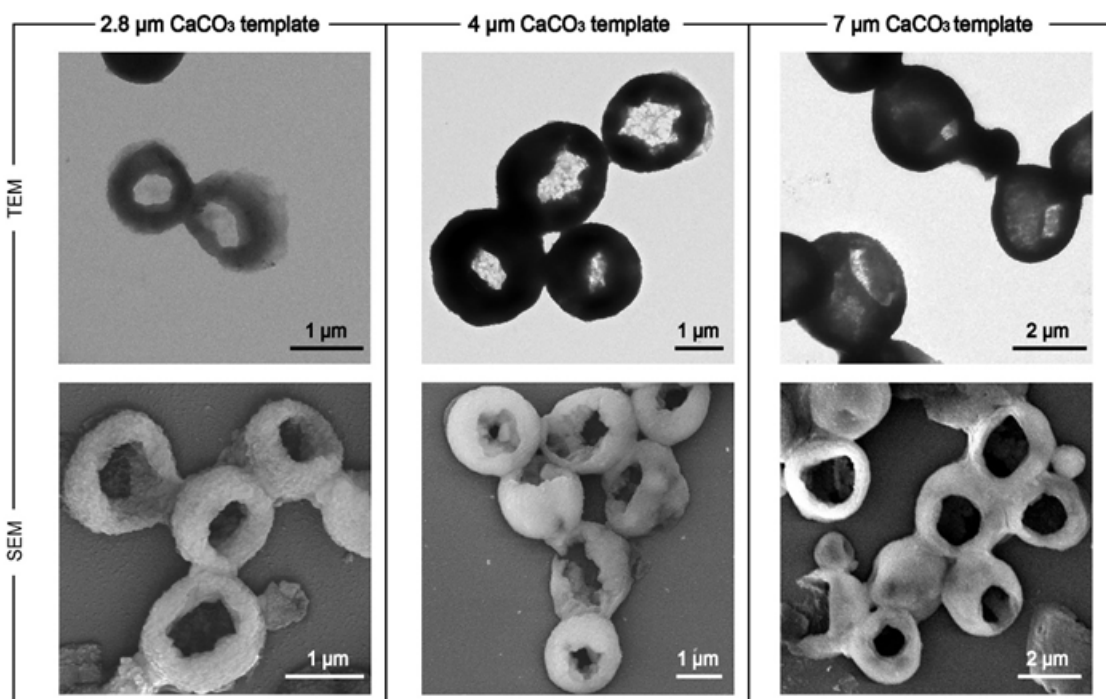


Figure S4.4. TEM and SEM images of the different yDNA–TA particles synthesized using CaCO_3 templates of different sizes.

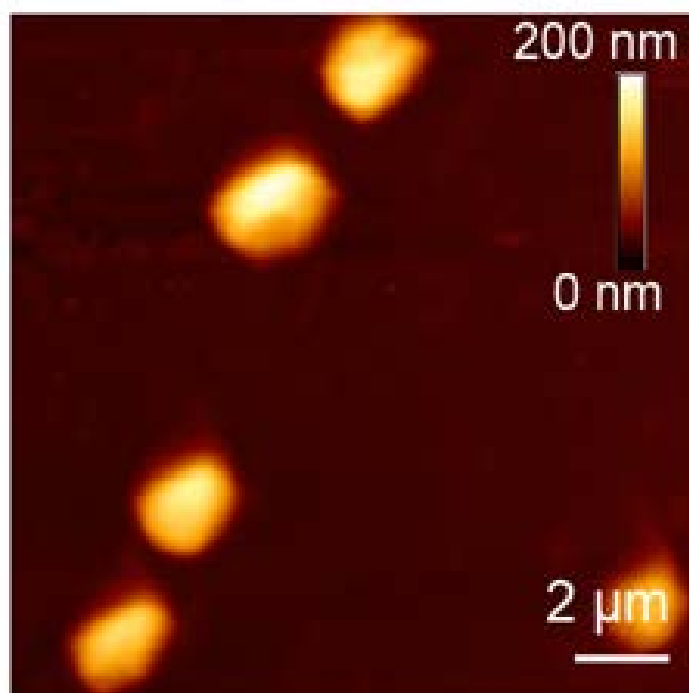


Figure S4.5. AFM image of the TA-yDNA particles. The particles were prepared by reversing the deposition order of the building blocks (that is TA was deposited followed by yDNA).

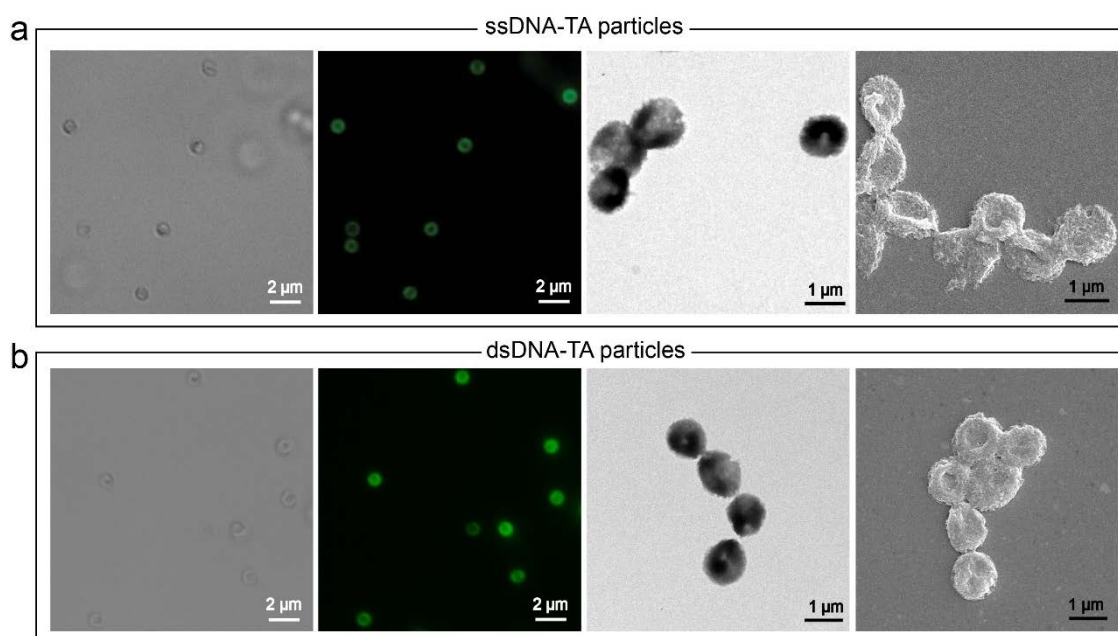


Figure S4.6. Differential interference contrast (DIC), CLSM, TEM, and SEM images of the (a) ssDNA-TA and (b) dsDNA-TA particles.

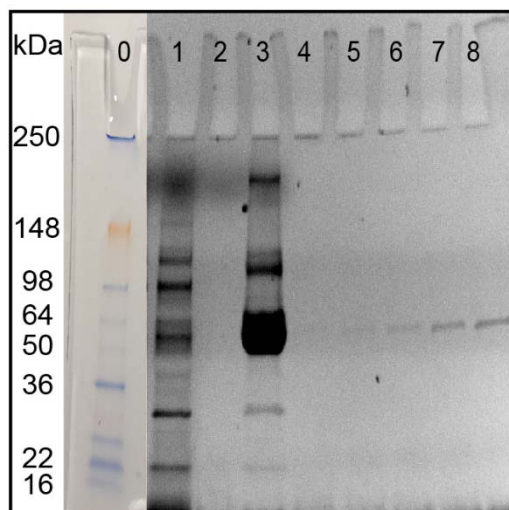


Figure S4.7. SDS-PAGE analysis of the proteins recovered from the yDNA–TA capsules following incubation with DMEM containing 10% FBS. Ladder (lane 0); proteins extracted from 2×10^8 yDNA–TA capsules (lane 1); DMEM (lane 2); DPBS containing 10% FBS (lane 3); BSA standards: 50 ng (lane 4), 100 ng (lane 5), 200 ng (lane 6), 500 ng (lane 7), and 1 μ g (lane 8).

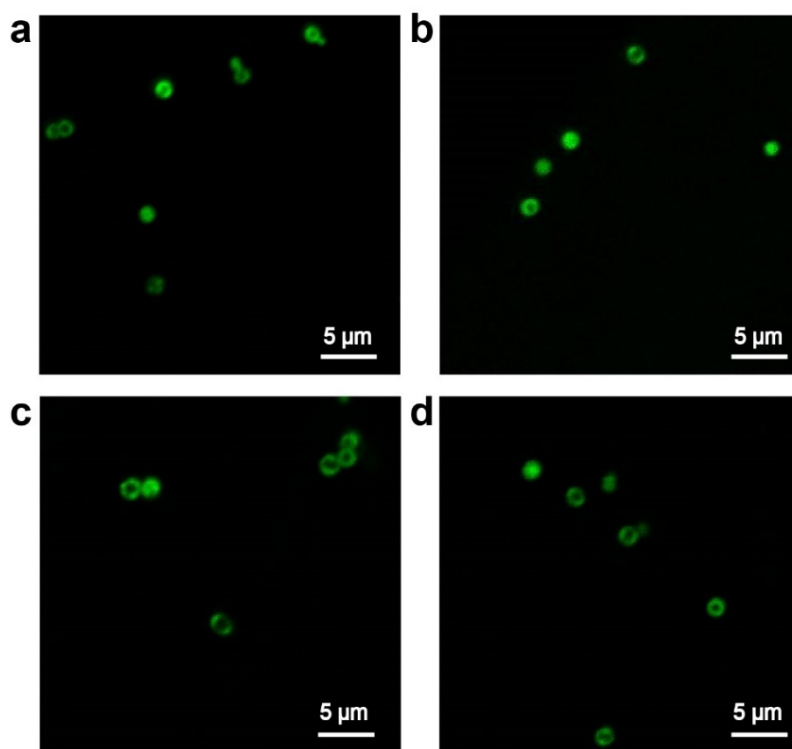


Figure S4.8. CLSM measurements of the stability of the yDNA–TA particles. The yDNA–TA particles were incubated in cell culture medium containing 10% FBS at 37 °C for (a) 2, (b) 4, (c) 8, and (d) 24 h prior to CLSM imaging.

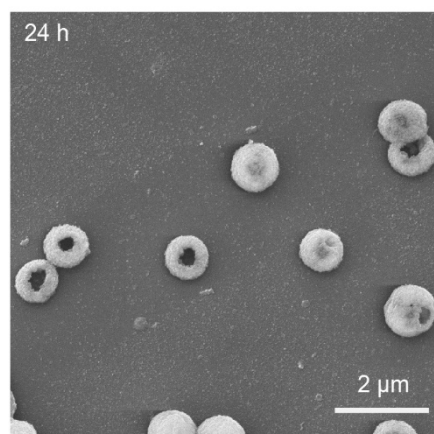


Figure S4.9. SEM image of the yDNA-TA particles incubated in 10% FBS at 37 °C for 24 h.

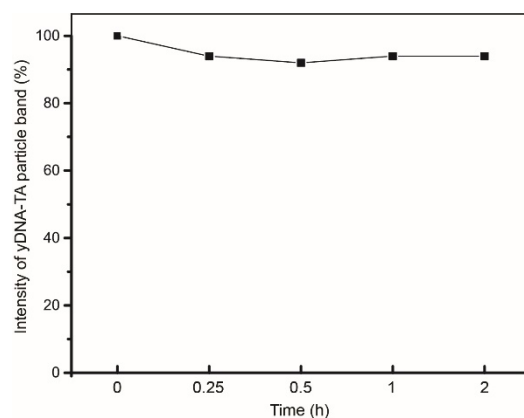


Figure S4.10. Intensity profile of the yDNA-TA particle band in agarose gel following incubation of the particles in 10% FBS medium at 37 °C.

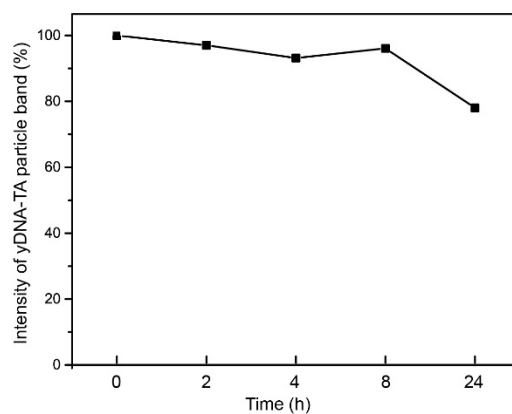


Figure S4.11. Intensity profile of the yDNA-TA particle band in agarose gel following incubation of the particles in DNase (1 U mL⁻¹) at 37 °C.

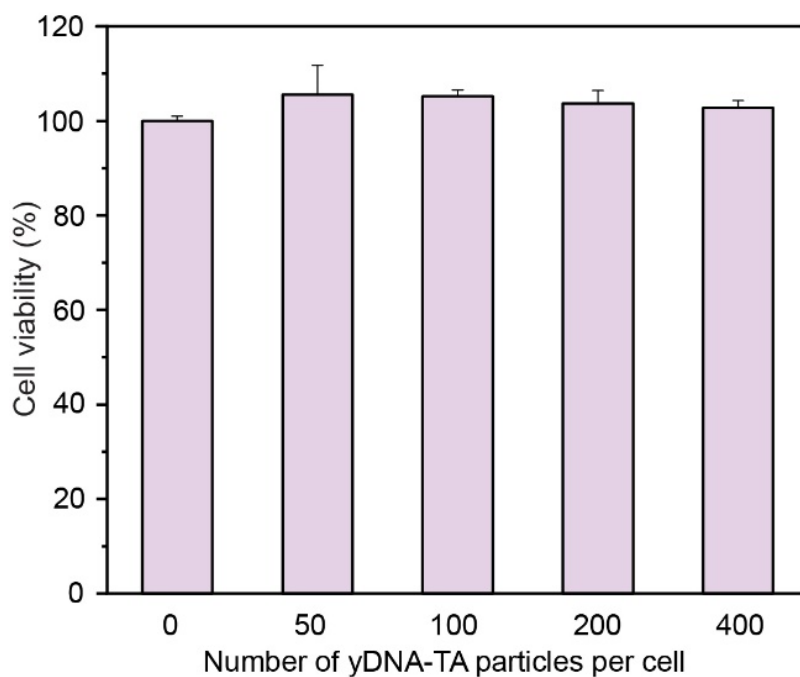


Figure S4.12. MTT assay of RAW264.7 cell viability measured after incubation of the yDNA–TA particles for 24 h at different particle-to-cell ratios.

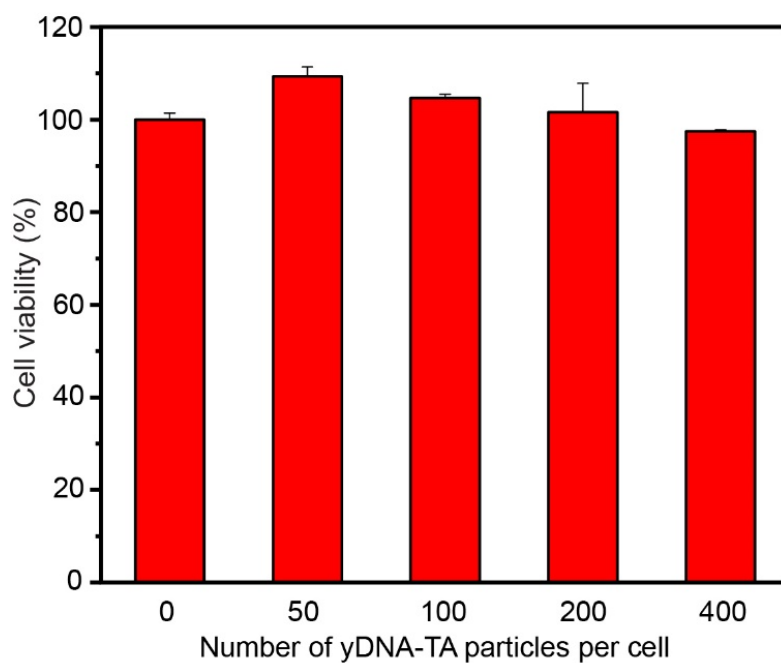


Figure S4.13. MTT assay of HeLa cell viability measured after incubation of the yDNA–TA particles for 24 h at different particle-to-cell ratios.

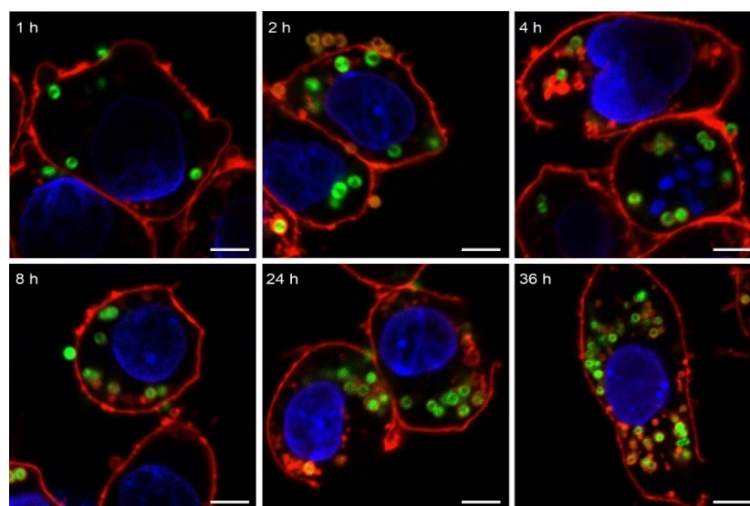


Figure S4.14. CLSM images of cells after incubation with the yDNA–TA particles for 1, 2, 4, 8, 24, and 36 h at a particle-to-cell ratio of 100:1. Green, AF488-labeled yDNA–TA particles; blue, nuclei; red, cell membrane. Scale bars: 5 μ m.

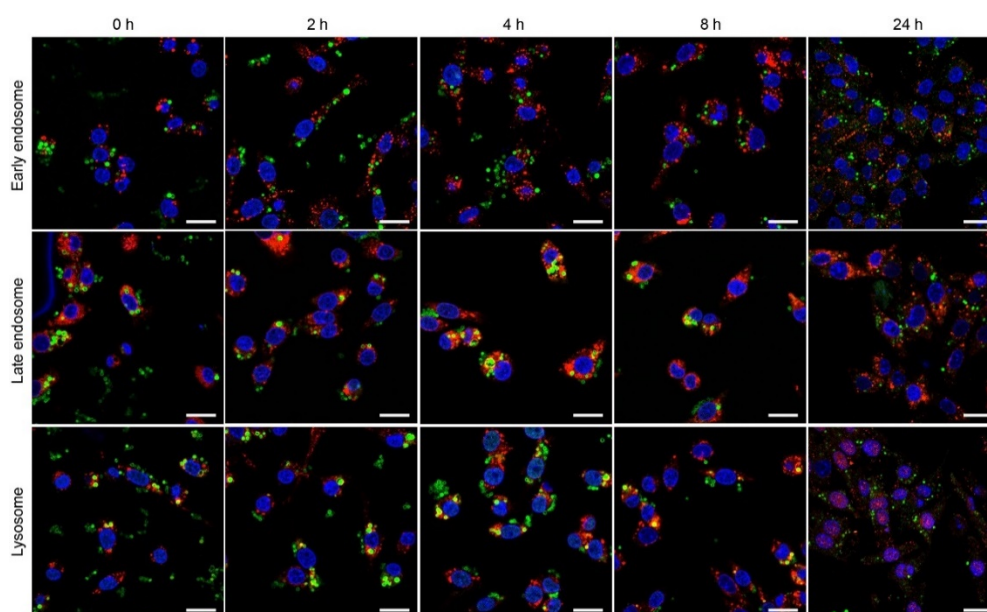


Figure S4.15. CLSM imaging of the intracellular trafficking of the yDNA–TA particles in RAW264.7 cells at varying incubation times of 0, 2, 4, 8, and 24 h (following initial incubation for 2 h). The particle-to-cell ratio was 100:1. Compartments in cells (red) were stained with EEA1 monoclonal antibody (early endosomes), anti-Rab7 monoclonal antibody (late endosomes), or anti-LAMP1 monoclonal antibody (lysosomes). Green, AF488-labeled yDNA–TA particles; blue, nuclei; red, early endosomes/late endosomes and lysosomes. Scale bars: 20 μ m.

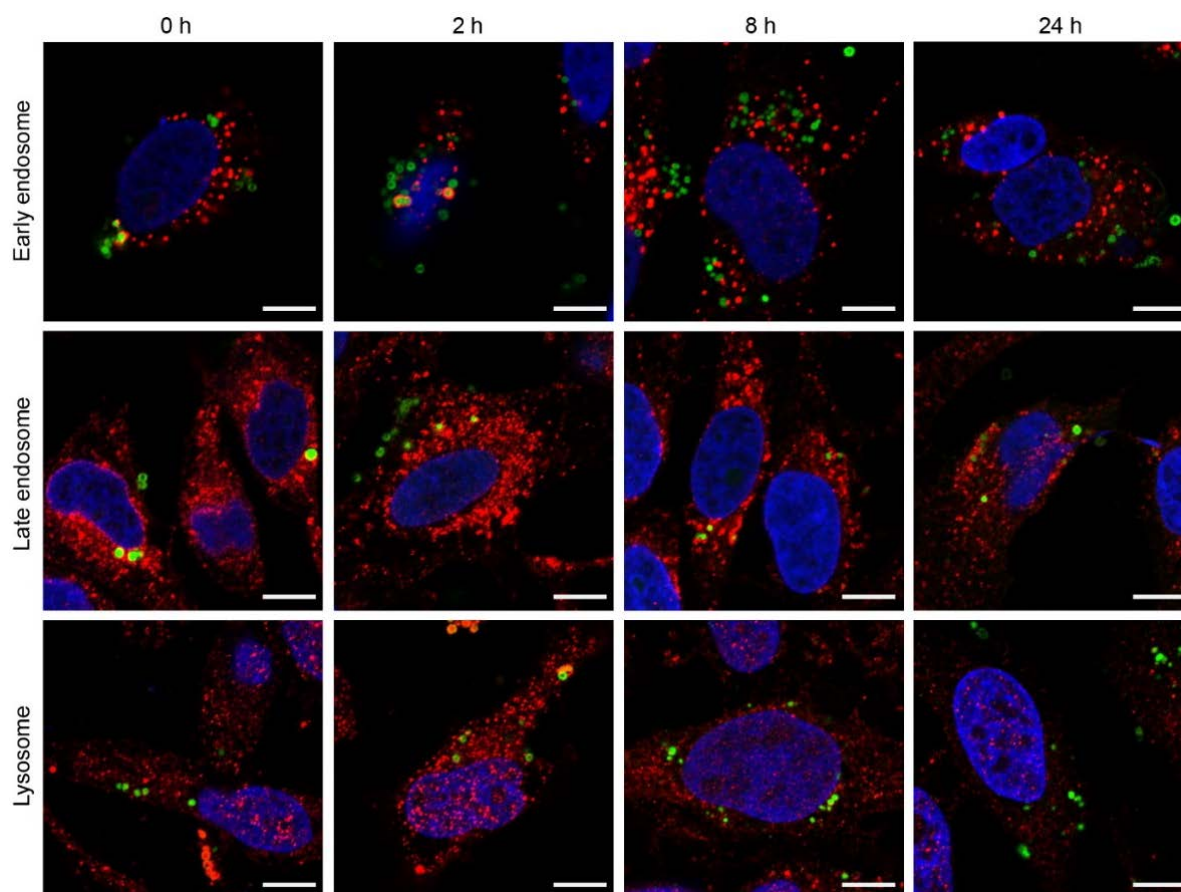


Figure S4.16. CLSM imaging of the intracellular trafficking of the yDNA–TA particles in HeLa cells at varying incubation times of 0, 2, 8, and 24 h (following initial incubation for 2 h). The particle-to-cell ratio was 100:1. Compartments in cells (red) were stained with EEA1 monoclonal antibody (early endosomes), anti-Rab7 monoclonal antibody (late endosomes), or anti-LAMP1 monoclonal antibody (lysosomes). Green, AF488-labeled yDNA–TA particles; blue, nuclei; red, early endosomes/late endosomes and lysosomes. Scale bars: 10 μ m.

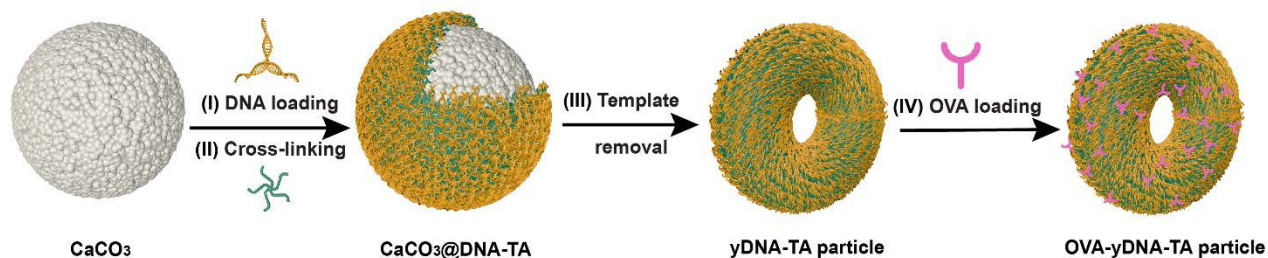


Figure S4.17. Schematic representation of the assembly of OVA–yDNA–TA particles: (I) loading of CaCO_3 particles with yDNA; (II) cross-linking by TA; (III) removal of CaCO_3 core template to yield yDNA–TA particles; and (IV) post-synthesis loading of OVA.

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Chapter 5

Polyphenol-Based Nanoparticles for Intracellular Delivery of Nucleic Acids via Supramolecular Assembly

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5.1 Aim

Intracellular delivery of therapeutic nucleic acids, including single and double-stranded oligonucleotides, is a prospective strategy to regulate cellular behavior and response to infections and cancer. Despite the development of various nanoparticle-based intracellular delivery systems, the engineering of universal, facile, reproducible, and efficient nanocarriers for the delivery of nucleic acids remains challenging. Herein, a facile and versatile supramolecular assembly approach to fabricate DNA–polyphenol nanoparticles which could effectively deliver nucleic acids is reported.

5.2 Introduction

The intracellular delivery of exogenous and genetically engineered nucleic acids is a promising therapeutic method for the treatment of various chronic diseases, infections, and cancers.^{1,2} In particular, non-viral DNA delivery systems have attracted great attention, owing to their ability to overcome the toxic drawbacks of viral vectors.^{3,4} Although micrometer-sized particles have shown in some cases a higher delivery efficiency, nano-sized particles are in general considered ideal candidates for *in vivo* nucleic acid delivery.^{5,6} Non-viral DNA delivery systems are primarily based on condensation of DNA molecules by positively charged lipids and polymers. Nanometer-sized complexes could be spontaneously formed via electrostatic interaction between DNA with the cationic species.⁷ The complexation between nucleic acids and lipids or polymers is important to avoid the enzymatic degradation of DNA by nucleases and enable its internalization in the targeted cells by endocytosis. However, due to their unspecific electrostatic and hydrophobic interactions with cell membranes, synthetic cationic lipids and polymers have also shown undesirable collateral and off-target effects.⁸ In particular, lipoplexes and polyplexes have shown toxicity and invoked innate immune responses *in vitro* and *in vivo*, which interferences with their metabolic stability and activity.⁹ Supramolecular assembly of nucleic acids with natural and degradable biomolecules may provide an innovative strategy for engineering less toxic and efficient nucleic acid delivery vehicles.

Tannic acid (TA) belongs to the category of plant/derived polyphenols and has attracted growing interest in biomaterial science, owing to the broad adherent properties and its high degradability and biocompatibility.¹⁰ Since TA has a multibranched molecular structure containing ten galloyl groups, it can interact with different metal ions, polymers, and biomolecules via multiple intermolecular interactions.^{11,12} The supramolecular assembly of tannic acid is typically regulated by a variety of interactions, such as hydrogen bonding, metal

coordination, and electrostatic interactions.¹³ For example, the strong H-bonding between TA and PEG allowed the formation of nanocomplexes that have shown great potential in the delivery of proteins.¹⁴ The preparation of a DNA/TA hydrogel stabilized by H-bonding between high molecular weight DNA and TA have been also reported.¹⁵ The supramolecular assembly properties of TA facilitated the design of vectors for transporting various drugs, enzymes, and proteins.¹⁶⁻¹⁸ To our knowledge, the supramolecular assembly of unmodified tannic acid and low molecular weight DNA to obtain nanocomplexes for intracellular delivery of oligonucleotides has not been reported.

Herein, we demonstrate a facile strategy for intracellular delivery of immunostimulatory nucleic acids by leveraging on one-pot supramolecular assembly of TA, DNA, and PEG. Oligonucleotides containing immunostimulatory unmethylated cytosine-guanine (CpG) motifs were used to prepare hybrid TA-DNA-PEG and TA-DNA nanoparticles. We show that the size of these nanoparticles can be tailored by using PEG chains with different molecular weights in the range of 1,000-20,000 g/mol. To control the kinetics of the assembly process, the mixture of TA and DNA was heated up to 95 °C and allowed to cool down slowly at room temperature. This annealing process enabled the formation of uniform and stable nanoparticles composed of TA and DNA without involving synthetic PEG chains in the supramolecular complex. The obtained nanoparticles showed limited cytotoxicity to macrophage cells, could be internalized into cells efficiently, trafficked via endo/lysosomal vesicles, and induce an immunostimulatory effect by the production of high levels of cytokines in macrophages. Moreover, to demonstrate the versatility of this approach, hybrid nanoparticles have been prepared using TA and the adjuvant agent dsRNA (polyIC) and OVA peptide. Compared to free polyIC RNA and OVA peptide, the hybrid nanoparticles stimulated higher cytokine production in macrophage cells. Collectively, this work highlights the potential of TA-based nanoparticles as a platform for co/delivery of nucleic acids and proteins.

5.3 Experimental Section

5.3.1 Materials

Materials: All oligonucleotides used in this study were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China). Tannic acid (TA), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), Dulbecco's phosphate-buffered saline (DPBS), and fetal bovine serum (FBS), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trihydrochloride (Hoechst 33342),

Dulbecco's modified Eagle's medium (DMEM), Alexa Fluor 488–wheat germ agglutinin conjugate (AF488-WGA), Alexa Fluor 594–wheat germ agglutinin conjugate (AF594-WGA), and trypsin were obtained from Life Technologies. Enzyme-linked immunosorbent assay (ELISA) kit of tumor necrosis factor (TNF)- α was purchased from Invitrogen. All chemicals were used as received without further purification. Ultrapure water with a resistivity of greater than 18 M Ω cm was used in all experiments and obtained from a three-stage Millipore Milli-Q Plus 185 purification system.

5.3.2 Synthesis of TDP NPs

DNA (10 μ L, 100 nM) and TA (20 μ L, 1 mg mL⁻¹) were added to the 150 μ L MilliQ for 1 min incubation. Then PEG (20 μ L, 1 mg mL⁻¹) was added into the solution and incubated at room temperature for 5 min. TA-DNA-PEG was obtained without further purification. TA-polyIC-PEG and TA-OVA-PEG nanoparticles were prepared the same way by using polyIC (1 mg mL⁻¹) and OVA (1 mg mL⁻¹).

5.3.3 Characterization of TDP NPs

The ζ -potential and size distribution of the particles were measured on a Zetasizer Nano-ZS instrument. STORM images of CY-5-labeled TDP NPs in an aqueous buffer were acquired. The images were acquired on a Nikon N-STORM system and fluorophores were excited by 647 nm laser lines. Fluorescence was collected through a Nikon 100 $\times$ 1.4 NA oil immersion objective and passed through a quad-band-pass dichroic filter.

5.3.4 Synthesis of TD NPs

The TD NPs were synthesized via a facile one-pot self-assembly method. A 40 μ L volume of an aqueous solution of tannic acid (5 mg mL⁻¹) was added to 60 μ L of an aqueous solution of 38 μ M DNA. After incubation at 95 $^{\circ}$ C for 3 h, the TD NPs were obtained by centrifugation at 13000 rpm for 15 min. To probe the impact of the TA:DNA molar ratio, the amount of DNA was consistent, just increasing the amount to tannic acid.

5.3.5 Characterization of TD NPs

TEM analysis was conducted at an operation voltage of 120 kV. Prior to TEM imaging, 5 μ L sample was deposited on formvar-carbon-coated copper grids, which were precleaned using plasma. Prior to AFM imaging, 10 μ L of sample solution was dropped onto a clean glass substrate overnight and imaged in tapping mode. The dried samples were sputter-coated with gold prior to SEM imaging. STORM images of AF488-labeled TD NPs were acquired as the

same method above.

5.3.6 Cell Culture

RAW264.7 cells with a passage number of 36-42 were cultured in complete DMEM supplied with 10% FBS at 37 °C, 5% CO₂, and 95% humidity.

5.3.7 MTT Assay

RAW264.7 cells were plated in a 96-well culture plate at a density of 1×10^4 cells/well overnight. Then cells were incubated with TDP NPs at different DNA concentrations for 24 h, the culture medium was removed. MTT solution (150 μ L, 0.5 mg mL⁻¹) was added to each well and incubated at 37 °C for 4 h. Then 50 μ L DMSO was added to dissolve formazan. A microplate reader was used to measure the absorbance of the supernatant at 570 nm. The cytotoxicity of TD NPs was studied by the same method.

5.3.8 Cell Association

RAW264.7 cells were plated in a 24-well culture plate at a density of 5×10^4 cells/well overnight. Cells were incubated with TNP NPs prepared by CY-5-labeled DNA (DNA: 100 nm) in fresh medium for 1, 2, 4, 8, and 24 h. Then, cells were harvested by trypsinization and washed 3 times with PBS at 400 g for 5min. The cells were resuspended in DPBS and at least 1×10^4 cells were analyzed by flow cytometry. The cell association of TD NPs was studied by the same method.

5.3.9 Cell Internalization

RAW264.7 cells were plated in Labtek 8-well slides at 3×10^4 cells/well and incubated overnight. AF488-labeled TDP NPs were added to incubate with cells. After incubation for 8 h, wash with PBS three times and then fix the cells with 4% paraformaldehyde for 15 min. The membrane was stained with AF488-WGA (5 μ g mL⁻¹) for 5 min, and the nucleus was stained with Hoechst 33342 (1 μ g mL⁻¹) for 10 min. Finally, cells were imaged with a confocal microscope.

5.3.10 Intracellular Trafficking Evaluated by CSLM

RAW264.7 cells were plated at 3×10^4 cells/well in Labtek 8-well chamber slides and incubated overnight. AF488-labeled TDP NPs were added to the cells and then incubated for 24 h at 37 °C. After incubation, the treated cells were gently washed 3 times with DPBS and stained with LysoTracker Green for endosome/lysosome labeling. The nucleus was stained

with Hoechst 33342 ($1 \mu\text{g mL}^{-1}$) for 10 min. Live cell imaging was performed by a confocal microscope. The intracellular trafficking of TD NPs was studied by the same method.

5.3.11 Cytokine Secretion

RAW264.7 cells were plated in 96-well plates at a density of 1×10^4 cells/ well overnight. Then, cells were incubated with CpG and TCP NPs (CpG:100 nM); polyIC and TPP NPs (polyIC: $1 \mu\text{g mL}^{-1}$); OVA and TOP NPs (OVA: $1 \mu\text{g mL}^{-1}$) in fresh medium. Cells were incubated at 37°C for 24 h and the supernatant was collected for TNF- α analysis. The cytokine release from cells stimulated by TD NPs was studied by the same method.

5.4 Results and Discussion

5.4.1 Synthesis and Characterization of TDP NPs

To prepare TA-DNA-PEG nanoparticles hereafter referred to as TDP NPs, we first mixed single-stranded DNA ($100 \mu\text{M}$) with TA (1 mg mL^{-1}) in MQ water for 1 min. The ratio between DNA and TA was according to Chapter 4. Next, PEG at a final concentration of 0.1 mg mL^{-1} was added to the mixture and the solution was kept under stirring for 1 min (**Figure 5.1a**). Different molecular weight PEG, in the range $1\text{-}20\text{k g mol}^{-1}$, were used for the preparation of TDP NPs. TDP NPs formed by mixing the three components for 1 min without further purification. Dynamic light scattering (DLS) analysis shows that TDP NPs with an average hydrodynamic diameter of 54 nm (PDI: 0.202), 60 nm (PDI: 0.251), 105 nm (PDI: 0.447), 170 nm (PDI: 0.115), and 270 nm (PDI: 0.298) were obtained by using PEG 20k, PEG 10k, PEG 5k, PEG 2k, and PEG 1k respectively (**Figure 5.1b**). These results suggest that higher molecular weight PEG molecules lead to the formation of smaller nanoparticles. To confirm that DNA strands were embedded in TDP NPs, TDP NPs were assembled using CY-5-labeled DNA and imaged by using super-resolution microscopy (STORM) in the original hydrated state. As observed in **Figure 5.1c**, the single DNA molecules embedded into the nanoparticles were localized and the STORM image of TDP_{2k} NPs was acquired with nanoscale resolution. The size of TNP_{2k} NPs observed by STORM was $\sim 180 \text{ nm}$, which is consistent with DLS measurement. As confirmed from the DLS analysis, no evidence of aggregation or disassembly of the TDP_{20k} NPs was observed after incubation in different pH buffered solutions (**Figure 5.1d**). This confirmed that the TNP NPs remain stable under physiological pH environments. The surface charge of the TDP_{20k} NPs measured in 5 mM PBS was $\sim -17 \text{ mV}$ at pH 7.4, -14

mV at pH 6.0 and -7 mV at pH 4.0 (**Figure 5.1e**), This change in surface charge is probably caused by the protonation of TA hydroxyl groups (pK 6).

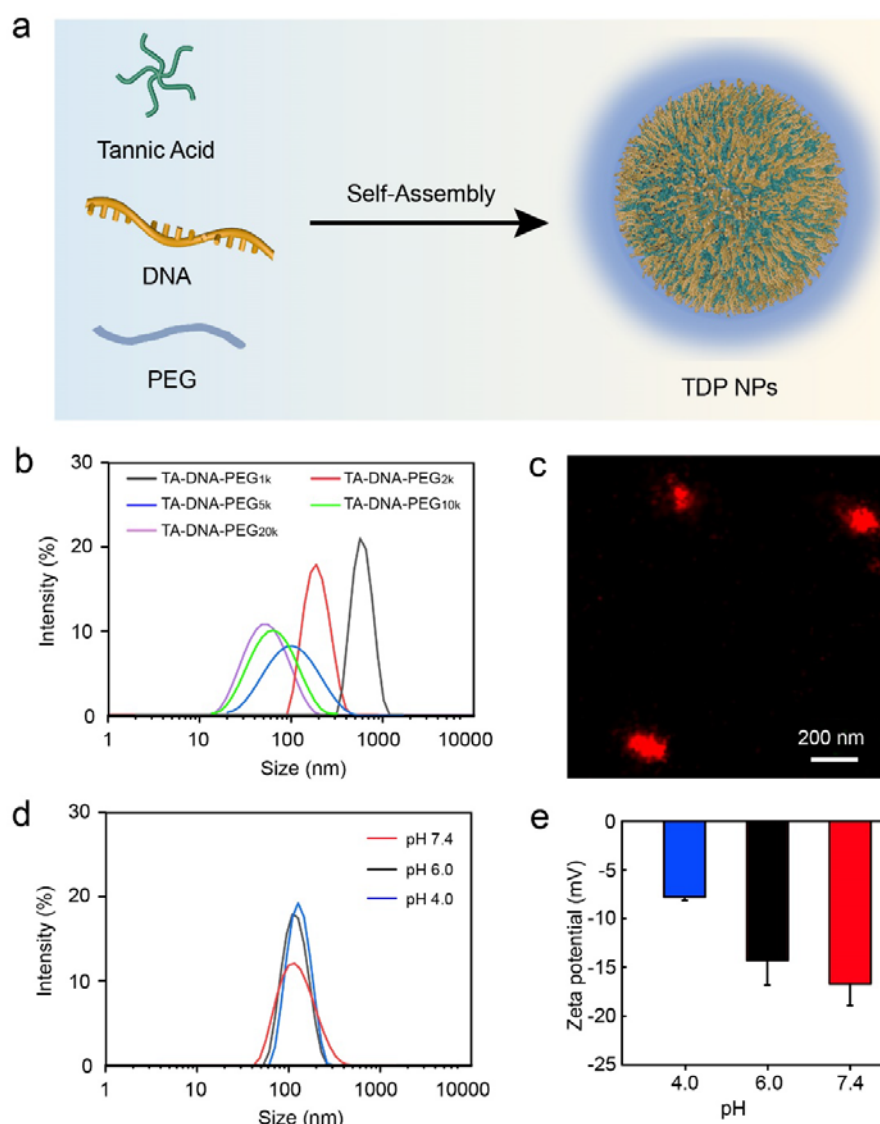


Figure 5.1. Preparation and characterization of TDP NPs. (a) Schematic illustration of the assembly of TDP NPs. (b) Size distribution of TDP NPs obtained by using PEG with different molecular weights. (c) STORM image of TDP_{2k} NPs. (d) Size distribution of TDP_{20k} NPs as a function of pH. (e) Zeta-potential of TDP_{20k} NPs as a function of pH. Data are shown as the mean $\pm$ SD ($n = 3$).

The rapid nano-complexation between TA and DNA is driven by both hydrogen bonding and π - π stacking interactions. In our previous study, we have shown that TA acts as molecular glue in connecting phosphodiester bonds on DNA chains by hydrogen bonds. In addition, our studies suggested that π - π stacking interactions between the galloyl groups of TA and DNA purine and pyrimidine residues can occur. We postulate that PEG chains are engaged by the

TA/DNA nanocomplex via hydrogen bonding with galloyl moieties to form a tricomponent system. Liquid/coacervate phase transition diagrams of TA/PEG mixtures were reported at different concentrations.¹⁹ However, the effect of PEG molecular weight was not investigated. Our findings suggest that higher molecular weight PEG chains can reduce the size of the PEG/TA/DNA nanocomplex via competing intermolecular interactions with TA. Overall, we have developed a simple method to form stable TA/DNA/PEG nanoparticles by exploiting the supramolecular assembly properties of TA.

5.4.2 Cytotoxicity, Cellular Interaction and Immunostimulatory Effect of TDP NPs

TDP NPs containing immunostimulatory unmethylated cytosine-guanine (CpG) motifs (TCP NPs) were prepared by the same method. It is reported that the size of nanoparticles likely plays a role in provoking an immune response. However, no universal relationship between immune response and different types of nanoparticles was derived.²⁰ Other properties of nanoparticles, such as charge, composition, and surface modification, can contribute to the immunostimulatory activity of nanoparticles. Therefore, as a proof of concept, TCP_{2k} NPs of 170 nm in size were selected for further investigation.

To investigate whether TCP_{2k} NPs efficiently elicit cytokine production from macrophages, CY-5-labeled TCP_{2k} NPs were incubated with RAW264.7 cells. The cytotoxicity of TCP_{2k} NPs to RAW264.7 cells was first tested. TCP_{2k} NPs showed no detectable cytotoxicity at the different TCP_{2k} NPs concentrations after incubation for 24 h (**Figure 5.2a**). The association of TCP_{2k} NPs with RAW264.7 cells showed in a time-dependent manner, and the percentage of TCP_{2k} NPs -associated cells reached 50% after 2 h and plateaued at 95% after 8 h (**Figure 5.2b**). Confocal laser scanning microscopy (CLSM) was used to further investigate the cellular internalization and trafficking of TCP_{2k} NPs. After incubation for 8 h, A strong fluorescence signal was detected in the cells after incubation with CY-5-labeled TCP_{2k} NPs for 8 h, suggesting that TCP_{2k} NPs were internalized into RAW264.7 cells efficiently (**Figure 5.2c**). To verify the endo/lysosomal escape of TCP_{2k} NPs, CY5-labeled TCP_{2k} NPs were incubated with RAW264.7 cells 24 h, and their intracellular distribution and colocalization with endocytic vesicles were examined by using LysoTracker staining of late endosomes and lysosomes. The cells displayed punctate as well as diffuse cytosolic red fluorescence (**Figure 5.2d**). TCP_{2k} NPs did not show colocalization with acidic compartments (endo/lysosomes) and lysosomes at 24 h, as the relatively Pearson's correlation coefficient R-value was 0.24. The

amphiphilic PEG molecules can potentially rupture the endosomes/lysosomes membrane and boost the lysosomal escape. These results show that TCP_{2k} NPs are non-cytotoxic to RAW264.7 cells, can be efficiently uptake by cells within 8 h and escape from endo/lysosome and localize in the cytosol within 24 h.

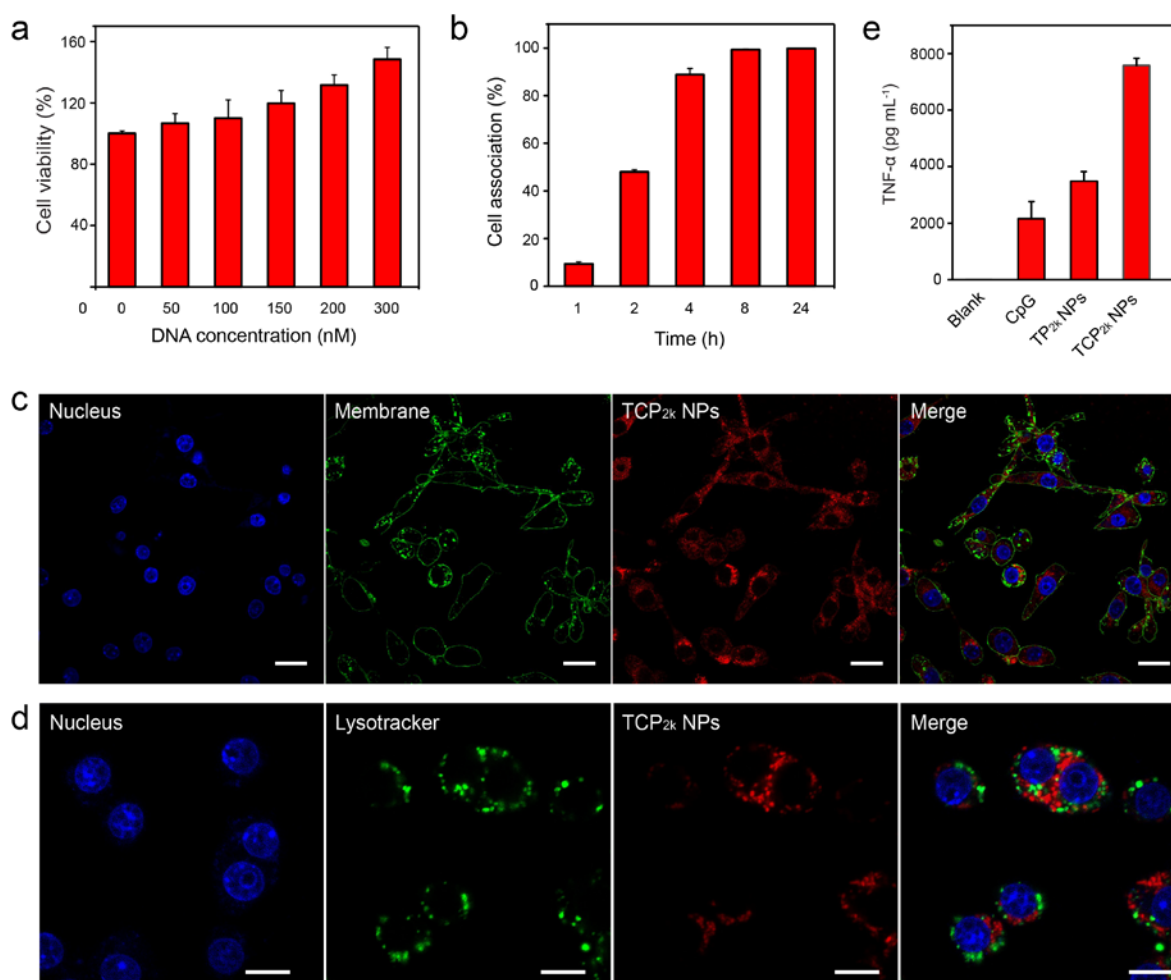


Figure 5.2. Cytotoxicity, cellular internalization, and immunostimulatory effect of TCP_{2k} NPs. (a) MTT assay of cell viability. TCP_{2k} NPs were incubated with RAW264.7 cells at different DNA concentrations for 24 h. (b) Cell association kinetics of TCP_{2k} NPs after incubation for 1, 2, 4, 8, and 24 h. (c) CLSM images of RAW264.7 cells after incubation with TCP_{2k} NPs for 8 h. Scale bars: 20 μ m. (d) CLSM images of a LysoTracker colocalization assay performed in RAW264.7 cells after incubation with TCP_{2k} NPs for 24 h. Red, CY-5-labeled TDP_{2k} NPs; blue, nuclei; green, endo/lysosome. Scale bars: 10 μ m. (e) TNF- α release stimulated by TCP_{2k} NPs after incubation for 24 h with RAW264.7 cells (CpG: 100 nM). Data are shown as the mean $\pm$ SD ($n = 3$).

We then investigated the potential of TCP_{2k} NPs to deliver the CpG motifs inside endosomes. Toll-like receptor 9 (TLR9) located in the endosome of immune cells can recognize CpG motifs and then trigger the secretion of several cytokines from immune cells, such as tumor necrosis factor (TNF)- α .²¹ RAW264.7 cells were incubated with TCP_{2k} NPs at 37 °C for 24 h (CpG:100 nM). The immunostimulatory effect of TCP_{2k} NPs and the control samples (CpG and TP_{2k} NPs) were determined by measuring the TNF- α production in the culture medium supernatant using ELISA. TCP_{2k} NPs induced 4-fold higher TNF- α production compared to pure CpG (**Figure 5.2e**). Of note, TP_{2k} NPs composed of TA and PEG could stimulate cytokine production. As TA itself was not found immunogenic in our previous study, we ascribed this unspecific production of cytokines to the presence of PEG. It is widely reported that PEG provokes the activation of macrophages.²² These results showed that the intracellular trafficking pathway of TCP_{2k} NPs allows the effective engagement of CpG with TLR9 in the endosomes to ultimately elicit an immunostimulatory effect in RAW264.7 cell.

To further demonstrate the potential of TA for the delivery of other biomolecules, a double-stranded RNA (dsRNA) and peptide were used in the preparation of nanoparticles. PolyIC is the synthetic analogue of dsRNA, which can be recognized by Toll-like Receptor 3 (TLR3) located on the plasma membrane and endocytic vesicles and trigger various cytokines production.²³ OVA peptide (OVA_p) is a model antigen for the vaccine, which is known to activate immune cells.²⁴ PolyIC and OVA_p were mixed with TA and PEG (2k Da) to synthesize TA-polyIC-PEG nanoparticles (TPP_{2k} NPs) and TA-OVA_p-PEG nanoparticles (TOP_{2k} NPs) respectively. By DLS analysis, TPP_{2k} NPs were found to be 175 nm in diameter (**Figure 5.3a**). To investigate whether polyIC-containing TPP_{2k} NPs induce cytokine production from macrophages, the nanoparticles (polyIC concentration: 1 $\mu\text{g mL}^{-1}$) were incubated with RAW264.7 cells at 37 °C for 24 h. Then the secretion of TNF- α in medium from stimulated RAW264.7 cells was measured using ELISA. Incubation with pure polyIC induced negligible secretion of TNF- α in RAW264.7 cells, due to its low penetration efficiency into cells (**Figure 5.3b**). In contrast, TP_{2k} NPs stimulated a high level of TNF- α in RAW264.7 cells. TPP_{2k} NPs stimulated significantly higher cytokine production than TP_{2k} NPs. This proved that polyIC was efficiently delivered by TPP_{2k} NPs into RAW264.7 cells and induces an immunostimulatory effect. As for OVA_p delivery study, TOP_{2k} NPs with a diameter of 210 nm were observed (**Figure 5.3c**). A similar trend in cytokine release was observed, where TOP_{2k} NPs exhibited the highest stimulatory activity (OVA concentration: 1 $\mu\text{g mL}^{-1}$) (**Figure 5.3d**). Collectively, these results showed that different types of biological molecules can be

incorporated into TA and PEG complex to form uniform functional nanoparticles. Moreover, these nanoparticles efficiently delivered their functional cargo inside the cells and trigger an immune response.

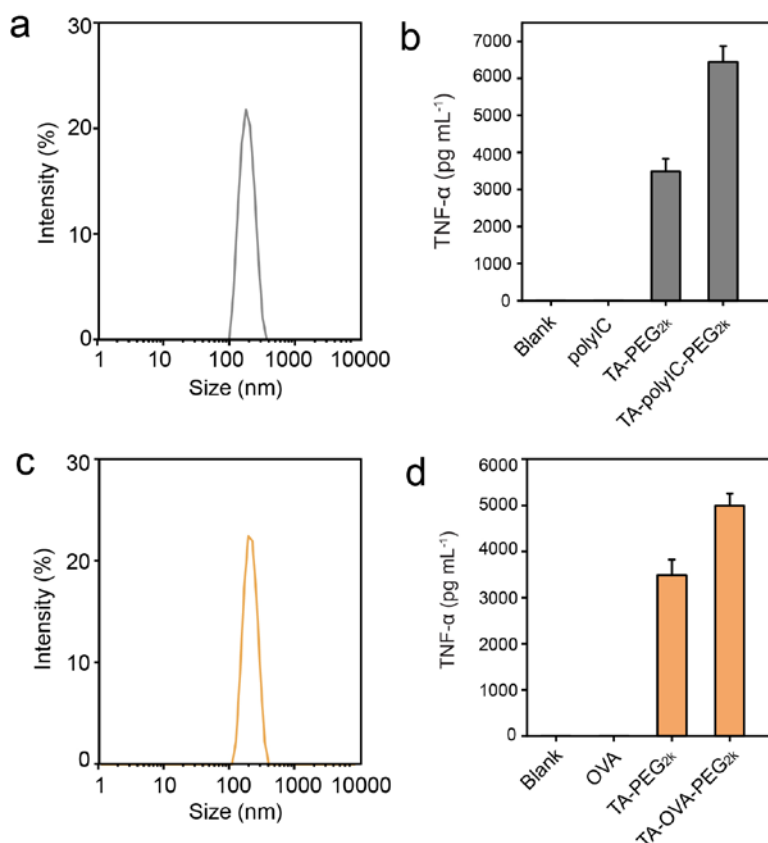


Figure 5.3. (a) Size distribution profile of TPP_{2k} NPs. (b) TNF-α release stimulated by TPP_{2k} NPs after incubation for 24 h with RAW264.7 cells. (c) Size distribution profile of TOP_{2k} NPs. (d) TNF-α release stimulated by TOP_{2k} NPs after incubation for 24 h with RAW264.7 cells. Data are shown as the mean ± SD (n = 3).

5.4.3 Synthesis and Characterization of TD NPs

Since TA/PEG-based nanoparticles have shown unspecific immune response, we developed an alternative method to prepare TA-DNA nanoparticles (TD NPs) without resorting to PEG. To prepare TD NPs, we first mixed TA (5 mg mL⁻¹) with a single-stranded DNA (76 μM) in MilliQ water (**Figure 5.4a**). To control the kinetics of the assembly process, the mixture of TA and DNA was heated up to 95 °C for 3 h and allowed to cool down slowly at room temperature. The thermal process enables a controlled assembly of TA and DNA molecules into uniform nanoparticles.

The nanoparticles were purified by centrifugation at 13,000 rpm for 15 min and the supernatant was discarded. The obtained nanoparticles were analyzed by UV-vis and fluorescence spectroscopy. The fluorescence peak at 531 nm peak, indicated the presence of DNA in the TD NPs (**Figure 5.4b**). The UV-vis spectrum of the TD NPs featured the phenol group's peak at 280 nm, ascribed to TA (**Figure 5.4c**). Well dispersed NPs approximately 100 nm in size were observed by SEM and TEM in an air-dried condition (**Figure 5.4d and e**). AFM images displayed uniform air-dried TD NPs with a thickness of 15 nm (**Figure 5.4f and g**).

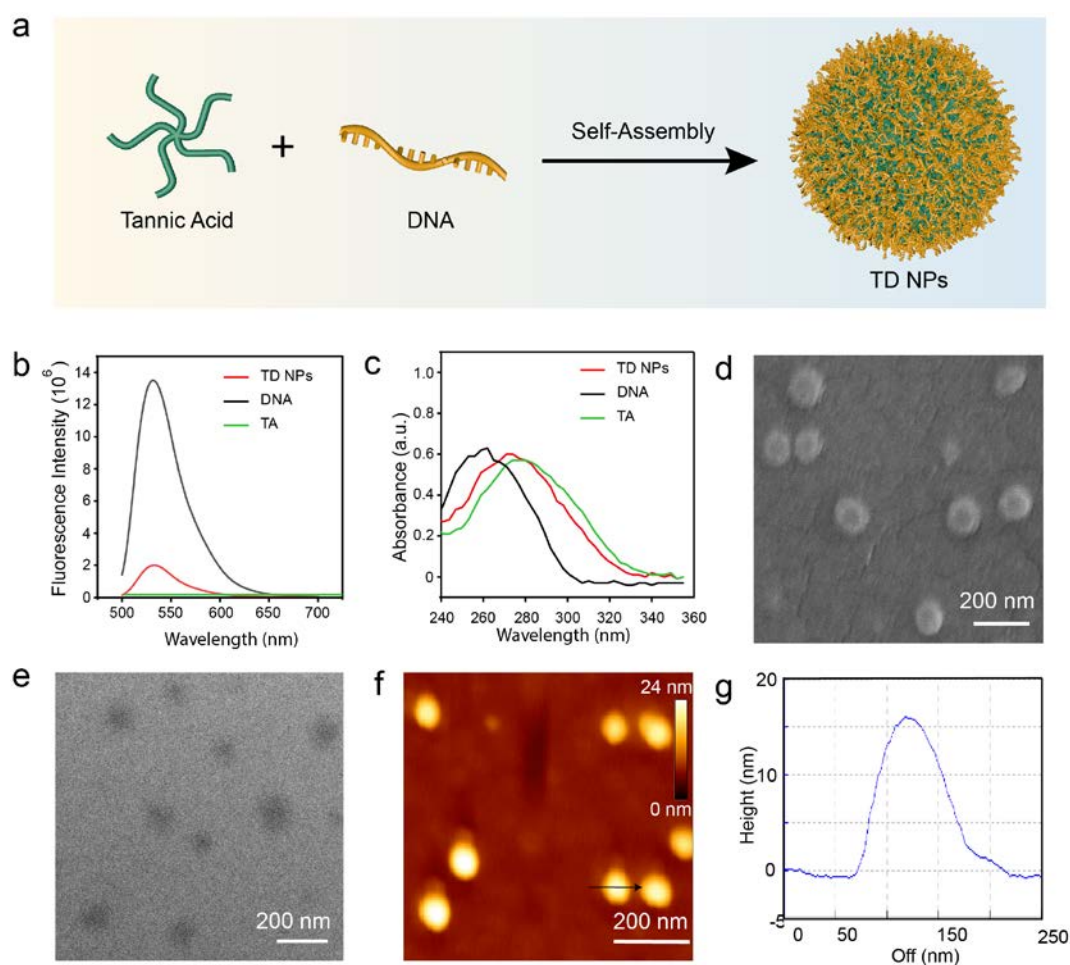


Figure 5.4. Preparation and characterization of TD NPs. (a) Schematic representation of the preparation of TD NPs through self-assembly of TA and DNA. (b) Fluorescence spectrum, $\lambda_{\text{ex}} = 531$ nm, (c) UV-vis spectrum, (d) SEM image, (e) TEM image, (f) AFM image, and (g) thickness of TD NPs.

To give a deeper insight into the size and composition of TD NPs, at a molecular level, TD NPs were assembled using AF488-labeled DNA (labeling ratio, 1:1) and analyzed by STORM. To obtain an optimal signal density for STORM imaging 1/4 labeled DNA strands were used

in the preparation of TD NPs. **Figure 5.5a** showed the size uniformity of the TD NPs with an average diameter of ~ 230 nm. The size distribution of TD NPs was quantified by plotting the frequency vs the diameter measured with 50 nm resolution (**Figure 5.5b**). The number of DNA molecules embedded in the TD NPs was quantified by counting the single-molecule localizations per particle (**Figure 5.5c**). Approximately 2300 blinking DNA molecules were counted in one TD NP, corresponding to the actual 9200 DNA strands embedded in one TD NP.

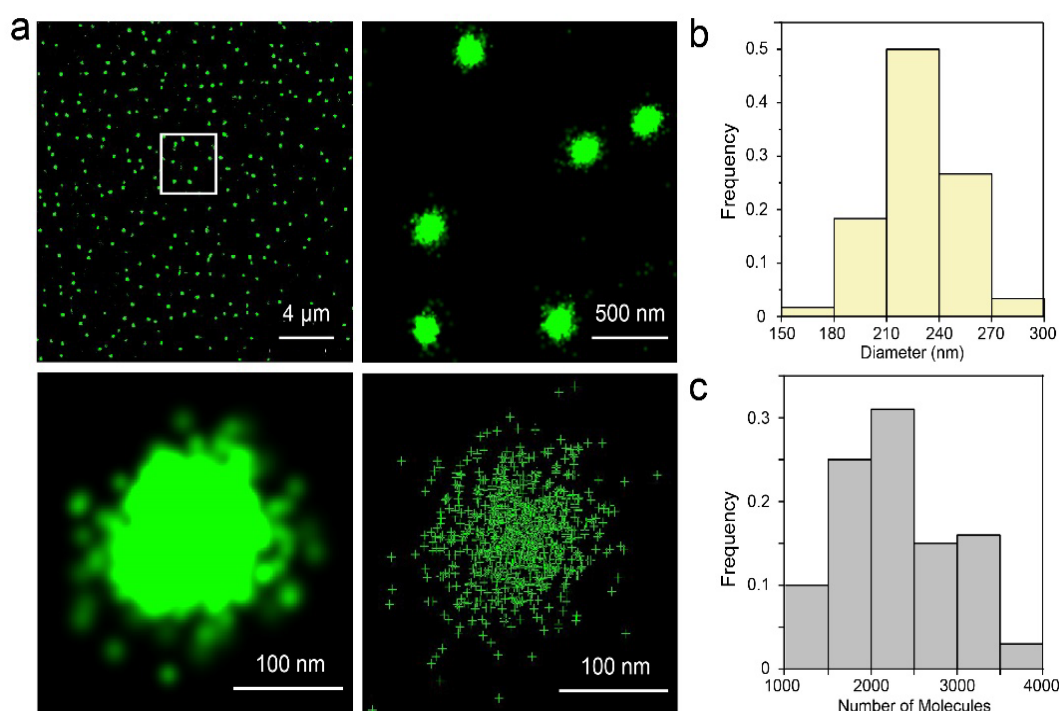


Figure 5.5. (a) STORM images of TD NPs and the magnified cluster image with molecules shown in cross representation. (b) Size distribution of TD NPs. (c) Number of molecules per TD NP.

Importantly, the TD NPs can be generated with high precision via controlling TA: nucleotide molar ratio (T/N). Owing to the ability to simultaneously measure the size and composition of TD NPs, STORM allowed individual TD NPs to be resolved during their formation. **Figure 5.6a** showed a large amount of green signal corresponding to DNA are not associated with the TD NPs when T/N ratio of 1 was used. However, with increasing T/N ratio from 4 to 12, the free DNA molecules imaged on the backgrounds tend to disappear, indicating that more DNA molecules were incorporated in the nanoparticles. Uniform and spherical nanoparticles were imaged when the T/N ratio was changed during the complexation. The diameter of the assembled nanoparticles was 290 ± 19 nm at the T/N ratio of 4, while decreased to 230 ± 22

nm at the T/N ratio of 7, 219 ± 22 nm at the T/N ratio of 10, and 170 ± 15 nm at T/N ratio of 12 (**Figure 5.6b**). In general, higher T/N ratio resulted in the formation of TD NPs with smaller size. The sizes of TD NPs imaged by STORM at different T/N ratios were compared on frequency histograms (**Figure 5.6c**).

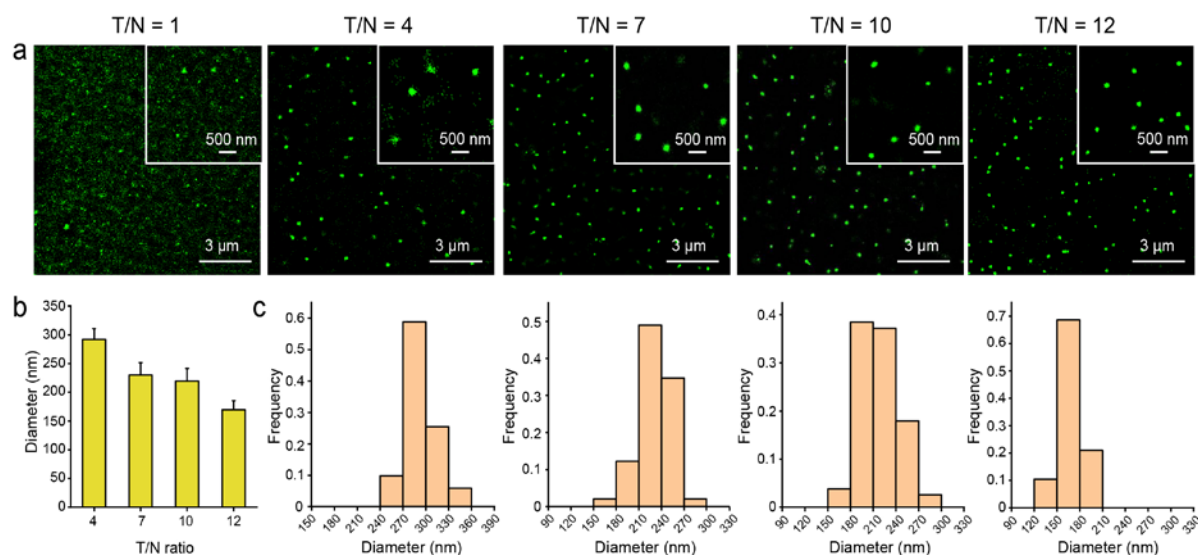


Figure 5.6. (a) STORM images of TD NPs prepared at T/N ratios of 1, 4, 7, 10, and 12. (b) Size of TD NPs quantified from the STORM images at each T/N ratio. (c) Frequency histograms of the size of TD NPs quantified from the STORM images at each T/N ratio.

5.4.4 Cytotoxicity, Cellular Interaction and Immunostimulatory Effect of TD NPs

The cytotoxicity of TD NPs to RAW264.7 cells was measured after incubation with TD NPs at different DNA concentrations for 24 h. TD NPs showed negligible cytotoxicity to RAW264.7 cells (**Figure 5.7a**). The percentage of AF488-labeled TD NPs -associated cells reached 30% after 4 h and plateaued at 70% after 24 h (**Figure 5.7b**). These results suggested a slow uptake process in RAW264.7 cells. To investigate the cellular internalization of TD NPs, RAW264.7 cells were incubated with AF488-labeled TD NPs for 24 h. A strong fluorescence signal was observed in the cells, indicating that TD NPs were internalized into RAW264.7 cells efficiently (**Figure 5.7c**). TD NPs showed the colocalization with acidic compartments (endo/lysosomes) at 24 h, as the relatively Pearson's correlation coefficient R-value was 0.8. This indicates that either the nanoparticles have no ability to escape endo/lysosomes due to the limited buffering capacity of TA or a prolonged incubation time is required.

These results showed that TD NPs have negligible cytotoxicity to RAW264.7 cells, can be uptaken by cells, and remain confined in endo/lysosomes up to 24 h.

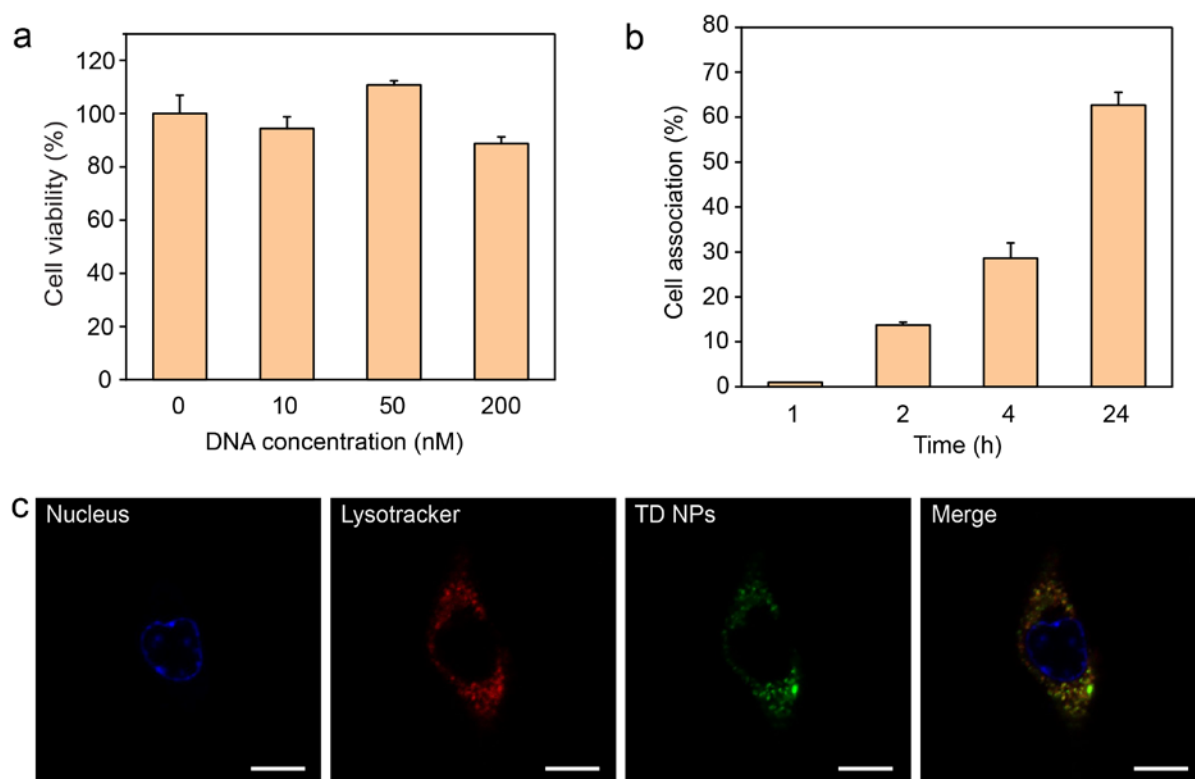


Figure 5.7. Cytotoxicity and cellular interaction of TD NPs. (a) MTT assay of cell viability. TD NPs were incubated with RAW264.7 cells at different DNA concentrations for 24 h. (b) Cell association kinetics of TD NPs after incubation for 1, 2, 4, and 24 h. (c) CLSM images of a LysoTracker colocalization assay performed in RAW264.7 cells after incubation for 24 h with TD NPs. Green, AF488-labeled TD NPs; blue, nucleus; and red, endo/lysosome. Scale bars: 10 μ m. Data are shown as the mean $\pm$ SD (n = 3).

The potential of TD NPs to deliver CpG motif was investigated. CpG motif contained TD NPs (TC NPs) were synthesized by the same method. TC NPs (CpG concentration: 100 nM) were incubated with RAW264.7 cells at 37 °C for 24 h. Compared to free CpG, TC NPs induced a significantly higher level of TNF- α in RAW264.7 cells (**Figure 5.8a**). The cytokine production of TC NPs was time-dependent (**Figure 5.8b**) and, after 8 h incubation, limited production of cytokine was observed. A dramatic increase in TNF- α secretion was observed after 24 h incubation. These results are consistent with the slow internalization process noticed in Figure 5.7b. The compact structure of TC NPs may protect DNA from the degradation by endo-lysosomal DNase and therefore the CpG motifs require prolonged residence time to be released

and function inside the endosomal compartments. Collectively, these results showed that CpG motifs could be delivered by TD NPs and activate macrophages to secrete cytokines.

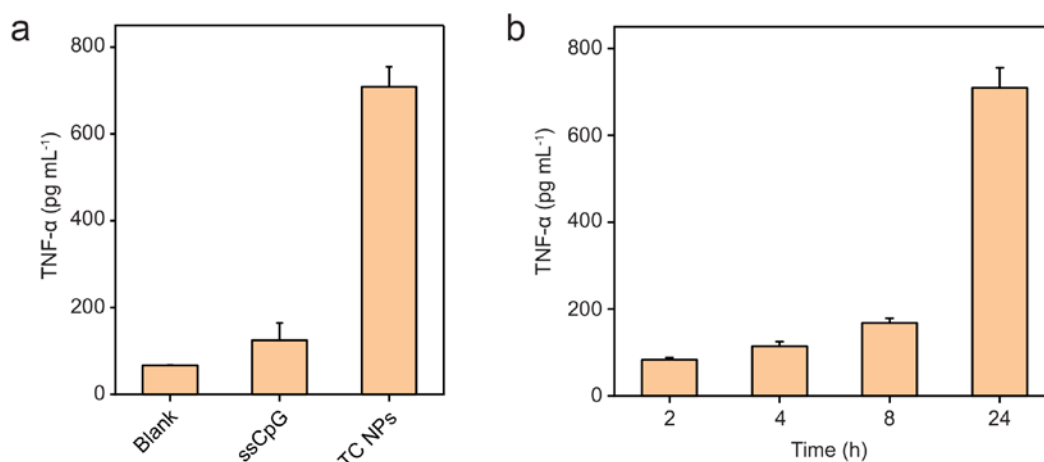


Figure 5.8. Cytokine release from RAW264.7 cells stimulated by TC NPs. (a) TNF- α release stimulated by TC NPs after incubation for 24 h with cells (CpG: 100 nM). (b) TNF- α secretion from cells incubated with TC NPs for 2, 4, 8, and 24 h. Data are shown as the mean $\pm$ SD ($n = 3$).

5.5 Conclusion

In summary, we easily produced nanoparticles by one-pot and organic solvent-free supramolecular assembly of TA, DNA, and PEG. Hybrid TA-DNA-PEG particles bearing CpG sequences could be effectively internalized by macrophages, escape from endo/lysosome and promote a high level of cytokine production, compared to free CpG. PolyIC- and OVA peptide-loaded nanoparticles induced higher cytokine release compared to free polyIC and OVA peptides, respectively. Furthermore, uniform and stable nanoparticles composed of TA and DNA without involving synthetic PEG chains were prepared. By tuning TA/DNA ratio, different size nanoparticles could be obtained. The nanoparticles can enhance the cellular uptake of CpG and secretion of cytokines in macrophages. Overall, the engineered DNA-based particles have great potential for co-delivering nucleic acids and protein antigens in immune cells to enhance the immunological response against infectious diseases and cancer.

5.6 References

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Chapter 6

Summary and Perspectives

Nanotechnology has emerged as a promising strategy for engineering a new generation of vaccines. Various micro- and nanoparticles have been reported as vaccine delivery systems, however, highly biocompatible, biodegradable and effective systems are still needed. Nanoengineered DNA particles are expected to be a suitable platform for vaccine delivery.

In Chapter 3, we have demonstrated a facile method to fabricate DNA capsules composed entirely of DNA strands, based on template-mediated assembly. DNA capsules can be endowed with immunostimulatory properties by incorporating the CpG into the oligonucleotide building blocks. Macrophages could uptake DNA capsules effectively and produced cytokines upon CpG immune stimulation. Moreover, DNA capsules could be degraded by intracellular DNase and accumulated in early and late endosomes, where TLR9 is located. DNA capsules not only act as adjuvants, but allow encapsulation and co-delivery of different cargoes, to achieve synergistic immunostimulatory effects. We anticipate that rationally designed DNA capsules will provide new platforms for future vaccine and immunotherapy applications.

In Chapter 4, we have developed a simple and general method for synthesizing various DNA-based microparticles by using polyphenols and DNA with diverse physicochemical properties and biological functions. Specifically, immunostimulatory DNA-TA particles can be synthesized by incorporating CpG sequence into the DNA building blocks to stimulate the immune system. CpG-TA particles exhibited excellent resistance against enzymatic degradation due to their compact structure. Moreover, these particles can be effectively internalized by macrophages and then induce cytokine production. CpG-TA particles can not only serve as adjuvants, but also act as carriers for antigen, highlighting the potential of CpG-TA particles as a vaccine platform. The ready access to DNA-TA particles combined with their remarkable stability and intracellular delivery capacity provides opportunities to advance the field of DNA-based nanobiotechnology and nanomedicine.

In Chapter 5, PEG was used in the synthesis of TA-based nanoparticles. TA-DNA-PEG nanoparticles were formed by mixing TA, DNA, and PEG in one system. Different molecular weight PEG resulted in different size TA-DNA-PEG nanoparticles. These nanoparticles showed high cellular uptake efficiency and could escape from endo/lysosome. Moreover, co-delivery of antigen/adjuvant by these systems increased cytokine production of macrophages compared to free antigen/adjuvant. Furthermore, nanoparticles assembled via direct interaction between TA and DNA were fabricated. By changing the molar ratio of TA to DNA, various sizes of TA-DNA nanoparticles could be formed. This work shows the potential of polyphenol-based nanoparticles for intracellular drug delivery.

In summary, this thesis has demonstrated i) DNA microcapsules composed of solely DNA for adjuvant delivery; ii) the application of TA-DNA microparticles for effective intracellular antigen and adjuvant co-delivery; and iii) TA-DNA self-assembled nanoparticles for the development of various antigen and adjuvant delivery.

The hollow, flexible and hydrated DNA microcapsules composed solely of DNA were deformed and degraded upon cellular uptake and the CpG motifs remained entrapped inside the endosomes. Compact and rigid DNA-TA microparticles were more stable than DNA microcapsules upon cellular uptake and they could escape from endo/lysosomes to release the cargo in the cytosol. TA-DNA nanoparticles showed lower cell internalization efficiency and cytokine production compared to DNA microcapsules and TA-DNA microparticles.

The study presented in this thesis can be further expanded in the field of *in vivo* delivery and activity of vaccines. We expect that the microparticles containing CpG could be efficiently internalized by peripheral APCs, which will then migrate to the lymph node where most immune cells accumulate. Furthermore, targeting of APCs can be achieved by modifying the microparticles with antibodies to enhance the internalization efficiency. As the self-assembled TA-DNA nanoparticles can be easily fabricated with different sizes, we can investigate how nanoparticle size affects the behavior of the nanoparticle *in vivo*, such as biodistribution, circulating time, and immune-stimulatory effect. We can also use this nanoparticle to deliver siRNA, mRNA, DNA plasmids, or proteins.

Nanotechnology platforms have extensively been used in vaccine development for several years. However, the question is: why only a few particle-based vaccine delivery systems are currently present on the market? The most effective vaccine against COVID-19 is mRNA-based vaccine that employs lipid nanoparticles to deliver mRNA in cells. Nevertheless, synthetic lipid components in vaccine formulations may induce side effects due to unspecific hydrophobic and electrostatic interactions with cells. The design of vaccine delivery systems for human use should be driven by the following principles: safety, cost, reproducible, and simple fabrication techniques. Lipid nanoparticles are cheap and easy to prepare but still have a safety concern. So safer, cheap, and easy to prepare delivery systems are highly needed to replace lipid nanoparticles.

The rapid development of vaccines against COVID-19 is a great public health achievement. Though these vaccines have efficiently prevented the spread of corona virus, large-scale manufacture is still highly demanded. Moreover, single-dose vaccines are in high demand. The

increasing possibilities for pre-emptive and reactive vaccine design, faster development and manufacturing options will allow us to rapidly respond to emerging viruses. As such, the investments made now in vaccine platform development and manufacturing will pay off and we are able to respond more quickly.

The TA-based nanoparticle has shown great potential to act as an efficient vaccine delivery system. Lipid-based formulations can potentially be replaced by TA-based platforms as TA is likely safer than cationic lipids. TA is a natural, abundant, cheap and biodegradable natural compound that exists in many plants widely. The galloyl chemical groups allow TA to interact with various materials, such as DNA, RNA, peptides, and proteins. Micro- and nanometer-sized particles can be easily generated by the versatile template-mediated and supramolecular assembly techniques. These findings highlight the potential use of TA as a carrier for mRNA- and DNA-based vaccines.

Finally, more effort should be devoted to understanding the structure/function relationship of TA-based particles. The fundamental study of particle-based vaccine is as important as the applications. There are still many aspects that need a deeper investigation, such as how the physical properties affect the behavior of the particle in vivo, inside the host cell, and how the size, shape, surface charge, softness of particles influence particle-cell interactions, biodistribution, circulation time, targeting efficiency and functionality.